UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

Matheus Dellaméa Baldissera

AVALIAÇÃO DA ATIVIDADE DE TRÊS COMPOSTOS DO ÓLEO DE MELALEUCA SOBRE O *Trypanosoma evansi*

Santa Maria, RS

2016

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Dissertação de mestrado apresentada ao curso de Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Mestre em Farmacologia**

Orientador: Prof.ª Dr.ª Silvia Gonzalez Monteiro

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Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Baldissera, Matheus Dellaméa
Avaliação da atividade de três compostos do óleo de melaleuca sobre o Trypanosoma evansi. / Matheus Dellaméa Baldissera.-2016.
63 f.; 30cm

Orientadora: Silvia Gonzalez Monteiro
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós-Graduação em Farmacologia, RS, 2016

1. Melaleuca alternifolia 2. "Surra" 3. Plantas medicinais 4. Tripanossomose I. Monteiro, Silvia Gonzalez II. Título.

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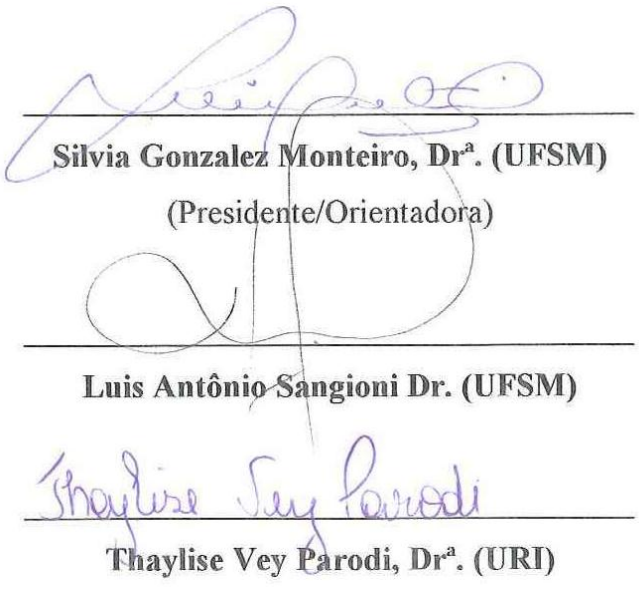
Aprovado em 15 de fevereiro de 2016:



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DEDICATÓRIA

Aos meus pais

Sérgio e Cláudia, pela confiança, apoio, carinho e por terem me guiado pelo caminho do bem. Amo muito vocês.

AGRADECIMENTOS

Primeiramente gostaria de agradecer a minha orientadora Dr.ª Silvia Gonzalez Monteiro pela confiança, profissionalismo, ensinamentos e por abrir as portas para que eu pudesse seguir meu caminho.

Aos meus amigos e colegas de laboratório Thirssa H. Grando, Mariangela F.de Sá, Lucas T. Gressler, Bianca C. Z. Porto, Luciana Dalla Rosa, Guilherme do Carmo, Luciana Cossetin e Andressa Schafer por todos os momentos descontraídos na sala de estudo, além de todo o apoio e auxílio na execução do estudo. Obrigado pela amizade.

A toda equipe LAPAVET.

A minha namorada Carine de Freitas Souza, pelo apoio e carinho.

Ao programa de Pós-Graduação em Farmacologia que possibilitou a conclusão desta minha etapa profissional.

A CAPES pelo auxílio financeiro a mim concedido meu agradecimento.

Um agradecimento em especial ao meu grande amigo Drº Aleksandro Schafer da Silva, o "Aleks". Meu muito obrigado pelo conhecimento concedido, amizade, e pela motivação em pesquisar cada vez mais.

Por fim, agradeço a minha família, minha mãe e ao meu pai que sempre me incentivaram nos estudos.

“O investigador sofre decepções, os longos meses passados em uma direção errada, os fracassos. Mas as falhas também são úteis, porque, se bem analisadas, podem levar ao sucesso. E para o pesquisador não há alegria comparável à de uma descoberta, ainda que pequena”.

“Sir Alexander Fleming”

RESUMO

AVALIAÇÃO DA ATIVIDADE DE TRÊS COMPOSTOS DO ÓLEO DE MELALEUCA SOBRE O *Trypanosoma evansi*

AUTOR: Matheus Dellaméa Baldissera
ORIENTADOR: Silvia Gonzalez Monteiro

O objetivo deste estudo foi avaliar a atividade tripanocida do terpinen-4-ol, gama-(γ)-terpinen e alfa-(α)-terpinen contra o *Trypanosoma evansi in vitro* e *in vivo.* Os testes *in vitro* foram realizados em meio de cultura contendo *T. evansi*, utilizando-se três concentrações de cada composto (0.5, 1.0 e 2.0%) de forma individual e em associação. A contagem de tripomastigotas vivas foi realizada em câmara de Neubauer após 1, 3, 6 e 9 horas pós-incubação. A partir dos testes *in vitro*, foi possível observar a morte dos parasitos de forma concentração-dependente, exceto o composto α-terpinen que foi capaz de eliminar todos os parasitos 1 h pós-incubação em todas as concentrações testadas. Para realização dos ensaios *in vivo*, primeiramente foram utilizados 70 camundongos divididos em 10 grupos (A ao J), com sete animais cada grupo. O grupo A serviu como controle negativo (animais sadios); grupos de B a J foram infectados com 0,1 mL de sangue contendo 10^5 tripomastigotas com *T. evansi*. O grupo B serviu como controle positivo; grupos C, D e E receberam terpinen-4-ol, γ-terpinen e α-terpinen via oral na dose de 1.0 mL kg^{-1}, respectivamente. O grupo F recebeu a associação dos compostos terpinen-4-ol + γ-terpinen, grupo G recebeu a associação dos compostos terpinen-4-ol + α-terpinen; grupo H recebeu a associação do γ-terpinen + α-terpinen via oral na dose de 1.0 mL kg^{-1} (0.5 mL kg^{-1} de cada composto). O grupo I recebeu a associação dos compostos terpinen-4-ol + γ-terpinen + α-terpinen via oral na dose de 1.0 mL kg^{-1} (0.335 mL kg^{-1} de cada composto). O grupo J foi tratado com aceturato de diminazeno na dose terapêutica (dose única de 3.5 mg kg^{-1}, via intramuscular) para validação do teste. Foi possível verificar que os animais do grupo E (tratados com α-terpinen) apresentaram um aumento da longevidade quando comparados ao grupo B, porém sem eficácia curativa, pois todos os animais deste grupo morreram durante o período de observação da parasitemia. A partir disto, um segundo experimento foi realizado testando a associação entre o α-terpinen e o aceturato de diminazeno. Foram utilizados 28 camundongos divididos em 4 grupos (A, B, C e D; n=7). O grupo A foi formado por animais não infectados (controle negativo), o grupo B foi formado por animais infectados (controle positivo), o grupo C foi formado por animais infectados e tratados com aceturato de diminazeno (dose única de 3.5 mg kg^{-1}, via intramuscular), e o grupo D foi formado por animais infectados e tratados com associação de aceturato de diminazeno (dose única de 3.5 mg kg^{-1}, via intramuscular) mais α-terpinen (1.0 mL kg^{-1} via oral durante 10 dias). A partir deste resultado, foi possível observar um aumento da eficácia curativa (57.14 %) no grupo D quando comparado ao grupo C, que obteve apenas 14.28 % de eficácia curativa. Baseado nestes resultados, o α-terpinen foi o composto presente no óleo essencial de *M. alternifolia* que apresentou atividade tripanocida.

Palavras-chave: *Melaleuca alternifolia*. "Surra". Plantas medicinais. Tripanossomose.

ABSTRACT

EVALUATION OF THE ACTIVITY OF THREE COMPOUNDS OF MELALEUCA OIL AGAINST *Trypanosoma evansi*

AUTHOR: MATHEUS DELLAMÉA BALDISSERA
ADVISOR: SILVIA GONZALEZ MONTEIRO

This study aimed the evaluation of the trypanocidal activity of terpinen-4-ol, γ-terpinene and α-terpinene against *Trypanosoma evansi in vivo* and *in vitro*. The *in vitro* assay was performed in culture medium of *T. evansi*, using three different concentrations of each compound (0.5, 1.0 and 2.0%), individually and in synergism. Motile (live) trypomastigotes were counted using a Neubauer chamber after 1, 3, 6 and 9 hours of incubation. Death of parasites occurred in a concentration-dependent manner, except for α-terpinene that was able to eliminate all parasites after 1 hour of incubation at all concentrations tested. Seventy mice were equally assigned into 10 groups (A to J) for the *in vivo* tests. Group A served as a negative control (healthy animals); groups B - J were infected with 0.1 mL of blood containing 10^5 *T. evansi* trypomastigotes. Group B served as a positive control; groups C, D and E received oral doses of 1.0 mL kg^{-1} of terpinen-4-ol, γ-terpinene and α-terpinene, respectively. Associations of the three compounds were orally administered to groups F – I: terpinen-4-ol + γ-terpinene to group F, terpinen-4-ol + α-terpinene to group G, α-terpinene + γ-terpinene at 1.0 mL kg^{-1} (0.5 mL kg^{-1} for each compound) to group H, terpinen-4-ol + γ-terpinene + α-terpinene at 1.0 mL kg^{-1} (0.335 mL kg^{-1} of each compound) to group I. A therapeutic dose of diminazene aceturate (single dose of 3.5 mg kg-1, intraperitoneally) was administered to Group J aiming test validation. Animals in group E (treated with α-terpinene) showed an increase of longevity when compared to animals in group B. However, no curative effectiveness was observed, since all animals in this group died during the observation period. In order to evaluate the association between α-terpinene and diminazene aceturate, twenty-eight mice were assigned into 4 groups (A, B, C and D, n = 7). Group A included animals uninfected (negative control); group B was composed by infected animals (positive control); group C consisted of infected animals treated with diminazene aceturate (single dose of 3.5 mg kg^{-1}, im); and group D consisted of infected animals treated with an association of diminazene aceturate (single dose of 3.5 mg kg^{-1}, im) and α-terpinene (1.0 ml kg^{-1}, orally administered for 10 days). It was observed, as a result of this experiment, an increase in the curative effectiveness (57.14%) in group D when compared to group C, which showed only 14.28% of curative effectiveness. Based on these results, α-terpinene was the compound present in *M. alternifolia* essential oil that showed trypanocidal activity.

Keywords: *Melaleuca alternifolia*. "Surra". Medicinal Plants. Tripanosomiasis.

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SUMÁRIO

APRESENTAÇÃO

Os resultados e a discussão que fazem parte desta dissertação estão apresentados sob a forma de manuscrito publicado (*Experimental Parasitology*), o qual se encontra no item **MANUSCRITO.** As secções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se no próprio manuscrito e representam a íntegra deste estudo.

As **REFERÊNCIAS BIBLIOGRÁFICAS** se referem somente as citações que aparecem nos itens **INTRODUÇÃO e REVISÃO BIBLIOGRÁFICA** desta dissertação.

1 INTRODUÇÃO

O *Trypanosoma evansi* é um protozoário hemoflagelado pertencente a seção Salivaria, agente causador da patologia conhecida como “Mal das Cadeiras” ou “Surra” em equinos (HERRERA et al., 2004). Geograficamente é distribuído por todas as áreas tropicais e subtropicais do mundo, parasitando diferentes espécies de animais domésticos e silvestres, e raramente humanos (SILVA et al., 2002; JOSHI et al., 2005). A transmissão da doença ocorre em sua maior parte por meio de moscas hematófagas (*Tabanus* spp. *Stomoxys* spp.), sendo também possível a transmissão por morcegos hematófagos (*Desmodus rotundus*) (HOARE et al., 1972). A doença é caracterizada pela rápida perda de peso, variáveis graus de anemia, febre intermitente, edema dos membros pélvicos e distúrbios locomotores (HERRERA et al., 2004; RODRIGUES et al., 2005).

Na ausência de vacinas, a terapia farmacológica atual é possivelmente a principal forma de controle terapêutico da patologia, baseada principalmente em quatro diferentes fármacos: suramina, aceturato de diminazeno, quinapiramina e melarsomina (BRUN et al., 1998). Entretanto, os tratamentos com estes medicamentos estão distantes de serem satisfatórios devido aos baixos índices terapêuticos, elevada toxicidade, efeitos secundários inaceitáveis, surgimento de cepas resistentes e elevado custo principalmente para países subdesenvolvidos. Assim existe uma necessidade de busca por novas drogas com maior eficácia e menores efeitos tóxicos aos hospedeiros (URBINA, 2010).

Atualmente, diversos autores têm relatado resultados promissores com o emprego de compostos naturais extraídos de plantas no controle de diferentes parasitos sanguíneos, como *T. evansi, Trypanosoma cruzi* e *Leishmania* spp. (MUGANZA et al., 2015; SILVA et al., 2015). Estudos têm demonstrado resultados satisfatórios com a utilização de óleos essenciais no tratamento das tripanossomoses, reduzindo a parasitemia e prevenindo danos associados a infecção. Estes fatores têm levado a busca por princípios ativos mais eficazes e menos tóxicos, que combatam o agente etiológico e os efeitos da infecção.

Recente estudo publicado pelo nosso grupo de pesquisa demonstrou o efeito tripanocida *in vitro* e *in vivo* do óleo essencial de *Melaleuca alternifolia* sobre o *T. evansi.* A análise cromatográfica revelou um total de 15 compostos presentes neste óleo, sendo os três principais o terpinen-4-ol, γ-terpinen e α-terpinen, com 41.98, 20.14 e 9.85% da composição total do óleo essencial de *M. alternifolia,* respectivamente (BALDISSERA et al., 2014). O terpinen-4-ol é conhecido por apresentar propriedades antiparasitárias (GRANDO et al., 2015) e antimicrobianas (CUARON et al., 2013) devido a capacidade de causar danos na

membrana, levando a perda de íons potássio e inibição da cadeira respiratória (CARSON et al., 2002). Na literatura a ação do γ-terpinen e α-terpinen em atividades biológicas são escassas, mas podemos destacar a atividade antioxidante (NAJAFLAN e ZAHEDIFAR, 2015) e antifúngica (SALEM et al., 2016). Neste contexto, este estudo teve como objetivo avaliar a atividade tripanocida *in vitro* e *in vivo* dos três compostos majoritários do óleo essencial de *M. alternifolia* individual e em sinergismo sobre *T. evansi.*

2 REVISÃO DE LITERATURA

2.1 *TRYPANOSOMA EVANSI*

2.1.1 Aspectos gerais e distribuição

Trypanosoma evansi é um protozoário flagelado pertencente ao reino Protozoa, filo Euglenozoa, ordem Kinetoplastida, família Trypanosomatidae (SILVA et al., 2002). O mesmo pertence a seção Salivaria, ou seja, aqueles transmitidos por picadas de vetores biológicos (HOARE, 1972). Foi o primeiro tripanossoma patogênico descoberto em 1880 por Griffith Evans, um médico veterinário do Reino Unido, encontrados no sangue de cavalos e camelos (MAUDLIN et al., 2004).

Este protozoário é classificado como monomórfico, ou seja, apresenta apenas a forma infectante (tripomastigota) durante toda sua vida (BRUN et al., 1998). Apresenta forma alongada e achatada com núcleo evidente, com comprimento variando entre 24 ± 4 µm e largura entre 1,5 ± 0,5 µm. Possui uma membrana ondulante presente em toda extensão do protozoário, bem como um flagelo na porção terminal (SILVA et al., 2002). O cinetoplasto pode ou não estar presente, uma vez que estirpes brasileiras não possuem esta organela (VENTURA et al., 2002) (Figura 1), o que torna o parasito mais resistente ao tratamento.

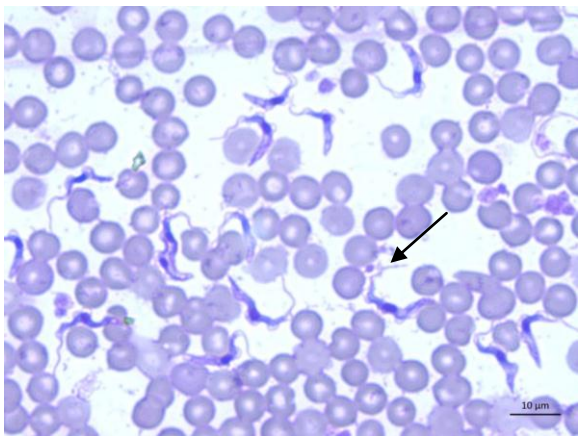

Figura 1 – Formas tripomastigotas em esfregaço sanguíneo de um camundongo infectado experimentalmente com *Trypanosoma evansi* (seta)

Fonte: Arquivo pessoal

A doença causada pelo *T. evansi* é conhecida globalmente como “Surra”, e na América Latina pode ser também denominada “Mal das Cadeiras”. Desde a descoberta do parasito por Griffith Evans em 1880, foram relatados casos de tripanossomose por *T. evansi* em vários continentes. O *T. evansi* possui a maior distribuição geográfica entre todas as tripanossomoses (DESQUENES et al., 2013), sendo encontrado em todas as áreas tropicais e subtropicais do mundo, podendo ocorrer na África, Índia, Malásia, Indonésia, China, Rússia, Filipinas, América Central e América do Sul (Figura 2).

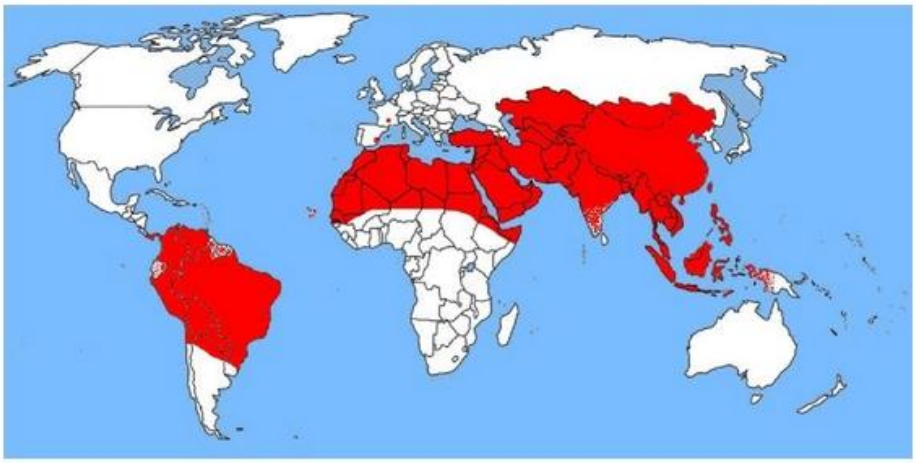

Figura 2 – Distribuição geográfica do *Trypanosoma evansi* no mundo.

Fonte: Desquenes et al., (2013)

Este protozoário teve sua origem no continente africano, sendo introduzido no continente americano pelos primeiros colonizadores europeus. Conforme HOARE (1972), a chegada do *T. evansi* na América do Sul deve-se a importação de cavalos provindos da Espanha, tendo casos descritos no Brasil, Bolívia, Colômbia, Guiana Francesa, Peru, Suriname, Venezuela e Argentina. Casos de infecções naturais por *T. evansi* foram relatados no Rio Grande do Sul (COLPO et al., 2005; FRANCISCATO et al., 2007), Mato Grosso do Sul (BRANDÃO et al., 2002), Santa Catarina (DA SILVA et al., 2008), e no Pantanal, onde é endêmica, com casos recorrentes (SILVA et al., 2002). Desde então, esta doença tem causado numerosos surtos com mortes em equinos, resultando em elevados prejuízos principalmente aos criadores desses animais (SILVA et al., 2002).

A existência de uma grande variedade de reservatórios silvestres e domésticos, a capacidade de difundir-se silenciosamente através de portadores saudáveis e a possibilidade de transmissão oral em animais que se alimentam de carcaças de outros animais parasitados

(RAMIREZ et al., 1972), e transmissão em cães e capivaras, através de brigas entre animais infectados e não infectados são os principais motivos para a manutenção da doença. Capivaras (*Hydrochaeris hydrochaeris*), quatis (*Nasua nasua*) e morcegos hematófagos são considerados os principais reservatórios silvestres (SILVA et al., 2002).

2.1.2 Ciclo biológico e transmissão

O ciclo de vida do *T. evansi* consiste na transmissão das formas tripomastigotas de forma mecânica de um hospedeiro parasitado para outro não parasitado, que são transmitidos por insetos hematófagos durante o repasto sanguíneo. Os principais vetores pertencem aos gêneros *Tabanus* sp., entretanto insetos dos gêneros *Stomoxys* sp., *Haematopota* sp. e *Lyperosia* sp. podem transmitir o parasito (SILVA, 2002). Em moscas do gênero *Stomoxys*, a sobrevivência do parasito no aparelho bucal pode chegar a 480 minutos (SUMBA et al., 1998). Quanto menor a diferença de tempo entre os repastos sanguíneos, maiores são as possibilidades da passagem do parasita para um novo hospedeiro (HOARE, 1972). Segundo um modelo matemático de transmissão do agente por tabanídeos proposto por DESQUESNES et al. (2009), para que ocorram frequentes surtos em uma determinada população, a prevalência de animais infectados deve estar em torno de 10 a 15% do total. De acordo com os autores, nesse modelo novos surtos podem acontecer em períodos de 3 a 5 anos. Condições estressantes como alterações climáticas e alimentares podem favorecer a ocorrência da doença. *T. evansi* multiplica-se na corrente circulatória de morcegos hematófagos do gênero *D. rotundus*, onde permanecem por até um mês, tornando esses mamíferos importantes transmissores e hospedeiros da doença principalmente na América Central e do Sul (HOARE, 1972).

Ainda, existe a possibilidade de transmissão oral em carnívoros que se alimentam da carcaça de animais infectados ou através de ferimentos ocasionados por brigas (RAMIREZ et al., 1979, BAZZOLI et al., 2002). Está via pode ser considerada importante na dispersão da infecção em cães, quatis e capivaras, que podem ser infectados em consequência das brigas. Quatis e capivaras tem um comportamento agressivo o que pode levar a transmissão do protozoário entre eles, mantendo a infecção no grupo social, já que a forma crônica da doença causada por *T. evansi* foi identificada nesses animais, que são reservatórios do agente. Cães e ruminantes também podem atuar como reservatórios do *T. evansi* quando o curso da doença for crônico (HERRERA et al., 2004). Recentemente, um estudo conduzido por

CAMPIGOTTO et al. (2015) relataram transmissão venérea da infecção em ovelhas prenhas, também com forte possibilidade de transmissão através do colostro e leite.

A doença afeta um grande número de animais domésticos e selvagens, entre eles: cavalos, camelos, bovinos, gatos, caprinos, suínos, cães, búfalos, elefantes, capivaras, quatis, antas, tatus, marsupiais, veados e pequenos roedores silvestres (LEVINE, 1973; SILVA et al., 2002; ATARHOUCH et al., 2003; HERRERA et al., 2004). Por fim, o primeiro caso humano foi relatado em 2005 em um fazendeiro na Índia, onde o mesmo apresentava as formas tripomastigotas no sangue associada a episódios febris (JOSHI et al, 2005). Porém, a infecção em humanos é rara devido a presença de uma apoliproteína ligada a lipoproteínas de alta densidade, chamada apolipoproteína L-1 (APOL1), que é considerada um fator tripanolítico. A mesma penetra no protozoário por endocitose promovendo a formação de poros na membrana lisossomal, induz o rompimento desta estrutura provocando a morte celular (VANHAME et al., 2003).

2.1.3 Patogênese

A patogenicidade do protozoário no hospedeiro varia de acordo com a espécie animal infectada, estirpe do flagelado, fatores não específicos que estão afetando o animal concomitantemente a infecção (estresse, outras patologias) e condições epizootiológicas locais (HOARE, 1972). No hospedeiro, o protozoário se divide assexuadamente por fissão binária nos espaços teciduais extracelulares, iniciando a multiplicação já no local da picada (pele), seguida pela invasão dos parasitos na corrente circulatória e sistema linfático, levando a respostas febris e induzindo uma resposta inflamatória (CONNOR e VAN DEN BOSSCHE, 2004). O *T. evansi* possui uma interessante ferramenta para evadir as defesas do hospedeiro, a expressão das glicoproteínas variáveis de superfície, ou “variant surface glycoproteins” (VSGs). Toda a superfície do protozoário (aproximadamente 95%) é recoberta por VSGs, que se alteram e burlam a resposta imune humoral do hospedeiro (PAYS et al., 2004). Os picos de parasitemia ocorrem devido a variações antigênicas na superfície do parasito, ou seja, das VSGs. Conforme os anticorpos são produzidos, há eliminação do clone corrente, mas sucessivos novos padrões de antígenos de superfície são gerados para evadir a resposta do hospedeiro (LUCAS et al., 1992), dificultando o desenvolvimento de vacinas.

2.1.4 Sinais clínicos, alterações patológicas e diagnóstico

No início da doença os sinais clínicos são geralmente inespecíficos e depende das lesões induzidas nos diferentes órgãos e tecidos, bem como do tipo de hospedeiro e virulência da cepa (SILVA et al., 2002). Geralmente, a fase aguda da infecção é caracterizada pelo surgimento de febre intermitente, edema subcutâneo, anemia progressiva, cegueira, letargia e alterações hemostáticas. Os animais afetados agudamente podem morrer dentro de semanas ou poucos meses. No entanto, as infecções crônicas podem durar anos (BRUN et al., 1998). Durante a fase crônica, os parasitos podem invadir o sistema nervoso central e causar agravamento dos sinais clínicos, sendo observado caquexia, edema, incoordenação motora e paralisia dos membros posteriores (BRANDÃO et al., 2002; SILVA et al., 2002; RODRIGUES et al., 2005) (Figura 3). Os sinais neurológicos têm sido descritos na fase terminal da doença, principalmente em equinos, bovinos, veados e búfalos infectados naturalmente (TUNTASUVAN et al., 1997; TUNTASUVAN e LUCKINS, 1998; TUNTASUVAN et al., 2003; RODRIGUES et al., 2005). Recentemente, alterações neurológicas associadas ao comportamento foram relatadas em ratos infectados experimentalmente na fase aguda da infecção. Diminuição de memória e aumento da atividade depressora foram observados, associadas com a diminuição de enzimas do metabolismo energético, como a piruvato quinase, importante na produção de glicose para o cérebro, o qual demanda de grande aporte energético (BALDISSERA et al., 2015), sugerindo que alterações neurológicas também podem ser observadas na fase aguda.

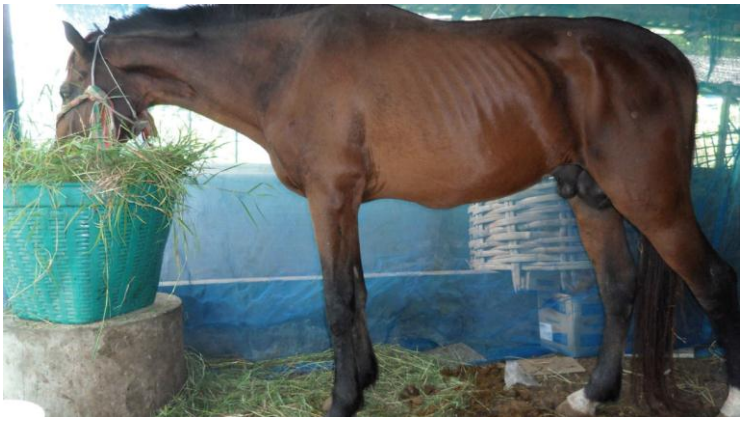

Figura 3 – Cavalo naturalmente infectado com *Trypanosoma evansi* apresentando perda de peso e edema testicular.

Fonte: Desquenes et al. (2013).

A principal alteração hematológica identificada em animais com tripanossomose é a anemia acentuada (CONNOR e VAN DEN BOSSCHE, 2004). A doença é marcada pela diminuição no valor de hematócrito, na concentração de hemoglobina e no número de eritrócitos totais. O principal mecanismo responsável pela anemia seria a liberação de hemolisinas e enzimas pelos tripanossomas, que induziram lesões diretamente na membrana dos eritrócitos, aumentando a fragilidade dos mesmos, juntamente com lesões induzidas pelos antígenos do parasito que se aderem à superfície dos eritrócitos e a adesão dos complexos antígeno-anticorpo, que irão promover a eritrofagocitose (CONNOR e VAN DEN BOSSCHE, 2004). Além disso, estudos relatam que a membrana dos eritrócitos dos animais infectados apresenta uma maior fragilidade osmótica, o que a torna mais susceptível a lise (MIJARES et al., 2010). Recentemente, a anemia também foi atribuída à peroxidação lipídica, pois o aumento de radicais livres acarreta danos à membrana eritrocitária (WOLKMER et al., 2009).

A identificação direta do agente pode ser realizada na fase aguda da doença, através da análise de esfregaço sanguíneo ou aspirado de linfonodos em microscópio. A busca por protozoários também pode ser realizada analisando-se uma gota de sangue entre lâmina e lamínula (busca por parasitos móveis), ou corando-se o esfregaço sanguíneo com Giemsa (KUBIAK e MOLFI, 1954). Alternativamente, métodos mais sensíveis podem ser utilizados para a detecção do *T. evansi*, como a reação em cadeia da polimerase (PCR). Métodos sorológicos para identificação de anticorpos específicos anti-*T. evansi* no soro também podem ser utilizados, através das técnicas de ELISA (“enzyme-linked immunosorbent assay”) e CAAT (“card agglutination test for trypanosomosis”) (HILALI et al., 2004).

2.1.5 Tratamento

O tratamento farmacológico é o mais importante método de combate e controle das tripanossomoses em animais domésticos. A terapêutica é baseada no emprego de quatros fármacos: suramina, aceturato de diminazeno, quinapiramina e melarsomina (BRUN et al., 1998). A escolha do fármaco, da via de aplicação e da dose depende da espécie animal infectada. Entretanto, por apresentar o maior índice terapêutico, o aceturato de diminazeno é o mais utilizado entre os fármacos disponíveis atualmente. Devido a seu constante uso, problemáticas durante o tratamento, como elevada toxicidade e resistência tem sido reportado por diversos autores. Em camelos, sinais de intoxicação como salivação e convulsão foram observados, associado ao aumento de biomarcadores hepáticos e renais, sugerindo dano no

fígado e rim quando tratados na dose de 3.5 mg kg^{-1} via intramuscular (HOMEIDA et al., 1981). Nesse trabalho, devido a recidiva, a dose foi aumentada para 10 e 40 mg kg^{-1}, ocasionando congestão e focos hemorrágicos nos tecidos hepático e renal, onde o camelo tratado na maior dose (40 mg kg^{-1}) foi a óbito 4 horas após o tratamento.

Estudos realizados por TUNTASUVAN et al. (2003) com o uso desse medicamento em equinos e mulas experimentalmente infectados com *T. evansi*, demonstrou ser eficaz somente na primeira aplicação realizada, eliminando os parasitos do sangue periférico. Porém, no segundo tratamento, 50% dos equinos e 25 % das mulas continuaram positivos para *T. evansi*. Além disso, esse medicamento demonstrou toxicidade após a administração. Estudo realizado por DOYLE et al. (2007), utilizando ratos *Wistar* experimentalmente infectados com *T. evansi* com dose única de aceturato de diminazeno (3,5 mg kg^{-1} via intramuscular), observou que os tripanossomas foram eliminados da corrente sanguínea em até dois dias, porém, após 15 a 30 dias ocorria recidiva da parasitemia.

A resistência ao aceturato está associada a expressão de um gene, o TeDR40. WITOLA et al. (2005) demonstraram que este gene tem a expressão aumentada em 1000 vezes em parasitos resistentes. Outro fator que também pode estar relacionado ao aparecimento de resistência é a ausência de cinetoplasto nas cepas brasileiras, uma vez que um dos mecanismos de ação do aceturato é sobre o cinetoplasto (GONZALEZ et al., 1997).

O reaparecimento dos tripanossomas na corrente sanguínea pode dar-se à sobrevivência dos parasitos devido à resistência ou ao resultado do escape à ação dessas drogas, possivelmente quando os parasitos se alojam no sistema nervoso central e, após excreção do fármaco, reinvadem a circulação sanguínea, já que esses medicamentos não ultrapassam a barreira hematoencefálica (KENNEDY et al., 1997). Devido as recidivas, novos tratamentos com doses superiores precisam ser realizados, o que também contribui para o surgimento de resistência e casos de toxicidade.

2.2 PRODUTOS NATURAIS NO TRATAMENTO DAS TRIPANOSSOMOSES

As plantas tem sido uma fonte de medicamentos para humanos e animais desde os tempos ancestrais (ODHIAMBO et al., 2011). Com o avanço da ciência farmacêutica, os fitoquimicos foram identificados como responsáveis por muitas atividades biológicas até então desconhecidas. Com o avanço das técnicas de identificação e isolamento de plantas e compostos, muitos destes foram utilizados para a fabricação de drogas contra doenças infecciosas (KASILO et al., 2010). Estudo realizado por NEWMAN et al. (2003)

demonstraram que entre os anos de 1981 e 2002, 75% dos fármacos desenvolvidos para o tratamento de doenças infecciosas pode ser atribuída a origens naturais. Dentro deste contexto, pode ser destacado o uso de óleos essenciais. As plantas geralmente possuem uma quantidade elevada de óleos essenciais, que são misturas complexas de compostos voláteis e semi-voláteis que exibem amplo espectro farmacológico, como antibacteriano (SANTOS et al., 2014), antiviral (LEE et al., 2009) e antiparasitário (ANTHONY et al., 2005).

Atualmente, estudos tem demonstrado a ação dos óleos essenciais no combate as tripanossomoses (SANTORO et al., 2007; DA SILVA et al., 2013). Pode-se citar diversos óleos essenciais já pesquisados com ação sobre *T. cruzi*, como: *Eucalyptus globulus* (nome popular: eucalipto), *Citrus limon* (nome popular: limão), *Rosmarinus officinalis* (nome popular: alecrim) e *Eugenia uniflora* (nome popular: pitanga), entre outros (AZEREDO et al., 2014).

Nosso grupo de pesquisa tem utilizado óleos essenciais ou extratos de plantas no combate ao *T. evansi in vitro* e utilizando modelos animais com roedores. Estudos utilizando curcumina (GRESSLER et al. 2015), óleo essencial de *Achyrocline satureioides* e *M. alternifolia* (BALDISSERA et al., 2014; DO CARMO et al., 2015) apresentaram promissores resultados *in vivo.* Os mecanismos de ação dos óleos essenciais não são bem elucidados, mas em geral, a interação com a membrana mitocondrial gera radicais livres que oxidam as estruturas dos parasitos, levando a morte por apoptose ou necrose (TARIKO et al., 2010).

Baseado nos estudos acima citados, e na necessidade de desenvolvimento de novas opções terapêuticas, óleos essenciais podem ser considerados promissores agentes tripanocida, abrindo perspectiva para a descoberta de novos fármacos de origem natural no tratamento das tripanossomoses.

2.3 ÓLEO ESSENCIAL DE *M. alternifolia*

O óleo essencial de melaleuca, conhecido popularmente como TTO (“tea tree oil”), é obtido a partir da destilação das folhas da planta nativa australiana *M. alternifolia* (Mirtacea). O óleo possui aproximadamente 100 compostos ativos diferentes que são obtidos por hidrodestilação ou destilação por arraste de vapor. Os principais constituintes são: terpinen-4-ol, γ-terpinen, α-terpinen, 1.8-cienol e terpinolene (CARSON et al., 2006; BALDISSERA et al., 2014). Conforme a farmacopeia e a ISO4730, um teor mínimo de 30 % de terpinen-4-ol é exigido para confirmar a genuidade do óleo (CARSON et al., 2006).

Várias propriedades terapêuticas têm sido descritas na literatura, com destaque para atividade antibacteriana (ANDRADE et al., 2015), antifúngica (HOMA et al., 2015); inseticida (KLAUCK et al., 2015) e antiparasitário contra *T. evansi* (BALDISSERA et al., 2014). Este último estudo demonstrou a ação tripanocida *in vitro* e *in vivo* do óleo essencial de *M. alternifolia* sobre o *T. evansi.* Animais infectados com *T. evansi* e tratados com TTO apresentaram aumento da longevidade quando comparado a animais infectado não tratados (controle positivo), entretanto, sem eficácia curativa, pois todos os animais foram a óbito durante o experimento. Devido a este resultado, foi avaliado o efeito sinérgico do TTO com o aceturato de diminazeno. Esta associação apresentou 100 % de eficácia curativa, comparada a 33 % de eficácia curativa apresentada pelo aceturato de diminazeno. A análise cromatográfica do estudo revelou a presença de um total de 15 componentes, sendo os três principais o terpinen-4-ol (41.98 %), γ-terpinen (20.15 %) e α-terpinen (9.85 %) (BALDISSERA et al., 2014).

2.4 COMPOSTOS MAJORITÁRIOS DO ÓLEO ESSENCIAL DE *M. alternifolia*

A pesquisa de metabólitos secundários de plantas tem despertado interesse devido a capacidade de proteger as plantas contra insetos herbívoros e contra microrganismos patogênicos (WINK, 1988; ISMAN 2006). Os óleos essenciais contêm uma grande quantidade de metabólitos secundários, como os terpenos, que são conhecidos principalmente devido a seu efeito inseticida (BAKKALI et al., 2008, YANG et al., 2003), classe esta a qual pertencem os três compostos majoritário do TTO.

O terpinen-4-ol (massa molecular: 154.25 g/mol) é o composto majoritário do TTO. O mesmo é amplamente utilizado na indústria farmacêutica no campo dos cosméticos, e seus efeitos farmacológicos incluem a atividade antibacteriana, antifúngica, anti-inflamatória e analgésica (REICHLING et al., 2006). O mesmo é considerado o composto responsável pelas atividades biológicas do TTO e estudos revelaram que seu mecanismo de ação consiste no comprometimento da integridade da membrana celular, ocorrendo assim uma perda de material intracelular, incapacitando células microbianas de manter a homeostase, ou devido a inibição da respiração celular (SOUZA, 2014).

O γ-terpinen e α-terpinen (massas moleculares: 136.23 g/mol) representam o segundo e terceiro composto com maior proporção no óleo de TTO. A diferença entre os compostos é observada devido a diferença da posição da dupla ligação (Figura 4). Estudos com atividades biológicas e propriedades terapêuticas com estes compostos usados de forma isolada são

muito escassos, mas recentemente investigações com estes compostos foram realizados, demonstrando o interesse dos pesquisadores no uso dos metabólitos secundários de plantas.

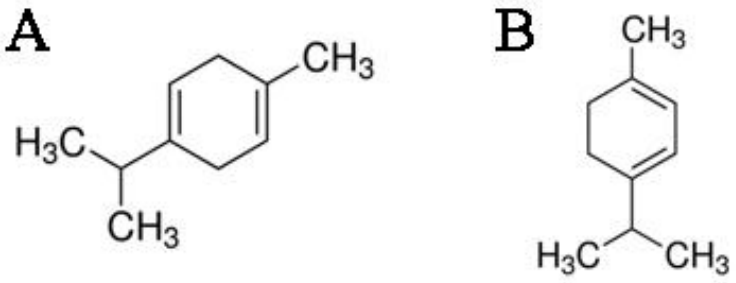

Figura 4 – Diferença da posição da dupla ligação entre os compostos γ-terpinen (A) e α-terpinen (B).

Fonte: domínio público (Sigma-Aldrich).

Podemos citar o efeito antitérmico do γ-terpinen extraído do óleo essencial de *Canarium schweinfuthii* (família Burseraceae) (NAGAWA et al., 2015), e o efeito imuno-modulatório do γ-terpinen extraído do óleo essencial de *Eucalyptus* ssp., (família Mirtaceae), devido a capacidade de diminuir parâmetros de inflamação como edema, produção de citocinas pró-inflamatórias e a migração celular no sítio da inflamação (RAMALHO et al., 2015).

Efeito repelente do α-terpinen extraído de seis diferentes espécies da planta *Murraya* ssp. (família Rutaceae) contra *Tribolium castaneum* (besouro castanho) foi observado por (YOU et al., 2015), apresentando 82 % de efeito repelente na dose de 78 nL/cm.^{2} Efeito antioxidante do composto também foi observado quando extraído da planta *Satureja hortensis* (família Lamiaceae) na dose de 0.05 g kg^{-1} (NAJAFLAN e ZAHEDIFAR, 2015).

3 MANUSCRITO

(Artigo aceito no periódico *Experimental Parasitology*)

In vitro and *in vivo* action of terpinen-4-ol, γ-terpinene, and α-terpinene against *Trypanosoma evansi*

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In vitro and *in vivo* action of terpinen-4-ol, γ-terpinene, and α-terpinene against *Trypanosoma evansi*

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ABSTRACT

This study aimed to evaluate the susceptibility *in vitro* and *in vivo* of *Trypanosoma evansi* to terpinen-4-ol, γ-terpinene and α-terpinene, the three main compounds of tea tree oil (*Melaleuca alternifolia*) with known efficacy in the treatment of trypanosomosis. *In vitro,* a trypanocidal effect of terpinen-4-ol, γ-terpinene, and α-terpinene was observed when used alone or associated at 0.5, 1 and 2% concentrations i.e., the α-terpinene showed a faster trypanocidal effect when compared to chemotherapy (diminazene aceturate - D.A.). *In vivo* studies were performed in two experiments: I and II where experiment I used *T. evansi* infected mice treated with terpinen-4-ol, γ-terpinene and α-terpinene alone (at a dose of 1.0 mL kg^{-1}.) or associated (two compounds, dose of 0.5 mL kg^{-1} of each compound; tree compounds, dose of 0.335 mL kg^{-1} of each compound); Treatment with α-terpinene was able to extend animal longevity, but showed no curative efficacy. In experiment II, *T. evansi* infected mice were treated with D.A. associate with α-terpinene, where a curative efficacy of 57.14% was found, a much better result when D.A. was used alone (14.28%). In summary, α-terpinene associated with D.A. can be used as an alternative treatment for *T. evansi* infection. The compound α-terpinene from *M. alternifolia* essential oil is the one responsible for the trypanocidal effect, a fact confirmed by *in vitro* results and the increased longevity observed on treated mice.

Keywords: Trypanosomosis, TTO, α-terpinene, Major compounds

1 INTRODUCTION

The genus *Melaleuca* belonging to the Myrtaceae family, consist of approximately 230 species, including *Melaleuca alternifolia*, also called “tea tree oil” (TTO) a plant native to Australia (Craven, 1999). TTO has documented antibacterial, antifungal, and anti-inflammatory properties (Hart et al., 2000; Carson et al., 2006). Furthermore, *M. alternifolia* has been used as an antiprotozoal agent, showing efficacy against *Trypanosoma evansi.* Recently, we focused our attention on the chemical composition of the essential oil from *M. alternifolia* and its potential activity against trypomastigote form of *T. evansi* (Baldissera et al., 2014a). Gas chromatograph-mass spectrometry (GC-MS) analyses revealed the presence of 15 compounds, where terpinen-4-ol (41.98%), γ-terpinene (20.15%), and α-terpinene

(9.85%) were the tree major compounds, which trypanocidal effectiveness has *not yet* been established.

Trypanosoma evansi is the etiologic agent of the popularly disease known as "*Surra"* in horses, the most widely distributed animal disease in Africa, also affecting animals in Asia and Latin America resulting in important economic losses (Dobson et al., 2009). In Brazil, the most common drug used to treat trypanosomiasis in domestic animals is diminazene aceturate (D.A.) (Peregrine, 1994). D.A. is capable of eliminating protozoans in the bloodstream a few hours after treatment (Peregrine and Mamman, 1993). Nevertheless, it has no curative efficacy in many situations, where relapses of parasitemia sometimes may occur. This situation usually happens when the trypanosomes pass through the blood-brain barrier, being localized in the central nervous system, a well-known refuge area for *T. evansi* during the drug residual period in the blood-stream. D.A. is unable to cross the blood-brain barrier in sufficient quantities to kill all the parasites (Masocha et al., 2007). In addition, D.A. used to treat *T. evansi* is considered hepatotoxic and nephrotoxic (Spinosa et al., 1999) and their prolonged use may worse the disease.

Currently, researchers are seeking plant-derived compounds to be used as novel trypanocidal drugs (Rodrigues et al., 2015). Although numerous studies have identified plant extracts and/or purified compounds with trypanocidal activity, effective alternative therapeutics for *T. evansi* have not yet been developed, moreover, trypanocidal effect of tea tree oil has been observed (Baldissera et al., 2014a). Thus, this study aimed to evaluate *in vitro* and *in vivo T. evansi* susceptibility to terpinen-4-ol, γ-terpinene and α-terpinene, mains compounds of tea tree oil.

2 MATERIALS AND METHODS

2.1 *trypanosoma evansi* isolate

This study was set up in two consecutive experiments (I and II), and the same *T. evansi* inoculum isolated from dogs (Colpo et al. 2005) was used in both experiments. Three rats (R_a, R_b and R_c) were infected intraperitoneally with trypomastigote from contaminated blood kept in liquid nitrogen. This initial procedure was performed to obtain a large amount of viable parasites for *in vitro* (R_a), and *in vivo* tests (R_b) and II (R_c).

2.2 Essential oil components

Terpinen-4-ol, γ-terpinene and α-terpinene were purchased from Sigma-Aldrich Corporation (St. Louis, United States), and its purity was ≥ 97%, ≥ 95 %, and 85 %, respectively.

2.3 *In vitro* tests

The culture media for *T. evansi* was adapted (Baltz et al., 1985) as previously published by Baldissera et al. (2013). The parasites were acquired from rat R_a previously infected with 5.0 x 10^6 trypomastigotes/μL of *T. evansi*. After isoflurane anesthesia, blood was collected by cardiac puncture, and stored in tubes containing EDTA (ethylenediamine tetraacetic acid). For trypomastigote separation, the blood was diluted in complete culture media (1:1 v/v), stored in microcentrifuge tubes, and centrifuged during 10 minutes at 400 x g. The supernatant was removed and resuspended in culture media and the number of parasites was counted in a *Neubauer* chamber. The culture media with the parasites was distributed in microtiter plates (270 μL/well), followed by the addition of 5 μL of terpinen-4-ol, γ-terpinene and α-terpinene (alone or associated) at concentrations of 0.5, 1 and 2% (diluted in culture media). A chemotherapy control (diminazene aceturate (D.A.) at a dilution of 0.5% in culture media) was carried out, at the same volume (5 μL). The tests were performed in duplicates and the parasites were counted at 1, 3, 6 and 9 hours after the onset of the experiment in *Neubauer* chambers. The microtiter plates were placed in incubator at 37 °C**.**

2.4 Experiment I

2.4.1 Animal model

Seventy mice, female, conventional, outbred strain, heterogenic, 60-day-old mice weighing an average of 27 (± 0.6) grams were used as the experimental model. The animals were maintained under controlled light and environment (12:12 h light-dark cycle, 23 ± 1 °C, 70 % relative humidity) with free access to food and water. All animals were subject to a period of 15 days for adaptation.

2.4.2 Experimental design and parasitemia estimation

The mice were divided into ten groups (A-J) with seven animals each. The group A was composed by healthy and uninfected animals (negative control). Animals in the groups B to J were inoculated intraperitoneally with 100 μL of blood from R_b containing 3.0 x 10^5 trypanosomes (Day 0). The group B was used as positive control without treatment, while mice from the groups C, D and E received terpinen-4-ol, γ-terpinene and α-terpinene, respectively, in dose of 1.0 mL kg^{-1}. The groups F to I received the association between compounds. The groups F, G, H and I received the association between terpinen-4-ol and γ-terpinene; terpinen-4-ol and α-terpinene; γ-terpinene and α-terpinene (dose of 0.5 mL kg^{-1} of each compound); and terpinen-4-ol, γ-terpinene and α-terpinene (dose of 0.335 mL kg^{-1} of each compound), respectively. The group J was treated with a single dose of D.A. (3.5 mg kg^{-1}) intramuscularly one hour after infection. The compounds were administered orally for ten days, starting one hour after infection.

Peripheral blood from the tail coccygeal vein of rats was examined daily for scoring degree of parasitemia. Each slide was prepared with fresh blood, stained by the Romanowski method, and visualized at a magnification of 1,000 x according to the method described by Da Silva et al. (2006). The animals were observed for up to 90 days.

2.4.3 Treatment efficacy

Treatment efficacy was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity and animal mortality were also reported.

2.5 Experiment II

2.5.1 Animal model

Twenty-eight, female, conventional, outbred strain, heterogenic, 60-day-old mice weighing an average of 25 (± 0.3 grams) were used as the experimental model. The animals were maintained under controlled light and environment (12:12 h light-dark cycle, 23 ± 1 °C, 70% relative humidity) with free access to food and water. All animals were subject to a period of 15 days for adaptation.

2.5.2 Experimental design and parasitemia estimation

The mice were divided into four groups (A-D) with seven animals each. The group A was composed by healthy and uninfected animals (negative control). Animals in the groups B to D were inoculated intraperitoneally with 100 μL of blood from R_c containing 4.0 x 10^5 trypanosomes (Day 0). The group B was used as a positive control, without treatment. The group C was treated with D.A. with a single dose of 3.5 mg kg^{-1} intramuscularly. The group D received an association of the following D.A. (single dose- of 3.5 mg kg^{-1} - intramuscularly) and α-terpinene (ten daily doses of 1.0 mL kg^{-1} – orally). One hour after trypomastigotes inoculation (Day 0), the treatment was initiated. The mice were observed for up to 90 days. The evolution of parasitemia and the effect of the treatment were daily monitored through blood smears, as well as describe above for Experiment I.

2.5.3 Treatment efficacy

It was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity, animal mortality and therapeutic success were also observed.

2.6 Statistical analyses

Normality and variance homogeneity were verified using the Shapiro-Wilk test. Thus, the bilateral one-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis were performed. Differences among the groups were rated significant with $p < 0.05$. All analyses were carried out in an IBM-compatible computer using the Statistical Package for the Social Sciences (SPSS) software 20. Results are presented as means and ± standard deviation of mean.

3 RESULTS

3.1 *In vitro* test

The results showed a dose-dependent trypanocidal effect of terpinen-4-ol (Figure 1a), γ-terpinene (Figure 1b) and α-terpinene (Figure 1c). At 1 h post-treatment, a reduction of 32,

44 and 66% in the number of live trypomastigotes was observed at terpinen-4-ol concentrations of 0.5, 1 and 2%, respectively, when compared to the control group. After 3 h post-treatment, there were no living trypanosomes in 1 and 2% concentrations of terpinen-4-ol. A reduction of live trypomastigotes was observed at the concentration of 0.5% of terpinen-4-ol when compared with the control group. Using γ-terpinene, after 1 h there were no living trypomastigotes in 2% concentration. A reduction of 32% and 55% in the number of live trypomastigotes was observed at γ-terpinene concentrations of 0.5, 1% respectively, when compared to the control group. After 3 h of the assay, there were no living trypanosomes in 1% concentration a reduction of 78% in the number of live trypomastigotes was observed at γ-terpinene concentration of 0.5%. After 6 h of the assay, there were no living trypomastigotes in 0.5% concentration of γ-terpinene. Using α-terpinene, after 1 h post-treatment, there were no living trypanosomes at 0.5, 1, and 2% concentrations.

A dose-dependent trypanocidal effect was observed when essential oil compounds were used on an association (Figure 2). At a 1 h post-treatment, a reduction of 9, 20 and 55% in the levels of live trypomastigotes was observed at terpinen-4-ol + γ-terpinene concentrations of 0.5, 1, and 2%. After 3 h post-treatment, there were no living trypanosomes in 1 and 2% concentrations. A reduction of 69% of live trypomastigotes was observed at the concentration of 0.5% when compared with the control group. After 6 h of the assay, there were no living trypomastigotes in 0.5% concentration. Using terpinen-4-ol + α-terpinene, after 1 h there were no living trypomastigotes in 2% concentration. A reduction of 85 and 75% in the number of live trypomastigotes was observed when terpinen-4-ol + α-terpinene at concentrations of 1 and 0.5% were used, respectively when compared to the control group. After 3 h of the assay, there were no living trypomastigotes in 1 and 0.5% concentrations. Using α-terpinene + γ-terpinene, after 1 h post-treatment, a reduction of 12, 35 and 75% in the number of live trypomastigotes was observed at concentrations of 0.5, 1 and 2%, respectively, when compared to the control group. After 3 h post-treatment, there were no living trypanosomes in the 2% concentration group. A reduction of 65 and 86% of live trypomastigotes was observed at the concentration of 1 and 0.5% when compared with the control group. After 6 h of the assay, there were no living trypomastigotes in 1 and 0.5% concentrations. Using terpinen-4-ol + α-terpinene + γ-terpinene, after 1 h, there was no living trypomastigotes in the 2% concentration. A reduction of 88 and 77% in the number of live trypomastigotes was observed in concentrations of 1 and 0.5%, respectively, when compared to the control group. After 3 h of the assay, there were no living trypomastigotes in 1 and 0.5% concentrations.

Using D.A., a reduction of 10 and 37.5% of living trypomastigotes were observed after 1 and 3 h post-treatment, respectively. After 6 h, no living trypomastigotes were observed.

3.2 Experiment I

Prepatent period increased in infected group treated with D.A. compared to infected and untreated mice (positive control) (Table 1). Longevity data have been shown in Table 1. The group A longevity was the longest (90 days). Mice from the group E, treated with α-terpinene had longevity of 12.1 days, statistically different from the group B (Table 1). This group showed no curative effect, only increased longevity compared to the group B.

3.3 Experiment II

Prepatent period increased on those groups infected by the parasite and treated either with D.A. or D.A. combined with α-terpinene, compared to infected and untreated mice (positive control) (Table 2). Longevity of the group A was of 90 days (exactly the days the experiment lasted). Longevity of the groups A, B, C and D were 90, 4.3, 58.9 and 81 days, respectively. Mice from the group C (treated with D.A.) had increase longevity and showed 14.28% of curative efficacy. However, mice from the group D (treated with D.A. combined with α-terpinene) had increased longevity and showed 57.14% of curative efficacy.

4 DISCUSSION

In the development of new treatments for trypanosomosis, research evaluating active components present in essential oils was found to be very promising. Among these components, a number of essential oils have shown trypanocidal activity against the trypomastigote form of *T. evansi* either *in vitro* or *in vivo* tests. Studies with essential oils of *Copaifera* spp. (Baldissera et al., 2014b), *Achyrocline satureioides* (Baldissera et al., 2014c) and *M. alternifolia* (Baldissera et al., 2014a) demonstrated the potential of these substances in the search for new trypanocidal agents. In a recent study, the trypanocidal effect *in vitro* and *in vivo* of *M. alternifolia* essential oil was demonstrated (Baldissera et al., 2014a). However, a main question arouses regarding which major component has the trypanocidal activity, or if this activity would be a result of a combination of two or more components. Therefore, the

components were tested alone or associated using initially an *in vitro* model with *T. evansi* trypomastigotes.

This was the first study to investigate the performance of terpinen-4-ol, γ-terpinene, and α-terpinene against *T. evansi.* These components had apparently a faster trypanocidal effect than the D.A. *in vitro.* Previous studies using terpenes obtained from other plants showed trypanocidal and leishmanicidal *in vitro* action against strains of *Trypanosoma cruzi* and *Leishmania amazonensis* (Rosa et al., 2003; Costa et al., 2011). *In vitro* test revealed that α-terpinene was the treatment that showed the best *in vitro* trypanocidal activity, however, studies on the biological effect of α-terpinene when used alone are scarce. One of the few studies using this compound demonstrated its larvicidal activity against larvae of *Culex quinquefasciatus* at a dose of 250 mg L^{-1} (Pavela, 2015). Rosa et al. (2003) demonstrated leishmanicidal activity of an important terpene obtained from *Croton cajucara* essential oil. The linalool *in vitro* caused a 50% growth reduction of *L. amazonensis* at dose of 8.3 ng mL^{-1} for promastigote and 8.4 ng mL^{-1} for amastigote forms. The mechanism responsible for trypanocidal action is not clearly understood, but the terpenes target the mitochondria, leading to increased reactive oxygen species through the electron transport chain, causing damage to the cell membrane, resulting in the death of the parasite (Saeidnia et al., 2013).

Based on these previous promising *in vitro* results, we have designed an *in vivo* experiment using mice infected by *T. evansi* as the experimental model. However, the therapeutic protocol used had no curative efficacy for all the groups, but in a group treated with α-terpinene alone (group E) an increase in animal longevity was observed. The association with α-terpinene and D.A caused an increase on animal longevity, and a 57.4% curative efficacy compared to the group C (treated only with D.A.) that showed only 14.28% of curative efficacy. Therefore, the α-terpinene alone would not be successful in the treatment against *T. evansi*, but when associated with D.A. we observed a synergic effect, which could result on a new option for treatment.

According to Baldissera et al. (2014a) the terpinen-4-ol corresponds to 41.98% of the total compounds present in *M. alternifolia* essential oil, and it has no *in vivo* action against *T. evansi*, which breaks the believe that always the largest component is the one responsible for the biological activity. De Medeiros et al. (2011) demonstrated that major constituents extracted from essential oils have better activity when used alone, not associated to others, like it was observed in this study. The differences between the pharmacological activities of essential oils and their isolated compounds may result from mutual antagonizing effects

among both compounds, which may influence the reabsorption rate of them and therefore alter the bioavailability of active components (Bakkali et al., 2008).

The α-terpinene has *in vitro* trypanocidal activity against *T. evansi* and *in vivo* it is responsible for increased longevity. However, it is not effective in the treatment of mice experimentally infected by *T. evansi.* This component when associated with D.A. better efficacy, and it can be used an alternative treatment for *T. evansi* infections. In summary, the α-terpinene alone is the component responsible for the trypanocidal effect in *M. alternifolia* essential oil among those compounds tested in this study, without discarding the possibility that other components present in tea tree oil might also have some trypanocidal action.

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Table 1 - Experiment I – Mean and standard deviation of the prepatent period, longevity and mortality after treatment with terpinen-4-ol, γ-terpinene and α-terpinene on their isolated and associated forms, and diminazene aceturate (D.A) in mice infected by *T. evansi.*

Groups (n=7)	Treatment	Prepatent period (Day)	Longevity (Day)	Mortality (n)
A	Negative control	-	90.0^{a} (± 0.0)	0/7
B	Positive control	1.0^{c} (± 0.0)	3.0^{d} (± 0.0)	7/7
C	Terpinen-4-ol	1.1^{c} (± 0.3)	3.4^{d} (± 0.5)	7/7
D	γ-terpinene	1.0^{c} (± 0.0)	3.2^{d} (± 0.5)	7/7
E	α-terpinene	2.2^{bc} (± 1.6)	12.1^{c} (± 2.0)	7/7
F	Terpinen-4-ol + γ-terpinene	1.0^{c} (± 0.0)	3.4^{d} (± 0.5)	7/7
G	Terpinen-4-ol + α-terpinene	1.0^{c} (± 0.0)	3.5^{d} (± 0.5)	7/7
H	γ-terpinene + α-terpinene	1.1^{c} (± 0.38)	7.2^{d} (± 3.0)	7/7
I	Terpinen-4-ol + γ-terpinene + α-terpinene	1.0^{c} (± 0.0)	3.2^{d} (± 0.5)	7/7
J	D.A. (3.5 mg Kg^{-1})	44.9^{a} (± 3.7)	63.8^{b} (± 3.2)	7/7

Means followed by same letter in the same columns do not differ significantly in the Tukey post hoc test. The experiment lasted 90 days after infection

Tabela 2 - Experiment II- Mean and standard deviation of the prepatent period, animal longevity and mortality using α-terpinene alone or associated with D.A. in mice experimentally infected by *T. evansi.*

Groups (n=7)	Treatment	Prepatent period (Day)	Longevity (Day)	Mortality (n)	Therapeutic successful (%)
A	Negative control	-	90.0^{a} (± 0.0)	0/7	-
B	Positive control	1.0^{c} (± 0.5)	4.3^{c} (± 0.5)	7/7	-
C	D.A. (3.5 mg Kg^{-1})	50.9^{b} (± 5.2)	58.9^{b} (± 3.2)	6/7	14.28
D	D.A. (3.5 mg Kg^{-1}) + α-terpinene	70.0^{a} (± 3.7)	81.0^{a} (± 6.0)	3/7	57.14

Means followed by same letter in the same columns do not differ significantly in the Tukey post hoc test. The experiment lasted 90 days after infection.

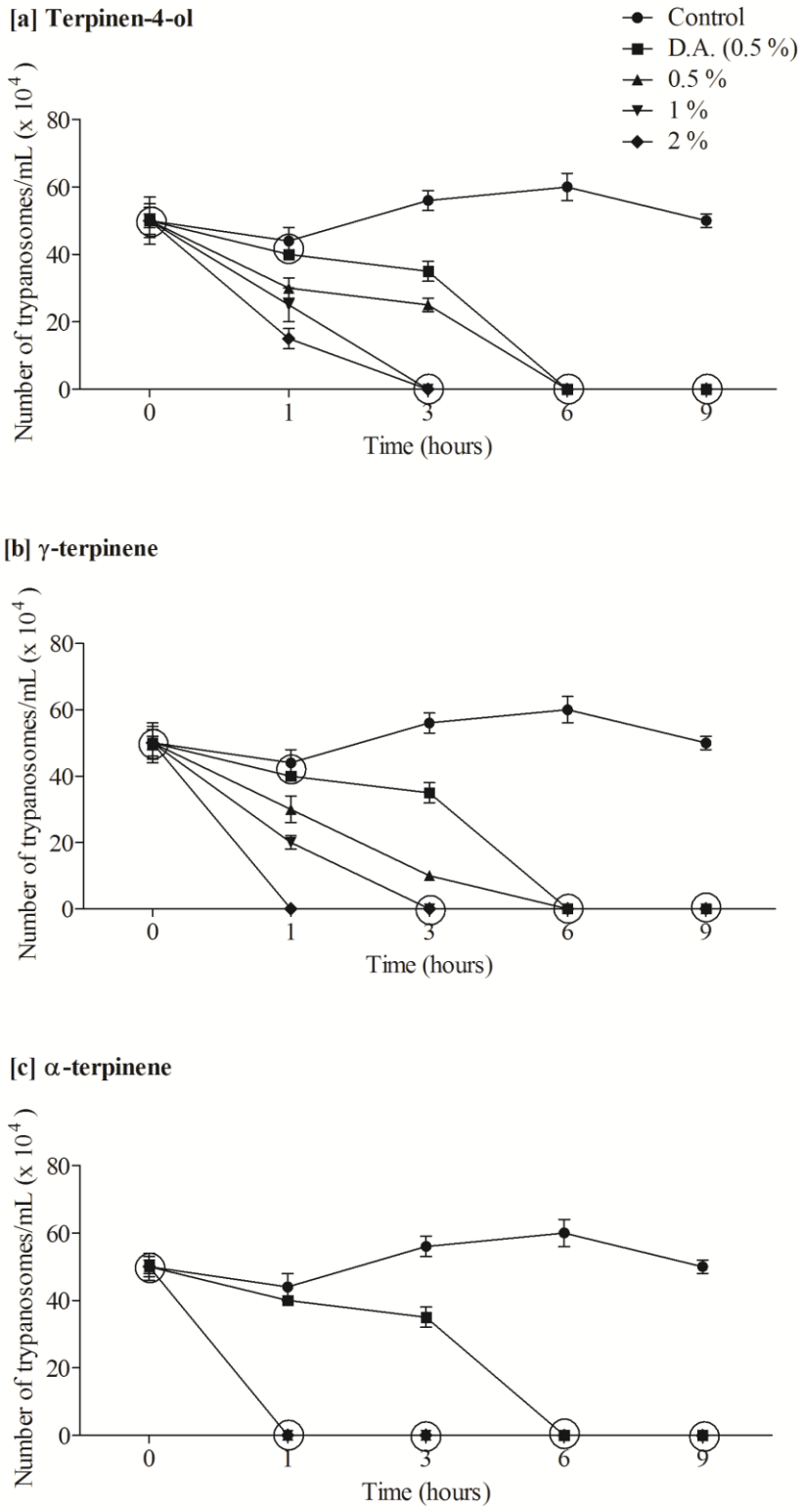

Fig. 1: *In vitro* activity of different concentrations of terpinen-4-ol (a); γ-terpinene (b) and α-terpinene (c) against *Trypanosoma evansi*. Results within a circle were not statistically different (P>0.05), at the same time (h).

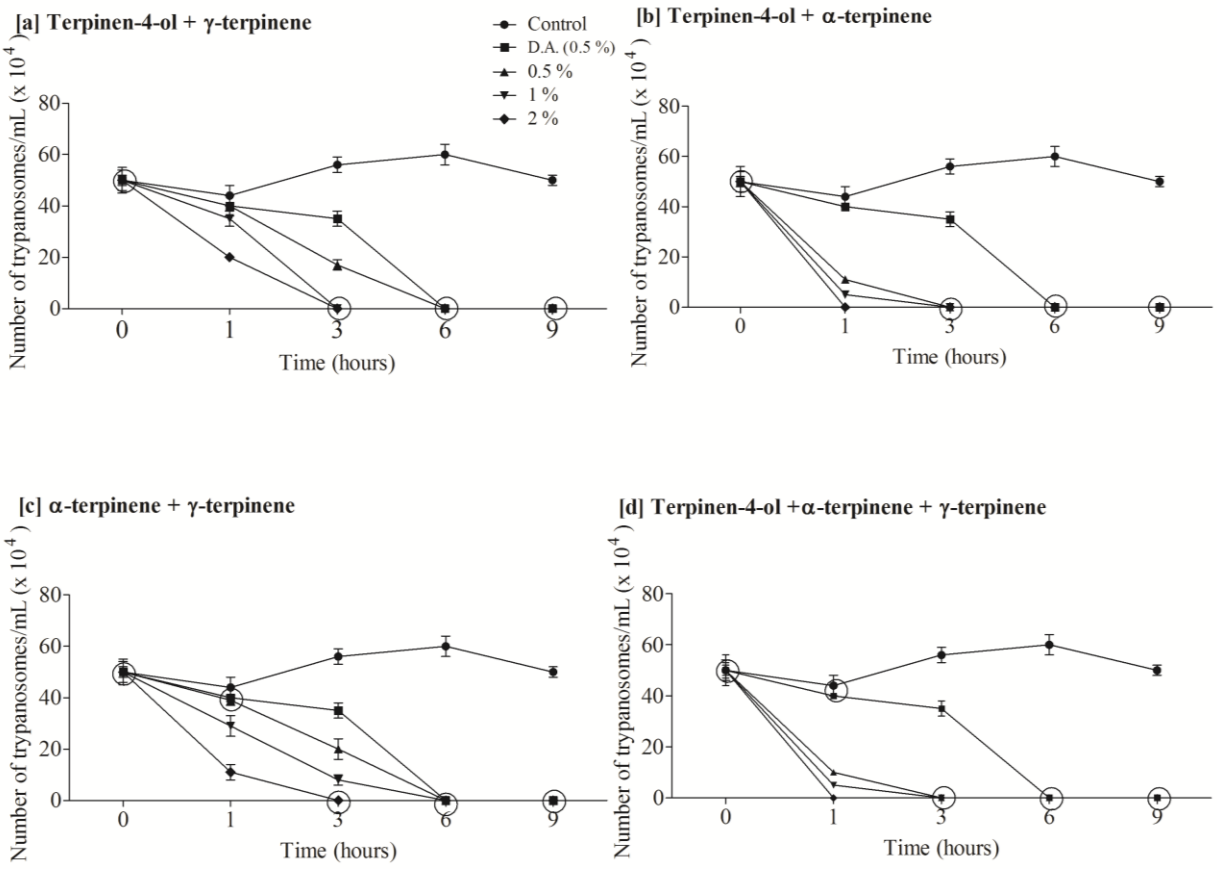

Fig. 2: *In vitro* activity of different concentrations of terpinen-4-ol + γ-terpinene (a); terpinen-4-ol + α-terpinene (b); α-terpinene+ γ-terpinene (c) and terpinen-4-ol + α–terpinene + γ-terpinene (d) against *Trypanosoma evansi*. Results within a circle were not statistically different (P>0.05), at the same time (h).

4 CONCLUSÕES

- Os três compostos majoritários do óleo essencial de *M. alternifolia* possuem atividade tripanocida *in vitro* contra o *T. evansi*, com destaque ao composto α-terpinen;
- *In vivo*, o composto α-terpinen apresentou uma longevidade 4 vezes superior ao controle positivo, porém sem eficácia curativa;
- O aceturato de diminazeno, quando associado ao α-terpinen, apresentou 100 % de eficácia curativa;
- O composto α-terpinen é o possivelmente o principal responsável pela atividade tripanocida contra *T. evansi*, sem descartar a possibilidade de que outros compostos não estudados exerçam ação sobre o protozoário.

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ANEXO A – DOCUMENTOS DE APROVAÇÃO PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE FEDERAL DE SANTA MARIA

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Avaliação da atividade tripanocida de três compostos majoritários (terpinen-4-ol, α-terpinen e γ-terpinen) presentes no óleo de malaleuca sobre o Trypanosoma evansi: teste in vitro e in vivo."

Número do Parecer: 117/2014

Pesquisador Responsável: Prof.ª Dr.ª Silvia Gonzalez Monteiro

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DE APROVAÇÃO: 12/11/2014.

Santa Maria, 12 de novembro de 2014.

Sonia Lucia Loro

Prof.ª Dr.ª Vania Lucia Loro
Vice-Coordenadora da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

UNIVERSIDADE FEDERAL DE SANTA MARIA

PRÓ-REITORIA DE PÓS-GRADUAÇÃO EM PESQUISA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

Santa Maria, 30 de Abril de 2015.

Prezada Prof.ª Dr.ª Silvia Gonzalez Monteiro

EMENDA AO PROJETO: **nº 117/2014** – "Avaliação da atividade tripanocida de três compostos majoritários (terpinen-4-ol, a-terpinen e y-terpinen) presentes no óleo de Melaleuca sobre o Trypanosoma evansi: teste in vitro e in vivo."

Sua solicitação de EMENDA ao projeto acima citado foi avaliada e teve parecer APROVADO, tendo em vista que o formulário de solicitação de EMENDA deixa claro que os pesquisadores irão obter dados mais completos que para a avaliação dos resultados obtidos. Dessa forma, ficam contemplados os princípios que norteiam as boas práticas de experimentação animal onde o refinamento consiste na obtenção de maior número de parâmetros sem o aumento do número de animais.

Foram autorizados: 28 camundongos 25-30 gramas (60 dias).

A entrega dos animais estará condicionada a disponibilidade do biotério.

Atenciosamente

Comissão de Ética no Uso de Animais- UFSM

ANEXO B – TRYPANOCIDAL ACTION OF TEA TREE OIL (*Melaleuca alternifolia*) AGAINST *Trypanosoma evansi IN VITRO* AND *IN VIVO* USED MICE AS EXPERIMENTAL MODEL. EXPERIMENTAL PARASITOLOGY. 2014 JUL; 141:21-27

Experimental Parasitology 141 (2014) 21–27

Contents lists available at ScienceDirect

Experimental Parasitology

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

Trypanocidal action of tea tree oil (*Melaleuca alternifolia*) against *Trypanosoma evansi in vitro* and *in vivo* used mice as experimental model

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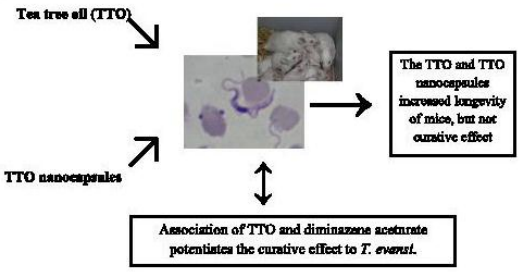
HIGHLIGHTS

- *Trypanosoma evansi* is the etiologic agent of trypanosomosis.
- The parasite has been recorded several cases of resistance to antiprotozoal.
- Tea tree oil (TTO) and tea tree oil nanocapsules (TTO nanocapsules) have trypanocidal action *in vitro*.
- Animals that received TTO and TTO nanocapsules have extended longevity in mice.
- Association of TTO and diminazene aceturate potentiates the curative effect to *T. evansi* in mice.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 14 November 2013
Received in revised form 16 February 2014
Accepted 4 March 2014
Available online 19 March 2014

Keywords:
TTO
Trypanocidal effect
Trypanosoma evansi

ABSTRACT

This study aimed to evaluate the *Trypanosoma evansi* susceptibility to tea tree oil (TTO – *Melaleuca alternifolia*) and tea tree oil nanocapsules (TTO nanocapsules) *in vitro* and *in vivo* tests. *In vitro*, we observed a mortality curve of trypomastigotes proportional to dose, i.e., the TTO and TTO nanocapsules have trypanocidal effect. Treatment with TTO *in vivo* was assessed in experiments (I and II). For Experiment I, *T. evansi* infected mice were treated with TTO and/or combinations of essential oil with chemotherapy (diminazene aceturate – D.A.). Treatment with TTO at a dose of 1 mL kg^{-1} was able to extend animal longevity, but had no curative efficacy. However, when TTO was combined with D.A. a disease curative efficacy of 100% for disease was observed, a much better result than the D.A. treatment (33.3%). In Experiment II, *T. evansi* infected mice were treated with TTO nanocapsules with doses of 0.3, 0.6 and 0.9 mL kg^{-1}. Animals treated with 0.9 mL kg^{-1} showed higher longevity however without curative effect. Active compounds present in natural products, such as *M. alternifolia*, may potentiate the treatment of trypanosomosis when associated with other trypanocidal drugs.

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http://dx.doi.org/10.1016/j.exppara.2014.03.007
0014-4894/

1. Introduction

Trypanosoma evansi is a flagellate protozoan, the etiologic agent of a disease known as *Mal das cadeiras* or *Surra* in horses (Silva et al., 2002). Trypanosomes multiply by binary fission in the blood of mammals (Brun et al., 1998), where trypomastigotes are mechanically transmitted among animals by bloodsucking insects, mainly tabanids (*Tabanus* sp., *Chrysops* sp., and *Hematopota* sp.) (Hoare, 1972). The disease caused by *T. evansi* is characterized by rapid weight loss, variable degrees of anemia, intermittent fever and swollen hind limbs (Herrera et al., 2004; Rodrigues et al., 2005). The treatment of trypanosomosis caused by *T. evansi* is based on diminazene aceturate (D.A.) (Peregrine and Mamman, 1993). In many cases, D.A. treatment may not be effective, leading to recurrent parasitemia, as well as is hepatotoxicity, and nephrotoxicity (Spinosa and Bernardi, 1999; Silva et al., 2002). As a result, researchers have tested natural products such as oils of copaiba, andiroba and aroeira, and propolis extract (Gressler et al., 2012; Dornelles et al., 2013; Baldissera et al., 2013).

Tea tree oil (TTO) essential oil is derived from an Australian native plant known as *Melaleuca alternifolia*. Medicinal TTO is a small Myrtaceous tree with sub-dermal foliar oil glands containing a valuable essential oil composed mainly by monoterpernes (Keszei et al., 2010). TTO has documented insecticidal, acaricidal, repellent (Walton et al., 2004; Iori et al., 2005; Callander and James, 2012), antibacterial, antifungical, anti-inflammatory, and analgesic properties (Carson and Riley, 1993; Hart et al., 2000; Hammer et al., 2004; Carson et al., 2006). Recently, researchers found that polymeric nanocapsules of tea tree oil (TTO nanocapsules) enhance the antifungal effect of the oil (Flores et al., 2013). The nanotechnology has been used in order to improve its efficiency using reduced volumes. This is possible by molecule manipulation to form new structural organization of their atoms (Martinez-Martin et al., 2011).

Nanotechnology is one of the most promising areas of research in modern science. Therefore, this study aimed to evaluate, for the first time, *in vitro* and *in vivo T. evansi* susceptibility to TTO and TTO nanocapsules.

2. Materials and methods

2.1. T. evansi isolate

This study was set up in two consecutive experiments (I and II). The same *T. evansi* isolate (LPV-2005) (Colpo et al., 2005) was used in both experiments. Three rats (R_1, R_2, and R_3) were infected intraperitoneally with trypomastigote contaminated blood kept cryopreserved in liquid nitrogen. This procedure was performed to obtain a large amount of viable parasites for *in vitro* tests (R1), and to infect the experimental groups I (R2) and II (R3).

2.2. Plant material, essential oil components, and nanotechnology

M. alternifolia oil was purchased from *Importadora Química Delaware Ltda*, Brazil. To adjust the dose to be used for *in vitro* tests and for each animal it was necessary to dilute the tea tree oil in dimethyl sulfoxide (DMSO – 1/10v). *M. alternifolia* essential oil was analyzed to quantify and qualify its components.

2.2.1. TTO characterization

Oil composition and yield was analyzed using the gas chromatography (GC) carried out using an Agilent Technologies 6890N GC-FID system, equipped with DB-5 capillary column (30 m × 0.25 mm × 2.5 μm film thickness) connected to a flame ionization detector (FID). The injector and detector temperatures were set to 250 °C. The carrier gas was helium, at a flow rate of 1.3 mL/min. The thermal programmer was 100–280 °C on a rate of 10 °C/min. Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors. The injection volume of the TTO was 1 μL (Hammer et al., 2000; Boligon et al., 2013). GC–Mass Spectroscopy (GC–MS) analyses were performed on an Agilent Technologies AutoSystem XL GC–MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (250 °C). The transfer line temperature was 280 °C. Helium was used as carrier gas (1.5 mL/min) and the capillary columns used were an HP 5MS (30 m × 0.25 mm × 2.5 μm film thickness) and an HP Innowax (30 m × 0.32 mm i.e., film thickness 0.50 mm). The temperature programed was the same as that used for the GC analyses. Essential oil injected volume was 1 μL.

Identification of TTO components was performed on the basis of retention index (RI), determined with reference of the homologous series of *n*-alkanes, C_7–C_{30}, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature date Adams (1995). The relative amounts of individual components were calculated based on the CG peak area (FID response).

2.2.2. TTO nanoparticles

TTO nanoparticles were obtained from Inventiva® (Porto Alegre, Brazil). Briefly, solid lipid nanoparticles were prepared with 7.5% of tea tree oil using a patented method (Inventiva®), based on high pressure homogenization. Cetyl palmitate was used as solid lipid and polisorbate 80 as surfactant. Total solid content was 18.6%. Particle size and zeta potential were evaluated in diluted samples (500×) using Zeta Sizer Nanoseries, Malvern. The pH was assessed by direct use of Digimed potentiometer.

2.3. In vitro tests

The culture medium for *T. evansi* was adapted (Baltz, 1985) as previously published by Baldissera et al. (2013). The trypomastigotes were acquired from the infection of one rat (R_1) with a *T. evansi* isolate. Five days post-infection R_1 showed high parasitemia (7.5×10^6 trypanosomes/μL) and it was anesthetized with isoflurane for blood collection by cardiac puncture stored in EDTA tubes. For blood separation, each 200 μL was diluted in complete culture medium (200 μL), stored in microcentrifuge tubes and centrifuged during 10 min at 400*g*. The supernatant was removed and resuspended in culture medium and the number of parasites was counted in a *Neubauer* chamber.

The culture medium with the parasites was distributed in microtiter plates (270 μL/pool), followed by the addition of 5 μL of tea tree oil (diluted in DMSO) at concentrations of 0.125%, 0.25%, 0.5%, 1.0% and 2.0%. The TTO nanocapsules were used at concentrations of 0.125%, 0.5%, 1.0% and 2.0%. Two negative controls (DMSO and NE-control (cetyl palmitate and polisorbate nanocapsules)) and a positive control (D.A. at a dilution of 0.5% DMSO) were also carried out, at the same volume (5 μL). The tests were performed in duplicates and the parasites were counted at 1, 3, 6 and 12 h after the onset of the experiment in *Neubauer* chambers. The microtiter plates were placed in a 5% CO2 incubator at 37 °C.

2.4. Experiment I

2.4.1. Animal model

Thirty-six, male, 60-day-old-mice weighing an average of 22 ± 0.7 g were used as the experimental model. They were kept in cages with six males each, housed on a light/dark cycle of 12 h, in an experimental room with controlled temperature and humidity (25 °C; 70%, respectively). They were fed with

commercial feed, and water *ad libitum*. All animals were submitted to a period of 15 days for adaptation.

2.4.2. Experimental design and parasitemia estimation

The mice were divided into six groups (A–F) with six animals each. The Group A was unique composed only by healthy and uninfected animals (negative control). Animals in the groups B–F were inoculated intraperitoneally with 0.1 mL of blood from R_2 containing 7.9×10^5 trypanosomes (Day 0). The Group B was used as a positive control without treatment, while mice from the groups C and D received treatment with *M. alternifolia* oil (TTO) in doses of 0.8 and 1.0 mL kg^{-1}, respectively (administered by oral gavage). Group E was treated with diminazene aceturate (D.A.) at 3.5 mg kg^{-1} intramuscularly. Mice from the group F received a combination of the following D.A. (3.5 mg kg^{-1} – intramuscularly) and TTO (1.0 mL kg^{-1} – orally). Twenty-four hours after trypomastigotes inoculation (Day 0) the treatment was initiated with dose intervals of 24 h for three days (doses defined based on the literature and pilot study). The mice were observed for up to 90 days.

The evolution of parasitemia and the effect of the treatment were daily monitored through blood smears. Each slide was prepared with fresh blood collected from the tail vein, stained by the panoptic method, and visualized at a magnification of 1000× (Da Silva et al., 2006).

2.4.3. Treatment efficacy

Treatment efficacy was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity and animal mortality were also observed.

2.4.4. Sample collection and molecular analysis

On day 90 of the experiment, survival animals (from the groups A, E and F) were anesthetized with isofluorane in an anesthesic chamber for bleeding by cardiac puncture. The blood collected was placed in tubes with anticoagulant (EDTA), and diluted with ethanol (v/v). Thereafter, animals were euthanized by decapitation following recommendations of the Ethics Committee and their brains were preserved in ethanol. Blood and brain were used to prove that the animals were free of *T. evansi* infection. Therefore, the DNA extraction of samples following by PCR *T. evansi*-specific was done (Ventura et al., 2002).

2.5. Experiment II

2.5.1. Animal model

Thirty, male, 60-day-old mice weighing an average of 23 ± 0.6 g were used as the experimental model. They were kept in cages with six animals each, housed on a light/dark cycle of 12 h in an experimental room with controlled temperature and humidity (25 °C; 70%, respectively). They were fed with commercial feed and water *ad libitum*. All animals were submitted to a period of 10 days for adaptation.

2.5.2. Experimental design, parasitemia estimation, and treatment efficacy

The mice were divided into five groups (A–E) with six animals each. Group A was unique composed by uninfected animals. Animals in the groups B–E were inoculated intraperitoneally with 0.1 mL of blood from R_3 containing 1.1×10^6 trypanosomes. The Group B was used as a positive control without treatment (positive control), while mice from the groups C, D, and E received *M. alternifolia* oil nanocapsules (TTO nanocapsules) treatment in doses of 0.3, 0.6 and 0.9 mL kg^{-1}, respectively (administered by oral gavage and defined in pilot study). Twenty-four hours after trypomastigotes inoculation (Day 0) the treatment was initiated with dose intervals of 24 h for 3 days. The mice were observed for up to 20 days. Parasitemia evolution and treatment efficacy was daily monitored through blood smears, as described previously in Experiment I.

Treatment efficacy was determined by the number of mice that did not show clinical signs after infection by *T. evansi*. Prepatent period, longevity and animals mortality were also observed.

2.6. Statistical analysis

Data of the prepatent period and longevity were submitted to analysis of variance followed by Duncan test ($P < 0.05$).

3. Results

3.1. TTO characterization

Fifteen components representing 95.86% of the total composition were identified in TTO essential oil (Fig. 1). The results indicated that terpinen-4-ol (41.98%) was the most abundant compound, followed by γ-terpinene (20.15%), α-terpinene (9.85%), 1.8-cineole (6.03%) and terpinolene (4.15%) as showed in Fig. 1.

3.2. TTO nanocapsules

The TTO nanocapsules were evaluated regarding their physical and chemical properties. The particle size was 287 (±2 nm) and the polydispersion index was 0.203 (±0.022) with a zeta potential of −14.2 ± 1.7 mV.

3.3. In vitro test

The results of trypanocidal effect of TTO and TTO nanocapsules on *T. evansi* trypomastigotes (Fig. 2). After 1 h, there were no living trypomastigotes in 2% and 1% concentrations. A reduction of live trypomastigotes was observed at the concentrations of 0.5%, 0.250% and 0.125%, respectively, when compared with the control group. After 3 h of the assay, there were no living trypomastigotes in D.A. treat tubes-test, and a reduction of live trypomastigotes at the concentrations of 0.5%, 0.250% and 0.125%, when compared with the control group was observed. After 6 h of the assay, there were no living trypomastigotes in 0.5% concentration, and a reduction of live trypomastigotes was observed when concentrations of 0.250% and 0.125%, respectively were used, compared to the control group. After 12 h of the assay, a reduction of live trypomastigotes was observed at the concentration of 0.125%, compared to the control group (Fig. 2A).

The TTO nanocapsules results are shown in Fig. 2B. After 1 h, there were no living trypomastigotes in 2% concentration, a reduction of live trypomastigotes was observed at the concentrations of 1%, 0.5% and 0.125%, respectively, when compared to the control group. After 3 h of the assay, there were no living trypomastigotes in D.A. and 1% concentration of oil, and a reduction of live trypomastigotes at the concentrations of 0.5% and 0.125% was observed when compared to the control group. After 6 h of the assay, there was no living trypomastigotes forms in 0.5% concentration, and a reduction of live trypomastigotes were observed at concentration of 0.125%, compared with the control group. After 12 h of the assay, there were no living trypomastigotes forms in 0.125% concentration, compared to the control group. The nanocapsule control showed no significant difference compared to the control group after 12 h of the assay.

3.4. Experiment I

There were no differences between groups regarding the prepatent period (Table 1). Longevity of the group A was exactly

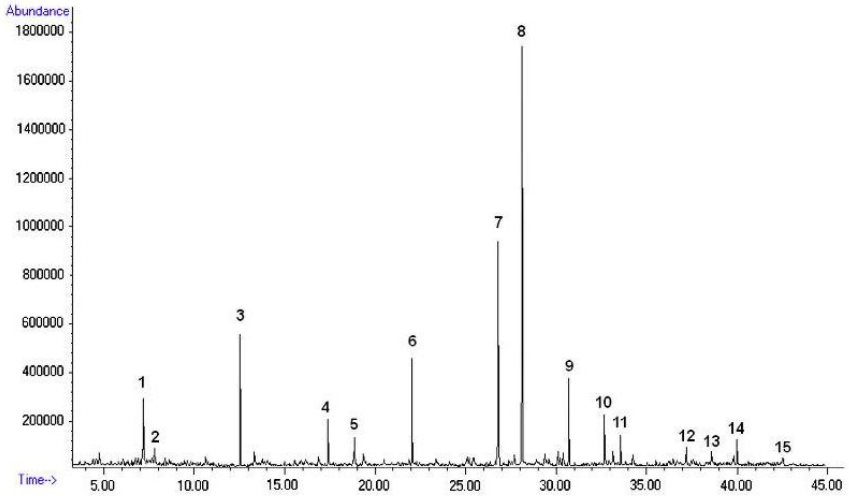

Fig. 1. Peaks of compounds present in TTO essential oil: (1) α-Pinene, (2) Sabinene, (3) α-Terpinene, (4) p-Cymene, (5) Limonene, (6) 1.8-Cineole, (7) γ-Terpinene, (8) Terpinen-4-ol, (9) Terpinolene, (10) α-Terpineol, (11) Aromadendrene, (12) Ledene, (13) δ-Cadinene, (14) Globulol, and (15) Viridiflorol quantified by the method of chromatography.

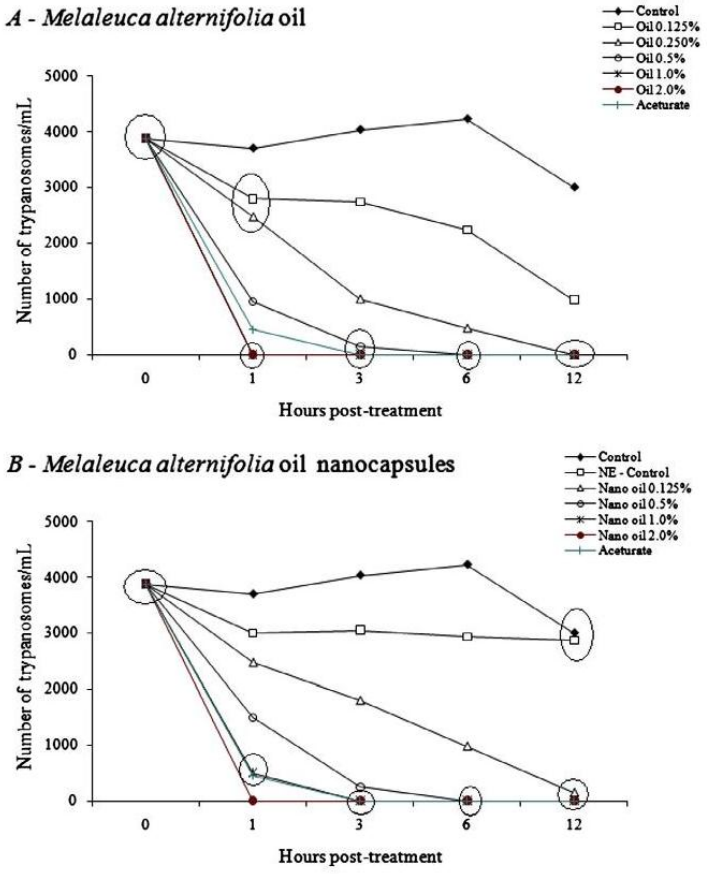

Fig. 2. *In vitro* activity of different concentrations of tea tree oil (*Melaleuca alternifolia*) against *Trypanosoma evansi*. The results within a circle are not statistically different ($P > 0.05$), at the same time (h).

Table 1
Experiment I – Mean and standard deviation of the prepatent period, longevity, mortality, and therapeutic success using treatment with tea tree oil (TTO – *Melaleuca alternifolia*) and diminazene aceturate (D.A.) in mice experimentally infected by *T. evansi*.

Groups ($n = 6$)	Treatment[#]	Prepatent period (Day)	Longevity (Day)	Mortality (n)	Therapeutic success (%)[#]
A	Negative control	–	90.0^{a} (±0.0)	0/6	–
B	Positive control	1.0^{a} (±0.0)	4.8^{d} (±2.6)	6/6	–
C	TTO (0.8 mL/kg)	1.0^{a} (±0.0)	7.5^{d} (±4.8)	6/6	0.0
D	TTO (1.0 mL/kg)	1.0^{a} (±0.0)	18.2^{c} (±6.6)	6/6	0.0
E	D.A. (3.5 mg/kg)	1.0^{a} (±0.0)	47.2^{b} (±33.5)	4/6	33.3
F	D.A. (3.5 mg/kg) + TTO (1.0 mL/kg)	1.0^{a} (±0.0)	90.0^{a} (±0.0)	0/6	100.0

Means followed by same letter in the same column do not differ significantly in the Duncan test. The experiment lasted 90 days post-infection.
[#] Survival animals in the end of experiment, showed negative PCR and blood smears.

Table 2
Experiment II – Mean and standard deviation of the prepatent period, longevity, mortality and therapeutic success using treatment with tea tree oil nanocapsules (TTO nanocapsules – *Melaleuca alternifolia*) in mice experimentally infected by *T. evansi*.

Group ($n = 6$)	Treatment	Prepatent period (Day)	Longevity (Day)	Mortality (n)	Therapeutic success (%)
A	Negative control	–	20.0^{a} (±0.0)	0/6	–
B	Positive control	1.0^{a} (±0.0)	5.1^{c} (±1.1)	6/6	–
C	TTO nanocapsules (0.3 mL/kg)	1.0^{a} (±0.0)	5.8^{c} (±1.2)	6/6	0.0
D	TTO nanocapsules (0.6 mL/kg)	1.0^{a} (±0.0)	6.5^{c} (±1.5)	6/6	0.0
E	TTO nanocapsules (0.9 mL/kg)	1.16^{a} (±0.4)	14.6^{b} (±4.5)	6/6	0.0

Means followed by same letters in the same column do not differ significantly in the Duncan test.

represented by the days that the experiment lasted (90 days). Longevity in the groups B, C, D, E and F were 4.8, 7.5, 18.2, 47.2, and 90 days, respectively. Mice from the group F had longevity of 90 days, statistically different from group B (Table 1). The groups C and D had no curative efficacy, but increased longevity compared to the group B. It was observed a dose-depend effect, because a TTO dose increase resulted on longevity increase in animals from the groups C and D. The association of D.A. and TTO (the group F) showed a synergistic effect, increasing the longevity of the animals when compared to those animals treated only with D.A. (the group E) (Table 1). Survival mice (groups E and F) in the end of experiment (day 90), showed negative PCR and blood smears. This proves the effectiveness of treatment for these animals.

3.5. Experiment II

There were no differences between groups regarding the prepatent period (Table 2). Longevity of the group A was exactly represented by the days that the experiment lasted (20 days). Longevity in the groups A, B, C, D, and E were 20, 5.1, 5.8, 6.5, and 14.6 days, respectively. Mice from the group A had longevity of 20 days, statistically different from the group B (Table 2). The groups C and D had no curative efficacy and no increase in longevity compared to the group B. A dose-depend effect was also observed on longevity when comparing the groups C and D.

4. Discussion

This study observed a trypanocidal effect *in vitro* and *in vivo* of TTO in its pure and nanostructured form. TTO nanocapsules had apparently a faster trypanocidal effect than the pure form of the oil, and even than the D.A. The TTO essential oil is composed by terpenes, particularly monoterpenes and sesquiterpenes, compounds formed by a great number of secondary organic metabolites (Langenheim, 2003), that due to its small molecular weight, are usually found in essential oils (Trapp and Croteau, 2001). It is important to emphasize that terpernes, monoterpenes and sesquiterpenes obtained from other plants or synthesized, had already proven *in vitro* trypanocidal effect against strains of *T. cruzi* (Cunha et al., 2003; Fernandes and Freitas, 2007; Costa et al., 2011).

This was the first study to investigate the performance of lipid nanocapsules containing TTO essential oil on trypanosomes. The results of trypanocidal effect of TTO and TTO nanocapsules against *T. evansi* trypomastigotes *in vitro* was proportional to dose. Reduction of *Trypanosoma brucei brucei* in culture medium has been reported after 24 h incubation in the presence of TTO (Valdés et al., 2008). In this study, the lowest concentration of TTO nanocoated showed greater efficacy than the free oil. This result can be explained by a greater interaction between the nanoparticle and the parasite membrane, facilitating oil penetration by its reduced size (Lherm et al., 1987). According to Flores et al. (2013) nanocapsules and nanoemulsions of TTO were able to reduce fungal growth *in vitro* using strains of *Trichophyton rubrum*. According to these researchers this was due to improved penetration of oil into the cell, with easy access to the fungus (Flores et al., 2013).

In the experiment I was observed a significant longevity increase and great therapeutic results. The higher dose of TTO increased longevity compared to the group B, but had no curative efficacy. However, the association of D.A. with the highest dose of TTO increased longevity compared to the group B, and had 100% therapeutic efficacy, compared to group E (treated only with D.A.), with 33% of therapeutic efficacy. In addition, it was observed in the experiment II a significant increase in the longevity of mice infected by *T. evansi* when treated with TTO nanocapsules only at the dose of 0.9 mL kg^{-1}. Animals from the group E showed increased longevity when compared to the group B, 14.6 and 5.1 days, respectively, but without curative effect. Therefore, the TTO oil alone would not be successful in the treatment of trypanosomosis, but when the oil is associated with a chemical drug such D.A., a synergic effect occurs, which could result on a new option for this disease treatment.

As Carson et al. (2006), terpinen-4-ol is the major compound present in the TTO oil, corresponding to approximately 40%, followed by γ-terpinene. As a result, our primary hypothesis is that these two components were responsible for reducing the number of trypomastigotes *in vitro* and *in vivo*. Two studies reported TTO antiprotozoal activity (Viollon et al., 1996; Mikus et al., 2000), similarly to our findings. TTO treatment *in vitro* caused a 50% reduction of *Leishmania major* and *Trypanosoma brucei* growth at

concentration of 403 and 0.5 mg mL^{-1}, respectively. The compound terpin-4-ol was responsible for the death of trypomastigotes of *T. brucei* (Mikus et al., 2000). Another study reported that TTO killed *in vitro* the parasite *Trichomonas vaginalis* at a concentration of 300 mg mL^{-1} (Viollon et al., 1996). In addition to the previously reported antiprotozoal activity in this study, the terpinen-4-ol may also act as antibacterial, antifungal and antiviral agent (Shapiro et al., 1994; Bishop, 1995; Hammer et al., 2000; Banes–Marshall et al., 2001).

The mechanism of action responsible for the trypanocidal activity of terpinen-4-ol has not been elucidated; however, the fungicidal activity is due to membrane damage which leads to K^+ ions and small molecules losses, followed by inhibition of the chain respiratory (Cox et al., 2000; Carson et al., 2002). According to researchers, loss of intracellular material, and failure to maintain cell homeostasis, leads to the loss of membrane integrity in bacteria (Cox et al., 2001). Another possible mechanism responsible for TTO trypanocidal activity may be due to sesquiterpenes. Saeidnia et al. (2013), claim that these compounds obtained synthetically or naturally from plants have trypanocidal activity against various species of *Trypanosoma* spp. The trypanocidal activity of sesquiterpene compounds targets the mitochondria, leading to increased reactive oxygen species through the electron transport chain, causing damage to the cell membrane and DNA, resulting in the death of the parasite (Desoti et al., 2012; Saeidnia et al., 2013).

The TTO, in its conventional and nanostructured forms has a trypanocidal activity *in vitro* and *in vivo* against *T. evansi* and also leads to higher animal longevity, but it was not effective in the treatment of mice experimentally infected by *T. evansi*; only presenting a slight control of parasitism in the early course of the infection. TTO oil when associated with diminazene aceturate achieved therapeutic success, becoming an alternative method to treat and control infections caused by *T. evansi*.

Ethical approval

The procedure was approved by the Animal Welfare Committee of Universidade do Estado de Santa Catarina under number 1.51.13.

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ANEXO C – *IN VITRO* AND *IN VIVO* ACTION OF TERPINEN-4-OL, Γ-TERPINENE, AND A-TERPINENE AGAINST *Trypanosoma evansi.* Experimental Parasitology. 2016 jan; 162:43-48

Experimental Parasitology 162 (2016) 43–48

Contents lists available at ScienceDirect

Experimental Parasitology

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

Full length article

In vitro and *in vivo* action of terpinen-4-ol, γ-terpinene, and α-terpinene against *Trypanosoma evansi*

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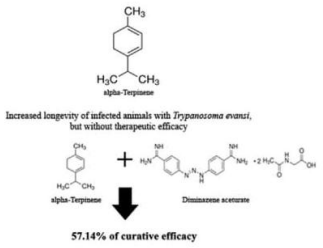
HIGHLIGHTS

- *In vitro,* a trypanocidal effect compounds was observed when used alone or associated.
- *In vitro,* the α-terpinene showed faster trypanocidal effect compared to chemotherapy.
- α-terpinene was able to extend animal longevity, but showed no curative efficacy.
- Chemotherapy associate with α-terpinene showed a curative efficacy of 57.14%.
- α-terpinene from *M. alternifolia* is the one responsible for the trypanocidal effect.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form
5 January 2016
Accepted 6 January 2016
Available online 8 January 2016

Keywords:
Trypanosomosis
TTO
α-terpinene
Majority compounds

ABSTRACT

This study aimed to evaluate the susceptibility *in vitro* and *in vivo* of *Trypanosoma evansi* to terpinen-4-ol, γ-terpinene and α-terpinene, the three main compounds of tea tree oil (*Melaleuca alternifolia*) with known efficacy in the treatment of trypanosomosis. *In vitro,* a trypanocidal effect of terpinen-4-ol, γ-terpinene, and α-terpinene was observed when used alone or associated at 0.5, 1 and 2% concentrations i.e., the α-terpinene showed a faster trypanocidal effect when compared to chemotherapy (diminazene aceturate - D.A.). *In vivo* studies were performed in two experiments: I and II where experiment I used *T. evansi* infected mice treated with terpinen-4-ol, γ-terpinene and α-terpinene alone (at a dose of 1.0 mL kg^{-1}) or associated (two compounds, dose of 0.5 mL kg^{-1} of each compound; tree compounds, dose of 0.335 mL kg^{-1} of each compound); Treatment with α-terpinene was able to extend animal longevity, but showed no curative efficacy. In experiment II, *T. evansi* infected mice were treated with D.A. associate with α-terpinene, where a curative efficacy of 57.14% was found, a much better result when D.A. was used alone (14.28%). In summary, α-terpinene associated with D.A. can be used as an alternative treatment for *T. evansi* infection. The compound α-terpinene from *M. alternifolia* essential oil is the one

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http://dx.doi.org/10.1016/j.exppara.2016.01.004

responsible for the trypanocidal effect, a fact confirmed by *in vitro* results and the increased longevity observed on treated mice.

1. Introduction

The genus *Melaleuca* belonging to the Myrtaceae family, consist of approximately 230 species, including *Melaleuca alternifolia*, also called "tea tree oil" (TTO) a plant native to Australia (Craven, 1999). TTO has documented antibacterial, antifungal, and anti-inflammatory properties (Hart et al., 2000; Carson et al., 2006). Furthermore, *M. alternifolia* has been used as an antiprotozoal agent, showing efficacy against *Trypanosoma evansi.* Recently, we focused our attention on the chemical composition of the essential oil from *M. alternifolia* and its potential activity against trypomastigote form of *T. evansi* (Baldissera et al., 2014a). Gas chromatograph-mass spectrometry (GC–MS) analyses revealed the presence of 15 compounds, where terpinen-4-ol (41.98%), γ-terpinene (20.15%), and α-terpinene (9.85%) were the tree major compounds, which trypanocidal effectiveness has not yet been established.

Trypanosoma evansi is the etiologic agent of the popularly disease known as "*Surra*" in horses, the most widely distributed animal disease in Africa, also affecting animals in Asia and Latin America resulting in important economic losses (Dobson et al., 2009). In Brazil, the most common drug used to treat trypanosomiasis in domestic animals is diminazene aceturate (D.A.) (Peregrine, 1994). D.A. is capable of eliminating protozoans in the bloodstream a few hours after treatment (Peregrine and Mamman, 1993). Nevertheless, it has no curative efficacy in many situations, where relapses of parasitemia sometimes may occur. This situation usually happens when the trypanosomes pass through the blood–brain barrier, being localized in the central nervous system, a well-known refuge area for *T. evansi* during the drug residual period in the blood-stream. D.A. is unable to cross the blood–brain barrier in sufficient quantities to kill all the parasites (Masocha et al., 2007). In addition, D.A. used to treat *T. evansi* is considered hepatotoxic and nephrotoxic (Spinosa et al., 1999) and their prolonged use may worse the disease.

Currently, researchers are seeking plant-derived compounds to be used as novel trypanocidal drugs (Rodrigues et al., 2015). Although numerous studies have identified plant extracts and/or purified compounds with trypanocidal activity, effective alternative therapeutics for *T. evansi* have not yet been developed, moreover, trypanocidal effect of tea tree oil has been observed (Baldissera et al., 2014a). Thus, this study aimed to evaluate *in vitro* and *in vivo T. evansi* susceptibility to terpinen-4-ol, γ-terpinene and α-terpinene, mains compounds of tea tree oil.

2. Materials and methods

2.1. Trypanosoma evansi isolate

This study was set up in two consecutive experiments (I and II), and the same *T. evansi* inoculum isolated from dogs (Colpo et al., 2005) was used in both experiments. Three rats (R_a, R_b and R_c) were infected intraperitoneally with trypomastigote from contaminated blood kept in liquid nitrogen. This initial procedure was performed to obtain a large amount of viable parasites for *in vitro* (R_a), and *in vivo* tests (R_b) and II (R_c).

2.2. Essential oil components

Terpinen-4-ol, γ-terpinene and α-terpinene were purchased from Sigma–Aldrich Corporation (St. Louis, United States), and its purity was ≥97%, ≥95%, and 85%, respectively.

2.3. In vitro tests

The culture media for *T. evansi* was adapted (Baltz et al., 1985) as previously published by Baldissera et al. (2013). The parasites were acquired from rat R_a previously infected with 5.0×10^6 trypomastigotes/μL of *T. evansi*. After isoflurane anesthesia, blood was collected by cardiac puncture, and stored in tubes containing EDTA (ethylenediamine tetraacetic acid). For trypomastigote separation, the blood was diluted in complete culture media (1:1 v/v), stored in microcentrifuge tubes, and centrifuged during 10 min at 400 × g. The supernatant was removed and resuspended in culture media and the number of parasites was counted in a *Neubauer* chamber. The culture media with the parasites was distributed in microtiter plates (270 μL/well), followed by the addition of 5 μL of terpinen-4-ol, γ-terpinene and α-terpinene (alone or associated) at concentrations of 0.5, 1 and 2% (diluted in culture media). A chemotherapy control (diminazene aceturate (D.A.) at a dilution of 0.5% in culture media) was carried out, at the same volume (5 μL). The tests were performed in duplicates and the parasites were counted at 1, 3, 6 and 9 h after the onset of the experiment in *Neubauer* chambers. The microtiter plates were placed in incubator at 37 °C.

2.4. Experiment I

2.4.1. Animal model

Seventy mice, female, conventional, outbred strain, heterogenic, 60-day-old mice weighing an average of 27 (±0.6) grams were used as the experimental model. The animals were maintained under controlled light and environment (12:12 h light–dark cycle, 23 ± 1 °C, 70% relative humidity) with free access to food and water. All animals were subject to a period of 15 days for adaptation.

2.4.2. Experimental design and parasitemia estimation

The mice were divided into ten groups (A-J) with seven animals each. The group A was composed by healthy and uninfected animals (negative control). Animals in the groups B to J were inoculated intraperitoneally with 100 μL of blood from R_b containing 3.0×10^5 trypanosomes (Day 0). The group B was used as positive control without treatment, while mice from the groups C, D and E received terpinen-4-ol, γ-terpinene and α-terpinene, respectively, in dose of 1.0 mL kg^{-1}. The groups F to I received the association between compounds. The groups F, G, H and I received the association between terpinen-4-ol and γ-terpinene; terpinen-4-ol and α-terpinene; γ-terpinene and α-terpinene (dose of 0.5 mL kg^{-1} of each compound); and terpinen-4-ol, γ-terpinene and α-terpinene (dose of 0.335 mL kg^{-1} of each compound), respectively. The group J was treated with a single dose of D.A. (3.5 mg kg^{-1}) intraperitoneally 1 h after infection. The compounds were administered orally for ten days, starting 1 h after infection.

Peripheral blood from the tail coccygeal vein of rats was examined daily for scoring degree of parasitemia. Each slide was

prepared with fresh blood, stained by the Romanowski method, and visualized at a magnification of 1000× according to the method described by Da Silva et al. (2006). The animals were observed for up to 90 days.

2.4.3. Treatment efficacy

Treatment efficacy was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity and animal mortality were also reported.

2.5. Experiment II

2.5.1. Animal model

Twenty-eight, female, conventional, outbred strain, heterogenic, 60-day-old mice weighing an average of 25 (±0.3 g) were used as the experimental model. The animals were maintained under controlled light and environment (12:12 h light–dark cycle, 23 ± 1 °C, 70% relative humidity) with free access to food and water. All animals were subject to a period of 15 days for adaptation.

2.5.2. Experimental design and parasitemia estimation

The mice were divided into four groups (A-D) with seven animals each. The group A was composed by healthy and uninfected animals (negative control). Animals in the groups B to D were inoculated intraperitoneally with 100 μL of blood from R_c containing 4.0×10^5 trypanosomes (Day 0). The group B was used as a positive control, without treatment. The group C was treated with D.A. with a single dose of 3.5 mg kg^{-1} intraperitoneally. The group D received an association of the following D.A. (single dose-of 3.5 mg kg^{-1} – intraperitoneally) and α-terpinene (ten daily doses of 1.0 mL kg^{-1} – orally). One hour after trypomastigotes inoculation (Day 0), the treatment was initiated. The mice were observed for up to 90 days. The evolution of parasitemia and the effect of the treatment were daily monitored through blood smears, as well as describe above for Experiment I.

2.5.3. Treatment efficacy

It was determined by the number of mice that did not show clinical signs of *T. evansi* infection after treatment. Prepatent period, longevity, animal mortality and therapeutic success were also observed.

2.6. Statistical analyses

Normality and variance homogeneity were verified using the Shapiro–Wilk test. Thus, the bilateral one-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis were performed. Differences among the groups were rated significant with $p < 0.05$. All analyses were carried out in an IBM-compatible computer using the Statistical Package for the Social Sciences (SPSS) software 20. Results are presented as means and ±standard deviation of mean.

3. Results

3.1. In vitro test

The results showed a dose-dependent trypanocidal effect of terpinen-4-ol (Fig. 1a), γ-terpinene (Fig. 1b) and α-terpinene (Fig. 1c). At 1 h post-treatment, a reduction of 32, 44 and 66% in the number of live trypomastigotes was observed at terpinen-4-ol concentrations of 0.5, 1 and 2%, respectively, when compared to the control group. After 3 h post-treatment, there were no living trypanosomes in 1 and 2% concentrations of terpinen-4-ol. A reduction of live trypomastigotes was observed at the

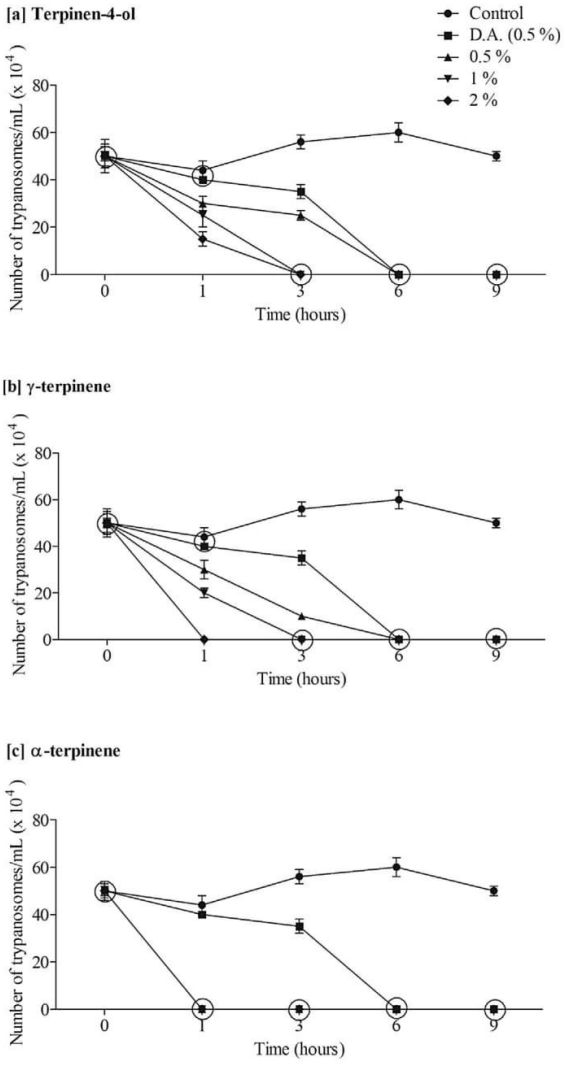

Fig. 1. *In vitro* activity of different concentrations of terpinen-4-ol (a); γ-terpinene (b) and α-terpinene (c) against *Trypanosoma evansi*. Results within a circle were not statistically different (P > 0.05), at the same time (h).

concentration of 0.5% of terpinen-4-ol when compared with the control group. Using γ-terpinene, after 1 h there were no living trypomastigotes in 2% concentration. A reduction of 32% and 55% in the number of live trypomastigotes was observed at γ-terpinene concentrations of 0.5, 1% respectively, when compared to the control group. After 3 h of the assay, there were no living trypanosomes in 1% concentration a reduction of 78% in the number of live trypomastigotes was observed at γ-terpinene concentration of 0.5%. After 6 h of the assay, there were no living trypomastigotes in 0.5% concentration of γ-terpinene. Using α-terpinene, after 1 h post-treatment, there were no living trypanosomes at 0.5, 1, and 2% concentrations.

A dose-dependent trypanocidal effect was observed when essential oil compounds were used on an association (Fig. 2). At a 1 h post-treatment, a reduction of 9, 20 and 55% in the levels of live

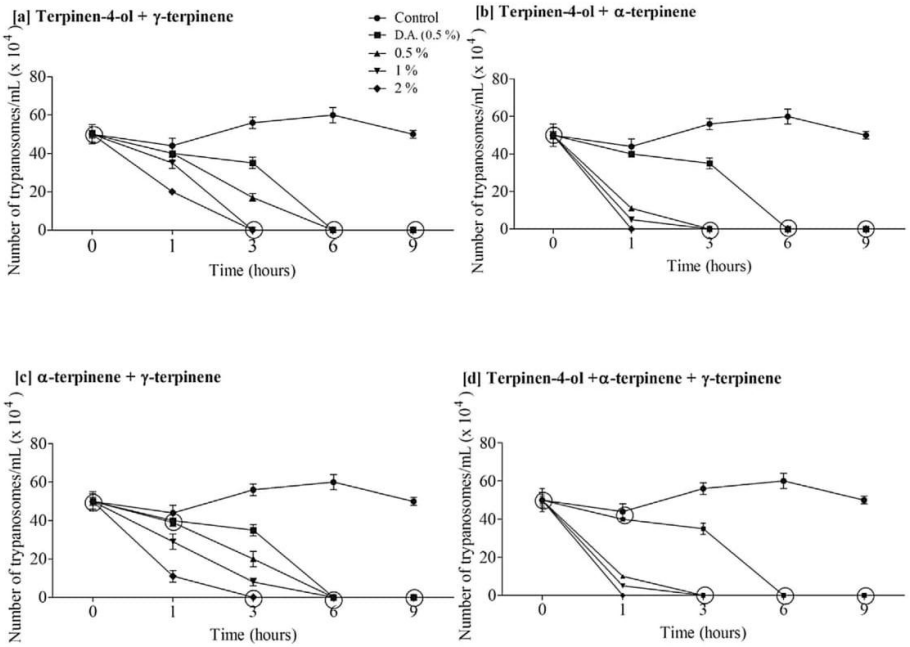

Fig. 2. *In vitro* activity of different concentrations of terpinen-4-ol + γ-terpinene (a); terpinen-4-ol + α-terpinene (b); α-terpinene+ γ-terpinene (c) and terpinen-4-ol + α–terpinene + γ-terpinene (d) against *Trypanosoma evansi*. Results within a circle were not statistically different (P > 0.05), at the same time (h).

trypomastigotes was observed at terpinen-4-ol + γ-terpinene concentrations of 0.5, 1, and 2%. After 3 h post-treatment, there were no living trypanosomes in 1 and 2% concentrations. A reduction of 69% of live trypomastigotes was observed at the concentration of 0.5% when compared with the control group. After 6 h of the assay, there were no living trypomastigotes in 0.5% concentration. Using terpinen-4-ol + α-terpinene, after 1 h there were no living trypomastigotes in 2% concentration. A reduction of 85 and 75% in the number of live trypomastigotes was observed when terpinen-4-ol + α-terpinene at concentrations of 1 and 0.5% were used, respectively when compared to the control group. After 3 h of the assay, there were no living trypomastigotes in 1 and 0.5% concentrations. Using α-terpinene + γ-terpinene, after 1 h post-treatment, a reduction of 12, 35 and 75% in the number of live trypomastigotes was observed at concentrations of 0.5, 1 and 2%, respectively, when compared to the control group. After 3 h post-treatment, there were no living trypanosomes in the 2% concentration group. A reduction of 65 and 86% of live trypomastigotes was observed at the concentration of 1 and 0.5% when compared with the control group. After 6 h of the assay, there were no living trypomastigotes in 1 and 0.5% concentrations. Using terpinen-4-ol + α-terpinene + γ-terpinene, after 1 h, there was no living trypomastigotes in the 2% concentration. A reduction of 88 and 77% in the number of live trypomastigotes was observed in concentrations of 1 and 0.5%, respectively, when compared to the control group. After 3 h of the assay, there were no living trypomastigotes in 1 and 0.5% concentrations.

Using D.A., a reduction of 10 and 37.5% of living trypomastigotes were observed after 1 and 3 h post-treatment, respectively. After 6 h, no living trypomastigotes were observed.

3.2. Experiment I

Prepatent period increased in infected group treated with D.A. compared to infected and untreated mice (positive control) (Table 1). Longevity data have been shown in Table 1. The group A longevity was the longest (90 days). Mice from the group E, treated with α-terpinene had longevity of 12.1 days, statistically different from the group B (Table 1). This group showed no curative effect, only increased longevity compared to the group B.

3.3. Experiment II

Prepatent period increased on those groups infected by the parasite and treated either with D.A. or D.A. combined with α-terpinene, compared to infected and untreated mice (positive control) (Table 2). Longevity of the group A was of 90 days (exactly the days the experiment lasted). Longevity of the groups A, B, C and D were 90, 4.3, 58.9 and 81 days, respectively. Mice from the group C (treated with D.A.) had increase longevity and showed 14.28% of curative efficacy. However, mice from the group D (treated with D.A. combined with α-terpinene) had increased longevity and showed 57.14% of curative efficacy.

4. Discussion

In the development of new treatments for trypanosomosis, research evaluating active components present in essential oils was found to be very promising. Among these components, a number of essential oils have shown trypanocidal activity against the trypomastigote form of *T. evansi* either *in vitro* or *in vivo* tests. Studies with essential oils of *Copaifera* spp. (Baldissera et al., 2014b), *Achyrocline satureioides* (Baldissera et al., 2014c) and *M. alternifolia* (Baldissera et al., 2014a) demonstrated the potential of these substances in the search for new trypanocidal agents. In a recent study, the trypanocidal effect *in vitro* and *in vivo* of *M. alternifolia* essential oil was demonstrated (Baldissera et al., 2014a). However, a main question arouses regarding which major component has the

Table 1
Experiment I – Mean and standard deviation of the prepatent period, longevity and mortality after treatment with terpinen-4-ol, γ-terpinene and α-terpinene on their isolated and associated forms, and diminazene aceturate (D.A) in mice infected by *T. evansi*.

Groups (n = 7)	Treatment	Prepatent period (Day)	Longevity (Day)	Mortality (n)
A	Negative control	–	90.0^{a} (±0.0)	0/7
B	Positive control	1.0^{c} (±0.0)	3.0^{d} (±0.0)	7/7
C	Terpinen-4-ol	1.1^{c} (±0.3)	3.4^{d} (±0.5)	7/7
D	γ-terpinene	1.0^{c} (±0.0)	3.2^{d} (±0.5)	7/7
E	α-terpinene	2.2^{bc} (±1.6)	12.1^{c} (±2.0)	7/7
F	Terpinen-4-ol + γ-terpinene	1.0^{c} (±0.0)	3.4^{d} (±0.5)	7/7
G	Terpinen-4-ol + α-terpinene	1.0^{c} (±0.0)	3.5^{d} (±0.5)	7/7
H	γ-terpinene + α-terpinene	1.1^{c} (±0.38)	7.2^{d} (±3.0)	7/7
I	Terpinen-4-ol + γ-terpinene + α-terpinene	1.0^{c} (±0.0)	3.2^{d} (±0.5)	7/7
J	D.A. (3.5 mg kg^{-1})	44.9^{a} (±3.7)	63.8^{b} (±3.2)	7/7

Means followed by same letter in the same columns do not differ significantly in the Duncan post hoc test. The experiment lasted 90 days after infection.

Table 2
Experiment II- Mean and standard deviation of the prepatent period, animal longevity and mortality using α-terpinene alone or associated with D.A. in mice experimentally infected by *T. evansi*.

Groups (n = 7)	Treatment	Prepatent period (Day)	Longevity (Day)	Mortality (n)	Therapeutic successful (%)
A	Negative control	–	90.0^{a} (±0.0)	0/7	–
B	Positive control	1.0^{c} (±0.5)	4.3^{c} (±0.5)	7/7	–
C	D.A. (3.5 mg kg^{-1})	50.9^{b} (±5.2)	58.9^{b} (±3.2)	6/7	14.28
D	D.A. (3.5 mg kg^{-1}) + α-terpinene	70.0^{a} (±3.7)	81.0^{a} (±6.0)	3/7	57.14

Means followed by same letter in the same columns do not differ significantly in the Duncan post hoc test. The experiment lasted 90 days after infection.

trypanocidal activity, or if this activity would be a result of a combination of two or more components. Therefore, the components were tested alone or associated using initially an *in vitro* model with *T. evansi* trypomastigotes.

This was the first study to investigate the performance of terpinen-4-ol, γ-terpinene, and α-terpinene against *T. evansi*. These components had apparently a faster trypanocidal effect than the D.A. *in vitro*. Previous studies using terpenes obtained from other plants showed trypanocidal and leishmanicidal *in vitro* action against strains of *Trypanosoma cruzi* and *Leishmania amazonensis* (Rosa et al., 2003; Costa et al., 2011). *In vitro* test revealed that α-terpinene was the treatment that showed the best *in vitro* trypanocidal activity, however, studies on the biological effect of α-terpinene when used alone are scarce. One of the few studies using this compound demonstrated its larvicidal activity against larvae of *Culex quinquefasciatus* at a dose of 250 mg L^{-1} (Pavela, 2015). Rosa et al. (2003) demonstrated leishmanicidal activity of an important terpene obtained from *Croton cajucara* essential oil. The linalool *in vitro* caused a 50% growth reduction of *L. amazonensis* at dose of 8.3 ng mL^{-1} for promastigote and 8.4 ng mL^{-1} for amastigote forms. The mechanism responsible for trypanocidal action is not clearly understood, but the terpenes target the mitochondria, leading to increased reactive oxygen species through the electron transport chain, causing damage to the cell membrane, resulting in the death of the parasite (Saeidnia et al., 2013).

Based on these previous promising *in vitro* results, we have designed an *in vivo* experiment using mice infected by *T. evansi* as the experimental model. However, the therapeutic protocol used had no curative efficacy for all the groups, but in a group treated with α-terpinene alone (group E) an increase in animal longevity was observed. The association with α-terpinene and D.A caused an increase on animal longevity, and a 57.4% curative efficacy compared to the group C (treated only with D.A.) that showed only 14.28% of curative efficacy. Therefore, the α-terpinene alone would not be successful in the treatment against *T. evansi*, but when associated with D.A. we observed a synergic effect, which could result on a new option for treatment.

According to Baldissera et al. (2014a) the terpinen-4-ol corresponds to 41.98% of the total compounds present in *M. alternifolia* essential oil, and it has no *in vivo* action against *T. evansi*, which breaks the believe that always the largest component is the one responsible for the biological activity. De Medeiros et al. (2011) demonstrated that major constituents extracted from essential oils have better activity when used alone, not associated to others, like it was observed in this study. The differences between the pharmacological activities of essential oils and their isolated compounds may result from mutual antagonizing effects among both compounds, which may influence the reabsorption rate of them and therefore alter the bioavailability of active components (Bakkali et al., 2008).

The α-terpinene has *in vitro* trypanocidal activity against *T. evansi* and *in vivo* it is responsible for increased longevity. However, it is not effective in the treatment of mice experimentally infected by *T. evansi*. This component when associated with D.A. better efficacy, and it can be used an alternative treatment for *T. evansi* infections. In summary, the α-terpinene alone is the component responsible for the trypanocidal effect in *M. alternifolia* essential oil among those compounds tested in this study, without discarding the possibility that other components present in tea tree oil might also have some trypanocidal action.

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