Ministério da Saúde

**FIOCRUZ**
**Fundação Oswaldo Cruz**

**INSTITUTO OSWALDO CRUZ**

**Pós-Graduação em Biologia Celular e Molecular**

*GIANI FRANÇA SANTORO*

# *Trypanosoma cruzi*: Investigação sobre óleos essenciais como potenciais agentes tripanocidas

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências

**Orientador:** Prof. Dr. Maurilio José Soares

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**ORIENTADOR: Prof. Dr. Maurilio José Soares**

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**RESUMO**

Nifurtimox e benznidazol, as drogas usadas no tratamento da doença de Chagas, apresentam vários efeitos colaterais e tem sua eficácia limitada, especialmente na fase crônica. Diversos produtos naturais têm demonstrado potencial antiparasitário em laboratório e, nesse sentido, compostos obtidos de plantas aparecem como agentes potenciais para o desenvolvimento de novas drogas antiparasitárias. Assim, no presente trabalho investigamos o efeito de óleos essenciais obtidos de *Origanum vulgare* L. (orégano), *Thymus vulgaris* L. (tomilho), *Achillea millefolium* L. (mil-folhas), *Syzygium aromaticum* L. (cravo) *Ocimum basilicum* L. (manjericão) e *Cymbopogon citratus* (DC) Stapf (capim-limão), e de seus principais constituintes, sobre a proliferação e ultra-estrutura de *Trypanosoma cruzi*.

Destilação a vapor foi usada para isolar os óleos essenciais, com análise química realizada por cromatografia gasosa acoplada a espectrometria de massa (CG-EM) através de colaboração com o Departamento de Química da UFLA (Lavras, MG). Epimastigotas de cultura e tripomastigotas sangüíneos foram incubados por 24 h com diferentes concentrações dos óleos essenciais e de alguns de seus principais constituintes sendo quantificado o valor de $IC_{50}$. Nossos dados indicam que óleos essenciais são eficazes contra *T. cruzi* apresentando a seguinte ordem decrescente de atividade sobre epimastigotas ($IC_{50}$/24 h expresso µg/mL): tomilho (77,0) > cravo (99,5) > manjericão (102,0) > capim-limão (126,5) > mil folhas (145,5) > orégano (175,0 µg/mL). Nos ensaios com formas tripomastigotas sangüíneas os óleos mais efetivos foram: capim-limão (15,5) > tomilho (38,0) > cravo (57,5) > orégano (115,0) > mil-folhas (228,0) > manjericão (467,5). Assim, com exceção dos óleos de manjericão e de mil-folhas, os demais óleos testados foram mais ativos sobre tripomastigotas do que sobre epimastigotas.

Tratamento com os principais constituintes dos óleos essenciais de capim-limão (citral), manjericão (linalol), cravo (eugenol) e tomilho (timol) também demonstrou efeito tripanocida. No caso de citral, timol e eugenol, as formas tripomastigotas foram as mais suscetíveis. O óleo de capim-limão foi eficiente no tratamento de macrófagos peritoneais infectados, inibindo a proliferação de amastigotas intracelulares e apresentando para o parâmetro percentagem de infecção um valor de $IC_{50}$/48 h de 12,1 µg/mL.

Experimentos de citometria de fluxo após incubação de epimastigotas e tripomastigotas com óleos essenciais de orégano e tomilho demonstraram que a permeabilidade da membrana celular foi afetada somente em concentrações 4 vezes maiores que a concentração correspondente ao $IC_{50}$/24 h. Por outro lado, uso de concentrações de óleos correspondentes ao $IC_{50}$/24h não resultou em permeabilização celular. Microscopia eletrônica de varredura (MEV) de parasitas tratados com todos os óleos essenciais não demonstrou alterações na membrana plasmática. Entretanto, havia inchaço do corpo do parasito, quando comparado a parasitas do grupo controle. Observação por microscopia eletrônica de transmissão (MET) mostrou inchaço citoplasmático, porém com manutenção na integridade da membrana dos parasitos. Estes dados indicam que os óleos permeam as membranas e atuam sobre organelas ou vias metabólicas citoplasmáticas.

Os óleos essenciais de tomilho e capim-limão foram muito mais ativos sobre os parasitas do que sobre macrófagos peritoneais apontando para a realização de ensaios *in vivo*. Nossos resultados indicam que óleos essenciais e seus constituintes são promissores agentes anti-*T. cruzi*, abrindo perspectivas para descoberta de drogas mais eficazes de origem vegetal para o tratamento de doenças parasitárias.

**ABSTRACT**

Nifurtimox and benznidazole, currently used for the treatment of Chagas disease, present several side effects and have limited efficacy, especially in the chronic phase of the disease. Several natural products have revealed anti-parasitic potential in the laboratory and, in this sense, plant-derived compounds appear as potential targets for the development of new anti-parasitic drugs. Thus, in the present work we have investigated the effect of essential oils obtained from *Origanum vulgare* L. (oregano), *Thymus vulgaris* L. (thyme), *Achillea millefolium* L. (yarrow), *Syzygium aromaticum* L. (clove), *Ocimum basilicum* L. (basil) and *Cymbopogon citratus* (DC) Stapf (lemongrass), and of their main constituents, on proliferation and ultrastructure of *Trypanosoma cruzi*.

Steam distillation was used to isolate the essential oils, with chemical analyses performed by gas chromatography C coupled to mass spectrometry (GC-MS) in collaboration with the Departamento de Química of UFLA (Lavras, MG). Epimastigotes and bloodstream trypomastigotes were incubated for 24 hours with different concentrations of the essential oils and with some of the main constituents of the oils, being quantified the $IC_{50}$/24h. Our data indicate that essential oils are effective against *T. cruzi*, presenting the following order of decreased activity on epimastigotes ($IC_{50}$/24 h expressed as µg/mL): thyme (77.0) > clove (99.5) > basil (102.0) > lemongrass (126.5) > yarrow (145.5) > oregano (175.0). In the assays with bloodstream trypomastigotes, the more active oils were: lemongrass (15.5) > thyme (38.0) > clove (57.5) > oregano (115.0) > yarrow (228.0) > basil (467.5). Thus, except for basil and yarrow, the essential oils were more active on trypomastigotes than on epimastigotes.

Treatment with the main constituents of lemongrass (citral), basil (linalool), clove (eugenol) and thyme (thymol) essential oils also demonstrated trypanocidal effect. The lemongrass oil was effective in the treatment of infected macrophages, inhibiting intracellular amastigote proliferation, and presenting for the parameter % of infection an $IC_{50}$/2 days of 12.1 µg/mL.

Flow cytometry experiments after incubation with oregano and thyme essential oils demonstrated that the cell membrane permeability of treated epimastigotes and trypomastigotes was affected only at concentrations at least 4 times higher than that of the corresponding $IC_{50}$/24h. On the other hand, use of corresponding $IC_{50}$/24h value for each essential oil did not result in cell permeabilization. Scanning electron microscopy (SEM) of treated cells demonstrated apparently no plasma membrane alteration with all used oils. However, there was swelling of the parasite body, as compared to control cells. Observation by transmission electron microscopy (TEM) showed cytoplasmic swelling, however with maintenance of the parasite membrane integrity, as compared to control cells. These data indicate that the oils permeate the membranes and act on cytoplasmic organelles or metabolic pathways.

Thyme and lemongrass oils were much more active against the parasites when compared to the effect on peritoneal macrophages, pointing out to further assays of treatment of experimentally infected mice. Our results indicate that essential oils and their constituents are promising anti-*T. cruzi* agents, opening perspectives to the discovery of more effective drugs of vegetal origin for the treatment of parasitic diseases.

# INTRODUÇÃO

## 1. O *Trypanosoma cruzi* e sua biologia celular

O *Trypanosoma cruzi* é um protozoário hemoflagelado da ordem Kinetoplastida, família Trypanosomatidae (Hoare & Wallace, 1966). Este parasita possui três formas evolutivas distintas (Fig. 1), que se diferenciam morfologicamente pela posição de emergência do flagelo, pelo tamanho e pela posição do cinetoplasto relativa ao núcleo (Prata, 2001).

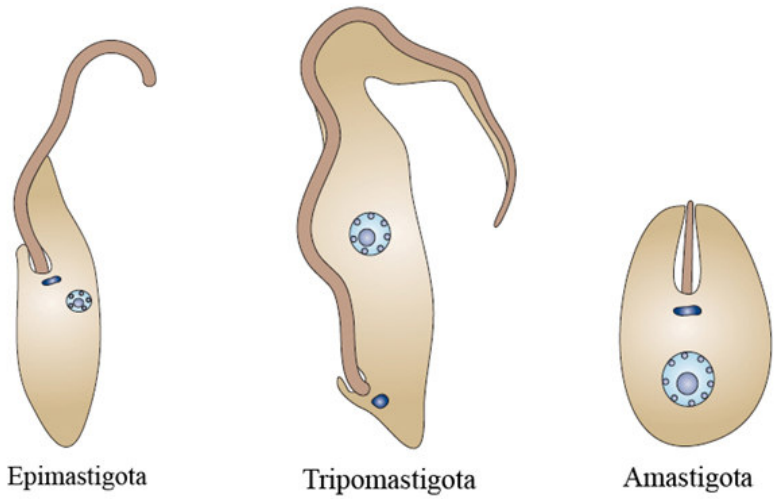



**Figura 1** - Desenho esquemático das três formas evolutivas do *Trypanosoma cruzi*. Adaptado de Docampo et al., 2005.

As formas tripomastigotas têm aproximadamente 15 μm de comprimento e são fusiformes. O cinetoplasto possui a forma redonda e encontra-se na região posterior ao núcleo. São as formas infectivas, não-multiplicativas, do parasita, sendo encontradas no sangue do hospedeiro (homem, mamífero) e no intestino posterior do inseto vetor (Hemiptera: Reduviidae: Triatominae). O flagelo emerge da região posterior do corpo e se dirige para a região anterior, aderido à membrana plasmática.

As formas epimastigotas têm cerca de 20 μm de comprimento e são também fusiformes. O cinetoplasto é em forma de barra e encontra-se na região anterior ao núcleo. Estas são as formas proliferativas no intestino do triatomíneo e as predominantes em culturas acelulares. Por esta razão, são as formas mais freqüentemente utilizadas em estudos bioquímicos e de biologia celular (Prata, 2001).

As formas amastigotas possuem aproximadamente 2 μm de diâmetro, são arredondadas, não possuem um flagelo emergente, e seu cinetoplasto apresenta-se em forma de barra. Estas formas se multiplicam por divisão binária no interior das células do hospedeiro (homem, mamífero) e se diferenciam em tripomastigotas, que com a ruptura da célula hospedeira são liberadas no sangue, podendo então invadir novas células (Prata, 2001). As formas amastigotas e tripomastigotas podem ser obtidas em culturas *in vitro* de células musculares, fibroblastos, macrófagos e outras células (Morello, 1988).

O *T. cruzi* foi descrito pelo pesquisador brasileiro Carlos Chagas em 1909, que já em sua descrição original demonstrou a existência das três formas evolutivas e sua grande diversidade morfológica (Fig. 2).

Este parasita possui organelas citoplasmáticas que são normalmente encontradas em células eucarióticas, além de possuir algumas próprias (De Souza, 1984, 2002). A sua superfície celular possui glicocálice, bicamada lipídica e microtúbulos subpeliculares. O glicocálice é muito fino nas formas epimastigotas e amastigotas e um pouco mais denso em tripomastigotas. Os glicoconjugados estão distribuídos em toda a superfície do corpo e do flagelo. Entretanto, há uma concentração maior na região ao redor do citóstoma (De Souza, 1999).

A membrana plasmática é composta por proteínas integrais, sendo que estas estão presentes em maior número na face interna da membrana de epimastigotas do que em amastigotas e tripomastigotas. Os lipídeos e carboidratos estão dispostos na superfície externa da membrana (Benchimol et al., 1977; De Souza et al., 1978).

O citoesqueleto é basicamente formado por microtúbulos subpeliculares dispostos por todo o corpo do parasita, localizados logo abaixo da membrana plasmática. Estes microtúbulos estão conectados uns aos outros e à membrana plasmática por filamentos curtos. Esta associação é responsável pela rigidez da célula e pela dificuldade encontrada no rompimento da célula por meios mecânicos (De Souza, 1984, 1999, 2002).

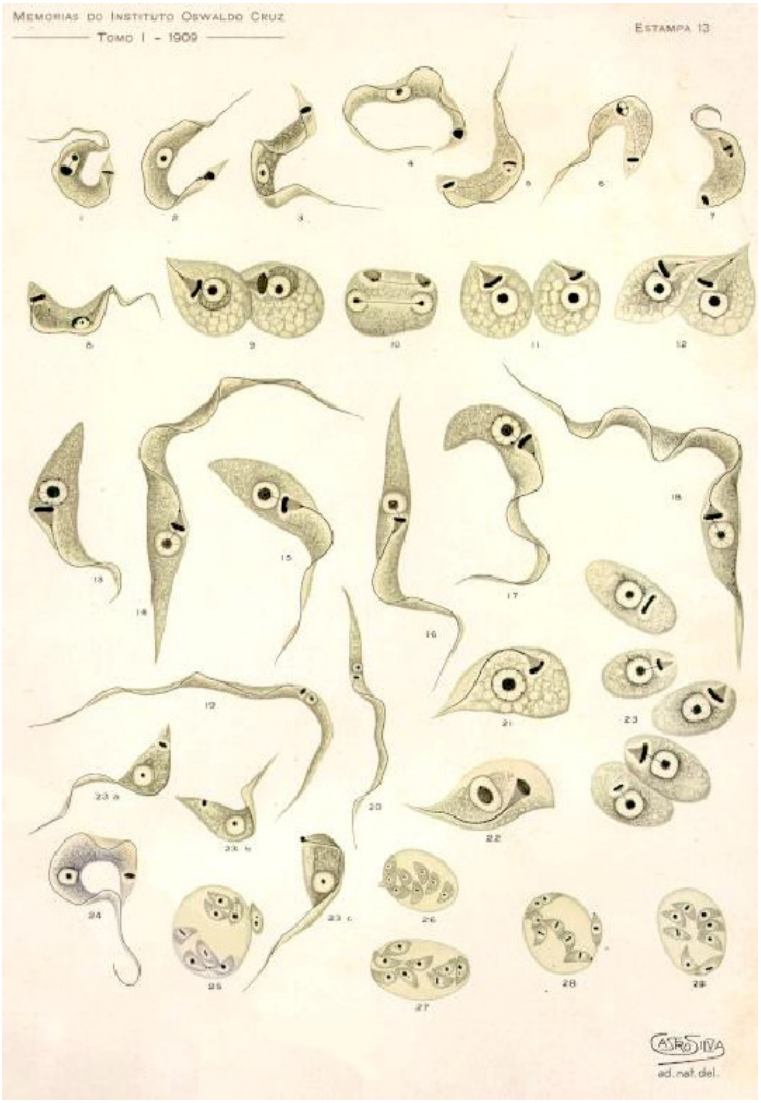



**Figura 2** - Prancha original do artigo de Carlos Chagas descrevendo o *Trypanosoma cruzi*. A legenda original da figura é: Preparados do *Schizotrypanum cruzi*. Fixação a húmido pelo sublimado-álcool (SCHAUDINN). Coloração pela hematoxilina férrea (ROSENBUSCH). Obtido do sítio: Biblioteca virtual Carlos Chagas:http://www.prossiga.br/chagas/

O flagelo está envolvido em no mínimo dois processos biológicos importantes: o movimento celular e a adesão do protozoário à superfície das células do hospedeiro e à membrana perimicrovilar interna do intestino do hospedeiro invertebrado. O flagelo emerge de uma invaginação da membrana plasmática, a bolsa flagelar. Através desta estrutura, o parasita secreta macromoléculas e absorve nutrientes do meio externo (De Souza, 1984, 1999, 2002). O flagelo é formado por um axonema do tipo “9+2” microtúbulos, além de uma estrutura paraxial formada por uma rede de filamentos.

A mitocôndria do *T. cruzi* é única, ramificada e se estende por todo corpo do parasita, apresentando cristas e enzimas como nas outras células eucarióticas (Meirelles & De Souza, 1982). Na região da matriz mitocôndria localizada próximo ao corpo basal existe uma disposição complexa de fibrilas de DNA que forma a estrutura conhecida como cinetoplasto (kDNA) (Borst & Hoeijmakers, 1979). O DNA do cinetoplasto consiste de uma rede de 20.000 a 30.000 moléculas de mini-círculos e dezenas de maxi-círculos associados (Shapiro & Englund, 1995).

O núcleo é alongado nas formas tripomastigotas e localizado na porção central do parasito, enquanto que nas formas amastigotas e epimastigotas é arredondado. Possui uma membrana nuclear típica com poros, seu nucléolo é central, com cromatina periférica. Durante a divisão celular a membrana nuclear permanece intacta e desaparecem a cromatina e o nucléolo, sendo que este reaparece nas fases finais da divisão celular (De Souza, 1984, 1999, 2002).

O retículo endoplasmático está distribuído em todo o citoplasma da célula, podendo fazer contato com a membrana plasmática e microtúbulos subpeliculares como demonstrado em Leishmania (Pimenta & De Souza, 1985). O complexo de Golgi contêm poucas cisternas empilhadas e está localizado na porção anterior do corpo, próximo à bolsa flagelar. Em geral as cisternas são perpendicularmente orientadas em relação ao cinetoplasto e paralelas à bolsa flagelar. Normalmente as faces *cis* e *trans* do Golgi podem ser reconhecidas, a cisterna *trans* sendo normalmente mais dilatada (De Souza, 2002).

O glicosomo é uma organela em geral arredondada, com um diâmetro de 0,3 µm, pertencente ao grupo dos peroxisomos, contendo catalase em algumas espécies e enzimas envolvidas na oxidação de aminoácidos e lipídeos (Michels & Opperdoes, 1991). Uma característica desta organela é concentrar e compartimentalizar enzimas envolvidas na via glicolítica, aumentando a eficiência deste processo (De Souza, 1999).

Os acidocalcisomos são limitados por uma unidade de membrana e estão distribuídos por todo o corpo do parasita. Demonstrou-se por estudos fisiológicos que em *T. cruzi* e outros tripanosomatídeos esta é uma organela acídica com altas concentrações de cálcio (Docampo et al., 1995). Estudos de micro-análise de raios-X confirmaram o acúmulo de cálcio e fósforo dentro dos acidocalcisomos (Scott et al., 1997). Estudos imunocitoquímicos mostraram a presença de uma $H^+$-ATPase vacuolar, $Ca^{2+}$-ATPase e uma pirofosfatase na membrana desta organela (Benchimol et al., 1998; Scott et al., 1998).

A bolsa flagelar é uma invaginação da membrana plasmática que estabelece uma continuidade direta com a membrana do flagelo (Webster & Russel, 1993). Evidências mostram que a bolsa flagelar é uma região altamente especializada da superfície de tripanosomatídeos: (a) é a única região que não apresenta a camada de microtúbulos associados à membrana; (b) a membrana interna da bolsa difere da membrana interna do corpo do parasito e da membrana flagelar em termos da distribuição de partículas intra-membranosas e localização de proteínas, incluindo algumas enzimas (De Souza, 1989); (c) existem muitas evidências morfológicas e citoquímicas mostrando que a bolsa é o local onde ocorre intensa atividade endocítica e exocítica.

Em formas epimastigotas é observado um citóstoma/citofaringe. É uma invaginação afunilada da membrana plasmática na região anterior da célula, a qual pode alcançar a região nuclear. A abertura da invaginação é o citóstoma, que pode alcançar um diâmetro de 0,3 μm. Alguns microtúbulos subpeliculares seguem a invaginação da membrana plasmática formando a citofaringe. Existe uma região especializada da membrana plasmática do parasita que começa na abertura do citóstoma e se projeta para a região da bolsa flagelar.

As formas epimastigotas apresentam vários reservosomos, localizados na região posterior da célula. A morfologia do reservosomo pode variar, mas normalmente é uma organela esférica, delimitada por uma unidade de membrana com diâmetro médio de 0,7 μm. Sua matriz elétron-densa é composta principalmente de proteínas e contém inclusões lipídicas (Soares & De Souza, 1988). A determinação do pH da organela usando o marcador DAMP (3-(2,4-dinitroanilino-3´-amino-*N*-propilamina) indicou um valor de pH 6,0, sugerindo assim que o reservosomo corresponde a um compartimento pré-lisosomal, embora nenhuma atividade de fosfatase ácida tenha sido detectada nesta organela (Soares et al., 1992). Outra característica desta organela é o acúmulo de grande quantidade de cruzipaína, a principal cisteíno protease de *T. cruzi* (Cazzulo et al., 1997; Soares, 1999). O reservosomo tem um papel importante no processo de metaciclogênese. Estudos demonstraram que seu conteúdo é consumido ao longo da diferenciação, ocorrendo o desaparecimento dessa organela quando formas epimastigotas se transformam em tripomastigotas (Soares et al., 1989).

As organelas citoplasmáticas típicas de uma forma epimastigota de *T. cruzi* estão representadas na Figura 3.

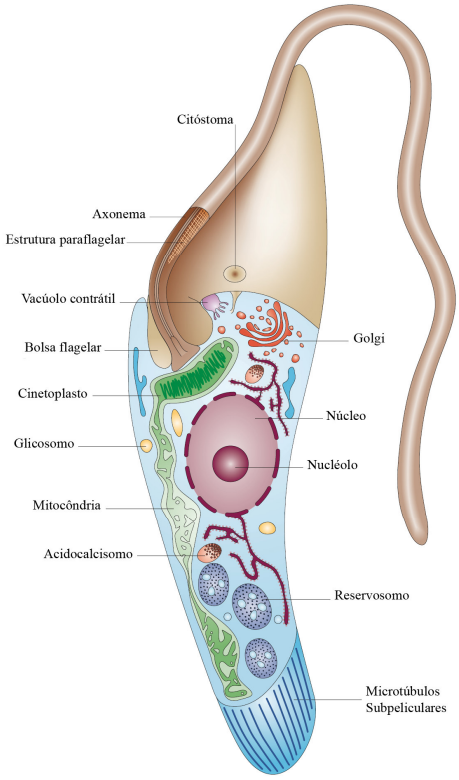



**Figura 3** - Desenho esquemático mostrando as organelas citoplasmáticas em uma forma epimastigota de *T. cruzi*. Adaptado de Docampo et al, 2005.

## 2. Ciclo de vida

O inseto vetor do *Trypanosoma cruzi* pertence à subfamília Triatominae, que possui hábito hematófago. No inseto a proliferação do parasito se dá no lúmen do intestino, sob a forma epimastigota. Estes se diferenciam para tripomastigotas metacíclicas na porção terminal do intestino. Estas últimas formas, não proliferativas, serão eliminadas nas fezes do inseto na hora da alimentação no mamífero (Brack, 1968; Brener & Alvarenga, 1976). No hospedeiro vertebrado as formas metacíclicas penetram em macrófagos e em células de tecido (Deutschlander et al., 1978), escapam do vacúolo fagolisosomal e se diferenciam em amastigotas. As formas amastigotas se multiplicam por divisão binária no citoplasma da célula hospedeira e depois de alguns ciclos de reprodução se diferenciam para formas tripomastigotas, que serão liberadas na circulação sangüínea, podendo então invadir novas células ou tecidos ou então serem ingeridas pelo inseto, transformando-se novamente em epimastigotas, completando assim o ciclo de vida do *T. cruzi* (Fig. 4).

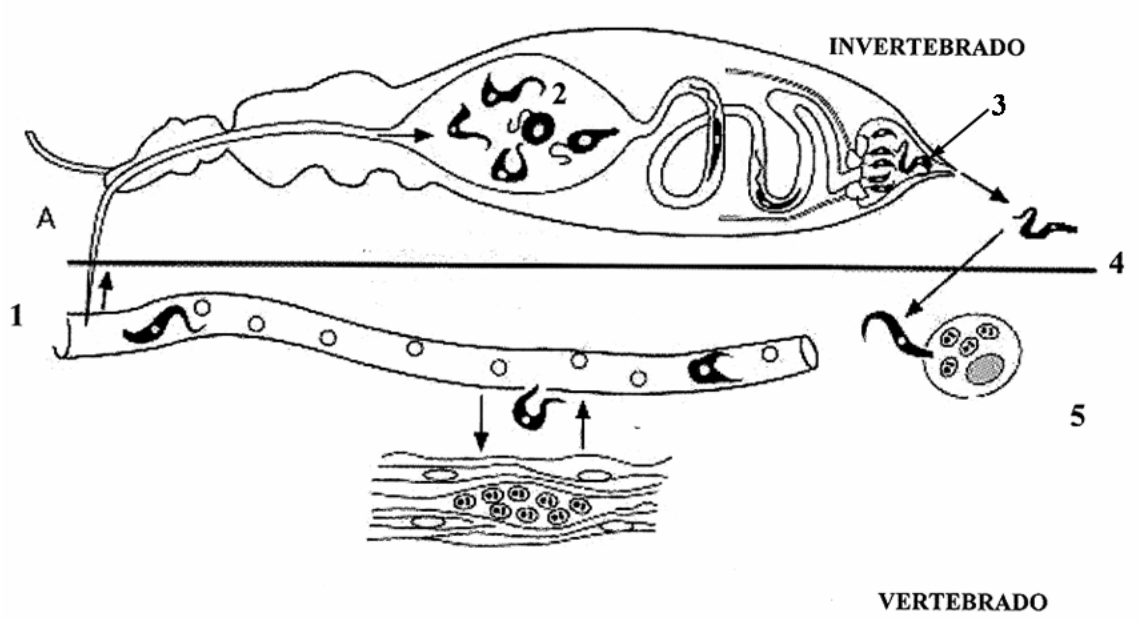



**Figura 4** – Ciclo de vida de *T. cruzi*: 1) formas tripomastigotas sangüíneas; 2) formas epimastigotas no lúmen do intestino; 3) formas tripomastigotas metacíclicas na porção terminal do intestino; 4) formas tripomastigotas eliminadas nas fezes do inseto durante a alimentação no mamífero; 5) formas metacíclicas penetram em células, proliferam, escapam do vacúolo fagolisosomal e se diferenciam em amastigotas. Estas se multiplicam por divisão binária no citoplasma da célula hospedeira e depois de alguns ciclos de reprodução se diferenciam para formas tripomastigotas, que serão liberadas na circulação sanguínea, podendo então invadir novas células ou tecidos ou então serem ingeridas pelo inseto, transformando-se novamente em epimastigotas, completando assim o ciclo de vida do *T. cruzi*.

## 3. Doença de Chagas

Também conhecida como tripanosomíase americana, a doença de Chagas é uma das principais doenças de saúde pública de interesse na América Latina, sendo endêmica em áreas da América Central e do Sul e no México (Castro et al., 2006). Está em segundo lugar, depois da malária, em prevalência e mortalidade devido a doenças associadas a inseto vetor (WHO, 2002). A doença atinge cerca de 16-18 milhões de pessoas no mundo. No Brasil atinge 5 milhões de pessoas e aproximadamente 200 mil novos casos surgem por ano (Dias & Coura, 1997; WHO, 2002). Relatos da Organização Mundial da Saúde indicam que a taxa de mortalidade varia de 8% a 12% dependendo do país, idade, condições de saúde do paciente e tratamento recebido (WHO, 2002).

A doença de Chagas, além de ser transmitida pelo inseto vetor, pode ocorrer também através de transfusão de sangue contaminado, que atinge cerca de 5 a 20% de casos e também por transmissão congênita, que representa 0,5 a 8% dos casos. A transmissão por órgãos transplantados foi relatada na América Latina (Carvalho et al., 1997) e países não-endêmicos como os EUA já apresentaram casos da doença (Chagas Disease after Organ Transplantation. 2001. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5110a3.htm). A transmissão oral de *T. cruzi* por ingestão de tripomastigotas não é comum, mas é possível, já tendo sido demonstrada em animais infectados experimentalmente e em acidentes de laboratório (Aufderheide et al., 2004). Além disso, este tipo de transmissão pode estar correlacionado à ingestão de alimentos contaminados por urina ou fezes de hospedeiros silvestres e/ou de vetores infectados (Shikanai-Yasuda et al., 1991; Gus et al., 1993; Camandaroba et al., 2002; Dias, 2006). Em 2005, ocorreu no Estado de Santa Catarina um surto da doença através da ingestão de caldo de cana contendo o parasita (Lewinsohn, 2005a,b,c).

A doença de Chagas tem três formas de apresentação: fase aguda, indeterminada e fase crônica. A fase aguda da doença se inicia logo após a infecção com um período de incubação de cerca de 7 dias, podendo haver sintomas como febre ou outras manifestações não específicas ou mesmo ser assintomática. Entretanto, miocardite ou meningoencefalite podem ocorrer durante esta fase, particularmente em crianças e pessoas imunocomprometidas (Moraes-Souza, 1999). A taxa de mortalidade causada por estes sintomas severos é aproximadamente 10% (WHO, 1991). Esta fase se caracteriza por uma alta parasitemia e pela presença de infiltrados inflamatórios em diversos tecidos (revisto em Lopes & Chapadeiro, 1997). Na ausência de um tratamento específico, os sintomas persistem por cerca de dois meses e levam a uma mortalidade de 2 a 8%, especialmente em crianças.

O indivíduo infectado permanece um bom tempo da vida em uma fase indeterminada da doença. Aproximadamente um terço dos indivíduos nesta fase desenvolverá a doença de Chagas crônica. A fase indeterminada foi definida baseada em alguns critérios (i) demonstração parasitológica e/ou teste positivo para anticorpo IgG específico contra o parasita; (ii) ausência de sintomas e sinais da doença de Chagas; (iii) ausência de anormalidades de ECG (eletrocardiograma); (iv) tamanho regular do coração, do esôfago e cólon por raios-X. Após anos ou décadas da infecção sub-clínica, 10-50% (de acordo com a área endêmica ou o modo de infecção) dos sobreviventes da fase aguda da doença desenvolve a fase crônica,

que é caracterizada por cardiopatia potencialmente letal ou mega-síndromes (megaesôfago e megacólon) (Coura & De Castro, 2002). Vale a pena ressaltar que mesmo pessoas que permanecem assintomáticas podem estar infectadas, com baixos níveis de parasita no sangue e outros tecidos. Uma "fase crônica recente" é considerada quando a infecção ocorreu nos últimos dez anos ou se as vítimas são crianças com menos de 12 anos (Coura & De Castro, 2002). Pacientes com mais de 10 anos de infecção são considerados "fase crônica tardia". Mesmo após o controle da doença, ela pode ser reativada em situações de imunossupressão, levando ao comprometimento cardíaco, nervoso e cutâneo.

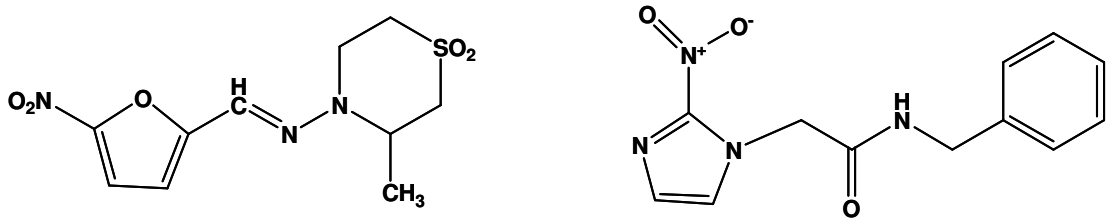
**4. Quimioterapia da doença de Chagas**

Os primeiros compostos usados experimentalmente para o tratamento da doença de Chagas foram descobertos em 1909 e eram à base de arsênico e antimônio.

Os fármacos mais freqüentemente usados para o tratamento da tripanosomíase são os compostos nitroheterocíclicos, o nifurtimox (Lampit® Bayer, 4[(5-nitrofurfurilideno)amino]3-metiltiomorfolina-1,1-dióxido) e o benznidazol (Rochagan® Roche, *N*-benzil-2-nitroimidazol-1-acetamida). Estudos revelaram a base molecular da atividade e toxicidade desses compostos sobre o *T. cruzi* (DoCampo, 1990). O nifurtimox atua via redução do grupo nitro gerando o radical nitro-ânion instável, o qual torna a reagir para produzir metabólitos de oxigênio reduzidos altamente tóxicos (ex: ânion superóxido, peróxido de hidrogênio). O mecanismo de detoxificação de *T. cruzi* para metabólitos de oxigênio é deficiente, particularmente para peróxido de hidrogênio, sendo então mais sensível ao estresse oxidativo que as células de mamífero (DoCampo, 1990). O benznidazol parece atuar por um mecanismo diferente (estresse redutivo), o qual envolve modificação covalente de macromoléculas ou nitro-redução de intermediários (DoCampo, 1990).

Tanto nifurtimox e benznidazol (Figura 5) têm atividade significativa na fase aguda, com cura parasitológica em mais de 80% dos pacientes tratados (a cura parasitológica é definida como o resultado negativo para todos os testes sorológicos e parasitológicos) (Cançado, 1999). Entretanto, a eficácia do tratamento varia de acordo com a região geográfica, provavelmente devido à diferença na susceptibilidade das diferentes cepas de *T. cruzi* às drogas (Andrade et al., 1992; Kirchhoff, 1999). Também já foi demonstrado que benznidazol é eficaz na fase

crônica inicial (Sosa Estani et al., 1998), mas com limitada eficácia na fase crônica tardia.




**Nifurtimox** **Benznidazol**

**Figura 5 -** Estrutura do Nifurtimox e do Benznidazol

O nifurtimox e o benznidazol causam toxicidade sistêmica e efeitos adversos que incluem anorexia, náusea, vômito, febre, dermatites, dores musculares e polineuropatia periférica (Kirchhoff, 2000). Além disso, existem relatos de mutagênese e dano ao DNA (Zahoor et al., 1987). A administração destes fármacos durante a fase crônica da doença é discutida, pelo fato da dificuldade de monitoramento de sua eficácia terapêutica (Coura, 1996). Portanto, a busca de agentes quimioterápicos mais eficazes é de grande interesse para a pesquisa em doença de Chagas.

## 5. Novos fármacos para o tratamento da doença de Chagas

Tanto nifurtimox como benznidazol apresentam sérios efeitos colaterais, levando por vezes à suspensão ou ao abandono do tratamento. Outro problema associado a estes compostos é a descontinuidade da comercialização de nifurtimox (década de 80), e a transferência pela Roche para o LAFEPE dos direitos de produção de benznidazol, que ainda não está disponível para comercialização. Desta forma, torna-se necessário o desenvolvimento de novos fármacos que sejam mais eficazes e menos tóxicos que os atualmente utilizados. Existe uma considerável quantidade de pesquisas de aspectos bioquímicos e fisiológicos do *T. cruzi*. Alguns estudos levam ao desenvolvimento de alvos específicos para uma melhor atuação dos fármacos. As abordagens quimioterápicas racionais estão sendo focadas nas diferenças entre o patógeno e o hospedeiro. Uma delas é a inibição seletiva de uma

via biosintética específica para a sobrevivência do parasito e que não apresente efeito tóxico para o hospedeiro.

Entre os diversos fármacos pesquisados como possíveis agentes quimioterápicos, há os que atuam em vias metabólicas conhecidas. Dentre estes podemos citar: (a) inibidores da biosíntese de esterol (Urbina & Docampo, 2003; Croft et al., 2005; Steverding & Tyler, 2005; Lockman & Hamilton, 2005; Linares et al., 2006; Maya et al., 2006), (b) inibidores de cisteíno proteases, que são proteases essenciais para o parasito (Docampo, 2001; Rodriguez, 2001; Urbina & Docampo, 2003; Croft et al., 2005; Lockman & Hamilton, 2005; Maya et al., 2006), (c) inibidores de endopeptidases, que catalisam a clivagem específica de ligações peptídicas dos resíduos prolina da região carboxi-terminal (Grellier et al., 2001; Lockman & Hamilton, 2005), e (d) inibidores do metabolismo de pirofosfato (Urbina & Docampo, 2003; Lockman & Hamilton, 2005; Maya et al., 2006).

Outros alvos para quimioterapia podem ser os inibidores do metabolismo de purinas, uma vez que os tripanosomatídeos são deficientes na biossíntese *de novo* de purinas (Urbina & Docampo, 2003; Lockman & Hamilton, 2005; Maya et al., 2006) e os inibidores do metabolismo de tripanotiona, que é uma enzima específica de tripanosomatídeos e atua na proteção contra espécies reativas de oxigênio (Docampo, 2001; Rodriguez, 2001; Lockman & Hamilton, 2005; Linares et al., 2006; Maya et al., 2006). Há ainda outra via interessante usada como alvo quimioterápico, a via glicolítica, uma vez que amastigotas de *T. cruzi* não possuem o ciclo do ácido tricarboxílico funcional, sendo então dependentes da glicólise para produção de energia (Lockman & Hamilton, 2005; Linares et al., 2006). Algumas destas pesquisas demonstram resultados promissores, outras ainda necessitam de mais estudos.

**6. Estudos com produtos vegetais**

Nas últimas décadas houve um movimento chamado de “volta à natureza”, com a re-descoberta da fitoterapia, reforçada pelo sucesso obtido por produtos naturais com taxol para o tratamento de câncer e artemisinina para malária. As plantas já forneceram muitas substâncias para a medicina no passado e permanecem como importante fonte no desenvolvimento de novos fármacos a serem utilizados como agentes quimioterápicos. Diversos estudos já demonstraram a atividade biológica de algumas plantas sobre parasitos (Phillipson, 2003).

Dados recentes mostram que as pesquisas sobre atividade antimicrobiana utilizando plantas medicinais, mais que dobraram entre os anos de 1995 a 2004, com 307 artigos relacionados a este tema neste período, quando comparado ao período entre 1966 a 1994, com 115 artigos, demonstrando o aumento do interesse por este tipo de pesquisa (Rios & Recio, 2005).

Estudos têm demonstrado a ação de diversas plantas e produtos naturais como potentes agentes antihelmínticos (Githiori et al.,2006), antifúngicos (De Boer et al., 2005), antibacterianos (Mahady, 2005) e antiprotozoários (Salem & Werbovetz, 2006).

A atividade tripanocida de extratos vegetais brutos (Caceres et al. 1998; Mafezoli et al. 2000; Schmeda-Hirschmann et al. 2001; Mesquita et al., 2005; Billo et al., 2005; Ndjakou Lenta et al., 2006) bem como de compostos isolados dos mesmos, como alcalóides (Morello et al., 1994; Chataing et al., 1998; Fournet et al. 1998; Ferreira et al., 2007), taxóides (Baum et al., 1981; Dantas et al., 2003), acetogeninas ((Waechter et al., 1998), lignanas (Bastos et al., 1999), terpenos (Neira et al., 1998; Batista et al., 1999; Fournet et al. 1994; Brengio et al., 2000; Cunha et al., 2006) e quinonas (De Castro et al., 1994; Morello et al., 1995; Pinto et al., 1997; Alves et al., 1999) têm sido evidenciados por diversas pesquisas (revisto em Coura & De Castro, 2002 e em Fournet & Munoz, 2002).

**7. Efeito microbicida de óleos essenciais**

A flora brasileira apresenta um imenso potencial para a produção de compostos primários e secundários, sendo que cerca de 16% das 500.000 espécies de plantas que existem no mundo encontra-se na floresta Amazônica. Entretanto, a pesquisa de substâncias ativas derivadas de plantas no Brasil ainda é muito incipiente: estima-se que até 1980 menos de 1% das espécies da flora brasileira eram conhecidas quanto aos seus constituintes químicos (Gottlieb & Mors, 1980). Plantas produzem uma grande variedade de metabólitos secundários com proteção natural contra ataque de insetos e microbianos e muitos destes metabólitos tem aplicações médicas ou em alimentos (Wallace, 2004).

Os óleos essenciais extraídos de plantas são usados há muito tempo na medicina como antibactericidas e antifúngicos. São constituídos principalmente por terpenos, hidrocarbonetos cíclicos, aldeídos ou derivados de ésteres, sendo uma

característica importante dos óleos essenciais e de seus constituintes a hidrofobicidade (Burt 2004).

As propriedades antimicrobianas de óleos essenciais vêm sendo evidenciadas há algum tempo (Shelef, 1983) e recentemente o interesse nestas substâncias aumentou consideravelmente (Nychas, 1995). Os óleos essenciais, ou seus componentes, têm um amplo espectro de efeitos farmacológicos, com antibacterianos (Deans & Ritchie, 1987; Carson et al., 1995; Mourey & Canillac, 2002), antivirais (Bishop, 1995), antihelmínticos (Pandey et al., 2000; Pessoa et al., 2002) e antiprotozoários (Mikus et al., 2000; Tchoumbougnang et al., 2005). Estas características são relacionadas possivelmente à função destes compostos nas plantas (Guenter, 1948; Mahmoud & Croteau, 2002). Geralmente os óleos essenciais com propriedades antibacterianas têm alta percentagem de compostos fenólicos como carvacrol, eugenol ou timol (Burt, 2004).

A investigação das atividades antimicrobianas, o modo de ação e o uso potencial de óleos de plantas apresentam agora um interesse renovado. Diversos produtos naturais demonstraram potencial antiparasitário em laboratório e então os compostos derivados de plantas aparecem como alvos potenciais para o desenvolvimento de novas drogas (Kayser et al., 2003). Demonstrou-se a ação inibitória contra vários parasitos humanos tais como *Plasmodium* (Valentin et al., 1995; Tchoumbougnang et al., 2005), *Trypanosoma brucei*, *Leishmania major* (Mikus et al., 2000) e *Leishmania amazonensis* (Rosa et al., 2003; Monzote et al., 2006).

### 7.1. *Origanum vulgare* L. (orégano)

As plantas do gênero *Origanum* (família Labiatae) são normalmente caracterizadas pelas diferentes composições químicas de seus óleos essenciais. A variação na composição química do óleo essencial do orégano tem provavelmente um papel importante em sua propriedade antimicrobiana (Sivropoulou et al., 1996). O óleo essencial de *Origanum vulgare* L. (orégano) é muito utilizado no preparo de alimentos e apresenta atividade antimicrobiana (Burt & Reinders 2003; Burt et al., 2004) e antifúngica (Paster et al., 1995), atividade esta atribuída a hidrocarbonetos como γ-terpineno e *p*-cimeno e a compostos fenólicos como timol e carvacrol (Aligiannis et al. 2001; Lambert et al. 2001). Estes dois fenólicos podem ser combinados para o tratamento de infecções, uma vez que apresentam atividade inibitória sobre bactérias orais (Ditry et al., 1994).

### 7.2. *Thymus vulgaris* L. (tomilho)

O óleo essencial do *Thymus vulgaris* L. (tomilho) possui propriedades farmacológicas que já foram investigadas (Kohlert et al., 2002), além de apresentar apresenta atividade antimicrobiana (Burt & Reinders 2003; Burt et al., 2004) e antifúngica (Giordani et al., 2004). O óleo é geralmente usado em culinária principalmente por seu sabor, aroma e capacidade de preservação das propriedades dos alimentos. Estudos mostram que o óleo do tomilho inibe o crescimento de microrganismos patogênicos e possui uma forte atividade antimicrobicida, na maioria das vezes atribuída à presença de constituintes fenólicos (Dorman & Deans, 2000; Lambert et al., 2001).

### 7.3. *Achillea millefolium* L. (mil-folhas)

Partes aéreas de diferentes gêneros de *Achillea* L., incluindo a *Achillea millefolium* L. (mil-folhas) são muito usadas na medicina popular devido às inúmeras propriedades farmacológicas, tais como anti-inflamatória, anti-oxidante, anti-espasmódica e antiséptica (Goldberg et al., 1969; Falk et al., 1975; Candan et al., 2003). Uma variedade de aplicações médicas foi relacionada a diversas classes dos metabólitos secundários encontrados em espécies de *Achillea* (Chandler et al., 1982; Mitich, 1990). Um dos usos medicinais destas plantas, já bem conhecido, é na prevenção de infecções e o tratamento de feridas e de febres (Rohloff et al., 2000).

### 7.4. *Syzygium aromaticum* L (cravo)

Na medicina chinesa tradicional, o *Syzygium aromaticum* L (cravo), tem sido usado há muito tempo por suas propriedades para doenças digestivas e diarréia (Kim et al., 2003). O efeito antibacteriano do óleo essencial do cravo foi descrito em diversos estudos (Larhsini et al., 2001; Burt & Reinders, 2003).

### 7.5. *Ocimum basilicum* L. (manjericão)

O óleo essencial do *Ocimum basilicum* L. (manjericão) vem sendo utilizado em produtos alimentícios, perfumaria e odontológicos. O óleo essencial do manjericão e seus principais constituintes têm atividade antimicrobiana sob uma grande variedade de bactérias Gram-positivas e Gram-negativas e leveduras (Suppakul et al., 2003). Um componente importante de *O. basilicum* é o eugenol, um alilfenol, com atividades microbicida e analgésicas para humanos (Koeduka et al., 2006). Demonstrou-se o uso deste óleo inibindo o crescimento do tripanosomatídeo

*Herpetomonas samuelpessoai*, além de provocar alterações ultra-estruturais (Holetz et al., 2003).

### 7.6. *Cymbopogon citratus* (DC) Stapf (capim-limão)

O chá de folhas de *Cymbopogon citratus* (DC) Stapf (capim-limão) é popularmente usado no Brasil devido a suas propriedades analgésicas, antipiréticas e antiinflamatórias (Carlini et al., 1986). Além disso, o óleo essencial do capim-limão possui atividade antimicrobicida (Onawunmi 1989; Ibrahim, 1992), antifúngica (Viollon & Chaumont, 1994; Wannissom et al., 1996) e antibacteriana (Onawunmi et al., 1984). Já foi demonstrada a atividade antibacteriana e antifúngica do óleo do capim-limão e seus constituintes (Mishra & Dubey, 1994; Cimanga et al., 2002). Onawunmi e colaboradores (1984) já indicaram que o citral, principal constituinte do óleo essencial do capim-limão, é o responsável por suas principais propriedades. Estudos com o óleo essencial do capim-limão *in vivo* em *Plasmodium berghei* demonstraram uma significante redução de seu crescimento (Tchoumbougnang et al., 2005) e este óleo também apresentou efeito antiprotozoário sobre *Crithidia deanei,* um tripanosomatídeo de inseto (Pedroso et al., 2006).

Uma vez que diversos óleos têm apresentado ação antiparasitária, nos propusemos a investigar o efeito tripanocida dos seis óleos essenciais apresentados acima, todos com a composição química determinada, bem como dos principais constituintes dos óleos de tomilho (timol), cravo (eugenol), manjericão (linalol) e de capim-limão (citral).

# OBJETIVOS

**Objetivo geral**

Analisar a atividade dos óleos essenciais de orégano (*Origanum vulgare* L.), tomilho (*Thymus vulgaris* L.), mil-folhas (*Achillea millefolium* L.), cravo (*Syzygium aromaticum* L.), manjericão (*Ocimum basilicum* L.), e capim-limão (*Cymbopogon citratus* (DC) Stapf), extraídos por destilação de arraste a vapor e caracterizados quimicamente, bem como de seus principais constituintes, sobre as três formas evolutivas de *Trypanosoma cruzi*.

**Objetivos específicos**

- Determinar a composição química dos óleos essenciais por cromatografia gasosa e espectrometria de massa [em colaboração com o Departamento de Química da Universidade Federal de Lavras (UFLA), MG].
- Determinar a concentração que inibe 50% do crescimento das formas epimastigotas ($IC_{50}$) após tratamento com os óleos essenciais e seus principais constituintes.
- Determinar a concentração que lisa 50% das formas tripomastigotas quando tratadas com os óleos essenciais e seus principais constituintes.
- Avaliar as alterações morfológicas induzidas pelos óleos essenciais sobre as formas epimastigotas e tripomastigotas por microscopia eletrônica de varredura e microscopia eletrônica de transmissão.
- Verificar a viabilidade da membrana plasmática das formas epimastigotas e tripomastigotas tratadas com diferentes concentrações dos óleos essenciais de tomilho e orégano por citometria de fluxo usando iodeto de propídio.
- Avaliar efeito citotóxico dos óleos essenciais de tomilho e capim-limão sobre macrófagos peritoneais de camundongo suíço.
- Analisar o efeito do óleo essencial de capim-limão sobre a proliferação de formas amastigotas em macrófagos peritoneais de camundongo suíço.

# RESULTADOS

# Artigo 1

**Santoro GF, Cardoso MG, Guimarães LGL, Salgado APSP, Menna-Barreto RFS, Soares MJ (2007). Effect of oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) essential oils on *Trypanosoma cruzi* (Protozoa: Kinetoplastida). *Parasitology Research 100: 783-790.***

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ORIGINAL PAPER

# Effect of oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) essential oils on *Trypanosoma cruzi* (Protozoa: Kinetoplastida) growth and ultrastructure

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**Abstract** In the present work, we have investigated the effect of essential oils obtained from *Origanum vulgare* L. (oregano) and *Thymus vulgaris* L. (thyme) on growth and ultrastructure of diverse evolutive forms of *Trypanosoma cruzi*. Culture epimastigotes and bloodstream trypomastigotes were incubated for 24 h with different concentrations of oregano or thyme essential oils and with thymol (the main constituent of thyme), and the inhibitory concentration $(IC)_{50}$ was determined by cell counting. Crude extract of oregano essential oil inhibited epimastigote growth ($IC_{50}$/24 h=175 μg/ml) and also induced trypomastigote lysis ($IC_{50}$/24 h=115 μg/ml). Thyme essential oil presented $IC_{50}$/24 h values of 77 μg/ml for epimastigotes and 38 μg/ml for trypomastigotes, while treatment with thymol resulted in an $IC_{50}$/24 h of 62 μg/ml for epimastigotes and 53 μg/ml for trypomastigotes. Scanning electron microscopy of treated cells showed few morphological alterations at the plasma membrane. Observation by transmission electron microscopy showed cytoplasmic swelling with occasional morphological alterations in plasma and flagellar membrane. Our data indicate that oregano and thyme essential oils are effective against *T. cruzi*, with higher activity of thyme, and that thymol may be the main component responsible for the trypanocidal activity.

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## Introduction

Essential oils are complex natural mixtures of volatile secondary metabolites, isolated from plants or spices usually by steam distillation. The antimicrobial properties of spice essential oils were long recognized (Shelef 1983) and recently the interest on these substances has considerably increased (Nychas 1995). Essential oils, or their components have a broad spectrum of pharmacological effects, with antibacterial (Deans and Ritchie 1987; Carson et al. 1995; Mourey and Canillac 2002), antiviral (Bishop 1995), antihelmintical (Pandey et al. 2000; Pessoa et al. 2002), and antiprotozoal (Mikus et al. 2000; Tchoumbougnang et al. 2005) properties. These characteristics are possibly related to the function of these compounds in plants (Guenther 1948; Mahmoud and Croteau 2002).

The essential oils of *Origanum vulgare* L. (oregano) and *Thymus vulgaris* L. (thyme) were shown to exhibit a range of biological activities. Both are commonly used in foods, mainly for their flavor, aroma, and preservation properties. They were used to delay or inhibit growth of pathogenic microorganisms, as they present strong antimicrobial activity, mostly attributable to the presence of phenolic compounds, such as thymol and carvacrol, and to hydrocarbons, such as γ-terpinene and *p*-cymene (Sivropoulou et al. 1996; Dorman and Deans 2000; Aligiannis et al. 2001; Lambert et al. 2001). Thymol and carvacrol can be used

alone or in combination during treatment of oral infectious diseases, as they show an inhibitory activity on oral bacteria (Ditry et al. 1994).

Little has yet been done on the evaluation of essential oils against endemic tropical diseases. Few data are known on their effects on the hemoflagellate protozoan *Trypanosoma cruzi*. There are currently 18–20 million people infected with *T. cruzi*, and another 40 million people are at risk of acquiring the disease (WHO 2002). Chemotherapy for this disease remains unsatisfactory. Nitrofurans and nitroimidazoles were used in the acute phase, but they are not totally efficient. Both drugs have serious side effects, including vomiting, peripheral polyneuropathy, and allergic dermopathy (De Castro 1993; Rassi and Luquetti 1992; Croft et al. 1997), probably as a consequence of oxidative or reductive damage in the host tissues (Urbina and Docampo 2003). Some new drugs already in test for treating this parasitic infection may have a promising future (Stoppani 1999; Croft 1999), but the lack of selective cytotoxicity against *T. cruzi* makes the search for new products a priority in the medical field (Sepúlveda-Boza and Cassels 1996).

Thus, the use of natural products with recognized low toxicity and potent antioxidative activity may be more appropriate than synthetic antioxidants if their trypanocidal activity against the infective trypomastigote form of *T. cruzi* proves to be adequate (Campos et al. 2005). Therefore, in the present work we have investigated the effect of essential oils and constituents obtained from oregano and thyme on growth in the cell viability and ultrastructure of diverse evolutive forms of *T. cruzi*.

## Materials and methods

### Parasites

Epimastigote forms of *T. cruzi*, Y strain (Silva and Nussenzweig 1953), were maintained at 28°C in LIT (liver infusion–tryptose) medium (Camargo 1964) supplemented with 10% fetal calf serum.

Bloodstream trypomastigotes were obtained by cardiac puncture of infected Swiss albino mice at the peak of parasitemia (7 days postinfection). Blood was collected with 2-ml syringes containing 0.2 ml of 3.8% sodium citrate as anticoagulant. Blood samples were pooled in 15-ml tubes and then centrifuged for 15 min at 500×*g* to isolate the parasites from red blood cells and leukocytes. The pellet containing the parasites was maintained for 20 min at 37°C to allow the trypomastigotes to swim to the supernatant. The supernatant was then centrifuged for 10 min at 1,500×*g* to eliminate platelets and the isolated parasites were transferred to RPMI-1640 medium (Sigma Chemical, St. Louis, MO, USA), and supplemented with 10% fetal calf serum. After homogenization, they were resuspended and kept in the same medium until use.

### Essential oil extraction and analysis

Plant material was collected at the Horto de Plantas Medicinais of the Universidade Federal de Lavras, Brazil. Collections of oregano (*O. vulgare* L.) and thyme (*T. vulgaris* L.) were always performed in the morning around 08:00 at a mild temperature and in the absence of rain. Various collections were performed in the period from May to August 2004 to guarantee an adequate supply of essential oil. Fresh material was collected from the whole aerial portion of the plants and the essential oils were isolated by steam distillation, employing a modified Clevenger apparatus (Craveiro et al. 1981).

Qualitative analysis of both essential oils was performed by gas chromatography coupled to mass spectrometry using a Shimadzu CG-17A (Shimadzu, Kyoto, Japan) chromatograph coupled with a QP-5000 mass selective detector under the following operational conditions: capillary DB5 fused silica column (30 m×0.25 mm, 0.25 μm film thickness); injector temperature 220°C; column temperature set initially at 40°C and then programmed at 3°C/min to 240°C; carrier gas helium with linear gas velocity of 1.0 ml/min; split ratio 1:10; injected volume 1.0 μl (1% dilution in dichloromethane); and inlet pressure 100.2 kPa. Mass spectra were taken at 70 eV; decomposition speed 1.000; decomposition interval 0.50; fragments from 45 to 450 Da were decomposed. A mixture of hydrocarbons ($C_9H_{20}$ to $C_{26}H_{54}$) was injected under the same conditions. Identification of constituents was then performed by comparing the spectra obtained with those of the equipment data bank and by the Kovats index, calculated for each constituent as previously described (Adams 1995).

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A equipment, under the following operational conditions: capillary DB5 column, injector temperature 220°C; detector temperature 240°C; column temperature set initially at 40°C and then programmed at 3°C/min to 240°C; carrier gas nitrogen with linear gas velocity of 2.2 ml/min; split ratio 1:10; injected volume 1 μl (1% dilution in dichloromethane); and inlet pressure 115 kPa. Quantification of each constituent was estimated by area normalization (%).

### Determination of the inhibitory concentration ($IC_{50}$/24 h)

The essential oils were initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml. This solution was then dissolved in culture medium to obtain a

stock solution at 1 mg/ml. Under this condition, the DMSO was diluted at 0.01%, a concentration that is not toxic for the protozoa. Both solutions were maintained at −20°C. The stock solution was then diluted at different concentrations for the experiments.

Five-day-old culture epimastigotes ($5\times10^6$ cells/ml) were grown at 28°C in LIT medium supplemented with 10% fetal calf serum and then incubated for 24 h in the absence or presence of different concentrations (10 to 200 μg/ml) of oregano or thyme essential oils. The inhibitory concentration $(IC)_{50}$/24 h (concentration that inhibits 50% parasite growth) was then evaluated by counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

Bloodstream trypomastigote forms ($5\times10^6$ cells/ml) were grown at 37°C in RPMI-1640 medium supplemented with 10% fetal calf serum and then incubated for 24 h with different concentrations (10 to 400 μg/ml) of oregano or thyme essential oils. The $IC_{50}$/24 h (concentration that lyses 50% of the parasites) was then evaluated by counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

In addition, 5-day-old culture epimastigotes (grown at 28°C in LIT medium) and bloodstream trypomastigote forms (grown at 37°C in RPMI-1640 medium) were incubated for 24 h in the absence and presence of different concentrations (25 to 250 μg/ml) of thymol, the main constituent of thyme essential oil. The $IC_{50}$/24 h was then evaluated by counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

## Cytotoxicity

Mouse peritoneal macrophages were obtained from Swiss mice that are 18–20 g in weight. Macrophages were seeded on coverslips located in 24-well plates ($10^6$ cells/well) and maintained in RPMI-1640 medium at 37°C for 24 h. Thereafter, the cultures were washed in medium and incubated for 24, 48, or 72 h with different concentrations (3.9 to 250 μg/ml) of thyme essential oil. The coverslips were then stained with Giemsa and observed in a Nikon Eclipse E600 (Nikon, Tokyo, Japan) light microscope.

The use of mice to perform the above-mentioned experiments (isolation of bloodstream trypomastigotes and obtaining of peritoneal macrophages) was made in adherence to ethical standards of Fundação Oswaldo Cruz and was approved by an ethics committee (CEUA-FIOCRUZ, protocol no. P0099-01).

## Scanning electron microscopy

Epimastigotes and bloodstream trypomastigotes were incubated for 24 h with the respective $IC_{50}$/24 h of oregano or thyme oils. The cells were then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, adhered for 15 min to glass coverslips coated with 0.1% poly-L-lysine, washed in buffer and postfixed for 30 min with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2. Thereafter, the samples were dehydrated in acetone, critical point dried and mounted on scanning electron microscopy (SEM) stubs. The samples were coated with a 20-nm-thick gold layer and examined in a Zeiss (Oberkochen, Germany) DSM940 scanning electron microscope. Digital images were acquired and stored in a computer.

## Transmission electron microscopy

Epimastigotes and bloodstream trypomastigotes were treated for 24 h with the concentration corresponding to the $IC_{50}$/24 h value of oregano or thyme oils and then collected by centrifugation at 5,500×*g*, washed in 0.1 M phosphate buffer at pH 7.2 and fixed for 30 min with 2.5% glutaraldehyde in 0.1 M phosphate buffer. The cells were then washed three times with 0.1 M phosphate buffer and postfixed for 15 min with 1% osmium tetroxide/0.8% potassium ferricyanide/5 mM $CaCl_2$ in 0.1 M cacodylate buffer at pH 7.2 (Meirelles and Soares 2001). After rinsing in this same buffer, the cells were dehydrated in graded acetone, infiltrated overnight in an acetone–PolyBed 812 mixture (1:1) and embedded for 72 h at 60°C in PolyBed 812 (PolySciences, Warrington, PA, USA) resin. Ultrathin sections were stained with 5% uranyl acetate and lead citrate and observed in a Zeiss EM10C transmission electron microscope.

## Estimate of *Trypanosoma cruzi* cell membrane permeability by flow cytometry

Epimastigote forms ($5\times10^6$ cells/ml) were treated for 24 h with 88 to 1,400 μg/ml of oregano, or else with 39 to 624 μg/ml of thyme essential oils. Bloodstream trypomastigotes ($5\times10^6$ cells/ml) were treated for 24 h with 57.5 to 920 μg/ml of oregano, or else with 19 to 304 μg/ml of thyme essential oils. The parasites were then incubated for 15 min with 30 μg/ml of propidium iodide (PI) and the samples were kept on ice until analysis. Positive lysis control was obtained by treating the parasites with 0.0025% saponin for 15 min.

Data acquisition and analysis were performed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with the Cell Quest software (Joseph Trotter, Scripps Research Institute, San Jose, CA, USA). A total of 10,000 events were acquired in the region previously established as that corresponding to normal forms of *T. cruzi*. The values obtained were then analyzed with Windows Multiple Document Interface (version 2.8) software for flow cytometry.

## Results

The composition of *O. vulgare* L. (oregano) and *T. vulgaris* L. (thyme) essential oils used in our experiments was determined by comparing their relative retention times and the mass spectra of oil components with mass spectra from a data library. The main compounds identified in these oils and their relative proportions are listed in Table 1. Thymol was the main component of thyme, while 3-cyclohen-1-ol was the main component of oregano.

Our results showed that treatment of epimastigotes with different concentrations of oregano and thyme essential oils resulted in dose-dependent growth inhibition, with $IC_{50}$/24 h of about 175 and 78 μg/ml, respectively (Fig. 1a). Lower values were obtained with treatment of bloodstream trypomastigotes, with $IC_{50}$/24 h of about 115 and 38 μg/ml for oregano and thyme, respectively (Fig. 1b).

Because thyme was more effective to kill trypanosomes than oregano, we have incubated parasites with different concentrations of thymol, the main component of thyme, to evaluate whether it could be responsible for the cell lysis. Indeed, our data demonstrated that the mean $IC_{50}$/24 h for epimastigotes and trypomastigotes was about 50–60 μg/ml (Fig. 1c), which was significantly lower than that obtained with whole extracts on epimastigotes, and similar to that obtained on trypomastigotes.

At the ultrastructural level, observation by SEM revealed that treatment of epimastigotes with a concentration corresponding to the $IC_{50}$/24 h value of oregano and thyme essential oils resulted in apparently no plasma membrane alteration (Fig. 2b), with occasional rounding of the cell body (Fig. 2c) compared to control cells (Fig. 2a). Examination of oregano-treated parasites by transmission electron microscopy (TEM) showed slight morphological alterations in the plasma membrane (Fig. 2e) compared to control parasites (Fig. 2d). Intracellular organelles, including kinetoplast, nucleus, and reservosomes, appeared, in general, not to be affected by the drug treatment. Similar observations were made in thyme-treated parasites (data not shown).

**Table 1** Quantitative and qualitative composition of *O. vulgare* L. (oregano) and *T. vulgaris* L. (thyme) essential oils

| | Main constituent | Retention index | Percent |
|---|---|---|---|
| *O. vulgare* L. (oregano) | *p*-Cymene | 14.989 | 2.3 |
| | γ-Terpinene | 16.572 | 16.0 |
| | α-Terpineol | 18.564 | 12.3 |
| | **3-ciclohen-1-ol** | **22.613** | 26.2 |
| *T. vulgaris* L. (thyme) | Limonene | 15.154 | 5.2 |
| | **Thymol** | **28.551** | 80.4 |
| | Carvacrol | 28.818 | 6.0 |

Main components are marked in bold.

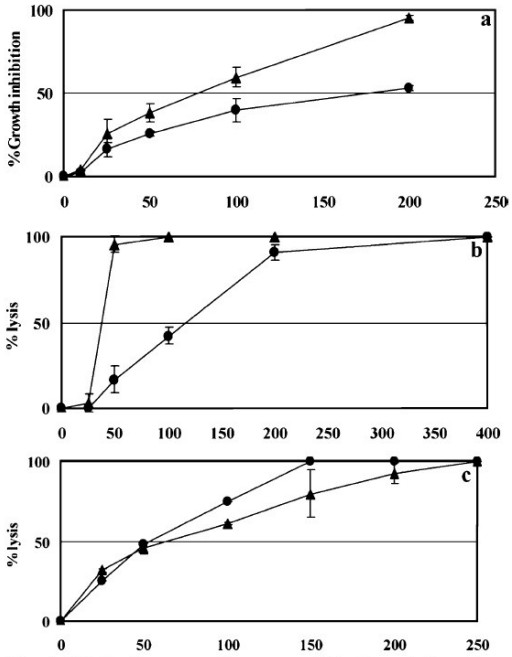


**Fig. 1** Effect of oregano, thyme essential oils, and thymol on epimastigote and trypomastigote forms of *T. cruzi* after a 24-h treatment, with determination of $IC_{50}$/24 h values. **a** Epimastigotes treated with concentrations from 25 to 250 μg/ml of oregano (*circles*) or thyme (*triangles*). **b** Bloodstream trypomastigotes treated with concentrations from 25 to 400 μg/ml of oregano (*circles*) or thyme (*triangles*). **c** Treatment of epimastigotes (*triangles*) and trypomastigotes (*circles*) with thymol, the main constituent of thyme, with concentrations from 25 to 250 μg/ml. Each point represents the mean±SD of three different experiments

Observation by SEM of thyme-treated trypomastigotes showed slight alteration in the cell shape (Fig. 3b) when compared to control cells (Fig. 3a). Similar observations were made in oregano-treated parasites (data not shown). Ultrastructural analysis by TEM of oregano- and thyme-treated cells demonstrated myelin figures inside the flagellum (Fig. 3d) and occasional cytoplasmic swelling (Fig. 3e) compared to control cells (Fig. 3c).

Incubation of mouse peritoneal macrophages for 24 to 72 h with thyme essential oil resulted in no cytotoxic effect with concentrations up to 62 μg/ml. This concentration was about 1.5 times higher than that required to affect the trypomastigote forms.

The flow cytometry experiments demonstrated that the cell membrane permeability of essential-oil-treated epimastigotes (Fig. 4a) and trypomastigotes (Fig. 4b) was affected only at concentrations at least four times higher than that of the corresponding $IC_{50}$/24 h. On the other hand, the use of

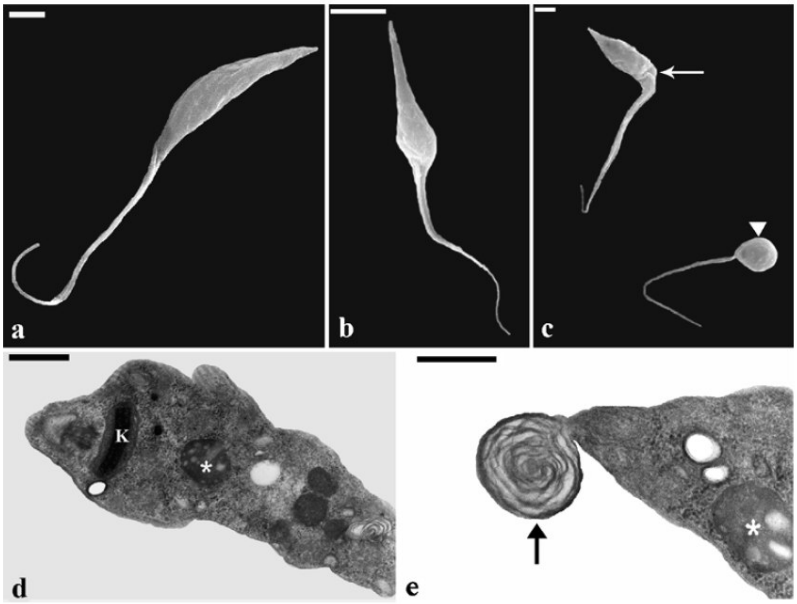


**Fig. 2** Effect of oregano and thyme essential oils on epimastigote forms of *T. cruzi* after 24-h treatment, as observed by SEM and TEM. **a** SEM of control untreated epimastigote showing the normal elongated morphology. **b** SEM of parasite treated with 175 μg/ml of oregano showing no morphological alteration compared to the control. **c** SEM of parasites treated with 78 μg/ml of thyme. Note the shorter size of a parasite (*arrow*) and a parasite with rounded body (*arrowhead*). **d** TEM of untreated epimastigote showing the normal morphology, with kinetoplast (*K*) and reservosomes (*asterisk*). **e** TEM of the formation of a membrane projection (*arrow*) in a parasite treated with 115 μg/ml of oregano. **a**–**c** Bar=2 μm. **d** Bar=0.5 μm. **e** Bar=0.2 μm

essential oil concentrations corresponding to the $IC_{50}$/24 h did not result in cell permeabilization: Most parasites preserved a morphology that could be recognized by the flow cytometer.

## Discussion

The antimicrobial properties of volatile essential oils and their constituents were assessed from a wide variety of plants (Deans and Ritchie 1987; Janssen et al. 1987;

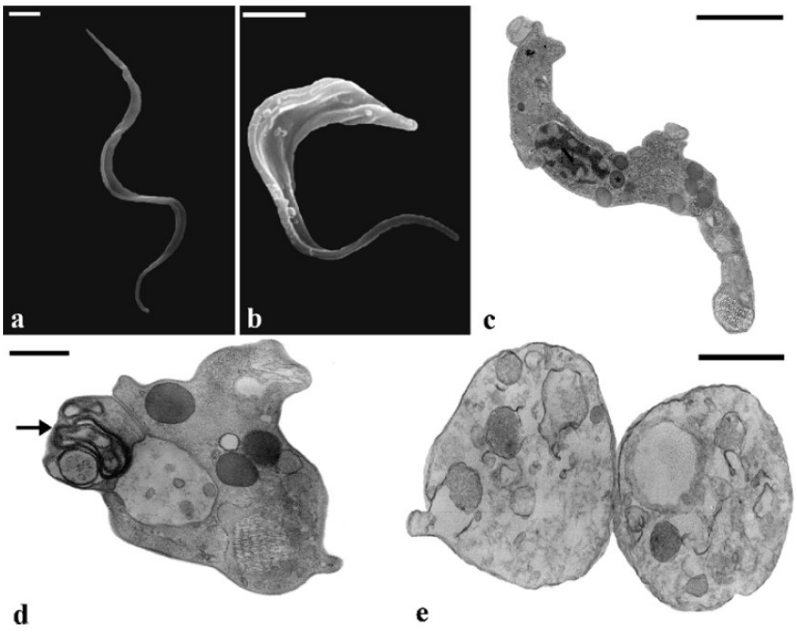


**Fig. 3** Effect of oregano and thyme essential oils on trypomastigote forms of *T. cruzi* after 24-h treatment, as observed by SEM and TEM. **a** SEM of untreated cell showing the typical morphology. **b** SEM of parasite treated with 38 μg/ml of thyme showing swelling of the body. **c** TEM of control cell with normal morphology showing the nucleus (*N*) and acidocalcisomes (*asterisk*). **d** TEM of parasite treated with 115 μg/ml of oregano. Note the myelin figure inside the flagellum (*arrow*). **e** TEM of trypomastigotes treated with 38 μg/ml of thyme showing cytoplasmic swelling. **a**, **b** Bar=2 μm. **c**–**e** Bar=0.5 μm

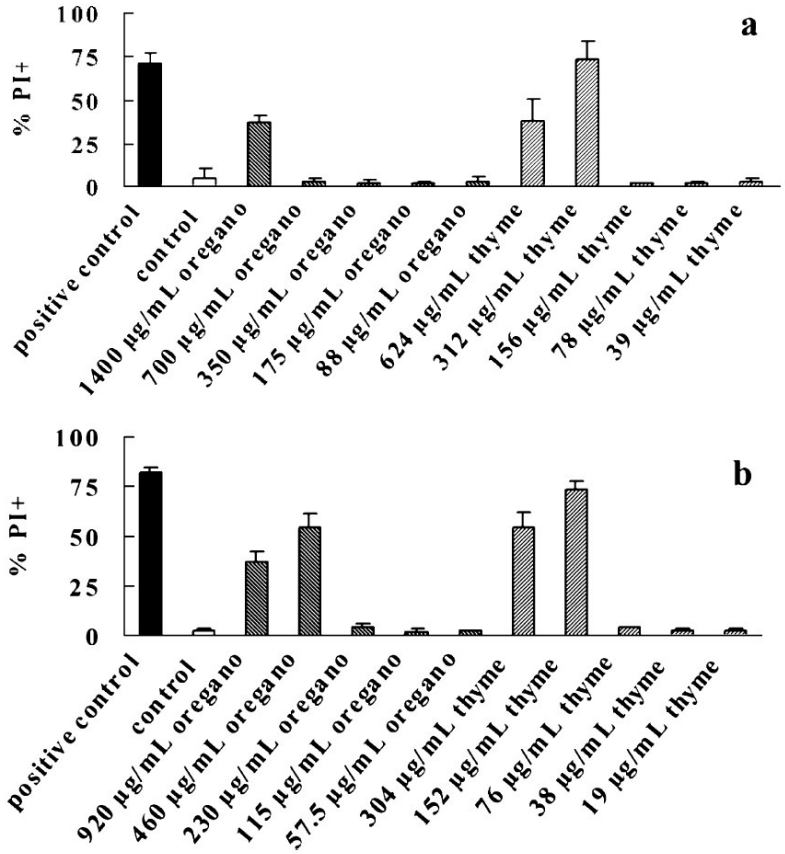


**Fig. 4** Flow cytometry analysis of *T. cruzi* epimastigotes (**a**) and trypomastigotes (**b**) treated with oregano and thyme essential oils and then labeled with PI. Treatment of epimastigotes with different concentrations of oregano and thyme essential oils demonstrate that permeability to PI occurs only at concentrations four times higher than that required to affect the parasite viability. Note that treatment with the $IC_{50}$/24 h concentration did not affect the membrane permeability. Positive control: parasites incubated with 0.0025% saponin for 15 min. Control: untreated parasites

Lis-Balchin and Deans 1997; Soylu et al. 2006). It is clear from these studies that these plant secondary metabolites have potential in medical procedures and applications in cosmetic, food (Shelef 1983; Friedman et al. 2002), and pharmaceutical industries (Janssen et al. 1987; Cai and Wu 1996; Mahmoud and Croteau 2002).

Investigation of antimicrobial activities, mode of action, and potential uses of plant volatile oils now presents a renewed interest. Several natural products have revealed antiparasitic potential in the laboratory and thus plant-derived compounds appear as potential targets for the development of new antiparasitic drugs (Kayser et al. 2003). In this sense, it was shown that oregano (*O. vulgare* L.) and thyme (*T. vulgaris* L.) essential oils present antimicrobial (Burt and Reinders 2003; Burt et al. 2005) and antifungal (Paster et al. 1995; Giordani et al. 2004) activities. Our data showed that the essential oils of oregano, thyme, and thymol were active against epimastigotes and trypomastigotes of the protozoan *T. cruzi* at an $IC_{50}$ that was similar to that obtained after treatment of the parasites in vitro with benznidazole ($IC_{50}$/24 h≅50 µg/ml), the drug currently in use for treatment of Chagas disease (Sepúlveda-Boza and Cassels 1996).

In our experiments we have used *T. cruzi* epimastigotes and trypomastigotes. In drug screening, tests are usually

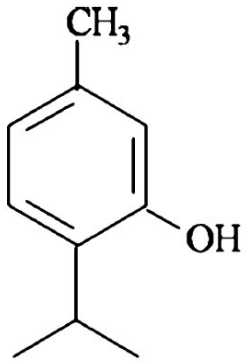


**Fig. 5** Molecular structure of thymol, the main component of *T. vulgaris* L.

performed on the epimastigote stage, as this evolutive form can be easily maintained in axenic cultures and thus, despite presenting different sensitivity compared to other stages (Sepúlveda-Boza and Cassels 1996), is useful to identify active compounds. Furthermore, our experiments were also carried out by using bloodstream trypomastigote forms, as these are the infective forms of the parasite to man. Our data showed that trypomastigotes were more sensitive than epimastigotes to the essential oils used. Different metabolic pathways may account for this different sensitivity, as trypomastigotes and epimastigotes are adapted to live in different environmental conditions.

Plants from the genus *Origanum* (Labiatae family) are often characterized by the presence of chemical differences with respect to both essential oil content and composition. The variation in chemical composition of *Origanum* essential oils is likely to play a role on the level of their antimicrobial properties (Sivropoulou et al. 1996). The main constituent of the oregano species used in our experiments was 3-cyclohen-1-ol, with a concentration of 26.2%. Both the low level of this compound and its chemical composition may be the explanation for low effectiveness of oregano essential oil in parasite killing.

The essential oil derived from *T. vulgaris* L. (Labiatae family) presents pharmacological properties that were already investigated (Kohlert et al. 2002). A previous study has demonstrated that thyme essential oil presents toxicity against the trypanosomatid protozoa *Trypanosoma brucei* and *Leishmania major* (Mikus et al. 2000). Our study showed that this oil is also effective against *T. cruzi*. Thymol, the main component of thyme (80.4%), may be the constituent responsible for the in vitro trypanocidal activity. However, the finding that trypomastigotes are more sensitive to thyme essential oil ($IC_{50}$=38 μg/ml) than to purified thymol (53 μg/ml) suggests that other thyme components might be even more active than thymol. The observation that the thyme essential oil was toxic to mouse peritoneal macrophages only at a concentration about 1.5 times higher than that required to affect the trypomastigote forms of *T. cruzi* indicates a potential use for this compound for further experiments.

The importance of the presence of the hydroxyl group in phenolic compounds such as carvacrol and thymol was confirmed (Knobloch et al. 1986; Dorman and Deans 2000; Ultee et al. 2002). The activity of thymol (Fig. 5) is attributed to the characteristic feature of the phenolic hydroxyl group, which is more acidic than that of the aliphatic hydroxyl group and to the presence of a system of delocalized electrons (Ultee et al. 2002). An important characteristic of essential oils and their components is their hydrophobicity (Burt 2004). Our flow cytometry data indicate that due to this characteristic, the essential oils permeate the cell membrane and kill the parasites by affecting cytoplasmic metabolic pathways or organelles, and not by compromising the parasite membrane integrity, which would result in cell membrane lysis.

In summary, our data, together with other data on treatment of trypanosomatid protozoa with plant essential oils (Holetzi et al. 2003; Rosa et al. 2003), open perspectives to the discovery of more effective drugs of vegetal origin for the treatment of parasitic diseases.

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## Artigo 2

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**_Trypanosoma cruzi_: Activity of essential oils from _Achillea millefolium_ L., _Syzygium aromaticum_ L. and _Ocimum basilicum_ L. on epimastigotes and trypomastigotes**

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**Abstract**

Trypanocidal activity of clove (*Syzygium aromaticum* L.), basil (*Ocimum basilicum* L.) and yarrow (*Achillea millefolium* L.) essential oils and some of their constituents (eugenol and linalool) was investigated on *Trypanosoma cruzi* epimastigote and bloodstream trypomastigote forms. Steam distillation was used to isolate the essential oils, with chemical analyses performed by gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS). The $IC_{50}$ (concentration that inhibits 50% parasite growth) of the oils and constituents upon *T. cruzi* was determined by cell counting in a Neubauer chamber. Cell morphology alterations were observed by scanning and transmission electron microscopy. Treatment with oils and constituents demonstrated that they inhibit parasite growth, with clove essential being the most effective one ($IC_{50}$ = 99.5 μg/ml for epimastigotes and 57.5 μg/ml for trypomastigotes). Ultrastructural alterations were observed mainly in the nucleus.

Key words: basil, clove, essential oil, *Trypanosoma cruzi*, ultrastructure, yarrow.

## 1. Introduction

American trypanosomiasis (Chagas' disease), caused by the trypanosomatid protozoan *Trypanosoma cruzi*, affects 16-18 million people, being 5 million only in Brazil and about 100 million people with infection risk (Dias and Coura, 1997; WHO, 2002). Recent surveys indicate that there are 200,000 new cases and 21,000 deaths associated with this condition every year (Morel, 2000; WHO, 2002). The drug most frequently used for treatment of Chagas' disease is benznidazole, a nitroimidazole derivative that was developed in the years 1960. This compound is active in the acute stage of Chagas' disease, but is of limited efficacy against an established chronic-stage of the disease. This drug has significant side effects, probably as a consequence of oxidative or reductive damage in the host tissues. These side effects, including anorexia, vomiting, peripheral polyneuropathy and allergic dermopathy (Kirchhoff, 1999), can in some cases lead to treatment discontinuation.

The functional role of spices and their constituents is a strong topic in food-related plant research. Spices, in fact, are actually not only evaluated for their seasoning properties, but are also appreciated for their bioactive efficacy as bacteriostatics, fungicides, antioxidants, and nutrients (Sacchetti et al., 2004). The aerial parts of different spices of the genus *Achillea* L., including *Achillea millefolium* L. (yarrow), are widely used in folk medicine due to numerous pharmacological properties, such as anti-inflammatory, antioxidant, antispasmodic, stomachic and antiseptic (Goldberg et al., 1969; Falk et al., 1975; Candan et al., 2003). In traditional Chinese medicine, clove (*Syzygium aromaticum* L.), has long been considered to have medicinal properties such as a stimulant against digestive disorders and diarrhea (Kim et al., 2003). The antibacterial effect of clove essential oil has been described in several studies (Larhsini et al., 2001; Burt and Reinders, 2003). The essential oil of basil (*Ocimum basilicum* L.) has been used extensively in food products, perfumery and dental products. Basil essential oils and their principal constituents were found to exhibit antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria, yeast, and mold (Suppakul et al., 2003).

There is an intense search for new trypanocidal drugs, and plant extracts and essential oils appear as a promising alternative. Thus, in this work we have analyzed the effect of basil, clove and yarrow essential oils on growth and ultrastructure of the hemoflagellate *T. cruzi*.

## 2. Materials and methods

### *2.1. Parasites*

Epimastigote forms of *T. cruzi*, Y strain (Silva and Nussenzweig, 1953), were maintained with weekly passages at 28 °C in LIT medium (Camargo, 1964) supplemented with 10% fetal bovine serum (FBS). Five-day-old epimastigotes (mid-log phase of growth) were used in all experiments.

Bloodstream trypomastigotes were obtained by cardiac puncture of infected Swiss albino mice, at the peak of parasitaemia (7 days post-infection). Blood was collected with 2 ml syringes containing 0.2 ml of 3.8% sodium citrate as anticoagulant. Blood samples were pooled in 15 ml tubes and then centrifuged for 15 min at 500*g* to isolate the parasites from red blood cells and leukocytes. The pellet containing the parasites was maintained for 20 min at 37 °C, to allow the trypomastigotes to swim to the supernatant. Thereafter, the supernatant was centrifuged for 10 min at 1,500*g* to eliminate platelets. The isolated parasites were transferred to RPMI-1640 medium (Sigma-Aldrich Co., St Louis, MO, USA), supplemented with 10% FBS. After homogenization, they were ressuspended in RPMI-1640 medium and kept at 37 °C in a 5% $CO_2$ incubator until use.

### *2.2. Plant material and essential oil isolation*

Yarrow (*Achillea millefolium* L.) was collected at the Medicinal Plants Garden of the Universidade Federal de Lavras (UFLA), Brazil (Voucher deposited at the Herbário ESAL, Dept. of Biology, UFLA; register number 17496). Collection was always performed in the morning around 08:00, during October 2005, at a temperature of 20 °C and absence of rain. Clove (*Syzygium aromaticum* L., syn. *Eugenia caryophyllata* L.) and basil (*Ocimum basilicum* L.) were purchased in the city market of Lavras, MG, during the same period. Clove was acquired from Comercial Santa Bárbara Ltda (Franca, SP, Brazil), while basil was acquired from Ki-flor Indústria e Comércio de Produtos Alimentícios Ltda (Campo Belo, MG, Brazil). The essential oils were isolated by steam distillation, employing a modified Clevenger apparatus (Craveiro et al., 1981).

### *2.3. Gas chromatography/mass spectrometry (GC/MS) analyses*

Qualitative analysis of essential oils was performed by gas chromatography coupled to mass spectrometry (GC/MS), using a Shimadzu CG-17A (Shimadzu Corporation, Kyoto, Japan) chromatograph coupled with a QP-5000 mass selective detector, under the following operational conditions: capillary DB5 fused silica column (30 m X 0.25 mm; 0.25 μm film thickness); injector temperature 220 °C; column

temperature set initially at 40 °C and then programmed at 3 °C/min to 240 °C; carrier gas helium, with linear gas velocity of 1.0 ml/min; split ratio 1:10; injected volume 1.0 µl (1% dilution in dichloromethane); inlet pressure 100.2 kPa. Mass spectra were taken at 70 eV; decomposition speed 1.000; decomposition interval 0.50; fragments from 45 to 450 Da were decomposed. A mixture of hydrocarbons ($C_9H_{20}$ to $C_{26}H_{54}$) was injected under these same conditions and identification of constituents was then performed by comparing the spectra obtained with those of the equipment data bank and by the Kovats index, calculated for each constituent as previously described (Adams, 1995).

*2.4. Gas chromatography/flame ionization gas chromatography (GC/FID)*

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) equipment, under the following operational conditions: capillary DB5 column, injector temperature 220 °C; detector temperature 240 °C; column temperature set initially at 40 °C and then programmed at 3 °C/min to 240 °C; carrier gas nitrogen, with linear gas velocity of 2.2 ml/min; split ratio 1:10; injected volume 1 µl (1% dilution in dichloromethane); inlet pressure 115 kPa. Quantification of each constituent was estimated by area normalization (%).

*2.5. Antitrypanocidal activity*

The essential oils were initially dissolved in dimethyl sulphoxide (DMSO) at a concentration of 100 mg/ml. This solution was then dissolved in culture medium to obtain a stock solution at 1 mg/ml. Under this condition, the DMSO was diluted at 0.01%, a concentration that is not toxic for the protozoa. Both solutions were stored at -20 °C. The stock solution was then diluted at different concentrations for the experiments.

Five-day-old culture epimastigotes (5 X $10^6$ cells/ml) were grown at 28 °C in LIT medium supplemented with 10% FBS and then incubated for 24 h in absence or presence of different concentrations (10 to 200 µg/ml) of clove, basil or yarrow essential oils. The $IC_{50}$ (concentration that inhibits 50% parasite growth) was then evaluated by counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

Bloodstream trypomastigote forms (5 X $10^6$ cells/ml) were grown at 37 °C in RPMI medium supplemented with 10% FBS and then incubated for 24 h with different concentrations (50 to 500 µg/ml) of clove, basil or yarrow essential oils. The

$IC_{50}$ (concentration that lyses 50% of the parasites) was then evaluated by counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

Five-day-old culture epimastigotes (grown at 28 °C in LIT medium) and bloodstream trypomastigote forms (maintained at 37 °C in RPMI medium) were also incubated for 24 h in absence and presence of different concentrations (50 to 250 μg/ml) of either eugenol (the main constituent of clove essential oil) or linalool (the main constituent of basil essential oil). The $IC_{50}$ was then evaluated by counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

Use of mice to perform the above-mentioned experiments (isolation of bloodstream trypomastigotes) was made in adherence to ethical standards of Fundação Oswaldo Cruz and was approved by an ethics committee (CEUA-FIOCRUZ, Protocol n° P0099-01).

*2.6. Analysis by scanning electron microscopy (SEM)*

Epimastigotes and bloodstream trypomastigotes were incubated for 24 h in the absence or presence of the respective $IC_{50}$ of clove, basil or yarrow essential oils. The cells were then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, adhered for 15 min to glass coverslips coated with 0.1% poly-L-lysine, washed in buffer and post-fixed for 30 min with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2. Thereafter, the samples were dehydrated in acetone, critical point dried and mounted on SEM stubs. The samples were coated with a 20-nm thick gold layer and examined in a Zeiss (Oberkochen, Germany) DSM940 scanning electron microscope. Digital images were acquired and stored in a computer.

*2.7. Ultrastructural analysis by transmission electron microscopy (TEM)*

Epimastigotes and bloodstream trypomastigotes were treated for 24 h in the absence or presence of the concentration corresponding to the $IC_{50}$ value of clove, basil and yarrow essential oils and then collected by centrifugation at 5,500*g*, washed in 0.1 M phosphate buffer, pH 7.2, and fixed for 30 min with 2.5% glutaraldehyde in 0.1 M phosphate buffer. The cells were then washed three times with 0.1 M phosphate buffer and post-fixed for 15 min with 1% osmium tetroxide / 0.8% potassium ferricyanide / 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2 (Meirelles and Soares, 2001). After rinsing in this same buffer, the cells were dehydrated in graded acetone, infiltrated overnight in an acetone-PolyBed 812 mixture (1:1) and

embedded for 72 h at 60 °C in PolyBed 812 (PolySciences, Warrington, PA, USA) resin. Ultra-thin sections were stained with 5% uranyl acetate and lead citrate and observed in a Zeiss EM10C transmission electron microscope.

## 3. Results

### *3.1. Qualitative and quantitative analysis of essential oils*

The chromatograms (Fig. 1) demonstrate the composition of *Syzygium aromaticum* L. (clove), *Ocimum basilicum* L. (basil) and *Achillea millefolium* L. (yarrow) essential oils used in our experiments. The compounds identified in these oils, their relative proportions and the Kovats indexes are listed in Table 1. The main components of clove, basil and yarrow essential oils were eugenol (86.34%), linalool (46.97%) and chamazulene (48.32%), respectively.

### *3.2. Antitrypanocidal activity of essential oils*

Treatment of epimastigotes with different concentrations of clove, basil and yarrow essential oils resulted in dose-dependent growth inhibition, with $IC_{50}$/24h of about 99.5 μg/ml, 102 μg/ml and 145.5 μg/ml, respectively (Fig. 2A). The $IC_{50}$/24h values obtained after treatment of bloodstream trypomastigotes were about 57.5 μg/ml, 467.5 μg/ml and 228 μg/ml for clove, basil and yarrow, respectively (Fig. 2B).

### *3.3. Antitrypanocidal activity of main constituent*

We have also determined the $IC_{50}$/24h value of the main constituent of the clove and basil essential oils after incubation with epimastigotes and bloodstream trypomastigotes. The values obtained for epimastigotes treated with eugenol and linalool were 246 μg/ml and 162.5 μg/ml, respectively (Fig. 3A), while treatment of bloodstream trypomastigotes resulted in $IC_{50}$/24h values of 76 μg/ml for eugenol and 264 μg/ml for linalool (Fig. 3B).

### *3.4. Ultrastructural analysis by scanning (SEM) and transmission electron microscopy (TEM)*

Epimastigotes treated with a concentration corresponding to the $IC_{50}$/24h value of basil essential oil and then observed by SEM demonstrated apparently no plasma membrane alteration. There was, however, rounding of the cell body (Fig. 4B), as compared to control cells (Fig. 4A). Similar observations were made on clove- and yarrow-treated parasites (data not shown).

Observation of treated cells at the ultrastructural level by TEM revealed that basil-treated epimastigotes presented cytoplasmic extraction and nuclear alteration, while the plasma membrane remained preserved (Fig. 4D), when compared to

control cells (Fig. 4C). There was shrinkage of the nuclear material, which became separated form the nuclear membrane (Fig. 4D). Similar observations were made on clove- and yarrow-treated parasites (data not shown).

Observation by SEM of clove-treated trypomastigotes showed swelling of the parasite body (Fig. 5B), when compared to control cells (Fig. 5A). Transmission electron microscopy analysis demonstrated that treatment of trypomastigotes with a concentration corresponding to the $IC_{50}$/24h value of yarrow essential oil resulted in altered nucleus (Fig. 5D), as compared to control cells (Fig. 5C). There was a loss of nuclear content, and masses of condensed chromatin appeared (Fig. 5D). Similar observations were made on clove and basil-treated parasites (data not shown).

**4. Discussion**

The chemical analyses obtained in the present study are similar to previous reports on essential oils of *Syzygium aromaticum* L. (Zheng et al., 1992), *Ocimum basilicum* (Suppakul et al., 2003) and *Achillea millefolium* (Rohloff et al., 2000) obtained in other countries.

It is well documented the antiprotozoal activity of diverse essential oils affecting protozoa such as *Leishmania*, *Trypanosoma brucei* and *Plasmodium* (Mikus et al., 2000; Rosa et al., 2003; Tchoumbougnang et al., 2005; Ueda-Nakamura et al., 2006). It has been demonstrated anti-plasmodial activity of diverse essential oils with $IC_{50}$ varying between 2.0 and 30 μg/ml (Boyom et al., 2003). Data obtained for our research group has also demonstrated that oregano and thyme essential oils present anti-*Trypanosoma cruzi* activity (Santoro et al., 2007). In the present study, best effects were obtained when *T. cruzi* was treated with clove essential oil, resulting in an $IC_{50}$/24h = 99.5 μg/ml for epimastigotes and 57.5 μg/ml for bloodstream trypomastigotes, thus showing that trypomastigotes are more susceptible to the treatment.

Eugenol is the major component of clove (*Syzygium aromaticum* L.) essential oil, but it is also an important chemical constituent of essential oils from many aromatic plants, such as *Dicipelium cariophyllatum*, *Pimenta dioica*, *Croton zehntneri* var. *eugenoliferum*, and *Croton zehntneri* (De Vincenzi et al., 2000). It has been shown that eugenol presents antibacterial activity (Nakamura et al., 1999). However, incubation of *T. cruzi* with eugenol alone resulted in $IC_{50}$/24h values = 246 μg/ml for epimastigotes and 76 μg/ml for trypomastigotes. These data, besides confirming the higher resistance of epimastigotes to clove and its main constituent (eugenol),

indicate that some synergism may exist with the different oil components. Recent data demonstrated that treatment with 100 μg/ml eugenol induced 100% death in *Leishmania amazonensis* (Ueda-Nakamura et al., 2006), thus suggesting that *Trypanosoma* parasites are more resistant to the oil action.

Linalool, a terpenic alcohol, is the main component of basil (*Ocimum basilicum* L.) essential oil (46.97%). It has been shown that linalool-rich essential oils extracted from several plants have antimicrobial properties (Mazzanti et al., 1998). Previous data have demonstrated that a linalool-rich essential oil is effective against *Leishmania amazonensis* promastigotes and amastigotes (Rosa et al., 2003), with 50% lethal dose of linalool = 22 ng/ml for amastigotes. In our experiments it was demonstrated that the $IC_{50}$/24h for linalool was 162.5 μg/ml for epimastigotes and 264 μg/ml for trypomastigotes. The little effect of linalool alone on *T. cruzi*, together with the high values needed to be effective against the parasites when using basil essential oil (102-467.5 μg/ml) do not indicate this oil as a possible candidate for further investigation.

A variety of medical applications has been related to several classes of secondary metabolites found in *Achillea* species (Chandler et al., 1982; Mitich, 1990). The well-known medicinal use of these plants includes the prevention of infections and treatment of wounds and fevers (Rohloff et al., 2000). As described elsewhere (Gherase et al., 2003), we have also identified chamazulene as one of the main constituents of yarrow (*Achillea millefolium* L.) essential oil. Our quantification analysis demonstrated similar concentrations (43%) as that obtained in the other study (53%), although the plants were collected in distinct regions of the world. However, in our experiments the yarrow essential oil did not demonstrate an effective trypanocidal action, as observed for clove.

The present study demonstrates that essential oils and their constituents exhibit antitrypanocidal properties in vitro. Then, they can represent a step forward in the search for novel antiprotozoal agents, at a time when there is an urgent need for novel drugs. Ongoing studies in our laboratory on the activity of essential oils on the amastigote forms and mammalian host cells (mouse peritoneal macrophages) show that the essential oil of thyme (Santoro et al., 2007, in Press) and lemongrass (Santoro et al., unpublished data) do not present cytotoxity for mouse macrophages at concentrations at least 1.5-2 times higher than the $IC_{50}$ concentrations for bloodstream trypomastigotes. Furthermore, our recent data on treatment of intracellular amastigotes with lemongrass essential oil (Santoro et al., unpublished

data) indicate that intracellular amastigotes are as susceptible as bloodstream trypomastigotes, while epimastigote forms are more resistant.

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**Legends to Figures**

Fig. 1. Chromatograms of essential oils by gas chromatography coupled to mass spectrometry (GC/MS), showing the Retention and Kovats indexes. (A) Clove. (B) Basil. (C) Yarrow.

Fig. 2. Effect of clove, basil and yarrow essential oils on *T. cruzi* epimastigote and trypomastigote forms after a 24 h treatment, with determination of $IC_{50}$/24h values. (A) Epimastigotes treated with concentrations from 10 to 200 μg/ml, showing the percent of growth inhibition. (B) Bloodstream trypomastigotes treated with concentrations from 50 to 500 μg/ml, showing the percent of cell lysis. Each bar represents the mean ± standard deviation of three different experiments.

Fig. 3. Effect of eugenol and linalool on *T. cruzi* epimastigote and bloodstream trypomastigote forms after a 24 h treatment, with determination of $IC_{50}$/24h values. (A) Epimastigotes treated with concentrations from 50 to 250 μg/ml of eugenol (■) or linalool (▲). (B) Bloodstream trypomastigotes treated with concentrations from 50 to 250 μg/ml of eugenol (■) or linalool (▲). Each point represents the mean ± standard deviation of three different experiments.

Fig. 4. Effect of basil essential oil on epimastigote forms, as observed by scanning (SEM) or transmission (TEM) electron microscopy, after treatment with 102 μg/ml ($IC_{50}$/24h) of essential oil. (A) Control parasites observed by SEM. (B) SEM of basil-treated epimastigote. Note the absence of plasma membrane alteration, but a rounding of the parasite body. (C) Untreated epimastigote showing normal organelles by TEM. (D) Treated epimastigote, showing altered nucleus (N). Note that the plasma membrane remains unaltered. N, nucleus; K, kinetoplast; F, flagellum. Figs. A and B: bar = 2 μm; Figs. C and D: bar =1 μm.

Fig. 5. Bloodstream trypomastigotes treated with the respective $IC_{50}$/24h of clove or yarrow essential oils. (A) Control trypomastigote form visualized by SEM with normal morphology. (B) Clove-treated trypomastigote (57.5 μg/ml). Note swelling of the parasite body. (C) Untreated bloodstream trypomastigotes showing typical organelles by TEM. (D) Bloodstream trypomastigote treated with yarrow

essential oil ($IC_{50}$/24h = 228 μg/ml) observed by TEM, showing altered nucleus (N). Note cytoplasmic extraction, while the plasma membrane remains unaffected, with the presence of subpellicular microtubules (arrowhead). N, nucleus; K, kinetoplast; F, flagellum; L, lipid inclusion. Figs. A and B: bar = 1 μm; Figs. C and D: bar = 0.5 μm.

**Table 1**: Quantative and qualitative composition of *Syzygium aromaticum* L. (clove), *Ocimum basilicum* L. (basil) and *Achillea millefolium* L. (yarrow) essential oils, as determined by GC/MS and GC/FID.

| | Main constituent | Retention Index (RI) | Kovats Index | % |
|---|---|---|---|---|
| *Syzygium aromaticum* L. (clove) | n.d | 10.451 | - | 0.59 |
| | n.d | 15.118 | - | 0.43 |
| | eugenol | 31.501 | 1356.0 | 86.34 |
| | trans-caryophyllene | 34.074 | 1415.4 | 8.20 |
| | α-humulene | 35.534 | 1453.7 | 0.83 |
| | eugenol acetate | 38.617 | 1524.2 | 3.58 |
| *Ocimum basilicum* L. (basil) | α-thujone | 10.412 | 935.0 | 0.85 |
| | camphene | 11.054 | 949.4 | 0.80 |
| | n.d | 12.274 | - | 0.62 |
| | myrcene | 12.383 | 991.8 | 1.33 |
| | α-terpinene | 13.201 | 1015.0 | 0.95 |
| | 1,8-cineole | 15.117 | 1030.2 | 14.97 |
| | fenchone | 17.918 | 1084.0 | 1.91 |
| | linalool | 18.907 | 1102.7 | 46.97 |
| | camphor | 20.820 | 1141.5 | 9.50 |
| | 4-terpineol | 22.466 | 1174.9 | 0.80 |
| | α-terpineol | 23.161 | 1187.9 | 2.53 |
| | eugenol | 31.153 | 1356.0 | 2.18 |
| | α-farnesene | 34.032 | 1435.2 | 2.88 |
| | n.d | 34.749 | - | 0.86 |
| | n.d | 35.520 | - | 0.90 |
| | γ-cadinene | 36.760 | 1511.4 | 5.32 |
| | δ-cadinene | 38.136 | 1521.6 | 1.39 |
| | δ-cadinol | 43.365 | 1639.9 | 5.14 |
| *Achillea millefolium* L. (yarrow) | germacrene D | 34.030 | 1481.0 | 6.10 |
| | chamazulene | 36.867 | 1723.9 | 48.32 |
| | n.d | 37.413 | - | 1.55 |
| | n.d | 37.888 | - | 5.25 |
| | n.d | 46.927 | - | 38.76 |

n.d: not detected. These peaks were detected by GC/FID, but were not detected by GC/MS.

**Figure 1**

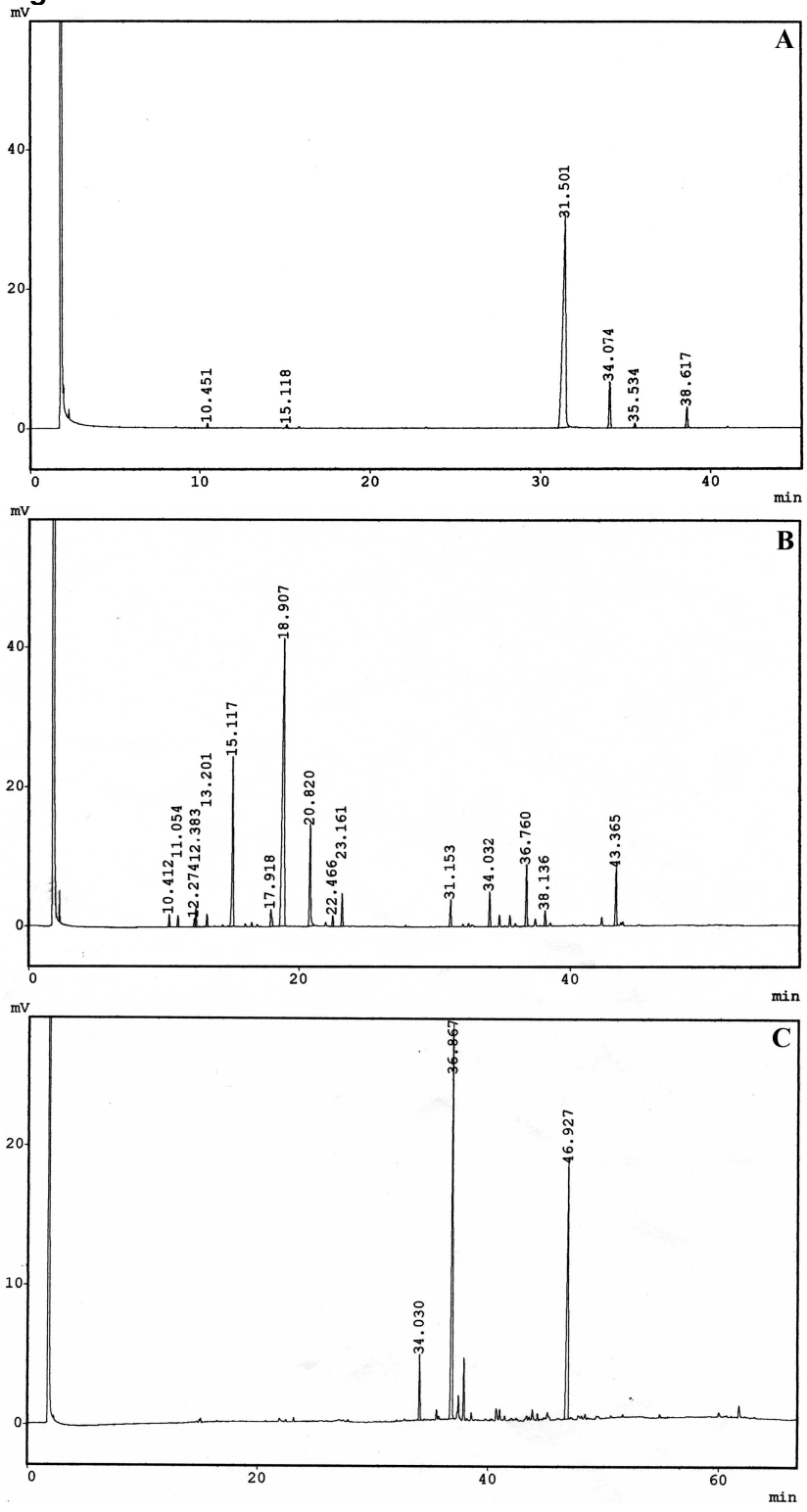
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**Figure 2**

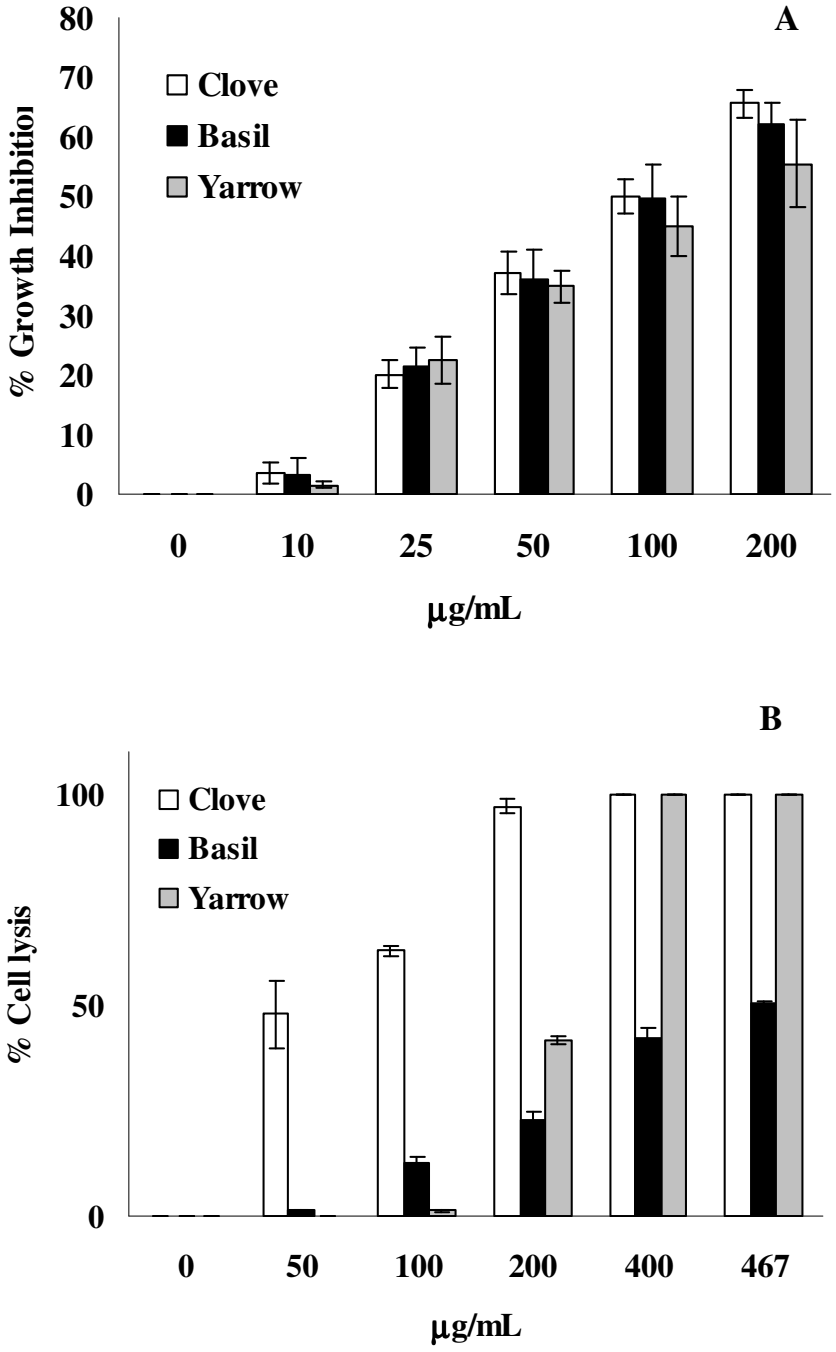
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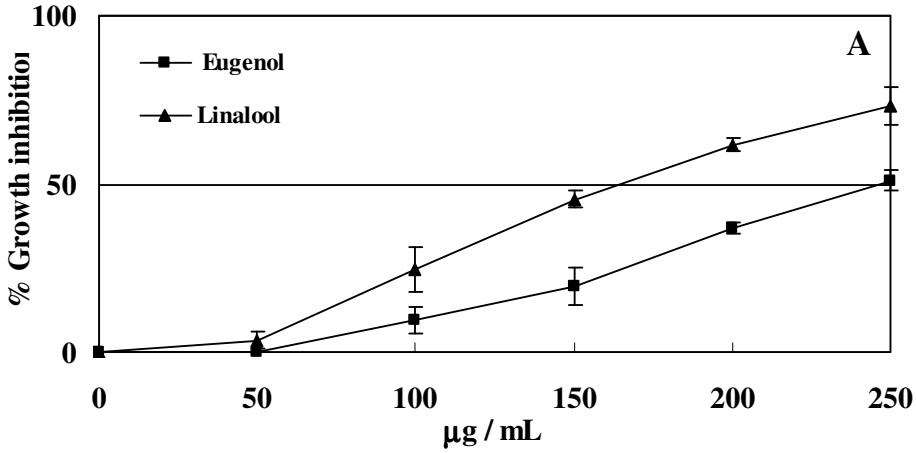
**Figure 3**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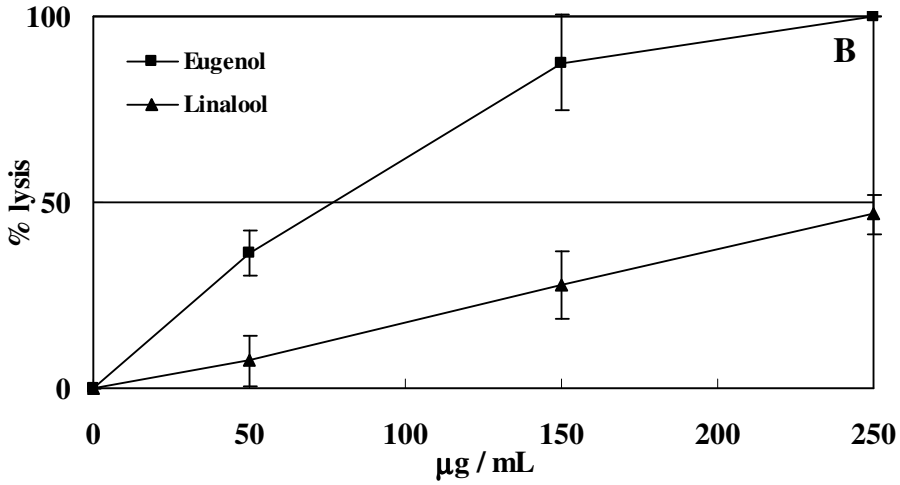
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**Figure 4**

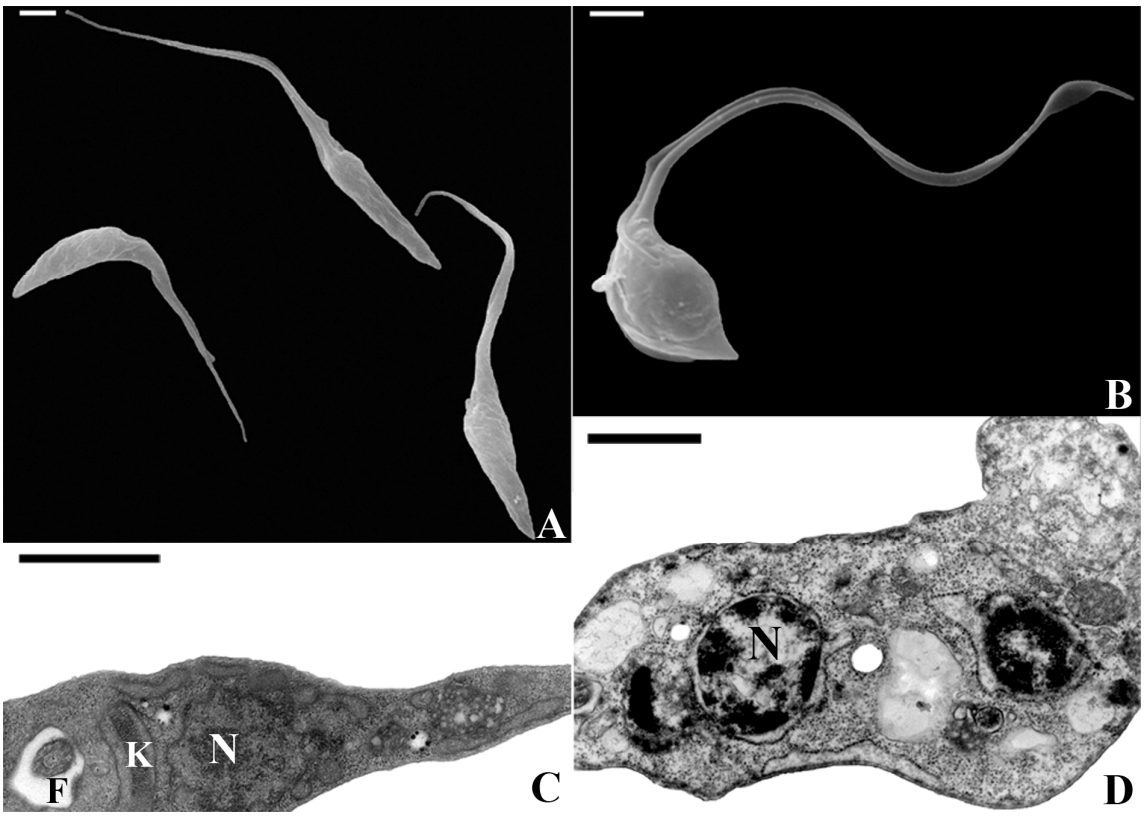
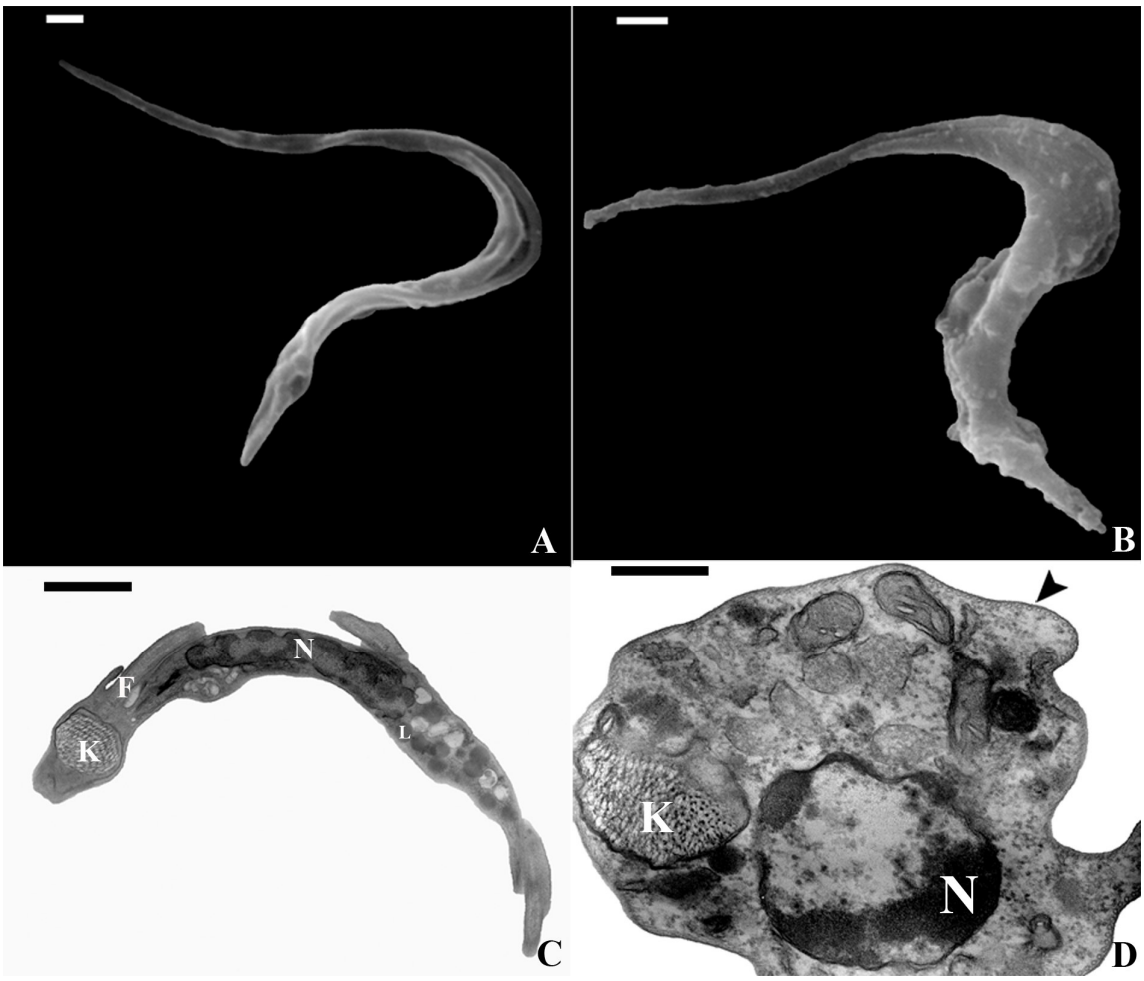


**Figure 5**



## Artigo 3

**Santoro GF, Cardoso MG, Guimarães LGL, Freire JM, Soares MJ (2007). Antiproliferative effect of the essential oil of *Cymbopogon citratus* (DC) Stapf (lemon grass) on intracellular amastigotes, bloodstream trypomastigotes and culture epimastigotes of *Trypanosoma cruzi* (Protozoa: Kinetoplastida). *Parasitology*, submetido para publicação.**

**Cópia da confirmação da submissão do Artigo 3**

07-Feb-2007

Dear Dr. Santoro:

A manuscript titled Antiproliferative effect of the essential oil of Cymbopogon citratus (DC) Stapf (lemongrass) on intracellular amastigotes, bloodstream trypomastigotes and culture epimastigotes of Trypanosoma cruzi (Protozoa: Kinetoplastida) (PAR-2007-0042) has been submitted by Dr. Maurilio Soares to Parasitology.

You are listed as a co-author for this manuscript.

Thank you for your participation.

Sincerely,
Parasitology Editorial Office

**Antiproliferative effect of the essential oil of *Cymbopogon citratus* (DC) Stapf (lemongrass) on intracellular amastigotes, bloodstream trypomastigotes and culture epimastigotes of *Trypanosoma cruzi* (Protozoa: Kinetoplastida)**

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**SUMMARY**

This study analyzes the anti-proliferative effect of lemongrass essential oil and its main constituent (citral) on all three evolutive forms of *Trypanosoma cruzi*. Steam distillation was used to obtain lemongrass essential oil, with chemical composition determined by gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS). The $IC_{50}$ (concentration that reduced the parasite population in 50%) of the oil and of citral upon *T. cruzi* was determined by cell counting in a Neubauer chamber, while morphological alterations were visualized by scanning and transmission electron microscopy. Treatment with the essential oil resulted in epimastigote growth inhibition with $IC_{50}$ = 126.5 µg/ml, while the $IC_{50}$ for trypomastigote lysis was 15.5 µg/ml. The $IC_{50}$/2 days for the percent of macrophage infection was 12.1 µg/ml, with a strong inhibition of intracellular amastigotes proliferation. Ultrastructural analysis demonstrated cytoplasmic and nuclear extraction, while the plasma membrane remained morphologically preserved. Our data show that lemongrass essential oil is effective against *T. cruzi* trypomastigotes and amastigotes, and that its main component, citral, is responsible for the trypanocidal activity. These results indicate that essential oils can be promising anti-parasitic agents, opening perspectives to the discovery of more effective drugs of vegetal origin for treatment of parasitic diseases.

Key words: *Cymbopogon citratus*, essential oil, lemongrass, *Trypanosoma cruzi*, ultrastructure.

## INTRODUCTION

Natural products are valuable sources of chemotherapeutic agents and have been traditionally used by native cultures to treat infectious diseases. In the last years interest in the research on natural products has intensified and it is not surprising the demonstration that many plant-derived compounds have anti-protozoan activity: for instance, it has been shown that various essential oils present inhibitory action against diverse human parasites such as *Plasmodium falciparum* (Valentin *et al*. 1995), *Trypanosoma brucei*, *Leishmania major* (Mikus *et al*. 2000), *Leishmania amazonensis* (Monzote *et al*. 2006) and *Trypanosoma cruzi* (Santoro *et al*. 2007).

*Cymbopogon citratus* (DC) Stapf (Gramineae) is an herb worldwide known as lemongrass. Tea made from its leaves is popularly used in Brazil due to its antispasmodic, analgesic, anti-inflammatory and antipyretic properties (Carlini *et al*. 1986). Furthermore, the essential oil of lemongrass has antimicrobial (Onawunmi, 1989; Ibrahim, 1992), antifungal (Viollon and Chaumont, 1994; Wannissom *et al*. 1996) and antibacterial (Onawunmi *et al*. 1984) activity. A study on the *in vivo* anti-malarial activity of *C. citratus* essential oil demonstrated a significant effect in reducing *Plasmodium berghei* growth (Tchoumbougnang *et al*. 2005). It has been also demonstrated that this essential oil presents anti-protozoan effect on the insect trypanosomatid *Crithidia deanei* (Pedroso *et al*. 2006).

The hemoflagellate parasite *T. cruzi* is the causative agent of American trypanosomiasis (Chagas disease), which affects 24 million people from Southern California (USA) to Argentina and Chile (WHO, 2002). According to World Health Organization reports, mortality rates vary from 8% to 12% depending on country, age and treatment received (WHO, 2002). The chemotherapy currently available is based on the nitroheterocyclic compounds nifurtimox and benznidazole. The mechanism of action of nifurtimox suggests that intracellular reduction of this compound generates nitro radicals, followed by redox cycling and hydrogen peroxide and anion superoxide production, while benznidazole does not depend on oxygen radicals. This treatment is inadequate because it presents serious toxic side effects. Furthermore, these compounds are not capable of achieving parasitological cure. Therefore, development of new, more efficient drugs is necessary.

Thus, in this paper we have evaluated the effect *in vitro* of the essential oil of *C citratus* (lemongrass) and its main constituent (citral) on growth, ultrastructure and infectivity of *T. cruzi* evolutive forms.

## MATERIALS AND METHODS

### *Parasite*

Culture epimastigote forms of *T. cruzi*, strain Y (Silva and Nussenzweig, 1953), were maintained with weekly passages at 28 ºC in LIT (Liver Infusion - Tryptose) medium (Camargo, 1964) supplemented with 10% inactivated fetal bovine serum (FBS). Five-day-old culture forms were used in the experiments.

Bloodstream trypomastigotes were obtained by cardiac puncture of infected Swiss albino mice, at the peak of parasitaemia (7 days post-infection). Blood was collected using 2 ml syringes containing 0.2 ml of 3.8% sodium citrate as anticoagulant. Blood samples were pooled in 15-ml tubes and then centrifuged for 15 min at 500*g* to isolate the parasites from red blood cells and leukocytes. The pellet containing the parasites was maintained for 20 min at 37 °C to allow the trypomastigotes to swim to the supernatant. The supernatant was then centrifuged for 10 min at 1,500*g* to eliminate platelets and the isolated trypomastigotes were transferred to RPMI-1640 medium (Sigma Chemical Co., St Louis, MO, USA), supplemented with 10% FBS. After homogenization, they were resuspended and kept in this same medium until use.

### *Plant material and essential oil isolation*

*C. citratus* (DC) Stapf (lemongrass) was collected at the Medicinal Plants Garden of the Universidade Federal de Lavras (UFLA), Brazil. Collection was always performed in the morning around 08:00, during October 2005, at a temperature of 20 °C and absence of rain. Essential oil was obtained by steam distillation, employing a modified Clevenger apparatus (Craveiro *et al*. 1981).

The essential oil and citral (isomeric mixture of geranial and neral) were initially dissolved in dimethyl sulphoxide (DMSO) at a concentration of 100 mg/ml. This solution was then dissolved in either LIT or RPMI-1640 culture media to obtain stock solutions at 1 mg/ml. Under this condition the DMSO was diluted at 0.01%, a concentration that is not toxic for the protozoa. All solutions were stored at -20 °C. The stock solutions were then diluted at different concentrations for the experiments.

### *Gas chromatography/mass spectrometry (GC/MS) analyses*

Analysis of the essential oil sample was performed by gas chromatography/mass spectrometry (GC/MS), with identification of constituents made by comparing the spectra obtained with those of the equipment data bank and

by the Kovat's index calculated for each constituent (Adams, 1995). The GC/MS analysis was performed with a Shimadzu CG-17A (Shimadzu Corporation, Kyoto, Japan) chromatograph coupled with a QP-5000 mass selective detector. Operating conditions were: capillary DB5 fused silica column (30 m X 0.25 mm; 0.25 µm film thickness); injector temperature 220 °C; column temperature set initially at 40 °C and then programmed at 3 °C/min to 240 °C; carrier gas helium, with linear gas velocity of 1.0 ml/min; split ratio 1:10; injected volume 1.0 µl (1% dilution in dichloromethane); inlet pressure 100.2 kPa. Mass spectra were taken at 70 eV; decomposition speed 1.000; decomposition interval 0.50; fragments from 45 to 450 Da were decomposed. A mixture of hydrocarbons ($C_9H_{20}$ to $C_{26}H_{54}$) was injected under these same conditions.

***Gas chromatography/flame ionization gas chromatography (GC/FID)***

The chemical constituents were quantified by GC/FID, using the same GC/MS column. The conditions were: capillary DB5 column, injector temperature 220 °C; detector temperature 240 °C; column temperature set initially at 40 °C and then programmed at 3 °C/min to 240 °C; carrier gas nitrogen, with linear gas velocity of 2.2 ml/min; split ratio 1:10; injected volume 1 µl (1% dilution in dichloromethane); inlet pressure 115 kPa. Quantification of each constituent was estimated by area normalization (%).

***Epimastigote and trypomastigote assay***

Five-day-old culture epimastigotes ($5x10^6$ cells per ml) were incubated for 24 h at 28 °C in absence or presence of different concentrations (25 to 200 µg/ml) of lemongrass essential oil or citral. The $IC_{50}$ (concentration that inhibited 50% parasite growth) was then evaluated by direct counting the cells in a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

Bloodstream trypomastigotes ($5x10^6$ cells per ml) were incubated for 24 h at 37 °C with different concentrations (25 to 250 µg/ml) of lemongrass essential oil or citral. The $IC_{50}$ (concentration that lysed 50% of the parasites) was then evaluated by using a Neubauer chamber. Each test was made in three experiments conducted in triplicate.

***Cytotoxicity assay***

Mouse peritoneal macrophages were obtained from Swiss mice with 18-20g. The cells were seeded on coverslips placed in 24-well plates ($10^6$ cells/well) and maintained at 37 °C for 24 h in RPMI-1640 medium. Thereafter, the cultures were washed with medium and incubated for 24, 48 or 72 h with different concentrations (3.9 to 250 μg/ml) of lemongrass essential oil. The coverslips were then stained with Giemsa and observed in a Nikon Eclipse E600 (Nikon, Tokyo, Japan) light microscope. Cytotoxicity was evaluated by comparing the morphology of treated and control macrophages.

Use of mice to perform the above mentioned experiments (isolation of bloodstream trypomastigotes and obtaining peritoneal macrophages) was made in adherence to the ethical standards of Fundação Oswaldo Cruz and was approved by an ethics committee (CEUA-FIOCRUZ, Protocol n° P0099-01).

***Intracellular amastigote assay***

Mouse peritoneal macrophages were seeded at $10^6$ cells per ml in 24-well plates containing glass coverslips and RPMI-1640 medium, and then maintained at 37 °C for 24 h to allow cell adhesion to the coverslips. The parasite-macrophage interaction analysis was performed by using two approaches: (a) adhered macrophages were pretreated for 24 h with different concentrations (7.5 to 60 μg/ml) of lemongrass essential oil and then the cultures were washed and infected with bloodstream trypomastigotes (ratio 10:1 parasites/host cell). After 3 h of interaction, non-internalized parasites were removed by washing with PBS, and fresh RPMI medium containing 10% FBS (RPMIS) was added; (b) untreated, adhered macrophages were washed and then infected with bloodstream trypomastigotes (ratio 10:1 parasites/host cell) as described above, and fresh RPMIS containing different concentrations (7.5 to 30 μg/ml) of lemongrass essential oil was added and changed daily.

At the specific times the coverslips were collected, fixed in Bouin's solution, stained with Giemsa and parasite infection was quantified using a Nikon Eclipse E600 light microscope. The percent of infected macrophages and the number of intracellular amastigotes per infected cell were evaluated by counting a total of 300 host cells in three different experiments. The $IC_{50}$ was estimated as the dose that reduced in 50% the percent of infection. The association index was obtained by

multiplying the percent of infected macrophages by the mean number of parasites per infected cell.

***Analysis by scanning (SEM) and transmission (TEM) electron microscopy***

Epimastigotes and bloodstream trypomastigotes were incubated for 24 h in the absence (control) or presence of the concentration corresponding to the $IC_{50}$ value of lemongrass essential oil for each form, collected by centrifugation at 5,500*g*, washed with 0.1 M phosphate buffer (pH 7.2), and then fixed for 30 min with 2.5% glutaraldehyde in 0.1 M phosphate buffer.

For SEM, the cells were adhered for 15 min to 0.1% poly-*L*-lysine coated glass coverslips, washed in buffer and then post-fixed for 30 min with 1% osmium tetroxide in 0.1 mol/ml cacodylate buffer, pH 7.2. Thereafter, the samples were dehydrated in acetone, critical point dried and mounted on SEM stubs. The samples were coated with a 20-nm thick gold layer and examined in a Zeiss (Oberkochen, Germany) DSM940 scanning electron microscope. Digital images were acquired and stored in a computer.

For TEM, the fixed cells were washed three times with 0.1 M phosphate buffer and post-fixed for 15 min with 1% osmium tetroxide / 0.8% potassium ferricyanide / 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2 (Meirelles and Soares, 2001). After rinsing in this same buffer, the cells were dehydrated in graded acetone, infiltrated overnight in an acetone-PolyBed 812 mixture (1:1) and embedded for 72 h at 60 °C in PolyBed 812 (PolySciences, Warrington, PA, USA) resin. Ultra-thin sections were stained with 5% uranyl acetate and lead citrate and observed in a Zeiss EM10C transmission electron microscope.

**RESULTS**

***Qualitative and quantitative analysis of lemongrass essential oil***

The compounds identified in the *C. citratus* essential oil, and their relative proportions, are listed in Table 1. The main components were geranial (38.9%) and neral (30.3%), isomeric forms of citral.

***Antitrypanocidal activity of the essential oil***

Incubation of *T. cruzi* epimastigotes and bloodstream trypomastigotes with lemongrass essential oil showed a dose-dependent effect. The value of $IC_{50}$/24h was

126.5 μg/ml for epimastigotes (Fig. 1A) and 15.5 μg/ml for trypomastigotes (Fig. 1B). At 50 μg/ml the lemongrass oil induced 100% lysis of the trypomastigotes.

***Antitrypanocidal activity of citral***

The activity of citral (main constituent of lemongrass essential oil) was also evaluated against epimastigotes and bloodstream trypomastigotes. The $IC_{50}$/24h value for epimastigotes was 42 μg/ml (Fig. 2A), about 3 times lower than that obtained with the whole essential oil. The $IC_{50}$/24h value for trypomastigote forms was 14.2 μg/ml (Fig. 2B), similar to that obtained with the whole essential oil. Again, in conformity to the results with the lemongrass essential oil, about 100% trypomastigote lysis was obtained with 50 μg/ml citral (Fig. 2B).

***Cytotoxicity and amastigote infectivity***

Incubation of mouse peritoneal macrophages for 24 to 72 h with lemongrass essential oil resulted in no cytotoxic effect with concentrations up to 31.2 μg/ml, as visualized by light microscopy examination. This concentration was about 2 times higher than the $IC_{50}$/24h value for trypomastigotes.

Pretreatment of macrophages with lemongrass essential oil did not reduce the percent of infection and the number of amastigotes/infected cell. Only at essential oil concentrations higher than 30 μg/ml after 48 h it was observed a small decrease in the percent of infection (data not shown). On the other hand, treatment of *T. cruzi*-infected macrophages with lemongrass essential oil led to a decrease in number of amastigotes/infected cells after 48 hours. The $IC_{50}$/48 h value for the percent of infection was 12.08 μg/ml. The association indexes after 48 h were reduced in 73% after treatment with 7.5 μg/ml, 90% after treatment with 15 μg/ml and 78% after treatment with 30 μg/ml (Fig. 3).

***Ultrastructural analysis by scanning (SEM) and transmission electron microscopy (TEM)***

Epimastigote forms treated with a concentration corresponding to the $IC_{50}$/24h value of lemongrass essential oil and then observed by SEM demonstrated apparently no plasma membrane alteration, but rounding of the cell body (Fig. 4B), as compared to control parasites (Fig. 4A). Occasionally small plasma membrane blebs seemed to detach from the parasite surface (Fig. 4B). Observation of treated

cells by TEM revealed that lemongrass-treated epimastigotes presented cytoplasmic and nuclear extraction (Fig. 4D), as compared to control cells (Fig. 4C). However, the plasma membrane remained morphologically preserved.

Trypomastigotes treated with the $IC_{50}$/24h of lemongrass essential oil and then observed by TEM also demonstrated cytoplasmic extraction (Fig. 4E), as compared to control cells (Fig. 4F). Although the plasma membrane remained intact, some membrane blebs could be occasionally observed (Fig. 4E).

**DISCUSSION**

In the search for new alternative drugs for the treatment of Chagas disease, phytocompounds such as plant extracts and essential oils appear as promising anti-proliferative agents. Several studies have already demonstrated the potential use of essential oils for killing trypanosomatid protozoa. Incubation of the monoxenic trypanosomatid *Herpetomonas samuelpessoai* showed that 91-100 μg/ml ($IC_{50}$/72h) of *Ocimum gratissimum* (alfavaca) essential oil inhibited parasite growth, leading to different ultrastructural alterations (Holetz *et al*. 2003). Treatment with the essential oil of *C. citratus* (lemongrass) resulted in a dose-dependent effect on growth of other lower trypanosomatid, *Crithidia deanei*, with $IC_{50}$/24h values between 60 and 120 μg/ml producing ultrastructural alterations in the parasites, such as vacuolization and alteration of the flagellar pocket membrane (Pedroso *et al*. 2006). Studies on *Leishmania* spp. with various essential oils such as *Croton cajucara* (Rosa *et al*. 2003), *O. gratissimum* (Ueda-Nakamura *et al*. 2006) and *Chenopodium ambrosioides* (Monzote *et al*. 2006) demonstrated that essential oils can be effectively used as potential new anti-leishmanial drugs. More recently it has been demonstrated that *Trypanosoma cruzi* growth was inhibited after incubation with oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) essential oils (Santoro *et al*. 2007). Our data showed that the essential oil of *C. citratus* was effective in killing *T. cruzi*, with low $IC_{50}$/24h (12-15 μg/ml) for both intracellular amastigotes and bloodstream trypomastigotes. Furthermore, this essential oil also inhibited epimastigote growth at low concentrations, inducing ultrastructural alterations in the parasites. Such high trypanocidal activity, of both the lemongrass essential oil and citral, its main component, indicates this essential oil as a good candidate for further phytoterapic analysis.

The antibacterial and antifungal activities of lemongrass essential oil and its components have been reported (Mishra and Dubey, 1994; Cimanga *et al*. 2002).

Lemongrass essential oil also showed significant anti-malarial activities after a four-day test *in vivo* with mice, with suppression of parasitaemia of 86.6% after treatment with 500 mg/kg body weight (Tchoumbougnang *et al*. 2005). It has been pointed out that the lemongrass essential oil properties are mainly due to citral (Onawunmi *et al*. 1984), which antifungal activity has been previously demonstrated (Kurita *et al*. 1981). It has been shown that the compounds neral and geranial (isomers of citral) were effective against epimastigotes of *T. cruzi* in the concentrations of 3.1 μM (Saeidnia *et al*. 2004). Our data demonstrated that incubation of *T. cruzi* epimastigotes and trypomastigotes with the citral constituent resulted in reduction of parasite growth at low concentrations, showing its high microbicidal activity.

No cytotoxic effects were observed when mouse peritoneal macrophages were incubated with lemongrass essential oil at concentrations corresponding to the $IC_{50}$ for trypomastigotes. The moderate toxicity against this mammalian cell type suggests that this essential oil may be used at safe dilutions for the host cells.

The association index when macrophages were first infected with *T. cruzi* trypomastigotes and then treated with the essential oil indicated a reduction in the number of infected host cells. Similar results have been described in assays with *Leishmania*-macrophage interaction and *C. cajucara* (Rosa *et al*. 2003) and *O. gratissimum* (Ueda-Nakamura *et al*. 2006) essential oils. Rosa and coworkers (2003) observed that treatment of the host cell with the essential oil induced an increase of 220% in nitric oxide production by the infected macrophages.

On the other hand, our data indicated that pretreatment of macrophages with lemongrass essential oil, followed by interaction with *T. cruzi*, did not influence the course of infection. It has been shown that pretreatment of macrophages with essential oils of *Croton cajucara* (Rosa *et al*. 2003) and *Ocimum gratissimum* (Ueda-Nakamura *et al*. 2006), followed by *Leishmania* infection resulted in reduction in the association indexes. Possibly, infective mechanisms related to the parasite species (cell entry mechanism and amastigote survival in the parasitophorous vacuole) may play a role in these differential results.

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**FIGURE LEGENDS**

**Figure 1.** Effect of lemongrass essential oil on *T. cruzi* epimastigotes and trypomastigotes after incubation for 24 h, with determination of $IC_{50}$/24h values. (A) Percent growth inhibition of epimastigote cultures treated with 25 to 200 μg/ml of the essential oil. (B) Bloodstream trypomastigotes treated with concentrations ranging from 25 to 200 μg/ml, showing the percent of cell lysis. Each bar represents the mean ± standard deviation of three different experiments.

**Figure 2.** Treatment of *T. cruzi* epimastigotes and bloodstream trypomastigotes for 24 h with citral, with determination of $IC_{50}$/24h values. (A) Epimastigotes incubated with concentrations from 50 to 200 μg/ml of citral. (B) Bloodstream trypomastigotes treated with concentrations from 50 to 250 μg/ml of citral. Each point represents the mean ± standard deviation of three different experiments.

**Figure 3.** Effect of lemongrass essential oil on the *T. cruzi*-macrophage interaction. Mouse macrophages were infected with the parasites and then treated for 48 h with 7.5 to 30 μg/ml essential oil. The association index showed a high decrease in all concentrations used, as compared to untreated cultures.

**Figure 4.** Effect of lemongrass essential oil on epimastigotes and bloodstream trypomastigotes, as observed by scanning (SEM) or transmission (TEM) electron microscopy, after treatment with $IC_{50}$/24h. (A) Untreated, control parasite observed by SEM. (B) SEM of lemongrass-treated epimastigote after incubation with 126.5 μg/ml essential oil. Note the rounding of the parasite body and a small vesicle (arrowhead) that appears to have been detached from the parasite plasma membrane. (C) Untreated epimastigote showing normal organelles by TEM. (D) Treated epimastigote, showing cytoplasmic extraction. The plasma membrane and the subpelicular microtubules remain unaltered (arrowhead). (E) Bloodstream trypomastigotes treated with the $IC_{50}$ (15.5 μg/ml) of lemongrass essential oil as observed by TEM, showing intense cytoplasmic extraction (asterisk) and formation of a membrane bleb (arrowhead). (F) Control, untreated bloodstream trypomastigote showing typical organelles. K, kinetoplast; N, nucleus. Figs. A and B: bar = 2 μm; Figs. C, D, E and F: bar = 1 μm.

**Table 1** - Quantitative and qualitative composition of *Cymbopogon citratus* (lemongrass) essential oil, as determined by GC/MS and GC/FID.

| Main constituent | Retention Index (RI) | % | Kovats Index |
|---|---|---|---|
| n.d | 15.295 | 0.54 | - |
| myrcene | 15.632 | 25.5 | 992.0 |
| n.d | 23.110 | 0.96 | - |
| n.d | 27.757 | 0.75 | - |
| n.d | 29.072 | 1.17 | - |
| neral | 33.274 | 30.28 | 1227.3 |
| n.d | 34.292 | 1.90 | - |
| geranial | 35.443 | 38.90 | 1268.0 |

n.d: not detected. These peaks were detected by GC/FID, but were not detected by GC/MS.

**Figure 1**

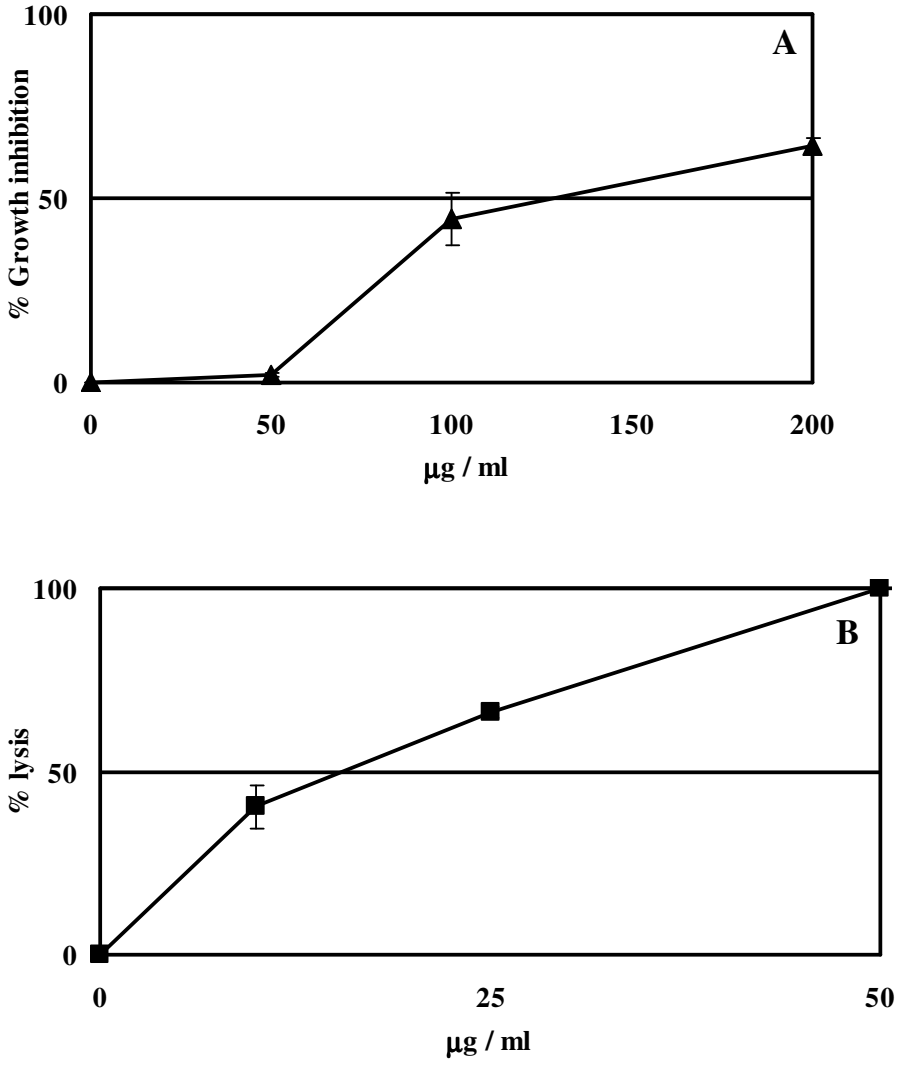
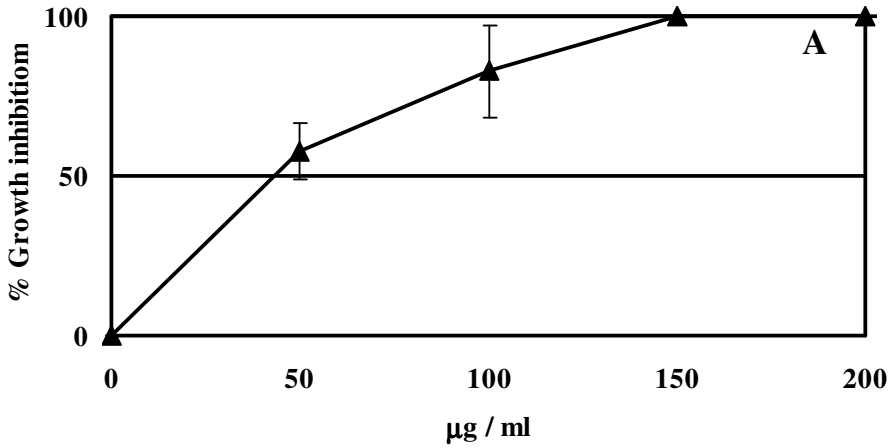

100
50
0
% Growth inhibition
A
0
50
100
150
200
µg / ml
100
50
0
% lysis
B
0
25
50
µg / ml

**Figure 2**


100
50
0
% Growth inhibitiom
A
0
50
100
150
200
µg / ml

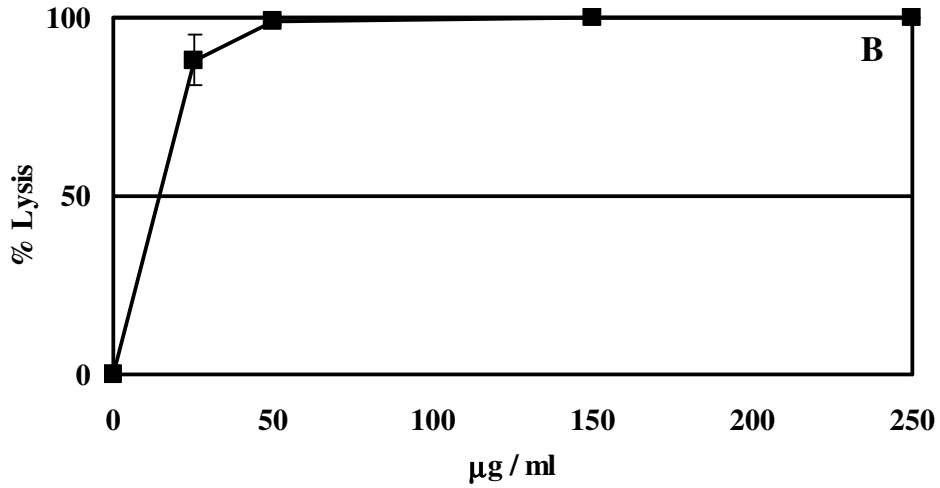

100
50
0
% Lysis
B
0
50
100
150
200
250
µg / ml

**Figure 3**

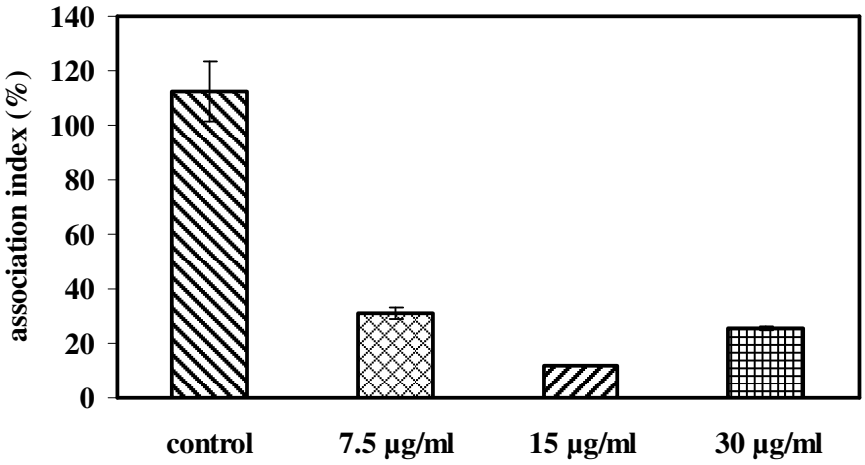
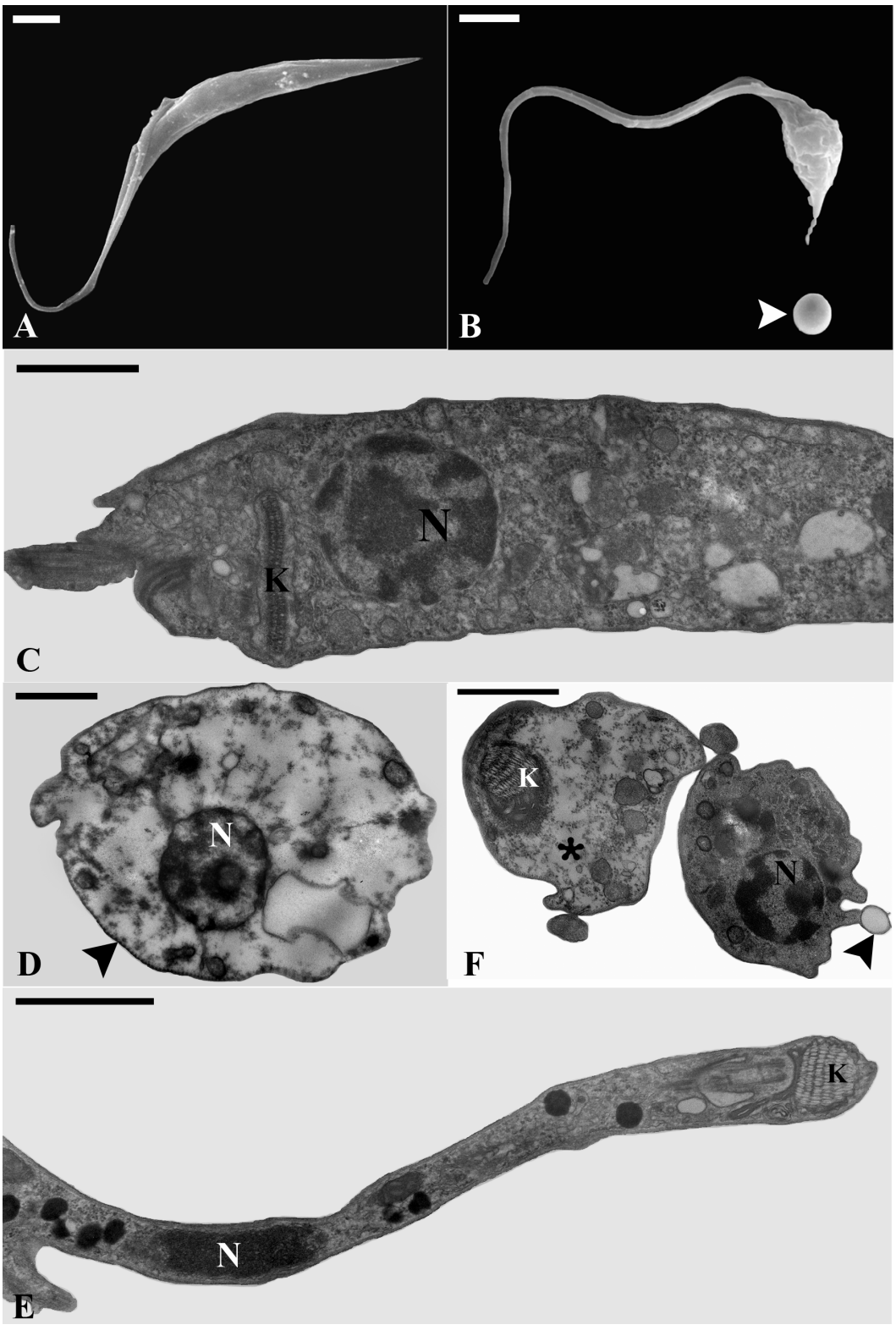

140
120
100
80
60
40
20
0
association index (%)
control
7.5 µg/ml
15 µg/ml
30 µg/ml

**Figure 4**



# DISCUSSÃO GERAL

A atividade microbicida de óleos essenciais e seus constituintes vem sendo investigada em uma enorme variedade de vegetais (Deans & Ritchie, 1987; Lis-Balchin & Deans, 1997; Soylu et al., 2006). Os dados da literatura mostram claramente que estes metabólitos secundários das plantas têm potencial utilização terapêutica, bem como aplicação nas indústrias de cosmética e alimentos (Shelef, 1983; Janssen et al., 1987; Friedman et al., 2002; Mahmoud & Croteau, 2002). A investigação da atividade antiparasitária e do mecanismo de ação de óleos essenciais têm sido objetos de estudo de um grande número de grupos de pesquisa (revisto em Kayser et al., 2003).

Após uma revisão dos nossos resultados, faremos uma análise dos Artigos 1 e 2 e do manuscrito submetido à publicação (Artigo 3), correlacionando-os com dados da literatura.

**1. Atividade tripanocida**

Após determinação quantitativa da composição dos óleos essenciais de orégano, tomilho, mil-folhas, cravo, manjericão e capim-limão (realizada em colaboração com o Departamento de Química da UFLA, Lavras, MG), realizamos uma triagem inicial da atividade dos mesmos sobre formas epimastigotas. Foi observada a seguinte ordem de atividade: tomilho > cravo > manjericão > capim-limão > mil-folhas > orégano. Nos ensaios com formas tripomastigotas sangüíneas os óleos mais efetivos foram: capim-limão > tomilho > cravo > orégano > mil-folhas > manjericão. Tripomastigotas, formas relevantes para a infecção humana, foram mais suscetíveis que epimastigotas, com exceção dos óleos de manjericão e de mil-folhas (Tabela 1). Experimentos com macrófagos infectados e posteriormente tratados com o óleo de capim-limão por 48 horas mostraram uma inibição de 50% no percentual de infecção na concentração de 12,1 µg/mL, indicando efeito sobre as formas intracelulares.

Quando analisamos componentes principais dos óleos de capim-limão (citral), tomilho (timol) e cravo (eugenol), observamos também que as formas tripomastigotas foram as mais suscetíveis. Apenas linalol, principal componente do óleo de manjericão, não apresentou grande alteração na atividade sobre as duas formas de *T. cruzi* (Tabela 2).

Tabela 1: Valores dos $IC_{50}$, em µg/mL dos óleos essenciais para *T. cruzi*

| Óleos Essenciais | Epimastigotas[a] | Tripomastigotas[a] | Amastigotas[b] |
|---|---|---|---|
| Capim-limão | 126,5 | 15,5 | 12,1 |
| Tomilho | 77,0 | 38,0 | nd |
| Cravo | 99,5 | 57,5 | nd |
| Orégano | 175,0 | 115,0 | nd |
| Mil-folhas | 145,5 | 228,0 | nd |
| Manjericão | 102,0 | 467,5 | nd |

nd: não determinado. [a]: $IC_{50}$/24h. [b]: $IC_{50}$/48h.

Tabela 2: Valores dos $IC_{50}$/24h, em µg/mL dos principais constituintes dos óleos essenciais para *T. cruzi*

| Componente (óleo essencial) | Epimastigotas | Tripomastigotas |
|---|---|---|
| Citral (capim-limão) | 42,0 | 14,2 |
| Timol (tomilho) | 62,0 | 53,0 |
| Eugenol (cravo) | 246,0 | 76,0 |
| Linalol (manjericão) | 162,5 | 264,0 |

**2. Toxicidade para células de mamíferos**

Os experimentos com os óleos essenciais de tomilho e capim-limão não demonstraram citotoxicidade para macrófagos após 3 dias de tratamento em concentrações duas vezes superiores quando comparados com o valor de $IC_{50}$/24h para tripomastigotas, indicando o uso potencial destes compostos para futuros experimentos *in vivo*.

**3. Análise por microscopia eletrônica**

As alterações ultra-estruturais observadas por microscopia eletrônica de varredura (MEV) mostraram-se semelhantes para todos os tratamentos com os óleos. Tanto epimastigotas quanto tripomastigotas apresentaram membranas plasmáticas íntegras, sendo observado inchaço no corpo dos parasitas. Com esta retração do corpo, o flagelo parecia ter um tamanho maior.

Por microscopia eletrônica de transmissão (MET) o tratamento com os óleos demonstrou que a membrana plasmática estava íntegra (como observado por MEV), mas as organelas citoplasmáticas apresentavam alterações, principalmente o núcleo e a mitocôndria. Além disso, o conteúdo citoplasmático apresentava-se bastante extraído.

Nossos dados sugerem que por sua característica hidrofóbica os óleos são capazes de permear a membrana plasmática e agir no metabolismo ou em organelas citoplasmáticas, levando então à lise dos parasitas. Marcação com iodeto de propídio e análise por citometria de fluxo mostraram que em epimastigotas e tripomastigotas tratados com os óleos de orégano e tomilho nas concentrações correspondentes ao $IC_{50}$ para cada forma do parasita não ocorria permeabilização da membrana plasmática. Esta permeabilização foi observada somente em concentrações 4 vezes maiores que os valores de $IC_{50}$.

## ARTIGO 1: Efeito do óleo essencial de orégano (*Origanum vulgare* L.) e tomilho (*Thymus vulgaris* L.) sobre o crescimento e ultra-estrutura de *Trypanosoma cruzi*. Parasitol Res 100: 783-790, 2007

Determinação da composição química dos óleos essenciais de orégano (*O. vulgare* L.) e de tomilho (*T. vulgaris* L.) mostrou como constituintes principais 3-ciclohexen-1-ol na percentagem de 26,2% e timol na percentagem de 80,4%, respectivamente (Fig. 1). O óleo de tomilho foi mais ativo que o de orégano sobre epimastigotas (2,3X) e tripomastigotas (3,1X), sendo a forma sangüínea a mais suscetível. Esta diferença na susceptibilidade entre as duas formas do parasita se deve, possivelmente, a diferenças em vias metabólicas, uma vez que estão adaptadas a ambientes completamente distintos.


3-cilcohexen-1-ol timol

**Figura 1** – Estrutura molecular do 3-ciclohexen-1-ol e do timol.

Timol (5-metil-2-(1-metiletil)fenol; 3-*p*-cimenol) apresentou uma atividade um pouco menor que a do óleo essencial de tomilho (respectivamente: $IC_{50}$/24h = 53 e 38 µg/mL), sugerindo que outros componentes possam também atuar sobre o parasita, mas que timol seria, pelo menos, um dos principais responsáveis pelo efeito tripanocida.

O constituinte principal do óleo de orégano foi 3-ciclohexen-1-ol, presente em uma percentagem relativamente baixa (26,2%), o que poderia explicar a menor atividade deste óleo quando comparado com o de tomilho (80,4% de timol).

Tratamento de epimastigotas com a concentração do $IC_{50}$ dos óleos de orégano e tomilho não resultou em alterações na membrana plasmática, ocorrendo porém um arredondamento no corpo dos parasitas, observado por MEV e MET. No caso de tripomastigotas tratados, houve aumento do volume dos parasitas (MEV e MET) e aparecimento de figuras de mielina e extração citoplasmática (MET). Análise por citometria de fluxo de epimastigotas e tripomastigotas tratadas com estes óleos mostrou permeabilização da membrana plasmática (medida por iodeto de propídeo) apenas em concentrações 4X superiores que os valores correspondentes do $IC_{50}$, indicando que estes óleos atravessam a membrana (característica hidrofóbica), interferindo no metabolismo do parasita, mas não comprometendo, pelo menos em uma etapa inicial, a integridade da membrana.

Já foi relatado na literatura que os óleos de orégano e de tomilho apresentam atividade bactericida (Burt & Reinders, 2003; Burt et al., 2005) e antifúngica (Paster et al., 1995; Giordani et al., 2004). Em relação ao óleo de tomilho foi observada anteriormente sua atividade sobre tripomastigotas de *Trypanosoma brucei*, sendo descrito que este óleo foi cerca de 50X mais tóxico sobre o parasita do que sobre células de linhagem HL-60 (Mikus et al., 2000). Nossos resultados mostram também atividade destes dois óleos sobre *T. cruzi*, assim como também de timol, o principal constituinte do óleo de tomilho.

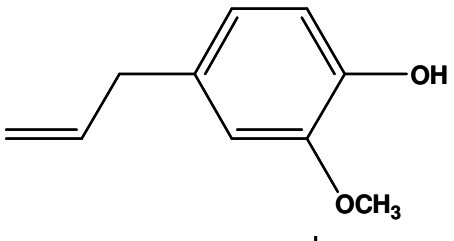
Sobre tripomastigotas, tanto o óleo de tomilho como timol apresentaram um valor de $IC_{50}$/24h semelhante ao de benznidazol (≅50 µg/mL), utilizado no tratamento clínico da doença de Chagas (Sepúlveda-Boza & Cassels, 1996). Além disso, estes óleos apresentaram baixa toxicidade para células mamíferas (macrófagos peritoneais de camundongo), sendo que até a concentração de 62 µg/mL do óleo de tomilho por 3 dias de tratamento não foram observdas alterações na morfologia, sendo esta concentração 1,5X maior que o $IC_{50}$ para tripomastigotas após apenas 1 dia de tratamento. Estes resultados estimulam a realização de experimentos *in vivo*.

**ARTIGO 2: *Trypanosoma cruzi*: Atividade de óleos essenciais de *Achillea millefolium* L., *Syzygium aromaticum* L. e *Ocimum basilicum* L. sobre epimastigotas e tripomastigotas. Exp Parasitol, (DOI: 10.1016/j.exppara.2007.01.018).**

A determinação da composição química dos óleos essenciais de cravo (*S. aromaticum* L.), manjericão (*O. basilicum* L.) e mil-folhas (*A. millefolium* L.) demonstrou que seus principais constituintes eram eugenol (83,34%), linalol (46,97%) e camazuleno (48,32%), respectivamente, sendo esta composição semelhante à descrita por outros autores (Zheng et al., 1992; Rohloff et al., 2000; Suppakul et al., 2003).

Os óleos de cravo, manjericão e mil-folhas demonstraram uma inibição dose-dependente da proliferação de epimastigotas com valores de $IC_{50}$/24h na faixa de 99,5 a 145,5 µg/mL. Sobre tripomastigotas o óleo de cravo foi cerca de 4,0X e 8,1X mais ativo do que, respectivamente, os de mil-folhas e majericão. Comparando-se as duas formas de *T. cruzi*, apenas no tratamento com o óleo de cravo a atividade sobre tripomastigotas foi superior àquela sobre epimastigotas.

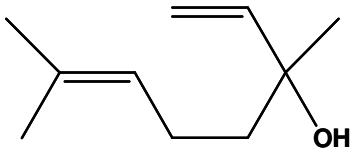
Eugenol [2-metoxi-4-(2-propenil)fenol] (Fig. 2) apresentou menor atividade sobre epimastigotas que o óleo de cravo do qual é principal constituinte, enquanto sobre tripomastigotas ambos (eugenol e óleo) apresentaram efeito similar. Outro dado é que eugenol foi mais ativo 3,2X sobre tripomastigotas em relação a epimastigotas. Eugenol é também um importante componente de uma variedade de outros óleos essenciais (De Vincenzi et al., 2000), apresentando atividade sobre bactérias (Nakamura et al., 1999) e sobre promastigotas de *Leishmania amazonensis* (Ueda-Nakamura et al., 2006).




eugenol

**Figura 2** – Estrutura molecular do eugenol

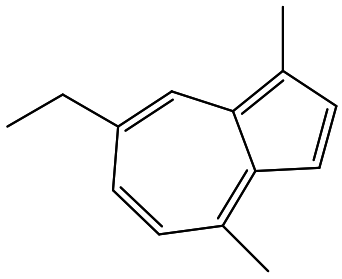
Linalol (3-7-dimetil-1,6-octadien-3-ol) (Fig. 3) apresentou em relação ao óleo de manjericão uma menor atividade sobre epimastigotas, porém sua atividade foi cerca de 1,8X maior que o óleo para tripomastigotas. Este composto é também extraído de vários óleos essenciais e apresenta atividade microbicida (Mazzanti et al., 1998). Estudos com promastigotas e amastigotas de *L. amazonensis* mostraram alta atividade do óleo essencial de *Croton cajucara*, rico em linalol, bem como do próprio composto (Rosa et al., 2003). Em nossos experimentos com *T. cruzi* o óleo de manjericão foi o que apresentou menor atividade sobre formas tripomastigotas, não estimulando estudos subseqüentes.




linalol

**Figura 3** – Estrutura molecular do linalol

Identificamos camazuleno (7-etil-1,4-dimetilazuleno) (Fig. 4) como um dos principais constituintes do óleo de mil-folhas, em percentagem semelhante à relatada por Gherase e colaboradores (2003), apesar das plantas terem sido obtidas em diferentes regiões geográficas. Entretanto, este óleo não foi dos mais efetivos sobre *T. cruzi*.



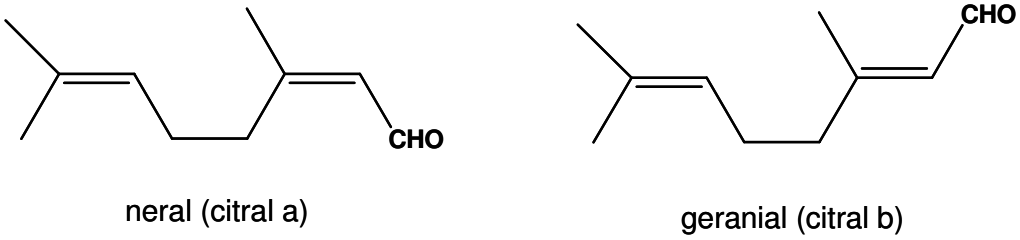
camazuleno

**Figura 4** – Estrutura molecular do camazuleno

A análise por MEV de epimastigotas e tripomastigotas tratadas também demonstrou, como nos outros óleos, a integridade da membrana plasmática, ocorrendo porém um inchaço no corpo dos parasitas. MET mostrou, em ambas as formas alterações no núcleo, com perda do conteúdo, e preservação da membrana, e no caso de tripomastigotas foi observada também extração citoplasmática.

**ARTIGO 3: Efeito antiproliferativo do óleo essencial de *Cymbopogon citratus* (DC), Stapf (capim-limão) em amastigotas intracelulares, tripomastigotas sanguíneos e culturas de epimastigotas de *Trypanosoma cruzi* (Protozoa: Kinetoplastidae). Parasitology, submetido à publicação.**

A determinação da composição química do óleo essencial de capim-limão (*C. citratus*) mostrou como constituintes principais geranial (citral-a) (38,9%) e neral (citral-b) (30,3%), formas isoméricas do citral (3,7-dimetil-2,6-octadienal) (Fig. 5).




neral (citral a) geranial (citral b)

**Figura 4** – Estrutura molecular do neral e do geranial

O óleo de capim-limão apresentou uma atividade 8,2X maior sobre formas tripomastigotas ($IC_{50}$/24h = 15,5 μg/mL) do que sobre epimastigotas ($IC_{50}$/24h = 126,5 μg/mL). Como já observado no **Artigo 1**, esta maior susceptibilidade de tripomastigotas se deve provavelmente a diferenças nas vias metabólicas entre as duas formas. Citral apresentou sobre epimastigotas uma atividade 3X superior em relação à do óleo de capim-limão, e semelhante em experimentos com tripomastigotas, indicando ser este composto um dos principais responsáveis pelo efeito tripanocida do óleo.

Tratamento de epimastigotas e tripomastigotas na mesma concentração do valor de $IC_{50}$, para cada uma das formas, mostrou por MEV integridade de membrana plasmática, mas com inchaço do corpo celular e por MET, extração citoplasmática e nuclear, sem alteração na membrana.

Incubação de macrófagos peritoneais com óleo de capim-limão por até 3 dias não demonstrou efeito citotóxico em concentrações acima de 31,2 μg/mL, cerca de 2X maior que o $IC_{50}$ para tripomastigotas após apenas 1 dia de tratamento. Pré-tratamento de macrófagos com o óleo, seguido da infecção com tripomastigotas, mostrou um pequeno decréscimo na percentagem de infecção apenas em concentrações superiores a 30 μg/mL, enquanto o número de parasitas/células infectadas não foi alterado em relação a culturas controle. Por outro lado, o

tratamento de macrófagos infectados mostrou um valor de $IC_{50}$/48h de 12,1 µg/mL para o parâmetro % de infecção.

O tratamento por 48 h de macrófagos infectados com óleo de capim-limão levou a uma acentuada redução no número de parasitas por células infectadas. Resultados semelhantes foram obtidos em estudos com *L. amazonensis* e óleos de *Croton cajucara* (Rosa et al., 2003) e de *Ocimum gratissimum* (Ueda-Nakamura et al., 2006). Nestes dois trabalhos, foi evidenciado que o pré-tratamento de macrófagos com os referidos óleos, seguido pela infecção, leva a uma redução acentuada nos índices de associação (% infecção x n° parasitas/célula infectada). Em nossos estudos com *T. cruzi* e óleo de capim-limão não observamos nenhum efeito deste pré-tratamento da célula hospedeira. Possivelmente, os mecanismos de infecção e vida intracelular para as duas espécies de tripanosomatídeos justifiquem tal diferença entre nossos resultados e os obtidos com *L. amazonensis*.

Tratamento com óleo de capim-limão induziu efeito inibitório sobre a proliferação do tripanosomatídeo *Crithidia deanei*, com valores de $IC_{50}$/24h entre 60 e 120 µg/mL, levando a vacuolização do parasita e alterações na membrana da bolsa flagelar (Pedroso et al., 2006). Este óleo apresenta também atividade sobre bactérias e fungos (Mishra & Dubey, 1994; Cimanga et al., 2002), e sobre *Plasmodium berghei*, neste caso com redução de 86,6% da parasitemia após tratamento de camundongos com 500 mg/kg de peso (Tchoumbougnang et al., 2005).

Citral é apontado como o principal componente do óleo essencial de capim-limão (Onawunmi et al., 1984), tendo sido demonstrada sua ação antifúngica (Kurita et al., 1981). Foi anteriormente demonstrado que neral e geranial, isômeros de citral, foram ativos sobre epimastigotas de *T. cruzi* na concentração de 3,1 µM (Saeidnia et al., 2004).

Nossos resultados mostraram que o óleo de capim-limão tem alta atividade tripanocida sobres formas amastigotas intracelulares e tripomastigotas, além de inibir a proliferação de epimastigotas. Esta alta atividade tanto do óleo como de seu principal componente, citral, aponta para este óleo essencial como um bom candidato para estudos *in vivo*.

Na busca de drogas alternativas para o tratamento da doença de Chagas, produtos naturais tais como extratos de plantas e óleos essenciais aparecem com agentes promissores. Vários estudos demonstraram a atividade de óleos essenciais sobre protozoários patogênicos como *L. amazonensis*, *T. brucei, Plasmodium*

*berghei* e *Plasmodium falciparum* (Mikus et al., 2000; Boyom et al., 2003; Rosa et al., 2003; Tchoumbougnang et al., 2005; Ueda-Nakamura et al., 2006). Incubação do tripanosomatídeo monoxênico *Herpetomonas samuelpessoai* com óleo de *O. gratissimum* (alfavaca) inibiu sua proliferação com um valor de $IC_{50}$/3 dias na faixa de 91-100 μg/mL, levando a várias alterações mitocondriais e vesiculação do complexo de Golgi (Holetz et al., 2003). Estudos de tratamento de *L. amazonensis* com óleos essenciais de *C. cajucara* (Rosa et al., 2003), *Ocimum gratissimum* (Ueda-Nakamura et al., 2006) e *Chenopodium ambrosioides* (estudos *in vitro* e *in vivo*) (Monzote et al., 2006a,b) mostraram o potencial destes produtos naturais como agentes leishmanicidas.

Assim, nossos resultados mostrando a atividade de vários óleos essenciais sobre *T. cruzi*, bem como de alguns de seus constituintes principais, juntamente com os relatos acima descritos, apontam para novas perspectivas para o desenvolvimento de compostos fitoterápicos para o tratamento de doenças parasitárias.

# CONCLUSÕES

- Seis óleos essenciais (tomilho, orégano, mil-folhas, manjericão, cravo e capim-limão) com composição química definida demonstraram efeito sobre *T. cruzi,* seja inibindo o crescimento de formas epimastigotas e amastigotas, seja lisando as formas tripomastigotas sangüíneas. Os mais ativos sobre formas tripomastigotas e amastigotas foram os de tomilho, cravo e capim-limão, indicando a necessidade da continuação do estudo *in vitro* destes óleos.

- A atividade dos constituintes isolados timol (de tomilho), linalol (de manjericão), eugenol (de cravo) e citral (de capim-limão) sobre tripomastigotas indicou que estes compostos têm papel no efeito dos óleos correspondentes, mas não necessariamente são os únicos responsáveis pela ação tripanocida.

- Análises por microscopia eletrônica (MEV e MET) e citometria de fluxo demonstraram que a ação destes óleos não afeta a permeabilidade da membrana, interferindo possivelmente em vias metabólicas do parasita.

- A baixa toxicidade para células de mamíferos (macrófagos) dos óleos de tomilho e capim-limão aponta para a realização de ensaios *in vivo*.

- Nossos resultados abrem novas perspectivas para o desenvolvimento de novos fármacos, mais eficazes e menos tóxicos, para o tratamento da doença de Chagas e podendo também ser utilizado no tratamento de outras parasitoses.

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