RAÍSSA BOCCHI PEDROSO

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Dissertação apresentada à Universidade Estadual de Londrina como requisito para obtenção do título de mestre em Microbiologia pelo curso de Pós-Graduação em Microbiologia.

Orientador Prof. Dr. Celso Vataru Nakamura

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À minha família: Odete,
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SUMÁRIO

1 INTRODUÇÃO

Durante o século passado a prática de herbalismo se tornou difundida pelo mundo devido, em parte, ao reconhecimento do valor da medicina tradicional, particularmente de origem asiática, o interesse de drogas derivadas de plantas superiores tem aumentado expressivamente nas últimas décadas. Isto é devido, em parte, ao reconhecimento dos valores das farmacopéias tradicionais e indígenas e à incorporação de alguns derivados destas fontes na prática farmacêutica (ELVIN-LEWIS, 2001). Plantas medicinais estão distribuídas mundialmente, mas são mais abundantes em países tropicais (CALIXTO, 2000).

Várias plantas medicinais têm sido estudadas para verificar atividades antimicrobianas. Holetz e colaboradores (2002) estudaram extratos de 13 plantas medicinais do Brasil que foram relatadas possuindo diferentes níveis de atividade antibacteriana e antifúngica. Plantas medicinais amplamente utilizadas na Nigéria e Costa do Marfim, também mostraram propriedades antiprotozoários (ADEWUNMIM et al., 2001 e OKPEKON et al., 2004).

Óleos essenciais são óleos aromáticos extraídos de várias partes das plantas (flores, bulbos, sementes, folhas, galhos, caule, frutos e raízes) que podem ser obtidos por expressão, fermentação e extração, mas o método de destilação a vapor é o mais comumente usado para produção comercial. Mais de 3000 óleos essenciais são conhecidos dos quais 300 são de importância econômica destinados principalmente para o mercado de perfumaria e alimentos (BURT, 2004). Dados da literatura indicam que esses óleos essenciais possuem atividade antiviral (BISHOP, 1995), antitoxigênica (JUGLAL et al., 2002), antibacteriana (WANNISSORN et al., 2005) e antiprotozoário (HOLETZ et al., 2003; UEDA-NAKAMURA et al., 2006), além de outras.

A maior parte dos esforços da pesquisa sobre o efeito de plantas em infecções parasitárias tem sido feita usando extratos alcoólicos ou aquosos, no entanto, óleos essenciais purificados de plantas também poderiam ser eficazes na prevenção dessas doenças parasitárias (ANTHONY, 2005). Propriedades como a baixa densidade (perto de 0,94 g/ml) e rápida difusão através da membrana celular podem favorecer as atividades dos óleos em parasitas intracelulares (BOYOM, 2003).

Cymbopogon citratus, descrito inicialmente como *Andropogon citratus* por De Candolle e re-classificado por Otto Stapf, pertence à família *Poaceae*, que engloba cerca de 500 gêneros e aproximadamente 8.000 espécies essencialmente herbáceas, denominadas genericamente de gramíneas. O gênero *Cymbopogon* engloba cerca de 30 espécies de gramíneas perenes aromáticas, sendo, a maioria destas, nativas da região tropical do Velho Mundo. O nome deste gênero, *Cymbopogon*, deriva de *kymbe* (barco) e *pogon* (barba); em referência ao arranjo da sua inflorescência (espiga) (GOMES et al., 2003). Originária do sudoeste asiático e, assim como outras espécies do gênero *Cymbopogon, C. citratus* encontra-se distribuída atualmente nas regiões tropicais e subtropicais (GUPTA e JAIN, 1978). Esta planta popularmente conhecida no Brasil como “Capim limão”, é comumente usada na medicina popular para o tratamento de distúrbios nervosos e gastrintestinais. Também é utilizada em outros países para o tratamento de estados febris (MELO et al., 2001). O óleo essencial obtido das folhas verdes de *C. citratus* é amplamente utilizado pela indústria de perfumes e cosméticos. Ele também é utilizado para síntese química devido a sua grande concentração de citral, uma mistura natural de dois isômeros de aldeídos, neral e geranial. Além disso, a literatura relata que a substância citral é a principal responsável pelas propriedades do óleo essencial (RAUBER et al., 2005).

No reino *Protozoa* a família *Tripanosomatidae* pertence à ordem Kinetoplastida. Esta família possui nove gêneros reconhecidos: *Trypanosoma, Leishmania, Endotrypanum, Phytomonas, Blastocrithidia, Crithidia, Herpetomonas, Leptomonas* e *Rhyncoidomonas.* Os quatro primeiros gêneros são de protozoários heteroxênicos, sendo *Trypanosoma, Leishmania e Endotrypanum* parasitas de insetos hematófagos e de vertebrados e o gênero *Phytomonas,* parasita de insetos fitófagos e de plantas. Os outros gêneros compreendem protozoários monoxênicos, parasitas de insetos, podendo também ocorrer em vermes e outros protozoários (CAMARGO, 1995). Os protozoários monoxênicos junto com o gênero *Phytomonas* são conhecidos como tripanosomatídeos inferiores (VICKERMAM, 1994).

Na ordem Kinetoplastida, encontram-se os protozoários com 1 ou 2 flagelos originados de uma abertura conhecida como bolsa flagelar. Esses organismos contêm ainda uma estrutura paraflagelar e uma estrutura proeminente conhecida como cinetoplasto, que corresponde a uma condensação de ácido desoxirribonucléico (DNA) localizada no interior de uma mitocôndria única e

ramificada por todo o corpo do protozoário. Organelas especiais do tipo peroxissoma (glicossoma) e microtúbulos subpeliculares são, também, estruturas características desses protozoários (DE SOUZA, 2000). Além disso, os tripanosomatídeos apresentam algumas características especiais fazendo que eles se constituam em excelentes modelos para o estudo de questões biológicas básicas, incluindo edição nuclear e "*trans-splicing*" mitocondrial do RNA mensageiro, organização do DNA extranuclear, variação antigênica, entre outros (DE SOUZA e MOTTA, 1999).

Tripanosomatídeos inferiores têm sido rotineiramente usados como modelos de laboratórios para estudos bioquímicos e moleculares devido a facilidade de serem cultivados em condições axênicas, alguns em meios quimicamente definidos e também por serem tradicionalmente classificados como não patogênicos (WALLACE, 1966). Recentemente foi relatado que *Crithidia deanei* e *Herpetomonas roitmani* podem infectar fibroblastos de pele de rato e que a presença do endossimbionte, nestes casos, pode ter influenciado nesta interação (SANTOS et al., 2004).

Protozoários do gênero *Crithidia* apresentam a forma coanomastigota no seu ciclo de vida. As espécies *C. deanei, C. desouzai* e *C. oncopelti* possuem um simbionte bacteriano no seu citoplasma (endossimbionte) que interfere em vários aspectos no metabolismo dos protozoários, sugerindo que vários metabólitos importantes para a célula eucariótica são sintetizados pela bactéria (FREYMULLER e CAMARGO, 1981; DE SOUZA e MOTTA, 1999; D´AVILA-LEVY et al., 2001; D´AVILA-LEVY et al., 2003). A presença do simbionte também interfere na distribuição espacial dos microtúbulos (DE SOUZA e MOTTA, 1999) e modula aspectos na superfície da membrana dos protozoários como a exposição de resíduos de carboidratos (ESTEVES et al., 1982; ODA et al., 1984, FARIA-e-SILVA et al., 1994) e a expressão de glicoproteínas (DIAS FILHO et al., 2005). A possibilidade de eliminação do endossimbionte com o uso de antibióticos (cura) tem aumentado o interesse no estudo desta inter-relação na espécie (D'AVILA-LEVY et al., 2003).

Todos os protozoários possuem carboidratos em suas superfícies celulares ligados a proteínas ou lipídeos de membrana, como parte do seu citoesqueleto ou em suas estruturas internas. Devido a isso a utilização de lectinas, que são proteínas não catalíticas que se ligam especificamente a resíduos de carboidratos, podem ser diretamente utilizadas nos testes de aglutinação desses

parasitas (JACOBSON e DOYLE, 1996). As reações específicas entre essas moléculas têm se tornado muito importante no estudo da inserção, destino, distribuição e funcionamento desses glicoconjugados em parasitas (GOLDSTAEIN et al., 1997). Os estudos de carboidratos de superfície celular, usando lectinas, já foram feitos em vários membros da família Trypanosomatidae como: *Trypanosoma*, *Leishmania*, *Herpetomonas*, *Phytomonas*, e *Crithidia.* Esta técnica tem sido proposta como importante ferramenta em análises comparativas dentro dessa família e também entre espécies de diferentes gêneros (DE SOUZA, 1989). Novas evidências também indicam que glicoconjugados de tripanosomatídeos são importantes em várias fases da interação parasita-hospedeiro como reconhecimento celular, adesão, penetração e sobrevivência no hospedeiro (DE SOUZA, 1995).

Doenças causadas por tripanosomatídeos são as grandes responsáveis por mortalidade em países subtropicais. O relato da presença de tripanosomatídeos inferiores em infecções cutâneas oportunistas em indivíduos imunocomprometidos (DEDET et al., 1995, BOISSEAU-GARSAUD et al., 2000) ou naqueles sem nenhuma história prévia de imunodepressão (BOISSEAU-GARSAUD et al., 2000), ser limitado o número de drogas disponíveis para o tratamento de tripanossomíase animal e humana e leishmaniose, além dos efeitos colaterais e fatores de resistência que os protozoários apresentam contra estas drogas, maior atenção deveria ser dada a extratos e compostos biologicamente ativos, isolados de plantas comumente usadas na medicina popular (ESSAWI e SROUR, 2000).

Benznidazol é uma droga utilizada na quimioterapia de fases aguda e intermediária da doença de Chagas, agindo via diferentes mecanismos que envolvem modificação covalente de macromoléculas por intermediários nitro-redutores (CASTRO et al., 2003). Anfotericina B é um antifúngico usado no tratamento de infecções sistêmicas e também para o tratamento de Leishmaniose. Essa droga interage com esteróis e ergosteróis de membrana do fungo assim como no protozoário que possui ergostanos como principal fonte de ergosterol de membrana (GOAD et al., 1984). Além de essas drogas necessitarem de um longo período de administração para o tratamento, também são dispendiosas, tóxicas e causam sérios efeitos colaterais nos pacientes. Alternativas terapêuticas são necessárias para o tratamento de doenças causadas por protozoários, e extratos e óleos essenciais de plantas comumente utilizadas na medicina popular devem ser investigados, pois são vistos como fontes potenciais para a produção de novas

drogas a serem utilizadas na quimioterapia antiprotozoários. tripanosomatídeos inferiores, como já mencionado no texto, sendo facilmente cultivados em culturas axênicas e não patogênicos, são ótimos modelos biológicos para a análise desses novos compostos.

2 OBJETIVOS

- Verificar o efeito do óleo essencial de *Cymbopogon citratus* no crescimento e viabilidade de *Crithidia deanei* com e sem endossimbionte.
- Comparar o efeito do óleo essencial nestes protozoários em relação às drogas benznidazol e anfotericina B.
- Avaliar alterações ultra-estruturais das células tratadas com o óleo essencial utilizando técnicas de microscopia eletrônica de transmissão.
- Analisar, com o emprego de lectinas, possíveis alterações na exposição de resíduos de carboidratos de membrana em *Crithidia deanei* com e sem endossimbionte tratadas com o óleo essencial.

3 CONCLUSÕES

- O óleo essencial de *Cymbopogon citratus* inibe o crescimento de *Crithidia deanei*. A amostra sem endossimbionte é mais sensível à ação do óleo essencial que a amostra contendo o endossimbionte. A concentração inibitória 50% (IC_{50}) para *C. deanei* sem endossimbionte foi de 60 µg/ml e de 120 µg/ml para a amostra com endossimbionte.
- No teste de viabilidade utilizando o corante trypan blue observou-se um menor número de células viáveis na cepa sem endossimbionte tratada com óleo essencial.
- Benznidazol não interfere no crescimento e nem na viabilidade desses protozoários possuindo um IC_{50} maior que 700 µg/ml para ambas amostras.
- Anfotericina B é necessária em baixas concentrações para inibir em 50% o crescimento de *C. deanei* com e sem ensdossimbionte (3,4 e 3,6 µg/ml, respectivamente).
- Através da microscopia eletrônica de transmissão observou-se que o óleo essencial e a anfotericina B parecem atuar de maneira similar, interferindo na integridade da membrana celular, principalmente na região da bolsa flagelar.
- O óleo essencial altera a composição de resíduos de açúcares de membrana de ambos os protozoários. Em *C. deanei* sem endossimbionte houve uma diminuição na aglutinação das células tratadas com *Liminus poliphemus* e um aumento na aglutinação de células tratadas com *Arachis hipogaeae*.

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ANEXOS

ANEXO I

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“Biological Activity of Essential Oil obtained from *Cymbopogon citratus* on *Crithidia deanei*”

Biological Activity of Essential Oil obtained from *Cymbopogon citratus* on *Crithidia deanei*

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SUMMARY

We report the effect of the essential oil of *Cymbopogon citratus* on ndosymbiont-harbouring and endosymbiont-free strains of the insect trypanosomatid *rithidia deanei* grown at 28°C in a chemically defined medium. A dose-dependent ntiprotozoan effect of the essential oil of *C. citratus* could be observed in both strains f *C. deanei*. The IC_{50} (50% inhibitory concentration) for symbiont-bearing and ymbiont-free strains was 120 and 60 µg/ml, respectively. The viability assay showed hat the symbiont-free strain is more sensitive to the presence of the essential oil, having ysed cells after 2 h of exposure at high concentrations. In addition, alterations of the ltrastructural and cell-surface carbohydrate residues of both strains of *C. deanei* treated ith essential oil were also evaluated. Both strains showed ultrastructural alterations in he cellular and flagellar pocket membranes, as revealed by transmission electron icroscopy. In the lectin assay, the essential oil influenced the expression of arbohydrates in symbiont-free *C. deanei*, as evidenced by a reduction of sialic acid esidues.

KEYWORDS: Atiprotozoa activity. *Crithidia deanei.* Medicinal plants. *Cymbopogon itratus*

INTRODUCTION

The family Trypanosomatidae harbour protozoans that are agents of important illnesses in humans and animals (such as the agents of leishmaniasis and Chagas´ disease), and in plants (*Phytomonas*). This family also includes some lower trypanosomatids such as *Crithidia*, *Blastocrithidia*, and *Herpetomonas,* monoxenous protozoans usually found in insect hosts and not considered capable of causing parasitic diseases in vertebrates (Wallace 1966). *Crithidia deanei*, which has a choanomastigote form, normally contains intracellular symbiotic bacteria, and, like other trypanosomatids, is easily cultured under axenic conditions. These insect trypanosomatids contain homologues of virulence factors of the pathogenic ones (D´Avila-Levy *et al.* 2003), and therefore have been used as laboratory models for biochemical and molecular studies (McGhee and Cosgrove 1980, Santos *et al.* 2004).

In trypanosomatids the possibility of elimination of the endosymbiont by antibiotic treatment has increased interest in the study of endosymbiont-harbouring species (De Souza and Motta 1999). The available data indicate that the presence of the endosymbiont induces morphological changes, interferes with several aspects of metabolism (Freymuller and Camargo 1981, De Souza and Motta 1999, D´Avila-Levy et al. 2001, 2003), and modulates the surface properties of the protozoan, such as exposure of carbohydrate residues (Esteves *et al.* 1982, Oda *et al.* 1984, Faria-e-Silva *et al.* 1994) and the expression of glycoproteins (Dias Filho *et al.* 2005).

The human diseases caused by *Trypanosoma cruzi* and *Leishmania* are responsible for mortality in tropical and subtropical countries. In addition, there are recent reports of trypanosomatids other than *Trypanosoma* and *Leishmania* present in some opportunistic cutaneous infections in immunocompromised individuals (Dedet *et al.* 1995, Boisseau-Garsaud *et al.* 2000) and those without any previous history of immunodepression (Boisseau-Garsaud *et al.* 2000). Drugs such as benznidazole (used in the acute and intermediate phases of Chagas' disease) and the polyene amphotericin B (used for leishmaniasis) are limited, and the side effects are drastic (Goad *et al.* 1984, Castro *et al.* 2003). Because of this, more attention should be given to extracts and biologically active compounds isolated from plants

commonly used in herbal medicine (Essawi and Srour 2000). The pratice of herbalism has become mainstream throughout the world. This is due in part to recognition of the value of traditional medical systems, and the identification of medicinal plants from indigenous pharmacopeias (Elvin-Lewis 2001). Medicinal plants are distributed worldwide, but they are most abundant in tropical countries (Calixto 2000).

Essential oils are aromatic oily liquids obtained from plant material. They can be obtained by expression, fermentation, or extraction, but steam distillation is the most commonly used method (Burt 2005). Some essential oils have antibacterial (Wannissorn *et al.* 2005), antifungal (Nakamura *et al.* 2004), antiviral (Bishop 1995), antitoxigenic (Juglal *et al.* 2002), and antiprotozoal (Holetz *et al.* 2003, Ueda-Nakamura *et al.* 2006) properties.

Cymbopogon citratus is a plant used in traditional folk medicine in Brazil for the treatment of nervous and gastrointestinal disturbances, and in various other countries to treat fevers (Melo *et al.* 2001). The volatile oil obtained from fresh leaves of this plant is widely used by the perfume and cosmetics industries. It has also been used in chemical synthesis, because of its high citral content (Rauber *et al.* 2005).

Here we report the effect of the essential oil from *C. citratus* on growth, viability, cell-surface carbohydrate residues, and ultrastructure of endosymbiontharbouring and endosymbiont-free *C. deanei* cultivated in defined medium at 28°C.

MATERIALS AND METHODS

Plant material. *C. citratus* was collected in Maringá, Paraná, Brazil, and identified. A voucher No. HUM 520 is deposited at the Maringá State University Herbarium. Fresh leaves from the plant were cut into pieces and steam-distilled by Clevenger´s apparatus. The essential oil was then stored at -20^{o}C until needed.

Microorganisms. Cultures of symbiont-bearing *Crithidia deanei* (ATCC 30255) were maintained by weekly transfers into a chemically defined medium (Mundin *et al.* 1974), added in 5-ml volumes to screw-capped tubes. The symbiont-free strain of *C. deanei* was maintained in the same defined medium supplemented with 0.03 g/l of nicotinamide (Sigma Chemical Company, St. Louis, MO, U.S.A.) (Mundin and Roitman 1977). Cells were grown at 28°C for 48 h and stored at 4°C.

Antiprotozoan activity of *Cymbopogon citratus* essential oil. For the experiment, symbiont-bearing and symbiont-free *C. deanei* were incubated in defined medium supplemented with 0.03 g/l of nicotinamide containing different concentrations of the essential oil, initially diluted in 2% Tween 80. Cells were grown in 13 x 100 mm tubes containing 1 ml of the medium, and the starting inoculum consisted of the protozoans in logarithmic growth phase (2 x 10^{6} cells/ml). After 24, 48, 72, and 96 h at 28°C, cell growth was estimated by counting in a haemocytometer (Improved Double Neubauer). All experiments were performed in triplicate. The results are expressed as log number (cells/ml) and as the percentage of growth inhibition at 48 h. Amphotericin B (FUNGISON®, Bristol-Myers Squibb, São Paulo, Brazil) and benznidazole (N-benzyl-2-nitro-1-imidazolacetamide, Roche Pharmaceuticals, Rio de Janeiro, Brazil) were prepared in the same defined medium and used as reference drugs.

Viability assay. In order to evaluate the viability of the protozoa treated with essential oil, each solution was added to eppendorfs containing 2 x 10^{7} cells in logarithmic growth phase and incubated at 28°C. After addition of the essential oil, 25 □l of protozoan suspension was removed at times of 0, 2, 4, 6, 8, 12, and 24 h, mixed to equal volumes of 0.4% trypan blue, and the cell viability was quantified by light microscopy. The preparations were made in duplicate. The

percentage of viability was determined by counting at least 200 cells (Berry *et al.* 1991).

Agglutination with lectins. The agglutination tests were made in 96-well plaques using a microtitrator. Equal volumes (25 □l) of the cell suspension (2 x 10_8 cell/ml), treated with IC_{50} (50% inhibitory concentration) of the essential oil, and the lectin were mixed, placed at room temperature (25°C) for 1 h, and read. The agglutination of the cells was always scored visually with a hand lens after gently resuspending the settled cells, and by observations using an inverted microscope (Zeiss - Axiovert 25). Agglutination inhibition assays were carried out at room temperature in the presence of specific monosaccharides. All lectins were purchased from Sigma Chemical Co. (St. Louis, MO).

Ultrastructure analysis. Both symbiont-bearing and symbiont-free *C. deanei* treated at the IC_{50} and IC_{90} (90% inhibitory concentration) from the *C. citratus* essential oil or amphotericin B in defined medium supplemented with 0.03 g/l of nicotinamide at 28°C for 48 h were collected by centrifugation, washed in PBS, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C. Cells were than rinsed with 0.1 M sodium cacodylate buffer and postfixed for 30 min at room temperature in 1% osmium tetroxide plus 0.8% potassium ferrocyanide and 5.0 mM $CaCl_2$, dehydrated in acetone, incubated in an acetone-epon mixture (2:1, 1:1, 1:2), and embedded in Epon resin. Ultrathin sections obtained in a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate, and observed in a Zeiss CEM-900 electron microscope.

RESULTS

ANTIPROTOZOAN ACTIVITY

A dose-dependent antiprotozoan effect of the essential oil of *C. citratus* on *C. deanei* is shown in Figure 1. An inhibitory effect of the essential oil was apparent in cells treated with high concentrations of the oil. Endosymbiont-free *C. deanei* (Fig. 1B) appeared to be more sensitive to the oil at concentrations over 100 μg/ml, compared to the endosymbiont-harbouring strain (Fig. 1A). In Figure 2 can be seen the inhibitory effect of the essential oil, benznidazole, and amphotericin B against both protozoa in the logarithmic phase (48 h) cultivated in defined medium. The IC_{50} and IC_{90} for symbiontbearing *C. deanei* treated with essential oil was 120 and 157 μg/ml, respectively (Fig. 2A). For the symbiont-free strain, the IC_{50} and IC_{90} were 60 and 92 μg/ml (Fig. 2B). Amphotericin B showed an inhibitory effect at concentrations lower than 5 μg/ml. In *C. deanei* with endosymbionts, the IC_{50} and IC_{90} were 3.4 and 4.7 μg/ml; and in the endosymbiont-free strain 3.6 and 5 μg/ml, respectively. For cells treated with benznidazole, much higher concentrations of the drug were necessary to reach the IC_{50}. For the symbiont-bearing strain, 841.7 μg/ml, and for the symbiont-free strain, 700 μg/ml were necessary. Tween 80 and dimethyl sulfoxide, the dilution agents, and petrolatum oil, used as indifferent oil, showed no effect on protozoan growth (data not shown).

VIABILITY

Viability of treated and control cells was assessed by a trypan blue dye exclusion test. The percentages of non-viable cells obtained by exposure of the protozoans to different concentrations of the essential oil and the drugs are shown in Figure 3. Endosymbiont-harbouring *C. deanei* had its viability reduced at high concentrations (500 and 250 μg/ml) after 8 h of exposure to the essential oil with only 5 and 9 % of viable cells, respectively (Fig. 3A). At concentrations lower than 100

μg/ml, viability was higher than 71.5% at 24 h. Endosymbiont-free *C. deanei* appeared to be more sensitive to the essential oil, because at 500 μg/ml, after 2 h of exposure all the cells were lysed, and at 250 μg/ml after 8 h, all the cells were non-viable (Fig. 3B). Concentrations below 100 μg/ml showed protozoan viability higher than 79%, after 24 h of incubation. Benznidazole did not interfere with the viability of both protozoans, even at concentrations as high as 1000 μg/ml. For amphotericin B, the endosymbiontfree strain was more sensitive than symbiont-harbouring cells (data not shown).

AGGLUTINATION OF LECTINS

The agglutination with lectins of both strains of *C. deanei*, symbiont-bearing and symbiont-free cells, control and treated with essential oil of *C. citratus* is shown in Table 1. The lectins are classified according to their sugar specificities, and the results are expressed as the minimum concentration of lectins required to agglutinate the cells. The binding reaction is considered to be more specific with cells which are agglutinated at the lowest lectin concentration. For symbiont-bearing *C. deanei* treated with essential oil, only *Dolichos biflorus* and *Glicine max*, D-GalNAc binding lectins, altered their minimum concentration required to agglutinate the cells with 125 and 15.6 μg/ml, respectively. For the symbiont-free strain, alterations in treated cells with essential oil were observed as a decrease in the binding specificity of the *Limulus polyphemus,* a sialic-acid-binding lectin, and increase of the binding specificity of *Arachys hypogaeae*, a D-Gal-binding lectin. *Artocarpus integrifolia* and *G. max*, D-GalNac-binding lectins, and *Lens culinaris,* a lectin that has binding sites complementary to D-mannose-like residues, also had their sugar-binding specificity increased in the presence of the essential oil. On the other hand, *D. biflorus* and *Canavalia ensiformis* (D-mannose-like binding lectin) had their sugar-binding specificity decreased. Agglutination was inhibited by 0.1 M of the respective specific monosaccharides (D-GalNAc, D-Gal, and α-D-methyl mannoside).

ULTRASTRUCTURE ANALYSIS

In order to determine ultrastructure changes in symbiont-harbouring and symbiont-free strains of *C. deanei* treated with the IC_{50} and IC_{90} of the essential oil of *C. citratus* and amphotericin B, transmission electron microscopy analysis was carried out. For the symbiont-harbouring strain treated with the essential oil at the IC_{50}, alterations at the membrane of the flagellar pocket with invaginations of this membrane and the presence of membraneous material, and an enlargement of the flagellar pocket were observed (Fig. 4B and 4C). When treated with essential oil at the IC_{90}, extensive vacuolisation and portions of the membrane detaching from the cell body (blebs) appeared (Fig 4E). The control cells had a proeminent nucleus with symbionts located close to it and near the flagellar pocket, endoplasmic reticulum was also well characterized (Fig 4A). For the symbiont-free *C. deanei,* the essential oil also affected the membrane of the flagellar pocket (Fig. 5B and 5C). Cells treated with IC_{50} showed small membrane fragments and enlargement of the flagellar pocket with membraneous material inside it. For cells treated with IC_{90}, cytoplasmic alterations, extensive vacuolisation and the presence of blebs were evident (Fig. 5F and 5G). Control cells had the nucleus located at the anterior end of the protozoa, and glycossomes are situated close to it. Also the kinetoplast could be observed inside the mitochondria and close to the flagellum (Fig 5A). When symbiont-harbouring and symbiont-free *C. deanei* were treated with amphotericin B, similar alterations were observed (Figs. 4D, 4F, 5D, 5E, and 5G). Blebs detaching from the outer membrane at the IC_{50} could only be seen in the symbiont-free-strain treated with amphotericin B (Fig. 5E).

DISCUSSION

A dose-dependent antiprotozoan effect of the essential oil of *C. citratus* in *C. deanei* could be observed, and this effect on the growth of both endosymbiontharbouring and symbiont-free showed differences in the growth inhibition. The concentration of essential oil necessary to inhibit endosymbiont-harbouring *C. deanei* was higher than that necessary to inhibit endosymbiont-free cells. Some investigators have reported that the presence of the endosymbiont interferes with the protozoan metabolism and induces morphological and biochemical changes (Freymüller and Camargo 1981, De Souza and Motta 1999). *Cymbopogon citratus* is an herb known worldwide as lemongrass, and the tea made from its leaves is popularly used in Brazil as an antispasmodic, analgaesic, anti-inflammatory, antipyretic, diuretic, and sedative (Carlini *et al.* 1986). Published reports indicate that the essential oil obtained from fresh leaves of this plant has antibacterial and antifungal properties (Onawunmim 1989, Lima et al. 1993, El-Kamil *et al.* 1998). Recently Luize *et al.* (2005) reported that a hydroalcoholic crude extract of *C. citratus* is active against promastigote and amastigote forms of *L. amazonensis* and *T. cruzi*, with inhibition rates over 90% at 100 □g/ml.

As *C. deanei* is a member of the family Trypanosomatidae, both drugs, benznidazole and amphotericin B were used to compare the effect of essential oil on the protozoans. Benznidazole is a drug used in the chemotherapy of the acute and intermediate phases of Chagas' disease, which is caused by *T. cruzi*. It acts via a different mechanism, which involves covalent modification of macromolecules by nitroreductive intermediates (Castro *et al.* 2003). Holetz *et al.* (2003) demonstrated that *Herpetomonas samuelpessoai* has natural resistance to benznidazole (IC_{50} higher than 3,840 μM). For *C. deanei* with and without endosymbionts the IC_{50} was 841.7 and 700 μg/ml, respectively, indicating possible resistance to this drug. Amphotericin B is a valuable drug used in the treatment of leishmaniasis. It interacts with protozoan membrane sterols, and preferentially with ergosterol (Goad *et al.* 1984). When *C. deanei* was treated with amphotericin B, concentrations lower than 5 μg/ml were sufficient to inhibit cell growth, indicating that this drug is efficacious against the protozoan.

At the ultrastructure level, both strains of *C. deanei* treated with the essential oil of *C. citratus* and amphotericin B showed alterations of the membrane of the flagellar pocket. These changes included invagination and the presence of membraneous material, and certain modifications in the cytoplasmic membrane, such as the presence of blebs. Alterations in trypanosomatid membranes have been reported for other compounds. Braga *et al.* (2004) reported alterations in the cytoplasmic outer membranes and an enlargement of the flagellar pocket of *T. cruzi* treated with squalene synthase inhibitors. Other alterations were observed by Rodrigues *et al.* (2005), who analyzed promastigote forms of *L. amazonensis* treated with BPQ-OH, a specific inhibitor of squalene synthase, which induced ruptures of the plasma membrane with disconnection from the subpellicular microtubules, the formation of elaborate structures, and intense membrane shedding. Other studies have reported analogous alterations in *Trypanosoma brucei* treated with *Kola acuminata* proanthocyanidins (Kubata *et al.* 2005). Santos *et al.* (2006) reported alterations of the cellular membrane, including the fragmentation of the flagellar pocket membrane in *Phytomonas serpens* treated with antipain and leupeptin (cystein peptidase inhibitors).

Due to these ultrastructure alterations, the expression of membrane carbohydrate residues was determined using the agglutination with lectins assay. All parasites have carbohydrates on their surfaces, as part of their cytoskeletons or in their internal structures, and because of this, lectins can be directly used in agglutination assays. Lectins have been defined as carbohydrate-binding proteins other than enzymes or antibodies (Jacobson and Doyle 1996). A study of cell-surface carbohydrates using lectins has been done on different members of the family Trypanosomatidae such as *Trypanosoma*, *Leishmania*, *Herpetomonas*, *Phytomonas*, and *Crithidia* (De Souza 1989). Esteves *et al.* (1982) studied the cell-surface carbohydrates in endosymbiontbearing and endosymbiont-free *C. deanei.* They observed that the agglutination pattern with the lectins for the symbiont-free organism was higher than for its counterpart. This pattern could also be observed in the present study. For symbiont-harbouring *C. deanei*, the essential oil increased the binding specificity of surface D-GalNAc residues observed with *G. max* and *D. biflorus* lectins. For the symbiont-free strain treated with the oil, there was depletion of the binding specificity for the sialic acid residues, observed with *L. polyphemus*, and an increase of its binding specificity for D-Gal and D-GalNac sugar residues.

Sialic acids are a family of nine carbon sugars that are found at the non-reducing end of glycoconjugates and are linked to galactose and N-acetyl-Dgalactosamine (Shauer and Kamerling 1997). This result indicates that the essential oil may have removed the sialic acid residues and exposed the other sugar residues, or that the essential oil may be interfering with the expression of these sialic acid glycoconjugates at the cell surface. Also for symbiont-free treated cells, there was a diminution of mannose residues, confirmed by *C. ensiformis* lectin.

In conclusion, this study of the effect of essential oil from *C. citratus* on the trypanosomatid *C. deanei* with and without endosymbionts demonstrated the importance of these protozoans as a biological model in the evaluation of the cellular alterations and the influence of the symbiont by herbal and commercial drugs. This model is capable of mimicking events in pathogenic microorganisms such as *T. cruzi*, *Leishmania,* and *Phytomonas*. These results can contribute to understanding of the drug's mechanism of action, opening new prospects of finding more effective, less toxic, and relatively inexpensive drugs of vegetable origin, in the treatment of diseases caused by trypanosomatids.

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Table 1 - Activity of lectins of various specificities for symbiont-harbouring and symbiont-free *Crithidia deanei* and cells treated with essential oil of *Cymbopogon citratus.*

Minimum concentration (µg/ml) required to agglutinate *Crithidia deanei*				
	Symbiont-harbouring		Symbiont-free	
	Control cells	Cells treated with essential oil	Control cells	Cells treated with essential oil
D-GlcNAc-binding lectins				
-Phytolaca americana	>500	>500	<7.8	15.6
-Triticum vulgares	>500	>500	125	62.5
D-GalNAc-binding lectins				
-Dolichos biflorus	>500	125	<7.8	62.5
-Wisteria floribunda	>500	>500	15.6	15.6
-Phaseolus vulgaris	>500	>500	15.6	<7.8
-Glicine max	62.5	15.6	31.2	<7.8
-Artocarpus integrifolia	<7.8	<7.8	250	15.6
D-Gal-binding lectins				
-Arachys hypogaeae	>500	>500	125	<7.8
D-Man-binding lectins				
-Canavalia ensiformis	>500	>500	<7.8	>500
-Lens culinaris	>500	250	62.5	15.6
L-fucose-binding lectins				
-Ulex europeus	>500	>500	62.5	31.2
Sialic acid-binding lectins				
-Limulus polyphemus	>500	>500	<7.8	31.2

Legend for Figures

Figure 1 - Growth curves of *Crithidia deanei* treated with essential oil of *Cymbopogon citratus*: (A) endosymbiont-harbouring; (B) endosymbiont-free. (♦) Control cells; (●) 50 µg/ml; (□) 100 µg/ml, (□) 150 µg/ml, (▲) 200 µg/ml, and (■) 250 µg/ml.

Figure 2 - Effect of essential oil from *Cymbopogon citratus* (■), benznidazole (▲), and amphotericin B (♦) on growth inhibition of *Crithidia deanei* in defined medium at 28$_{o}$C after 48 h. (A) endosymbiont-harbouring; (B) endosymbiont-free.

Figure 3 - Effect of essential oil on viability of *Crithidia deanei* strains: (A) endosymbiont-harbouring; (B) endosymbiont-free (B).

Figure 4 - Endosymbiont-harbouring *Crithidia deanei* cultured at 28°C for 48h. **A**control cells; **B** and **C-** Cells treated with IC_{50} of essential oil of *Cymbopogon citratus*; **D**- Cell treated with IC_{50} of amphotericin B; **E**- IC_{90} of cells treated with essential oil of *Cymbopogon citratus*; **F**- IC_{90} of cells treated with amphotericin B. **e**- endosymbiont; **f**flagellum; **fp**- flagellar pocket; **g**- glycossome; **k**- kinetoplast; **l**- lipid inclusion; **m**mitochondia, **n**, nucleus. Arrows indicate the presence of membraneous material at the flagellar pocket of treated cells at the IC_{50} and membranes detaching from the cell at the IC_{90}. Bar = 1µm.

Figure 5 - Endosymbiont-free *Crithidia deanei* cultured at 28°C for 48h. **A**- Control cells; **B** and **C-**Cells treated with IC_{50} of essential oil of *Cymbopogon citratus*; **D** and **E**cells treated with IC_{50} of amphotericin B; **F**- IC_{90} of cells treated with essential oil of *Cymbopogon citratus*; **G**- IC_{90} of cells treated with amphotericin B. **e**- endosymbiont; **f**flagellum; **fp**- flagellar pocket; **g**- glycossome; **k**- kinetoplast; **l**- lipid inclusion; **m**mitochondia, **n**, nucleus. Arrows indicate the presence of membraneous material at the flagellar pocket of treated cells and the presence of blebs. Bar = 1µm.

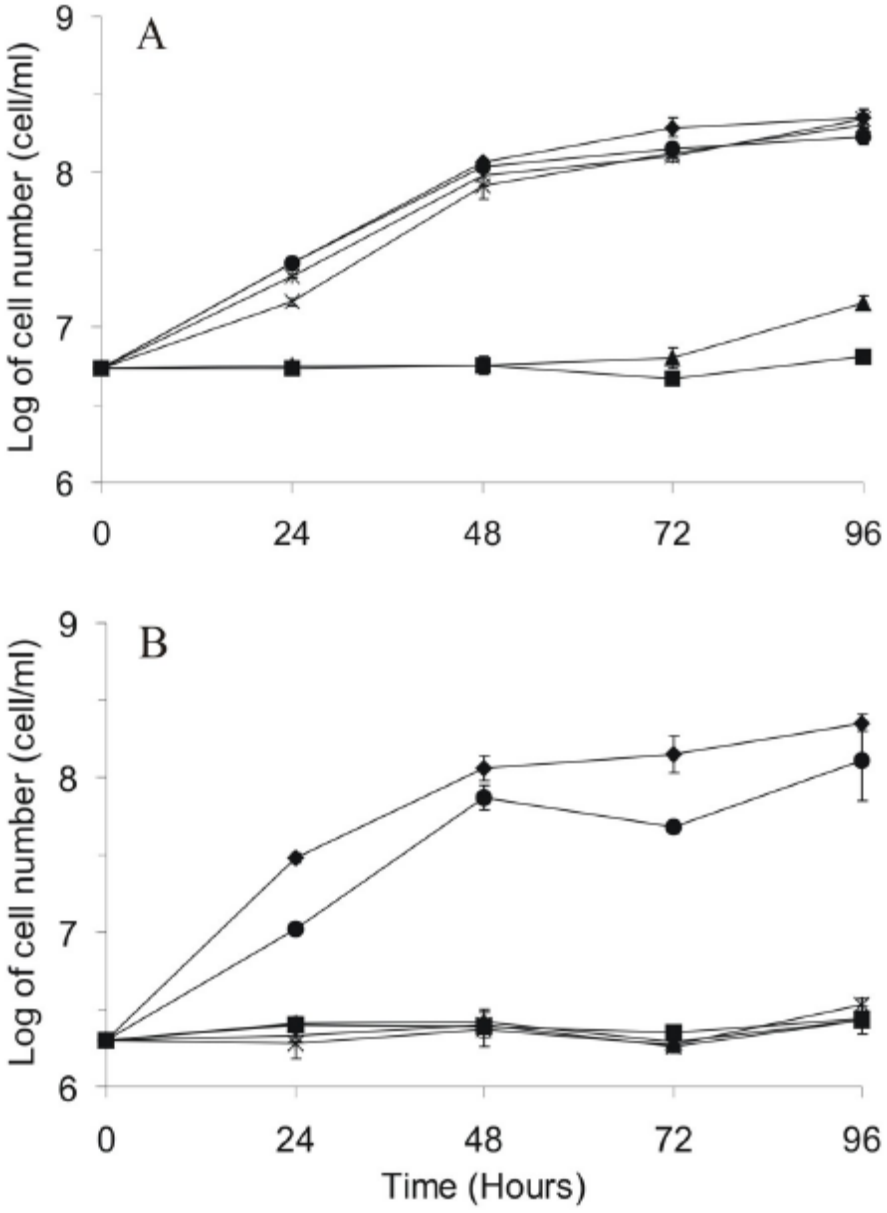

Figura 1

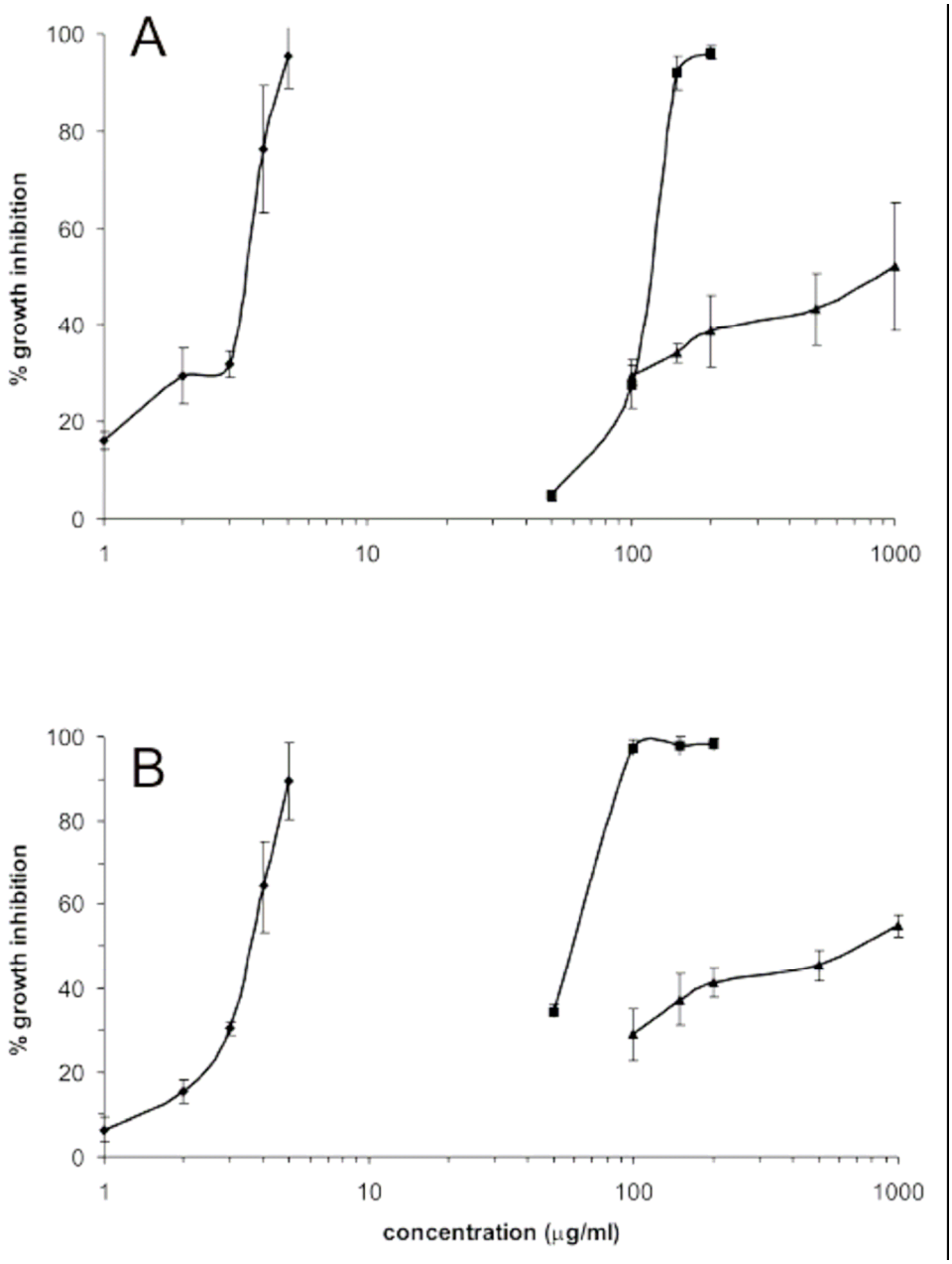
A
B
% growth inhibition
% growth inhibition
100
80
60
40
20
0
1
10
100
1000
concentration (μg/ml)

Figura 2

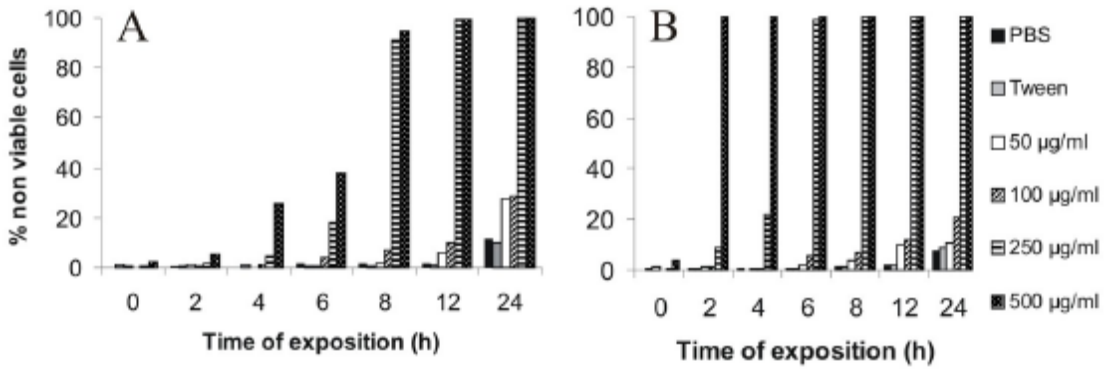
A
B
% non viable cells
100
80
60
40
20
0
0 2 4 6 8 12 24
Time of exposition (h)
PBS
Tween
50 µg/ml
100 µg/ml
250 µg/ml
500 µg/ml

Figura 3

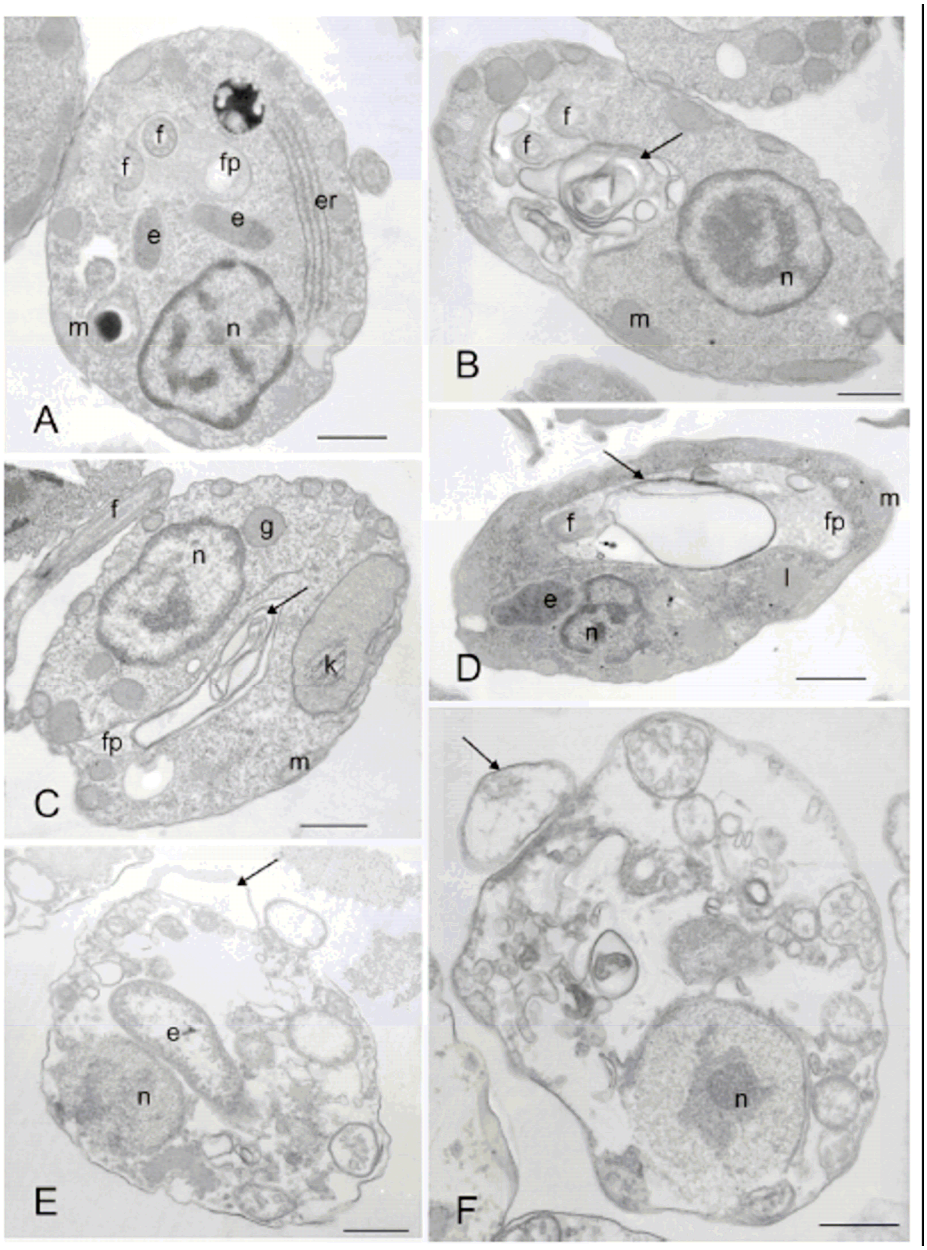

Figura 4

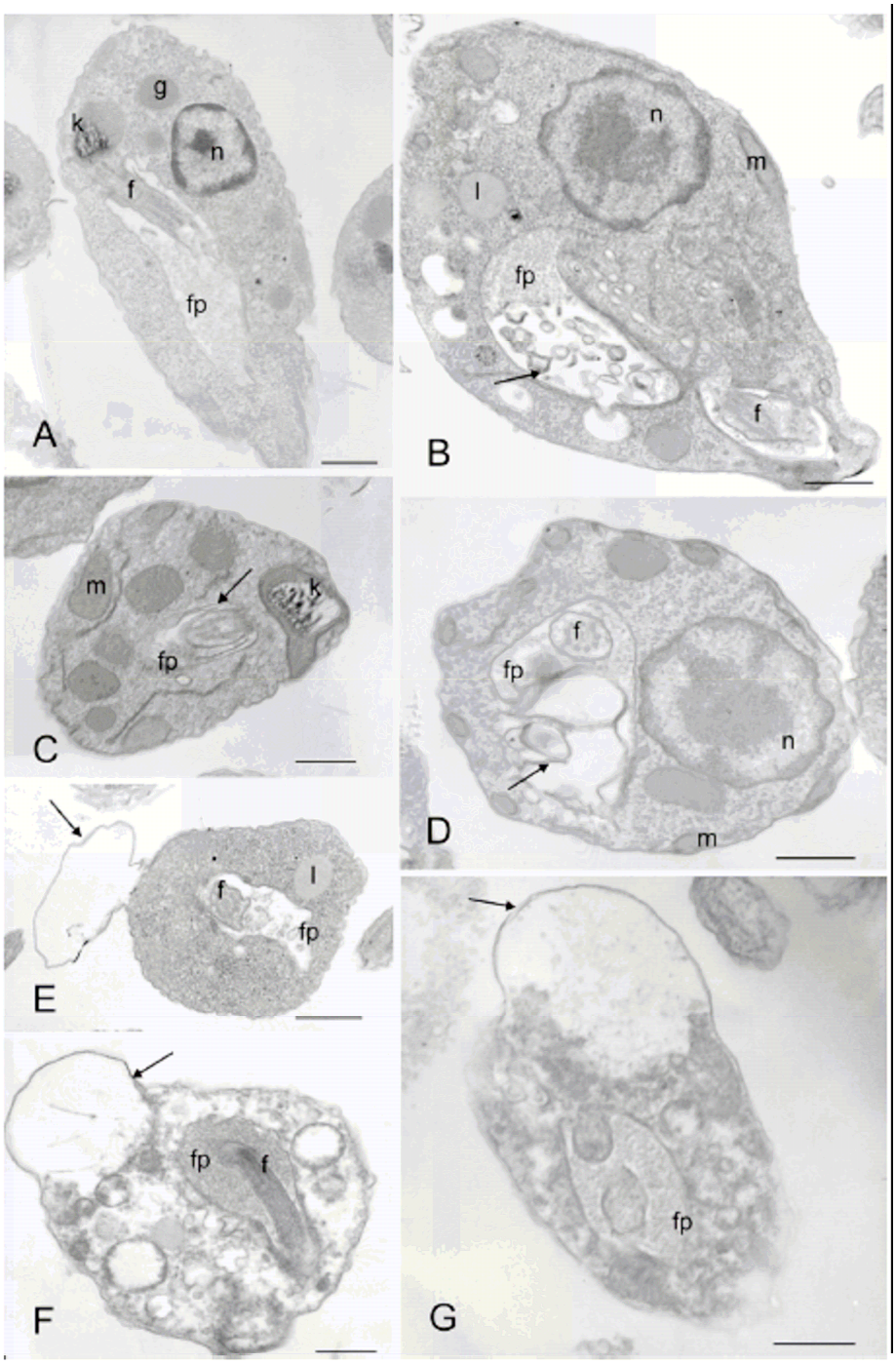

Figura 5