UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

TESE DE DOUTORADO

Atividade leishmanicida e imunomoduladora do óleo essencial e do isolado 6,7-dehidroroileanona derivados de *Tetradenia riparia* (Hochstetter) Codd

IZABEL GALHARDO DEMARCHI

Maringá
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito para obtenção do título de Doutor em Ciências da Saúde.

Área de concentração: Doenças Infecciosas e Parasitárias.

Orientadora: Prof.a Dr.a Maria Valdrinez Campana Lonardoni

Maringá
2015

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

D372a

Demarchi, Izabel Galhardo
Atividade leishmanicida e imunomoduladora do óleo essencial e do isolado 6,7-dehidroroileanona derivados de *Tetradenia riparia* (Hochstetter) Codd / Izabel Galhardo Demarchi. -- Maringá, 2015.
134 f. : il. col., figs., tabs.

Orientadora: Profa. Dra. Maria Valdrinez Campana Lonardoni.
Tese (doutorado) - Universidade Estadual de Maringá, Centro de Ciências da Saúde, Programa de Pós-Graduação em Ciências da Saúde, 2015.

1. *Leishmania*. 2. Leishmaniose cutânea. 3. *Tetradenia riparia*. 4. Citocinas. 5. Diterpeno. I. Lonardoni, Maria Valdrinez Campana, orient. II. Universidade Estadual de Maringá. Centro de Ciências da Saúde. Programa de Pós-Graduação em Ciências da Saúde. III. Título.

CDD 21.ed.616.9364

FOLHA DE APROVAÇÃO

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Aprovada em: 17 de julho de 2015.

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AGRADECIMENTO(S)

A Deus.

Aos meus pais Ivani A. Chichera Galhardo e Jorge Luiz Galhardo (*in memoriam*), pela confiança, dedicação, carinho, amor e incentivo para concluir os estudos durante toda a minha vida.

Ao meu marido Fábio Demarchi, que com muita paciência, amor e dedicação tornaram possível a realização deste sonho.

Aos meus avôs, primos, tios, sogros e cunhadas pelo amor e amizade. Aos meus sobrinhos, Giovanni, Mariana e Fabrícia por encherem meu coração de esperança, alegria e amor.

À minha orientadora, Profª. Drª. Maria Valdrinez Campana Lonardoni, pela sua dedicação, competência e profissionalismo, essenciais ao desenvolvimento dessa pesquisa, a quem tenho grande admiração, carinho e respeito.

Ao *teacher* phD. Jorge Juarez Vieira Teixeira pelo respeito, amizade, carinho e por ser considerado meu pai na profissão.

Aos pesquisadores Drs. Sandra Mara, Thaís Silveira, Kárin, Tatiane Perles, Melina, Quirino pela atenção e amizade. Aos animais de laboratório fica o meu eterno respeito.

Aos funcionários do laboratório de Imunologia clínica da Universidade Estadual de Maringá: Marinete, Paulo, Ivone, Marina, Zilda, Mírian e Carla pelo apoio, colaboração e amizade.

Aos colegas de minha turma de Doutorado em Ciências da Saúde.

A Mariana de Souza Terron e Mateus Vailant Thomazella pela dedicação prestada ao desenvolvimento deste trabalho e a todos os alunos de farmácia e biomedicina.

Aos colegas de laboratório: Cissi, Lilian, Taísa, Carol, Laís, Bruna, Dani, Diego Molina pelos momentos de descontração, amizade e apoio. A Dani e Carol, alunas que participaram do programa Ciências Sem Fronteiras e que corrigiram o inglês dos artigos, meu muito obrigada pela dedicação e correções.

A todos que participaram do projeto PET-saúde, em especial ao meu grupo do NIS Pinheiros.

Aos alunos, aos pacientes e servidores da saúde que colaboraram e se dedicaram ao nosso projeto.

A todos, que direta ou indiretamente ajudaram em meu trabalho, o meu sincero agradecimento.

EPÍGRAFE

“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos”.

(Isaac Newton)

Atividade leishmanicida e imunomoduladora do óleo essencial e do isolado 6,7-dehidroroileanona derivados de *Tetradenia riparia* (Hochstetter) Codd

RESUMO

Introdução: A leishmaniose cutânea é causada por protozoários do gênero *Leishmania* e é caracterizada por lesões na pele localizadas ou disseminadas. A espécie *Leishmania (Leishmania) amazonensis* é responsável pela forma clínica cutânea e cutânea difusa, a mais grave forma clínica e de difícil tratamento. Esta espécie geralmente mostra resistência terapêutica levando à falha da terapia e agravamento da doença. O tratamento atual para a leishmaniose pode provocar graves efeitos adversos e toxicidade. Neste contexto, os produtos naturais são considerados potenciais candidatos para a terapia alternativa da leishmaniose, pois além da atividade leishmanicida, possuem a capacidade de modular citocinas da resposta imune do hospedeiro, a qual é essencial para a resolução da doença. A planta *Tetradenia riparia* é utilizada como tradicionalmente na África para o tratamento de doenças inflamatórias e infecciosas. Os extratos e o óleo essencial derivados desta planta apresentam propriedades antioxidantes, anticarcinogênica e antimicrobiana. Embora esta planta seja utilizada como remédio popular por diferentes populações para a cura de uma diversidade de doenças, poucos estudos têm investigado os efeitos anti-*Leishmania* e imunomoduladores da planta *T. riparia.* O óleo essencial derivado de *T. riparia* (TrEO) é um rico complexo de terpenoides, incluindo os diterpenos (hidrocarbonetos ou oxigenados) relacionados com a atividade antimicrobiana dos óleos essenciais. O diterpeno 6,7-dehidroroileanona derivado de *T. riparia* (TrROY) foi recentemente descrito na literatura, e ainda há poucos estudos sobre as propriedades farmacológicas deste composto. Assim, neste estudo foi investigado: o potencial leishmanicida do TrEO e do TrROY sobre formas promastigotas e amastigotas de *L. (L.) amazonensis*; a citoxicidade de TrEO e TrROY sobre macrófagos murinos e eritrócitos humanos; a produção de nitrito e a expressão de óxido nítrico sintase induzível (iNOS) por macrófagos murinos infectados com *L.(L.) amazonensis* e tratados com TrEO; a imunomodulação de macrófagos murinos tratados com TrEO; e a modulação da expressão gênica e da produção de citocinas por macrófagos murinos tratados com TrEO e infectados com *L. (L.) amazonensis*. **Metodogia:** Os efeitos de TrEO e TrROY sobre formas promastigotas de *Leishmania* foram avaliados utilizando as metodologias de contagem microscópica convencional, de redução do XTT (2,3-bis-[2-metoxi-4-nitro-5-sulfofenil]-5-[(fenilamino), e ainda foi realizada a microscopia eletrônica de transmissão para verificar as alterações ultraestruturais das formas promastigotas tratadas com TrEO. Os efeitos citotóxicos de TrEO e TrROY para macrófagos murinos foram avaliados utilizando o método de XTT e de exclusão de *Trypan Blue*, e para eritrócitos humanos foi realizado o teste de hemólise a partir da leitura em espectrofotômetro. Para verificar os efeitos de TrEO e TrROY sobre as formas intracelulares de *Leishmania*, macrófagos peritoneais obtidos de camundongos BALB/c foram infectados com promastigotas, e posteriormente tratados com TrEO e TrROY, por microscopia convencional obteve-se a porcentagem de células infectadas e o número médio de formas amastigotas por macrófago. A técnica de reação em cadeia da polimerase quantitativa em tempo real (qPCR) também foi realizada para a verificar a quantidade de parasitos baseando-se na detecção do DNA de *Leishmania*. Para os estudos de imunomodulação, macrófagos peritoneais murinos foram infectados com *Leishmania* e tratados com 30 ng/mL de TrEO. Após os períodos de incubação de 3, 6 e 24 h, a produção de nitrito foi determinada por espectrometria utilizando o método de Griess, e a expressão da enzima óxido nítrico sintetase induzível (iNOS) e de citocinas (interleucinas, IL-1β, IL-2, IL-

4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-18, IL-33; interferon-γ, IFN-γ; fator de necrose tumoral, TNF; fator de crescimento de colônias de macrófagos e granulócitos, GM-CSF) foi verificada pelo método de PCR semi-quantitativo associado à transcriptase reversa. A produção de citocinas foi verificada por citometria de fluxo. Para a análise estatística considerou-se um intervalo de confiança de 95%. **Resultados**: TrEO e TrROY promovem a morte de promastigotas de *L. (L.) amazonensis* em até 72 h de incubação. TrEO foi mais efetivo que TrROY, a dose letal em 50% (DL_{50}) de TrEO foi de 0,8 µg/mL e de TrROY de 3 µg/mL. TrEO e TrROY não mostraram citotoxicidade sobre eritrócitos humanos, mas TrROY mostrou toxicidade para macrófagos murinos resultando em uma baixa seletividade das substâncias para o parasito em relação às células. Na concentração de 0,03 µg/mL, o TrEO foi capaz de modificar as ultraestruturas de promastigotas sugerindo o processo de morte celular por autofagia indicada pela presença de condensação da cromatina, formação de *blebbing* de membrana, perfis membranosos e fragmentação nuclear. Os macrófagos tratados com 0.03 µg/mL de TrEO e 10 µg/mL de TrROY mostraram uma redução de 65 e 48% no índice de infecção por *Leishmania*, respectivamente. TrEO e TrROY não induziram a expressão de iNOS e a produção de nitrito em macrófagos infectados com *Leishmania.* Embora, o TrEO não tenha modificado a via do óxido nítrico, TrEO modulou a expressão gênica e a síntese de citocinas de macrófagos murinos não infectados e tratados em todos os períodos estudados. A modulação da expressão gênica ocorreu somente nos períodos de 3 e 6 h, enquanto que os efeitos sobre a produção das citocinas foram observados até 24 h. A expressão e produção de 1β, IL-12, IL-17 e IFN-γ foram altamente induzidas por TrEO em 3 h, e somente a IL-1β foi expressa e produzida em altos níveis em 6 h, os quais foram reduzidos gradualmente até o período de 24 h. A IL-2 foi produzida em altos níveis no início da incubação, e GM-CSF e IL-17 mais tardiamente (24 h). TrEO inibiu significativamente a produção de IL-10 e IL-6 em macrófagos murinos. Em macrófagos infectados com *Leishmania* e tratados com TrEO, houve um significativo estímulo para produção de IFN-γ e inibição de IL-1β, IL-6, IL-17, IL-33, TNF, e de citocinas da resposta do tipo T_H2 (linfócitos T *helper*, IL-4, IL-5 e IL-10). Os níveis de IL-12 foram mantidos a níveis normais pelo tratamento com o TrEO. Enquanto que a infecção por *L. (L.) amazonensis* estimulou a produção de IL-10, IL-1β, IL-4, IL-5, IL-6, IL-17 e IL-33, e inibiu IL-12 e IFN-γ produzidos pelos macrófagos infectados e não tratados. **Conclusão**: TrEO e TrROY promovem a morte dos parasitos *L. (L.) amazonensis* possivelmente por alterações no metabolismo mitocondrial, respiratório e lipídico. O TrEO em baixas concentrações não apresenta citotoxicidade às células e é capaz de modular a expressão gênica e a produção de citocinas importantes da resposta imunológica. O perfil de citocinas induzido pelo tratamento com o TrEO na ausência da infecção está associado ao estímulo da resposta imune inata celular e a supressão de citocinas de células T_H2. Assim, TrEO poderia ser uma alternativa terapêutica para várias doenças em que a resposta imune celular é crucial para a sua resolução, como nas doenças infecciosas, autoimunes e no câncer. Os efeitos do TrEO sobre a leishmaniose, sugere que o TrEO é capaz de regular negativamente as citocinas T_H2 envolvidas com a progressão da leishmaniose, e aumenta IFN-γ que é essencial para a resolução da doença. Todos esses resultados sustentam o uso da planta *T. riparia* como medicamento popular para o tratamento de infecções parasitárias como a leishmaniose, e outras doenças que requerem a modulação da resposta imune. O TrEO poderia ser utilizado como terapia alternativa para o tratamento da leishmaniose ou concomitante aos medicamentos preconizados, por isso, sugere-se que ensaios *in vivo* e em humanos sejam conduzidos para garantir a sua eficácia e o uso seguro deste tratamento.
Palavras-chave: *Leishmania*. Leishmaniose cutânea. *Tetradenia riparia*. Citocinas. Diterpeno

Antileishmanial and immunomodulatory activitiy of the essential oil and 6,7-dehydroroyleanone from *Tetradenia riparia* (Hochstetter) Codd

Abstract

Introduction: Cutaneous leishmaniasis is caused by protozoa of the genus *Leishmania*, and it is characterized by skin lesions localized or disseminated. *Leishmania (Leishmania) amazonensis* is responsible agent for cutaneous and diffuse cutaneous, the most severe clinical and difficult to treat form. This species usually shows therapeutic resistance leading to therapy failure and worsening injury. The current treatment for leishmaniasis has caused serious side effects and toxicity. In this context, the natural products are considered potential candidates for alternative therapy for leishmaniasis, as well the leishmanicidal activity, products able to modulate cytokines of the host immune response are essential for the resolution of the disease. The *Tetradenia riparia* plant is used as traditionally in Africa for the treatment of inflammatory and infectious diseases. The extracts and the essential oil derived from this plant have antioxidant, anticarcinogenic and antimicrobial properties. Although this plant is used as a folk medice in different populations for curing a variety of diseases, few studies have investigated the anti-*Leishmania* and immunomodulatory effects of the *T. riparia* plant. The essential oil derived from *T. riparia* (TREO) is a rich complex of terpenoids, including diterpenes (or oxygenated hydrocarbons) related to the antimicrobial activity of essential oils. The diterpene 6,7-dehidroroileanona derived from *T. riparia* (TrROY) has recently been described in the literature, there are few studies on the pharmacological properties of this compound. In this study, we investigated: the leishmanicidal potential TrEO and TrROY on promastigotes and amastigotes of *L. (L.) amazonensis*; the cytotoxicity of TrEO and TrROY on murine macrophages and human erythrocytes; the production of nitrite and nitric oxide synthase mRNA expression (iNOS) by murine macrophages infected with *L. (L.) amazonensis* and treated with TrEO; the immunomodulation of murine macrophages treated with TrEO; and modulation of gene expression and cytokine production by murine macrophages treated with TrEO and infected with *L. (L.) amazonensis*. **Methodology**: The effects of TrEO and TrROY on *Leishmania* promastigotes were evaluated using three methods, the conventional microscopic, reduction of XTT (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino), and transmission electron microscopy to check the ultrastructural alterations of promastigotes treated with TrEO. The TrEO and TrROY cytotoxicity was evaluated using the XTT method and Trypan Blue exclusion test, and for human erythrocytes was performed hemolysis test from reading in spectrophotometer. To check the effects of TrEO and TrROY on intracellular forms of *Leishmania*, peritoneal macrophages obtained from BALB/c mice were infected with promastigotes, and subsequently treated with TrEO and TrROY. The percentage of infected cells and the average number of amastigotes per macrophage was obtained counting cells in conventional light microscopy. Quantitative real time polymerase chain reaction (qPCR) was also performed to determine the quantity of parasites based on the *Leishmania* DNA detection. For studies of immunomodulation, murine peritoneal macrophages were infected with *Leishmania*, and treated with 30 ng/mL of TrEO. After 3, 6 and 24 hours of incubation, nitrite production was determined by spectrometry using the Griess reagent, and expression of iNOS and cytokines (interleukins, IL-1β, IL -2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-18, IL-33, interferon-γ, IFN-γ, tumor necrosis factor, TNF; growth factor of colonies of granulocytes and macrophages, GM-CSF) was measured by semi-quantitative PCR method associated with the reverse transcriptase. Cytokine production was detected by flow

cytometry. For statistical analysis it was considered a 95% confidence interval. **Results**: TrEO and TrROY promote the *L. (L.)* amazonensis promastigote forms death within 72 h of incubation. TrEO was more effective than TrROY, which the 50% lethal dose (LD50) of TrEO was 0.8 µg/mL and TrROY 3 µg/mL. TrEO and TrROY did not show cytotoxicity on human erythrocytes, but TrROY showed toxicity to murine macrophages resulting in a low selectivity index. TrEO at the concentration of 0.03 µg/mL was able to modify the ultrastructures of the promastigotes suggesting autophagy process and cell death indicated by the presence of chromatin condensation, membrane blebbing formation, membranous profiles and nuclear fragmentation. Macrophages treated with 0.03 µg/mL of TrEO and 10 µg/ml of TrROY reduced the infection index from 177 (macrophages infected with *Leishmania*) to 65 and 48%, respectively. TrEO and TrROY subvert the inhibition of expression of iNOS and nitrite production in macrophages infected with *Leishmania*. TrEO modulated cytokine gene expression and synthesis by murine macrophage uninfected and treated in all periods studied. The modulation of gene expression occurred only at 3 and 6 h, while the effects on cytokine production were observed up to 24 h. The expression and production 1β, IL-12, IL-17 and IFN-γ were highly induced by TrEO at 3 h. IL-1β was expressed and produced in high levels in 6 h, which was gradually reduced to the period of 24 h. IL-2 was produced at high levels at the start of incubation, and GM-CSF and IL-17 later (24 h). TrEO significantly inhibited IL-10 and IL-6 production by murine macrophages. In macrophages infected with *Leishmania*, and treated with TrEO, IFN-γ was highly produced, and IL-1β, IL-6, IL-17, IL-33, TNF, and T_H2-type cytokine response (helper T lymphocytes, IL-4, IL-5 and IL-10) were inhibited. IL-12 levels were maintained at normal levels by treatment with TrEO. While infection with *L. (L.) amazonensis* stimulated IL-10 production, IL-1β, IL-4, IL-5, IL-6, IL-17 and IL-33, and inhibit IL-12 and IFN-γ produced by macrophages infected and not treated, TrEO treatment subvert it favoring the infection resolution. **Conclusion**: TrEO and TrROY promote the death of *L. (L.) amazonensis* possibly by mitochondrial, respiratory and lipid metabolism. TrEO at low concentrations did not show cytotoxicity to cells and is capable of modulating gene expression and production of major cytokines of the immune response. The profile of cytokines induced by TrEO in the absence of infection is associated with stimulation of the innate cellular immune response and suppression of T_H2 cell cytokines. Thus, TREO would be an alternative therapy for various diseases in which the immune response is crucial for its resolution as in infectious diseases, autoimmune and cancer. The effects of TrEO on leishmaniasis, suggests that it is able to suppress T_H2 cytokines involved with the progression of leishmaniasis, and enhances IFN-γ which is essential for the resolution of this disease. All these results support the use of *T. riparia* plant as a folk medicine for the treatment of parasitic infections such as leishmaniasis and other diseases which require modulation of the immune response. TrEO could be used as an alternative therapy for leishmaniasis. We suggested that in vivo tests in humans are performed to ensure its efficacy and safe use of this treatment for leishmaniasis or other disease.

Keywords: *Leishmania*. Cutaneous leishmaniasis. *Tetradenia riparia*. Cytokines. diterpene

Tese elaborada e formatada conforme as

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SUMÁRIO

CAPÍTULO I
Revisão Bibliográfica

Leishmanioses

As leishmanioses são doenças infecciosas negligenciadas que tem como agente etiológico os parasitos do gênero *Leishmania* pertencentes à ordem *Kinetoplastida* e à família Trypanosomatidae.A leishmaniose tem caráter zoonótico, pois é primariamente uma enzootia de animas silvestres. Os parasitos causadores da leishmaniose são protozoários digenéticos que tem ciclo biológico heteróxeno, ou seja, mais de um hospedeiro. Os hospedeiros vertebrados mamíferos incluem os canídeos, primatas e o homem, e os hospedeiros invertebrados são insetos da subfamília Phlebotominae, sendo os gêneros *Lutzomya* e *Plebotomus* (Brasil, 2006).

Os parasitos *Leishmania* são encontrados sob as formas promastigotas, forma flagelada, encontradas no meio extracelular da luz do trato digestivo dos flebotomíneos e que são inoculadas na pele dos mamíferos a partir do repasto sanguíneo durante a picada. Nos mamíferos, destacando-se o homem, assumem a forma amastigota, arredondada e imóvel (morfológica e bioquimicamente distinta das promastigotas), com multiplicação obrigatoriamente em células do sistema mononuclear fagocitário (SMF), principalmente macrófagos. A infecção dos insetos flebotomíneos ocorre através da picada da fêmea em um vertebrado, que durante o repasto sanguíneo ingere sangue que pode conter macrófagos parasitados pelas formas amastigostas (Killick-Kendrick*et al.*, 1991; Walters, 1993).

A leishmaniose cutânea (LC) atinge cerca de um milhão de pessoas no mundo, e ameaça 350 milhões de pessoas que vivem em áreas de risco, das quais a maioria se encontra em países em desenvolvimento. No Brasil, as principais espécies responsáveis pela LCsão *Leishmania (Leishmania) amazonensis* e *Leishmania (Viannia) braziliensis*; de leishmaniose visceral é *Leishmania (Leishmania) chagasi*. A espécie *L. (L.) amazonensis* é também o agente etiológico da leishmaniose cutânea difusa,forma clínica mais grave e destrutiva da doença (OMS, 2010; Marzochi e Marzochi, 1994).

A lesão cutânea é caracterizada, na maioria das vezes, por uma úlcera única na pele, geralmente grande, persistente e desfigurante. As manifestações e a cura clínica da doença dependem da resposta imunológica do hospedeiro e da infectividade das espécies de parasito (Silveira *et al.*, 1999).

Imunopatogenia da leishmaniose cutânea

Os parasitos são reconhecidos no sítio da infecção por células da imunidade inata como os macrófagos, *natural killers* e células dendríticas (Ebadi *et al.*, 2014). Respostas específicas são promovidas por essas células evitando a disseminação da infecção no organismo, e dependem da indução de citocinas pelas células.

As citocinas são proteínas que estimulam a ativação, regulação, diferenciação e migração celular para o combate de infecção. As citocinas são classificadas em grupos de família baseados na homologia estrutural de seus receptores celulares, e as principais são pertencentes à família das interleucinas (ILs), interferons (IFNs) e quimiocinas. Essas proteínas são essenciais para o desenvolvimento das respostas imunológicas (inata e adaptativa), para o crescimento, a diferenciação e morte celular, angiogênese e processos de reparo (Abbas, Lichtman e Pillai, 2011).Por sua ação moduladora, as citocinas podem interferir nas defesas antimicrobianas, recrutamento de células efetoras e reparação do tecido infectado. Após a entrada do patógeno, todos os mecanismos imunológicos são coordenados em poucas horas, e estes processos requerem grandes mudanças na expressão de RNA mensageiro (mRNA, *Messenger RNA*, ácido ribonucleico) e de proteínas. Alguns patógenos possuem a habilidade de interferir diretamente na síntese de proteínas do hospedeiro e inibir as respostas inatas (Argüello *et al.*, 2015). Na leishmaniose cutânea, a resposta imune do hospedeiro é um dos fatores mais importantes para o estabelecimento da infecção e cura da doença (OMS, 2010).

Entretanto, o parasito *Leishmania* tem a habilidade de modificar a resposta imune inata e adaptativa, inibindo os mecanismos antimicrobianos das células do hospedeiro, isso através do desequilíbrio da produção, ativação e diferenciação de células T auxiliares, também conhecidas como linfócitos T *helper* (T_H) (Gollob *et al.*, 2014; Silverman *et al.*, 2010). Devido à habilidade do parasito em modular negativamente a resposta imune do hospedeiro, o tratamento mostra falhas terapêuticas, favorecendo a persistência da infecção. A ativação das células da resposta imune celular, como as células T_H1 produtoras de IFN-γ, fator de necrose tumoral (*tumor necrosis factor*, TNF) e interleucinas como a IL-12, juntamente com macrófagos ativados, promovem a ativação de mecanismos microbicidas e morte do parasito (Tripathi *et al.*, 2007). No entanto, a localização intracelular do patógeno pode influenciar o efeito de drogas leishmanicidas. Ainda, o parasito tem capacidade de resistir aos mecanismos microbicidas intracelulares e impedir a fusão fagolisossomo. A

infecção por *L. (L.) amazonensis* induz a liberação de citocinas IL-10, IL-4 e IL-5 envolvidas com a resposta imune do tipo T_H2, considerada não protetora e diretamente relacionada com a progressão da doença (Duque *et al.*, 2014) (Figura 1).

O IFN-γ é uma das citocinas mais importantes para a resposta imune protetora contra *Leishmania*, devido a sua capacidade de estimular a síntese de espécies derivadasdo oxigênio (ROS, *reactive oxigen species*), e o óxido nítrico (NO, *nitric oxide*), uma das principais substâncias leishmanicidas. Esse estímulo se dá principalmente pela ativação da transcrição de genes que codificam enzimas necessárias para a geração de ROS, como a enzima sintase indutora do óxido nítrico (iNOS, *inducible nitric oxide synthase*) (Abbas, Lichtman e Pillai, 2011). Na leishmaniose, o aumento de iNOS pode influenciar a produção de NO e promover a eliminação do parasito. Portanto, para a resolução da infecção é necessário um balanço no perfil de citocinas da resposta imune do hospedeiro (Amoo, *et al.*, 2012; Oliveira, *et al.*, 2014) (Figura 1).

A Figura 1 é um esquemado processo imunológico na infecção por *L. (L.) amazonensis*. Inicialmente, as formas promastigotas de *Leishmania* são fagocitadas por macrófagos (ou outras células do sistema mononuclear fagocitário), transformando-se em amastigotas. Este processo promove a ativação, proliferação e diferenciação das células imunes que dependem principalmente das citocinas secretadas pelas células da resposta imunológica durante a infecção. Para a resolução da doença, é necessária a liberação de citocinas como o IFN-γ, IL-12 e IL-18 que ativam macrófagos e células T_H1, levando os macrófagos a secretarem substâncias microbicidas como o NO, e consequentemente à morte do parasito (Oliveira *et al*., 2014).

Os protozoários da espécie *L. (L.) amazonensis* são capazes de inibir essa resposta protetora e ainda promover a resposta imune do tipo T_H2 levando à persistência da infecção (Espirret al., 2014). As citocinas IL-1β, IL-4, IL-5, IL-6, IL-10 e o fator de crescimento de colônias de granulócito e macrófago (GM-CSF, granulocyte macrophage colony-stimulating factor) ativam as células T_H2 e à inibição da ativação dos macrófagos. Durante a infecção por *Leishmania*, os linfócitos T_H17 são ativados pela liberação da IL-6 e promovem a secreção de IL-17 e TNF, essas citocinas são pró-inflamatórias e responsáveis pelo recrutamento de neutrófilos aumentando o processo inflamatório. Pacientes infectados com *L. (L.) amazonensis a*presentam um perfil de citocinas do tipo T_H2 e T_H17 que levam à progressão

da doença e persistência do parasito, mesmo sob tratamento. Um perfil de resposta do tipo T_H1 e supressão de T_H2 favorece a cura da doença,(Oliveira *et al*., 2014; Espirr *et al*., 2014).

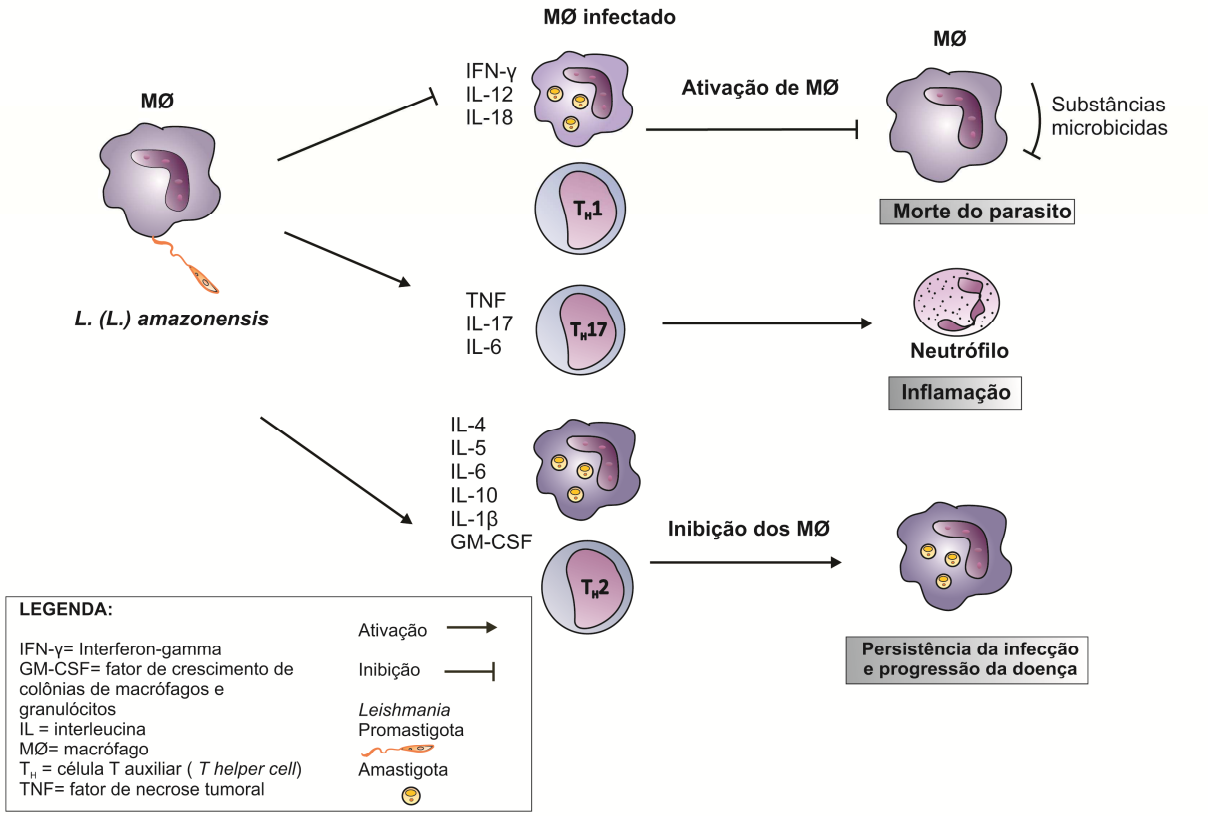


Figura 1. Resumo do perfil de citocinas e de resposta imunológica de macrófagos e células T na infecção por *L. (L.) amazonensis*.

FONTE: produção própria.

Tratamento da leishmaniose cutânea

Recentes estudos indicam que estratégias que modulam a resposta imune do hospedeiro, sendo chamadas de imunoterapia, podem resultar em um tratamento profilático ou terapêutico que favorece a cura da leishmaniose (Okwor e Uzonna, 2009). A Organização Mundial da Saúde (OMS) e o Ministério da Saúde do Brasil recomendam para o tratamento da leishmaniose cutânea os antimoniais pentavalentes como o Glucantime® (Sanofi-Aventis Farmacêutica Ltda., RJ, Brasil). Os antimoniais têm sido utilizados para o tratamento da leishmaniose desde 1945, quando o primeiro antimônio pentavalente foi sintetizado, o

estibogluconato de sódio (Pentostam®, GlaxoSmithKline, Inglaterra). Posteriormente, naquele mesmo ano, foi sintetizado outro composto pentavalente, o antimoniato de N-metil glucamina, o Glucantime®. Devido à alta toxicidade dos antimoniais, falhas terapêuticas e efeitos adversos, outros fármacos foram desenvolvidos e aplicados para o tratamento da leishmaniose, como a anfotericina B desoxicolato de sódio (AmB, Anforicin B), a qual tem sido utilizada como terapia de segunda escolha (Sundar e Chakravarty 2013;OMS, 2010). A eficiência da AmB no tratamento da leishmaniose foi relatada no final da década de 1950 e início dos anos 1960 (Sampaio *et al.*, 1960), mostrando um pequeno número de recidivas e melhor ação sobre as lesões em comparação com os antimoniais (Castro, 1972; Sampaio *et al.*, 1960; Sampaio e Marsden, 1997).

Os antimoniais podem estimular o sistema imune inato favorecendo a resolução da infecção (Ghosh*et al.*, 2013). Entretanto, nos últimos anos, muitos estudos têm reportado uma atividade leishmanicida ineficaz e efeitos adversos durante o tratamento com antimoniais e drogas de segunda escolha, como a anfotericina B, o que tem levado muitos pacientes a abandonaro tratamento da doença (Berman*et al.*, 1985; Berman, 1997). Dentre os efeitos adversos mais comuns como resultado da terapia com antimoniais estão a cardiotoxicidade e pancreatite, por isso o monitoramento da toxicidade é requerido quando o antimonial é utilizado (Sundar e Chakravarty*et al.*, 2010). A AmB é um agente antifúngico, antibacteriano e antiprotozoários, e usada clinicamente há muitas décadas. No entanto, o seu uso é limitado devido a sua nefrotoxicidade (Baginski e Czub*et al.*, 2009). Desta maneira, pesquisas têm sido conduzidas para a descoberta de novas drogas para o tratamento da leishmaniose com a finalidade de produzir um novo fármaco com grande potencial leishmanicida, menor toxicidade e com efeitos imunomoduladores benéficos (Monge-Maillo e Lopez-Velez, 2013; Oliveira *et al.*, 2011).

Terapia alternativa para a leishmaniose cutânea

As plantas medicinais possuem diversos metabólicos com atividade farmacológica, inclusive contra as leishmanioses (Chouhan *et al.*, 2014; Monzote *et al.*, 2011). Os estudos também mostram que os produtos naturais possuem uma baixa probabilidade de causar efeitos adversos; além de possuírem menor custo, maior acessibilidade e sustentabilidade, quando comparados aos tratamentos atuais preconizados para a leishmaniose (Singh *et al.*, 2014; Adebayo, 2013). Atualmente, a indústria farmacêutica tem se comportado com uma

relativa anergiaà esta doença, o arsenal de drogas leishmanicidas disponível permanece limitado, muitas vezes levando as pessoas de áreas endêmicas de leishmaniose a depender de medicamentos tradicionais usados para aliviar os seus sintomas. E assim, os estudos com as plantas medicinais utilizadas na preparação de remédios populares têm contribuído com a medicina moderna para a formulação de novos compostos farmacêuticos ativos (Sen e Chatterjee, 2011). Para o tratamento da leishmaniose, plantas com ambas as atividades leishmanicida e de imunomodulação podem ser utilizadas como terapia alternativa no tratamento da leishmaniose cutânea (Shale *et al.*, 1999; Nam *et al.*, 2008; Okem *et al.*, 2012).

A utilização de plantas medicinas pode ser uma estratégia para a modulação da secreção de citocinas, oferecendo novas abordagens para o tratamento da doença. Na leishmaniose, os produtos com atividade antileishmanial capazes de estimular a liberação de citocinas da resposta imune celular protetora e daquelas que regulam negativamente a imunidade supressora, poderiam ser uma alternativa para os casos de leishmaniose em que há resistência terapêutica, falhas ou efeitos adversos (Sen e Chatterjee, 2011).

***Tetradenia riparia* (Hochstetter) Codd**

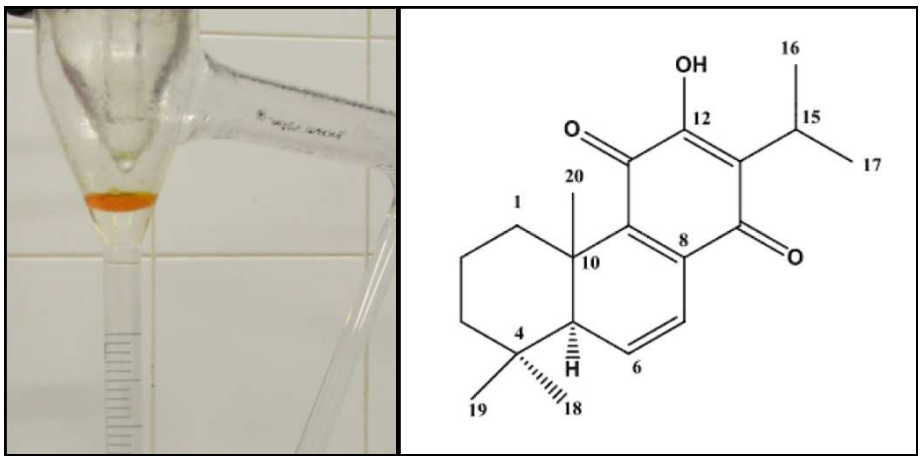
A planta *Tetradenia riparia* (Hochstetter) Codd pertence à família Lamiaceae é também conhecida como *Iboza riparia* N.E. BR. Ou *Moschosma riparium*. Esta planta é um arbusto herbáceo encontrado principalmente em regiões tropicais como a África (Polya, 2003, Shale, *et al.*, 1999, van Puyvelde, *et al.*, 1988, York, *et al.*, 2011 e 2012). Neste continente, a planta *T. riparia* é utilizada na medicina tradicional para o tratamento de doenças inflamatórias e infecciosas, ou seja, é utilizada como um medicamento popular por pessoas leigas para o tratamento de diversas doenças (Campbell, *et al.*, 1997; Cardoso, *et al.*, 2011; Gazim, *et al.*, 2010, 2011, 2014; Martins, 2008; van Puyvelde, *et al.*, 1986, 1987 e 1988).O uso de suas folhas e o óleo essencial foram descritos para o tratamento de malária, criptococose, candidíase e infecções respiratórias (Campbell, *et al.*, 1997; Okem, *et al.*, 2012; van Puyvelde, *et al.*, 1986; York, *et al.*, 2012). No Brasil, esta planta também é conhecida como falsa mirra, verbena e lavandula e é usada como incenso e planta ornamental (Martins, 2008; Gazim*et al.*, 2010; van Puyvelde*et al.*, 1988; York*et al.*, 2011) (Figura 2).

Figura 2. Estados vegetativos da planta *Tetradenia riparia*,localizada no herbário da Universidade Paranaense (UNIPAR), Umuarama, Paraná. A primeira figura é o estado vegetativo da planta durante as estações de primavera, verão e início do outono. A segunda figura é a planta no final do outono e início do inverno, enquanto que a terceira figura é o estado vegetativo durante o inverno.

FONTE: fotos cedidas pela professora Dra. Zilda CristianiGazim.

O óleo essencial derivado da planta *T. riparia* (TrEO, '*Tetradenia riparia* essentialoil') possui cor alaranjada e fragrância agradável e bem característica (Figura 3, A). O TrEO é uma mistura complexa de terpenoides, incluindo monoterpenos, sesquiterpenos e diterpenos (hidrocarbonos ou oxigenados)(Gazim, *et al.*, 2010). Os terpenoides oxigenados são as substâncias com maior atividade antimicrobiana (Gazim*et al.*, 2010 e 2014). Torquilho (1999) demonstrou a atividade leishmanioestática(capacidade de inibir o crescimento parasitário) do óleo essencial derivado da espécie *Moschoma riparium* Hochst sobre *L. (L.) amazonensis*,utilizando a metodologia de contagem microscópica dos parasitos em câmara de Neubauer. Apesar deste estudo, a atividade anti-*Leishmania* tem sido pouco estudada (Thomazella *et al.*, 2013; Demarchi *et al.*, 2013).

Recentemente, dois diterpenos foram isolados do TrEO e descritos por Gazim e colaboradores (Gazim *et al.*, 2014), os quais foram: 6,7-dehidroroileanona (Figura 3, B), substância descrita por Kusumoto *et al.*, 2009), e o composto inédito 9β,13-epoxi-7-abietano. Este novo diterpeno mostrou uma boa atividade citotóxica para diferentes linhagens de células tumorais humanas *in vitro*, enquanto que o 6,7-dehidroroileanona não foi citotóxico, mas mostrou alta atividade antioxidante (Gazim, *et al.*, 2014).Outros estudos mostraram uma atividade antiinflamatória do extrato de *T. riparia* sobre as ciclooxigenases1 (COX 1) e 2 (COX 2) (Okem *et al.*, 2012;Ndhlala *et al.*, 2011), mas os mecanismos protetores dos derivados da planta *T. riparia* ainda não estão elucidados.



(A) (B)

Figura 3. Óleo essencial de *T. riparia* (TrEO) obtidopelo processo de destilação por arraste a vapor em aparelho de Clevenger modificado (A) e estrutura química do diterpeno 6,7-dehidroroileanona derivado do TrEO (B).

Embora a planta *T. riparia* tenha uso tradicional para diferentes doenças, com um grande potencial antiinflamatório, antioxidante e antimicrobiano, (Campbell *et al.*, 1997; Gazim*et al.*, 2010 e 2014; Martins, 2008) poucos estudos foram encontrados na literatura sobre a sua atividade leishmanicida e imunomoduladora (Demarchi *et al.*, 2013; Thomazella *et al.*, 2013; Torquilho, 1999).

Métodos para estudo de drogas com atividade anti-*Leishmania* e imunomoduladora

A fim de investigar o potencial leishmanicida e imunomodulador do óleo essencial derivado de *T. riparia*, neste estudo avaliou-se a atividade leishmanicida do TrEO e do diterpeno 6,7-dehidroroileanona (TrROY, '*T. riparia* 6,7-dehydroroyleanone') sobre as formas promastigotas e amastigotas de *L. (L.) amazonensis*, a citotoxicidade sobre macrófagos murinos e eritrócitos humanos, a produção de NO, e expressão gênica do mRNA de iNOS em macrófagos murinos infectados e tratados com TrEO e TrROY. Além disso, foram analisados, o perfil de expressão gênica e a produção de citocinas em macrófagos murinos tratados com TrEO, e durante a infecção de macrófagos por *L. (L.) amazonensis* tratados e não tratados com TrEO.

No presente estudo, para os ensaios leishmanicidas utilizou-se a técnica de microscopia comum para a contagem de formas promastigotas e amastigotas, e também foi empregada a técnica colorimétrica de redução do XTT (2,3-bis-[2-metoxi-4-nitro-5-sulfofenil]-5-[(fenilamino) carbonil]-2H-tetra-zolium hidróxido). A contagem microscópica demanda muito tempo e é subjetiva, por isso está sujeita a erros. Esta técnica é mais utilizada para avaliar a concentração inibitória de drogas em 50% do crescimento das formas promastigotas (IC_{50}), considerada a atividade leishmaniostática, mas existem outros métodos que avaliam a atividade leishmanicida de drogas. Estes métodos, como o do XTT, permitem calcular a dose letal em 50% (LD_{50}), e estudar a toxicidade de drogas sobre o metabolismo mitocondrial do parasito (El-On, *et al.*, 2009, Williams, *et al.*, 2003).Além desta técnica, a microscopia eletrônica de transmissão (MET) pode ser útil para estudos de atividade de drogas anti-*Leishmania*, devido à sua capacidade de revelar alvos subcelulares dos parasitos e elucidar o mecanismo de ação final da droga testada, inferida pelas alterações induzidas por compostos antiparasitários. A luz emitida pelo microscópio eletrônico de transmissão demonstra a organização celular das ultraestruturas do parasito, como o núcleo, cinetoplasto, flagelo, mitocôndria e outras organelas que podem ser afetadas durante a exposição à droga. Assim, a partir da MET podem-se indicar as vias metabólicas alvos dos efeitos tóxicos ou letais de uma droga (Vannier-Santos e Castro, 2009).

A atividade anti-*Leishmania* para as formas amastigotas foi estudada pela infecção de macrófagos e pode ser avaliada *in vitro* para o rastreamento de drogas com efeito sobre a forma tecidual encontrada no hospedeiro. Para isso, os modelos mais utilizados para a pesquisa da atividade sobre amastigotas são as células peritoneais de camundongos ou macrófagos derivados de monócitos humanos (Gupta e Nishi, 2011). Neste estudo, macrófagos peritoneais de camundongos BALB/c foram infectados com *L. (L.) amazonensis* e tratados com TrEO, as amostras foram analisadas por microscopia óptica comum para determinar a porcentagem de células infectadas e o número médio de parasitos por macrófago. No entanto, este método de contagem requer muito tempo e a análise pode ser subjetiva, podendo não ter alto rendimento comparado aos outros modelos (Gupta e Nishi, 2011). Portanto, nessa pesquisa também foi aplicada a técnica quantitativa de reação em cadeia da polimerase (PCR, *polymerase chain reaction*) em tempo real (qPCR) para detecção de DNA de *Leishmania* em amostras de macrófagos murinos infectados com *Leishmania* e tratados com TrEO.

Para a citotoxicidade do TrEO e TrROY foram utilizados os ensaios de hemólise para eritrócitos humanos (% de lise das hemácias por análise em espectrofotômetro) (Valdez, *et al.*, 2009) e viabilidade de macrófagos murinospelos métodos de impregnação do corante *Trypan Blue* (Demarchi*et al.*, 2012) e do XTT (El-On, *et al.*, 2009; Williams, *et al.*, 2003). Para a análise da imunomodulação em macrófagos murinos infectados com *L. (L.) amazonensis* e tratados com TrEO, foi mensurada a produção denitrito, derivado do NO, pelo método de Griess (Green *et al.*, 1999). Considerando a enorme variedade nos padrões de expressão de genes de citocinas, isto é, elas podem ser constitutivamente expressadas ou ativadas ou ainda reprimidas quando uma célula é exposta a um sinal particular (SHANNON *et al.*, 2001), a expressão do Mrna de citocinas foi realizada utilizando a técnica de transcriptase reversa associada à PCR (RT-PCR, *reverse transcription PCR*). A reação de RT-PCR tem sido também utilizadapara investigar a modulação de citocinas da resposta imune por drogas durante a infecção por parasitos do gênero *Leishmania* (Espitia *et al.*, 2010;Melby *et al.*, 2001, 1998; Mendez *et al.*, 2005; Travi *et al.*, 2002; Silverman *et al.*, 2010). A técnica semi-quantitativa de RT-PCR trata-se de uma versão estendida da técnica "quantitative PCR", com uma etapa envolvendo o tratamento do RNA com a enzima transcriptase reversa antes de uma PCR convencional (Meide *et al.*, 2008).

Além da expressão gênica das citocinas, a quantificação dessas proteínas foi realizada por citometria de fluxo. Esta técnica tem sido amplamente utilizada para determinação das citocinas secretadas na leishmaniose e para avaliação da atividade imunomoduladora de drogas (Charret*et al.*, 2013; Cronemberger-Andrade *et al.*, 2014). Este método é utilizado para contagem e análise de partículas microscópicas suspensas em um fluido, mede as propriedades de dispersão da luz pelas partículas e a emissão de fluorescência pelos anticorpos monoclonais associados aosfluorocromos e ligados sobre a superfície de uma célula. As análises são multiparamétricas simultâneas. Neste estudo, foram utilizados os kits *multiplex Mouse Cytokine 10-Plex Panel kit*e*Mouse IL-17 Singleplex Bead Kit* (Invitrogen, Carlsbad, CA, EUA) para a determinação da concentração (pg/mL)de citocinas, e a análise foi realizada no citômetro de fluxo Luminex® 200™. O kit multiplex utiliza esferas de poliestireno (*beads*, 5,6 microns) magnéticas sensibilizadas com anticorpos monoclonais associados a dois diferentes fluorocromos com intensidades diferentes, permitindo a detecção de um grande número de moléculas analisadas em um curto tempo. No caso deste kit, ele permite a detecção de até 10 citocinas diferentes simultaneamente: IL-1β, IL-2, IL-4, IL-5,

IL-6, IL-10, GM-CSF, TNF e IFN-γ. O kit *Singleplex* foi utilizado para a detecção de IL-17 isoladamente. Neste caso, as *beads* eram apenas de poliestireno e não magnetizadas. As *beads* magnéticas permitem uma melhor eficiência da técnica e rapidez na execução do método (manual do fabricante; Nolan e Condello, 2013).

Os resultados desta pesquisa científica foram agrupados em três artigos originais, descritos no capítulo II desta tese de Doutorado, e as conclusões estão no capítulo III.

Objetivos gerais

- Investigar o potencial leishmanicida e imunomodulador do óleo essencial derivado de *T. riparia*;
- Estudar a atividade leishmanicida do diterpeno 6,7-dehidroroileanona (TrROY) e sua ação sobre a produção de nitrito.

Objetivos específicos

- Investigar a atividade leishmanicida do TrEO e do diterpeno 6,7-dehidroroileanona sobre as formas promastigotas e amastigotas de *L. (L.) amazonensis*,
- Avaliar a citotoxicidadede TrEO e TrROYsobre macrófagos murinos e eritrócitos humanos;
- Determinar a produção de nitrito induzida pelo tratamento com TrEO e TrROY;
- Verificar a expressão gênica do mRNA de iNOS em macrófagos murinos infectados e tratados com TrEO e TrROY;
- Determinar o perfil de expressão gênica e da produção de citocinas em macrófagos murinos tratados com TrEO;
- Verificar o perfil de citocinas de macrófagos infectados com *L. (L.) amazonensis* tratados e não tratados com TrEO.

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CAPÍTULO II

Artigos científicos

O capítulo II é formado por três artigos científicos redigidos em inglês e formatado de acordo com as normas dos periódicos de provável publicação.

Artigo 1 – Aceito em 22 de junho de 2015.
Título em inglês: 'Antileishmanial activity of essential oil and 6,7-dehydroroyleanone isolated from *Tetradenia riparia'*
Periódico: *Experimental Parasitology* (fator de impacto pelo *Journal Citation Reports 2014* de 1.859, classificação B1 no Qualis Medicina II da CAPES/CNPq).
As normas do periódico estão disponíveis em:
http://www.elsevier.com/journals/experimental-parasitology/0014-4894/guide-for-authors.

Artigo 2- Aceito em 19 de junho de 2015.
Título em inglês: 'Immunomodulatory activity of essential oil from *Tetrania riparia* (Hochstetter) Codd in murine macrophages'
Periódico: *Flavour and Fragrance Journal* (fator de impacto pelo *Journal Citation Reports 2014* de 1.97, classificação B1 no Qualis Medicina II da CAPES/CNPq).
As normas do periódico estão disponíveis em:
http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291099-1026/homepage/ForAuthors.html

Artigo 3 – Submetido em 25 de maio de 2015, sob revisão desde 19 de junho de 2015 com prazo para submissão da revisão em até 4 meses.
Título em inglês: 'Antileishmanial and immunomodulatory effects of the essential oil from *Tetradenia riparia* (Hochstetter) Codd'

Periódico: *Parasite Immunology* (fator de impacto pelo *Journal Citation Reports 2014* de 1.849, classificação B1 no Qualis Medicina II da CAPES/CNPq).

As normas do periódico estão disponíveis em:
http://onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291365-3024.

Artigo 1

Antileishmanial activity of essential oil and 6,7-dehydroroyleanone isolated from *Tetradenia riparia*

Izabel Galhardo Demarchi[a*], Mateus Vailant Thomazella[a], Mariana de Souza Terron[a], Lilian Lopes[a], Zilda Cristiani Gazim[b], Diógenes Aparício Garcia Cortez[c], Lucélia Donatti[d], Sandra Mara Alessi Aristides[a], Thaís Gomes Verzignassi Silveira[a], Maria Valdrinez Campana Lonardoni[a]

[a] *Laboratório de Imunologia Clínica da Universidade Estadual de Maringá, Maringá, Paraná, Brazil.*

[b] *Laboratório de Química de Produtos Naturais da Universidade Paranaense, Umuarama, Paraná, Brazil.*

[c] *Departamento de Farmácia e Farmacologia da Universidade Estadual de Maringá, Maringá, Paraná, Brazil.*

[d] *Laboratório de Biologia Adaptativa, Departamento de Biologia Celular, Universidade Federal do Paraná, Curitiba, Brazil.*

Corresponding author:

Name: Izabel Galhardo Demarchi

Affiliation: Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná State, Brazil

Email: bel_galhardo@yahoo.com.br

Telephone: 55 00 (xx44) 3261-4878/ fax numbers: 55 00 (xx44) 3261-4860

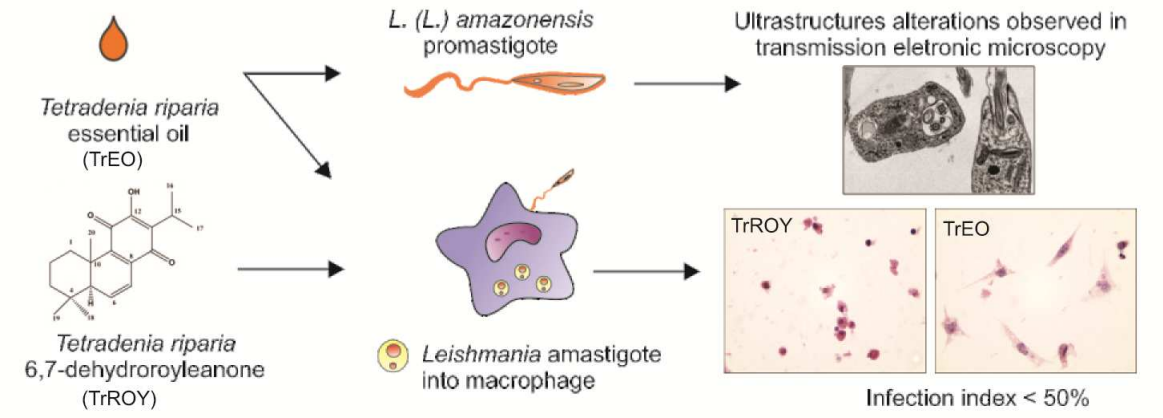
mail address: Av Colombo, 5790. Universidade Estadual de Maringá, Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC). CEP 87020-900 – Maringá – Paraná, Brazil

Type of paper: Original research

Highlights

- *T. riparia* essential oil induces ultrastructures changes on *Leishmania.*
- *T. riparia* 6,7-dehydroroyleanone acts against *L. (L.) amazonensis* infection.
- *T. riparia* essential oil did not induce iNOS mRNA expression in *Leishmania* infection.

Graphical abstract



Abstract

Tetradenia riparia plant is used as a traditional medicine in Africa for the treatment of inflammatory and infectious diseases as parasitic. Therapy for leishmaniasis caused by *Leishmania (Leishmania) amazonensis* specie often fails, and the conventional drugs are toxic, expensive, require a long period of treatment, and adverse effects are common. The alternative therapies using natural products are inexpensive and have few or any adverse reaction. These reasons are sufficient to investigate new natural therapeutic for leishmaniasis. We evaluated the potential of the essential oil (TrEO) and 6,7-dehydroroyleanone (TrROY) isolated from *T. riparia* on *L. (L.) amazonensis* promastigote and amastigote forms, cytotoxicity on human erythrocytes and murine macrophages, nitric production and inducible nitric oxide synthase (iNOS) mRNA expression. TrEO was the most effective to promote *Leishmania* promastigote death. After 72 h incubation, the lethal dose of TrEO and TrROY that promoted 50% *Leishmania* death (LD_{50}) were 0.8 µg/mL and 3 µg/mL, respectively. TrEO and TrROY were not cytotoxic to human erythrocytes, but TrROY was toxic to murine macrophages resulting in a low selectivity index. The transmission electronic microscopy showed that TrEO (0.03 µg/mL) was able to modify the promastigote ultrastructures, suggesting autophagy as chromatin condensation, blebbing, membranous profiles and nuclear fragmentation. Infected-macrophages treated with TrEO (0.03 µg/mL) or TrROY (10 µg/mL) had an infection index decreased in 65, and 48%. TrEO did not induce iNOS mRNA expression or nitrite production in macrophages infected with *Leishmania*. TrROY and mainly TrEO promoted *Leishmania* death. Other compounds derived from *T. riparia* and the essential oil could be explored to develop new alternative treatments for leishmaniasis.

Keywords: *Tetradenia riparia;* Lamiaceae family; diterpenoids; antiprotozoal activity; leishmaniasis; *Leishmania (Leishmania) amazonensis*

1. Introduction

Leishmaniasis are infectious diseases caused by parasites of the genus *Leishmania*, and have been treated with antimonials such as glucantime, but therapy often fails, and adverse effects are frequently observed. *Leishmania (Leishmania) amazonensis* is the etiologic agent of cutaneous and diffuse cutaneous leishmaniasis, the most severe and destructive clinical form of the disease. This species of parasite is also associated with therapeutic failure and disease recurrence (WHO, 2010). For these reasons, researchers have investigated natural products to discover new therapeutic strategies for leishmaniasis.

Tetradenia riparia (Hochstetter) Codd plant belongs to the Lamiaceae family, also known as *Iboza riparia* and *Moschosma riparium*. It is used as a traditional medicine in Africa for the treatment of inflammatory and infectious diseases. The plant is an herbaceous shrub that occurs throughout tropical Africa and other regions of the world (Polya, 2003, Shale, et al., 1999, van Puyvelde, et al., 1988, York, et al., 2011, York, et al., 2012). In Brazil, it is known as false myrrh, and it is mainly used as an ornamental plant and incense (Gazim, et al., 2010, Martins, 2008). Its leaves and essential oil have been used for the treatment of malaria, cryptococcosis, candidiasis, and respiratory infections (Campbell, et al., 1997, Okem, et al., 2012, van Puyvelde, et al., 1986, York, et al., 2012). Natural products are relatively inexpensive, accessible, and sustainable, and many of them do not have a high likelihood of causing serious adverse effects (Adebayo, 2013).

The essential oil from *T. riparia* (TrEO) is a complex mixture of terpenoids, including monoterpenes, sesquiterpenes, and diterpenes (hydrocarbons or oxygenated), the most representative class (Gazim, et al., 2010). *T. riparia* has been used as traditional medicine, also known as indigenous or folk medicine used by lay people, but few studies have evaluated its biological effects (Campbell, et al., 1997, Cardoso, et al., 2011, Gazim, et al., 2010, Gazim, et al., 2011, Gazim, et al., 2013, Martins, 2008, van Puyvelde, et al., 1988, Van

Puyvelde, et al., 1987, van Puyvelde, et al., 1986). The microbicidal and anti-inflammatory activity of *T. riparia* has scarcely been documented in the literature (Campbell, et al., 1997, Gazim, et al., 2010, Gazim, et al., 2013, Okem, et al., 2012, York, et al., 2011, York, et al., 2012). The diterpene 6,7-dehydroroyleanone isolated from *T. riparia* (TrROY) may have some biological effects such as antioxidant and antitermitic activity (Gazim, et al., 2013, Kusumoto, et al., 2009). The leishmaniostatic activity of TrEO against *L. (L.) amazonensis* was recently reported (Demarchi et al., 2013; Thomazella et al., 2013), but the potential effects of TrEO and the isolated compounds on *Leishmania* have not yet been investigated. Therefore, to determine their potential as an alternative therapy for leishmaniasis, we studied TrEO and TrROY effects on *L. (L.) amazonensis* promastigote and amastigote forms, cytotoxicity on human erythrocytes and murine macrophages, nitric production and inducible nitric oxide synthase (iNOS) mRNA expression.

2. Materials and methods

2.1 Plant material

T. riparia leaves were collected monthly between September 2006 and August 2007 in Umuarama, Paraná, Brazil (23º46’22”S and 53º16’73”W, 391 m). The plant was identified by Professor Ezilda Jacomasi of the Departamento de Farmácia of Universidade Paranaense (UNIPAR, Umuarama, Paraná, Brazil). A voucher specimen of plant was deposited in the UNIPAR Herbarium (code number 2502). The mean values for the maximum and minimum temperatures, precipitation, and relative humidity from September 2006 to August 2007 were reported by Gazim et al. (2010).

2.2 Essential oil extraction of T. riparia (TrEO)

The fresh leaves of *T. riparia* were used to extract the essential oil, which was obtained by hydrodistillation for 3 h using a Clevenger-type apparatus. The distilled oils were collected and dried over anhydrous sodium sulfate and stored in a freezer. The oil was analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 5973N GC-MS System that was operated in electron ionization mode and equipped with a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm, Agilent, PA, USA) that was used to inject 1 µl of a solution sample (Gazim, et al., 2010, Omolo, et al., 2004). The initial temperature of the column was 80°C. The column was gradually heated to 260°C at a rate of 4°C/min. The injector (splitless, 0.5 min) and transfer line temperatures were held at 260°C and 280°C, respectively. Helium (1.0 mL/min) was used as a carrier gas. The same temperature program was used for gas chromatography with a flame ionization detector (GC-FID). The identification of the compounds was based on comparisons of their retention time that were obtained using various n-alkanes (C7 - C25). Their electron impact mass spectra were compared with the Wiley library spectra and literature (Gazim, et al., 2010).

2.3 Isolating 6,7-dehydroroyleanone of T. riparia *(TrROY)*

Briefly, the TrEO (2 g) from leaves was subjected to column chromatography over a silica gel support and eluted with pentane, pentane-dichloromethane (9:1; 8:2; 7:3 and 1:1), dichloromethane-pentane (3:7), dichloromethane, dichloromethane-methanol (9:1; 7:3 and 1:1), and methanol, resulting in 29 fractions (Gazim, et al., 2013). Fraction 17 (11.7 mg) pentane-dichloromethane (8:2) were identified by ^{1}H, ^{13}C, DEPT, HSQC, HMBC and NOESY according to Gazim et al., (2014) and by comparison with literature data (Rodriguez, 2003). Fraction 17 (11.7 mg) orange crystalline powder gave an {M – H] – at m/z 313 was

identified as TrROY (Fig. 1). Spectral data corresponded with data previously described by Kusumoto (Kusumoto, et al., 2009).

2.4 Cytotoxicity assays

2.4.1 Haemolysis assay

Erythrocyte toxicity was determined as described by Valdez et al. (Valdez, et al., 2009). Briefly, a 6% suspension of fresh defibrinated human blood was prepared in sterile 1% glucose saline solution. 6,7-dehydroroyleanone was dissolved in 5% dimethylsulfoxide (DMSO; obtained from Sigma-Aldrich, St. Louis, MO, USA). DMSO concentration promoted haemolysis from 6% v/v, and at 5% did not cytotoxicity (results not shown). The compound was then serially distributed into culture plates at concentrations of 5.0-0.1 μg/mL for TrEO, 50-0.1 μg/mL for TrROY and 500-1 μg/mL for amphotericin B (AmB, Anforicin, Cristalia Laboratory, São Paulo, SP, Brazil). The compounds were incubated with erythrocytes in suspension at 37 °C. After 2 h, the samples were centrifuged at 300 × *g* for 3 min. Absorbance of the supernatant was determined at a wavelength 550 nm to estimate hemolysis. A solution of 4% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control, and the cell suspension was used as the negative control. The hemolysis was tested in duplicate. The results are expressed as a percentage of hemolysis based on the Haemolysis equation (%= 100 − [(*Ap*−*As*)/(*Ap*−*Ac*)×100]; where *Ap*, *As* and *Ac* are the absorbance of the positive control, test sample and negative control, respectively).

2.4.2 Macrophages Cytotoxicity

Peritoneal macrophages were obtained from BALB/c mice in accordance with the Ethics Committee on the Use of Experimental Animals of the Universidade Estadual de Maringá, Paraná State, Brazil (warrant no.133/2012).The peritoneal cavity was washed with 8 mL

sterile RPMI 1640 medium. The cell suspension was adjusted to 1×10^6 macrophages/mL. Next, 100 µl was plated in 96-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37°C, 5%CO_2, and non-adherent cells were removed by sterile phosphate buffered saline (PBS) washing. TrROY was diluted in DMSO and RPMI 1640 medium from 100 to 0.1 µg/mL, and TrEO from 3 µg/mL to 30 ng/mL. The DMSO did not exceed 0.005% v/v, and it had not macrophage cytotoxicity effect. Non-treated cultures were used as viability control. The plates were maintained at 37°C in a humid atmosphere containing 5% CO_2 during 24 h. The results were revealed using colorimetric cell viability XTT (2,3-bis[2-methyloxy-4- nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma Chemical Co; St. Louis, MO) (El-On, et al., 2009, Williams, et al., 2003). A XTT solution (500 µg/mL) was activated with 50 µg/mL phenazine methosulfate (PMS, Sigma Co Chemical; St. Louis, USA), and it was added over the cell monolayer to each well. After 3-5 h incubated at 37°C, with 5% CO_2 and protected from light, the result was measured at a wavelength 450/650 nm using a spectrophotometer plate reader (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The cytotoxicity concentration (CC_{50}) was defined as the dose of the compound that reduced 50% of the survival of macrophages comparing with untreated macrophages (viability control) (Cardoso et al., 2011). This test was done in duplicate.

2.5 Promastigote assay

2.5.1 Parasite strain and culture

L. (L.) amazonensis (MHOM/BR/1977/LTB0016) infection was induced by inoculating the left footpad of BALB/c mice with 1×10^7 parasites. Animals between 30 and 40 days of age were infected and after 30 days, they were sacrificed with 160 mg/Kg ketamine (Agner União, Embu-Guaçu, SP, Brazil) and 50 mg/Kg xylazine (Coopazine®, Invervet Schering-Plough, Cotia, SP, Brazil) . The lymph node fragments were incubated in 199 culture medium

supplemented with 10% fetal bovine serum, 1% human urine, 2 mM L-glutamine, and antibiotics (100 UI/mL penicillin and 0.1 mg/mL streptomycin). The cultures were incubated at 25ºC, and parasites were maintained by weekly transfers in 25 cm^2 culture flasks supplemented with 199 culture medium (Demarchi, et al., 2012).

2.5.2 XTT tetrazolium method

Promastigote viability was evaluated using the XTT method. Promastigotes (4×10^6 parasites/100 µl/well) from a logarithmic growth phase culture were seeded into flat-bottomed 96-well plastic tissue culture microplates in triplicate. TrEO, TrROY, and AmB were dissolved in 1.6% v/v DMSO in the first well and diluted in series from 50 µg/mL to 1 ng/mL in a 96-well culture plate. After 24, 48, and 72 h incubation at 25°C, 0.2 mg/mL XTT and 200 µM phenazine methosulfate (PMS, Sigma Co Chemical; St. Louis, USA) were added to each well and incubated for 3-5 h at 37°C. The result was measured at a wavelength 450/650 nm using a spectrophotometer plate reader (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The lethal dose (LD_{50}) was defined as the dose of the essential oil that reduced the survival of *Leishmania* parasites by 50% compared with untreated parasites (El-On, et al., 2009, Williams, et al., 2003). The therapeutic selectivity index ($TSI=CC_{50}/LD_{50}$) was calculated by the ratio of toxicity to macrophage vs. toxicity to the parasites after 24 h incubation. The test was done in triplicate.

2.5.3 Promastigote growth inhibition

Promastigotes were cultured in Schneider's insect medium (Sigma-Aldrich, St. Louis, MO, USA), pH 7.2, supplemented with 10% FCS (v/v) and 2 mM L-glutamine until they reached the logarithmic growth phase. TrEO and TrROY were diluted in DMSO from 50 µg/mL to 1 ng/mL on a cell culture plate with 96 wells (TPP® test plate, Switzerland). The

concentrations of DMSO not exceed 1.6% v/v DMSO in the first well, and it had no effect on the parasites. Next, 100 μL of the suspension containing 4×10^6 parasites/mL was distributed in each well of the culture plate. After 24 h at 25 °C, an aliquot of each well was added to a solution containing 10% eosin and 2% formalin, and the parasites were counted in a Neubauer chamber. All tests were performed in triplicate. The inhibitory concentration that caused a 50% decrease in survival (IC_{50}) of promastigotes was calculated. Values were compared to promastigotes cultures not treated. The results were evaluated by linear regression of the inhibition percentage (Demarchi et al., 2012). The selectivity index (SI= CC_{50}/IC_{50}) was calculated by ratio of toxicity concentration to macrophage vs. inhibitory concentration to the parasite growth after 24 h incubation.

2.5.4 Ultrastructural alterations

L. (L.) amazonensis promastigotes were grown in 199 medium during 3 days in 25 cm^2 culture vials at 25°C. The LD_{50} concentration of TrEO and 50% inhibitory concentration (IC_{50}= 30 ng/mL) (Demarchi, et al., 2011) was added to the cultures and incubated at 25°C for 24 h. After incubation, the IC_{50} was calculated according to the number of remaining parasites in a Neubauer chamber compared with control cultures without drugs and diluents (DMSO). The results were evaluated by linear regression of the inhibition percentage. An untreated culture was used as a negative control, and the ultrastructural changes were observed by transmission electron microscopy. For this, the samples were centrifuged at 12,000 g-force per minute for 10 min and fixed in Karnovsky solution 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at 4°C (Karnovsky, 1965). The material was fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Block staining was performed with 2% uranyl acetate for 2 h, and the blocks were dehydrated in a graded series of alcohol and acetone. Embedding was performed in Epon 812 resin (Luft, 1961).

Sections were obtained using an Sorval Porter Blum MT-2 ultramicrotome and concentrated in 2% uranyl acetate (Watson, 1958) and nitrate/acetate lead (Reynolds, 1963). The JEOL 1200EX II transmission electron microscopy was used for ultrastructural evaluation. The assays were conducted in duplicate.

2.6 Amastigote assay

2.6.1 Leishmania infection on macrophages to obtain amastigote forms

Peritoneal macrophages were obtained from BALB/c mice peritoneal cavity as already reported above. The cell suspension was adjusted to 1 x 10^6 macrophages/mL. Next, 500 µL were distributed on 13 mm-diameter sterile glass coverslips (Glastecnica, São Paulo, SP, Brazil), and placed in 24-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37°C. The non-adherent cells were removed by sterile PBS washing. Macrophages were infected with promastigote forms in the proportion of six parasites for each macrophage, and the plates were incubated for 4 h at 37°C in an atmosphere of 5% CO_2.

2.6.2. Infection index

TrEO and TrROY were diluted in DMSO and RPMI 1640 medium. The final concentration of DMSO did not exceed 0.05%, and no cytotoxicity effect on the macrophages was observed. TrEO and TrROY were added to cultures at concentrations from 300 µg/mL to 30 ng/mL, and 100 to 0.1 µg/mL, respectively. After 24 h at 37°C and 5% CO_2, the cells on coverslips were fixed in 95% ethanol and dyed with eosin and hematoxylin. At least 200 cells were counted in an optical microscope. The infection index was determined from the percentage of infected macrophages multiplied by the mean number of parasites per macrophage. The supernatant was stored at - 30°C to nitrite assay.

2.7 Inducible nitric oxide synthase (iNOS) expression and nitrit assay

2.7.1 Cells and treatment

Peritoneal macrophages were obtained from BALB/c mice. Briefly, the peritoneal cavity was washed with 8 mL sterile RPMI 1640 culture medium. The cell suspension was adjusted to 1 $\times 10^6$ macrophages/mL, 1 mL of which was added to each well of a 12-well culture plate. The plates were incubated for 1 h at 37°C, and non-adherent cells were removed by three sterile washes in PBS. The adherent cells were incubated in RPMI 1640 culture medium. After a few minutes, macrophages were subjected to the following conditions: (1) stimulated with 5 µg/mL lipopolysaccharide (LPS, Sigma-Aldrich, Brazil), (2) untreated and uninfected (negative control), (3) infected by the promastigote form of *L. (L.) amazonensis* at a proportion of six parasites for each macrophage, (4) treated with 30 ng/mL TrEO, (5) infected with the promastigote form of *L. (L.) amazonensis* for 4 h and treated with 30 ng/mL TrEO, (6) treated with 100-0.1 µg/mL TrROY, (7) infected with the promastigote form of *L. (L.) amazonensis* for 4 h and treated with 100-1 µg/mL TrROY. The final concentration of DMSO did not exceed 0.01%, and no cytotoxic effect on the macrophages was observed. The plates were maintained at 37°C in a humid atmosphere that contained 5% CO_2 for 3, 6, and 24 h. The supernatant was also removed and stored at –30°C for the nitrite assay. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to adhered cells for RNA extraction.

2.7.2 Nitrite assay

Nitric oxide was quantified by determining the accumulation of nitrite (NO^-_2) in the supernatants after 24, 48, and 72 h using the standard Griess assay (Green, et al., 1982). Readings were performed in a spectrophotometer at a wavelength 550/620 nm (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The experiments were performed in duplicate and on different days. The results are expressed as nitrite concentration (µM).

2.7.3 mRNA analysis by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA from the samples that were stored in Trizol reagent was extracted according to the manufacturer. cDNA was synthesized using 1 μg of total RNA as a template in a reverse transcription reaction (Superscript III reverse transcriptase, Invitrogen, Carlsbad, CA, USA). RNA quantification (ng/μl) and the determination of purity were performed using NANODROP 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc, USA), considering an optimal purity level ≥ 1.8. PCR primers were: iNOS (Genbank accession no. NM_010927.3, forward 5'-CTGCAGCACTTGGATCAGGAACTG-3'; reverse 5'-GGGAGTAGCCTGTGTGCACCTGGAA-3', 311 bp); Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA of *Mus musculus* was used as intern control (Genbank accession no. XM_001476707.3, forward 5´-CCACCATGGAGAAGGCTGGGGCTC-3′; reverse 5′-AGTGATGGCATGGACTGTGGTCAT-3′, 239 bp). The primers were chosen according to the BLAST tool (available in the Genbank database) and previous publications (Byrne, et al., 2011, Chen, et al., 2004). The PCR conditions were the following: 95ºC (5 min), 30 cycles of 95ºC (20 s) and 55ºC (25 s), followed by a standard denaturation curve. iNOS mRNA expression was evaluated by comparing the presence and absence of the expression between experimental conditions and incubation times. Semi-quantitative RT-PCR was conducted by quantifying the densitometry of the bands using ImageJ software (National Institutes of Health, USA). Each gene was normalized to GAPDH as a housekeeping gene (internal control) (Lee et al., 2011). The amplified DNA fragments were separated by 2% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator.

2.8 Statistical analysis

The values are presented as mean ± standard error of mean (SEM). Differences among means and values were tested for statistical significance using one-way analysis of variance (ANOVA) and the Tukey test. Analyses were performed using Statistic 7 software. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

The essential oil and the 6,7-dehydroroyleanone derived from *T. riparia* induced *Leishmania* promastigote forms death (Fig. 2). After 24, 48, and 72 h incubation, the LD_{50} of *T. riparia* essential oil was 0.5, 0.3, and 0.8 µg/mL, respectively (Fig. 2A). The LD_{50} of TrROY was 16.9, 14.9, and 3 µg/mL (Fig. 2B), respectively. LD_{50} AmB was less than 1.5 µg/mL in all times (Fig. 2C). No difference was found between LD_{50} of TrEO and AmB ($p > 0.05$). TrROY LD_{50} was higher than AmB and TrEO ($p < 0.001$). Thus, TrEO had better microbicidal activity than TrROY.

Essential oils are natural products that contain a complex mixture of pharmacological compounds. These compounds are most often terpenoids, which are hydrocarbons. The chemical composition of TrEO was analyzed by GC-MS in Gazim et al. (2010) showing the chemical identification and concentration (%) of compounds isolated from TrEO. The chemical composition revealed a complex terpenoids mixture such as diterpenes, sesquiterpenes and monoterpenes (Gazim, et al., 2010) with anti-inflammatory activity and microbicidal effects (Campbell, et al., 1997, Gazim, et al., 2010, Gazim, et al., 2013, Okem, et al., 2012, York, et al., 2011, York, et al., 2012). Antileishmanial activity of TrEO and isolated substances from this plant has been poorly described (Torquilho et al., 1999; Thomazella et al., 2013). Thus, the substances with anti-*Leishmania* activity remain unknown so far. We showed that a diterpene TrROY from TrEO is one of the substances with

antileishmanial activity. Other substances isolated from TrEO should be evaluated against *Leishmania*, and also interactions between isolated compounds could be performed to identify the substances with anti-*Leishmania* activity. The several biological effects of essential oils can be explained by the interactions between different bioactive components, which may lead to synergistic, additive, or antagonistic effects (Bassolé and Juliani, 2012).

The use of a compound isolated from essential oils may not have the same effect promoted by the essential oil, requiring a greater amount of the isolated compound to achieve the desired action (Bassole and Juliani, 2012). These reasons may explain the highest concentration of the isolated compound that was necessary to induce *Leishmania* death compared with TrEO. Checkerboard, graphical and Time-kill methods are widely used procedures to determine these interactions (Bassole and Juliani, 2012), but we have not yet assessed the interactions between the essential oil components.

Terpenoids have antiparasitic effects on different *Leishmania* species (Ogungbe and Setzer, 2013). Some diterpenoid compounds that are isolated from plants have been reported to have leishmanicidal activity, particularly against *L. amazonensis* (Dos Santos, et al., 2011, Jullian, et al., 2005, Santos, et al., 2013), but the effects of the diterpene TrROY on this species had not yet been demonstrated. Some studies showed that diterpenoids favor docking to glycerol-3-phosphate dehydrogenase (GPDH) of *L. mexicana* (Ogungbe and Setzer, 2013), which is also involved in the glycosomal and cytosolic glycolytic pathways of *Leishmania* (Zhang, et al., 2013). One of these pathways involves NADH that is produced by GPDH, which can be evaluated using the XTT assay (Ramirez-Macias, et al., 2012). This method has been used to study the mitochondrial metabolism and respiratory toxicity of drugs (El-On, et al., 2009, Williams, et al., 2003). We showed that the essential oil and TrROY derived from *T. riparia* probably promote *Leishmania* death through mitochondrial metabolism pathways.

TrEO at 5 µg/mL promoted 3.2% haemolysis in human erythrocytes (Fig. 2). TrROY exhibited low toxicity in human erythrocytes at 50 µg/mL (< 18% hemolysis) comparing with reference drug (AmB, 50 µg/mL, > 50% hemolysis) (Fig. 2). Gazim et al. (2014) showed that TrEO and TrROY were not cytotoxic to human melanoma, human nervous system and human colon cells line. Although TrROY was not toxic on human cells, we observed a cytotoxicity of this compound on murine macrophage (Table 1). TrROY CC50 was 0.53µg/mL, and the selectivity index (SI) and therapeutic SI (TSI) were 0.22 and0.03 , respectively. On the other hand, TrEO CC_{50} was 0.17 µg/mL resulting in 5.67 for SI and 0.34 for TSI. The TSI obtained with TrEO was ~ 11 folds higher concentration compared to observe with TrROY (Table 1). The cytotoxicity effects on murine macrophages can also be observed in Fig. 3.

TrEO at 30 ng/mL (IC_{50}) and 0.5 µg/mL (LD_{50}) inhibited 62% and 80% of *Leishmania* promastigote growth after 24 h, respectively. Both concentrations modified the morphology of *L. (L.) amazonensis* promastigote viewed by transmission electronic microscopy. The ultrastructural changes observed with the IC_{50} included intense cytoplasm vacuolization, membranous profiles inside the organelle, lipid vesicles, and membrane blebbing that suggested autophagy, thickening of the kinetoplast, chromatin condensation, and nuclear fragmentation (Fig. 4).

Nuclear fragmentation suggests apoptosis, and a vesicle that engulfs a part of the cytoplasm indicates autophagic cell death. The presence of multivesicular bodies and several vacuoles that have membrane profiles and cellular debris is related to the presence of secondary lysosomes and organelles that are likely involved in the degradation of damaged structures (Rodrigues and de Souza, 2008, Santos, et al., 2013). They may also correspond to the secretion of abnormal lipids that accumulate as a consequence of drug treatment and alterations in the physical properties of lipids and membranes, leading to the accumulation of

concentric membranes, and myelin-like structures. The lipid composition can induce irreversible changes in the structure and function of several organelles, leading to the appearance of autophagosomes to remove and recycle abnormal membrane structures and suggesting an intracellular remodeling process (Granthon, et al., 2007, Lira, et al., 2001). TrEO LD_{50} promoted the complete destruction of parasite membranes and other structures. These results indicate the high potential of this essential oil to induce *L. (L.) amazonensis* death.

Both TrEO and TrROY decreased the infection index of murine macrophages infected with *Leishmania* amastigote significantly. While the infection index 177 was obtained in macrophages infected and non-treated (positive control), TrEO index was 80 at 3 µg/mL, 73 (300 ng/mL) and 62 (30 ng/mL) reducing the infection in 55, 59 and 65%, respectively. TrROY infection index was 122 to 0.1µg/mL, 96 (1 µg/mL), 93 (10 µg/mL) and 105 (100 µg/mL) decreasing in 31, 46, 48 and 41%, respectively. Thus, TrEO at 30 ng/mL showed the most appropriate results because it has not presented toxicity into the cells and promoted promastigote and amastigote forms death.

TrEO showed the outcome and shows promise in leishmaniasis treatment. Recent research on essential oils has shown a successful approach to obtain new antileishmanial alternatives (Monzote, et al., 2007). Studies have showed the promising results of the essential oils from *Chenopodium ambrosioide* in BALB/c mice *Leishmania*-infected (Monzote, et al., 2009, Monzote, et al., 2010, Monzote, et al., 2014, Monzote, et al., 2007). The use of natural products could be an alternative to established cutaneous leishmaniasis therapy, but there are few studies showing the effectiveness of essential oils. Thus, TrEO and TrROY could be explored to develop a new alternative to leishmaniasis treatment. We suggest that *in vivo* studies should be performed to evaluate the therapeutic efficacy of essential oil and its compounds from *T. riparia* in cutaneous leishmaniasis.

Some medicinal plants have anti-inflammatory effects and can act on immune response, which can be demonstrated by reductions in iNOS expression and nitric oxide synthesis (Choudhari, et al., 2013, Jeong, et al., 2013). The *Leishmania* genus survives in macrophages, and cellular death is induced by reactive oxygen species, especially nitric oxide, which depends on iNOS expression (Amoo, et al., 2012, Oliveira, et al., 2014). In our study, iNOS mRNA was expressed by infected-macrophages after 3 and 6 h (Fig. 5 A), and the iNOS mRNA levels were higher in treated-macrophages than in infected-macrophages, but infected-macrophage treated with TrEO did not expressed iNOS mRNA (Fig. 5 B). After 24 h, iNOS mRNA was observed only in LPS-stimulated macrophages. The nitrite production was < 0.1 µM under all of the experimental conditions at 3 and 6 h. After 24 h, the nitrite level was 5 µM for LPS-stimulated macrophages, 1.8 µM to treated-macrophages, 1.7 µM to infected and treated-macrophages, while it was 0.8 µM to *Leishmania*-infected cells ($p < 0.001$) comparing with 1.5 µM to negative control (only macrophage). Thus, the infection reduced nitrite levels but enhanced iNOS mRNA expression in macrophages. These results is in concordance with studies that show the infection caused by *L. amazonensis* can decrease the production of nitric oxide as an escape mechanism of the immune host (Amoo, et al., 2012, Oliveira, et al., 2014). TrROY also did not change the iNOS mRNA expression and nitrite levels (1.8 µM) comparing with control.

This difference between iNOS mRNA expression and nitrite production shows that the parasite affected post-transcriptional nitric oxide synthesis events, which may result in parasite survival and escape from the immune response. Calegari et al. (Calegari-Silva, et al., 2009) reported that *L. amazonensis* has developed an adaptive strategy to escape from host defenses by activating the host transcriptional response, particularly nuclear factor κB, which in turn down regulates the expression of iNOS mRNA and favors the infection. In addition, *L. amazonensis* produces nitric oxide, which may lead to iNOS downregulation by macrophages

(Balestieri, et al., 2002, Genestra, et al., 2006). The nitric oxide (NO) synthesis required L-arginine, a crucial amino acid also used by *Leishmania* for polyamine-mediated parasite replication. Thus, *Leishmania* abducts L-arginine avoiding the NO production by macrophage, so other mechanisms are required to parasite killing as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitrogen species (NOx) and Th1 cytokines (Wanasen and Soong, 2008). In our study, *L. (L.) amazonensis* inhibited NO production, but TrEO and TrROY subverted it inducing normal levels. TrEO and TrROY increased iNOS mRNA expression, but they did not change nitrite production suggesting that TrEO did not act on iNOS and nitrite production during *Leishmania* infection, but TrROY and TrEO can modulate other immune mechanisms not investigated yet.

Previous studies have shown the anti-inflammatory effects of *T. riparia* extract on prostaglandins, cyclooxygenase 1 (COX 1), and cyclooxygenase 2 (COX 2), but its effect on iNOS has been unknown (Ndhlala, et al., 2011, Okem, et al., 2012). In leishmaniasis, this increase in iNOS may influence the nitric oxide production and promote elimination of the parasite. TrEO did not affect this mechanism but still acted against *Leishmania*. Other mechanisms of the immune response are involved in *Leishmania* death such as a balance of the cytokines of host immune response that could solve the curse of infection (Amoo, et al., 2012, Oliveira, et al., 2014). The main immunopathogenesis competencies of *L. amazonensis* are to carry the anergic diffuse cutaneous leishmaniasis at the T-cell hyposensitivity pole and with a higher T_H2-type immune response. This shift and imbalance in immunity leads to persistence of the disease and treatment failure (Silveira, et al., 2009). New drugs with antileishmanial and immunomodulation activity may be promising to treatment leishmaniasis, and TrEO can be one of this. Studies on the possible immunomodulatory activity of TrEO have been conducted by our group.

Despite the African and other population have used this plant for a variety diseases, the biological effects still remained to be investigated. We showed in vitro that the TrEO and TrROY have an ability to induce the *Leishmania* death by mitochondrial metabolism pathways, and induction of the secretion of abnormal lipids that accumulate as a consequence of drug treatment, and alterations in the physical properties of lipids and membranes. Although the isolated showed a high cytotoxicity in murine macrophages, the plant and the essential oil were not cytotoxicity against human cells. The cytotoxicity of TrROY must be considered before therapeutic application in leishmaniases. Other compound isolated from *T. riparia* may be investigated for the development of future leishmanicidal agents. For this, in vivo studies and other cytotoxicity assays must be conducted to ensure the safe use of this plant and its compounds to infections or inflammatory diseases. The isolation of antileishmanial compounds from the essential oil of *T. riparia* supports the traditional medicinal use of this plant as a treatment for parasitological infections as leishmaniasis.

The authors declare that they have no conflicting interests.

Acknowledgments

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

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FIGURES AND TABLES

Table 1. Antileishmanial and macrophage cytotoxicity effects of 6,7-dehydroroyleanone and the essential oil derived from *Tetradenia riparia*.

Treatment	Promastigote IC_{50}/24h (µg/ mL)	Promastigote LD_{50}/24h (µg/ mL)	Murine macrophage cytotoxicity CC_{50}/24h (µg/mL)	Selectivity index (SI)	Therapeutic Selectivity index (TSI)
6,7-dehydroroyleanone (TrROY)	2.45	16.9	0.53	0.22	0.03
T. riparia essential oil (TrEO)	0.03	0.5	0.17	5.67	0.34

IC_{50}= inhibitory concentration that reduces 50% parasite growth; LD_{50}= 50% lethal dose; CC_{50}= 50% cytotoxicity concentration; SI= CC_{50}/ IC_{50}; TSI= CC_{50}/ LD_{50}.

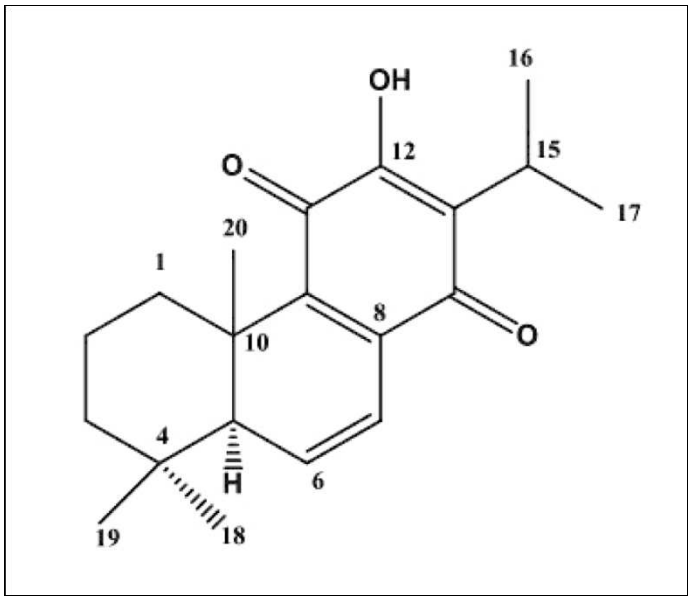


Fig.1. Structural formula of compound 6,7-dehydroroyleanone derived from *T. riparia* (TrROY).

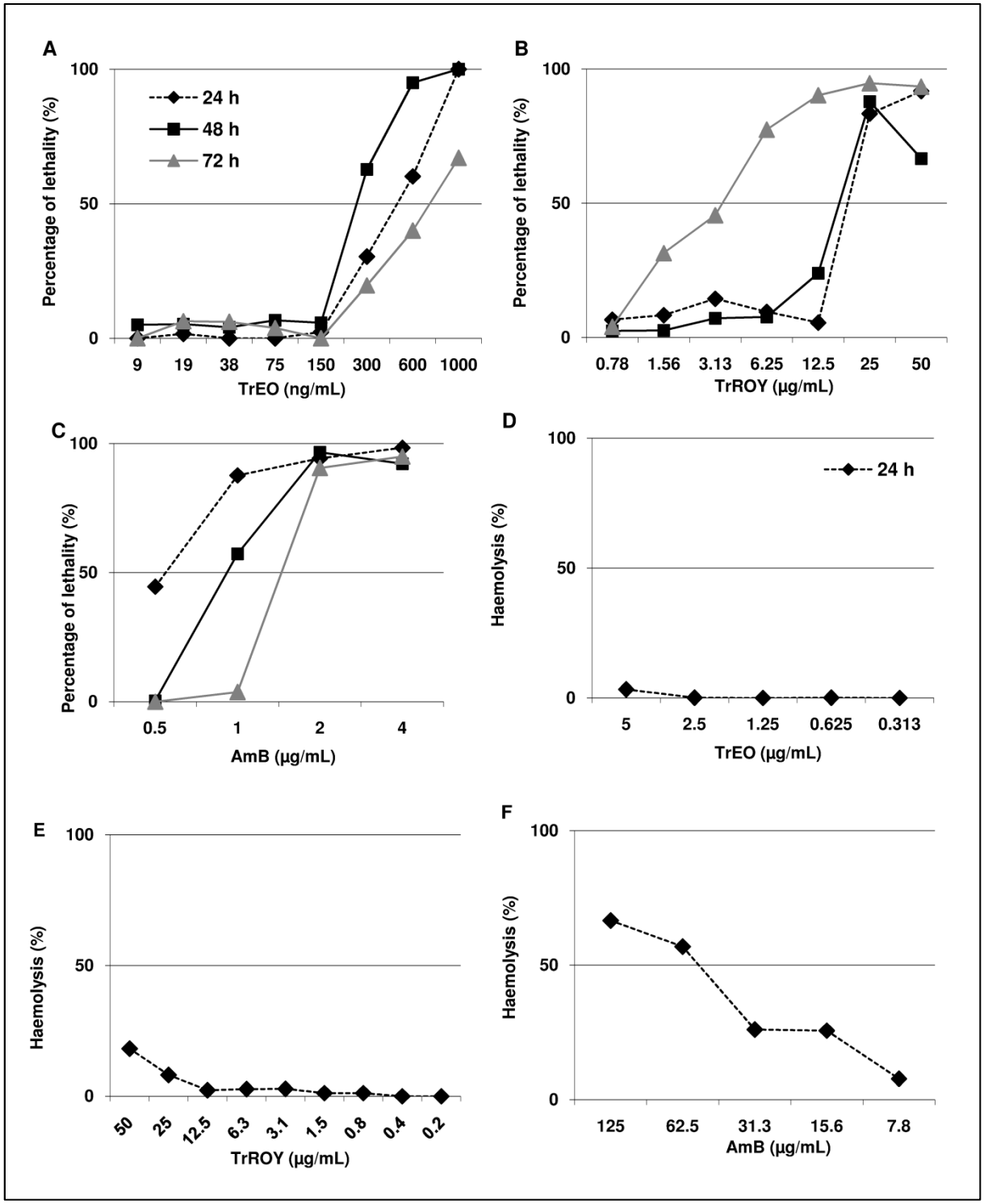
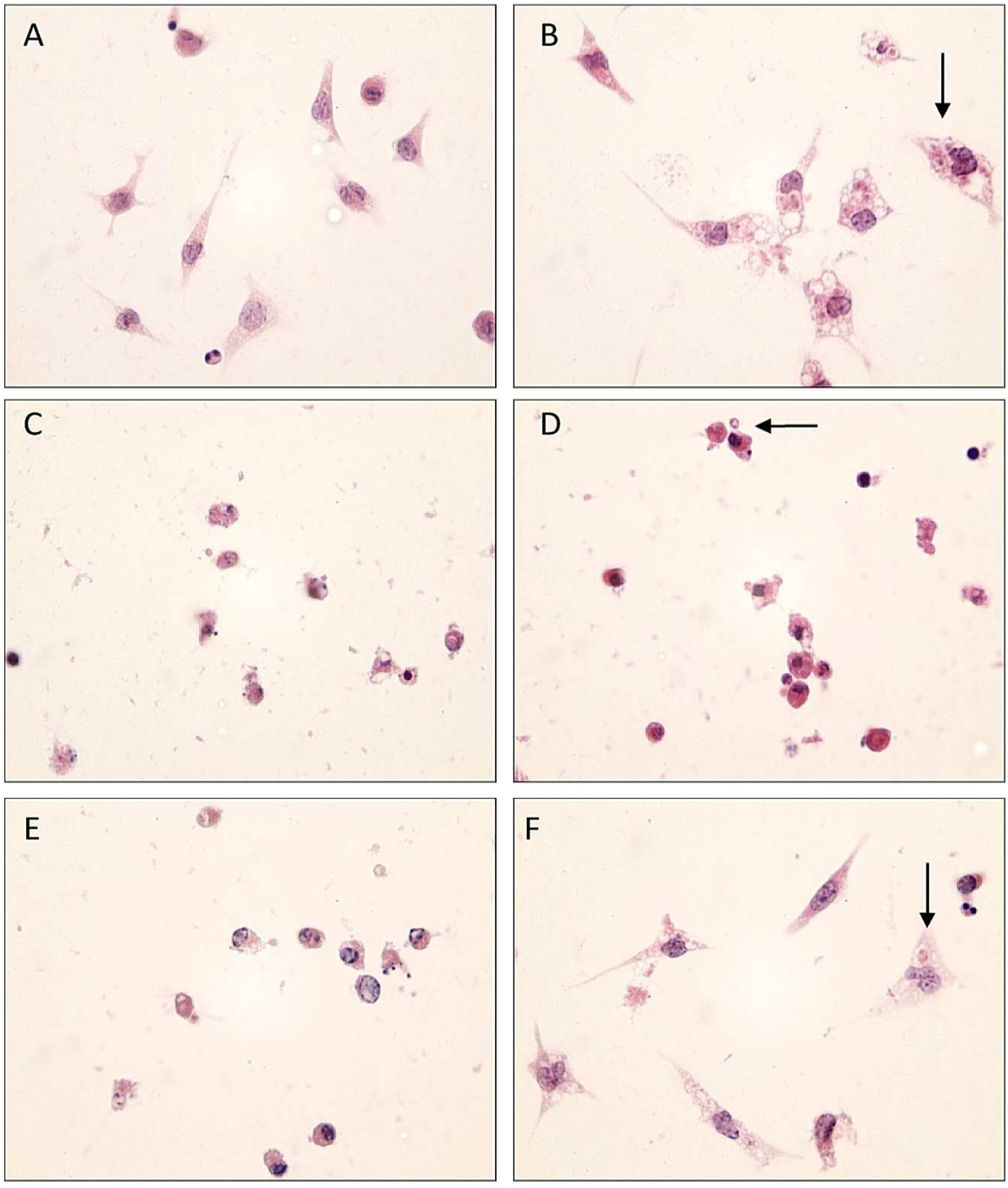


Fig. 2. Antileishmanial activity and haemolysis percentage of TrEO and TrROY. *Leishmania* promastigotes were incubated under the following conditions for 24, 48, and 72 h: (A) TrEO. (B) TrROY. (C) AmB (leishmaniasis reference drug). The lethality percentage was obtained using the XTT method. (D) Haemolysis percentage of TrEO (5.0-0.3 µg/mL) in erythrocytes. (E) Haemolysis percentage in erythrocytes treated with TrROY (50.0-0.2 µg/mL). (F) Haemolysis percentage of AmB (500-1 µg/mL) in erythrocytes. Each experiment was performed independently and in triplicate.



Condition tested	Infection Index (% of reduction)
(A) Macrophages non infected and non treated	-
(B) Macrophages infected with *L. (L.) amazonensis*	177
(C) Infected-macrophages treated with 100 µg/mL of TrROY	105 (41)
(D) Infected-macrophages treated with 0.1 µg/mL of TrROY	122 (31)
(E) Infected-macrophages treated with 3 µg/mL of TrEO	80 (55)
(F) Infected-macrophages treated with 30 ng/mL of TrEO	62 (65)

Fig. 3. Peritoneal macrophages of BALB/c mice infected and treated with TrEO and TrROY. The cells were stained with hematoxylin and eosin (100 x objective). Arrow indicates amastigote intracellular forms. All conditions were tested in duplicate and analyzed after 24 h of the incubation at 37°C with 5% CO_2.

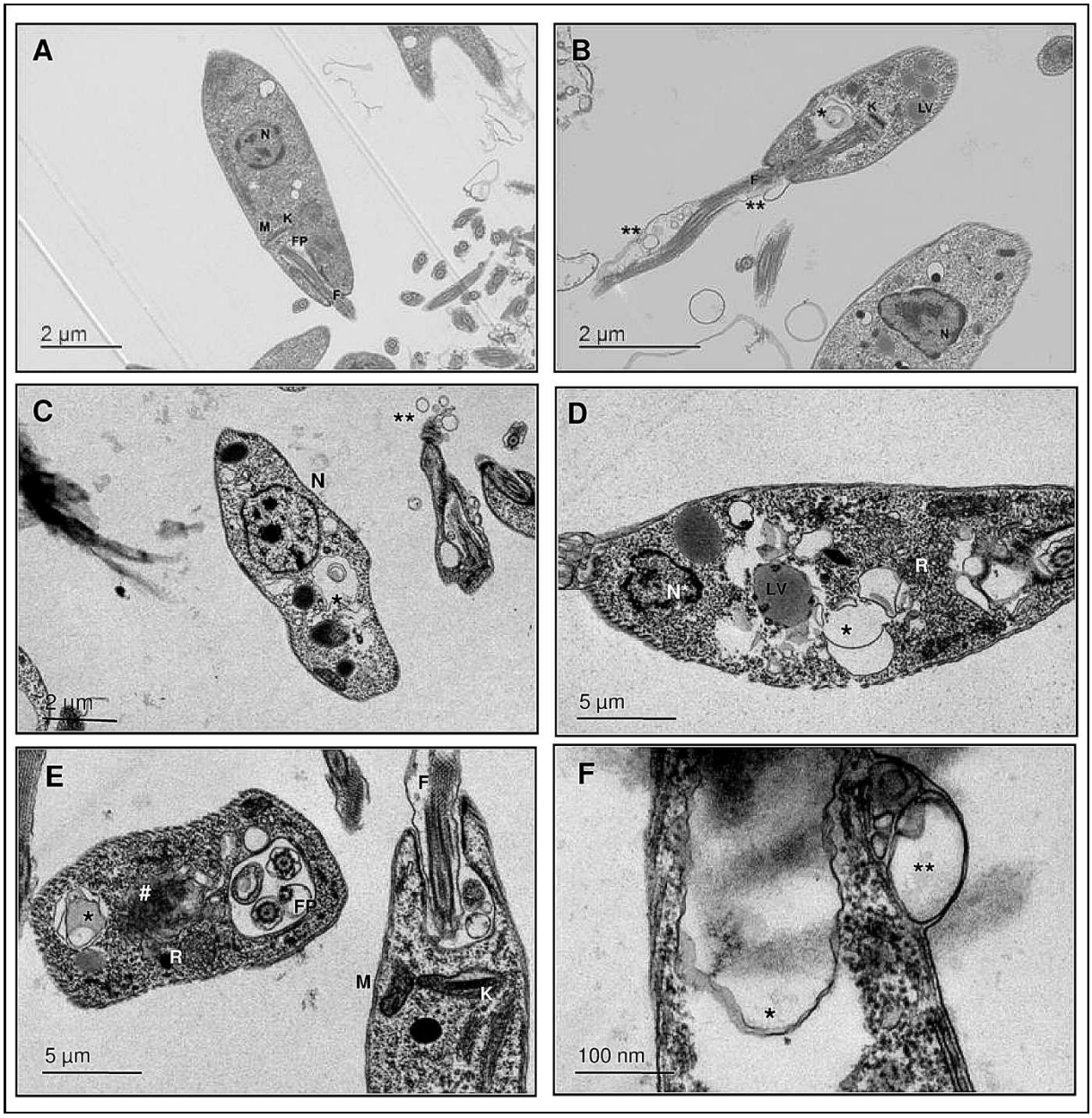


Fig. 4. Transmission electron microscopy of *L. (L.) amazonensis* treated with TrEO for 24 h. (A) *Leishmania* promastigotes. (B-F) Promastigotes treated with TrEO (30 ng/mL). N, nucleus; N*, abnormal chromatin condensation nuclear alterations; K, kinetoplast; M, mitochondria; FP, flagellar pocket; F, flagellum; V, vacuoles; LV, lipid vesicles; R, myelin-like figure appears in close association with the flagellar pocket membrane; *membranous profiles; **blebbing; #mitochondrial swelling.

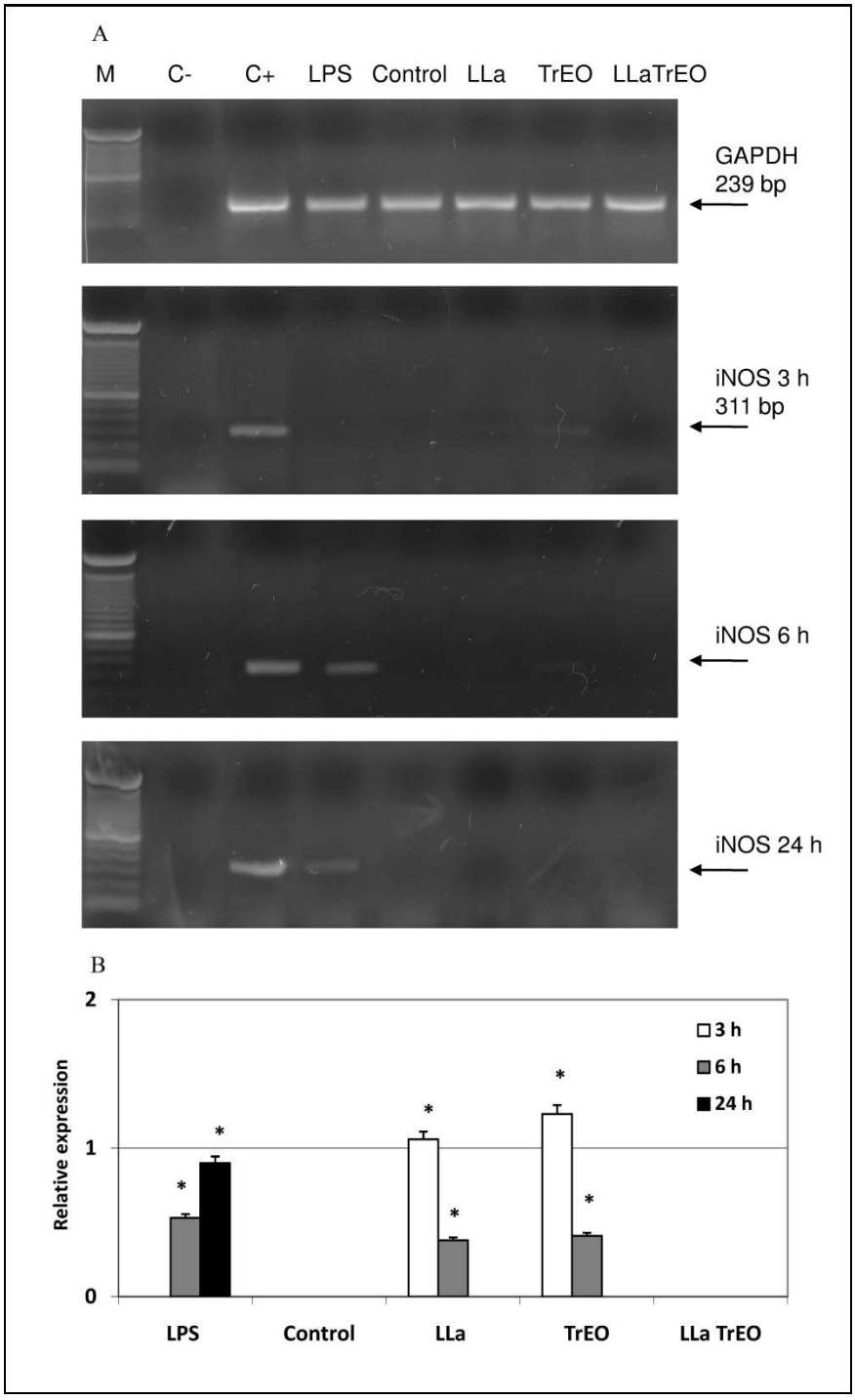


Fig. 5. iNOS mRNA expression using semi-quantitative RT-PCR. (A) DNA fragments were separated in 2% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator. (B) expression levels of iNOS mRNA were normalized to GAPDH as a housekeeping gene (internal control). M, molecular marker; C-, intern negative control (H_2O). C+, DNA obtained from *L. (L.) amazonensis* promastigotes cultured in 199 medium during 3 days. LPS, lipopolysaccharide-stimulated macrophages; Control, untreated and uninfected macrophages (negative control); LLa, macrophages infected with *L. (L.) amazonensis*; TrEO, macrophages treated with 30 ng/mL of TrEO; LLa TrEO, macrophages infected with *L. (L.) amazonensis* and treated with 30 ng/mL of TrEO. All of the conditions were analyzed with 3, 6, and 24 h incubation at 37°C. *$p < 0.05$.

Artigo 2

Immunomodulatory activity of essential oil from *Tetrania riparia* (Hochstetter) Codd in murine macrophages

Running title: Immunomodulatory activity of *Tetradenia riparia essential oil*

Izabel Galhardo Demarchi[1*], Mariana de Souza Terron[1], Mateus Vailant Thomazella[1], Raíssa B Pedroso[2], Zilda Cristiani Gazim[3], Diógenez Aparício Garcia Cortez[4], Sandra Mara Alessi Aristides[1], Thaís Gomes Verzignassi Silveira[1], Maria Valdrinez Campana Lonardoni[1].

[1] Departamento de Análises Clínicas e Biomedicina, Laboratório de Imunologia Clínica, Universidade Estadual de Maringá, Maringá, Paraná, Brazil.

[2] Pós-graduação em Biociências Aplicadas à Farmácia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil.

[3] Departamento de Farmácia, Laboratório de Química de Produtos Naturais da Universidade Paranaense, Umuarama, Paraná, Brazil.

[4] Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil.

*Corresponding author

Name: Izabel Galhardo Demarchi

Email: bel_galhardo@yahoo.com.br

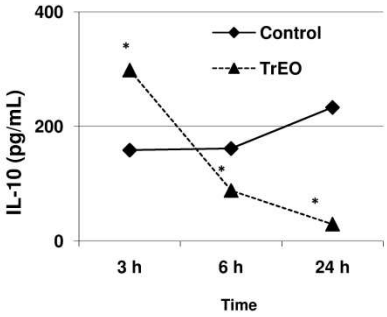
Affiliation: Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná State, Brazil

Telephone: 55 00 (xx44) 3261-4878/ fax numbers: 55 00 (xx44) 3261-4860

mail address: Av Colombo, 5790. Universidade Estadual de Maringá, Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC). CEP 87020-900 – Maringá – Paraná, Brazil

Graphical Table of Contents

TrEO induced proinflammatory cytokines that are associated with the innate cellular immune response. It may be an alternative therapy for several diseases, such as infectious diseases, that depend on the cellular immune response.



Immunomodulatory activity of essential oil from *Tetrania riparia* (Hochstetter) Codd in murine macrophages

Izabel G Demarchi, Mariana S Terron, Lilian Lopes, Mateus V Thomazella, Raíssa B Pedroso, Zilda C Gazim, Diogenes A G Cortez, Sandra M A Aristides, Thaís G V Silveira, Maria V C Lonardoni

Abstract*: Tetradenia riparia* is a plant that is used as a traditional medicine in South Africa to treat inflammatory and infectious diseases. The extracts and essential oil of this plant have shown antioxidant, anticarcinogenic, and antimicrobial properties. Some infections that resolve depend on the specific pathogens and host immune response balance, but only a few studies have screened the immunomodulatory effects of *T.riparia*. Thus, we studied the immunomodulatory effects of *T. riparia* essential oil (TrEO) on macrophages. Murine peritoneal macrophages were incubated with 30 ng/ml TrEO for 3, 6, and 24 h. Cytokine expression and production were determined by semiquantitative reverse-transcriptase polymerase chain reaction and flow cytometry, respectively. TrEO affected cytokine synthesis at all time points, whereas mRNA expression was altered only at 3 and 6 h. Interleukin (IL)-1β, IL-12, IL-17, and interferon-γ expression were highly induced by TrEO at 3 h. Only IL-1β expression was elevated at 6 h, and its production gradually increased until 24 h. IL-2 was produced at high levels in the initial response, and granulocyte monocyte colony stimulating factor and IL-17 were produced at high levels at 24 h. TrEO inhibited IL-10. These results indicate that TrEO can modulate proinflammatory cytokines and downregulate IL-10 and IL-6. This profile is associated with stimulation of the innate cellular immune response and T_H2 cell suppression. The essential oil from *T. riparia* may be an alternative therapy for carcinogenic, autoimmune, and infectious diseases in which cellular responses are critical for their resolution.

Keywords: essential oil; *Tetradenia riparia*; protective agents; cytokines; immune response

Introduction

The *Tetradenia riparia* (Hochstetter) Codd plant belongs to the Lamiaceae family. It is also known as *Iboza riparia* N.E. BR and *Moschosma riparium*. In South Africa, it is used as a traditional medicine for the treatment of inflammatory and infectious diseases. The plant is an herbaceous shrub that is found throughout tropical Africa and other regions of the world, including China, India, and Brazil. In these countries, it is known as false myrrh, lemon verbena, lavandula, and misty plume and used mainly for incense and as an ornamental plant [1-4]. Several infections, such as malaria, cryptococcosis, candidiasis, gastroenteritis, respiratory infections, and inflammatory disease, have been treated by the leaves and essential oil from *T. riparia* [3, 5-9].

The use of natural products worldwide is as old as human life itself, and its popularity has increased. Plants possess diverse metabolites that display a vast array of pharmacological activity [10], with the potential to be used against infections, inflammation, autoimmune diseases, and carcinogenic processes [7, 11, 12]. Additionally, natural products are cheaper, accessible, and sustainable and have a lower likelihood of causing serious adverse effects [10]. The extracts and essential oils of many plants have been screened for their antioxidant, antimicrobial, and immunomodulatory effects. They can also be used to resolve infectious diseases, neoplasms, and other illnesses [2, 13-15]. Some plants have immunomodulatory actions. Studies have reported the immunomodulatory effects of plants on macrophages, natural killer (NK) cells, dendritic cells, and T and B lymphocytes. Different infections require regulation of the immune response, in which cytokines play a central role. Furthermore, variations in cytokine expression and production are usually associated with immune-mediated diseases or inflammatory disorders [16].

Herbal medicines may be used as a strategy to modulate the secretion of cytokines, offering a novel approach for the treatment of different diseases [17]. We recently reported

the antileishmanial activity of essential oil from *T. riparia* (TrEO) against *Leishmania (Leishmania) amazonensis*. TrEO also induced the high mRNA expression of inducible nitric oxide synthase (iNOS) and normalized nitric oxide (NO) levels, thus subverting a downstream effect of *Leishmania* infection [18, 19]. Nevertheless, *T. riparia* is a traditional herbal medicine, and its biological and pharmacological activities have been poorly studied. Previous studies have reported the antiinflammatory effects of *T. riparia* extract on cyclooxygenase 1 (COX-1) and COX-2 [7, 12], but other protective mechanisms of derivatives of *T. riparia* remain to be investigated. Thus, we studied the immunomodulatory effects of TrEO in murine macrophages to elucidate its possible protective effects in the host.

Experimental

Obtaining T. riparia essential oil

T. riparia leaves were collected monthly between September 2006 and August 2007 in Umuarama, Paraná, Brazil (23º46’22”S and 53º16’73”W, 391 m). The plant was identified by Prof. Ezilda Jacomasi of the Department of Pharmacy, Universidade Paranaense (UNIPAR). A voucher specimen was deposited in the UNIPAR Herbarium (code no. 2502). The essential oil was extracted from the fresh leaves of *T. riparia* that were cultivated in the summer of 2006 and 2007 and obtained by hydrodistillation using a Clevenger type apparatus (3h). The mean maximum and minimum temperature, precipitation, and relative humidity by season from September 2006 to August 2007 were reported by Gazim et al. [20]

GC-MS and GC-FID analysis

The GC-MS analyses were performed using an Agilent 5973N GC-MS System operating in EI mode (electron ionization), equipped with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm, Agilent, Palo Alto, CA, USA) was used to inject 1 μl of a solution sample. The

initial temperature of the column was 80°C. The column was heated gradually to 260°C with a 4°C/min rate. The injector (splitless, 0.5 min), and transfer line temperature were held at 260 and 280°C, respectively. He (1.0 ml/min) was used as the carrier gas. Together with the sample, *n*-nonadecane was added as an internal standard. The same temperature program was used for GC-FID. The identification of the compounds was based on comparison of their retention indices (RI) [13] obtained using various *n*-alkanes (C7 - C25). Also, their EI-mass spectra were compared with the Wiley Library spectra and the literature [21, 22].

Macrophage cytotoxicity

Resident peritoneal macrophages were obtained from BALB/c mice in accordance with the Ethics Committee on the Use of Experimental Animals of the Universidade Estadual de Maringá, Paraná, Brazil (warrant no. 133/2012). The mice (30-40 days old) were euthanized by 40% CO_2 inhalation in a chamber at a moderate fill rate (AVMA Guidelines on Euthanasia, 2007). The peritoneal cavity was washed with 8 ml sterile RPMI 1640 medium. The cell suspension was adjusted to 1×10^6 macrophages/ml. Next, 100 µl was plated in 96-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37°C and 5% CO_2, and non-adherent cells were removed by washing with sterile phosphate-buffered saline (PBS). TrEO was diluted from 3 µg/ml to 30 ng/ml. The essential oil was diluted in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), the concentration of which did not exceed 0.005% v/v. This concentration of DMSO was previously shown not to have cytotoxic effects on macrophages. Untreated cultures were used as a viability control. The plates were maintained at 37°C in a humid atmosphere that contained 5% CO_2 for 24 h. Cytotoxicity in macrophages was determined using colorimetric cell viability XTT solution (2,3-bis[2-methyloxy-4- nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma Chemical Co, St. Louis, MO) [23, 24]. The XTT solution (500 µg/ml) was activated with 50

µg/ml of phenazine methosulfate (PMS; Sigma Co Chemical, St. Louis, USA) and added to the cell monolayer of each well. After 3-5 h incubation at 37°C with 5% CO_2while protected from light, the XTT reduction caused by *Leishmania* viability produces a formazan compound that was measured at a wavelength of 450/650 nm using a spectrophotometer plate reader (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The Trypan Blue test was also performed to verify cytotoxicity in macrophages. From the macrophage suspension, 500 µl was distributed on sterile 13 mm diameter glass coverslips (Glastecnica, São Paulo, SP, Brazil) and placed in 24-well culture plates (TPP, Switzerland). The plates were incubated for 1 h at 25°C, and non-adherent cells were removed by three washes with sterile PBS. The cultures were treated with TrEO at 3 µg/ml to 30 ng/ml and incubated at 37°C in a humid atmosphere that contained 5% CO_2 for 24 h. The coverslips were then stained with 1% Trypan Blue (Sigma-Aldrich, Saint Louis, MO, USA) and examined by microscopy. All of the tests were performed in duplicate, and the results are expressed as percent viability. The cytotoxicity concentration (CC_{50}) was defined as the dose of the compound that reduced the survival of the macrophages by 50% compared with untreated macrophages (viability control).

Immunomodulatory assay

Resident peritoneal macrophages were obtaining from BALB/c mice (sacrificed as mentioned above). Briefly, the peritoneal cavity was washed with 5-8 ml sterile RPMI 1640 culture medium. The cell suspension was adjusted to 1×10^6 macrophages/ml, and 1 ml of the suspension was added to each well of 12-well culture plates (TPP, Switzerland). The plates were incubated for 1 h at 37°C in a humid atmosphere that contained 5% CO_2, and non-adherent cells were removed by three washes with sterile PBS. Adherent cells were incubated in RPMI 1640 culture medium. After a few minutes, the macrophages were

subjected to the following conditions: (*i*) untreated macrophages and (*ii*) macrophages treated with 30 ng/ml TrEO diluted in DMSO (1:2) and RPMI 1640 medium. The maximum DMSO concentration did not exceed 0.01% and did not cause cytotoxicity in murine macrophages. The plates were maintained at 37°C in a humid atmosphere that contained 5% CO_2 for 3, 6, and 24 h. After these times, the supernatant was removed and stored at -80°C for the cytokine quantification assay. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added on adhered cells to extract nucleic acids and stored at -80°C.

Cytokine mRNA expression

Cytokine mRNA expression analyses were performed using semi quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). For this reaction, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using 1 µg of total RNA as a template in a reverse transcription reaction (Superscript III reverse transcriptase, Invitrogen, Carlsbad, CA, USA). The purity level and amount of material extracted were determined using a NANODROP 2000 UV-V is spectrophotometer (Thermo Fisher Scientific Inc, USA), considering an optimal purity level of $\geq$ 1.8. The standard PCR conditions are shown in Table 1. Primers were chosen according to the BLAST tool that is available in the GenBank database and previous publications [25-28]. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA of *Mus musculus* was used as an internal control. The positive internal control was DNA from macrophages that were stimulated with 5 µg/ml of lipopolysaccharide for 4 h (LPS, Sigma-Aldrich, St. Louis, MO, USA). The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis and revealed with an ethidium bromide transilluminator. Semi quantitative RT-PCR was performed by quantifying the band densitometry using ImageJ software (National Institutes of Health,

USA). Each gene was normalized to GAPDH as a housekeeping gene control (internal control) [29].

Cytokine quantification

The supernatants were centrifuged, and 50 µl of the sample was used for cytokine quantification (pg/ml) by flow cytometry based on streptavidin-biotin enzyme reaction. The multiplex Mouse Cytokine 10-Plex Panel kit and Mouse IL-17 Singleplex Bead Kit (Invitrogen, Carlsbad, CA, USA) were assayed in all samples. The determination was performed using a Luminex 200 System flow cytometer and xPONENT 3.1 software.

Statistical analysis

The *in vitro* assays were performed in duplicate. The statistical analysis was performed using the $\chi 2$ test, one-way analysis of variance (ANOVA), and the Tukey test. The analyses were conducted using Statistic 7 software. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

In the present study, TrEO was screened for cytokine modulation in murine macrophages (Fig. 1-3). Cytokines are soluble extracellular proteins or glycoproteins that are key intercellular regulators and mobilizers. Based on structural homology, their receptors are classified in family groups (e.g., interleukins, interferons, and chemokines). These proteins are critical for innate and adaptive immune responses, cell growth and differentiation, cell death, angiogenesis, and repair processes [30]. The extracts and essential oils of many plants have been screened for their immunomodulatory activity. Herbal medicines are one strategy

to modulate cytokine secretion, offering a novel approach for the treatment of different diseases [17].

Before conducting the immunomodulatory assay, we evaluated the cellular viability of TrEO in murine macrophages using two methods. All of the concentrations tested maintained approximately 90% cell viability according to the Trypan Blue method, which was similar to untreated macrophages (96% viability). The CC_{50} of TrEO was 165 ng/ml according to the XTT method, and high viability (97%) was maintained at 30 ng/ml (data not shown). Because of the high cell viability at 30 ng/ml, we chose this concentration to test the immunomodulatory effect of TrEO on macrophages.

At a low concentration, TrEO modulated the mRNA expression and production of cytokines associated with a cellular immune response. This response has been shown to protect against different infectious and some non-infectious diseases [31]. TrEO stimulated high proinflammatory cytokine expression in macrophages, the effects of which varied according to the incubation time. After 3 h , the mRNA expression of IL-1β, IL-12, IL-17, IFN-γ and also IL-10 (anti-inflammatory cytokine) was detected, but only IL-1β, IL-10 and IL-12 mRNA expression was high after 6 h. Tumor necrosis factor α (TNF-α), IL-18, and IL-33 mRNA expression was not modified at 3 or 6 h (Fig. 1-3). The gene expression profile that was promoted by TrEO was maintained for 24 h. However, TrEO modulated the synthesis of cytokines until 24 h.

TrEO increased IL-1β synthesis (Fig. 4), and gradually decreased IL-10 synthesis (Fig. 5) until 24 h. IL-6 levels increased at 3h, decreased at 6 h, and normalized after 24 h. TrEO induced proliferative cytokine production by first inducing IL-2 productionat 3 h and then inducing granulocyte macrophage colony-stimulating factor (GM-CSF) production at 24 h (Fig. 4). IL-17 production was high after 24 h. TrEO did not modify IL-12 (Fig. 4), TNF-α, or IL-5 production (Fig. 5). Murine macrophages that were treated with TrEO further

produced and expressed proinflammatory cytokines. All of these mediators stimulate the activation and differentiation of cells that are critical for the immune response by eliminating pathogenic microorganisms and abnormal cells. Macrophages are present in the natural immune response and have a primary function of engulfing foreign agents that enter the body. Their other functions include the elimination of pathogens, tumor cells, and other antigenic stimuli. Many studies have been conducted to discover therapeutic strategies to stimulate or inhibit cytokines that promote the activation or inhibition of these cells to control and resolve various diseases [32].

Essential oils of plants as those from the Lamiaceae family can stimulate cytokines that are associated with cellular immune responses to protect against intracellular pathogens, cancer, and autoimmune disorders. Antimicrobial, antioxidant, and acaricidal effects have been attributed to TrEO [2, 7, 12], but its immunomodulatory effects on cytokines have not been previously investigated. Our results showed that TrEO is a potent activator of cytokines that are important in the proliferation, activation, and differentiation of effector immune cells, such as macrophages.

TrEO modified IL-1β production and expression in macrophages. This interleukin is synthesized as a precursor and cleaved by inflammasome-activated caspase-1. Caspase activation triggers the autophagy process, which plays an important role in the release of this cytokine. This pathway leads to the elimination of abnormal cells and pathogens. IL-1β is a chemoattractant for granulocytes. It can increase the expansion and differentiation of lymphocytes and enhance the expression of cell adhesion molecules in leukocytes and endothelial cells [33]. IL-1β also appears to be important for the early immune response to infections that are caused by several pathogens, such as bacteria, protozoa, virus, and fungi [34-36]. In the present study, TrEO significantly induced IL-1β, which may contribute to the treatment of some infectious diseases.

IFN-γ and IL-12 are proinflammatory cytokines that are produced early by macrophages in antigenic responses. Antimicrobial death can be activated by IFN-γ production, which is the most potent macrophage activator that produces reactive oxygen species (ROS) and NO to promote the elimination of pathogens and cell death. Nitric oxide synthesis is regulated by iNOS [37]. In a previous study, we observed high iNOS mRNA expression after TrEO treatment and normal levels of NO in macrophages that were infected with *Leishmania* and uninfected cells that were treated with 30 ng/ml of TrEO. This parasite induces lower NO production by macrophages, thus contributing to persistent infection [19]. TrEO was able to subvert the downstream decrease in NO production that is promoted by the parasite [19]. Another important cytokine is IL-12, which promotes cell-mediated immunity via the stimulation of T_H1 lymphocytes to control inflammation. IL-12-deficient mice were shown to be susceptible to infectious intracellular pathogens, such as mycobacteria [39]. IL-12 is a potent inducer of INF-γ by NK cells. Through the activation of macrophages, NK cells, T_H1 cells, and IL-12 production are crucial for effective cancer treatment. The addition of exogenous IL-12 has been considered a therapeutic option for the treatment of tumors and inflammatory conditions [39, 40]. Considering that INF-γ and IL-12 are induced by TrEO, it may be considered a viable option for the treatment of these diseases.

IL-17 is a proinflammatory cytokine that is produced by T_H17 lymphocytes, mainly macrophages and other cells that regulate the innate immune response. Although IL-17 levels were low (<5 pg/ml) in all conditions, IL-17 was significantly expressed at the initial time of incubation and the production at 24 h. T_H17 cells are stimulated by IL-6, IL-1, and TNF that are secreted by CD4+ T cells in adaptive immune responses. These cytokines also stimulate antimicrobial gene expression in neutrophils. IL-17 production promotes neutrophil recruitment and can promote inflammation and local tissue damage at high concentrations [41]. IL-17-deficient mice are highly susceptible to extracellular pathogens (e.g., *Klebsiella*

pneumonia and *Candida albicans*) [42, 43]. In the present study, TrEO stimulated IL-17, a proinflammatory cytokine that is important for the control of fungi and extracellular infections.

Moreover, TrEO induced proliferative cytokine production by macrophages, including IL-2 at 3 h. This interleukin has been described as a growth factor that is importantly involved in T-cell activation *in vitro*, and it promotes CD8+ T-cell proliferation and differentiation *in vivo*. IL-2 is a key mediator of apoptosis in peripheral autoreactive T cells, which play an essential role in maintaining self-tolerance by CD4+CD25+ T regulatory (T reg) cells. Furthermore, some studies have indicated that IL-2 is the main inducer of suppressive T reg cell production, suggesting that IL-2 could be used therapeutically to treat several diseases, such as autoimmune diseases, neoplasias, and infectious diseases [44, 45]. Moreover, IL-2 is essential for stimulating NK cell growth and differentiation to eliminate tumor cells. IL-2 has been used for the treatment of melanoma and renal carcinoma. Finally, IL-2 can also modulate the immune response against infectious agents, such as hepatitis C virus, human immunodeficiency virus, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, and *Listeria monocytogenes* [46, 47].

In the present study, at the beginning of incubation with TrEO, IL-2 was produced by macrophages, whereas GM-CSF was secreted only after 24 h. GM-CSF plays relatively non-overlapping roles in the differentiation and maintenance of specific macrophage subsets that induce the M1 phenotype (classically activated macrophages). In the initial phase of the immune response, M1 has important functions, such as killing pathogens and inducing the synthesis of proinflammatory cytokines. GM-CSF can be a significant determinant of the magnitude and duration of both acute and chronic inflammatory pathology. GM-CSF can contribute to the mobilization of monocytes and activation of neutrophils [48, 49]. High levels of GM-CSF have been associated with the exacerbation of arthritis, autoimmune

encephalomyelitis, atherosclerosis, nephritis, lung inflammation, and cancer. GM-CSF is involved in disease mechanisms and could be a viable target for arthritis and cancer therapy [49, 50]. Thus, TrEO may also contribute to the treatment of infectious diseases and other conditions in which GM-CSF production is excessively high.

We found that IL-10 gradually decreased over time. This cytokine induces the differentiation and activation of T_H2 and T reg lymphocytes. This action can consequently inhibit or delay the proliferation, differentiation, and function of other T cell subpopulations [51]. The innate immune response down regulates effectors mechanisms and restores homeostasis in injured tissue via IL-10 and other cytokine families, mainly those that are released from macrophages, particularly the M2 subset (alternative activated macrophages) that is activated in the later phase of the response. T reg cells that are mediated by IL-10 play an important role in controlling infections that are caused by protozoa and helminthes. However, the excessive production of IL-10 can lead to high susceptibility to parasite infections [52, 53]. IL-10 also acts in autoimmune diseases, allergies [54], and neoplasms [55], inhibits the production of proinflammatory cytokines, and induces tissue healing by regulating extracellular matrix protein deposition and angiogenesis. These mechanisms suggest that IL-10 may be therapeutically beneficial [56]. TrEO can be a potential inhibitor of IL-10 and contribute to the treatment of parasitic infections in which the IL-10-mediated immune response is critical.

In the present study, IL-6 was produced at high levels only at 3 h. IL-6 is synthesized early in response to infectious agents that act on T_H17 activation and inhibit T reg cells. An increase in IL-6 secretion can be augmented by the influx of neutrophils and inflammatory monocytes to the site of infection. Additionally, both of these cell types have been shown to be targets of infection. IL-6 is also produced in the early stages of inflammation, and it is central in driving the acute-phase response. Some studies suggest that IL-6 may amplify the

inflammatory response to infections and can contribute to exacerbation of a “cytokine storm” in cases of inflammatory diseases [57]. These results indicate that TrEO can induce the production of proinflammatory cytokines, mainly those that are involved in infection.

TrEO induced the production of proinflammatory cytokine that are involved in effector cell activation, and the molecules that directly promote inflammation as acetyl cholinesterase (AChE), COX-1, and COX-2 were suppressed by TrEO [21]. Thus, TrEO modulated cytokines activation of T_H1 response and suppress T_H2 cytokines, which can explain the inhibition of the inflammatory process as COX and AChE routes. A previous study supported this possibility. In other study, TrEO was administered orally in mice and shown to have excellent analgesic effects, in which it inhibited abdominal constrictions that were induced by acetic acid [21]. Animal and human studies have shown that COX-2 is related to the pain and inflammation associated with osteoarthritis and rheumatoid arthritis. COX-1 is a constitutive enzyme with homeostatic functions, unlike COX-2 [7, 9, 12].

Epidemiological, animal, and *in vitro* studies have shown that COX-2 plays a role in the development of colon cancer. COX-2 promotes the development and progression of gastric cancer, likely through stimulation of the proliferation of gastric cancer cells, inhibition of apoptosis, and promotion of angiogenesis and lymphatic metastasis. It has also been shown to participate in cancer invasion and immunosuppression [65, 66]. *T. riparia* was reported to reduce COX-2, this enzyme is inducible in treating inflamed tissue, resulting in less gastric irritation compared with COX-1 inhibitors and decreasing the risk of gastric ulceration and spasmodic effects. All together, these findings support the use of *T. riparia* for the treatment of gastric diseases and inflammatory conditions. The aqueous extracts of this plant showed good inhibitory activity against COX enzymes. Plants with good antiinflammatory activity, particularly against COX-2, are required to determine the inhibitory potential against other proinflammatory mediators that induce the production of inducible nitric oxygen species,

proinflammatory cytokines, and inducible COX [67]. In the present study, TrEO induced protective cytokines that are associated with the cellular immune response, modulated proinflammatory cytokines, and downregulated IL-10 and IL-6. We previously showed that TrEO did not modulate the production of inducible nitric oxygen species [19].

In popular medicine, *T. riparia* leaves are used to treat respiratory ailments (e.g., coughs, colds, and sore throat), mouth ulcers, stomachaches, diarrhea, influenza, fever, malaria, and headaches [12]. Although some biological effects of some of the constituents of TrEO are known, the effects of other constituents and their interactions on multiple biochemical processes of cells and microorganisms remain unknown. 8(14),15-Sandaracopimaradiene-7α,18-diol has been isolated from *T. riparia* and shown to have significant antimicrobial activity against several bacteria and fungi [5]. Campbell et al. reported moderate antimalarial activity against two strains of *Plasmodium falciparum* [6]. We recently showed that TrEO promotes the death of *Leishmania amazonensis* and *L. braziliensis in vitro* [18] and induces iNOS mRNA expression but does not act on NO production by murine macrophages [19].

The immunomodulation of TrEO can be attributed to its complex mixture of biologically active components. TrEO is a complex mixture of terpenoids, including monoterpenes, sesquiterpenes, and diterpenes (hydrocarbons or oxygenated) [20]. In our study, the essential oil obtained in the summer season was detected 52 compounds , but 47 was identified (Table 2). The major class was oxygenated sesquiterpenes (38.85%) followed by oxygenated diterpenes (24.67%). The major constituents found in the oil was α-cadinol (14.38%); 14-Hydroxy-9-epi-caryophyllene (12.14%) both sesquiterpenes compounds. In the oxygenated diterpenes class was found the presence of two compounds majority: 9β,13β-epoxy-7-abietene (7.37%) and 6,7-dehydroroyleanone (14.89%). These compounds were

identified by NMR spectra and evaluated for cytotoxic, antioxidant and analgesic activity [15, 20].

Most of the antimicrobial activity of essential oils is attributable to oxygenated terpenoids and to a lesser extent hydrocarbon [2, 15]. Their interactions can have antagonistic, additive, or synergistic effects. Interactions between the compounds of essential oils can be investigated using macro/microdilution techniques, such as the CheckerBoard, graphical, and time-kill methods [11]. Such investigations could elucidate the mechanisms of action of the cellular and microbicidal effects of TrEO.

Considering the potential biological effects of oxygenated terpenoids, Gazim et al. [15] isolated a new natural diterpene-type abietane (9β,13β-epoxy-7-abietene) from TrEO and reported cytotoxic effects and no antioxidant activity. The diterpene-type abietane and TrEO inhibited the viability of the tumoral cell lines SF-295 and HCT-8 by more than 70% and the human melanoma cell line MDA-MB-435 by more than 55%. This indicates the high cytotoxicity potential of this essential oil and fraction against these tumor cell lines. Antioxidant activity has been reported for another diterpene, 6-7-dehydroroyleanone, with no cellular toxicity [15]. Thus, TrEO and its isolated compounds appear to be promising antimicrobial and anticarcinogenicagents. Nonetheless, futures studies need to be conducted to better understand the mechanisms and applicability of TrEO for disease treatment.

The present results provide further information on commercial essential oils that may be used by policymakers to develop regulations regarding the manufacture and validation of natural products. TrEO induced proinflammatory cytokines that are associated with the innate cellular immune response. It may be an alternative therapy for several diseases, such as infectious diseases, that depend on the cellular immune response. TrEO suppressed IL-10 and increased IFN-γ. *T. riparia* may represent an important component of folk medicine for modulating the immune response and treating infections. Its biological and pharmacological

effects must be studied *in vivo* and in humans to ensure its safety and establish treatment protocols to guide the selection of appropriate therapeutic regimens for specific clinical conditions.

The authors declare that they have no conflicting interests.

Acknowledgments

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

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Table 1. Set of specific genes primer.

Gene	Primer sequence		Product size (bp)	Conditions for PCR reaction (cycles)
IL-10 [25]	F1 R2	5'-TAC CTG GTA GAA GTG ATG CC-3' 5'- CAT CAT GTA TGC TTC TAT GC-3'	252	93 °C for 55 s, 61 °C for 45 s, 72 °C for 40 s (36)
IL-12 [25]	F1 R2	5'-CGTGCTCATGGCTGGTGCAAAG-3' 5' CTTCATCTGCAA GTTCTTGGGC-3'	315	93 °C for 55 s, 61 °C for 45 s, 72 °C for 40 s (36)
IL-1β [26]	F1 R2	5'-TTGACGGACCCCAAAAGATG-3' 5'-AGA AGGTGCTCATGTCCTCA-3'	204	95 °C for 20 s, 57 °C for 25 s, 72 °C for 45 s (30)
IL-33 [27]	F1 R2	5'-ATGGGTACCTTCCTCGCCATG-3' 5'-GAACGCACAGGCGTTTTACT-3'	187	95 °C for 15 s, 60 °C for 60 s, 72 °C for 45 s (35)
IL-17 [28]	F1 R2	5'-ACCGCAATGAAGACCCTGATAG-3' 5'-TTCCCTCCGCATTGACACAG-3'	84	95 °C for 30 s, 60 °C for 60 s, 72 °C for 30 s (35)
IL-18 [28]	F1 R2	5'-ACTGTACAACCGGAGTAATACGG3' 5'-AGTGAACATTACAGATTTATCCC-3'	440	95 °C for 30 s, 60 °C for 60 s, 72 °C for 30 s (36)
IL-4 [28]	F1 R2	5'-GGAGATGGATGTGCCAAACG-3' 5'-GCACCTTGGAAGCCCTACAG-3'	80	93 °C for 45 s, 55 °C for 45 s, 72 °C for 30 s (35)
TNF-α [25]	F1 R2	5'-ATGAGCACAGAAAGCATGATC-3' 5'-TACAGGCTTGTCACTCGAATT-3'	276	93 °C for 55 s, 61 °C for 45 s, 72 °C for 40 s (36)
IFN-γ [26]	F1 R2	5'-TGGAGGAACTGGCAAAAGGATGGT-3' 5'-TTGGGACAA TCTCTTCCCCAC -3'	336	95 °C for 20 s, 56 °C for 25 s, 72 °C for 45 s (35)
GAPDH [27]	F1 R2	5'-CCACCATGGAGAAGGCTGGGGCTC-3' 5'-AGTGATGGCATGGACTGTGGTCAT-3'	239	95 °C for 15 s, 60 °C for 60 s, 72 °C for 45 s (35)

F1: forward; R2: reverse; IL: interleukin; TNF: tumor necrosis factor, IFN: interferon, GAPDH: Glyceraldehyde-3 phosphate dehydrogenase.

Table 2. Chemical composition of essential oil obtained from leaves of *Tetradenia riparia*.

Peak	[a]Compound	[b]RI	Area %	Methods of Identification
	Monoterpene Hydrocarbons			
1	α-pinene	927	0.46	b, c
2	Limonene	1000	0.59	b, c
3	*Trans*-β-ocimene	1048	t	b, c
4	n.i	1076	t	b, c
5	n.i	1086	t	b, c
6	n.i	1095	t	b, c
	Oxygenated Monoterpenes			
7	Fenchone	1096	4.19	b, c
8	*Endo*-fenchol	1112	5.96	b, c
9	*Exo*-fenchol	1123	0.49	b, c
10	Camphor	1133	0.82	b, c
11	Borneol	1154	0.84	b, c
12	Terpinen-4-ol	1169	1.29	b, c
13	α-terpineol	1183	0.42	b, c
14	γ-terpeneol	1198	0.68	b, c
15	n.i	1230	0.20	b, c
16	n.i	1253	0.21	b, c
17	n.i	1271	0.41	b, c
	Sesquiterpene Hydrocarbons			
18	α-cubebene	1336	0.34	b, c
19	α-copaene	1363	0.30	b, c
20	β-elemene	1382	0.40	b, c
21	α-gurjunene	1401	0.61	b, c
22	β-Caryophyllene	1427	2.83	b, c
23	α-*trans*-Bergamotene	1440	0.32	b, c
24	α-humulene	1453	0.21	b, c
25	*allo*-Aromadendrene	1456	0.36	b, c
26	Germacrene-D	1481	2.18	b, c
27	*Cis*-β-guaiene	1486	0.33	b, c
28	Bicyclogermacrene	1508	0.46	b, c
29	α-muurolene	1532	3.42	b, c
30	α-(E,E)-Farnesene	1534	3.64	b, c
31	δ-amorphene	1544	3.98	b, c
32	δ-cadinene	1546	0.57	b, c
	Oxygenated Sesquiterpenes			
33	Muurolol 5-en-4α-ol	1560	0.30	b, c
34	Spathulenol	1568	0.46	b, c
35	Globulol	1587	0.28	b, c
36	Viridiflorol	1597	2.72	b, c

37	Guaiol	1599	0.23	b, c
38	Cadinol *epi*-α	1624	0.34	b, c
39	Aromadendrene *epoxi-allo*	1631	3.50	b, c
40	α-cadinol	1648	14.38	b, c
41	14-Hydroxy-9-epi-caryophyllene	1683	12.14	b, c
42	(2Z,6E)-Farnesol	1739	0.40	b, c
43	Guaiol acetate	1743	0.21	b, c
44	N-nonadecane	1900	3.89	b, c
	Oxygenated Diterpenes			
45	9β,13β-epoxy-7-abietene	1988	7.37	d
46	dehydroabietane	2141	0.25	b, c
47	n.i	2148	0.75	b, c
48	n.i	2229	0.28	b, c
49	abieta-7,13-dien-18-ol	2310	0.14	b, c
50	Abietol	2374	0.34	b, c
51	Manoyl oxide	2421	0.65	b, c
52	6,7-Dehydroroyleanone	2435	14.89	d
	Total identified	**98.14%**		
	Monoterpene hydrocarbons	**1.05%**		
	Oxygenated monoterpenes	**15.51%**		
	Sesquiterpene hydrocarbons	**19.91%**		
	Oxygenated sesquiterpenes	**38.85%**		
	Oxygenated diterpenes	**24.67%**		

[a]Compound listed in order of elution from a DB-5 column; [b]Identification based on retention index (RI); [c]Identification based on comparison of mass spectra; [d]Identification based on NMR spectra [15].

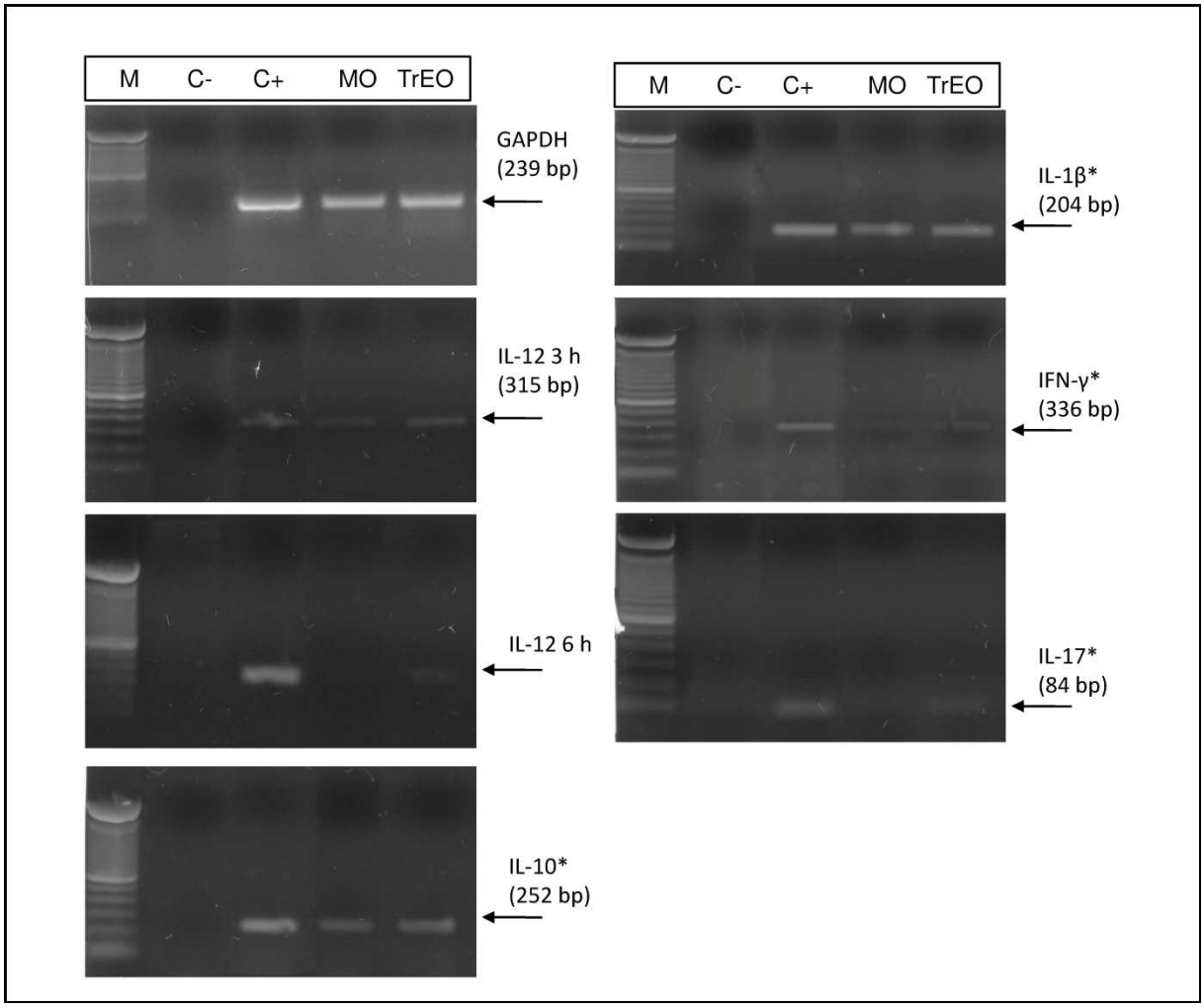
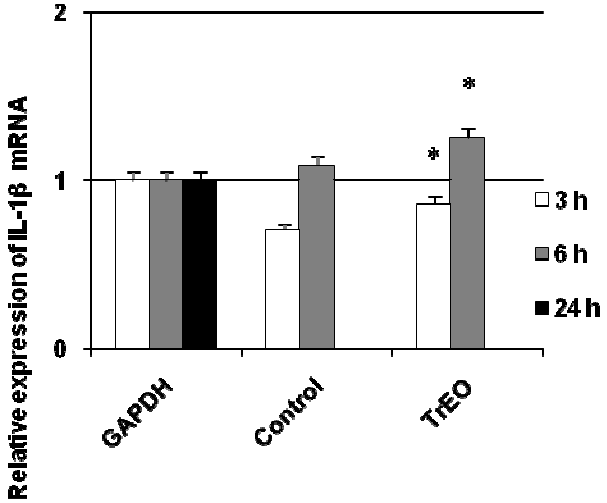
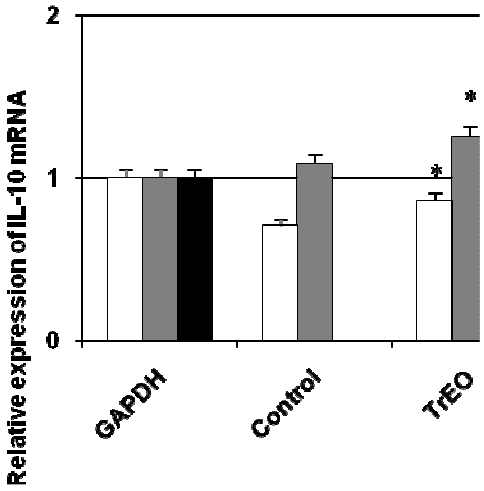
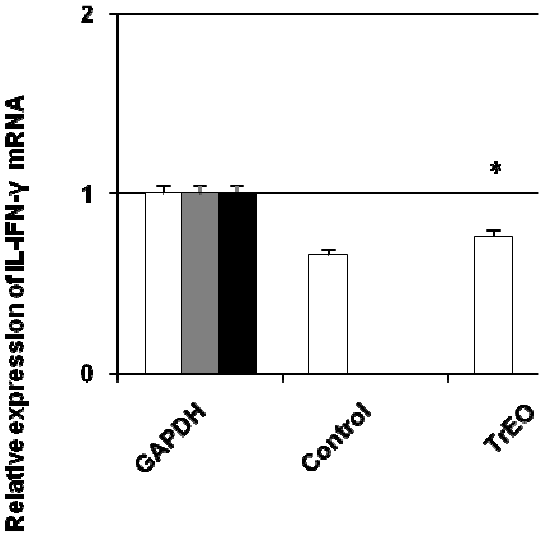
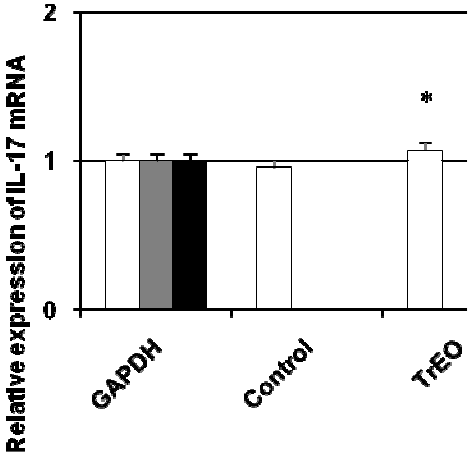


Figure 1. Cytokines mRNA expression by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). DNA fragments were separated in 1.5% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator . M, molecular marker; C-, intern negative control (H_2O). C+, DNA obtained from macrophages stimulated with 5 µg/mL of LPS; MO, macrophages (negative control); TrEO, macrophages treated with 30 ng/mL of *Tetradenia riparia* essential oil. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37 ° C. * p-value significant < 0.05.


Relative expression of IL-1β mRNA
2
1
0
*
*
3 h
6 h
24 h
GAPDH
Control
TrEO


Relative expression of IL-10 mRNA
2
1
0
*
*
GAPDH
Control
TrEO


Relative expression of IL-IFN-γ mRNA
2
1
0
*
GAPDH
Control
TrEO


Relative expression of IL-17 mRNA
2
1
0
*
GAPDH
Control
TrEO

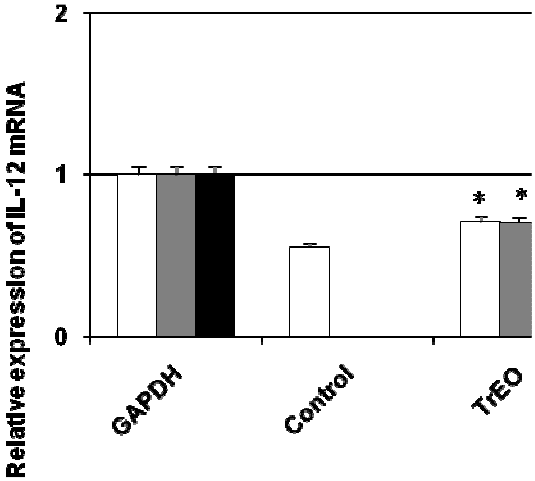

Relative expression of IL-12 mRNA
2
1
0
*
*
GAPDH
Control
TrEO

Figure 2. Expression levels of cytokines mRNA by semi-quantitative RT-PCR. Control, macrophages non-treated (negative control); TrEO, macrophages treated with 30 ng/mL of TrEO. iNOS mRNA expression levels were normalized to GAPDH as a housekeeping gene (internal control). All conditions tested were analyzed within 3, 6 and 24 h incubation at 37 ° C. * p-value significant < 0.05.

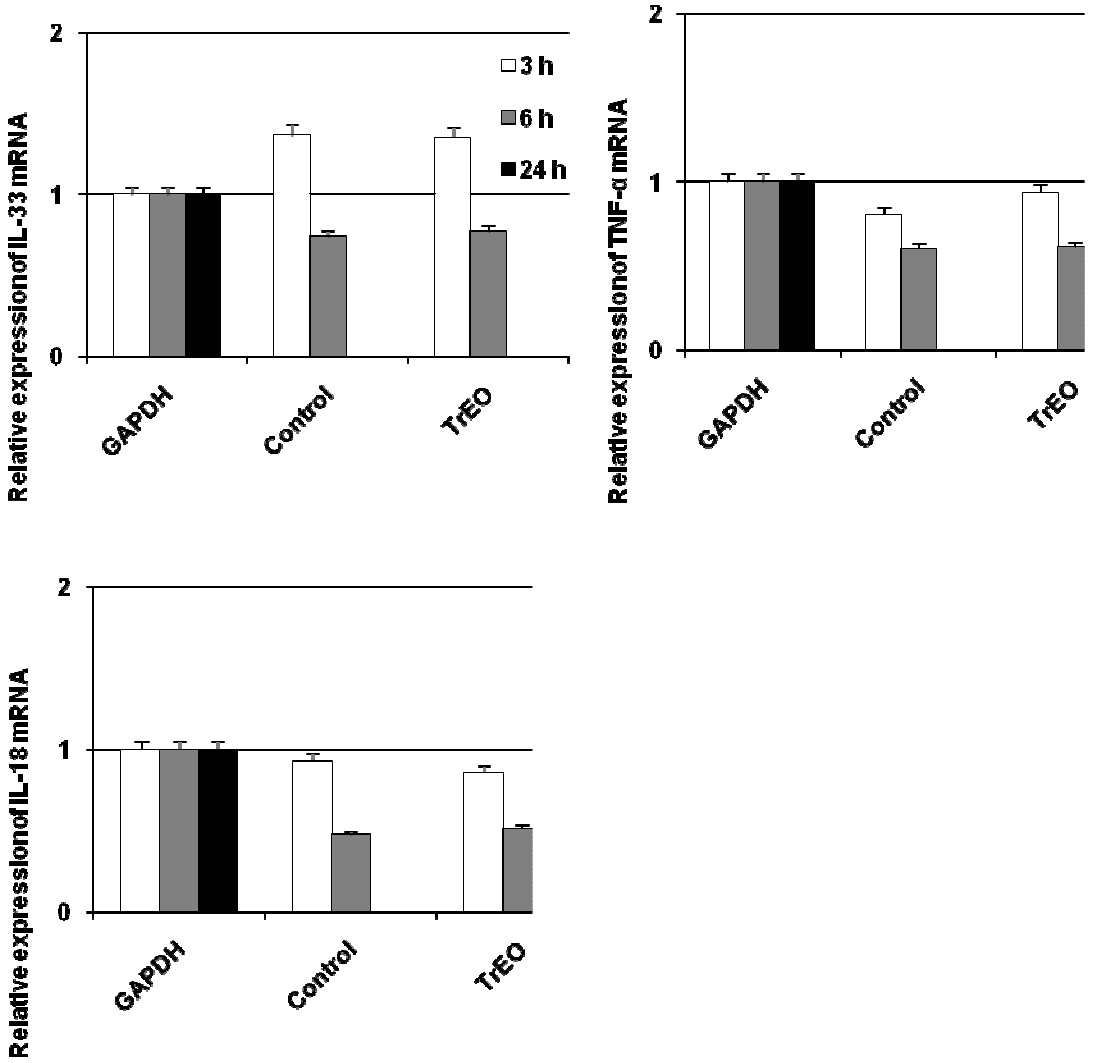


Figure 3. Cytokines mRNA expression not induced by TrEO using semi-quantitative RT-PCR. Control, macrophages non-treated (negative control); TrEO, macrophages treated with 30 ng/mL of TrEO. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37 ° C. * p-value significant < 0.05.

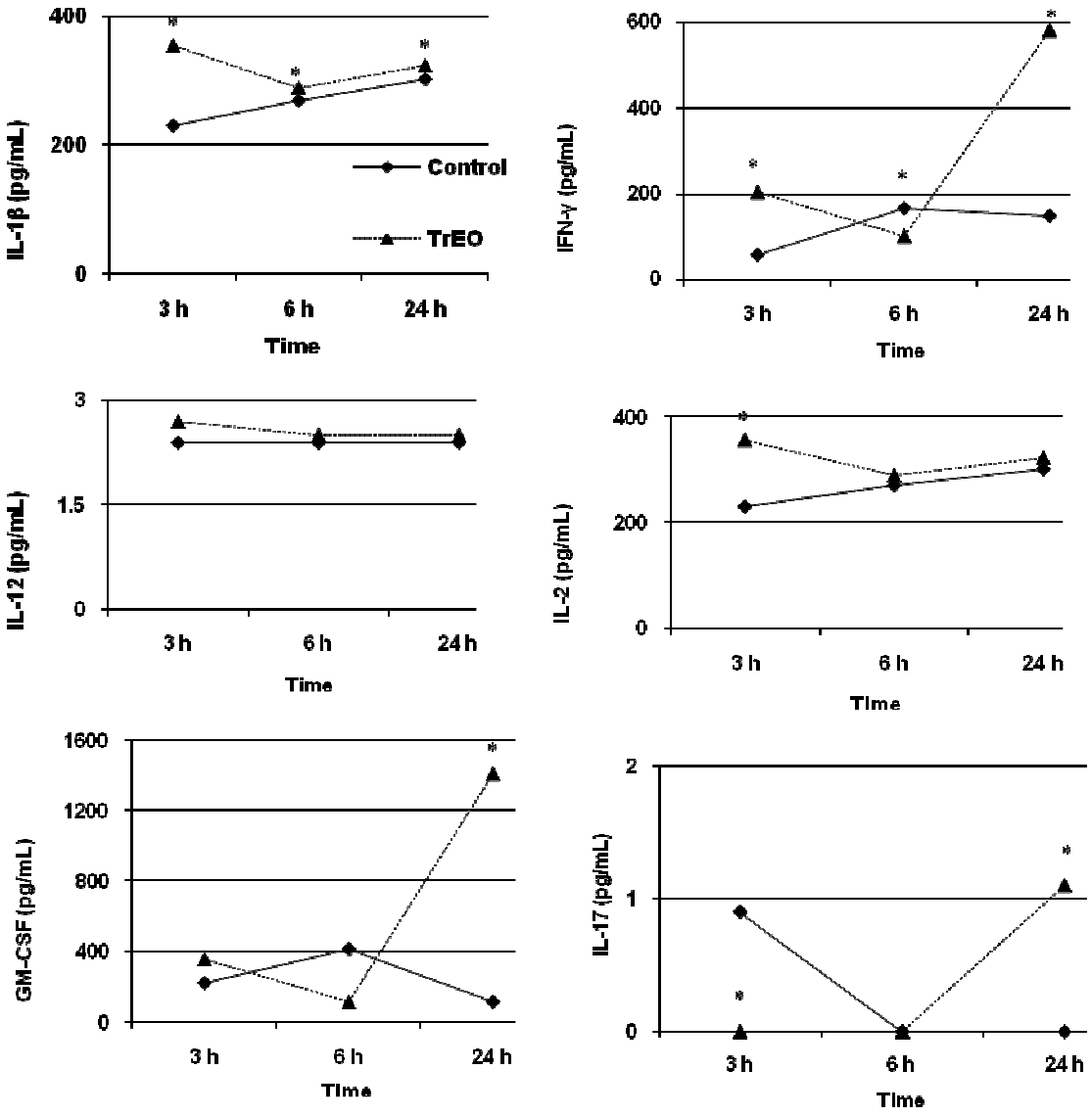


Figure 4. Cytokines produced by murine macrophages stimulated by TrEO using multiplex flow cytometry. Control, macrophages non-treated (negative control); TrEO, macrophages treated with 30 ng/mL of TrEO. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37 ° C. * p-value significant < 0.05 comparing negative control.

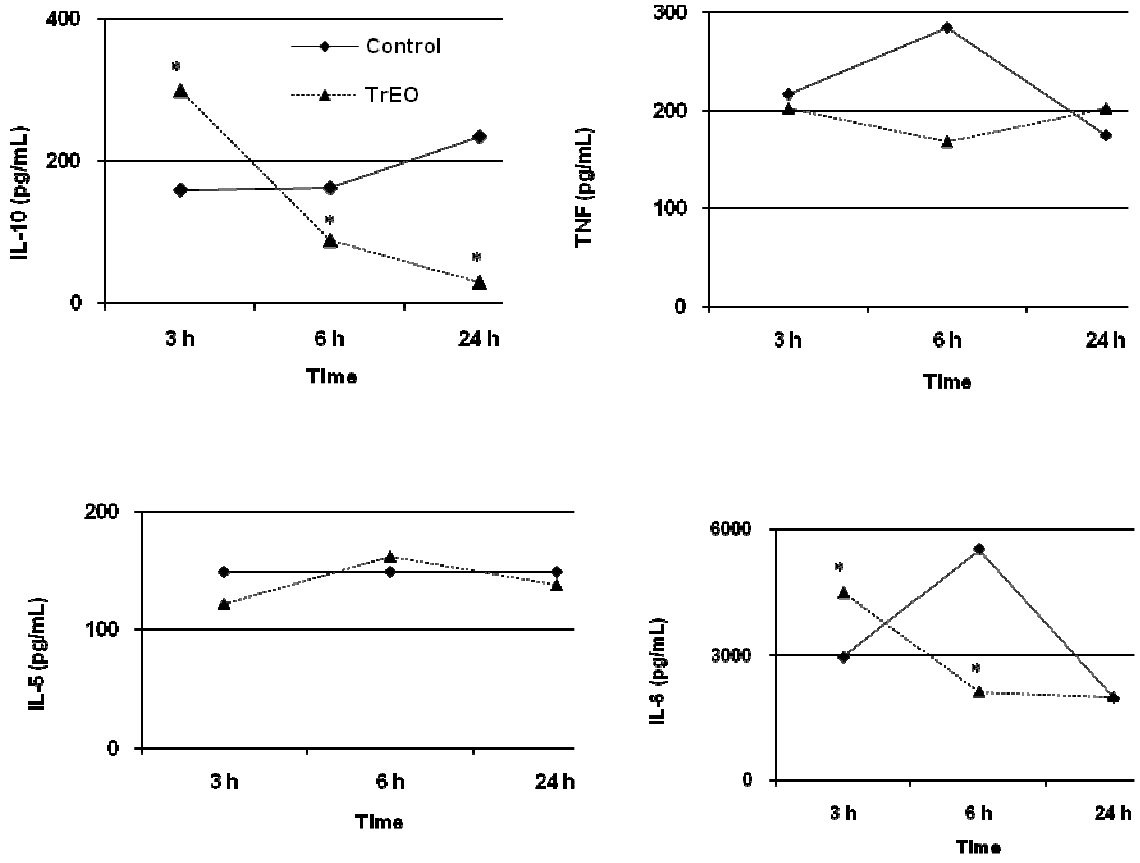


Figure 5. IL-2 and other cytokines produced by murine macrophages stimulated by TrEO. The production was evaluated using multiplex flow cytometry; IL-12, TNF, and IL-5 were not change. Control, macrophages non-treated and non-infected (negative control); TrEO, macrophages treated with 30 ng/mL of TrEO. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37° C. * p-value significant < 0.05 comparing negative control.

Artigo 3

Antileishmanial and immunomodulatory effects of the essential oil from *Tetradenia riparia* (Hochstetter) Codd

Izabel Galhardo Demarchi[*1], Mariana de Souza Terron[1], Mateus Vailant Thomazella[1], Camila Alves Mota[1], Zilda Cristiani Gazim[2], Diógenez Aparício Garcia Cortez[3], Sandra Mara Alessi Aristides[1], Thaís Gomes Verzignassi Silveira[1], Maria Valdrinez Campana Lonardoni[1].

[1] Departamento de Análises Clínicas e Biomedicina, Laboratório de Imunologia Clínica, Universidade Estadual de Maringá, Maringá, Paraná, Brazil.

[2] Departamento de Farmácia, Laboratório de Química de Produtos Naturais da Universidade Paranaense, Umuarama, Paraná, Brazil.

[3] Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil.

*Corresponding author

Name: Izabel Galhardo Demarchi

Email: bel_galhardo@yahoo.com.br

Affiliation: Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná State, Brazil

Telephone: 55 00 (xx44) 3261-4878/ fax numbers: 55 00 (xx44) 3261-4860

mail address: Av. Colombo, 5790. Universidade Estadual de Maringá, Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC). CEP 87020-900 – Maringá – Paraná, Brazil

Type of paper: Original paper

ABSTRACT

Cutaneous leishmaniasis caused by *Leishmania (Leishmania) amazonensis* species usually presents therapeutic resistance to antimonials; besides this therapy has shown many adverse effects and toxicity. In this context, natural products are regarded to be potent candidates for alternative leishmaniasis therapy. *Tetradenia riparia* plant has been used as a folk medicine by many populations to treat infectious and inflammatory diseases, but few studies have investigated the antileishmanial and immunomodulation effects of this plant. We investigated the potential of TrEO against *Leishmania* infection and the imunomodulatory effects that contribute to infection resolution. For this, peritoneal fluid cells were infected with *Leishmania* sp. After 4 h, the cells were treated with TrEO, and at 3, 6 and 24 h of incubation, the cytokines were screened using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and flow cytometry. Antileishmanial activity was evaluated after 24 h by microscopic counting and real-time PCR (quantitative, qPCR). . The treatment with 30 ng/mL induced 50% of the death of *Leishmania* amastigote forms after 24 h incubation using microscopic counting, and 15.2% by qPCR. Also, TrEO stimulated IFN-γ subverting the inhibition of it by *Leishmania* infection; and TrEO inhibited IL-1β, IL-6, IL-17, IL-33, TNF, and T_H2 cytokines (IL-10, IL-4 and IL-5) subverting the increased of these cytokines promoted by *Leishmania* infection. In addition, IL-12 levels were maintained by TrEO. In contrast, *L. (L.) amazonensis* infection stimulated the production of IL-10, IL-1β, IL-4, IL-5, IL-6, IL-17 and IL-33, while inhibited IL-12 and IFN-γ in macrophages. Thus, TrEO down-regulated T_H2 cytokines as IL-4 and IL-10, involved with the progression of the infection. TrEO also increased IFN-γ which is critical to the resolution of leishmaniasis.

Keywords: cutaneous leishmaniasis; *Leishmania (Leishmania) amazonensis; Tetradenia riparia*; cytokines; immune response

INTRODUCTION

Leishmaniases are a neglected infectious diseases which the etiologic agents are protozoan parasite belonging to the *Leishmania* genus (1). There is a broad variety of specie, which can infect animals and humans promoting skin lesions or visceral involvement. The clinic manifestations and clinic healing depend on host immune response and parasite species infectiveness. *Leishmania (Leishmania) amazonensis* is an agent of cutaneous (CL) and diffuse cutaneous leishmaniasis, the most severe and destructive clinic form (2). The CL clinical form affects about one million people all over the world and the host immune response is a crucial factor to control infection and pathogen death. However, *Leishmania* has an ability to modify the innate and adaptative immune responses inhibiting the antimicrobial mechanisms of the host cells by mediating an imbalance of T helper (T_H) cells (3-4). The recommended treatment has been failure and the infection persists, this may be related with the host immunity and this parasite ability,. The CL specific treatment is based on pentavalent antimonials and amphotericin B, which has been used as the second-line therapy (5). Antimonials and other antileishmanial drugs can stimulate the innate branch of immune system which favors the infection resolution (6). Despite this, in the last years, many studies have reported the undeniable leshmanicidal activity and side effects during treatment with antimonials and second-line drugs, which can lead patients to abandon the treatment. Therefore, researches have been conducted to discover new drugs to leishmaniasis with lesser toxicity profiles, immunomodulatory effects and potential leishmanicidal action (7-8).

Plants possess diverse metabolites that display a vast array of pharmacological activity with potential to be used against leishmaniasis (9-10). Natural products have not a high probability to cause serious adverse effects; further they are inexpensive, accessible and sustainable comparing with current treatment for leishmaniasis (11). In addition to antileishmanial action, extracts and essential oils derived from plants have been screened for

immunomodulatory activity. Studies suggest that products with both activities can be used alternatively to treat infectious diseases, neoplasm and other diseases in which the immune response is essential for the evolution of the illness such as cutaneous leishmaniasis (12-14).

Tetradenia riparia (Hochstetter) Codd plant belongs to the Lamiaceae family, it is also known as *Iboza riparia* and *Moschosma riparium*. It is used as a folk medicine in Africa, which is used to treat the inflammatory and infectious diseases. The plant is an herbaceous shrub that occurs throughout tropical Africa and in other regions of the world like Brazil (15-19). Its leaves and essential oil have shown antimicrobial activity for the treatment of several infections such as malaria, cryptococcosis and candidiasis. It also has been used for treating respiratory infections as an alternative therapy (19, 20-22). Most recently, our research group shown the in vitro antileishmanial activity of *T. riparia* essential oil (TrEO) and the expression of the inducible nitric oxide synthase (iNOS) mRNA, although TrEO did not induce nitric oxide production (NO) above normal levels in macrophages infected with *Leishmania* (23-24). Oxygen reactive and NO are important components of the host immune response to combat the *Leishmania* infection (25). The protective immune mechanisms of the essential oil from *Tetradenia riparia* for leishmaniasis have not been demonstrated until now. Thus, the purpose of this research was to evaluate the antileishmanial activity and immunomodulation of *T. riparia* essential oil (TrEO) on peritoneal fluid cells infected with *L. (L.) amazonensis*.

MATERIAL AND METHODS

Essential oil extraction of T. riparia

T. riparia leaves were collected monthly between September 2006 and August 2007 in Umuarama, state of Paraná, Brazil (23º46’22”S and 53º16’73”W, 391 m). The plant was identified by Professor Ezilda Jacomasi of the Departamento de Farmácia of Universidade

Paranaense (UNIPAR).A voucher specimen is deposited at the UNIPAR Herbarium (code number 2502). The mean values for maximum and minimum temperature, precipitation, and relative humidity by season from September 2006 to August 2007 were described by Gazim et al. (2010) (12). The fresh leaves of *T. riparia* were used to extract the essential oil, which was obtained by hydrodistillation for 3 h using a Clevenger-type apparatus. The distilled oils were collected and dried over anhydrous sodium sulfate and stored in a freezer. The oil was analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 5973N GC-MS System that was operated in electron ionization mode and equipped with a DB-5 capillary column (30 m x 0.25 mm x 0.25 µm, Agilent, PA, USA) that was used to inject 1 µl of a solution sample (12, 27). The initial temperature of the column was 80°C. The column was gradually heated to 260°C at a rate of 4°C/min. The injector (splitless, 0.5 min) and transfer line temperatures were held at 260°C and 280°C, respectively. Helium (1.0 mL/min) was used as a carrier gas. The same temperature program was used for gas chromatography with a flame ionization detector (GC-FID). The identification of the compounds was based on comparisons of their retention time that were obtained using various n-alkanes (C7 - C25). Their electron impact mass spectra were compared with the Wiley library spectra and literature (12).

Cytotoxicity

Peritoneal fluid cells were obtained from BALB/c mice in accordance with the Ethics Committee on the Use of Experimental Animals of the Universidade Estadual de Maringá, Paraná State, Brazil (warrant no.133/2012). Briefly, the peritoneal cavity was washed with 8 mL sterile RPMI 1640 medium. The cell suspension was adjusted to 1×10^6 cells/mL. Next, 100 µL was plated in 96-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37°C, 5% CO_2, and non-adherent cells were removed by sterile PBS washing. TrEO

was diluted from 3 µg/mL to 30 ng/mL in dimethylsulfoxide (DMSO, did not exceed 0.005% v/v, and it did not have cytotoxic effect). Non-treated cultures were used as viability control. The plates were maintained at 37°C in a humid atmosphere containing 5% CO_2 during 24 h. The cytotoxicity on cells was done using colorimetric cell viability XTT (2,3-bis[2-methyloxy-4- nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma Chemical Co; St. Louis, MO) (28-29). The XTT solution (500 µg/mL) was activated with 50 µg/mL phenazine methosulfate (PMS, Sigma Co Chemical; St. Louis, USA), and it was added over the cell monolayer to each well. After 3-5 h incubated at 37°C with 5% CO_2 and protected from light, the results were mensuared at 450/650 nm using a spectrophotometer plate reader (ASYS Expert Plus, ASYS Hitech GMBH, Austria). All tests were done in duplicate and the results were expressed as viability percentage. The cytotoxicity concentration (CC_{50}) was defined as the dose of the compound that reduced 50% of the survival of cells comparing to untreated cells (viability control).

Antileishmanial activity

Parasite strain and culture

L. (L.) amazonensis (MHOM/BR/1977/LTB0016) was kept by the inoculation of $1x10^7$ parasites in the hind left footpad of the BALB/c mice. Animals between 30 and 40 days of age were euthanized by inhalation of 40% CO_2 in a chamber at a moderate fill rate (AVMA Guidelines on Euthanasia, 2007). Their lymph nodes fragments were inoculated in 199 culture medium supplemented by fetal bovine serum (FCS) 10% (v/v), 1% human urine, 2 mM L-glutamine and antibiotics (100 UI/mL penicillin and 0.1 mg/mL streptomycin). The cultures were incubated at 25ºC and parasites maintained by weekly transfers in 25-cm^2 culture flasks with 199 insect medium supplemented.

Drug activity measured by microscopic counting

Peritoneal fluid cells were obtained from BALB/c mice as already reported above. The cell suspension was adjusted to $1x10^6$ macrophages/mL. Next, 500 µL were distributed on 13 mm-diameter sterile glass coverslips (Glastecnica, São Paulo, SP, Brazil) and placed in 24-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37° C. The non-adherent cells were removed by sterile PBS (phosphate buffered saline) washing. Macrophages were infected with promastigote forms in the proportion of six parasites for each macrophage and the plates were incubated for 4 h at 37°C in an atmosphere of 5% CO_2. TrEO was diluted in DMSO and RPMI 1640 medium. The final concentration of DMSO did not exceed 0.05% and no cytotoxicity effect on the macrophages was observed. After infection with *Leishmania*, TrEO was added to cultures at 30 ng/mL, and after 24 h at 37°C and 5% CO_2, the cells on coverslips were fixed in 95% ethanol and dyed with eosin and hematoxylin. At least, 200 cells were counted in an optical microscope. The infection index was determined by the percentage of infected macrophages multiplied by the mean number of parasites per macrophage.

Drug activity measured by conventional polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR)

Leishmania sp. has a conserved region of minicircle in the mitochondrial DNA that can be amplified using polymerase chain reaction (PCR) method. For this, DNA was extracted from samples conserved in Trizol reagent according to manufacturer. The PCR was performed with two oligonucleotides: A1 (5'-(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACCAACCCC-3') and A2 (5'-GGGGAGGGGCGTTCTGCGAA-3') (30). The reaction mixture contained 25 µM of each primer, 1 unit Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 50 mM $MgCl_2$ in a final

volume of 25 µL. The DNA amount of 5 µL was added to each test tube. The amplification conditions were: one cycle at 94°C for 5 min, 26 cycles at 94°C for 30 s, 50°C for 30 s and a final step at 72°C for 10 min. After the PCR, the product (~120 base pairs) was separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Leishmania mitochondrial DNA cited above was also measured by quantitative real-time PCR (qPCR) using the same primers A1 and A2. Each sample was tested in triplicate in a final volume of 10 µL, including 1 µL of template DNA, 5 µL of 1X Sybr® Green Select Master Mix (Applied Biosystems, Austin, TX, USA), and 25 µM of each primer. The amplification was run on the thermocycler StepOne Real-Time PCR System (Applied Biosystems, CA, USA). The optimized PCR amplification conditions were 94°C for 5 min and 40 cycles of 94°C for 30 s and 50°C for 30 s. The threshold of detection and the baseline were automatically determined using StepOne™ Software v2.1. Each sample amplification was performed in triplicate, and a blank consisting of the reaction mixture without DNA template was included in each qPCR run. A standard curve (positive control) qPCR was performed using *L. (L.) amazonensis* DNA from $1x10^5$ (high control) to 1 parasites/sample (low control). The parasites quantification of each sample was compared with standard curve. The DNA percentage reduction of *Leishmania*-infected and treated macrophages was calculated comparing with infected-macrophages.

Immunostimulatory effects of T. riparia essential oil

Resident peritoneal murine cells ($1x10^6$ cells) were submitted to the following conditions: a) infected by the promastigote forms of *L. (L.) amazonensis* in the proportion of six parasites for each cell (positive control); b) cells infected by promastigotes (6 parasites/1 cell) were incubated for 4 h to be infected and treated with 30 ng/mL of TrEO; c) non-treated and non-infected cells were regarded as negative control. TrEO was diluted in DMSO (1:2) and medium RPMI1640 until obtained 30 ng/mL (there was not cytotoxicity

concentration, view in results section). The maximum DMSO concentration did not exceed 0.01%, and it did not result in cell cytotoxicity. The plates were maintained at 37°C in a humid atmosphere containing 5% CO_2 for 3, 6 and 24 h. After these times, the supernatant was removed and stored at – 80°C to cytokine quantification assay. Trizol reagent (Invitrogen, USA) was added on adhered-cells to extract nucleic acid and stored at - 80°C.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. cDNA was synthesized using 1 μg of total RNA as a template in a reverse transcription reaction (Superscript III reverse transcriptase, Invitrogen, Carlsbad, CA, USA). The purity level and quantity of material extracted were done using the NANODROP 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc, USA), considering an optimal purity level ≥ 1.8. The standard PCR conditions are in Table 1. The primers were chosen according to BLAST tool available in Genbank database and publications (31-34). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA of *Mus musculus* was used as an intern control. The amplified DNA fragments were separated in 1.5% agarose gel electrophoresis and revealed with ethidium bromide transilluminator. Semi-quantitative RT-PCR was conducted by quantifying the bands densitometry using ImageJ software (National Institutes of Health, USA). Each gene was normalized against GAPDH as a housekeeping gene control (intern control) (35).

Cytokine quantification

The supernatants previously collected were centrifuged and 50 μL of the sample was used for cytokine quantification (pg/mL) by multiplex Mouse Cytokine 10-Plex Panel Kit and Mouse IL-17 Singleplex Bead Kit (Invitrogen, Carlsbad, CA, USA). The assay was read on

Luminex® 200™ System using xPONENT 3.1™ Software, and the samples were tested in quadruplicate of two independent experiments.

Statistical analysis

Analyzes were carried on using Statistic 7 software and p-value < 0.05 was considered to be significant. The statistical significance was done using chi-square test, one-way analysis of variance (ANOVA) and Turkey test.

RESULTS AND DISCUSSION

Essential oil from *T. riparia* was not cytotoxicity in murine macrophages at 30 ng/mL (> 95% viable cells), but at 0.2 µg/mL it showed 50% toxicity for the cells. Gazim et al. (2013) (36) showed that TrEO at 25 µg/mL was a potential growth inhibitor of tumoral cell lines SF-295, human melanoma cell (MDA-MB-435) and human colon cell line (HCT-8), although the mechanisms remain to be elucidated. So, the dose and route of the treatment using TrEO must be evaluated to each condition established. The results in this paper show that TrEO at 30 ng/mL induces 50% death of *Leishmania* amastigote forms after 24 h incubation (Figure 1). The infection index is 112 in each non- treated infected-macrophage, while the treatment with TrEO reduces the index to 54 at 30 ng/mL, 68 at 0.3 µg/mL and 79 at 3 µg/mL (data not shown). Based on this, TrEO was most effective in the lowest concentration against *L. (L.) amazonensis* infection. The possible reason for this might be due to the essential oil in high dose is cytotoxic to cells, allowing the action on parasite and not in macrophage. The parasite load using qPCR showed that *Leishmania*-infected macrophages treated with 30 ng/mL of TrEO had a reduction of 15.2% comparing with macrophages infected and non-treated ($p<0.001$) (data not shown). African population has used the leaves from *T. riparia* plant as a traditional medicine use for a broad range of diseases, especially

respiratory infection. The antimicrobial, antioxidant, acaricidal and antileishmanial activities were attributed to *T. riparia* (12, 21, 24, 26).

In primary screening for new antileishmanial drugs, in vitro assays play as important role. Amastigote-macrophages models require murine peritoneal macrophages or human monocyte-derived macrophages. This method is the most used assay for testing drugs against *Leishmania* sp. The drug test is usually measured by microscopic counting of the infected cells percentage multiplied by number of amastigote per cell through of 200-400 macrophages examined (37). In the present study, the qPCR method was also used as a reliable tool to evaluate the parasite load in amastigote assay, but the results of reduction of parasite burden obtained by real-time PCR and the number and percentage of infected macrophages in culture were discrepant. This difference between qPCR and microscopic counting as expected, because according to Reimão *et al*. (2011), the DNA quantification would not ensure a detection of only living parasites, since the drugs act resulting in living and dead microorganisms. Additionally, the DNA molecule has a high proper chemical stability and could hamper its degradation (38). Also, the microscopic assay demands time and the analysis are subjective which can lead to results not as accurate (37).

Torquilho *et al.* observed that essential oil extracted from *M. riparium* Hochst has a lethal dose of 50% (LD50) at 0.98 µg/mL in *L. amazonensis* promastigotes (39). Recently, the LD50 in *L. amazonensis* promastigote was observed to be higher than 0.2 µg/mL (24). In other study, the minimum inhibitory concentration (MIC) of extract leaves from *T. riparia* was measurement against some bacteria and fungus concluding that the most sensitive microorganisms were *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans* showing the lowest MIC values of 15.6, 7.8 and 31.2 µg/mL, respectively (12).

The pathogens are recognized in the infection site by innate immune cells such as macrophages, natural killers (NK) and dendritic cells (40). Specific responses are mounted

by these cells to avoid the infection to spread to the entire organism. Cytokines inductions are one of those responses, and their modulation impact the innate antimicrobial defenses, recruitment of effectors cells and repairing the infected tissue. After pathogen encounter, all immune mechanisms are coordinated early to take place in few hours, these processes require major changes in mRNA and protein expression. Some pathogens can interfere directly with continuous host protein synthesis and innate responses inhibition (41). In cutaneous leishmaniasis, the host immune response is one of the most important factor for infection development. *Leishmania* parasite has an ability to withstand, inhibit or deceive the antimicrobial effect on host macrophages by subverting induction of innate and adaptative immune responses through mediating an imbalance of T helper (T_H) cells (3).

The clinical forms of leishmaniasis are usually affected through upregulation of cellular immune responses involved with host macrophages activation and T_H1 cells producers of IFN-γ (42). The intracellular localization of the pathogen can affect antileishmanial drugs besides their ability to be internalized by host cells including rate of uptake, capability to resist an intracellular degradation and trafficking, and possibility of cells cytotoxicity. The screening for leishmanicidal drugs needs test with extracellular promastigotes to identify promising compound(s) which also should have a high therapeutic index against intracellular amastigotes, a process that is technically more complex (43). Thus, an antileishmanial product capable of stimulating protective immune response against intracellular pathogens and of inhibiting suppress immunity could be an alternative to deal with therapeutic resistance, failures and adverse effects. TrEO modulated both mRNA expression (Table 2, Figure 2-3) and production of various cytokines in peritoneal mice cells infected with *L. (L.) amazonensis* (Figure 4-5). TrEO mainly stimulated IFN-γ and inhibited some proinflammatory cytokines like IL-1β, IL-17, IL-33 and TNF. In addition, the production of T_H2 cytokines by *L. (L.) amazonensis* infection such as IL-10, IL-4, and IL-5

were also prevented after TrEO treatment. Furthermore, the proliferative cellular mediators, IL-2 and GM-CSF (Figure 5), were inhibited after treatment. Summarizing, TrEO contributes to reduce cytokines involved with the progression of the infection and increase IFN-γ (Figure 4), which is critical to the resolution of the disease (25). The results of this research showed that *L. (L.) amazonensis* species was able to modify the production and mRNA expression of important cytokines involved with persistent infection, including IL10, IL-1β and TNF mRNA expression (Figure 2-3) until 6 h, and their production (Figure 4) was stimulated by the infection until 24 h. IL-4 level was elevated in the infection, but the experimental conditions of this study were not able to express IL-4 mRNA. IL-33 mRNA expression levels were high in the disease, as well as the levels of IL-2, IL-17, IL-6, IL-5 and GM-CSF at different times (Figure 5). Therefore, IL12, IL-18, and IFN-γ expression were inhibited, and the production of IL-12 and IFN-γ were decreasing progressively after the infection (Figure 4).

While the parasite increases cytokines involved in the persistence of the infection such as IL-1β, TrEO decreases IL-1β production and mRNA expression in *Leishmania*-infected macrophages ($p < 0.05$), showing that the essential oil is able to subvert the IL-1β production induced by the *Leishmania* infection (Figure 2 and 4). According to Lima-Junior et al. (2013) the inflammasome-derived IL-1β induces resistance to *Leishmania amazonensis* in infected macrophages (45). IL-1β down-regulation was promoted by TrEO, which can confer advantage in the healing and parasite death mechanisms. IL-33 was also stimulated by the infection and inhibited by TrEO. This interleukin is a member of the IL-1-family of cytokines which can promote the proliferation and activation of T_H2 cells and suppress the T_H1 response (31, 46-47). There is a hypothesis that IL-33 synthesis occurs after tissue damage and in response to inflammation, acting as an alarming that recruits cells to local infection (31, 48-49). The role of IL-33 in leishmaniasis is still not very clear. IL-33 was associated

with human visceral leishmaniasis and suppresses T_H1 responses in the livers of BALB/c mice infected with *L. donovani* (50) . The authors also suggest that IL-33 could be a new susceptibility factor and a potential prognostic marker during visceral leishmaniasis (50). In the present study, IL-33 was highly expressed by macrophages infected with *L. amazonensis*, this result is in agreement with the study mentioned above. TrEO shows a potent action on IL-1β and IL-33 cytokines reducing their amount and expression, subverting the up-regulation promoted by *Leishmania*.

The IL-17, IL-6 and TNF production were declined in *Leishmania* infected-macrophages by the treatment with TrEO after 24 h was able to subvert thus inhibition promoted by infection (Figure 5). In contrast, *Leishmania* infection induced a high IL-17 and IL-6 levels in non-treated macrophages (Figure 3). These stimuli can promote the infection persistence by T_H17 cell activation. IL-17 is a proinflammatory cytokine produced by T_H17 and other cells, it induces the synthesis of IL-6, IL-1 and TNF, resulting in an intense inflammation because these cytokines can induce the neutrophil recruitment, increasing the inflammation and tissue damage local. TNF level was elevated in infected macrophages by *Leishmania*, this result is according to other study that showed the relationship between TNF increase and susceptibility to *L. (L.) amazonensis* infections due to the induction of persistent parasitic infection and tissue damage (51). TrEO inhibition of IL-17, IL-6 and TNF may contribute to reduction of local damage caused by neutrophil recruitment before the starting of infection control.

IL-6 is synthesized early in response to an infectious agent acting on T_H17 activations and inhibiting T regulatory cells favoring the infection (52). Further, an increase in TNF and IL-6 secretion can be augmented as an influx of neutrophils and inflammatory monocytes to infection and both of these cell types have been showing to be infection targets and aid in the establishment of the infection. Recently, Duque et al. (2014) shows that *Leishmania*

promastigotes promotes the degradation of synaptotagmins (Syts) resulting in the induction of the cytokine secretion as IL-6 and TNF in murine cells.. Syts are type-I membrane proteins responsible for regulating vesicle docking and fusion in exocytosis and phagocytosis by host cells (53). This regulation is possible due to the zinc metalloprotease GP63 of *Leishmania*, which directly degrades the Syt XI and it is excluded from *Leishmania parasitophorous* vacuoles by the promastigotes surface glycolipid lipophosphoglycan. Thus, the GP63 induces proinflammatory cytokine release and increases infiltration and inflammatory phagocytes (53), but TrEO treatment is able to inhibit these cytokines, contributing to parasite death mechanisms activation and reduction of local inflammation.

TrEO was able to stimulate IFN-γ and maintained IL-12 levels in the infection. IFN-γ and IL-12 are pro-inflammatory cytokines produced early by macrophages in antigenic response. The second one promotes cell-mediated immunity via stimulation of T_H1 lymphocytes and acts controlling the inflammation (54). And the first one is the most potent macrophage activator to produce the antimicrobial substances like oxygen reactive and nitric oxide (NO), promoting pathogens or cells death. IFN-γ is also important to control infection dissemination in intracellular agents like *Leishmania* and it can be a target for leishmaniasis therapy (25). Although TrEO induced IFN-γ and inducible nitric oxide synthase (iNOS) mRNA expression, higher production of NO was not detected by TrEO stimulus in previous study. The direct action of TrEO on *Leishmania* parasite death, and consequently antigen stimulus reduction could explain the normal NO production that was similar in non-infected and non-treated cells (23). It is may be a result of other death mechanisms stimulated by TrEO. Although NO is considered the most relevant microbicidal molecule, reactive oxygen species (ROS) are also related with disease susceptibility. The ROS are produced by the membrane-bound NADPH-dependent oxidases (NOX) and a study demonstrated that NOX deficient mice were more susceptible to *L. donovani* and *L. major* infection (55-56). Based on

this, other microbicidal process of TrEO should be better investigated in vitro and in vivo models to clarify all death mechanisms induced after the treatment with this essential oil.

In response to *L. (L.) amazonensis* infection, IL-2 was produced early in high levels by murine cells at 3 h. This result agrees with the increase of IL-2 observed in patients with cutaneous leishmaniasis caused by *L. (L.) amazonensis* (57). The high GM-CSF production was belatedly induced in infected cells. The GM-CSF appears to increase the infectivity of *L. amazonensis* by protecting promastigotes from heat-induce death and it is also a growth-factor for promastigotes of *Leishmania* (58-61). TrEO inhibits both mediators showing its importance for the early and late response to infection.

The synthesy levels of IL-10, IL-4, and IL-5 were lower on infected cells treated with TrEO comparing to non- treated infected macrophages ($p<0.05$). Those cytokines are known as down-regulators of immune response. They also stimulate the T_H2 response that induces the inhibition of *Leishmania* sp. death mechanisms by macrophages, leading to disease progression (25). Patients infected by *Leishmania amazonensis* produce high levels of IL-2, IL-4, IL-6, IL-10, TNF, and IL-17 (57) as well our results. This profile is related to the clinical course of human cutaneous leishmaniasis. The relation of those cytokines with *Leishmania* infection remains doubtful, because it is associated with remission and also with the development of relapses and more severe forms of the illness (57). IL-10 is a regulatory cytokine that can stimulate CD4+ CD25 T cells (T regs), which inhibit or decrease the proliferation, differentiation and function of other T cells subpopulations, which influence disease development (25). Also, IL-10 inhibits inflammatory mediators and induces activation of monocyte regulating the inflammatory immune response (62). Thus, this study shows that TrEO inhibits T_H2 and regulatory cytokines involved with the progression of the infection and stimulates IFN-γ, an important T_H1 mediator of the resolution of leishmaniasis (53).

In this study, IL-18 mRNA was less expressed after TrEO treatment (Figure 2-3). This cytokine primarily produced by macrophages and dendrites cells was initially described as an IFN-γ-inducing factor (63). It serves as a cofactor for IL-12-induced development of T_H1 immune response and optimizes IFN-γ production from effectors T_H1 cells. The IL-18 role is critical in the development of protective immunity against intracellular pathogens such as *L. major* (64-65). Although TrEO down-regulates IL-18 mRNA expression, the production of it by macrophages and other cells involved with protection against *L. (L.) amazonensis* should be investigated to evaluate the role of this cytokine in infection.

At 24 h, infected macrophages treated with TrEO returned to express high levels of IL-12, IL-18, IL-10 and TNF mRNA, suggesting that protective immunomodulation promoted by TrEO did not occur after 24 h. Considering that TrEO modulates harmful cytokines expression until 6 h and shows the remaining parasites and persistent infection after 24 h (Figure 1), the parasite persistence might stimulate these cytokines mRNA expression again. In conclusion, there are sufficient reasons to explain the high mRNA expression of IL-12, IL-18, IL-10 and TNF observed. For the maintenance of the TrEO immunomodulation, a new dose would be required successively or other therapeutic regimens should be tested.

The essential oils including those of the Lamiaceae family are able to stimulate cytokines that mediate the protective cellular immune response against intracellular pathogens, other agents, cancer and autoimmune disorders (13, 21, 26, 66-67). The immunomodulatory and antileishmanial effects induced by TrEO can be attributed to its complex mixture of compounds. Essential oils use to have large amount of terpenes and terpenoids constituents (12-13) and the interactions between them may result in antagonistic, additive or synergistic effects as showing in other essential oils (68) . TrEO is a complex mixture of terpenoids: monoterpenes, sesquiterpenes, and diterpenes (hydrocarbons or oxygenated) (12). Most of the antimicrobial activity of essential oils is found in the

oxygenated terpenoids and lesser extended hydrocarbons. In addition, the interactions between the compounds of the essential oil can be investigated using macro/microdilution techniques like CheckerBoard, graphical and Time-kill methods (68). The interaction studies could elucidate the mechanism action of the cellular process induced by TrEO during *Leishmania* infection.

In other study, the essential oil from *T. riparia* exhibited an excellent analgesic activity when it was administrated orally in mice and inhibiting the constrictions induced by acetic acid given by abdominal route (12). *T. riparia* leaves extract inhibited the percentage of the acetyl cholinesterase (AChE), COX 1, and COX 2 enzymes showing an anti-inflammatory effect (21, 26). In folks medicine, *T. riparia* leaves are used for treating respiratory illness (coughs, colds, and sore throat), mouth ulcers, stomach ache, diarrhea, influenza, fever, malaria and headaches (21). Therefore, all results suggest that this plant has a potential antileishmanial, analgesic and immunomodulatory effects that offer a stepping stone towards the documentation of commercial essential oils and information to policy makers on the process to regulate the manufacturing and validation of natural products as alternative for the leishmaniasis therapy.

The pharmaceutical industry has behaved with relative anergy, consequently the available therapeutic source of antileishmanial drugs remains limited, often leading people from leishmaniasis endemic areas to depend upon traditional medicines used to relieve the symptoms. The studies with medicinal plants used in the preparation of popular remedies have contributed with the modern medicine providing pharmaceutical active compounds (43).

Summarizing, TrEO treatment inhibits *L. (L.) amazonensis* infection in murine macrophages, down-regulates cytokines involved with the progression of the infection and it also up-regulates IFN-γ, which is relevant to the disease resolution (Figure 7). These results

support the traditional medicine usage of *T. riparia* for parasitic infections and others that require an immune response modulation. We suggest that the biological and pharmacological effects on leishmaniasis must be well known in vivo and in human to ensure safety use and establish the treatment protocols. In addition, the immunomodulation by essential oils or their compounds must be evaluated to guide the selection of the most appropriate therapeutic regimens according to the patient clinical conditions. As a conclusion, TrEO could be an alternative to leishmaniasis therapy considering the antileishmanial and immunomodulatory effects promoted by it.

ACKNOWLEDGMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

DISCLOSURES

None.

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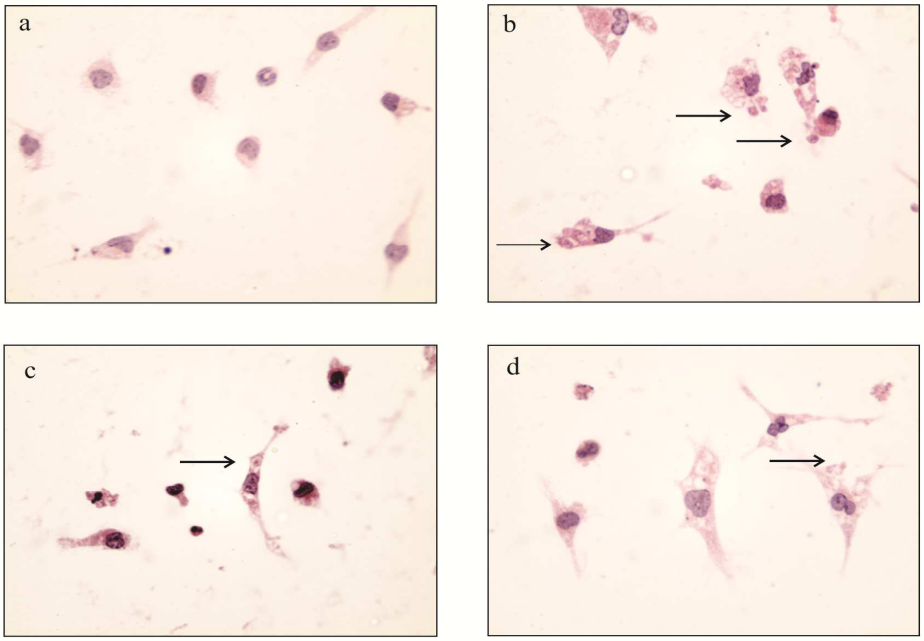
Table 1. Set of specific genes primer.

Gene		Primer sequence	Product size (bp)	Conditions for PCR reaction (cycles)
IL-10 (34)	F1 R2	5’-TACCTGGTAGAAGTGATGCC-3’ 5’-CATCATGTATGCTTCTATGC-3’	252	93°C for 55 s, 61°C for 45 s, 72°C for 40 s (36)
IL-12 (34)	F1 R2	5’-CGTGCTCATGGCTGGTGCAAAG-3’ 5’-CTTCATCTGCAA GTTCTTGGGC-3’	315	93°C for 55 s, 61°C for 45 s, 72°C for 40 s (36)
IL-1β (33)	F1 R2	5’-TTGACGGACCCCAAAAGATG-3’ 5’-AGA AGGTGCTCATGTCCTCA-3’	204	95°C for 20 s, 57°C for 25 s, 72°C for 45 s (30)
IL-33 (31)	F1 R2	5’-ATGGGTACCTTCCTCGCCATG-3’ 5’-GAACGCACAGGCGTTTTACT-3’	187	95°C for 15 s, 60°C for 60 s, 72°C for 45 s (35)
IL-17 (32)	F1 R2	5’-ACCGCAATGAAGACCCTGATAG-3’ 5’-TTCCCTCCGCATTGACACAG-3’	84	95°C for 30 s, 60°C for 60 s, 72°C for 30 s (35)
IL-18 (32)	F1 R2	5’-ACTGTACAACCGGAGTAATACGG-3’ 5’-AGTGAACATTACAGATTTATCCC-3’	440	95°C for 30 s, 60°C for 60 s, 72°C for 30 s (36)
IL-4 (32)	F1 R2	5’ GGAGATGGATGTGCCAAACG 3’ 5’-GCACCTTGGAAGCCCTACAG 3’	80	93°C for 45 s, 55°C for 45 s, 72°C for 30 s (35)
TNF (34)	F1 R2	5’-ATGAGCACAGAAAGCATGATC-3’ 5’-TACAGGCTTGTCACTCGAATT-3’	276	93°C for 55 s, 61°C for 45 s, 72°C for 40 s (36)
IFN-γ (33)	F1 R2	5’-TGGAGGAACTGGCAAAAGGATGGT-3’ 5’-TTGGGACAA TCTCTTCCCCAC-3’	336	95°C for 20 s, 56°C for 25 s, 72°C for 45 s (35)
GAPDH (31)	F1 R2	5’-CCACCATGGAGAAGGCTGGGGCTC-3’ 5’-AGTGATGGCATGGACTGTGGTCAT-3’	239	95°C for 15 s, 60°C for 60 s, 72°C for 45 s (35)

F1: forward; R2: reverse; IL: interleukin; TNF: tumor necrosis factor, IFN: interferon, GAPDH: Glyceraldehyde-3 phosphate dehydrogenase.

	IL-1β	IL-4	IL-6	IL-10	IL-12	IFN-γ	TNF	IL-17
Control								
3h	37.3±0	737.8±76.5	1761.5±188.7	159.0±58.8	2.5±0	58.8±16.4	215.9±167.0	0.9±0.0
6h	29.7±13.1	1175.1±384.2	7120.6±1074.8	161.8±94.9	22.4±34.5	167.4±31.2	284.6±286.0	0.0±0.0
24h	51.1±12.0	1181.4±196.0	1990.2±99.9	233.5±82.6	2.5±0	149.4±0	174.9±28.8	0.0±0.0
LLa								
3h	77.7±0	2187.8±262.5	8448.9±112.8	99.5±0	46.9±8.1	472.0±0	1400.4±308.7	0.1±0.0
6h	83.7±29.1	1263.6±419.0	2109.3±627.7	215.7±87.0	20.0±30.3	276.3±243.4	317.9±132.7	0.5±0.0
24h	77.7±0	992.5±71.7	2290.8±228.0	194.3±115.6	2.5±0	68.3±16.4	346.5±15.1	1.8±0.9
LLa TrEO								
3h	23.2±12.3	363.9±0.1	187.2±0	109.8±8.9	2.5±0	49.4±0.0	73.6±20.0	0.3±0.0
6h	14.6±0	202.5±0	1707.3±14.0	29.6±0	2.5±0	161.4±72.5	195.6±128.1	0.9±0.0
24h	30.7±11.3	1349.6±990.2	1263.6±563.4	128.5±130.5	3.3±1.4	425.3±122.5	172.3±105.8	0.3±0.2

Table 2. Cytokines determination (pg/mL) using flow cytometry. Mean ± standard deviation. LPS: lipopolysaccharide-stimulated macrophages; Control: macrophages non-treated and non-infected (negative control); LLa: macrophages infected by *Leishmania (L.) amazonensis* (6:1); LLa TrEO: macrophages infected by *Leishmania* sp. (6:1) and treated with 30 ng/mL of *T. riparia* essential oil. IL: interleukin; IFN-γ: interferon-gama; TNF: tumor necrosis factor; conditions tested were analyzed within 3, 6 and 24 h incubation at 37° C.



Condition tested	Infection Index (% of reduction)
(a) Macrophages non infected and non treated	-
(b) Macrophages infected with *L. (L.) amazonensis*	112
(c) Infected-macrophages treated with 3 µg/mL of TrEO	79 (29.5)
(d) Infected-macrophages treated with 30 ng/mL of TrEO	54 (51.8)

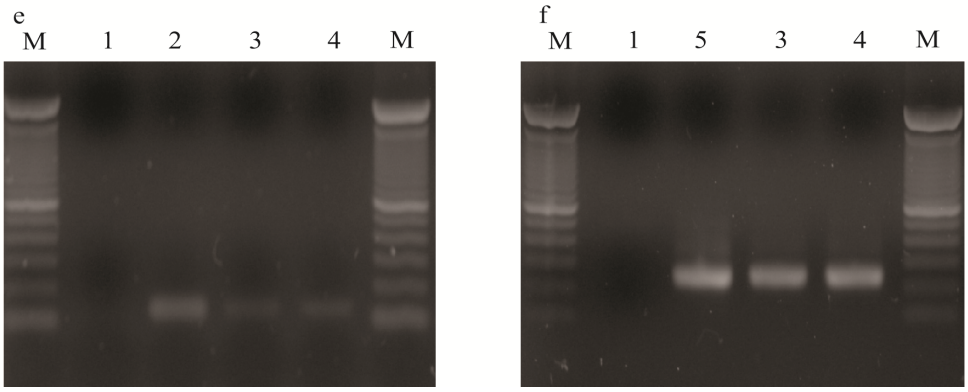


Figure 1. Antileishmanial activity of *Tetradenia riparia* essential oil. Peritoneal cells of BALB/c mice infected and treated with *T. riparia* essential oil (TrEO) (a-d). (a), non-infected and non-treated macrophages (negative control); (b), macrophages infected with *L. (L.) amazonensis* (6 parasites/macrophage); (c), infected-macrophages treated with 3 µg/mL of *T. riparia* essential oil (TrEO); (d), infected-macrophages treated with 30 ng/mL of TrEO. The cells were stained with hematoxylin and eosin (100 x objective). Arrow indicates amastigote intracellular forms. All conditions were tested in duplicate and analyzed after 24 h of the incubation at 37°C with 5% CO_2. (e-f), *Leishmania* (e) and GAPDH DNA expression (f) detected by semi-quantitative reverse transcription-polymerase chain reaction. DNA fragments were separated in 1.5% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator. (1), intern negative control (H_2O); (2), *L. (L.) amazonensis* promastigotes cultivated in 199 culture medium (control); (3), cells infected with *L. (L.) amazonensis* (6 parasites/macrophage); (4), cells infected with *L. (L.) amazonensis*. and treated with 30 ng/mL of TrEO; (5) non treated and non infected cells. All of the conditions were analyzed in 3, 6, and 24 h incubation at 37°C. *$p < 0.05$. bp: base pairs.

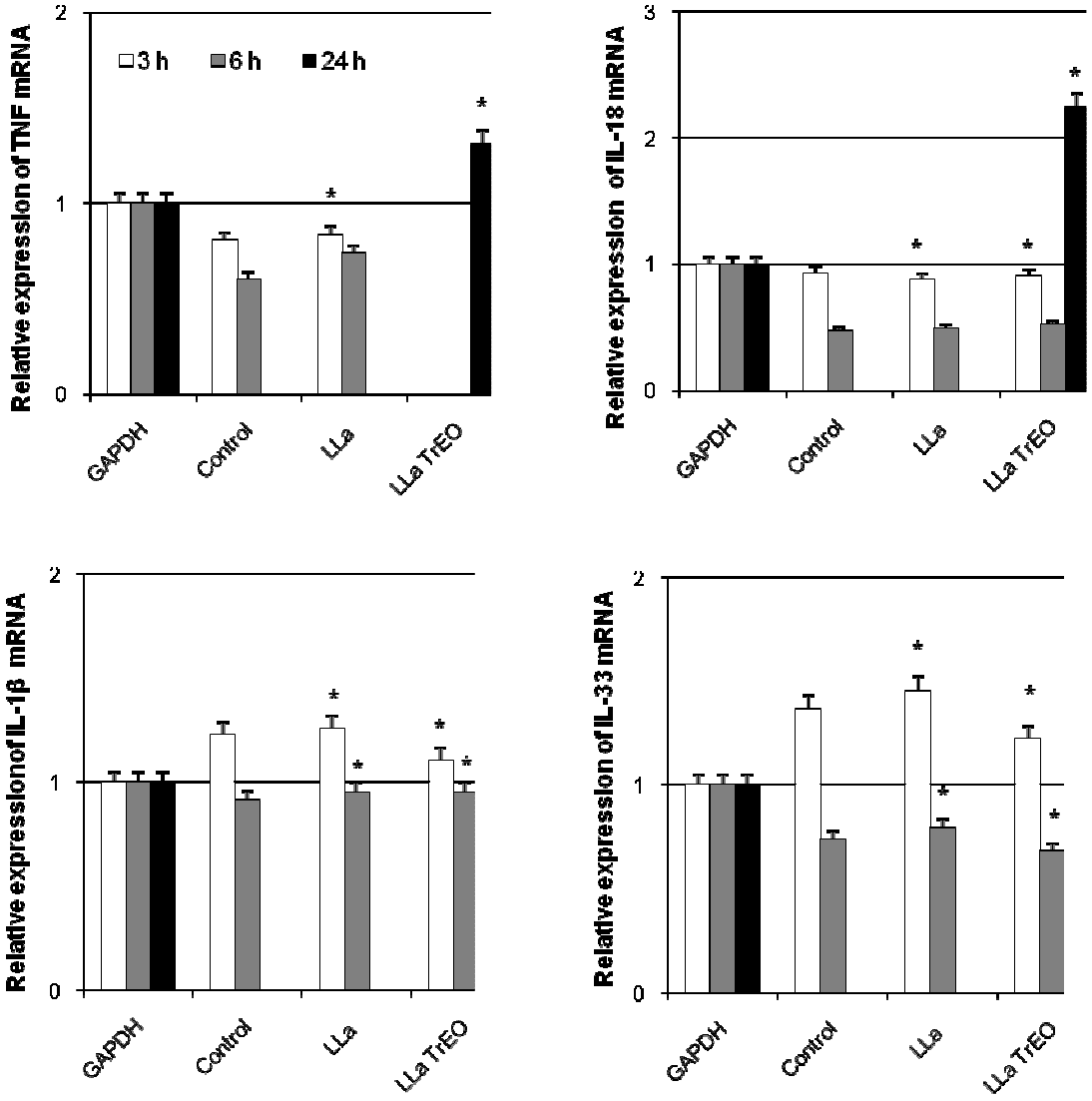


Figure 2. Expression levels of cytokines mRNA by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). LPS: lipopolysaccharide-stimulated macrophages; Control: macrophages non-treated and non-infected (negative control); LLa: macrophages infected by *L. (L.) amazonensis* (6:1); LLa TrEO: macrophages infected by *L. (L.) amazonensis* (6:1) and treated with 30 ng/mL of *TrEO*. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37° C. * p-value significant <0.05.

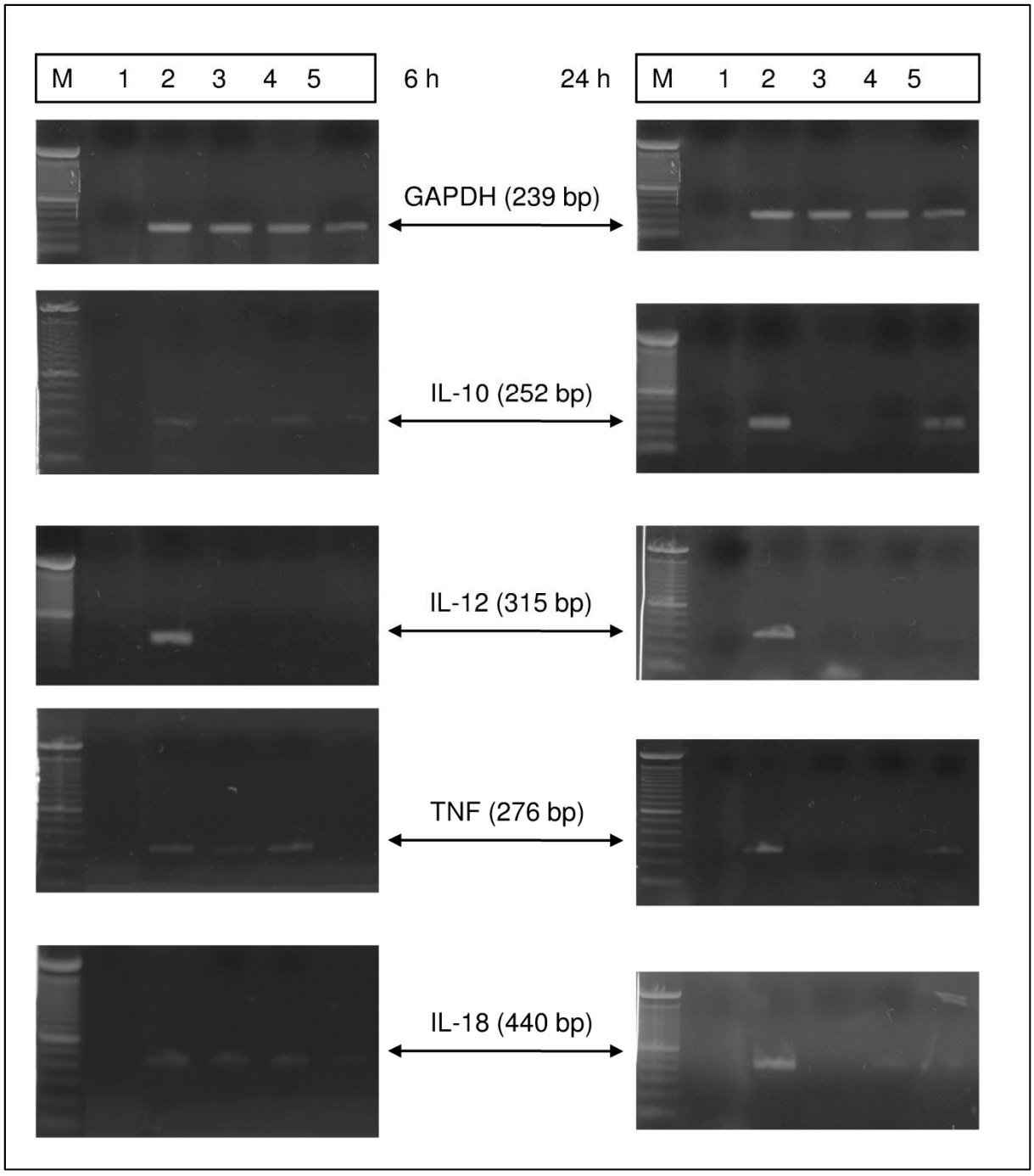


Figure 3. Cytokines mRNA expression induced by TrEO using semi-quantitative RT-PCR. DNA fragments were separated in 1.5% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator. (1), Negative intern reaction control (H_2O); (2), lipopolysaccharide-stimulated macrophages; (3), untreated and uninfected macrophages; (4) LLa, macrophages infected with *L. (L.) amazonensis*; (5) LLa TrEO, macrophages infected with *L. (L.) amazonensis* sp. and treated with 30 ng/mL of TrEO. Above, the expression was observed after 6 and 24 h incubation. bp: base pairs.

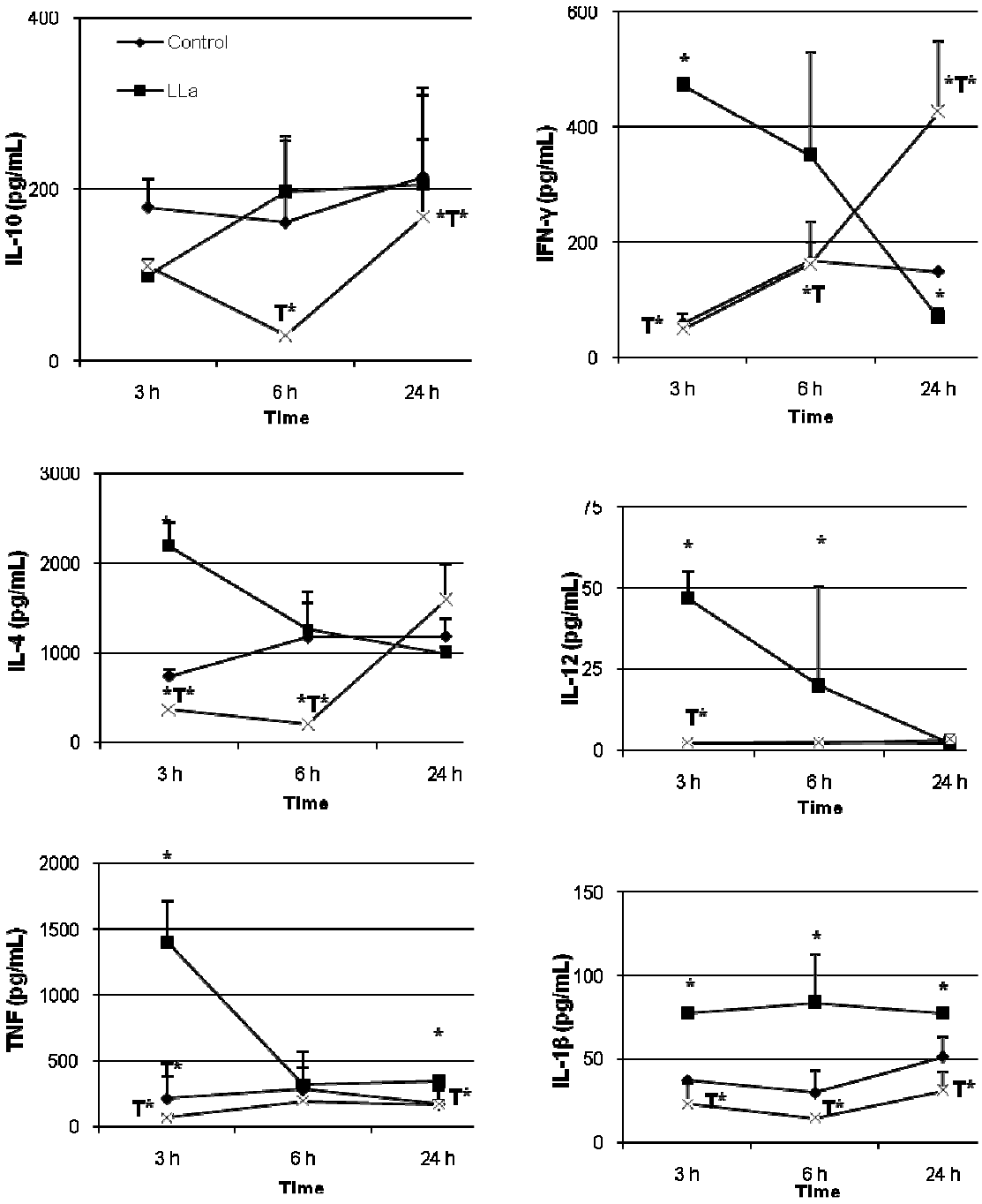


Figure 4. Essentials cytokines involved in *L. (L.) amazonensis* infection during treatment with *Tetradenia riparia* essential oil. Cytokines were quantified in macrophages by multiplex flow cytometry; LPS: lipopolysaccharide-stimulated macrophages; Control: macrophages non-treated and non-infected (negative control); LLa: macrophages infected by *L. (L.) amazonensis* (6:1); LLa TrEO: macrophages infected by *L. (L.) amazonensis* (6:1) and treated with 30 ng/mL of TrEO. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37°C. * p-value significant < 0.05 comparing negative control. T* p-value significant < 0.05 comparing *Leishmania*-infected macrophages.

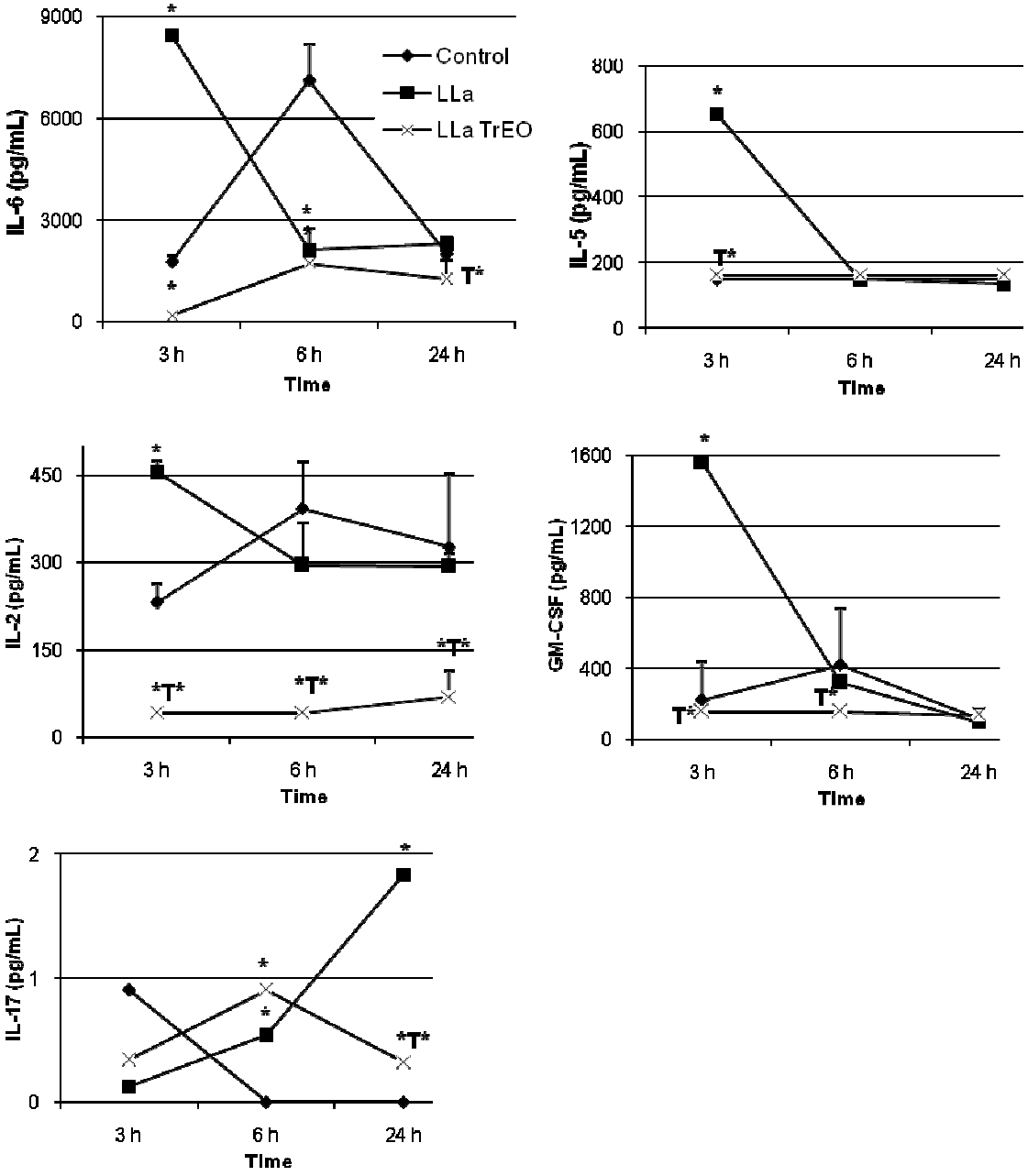


Figure 5. Interleukin 17 and other important cytokines production during *L. (L.) amazonensis* infection treated with *Tetradenia riparia* essential oil. Cytokines were quantified in macrophages by multiplex flow cytometry; LPS: lipopolysaccharide-stimulated macrophages; Control: macrophages non-treated and non-infected (negative control); LLa: macrophages infected by *L. (L.) amazonensis* (6:1); LLa TrEO: macrophages infected by *Leishmania* sp. (6:1) and treated with 30 ng/mL of essential oil. All conditions tested were analyzed within 3, 6 and 24 h incubation at 37°C. * p-value significant < 0.05 comparing negative control. T* p-value significant < 0.05 comparing *Leishmania*-infected macrophages.

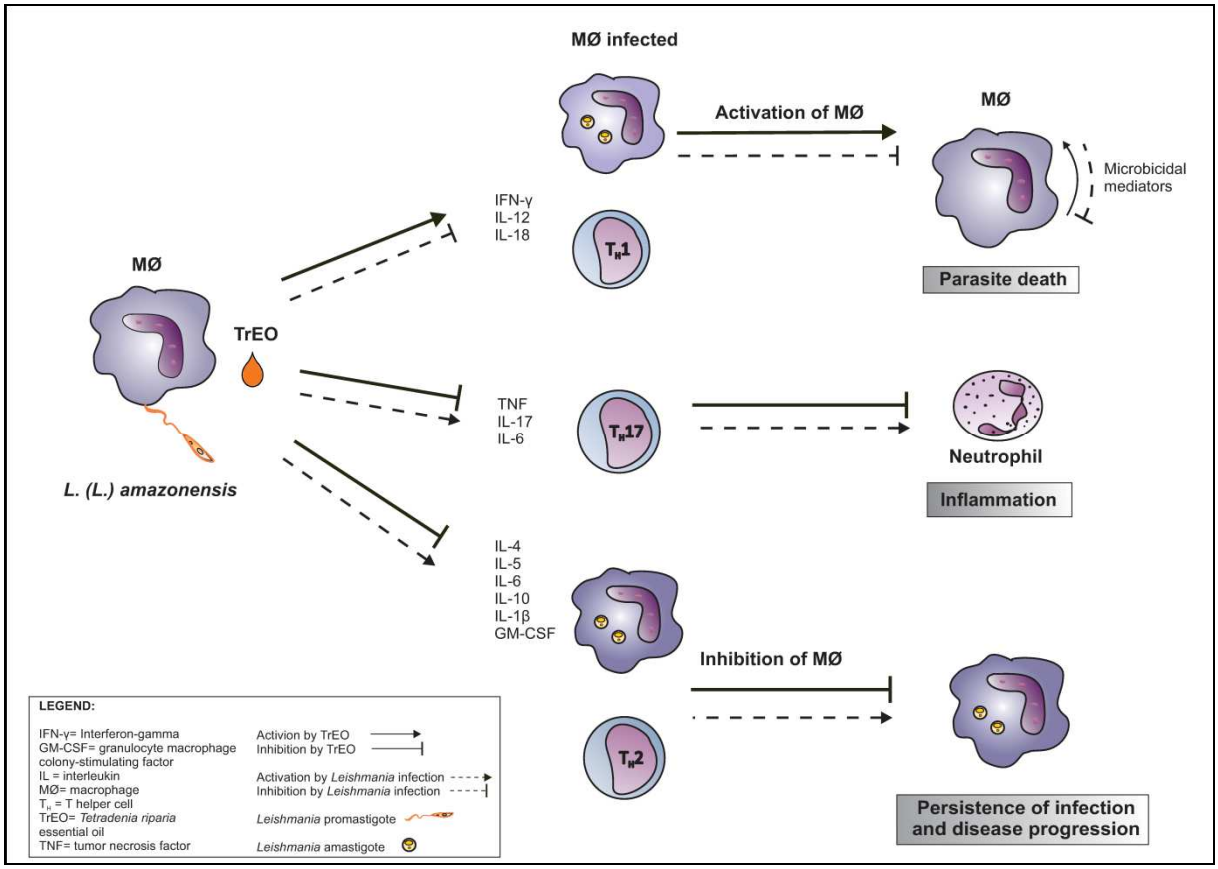


Figure 6. Hypothetical role of signaling events during treatment with *T. riparia* essential oil leading to the induction or inhibition of cells functions during *Leishmania* infection. *L. (L.) amazonensis* promastigotes are phagocytized by macrophages and other types of mononuclear phagocytic cells, where promastigotes transform in amastigote forms. The parasite and immunology contribute to disease outcome or infection persistence. The cytokine environment promotes the activation or inhibition of primary cells involved with cutaneous leishmaniasis that are macrophages, neutrophil, T helper 1(T_H), T_H17 and T_H2 cells. Interferon-gamma (IFN-γ), interleukin 12 (IL-12), and IL-18 production promote the activation of macrophage and T_H1 cell leading to microbicidal mediators secretion by macrophages, such as oxide nitric, reactive oxygen and other. This route leads to parasite death. Moreover, *L. (L.) amazonensis* infection can inhibit these protective cytokines secretion. *T. riparia* essential oil (TrEO) can subvert the inhibition promoted by the parasite and enhanced mainly IFN-γ secretion. IL-1β, IL-4, IL-6, IL-10, TNF and other induced by *Leishmania* infection are involved with macrophage inhibition, T_H2 and T_H17 cells activation favoring the parasite persistence and disease progression. TNF secretion can promote the neutrophil recruitment enhancing the inflammation. T_H2 and T_H17 cytokine profile induced by *L. (L.) amazonensis* infection is related with parasite persistence and disease progression. Moreover, after TrEO treatment, mainly TNF, IL-10 and IL-4 production were inhibited. Thus, T_H2 and T_H17 cytokines are inhibited, and protective cytokines are stimulated by TrEO that it contributes to *Leishmania* death, control of inflammation and disease outcome.

CAPÍTULO III

Conclusões e perspectivas futuras

Os resultados obtidos nos ensaios *in vitro* para a atividade do óleo essencial de *T. riparia* (TrEO) e do seu isolado 6,7-dehidroroileanona (TrROY) permitem concluir que:

O isolamento de compostos leishmanicidas como o TrROY a partir do TrEO sustenta o uso medicinal tradicional desta planta por algumas populações para o tratamento de infecções parasitológicas; TrEO e TrROY são capazes de subverter a inibição da produção de nitrito promovida pela infecção por *L. (L.) amazonensis*, e induziram a produção em níveis normais na ausência da infecção.

Na ausência de infecção, o TrEO foi capaz de induzir a expressão de mRNA e produção de citocinas pró-inflamatórias principalmente da resposta imune celular inata; TrEO também mostrou um grande potencial em suprimir a produção de IL-10 contribuindo para a regulação da imunidade na ausência da infecção e durante esta; TrEO estimulou uma maior expressão de mRNA e a produção de interferon-gamma (IFN-γ) favorecendo a resposta imune protetora contra a leishmaniose.

Considerando todos os efeitos leishmanicidas e imunomoduladores do óleo essencial derivado da planta *T. riparia,* este poderia ser utilizado como uma terapia alternativa para o tratamento da leishmaniose cutânea. Todos os resultados oferecem um trampolim para a documentação deste óleo essencial para o comércio e e informação aos decisores políticos no caminho para a regulamentação da produção e validação de produtos naturais para o tratamento da leishmaniose cutânea. Para isso, sugere-se que os efeitos biológicos e farmacológicos do TrEO e seu isolado sejam bem conhecidos *in vivo* e em humanos para assegurar o uso seguro destes produtos e para estabelecer os protocolos de tratamento. O potencial imunomodulador deste óleo essencial na ausência e presença de infecção deve ser considerado para orientar a seleção de regimes terapêuticos apropriados para cada condição clínica.

Como perspectivas futuras, acredita-se que a eficácia e segurança do tratamento com o TrEO paara a infecção por *L. (L.) amazonensis* seja avaliado também em modelos animais e posteriormente, em ensaios clínicos. Além disso, com a finalidade de se desenvolver novos agentes leishmanicidas, outros compostos devem ser isolados da planta *T. riparia* e testados inicialmente *in vitro* e *in vivo*, assim como a interação farmacológica entre os isolados. A utilização de produtos naturais, como o TrEO, como tratamento alternativo da leishmaniose

poderá contribuir com os indivíduos que apresentam falha terapêutica ou resistência parasitária, e em populações que utilizam plantas medicinais e não possuem acesso gratuito aos medicamentos para a cura da leishmaniose.