Acta Protozool. (2006) 45: 231 - 240

ACTA
PROTOZOOLOGICA

Biological Activities of Essential Oil Obtained from *Cymbopogon citratus* on *Crithidia deanei*

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Summary. We report the effect of the essential oil extracted from *Cymbopogon citratus* on endosymbiont-harbouring and endosymbiont-free strains of the insect trypanosomatid *Crithidia deanei* grown at 28°C in a chemically defined medium. A dose-dependent antiprotozoan effect of the essential oil of *C. citratus* could be observed on both strains of *C. deanei*. The IC_{50} values (50% inhibitory concentration) for symbiont-bearing and symbiont-free strains were 120 and 60 µg/ml, respectively. The viability assay showed that the symbiont-free strain is more sensitive to the presence of the essential oil, because lysed cells were observed after 2 h of exposure at higher concentrations. In addition, alterations in the ultrastructure and in the detection of cell-surface carbohydrate residues in both strains of *C. deanei* after treatment with the essential oil were also evaluated. Both strains showed ultrastructural alterations in the cellular and flagellar pocket membranes, as revealed by transmission electron microscopy. In the lectin assay, the essential oil influenced the expression of carbohydrates in symbiont-free *C. deanei*, as evidenced by a reduction of sialic acid residues on the cell surface.

Key words: antiprotozoan activity, *Crithidia deanei*, *Cymbopogon citratus*, medicinal plants.

INTRODUCTION

The family Trypanosomatidae harbours 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),

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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 (Freymüller 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 the 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 the presence of trypanosomatids other than *Trypanosoma* and *Leishmania* in certain opportunistic cutaneous infections in immunocompromised individuals (Dedet *et al.* 1995, Boisseau-Garsaud *et al.* 2000) and also in individuals with no 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) have limited action, 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 practice of herbalism has become mainstream throughout the world. This is due in part to the recognition of the value of traditional medical systems, and the identification of medicinal plants from indigenous pharmacopeias (Elvin-Lewis 2001). Medicinal plants occur 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 extracted from *C. citratus* on the growth, viability, cell-surface carbohydrate residues, and ultrastructure of endosymbiont-harbouring and endosymbiont-free strains of *C. deanei* cultured in a defined medium at 28°C.

MATERIALS AND METHODS

Plant material. *Cymbopogon 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°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 this experiment, symbiont-bearing and symbiont-free strains of *C. deanei* were incubated in the 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 13×100 mm tubes containing 1 ml of the medium, and the starting inoculum consisted of cells in logarithmic growth phase (2×10^6 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 the experiments were performed in triplicate. The results were expressed as log number (cells/ml) and as the percentage of growth inhibition at 48 h. Amphotericin B (FUNGIZON®, 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 microcentrifuge tubes containing 2×10^7 cells in the logarithmic growth phase, and incubated at 28°C. After addition of the essential oil, 25 µl of the protozoan suspension was removed after 2, 4, 6, 8, 12, and 24 h and was mixed in an equal volume of 0.4% trypan blue, and the cell viability was quantified by light microscopy. All 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 µl) of the

cell suspension (2×10^8 cell/ml), treated with the IC_{50} concentration (50% inhibitory concentration) of the essential oil and each lectin were mixed and then left at room temperature (25°C) for 1 h. The agglutination of the cells was always scored visually with a hand lens after the settled cells had been gently resuspended, and also by observations using an inverted microscope (Zeiss Axiovert 25). The 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 cells of *C. deanei* were treated with the IC_{50} and IC_{90} amounts (90% inhibitory concentration) of the *C. citratus* essential oil or amphotericin B in the defined medium supplemented with 0.03 g/l of nicotinamide at 28°C for 48 h. Cells 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. The cells were then 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 with a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate, and observed with a Zeiss CEM-900 electron microscope.

RESULTS

Antiprotozoan activity of the essential oil

A dose-dependent antiprotozoan effect of the essential oil from *C. citratus* on *C. deanei* is evident in Fig. 1. The inhibitory effect of the essential oil was apparent in the cells treated with the highest concentrations of the oil. The endosymbiont-free *C. deanei* (Fig. 1B) appeared to be more sensitive to the essential oil at concentrations over 100 µg/ml, compared to the endosymbiont-harbouring strain, in which the cell growth was inhibited at concentrations over 200 µg/ml (Fig. 1A). In Fig. 2, the inhibitory effect of the essential oil, benznidazole, and amphotericin B on both strains in the logarithmic phase (48 h) can be seen. The IC_{50} and IC_{90} concentrations for the symbiont-bearing strain treated with the essential oil were 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 cells of *C. deanei* containing the endosymbiont, the IC_{50} and IC_{90} concentrations were 3.4 and 4.7 µg/ml; and in the endosymbiont-free strain, 3.6 and 5 µg/ml, respectively. For benznidazole-treated cells, 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 an indifferent oil, had no effect on protozoan growth (data not shown).

Cell viability

The viability of treated and control cells were assessed by a trypan blue dye exclusion test. The percentages of non-viable cells obtained by exposing the protozoan cells to different concentrations of the essential oil and the drugs are shown in Fig. 3. The endosymbiont-harbouring strain of *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 more than 71.5% at 24 h. The endosymbiont-free *C. deanei* appeared to be more sensitive, because after 2 h of exposure at 500 µg/ml of the essential oil, all the cells were lysed, and after 8 h at 250 µg/ml, all the cells were non-viable (Fig. 3B). Concentrations below 100 µg/ml showed a protozoan viability over 79%, after 24 h of incubation. Benznidazole did not interfere with the viability of either strain, even at concentrations as high as 1000 µg/ml. For amphotericin B, the endosymbiont-free strain was more sensitive than the symbiont-harbouring cells (data not shown).

Agglutination test with lectins

The lectin agglutination of cells from both strains of *C. deanei*, treated or not with the essential oil, is shown in Table 1. The lectins are classified according to their sugar specificities, and the results were expressed as the minimum concentration of lectins required to agglutinate the cells. The binding reaction was more specific with cells which were agglutinated at the lowest lectin concentration. For symbiont-bearing cells of *C. deanei* treated with the essential oil, only *Dolichos biflorus* and *Glicine max*, D-GalNAc binding lectins, altered the minimum concentration required to agglutinate the cells, to 125 and 15.6 µg/ml, respectively. For the symbiont-free strain, alterations in cells treated with the essential oil were observed for many lectins, as a decrease in the binding specificity of *Limulus polyphemus*, a sialic acid-binding lectin, and an increase in 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

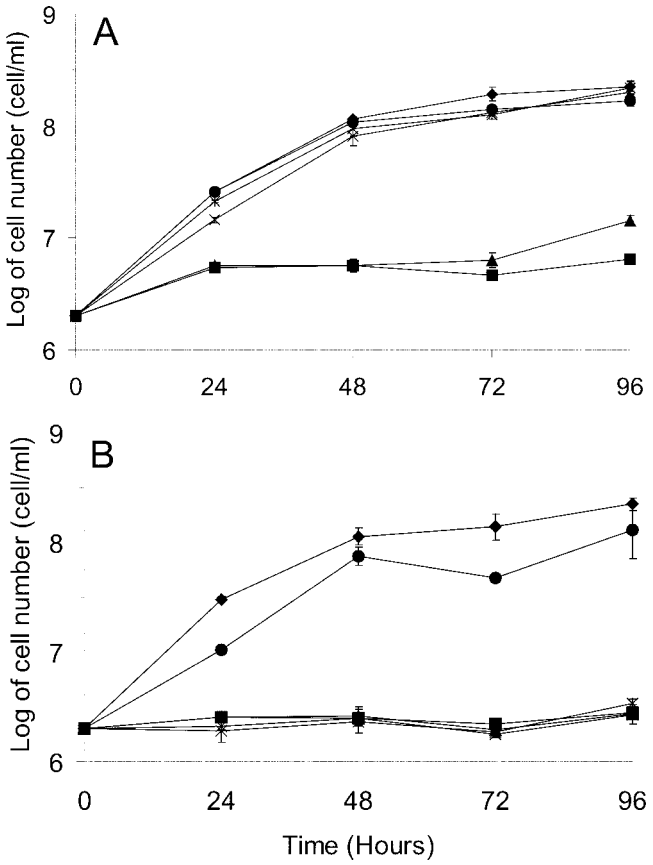


Fig. 1. Growth curves of strains of *Crithidia deanei* treated with the essential oil from *Cymbopogon citratus*: (A) endosymbiont-harbouring; (B) endosymbiont-free. Control cells - filled diamond; 50 µg/ml - filled circle; 100 µg/ml - open circle; 150 µg/ml - open square; 200 µg/ml - filled triangle; 250 µg/ml - filled square.

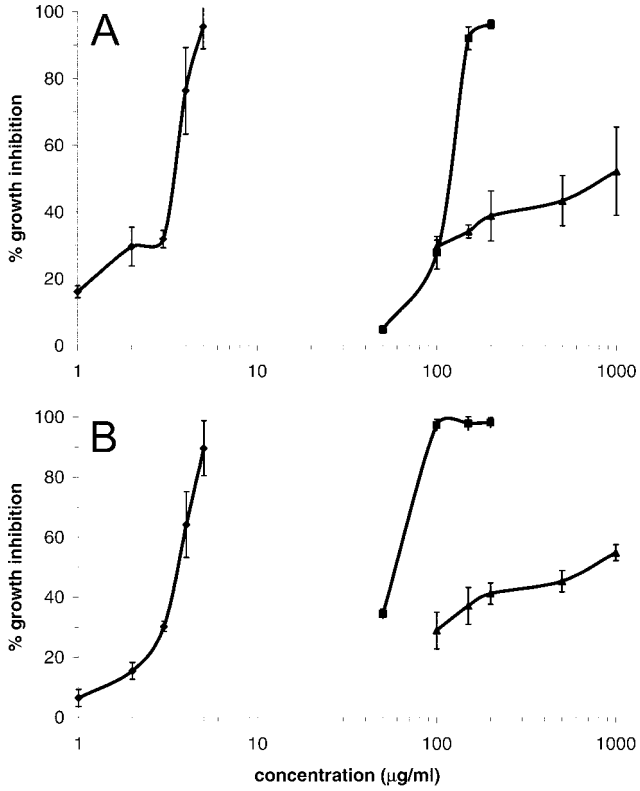


Fig. 2. Effect of the essential oil from *Cymbopogon citratus* (filled square), benznidazole (filled triangle), and amphotericin B (filled diamond) on the growth inhibition of strains of *Crithidia deanei* in defined medium at 28°C after 48 h. (A) endosymbiont-harbouring; (B) endosymbiont-free.

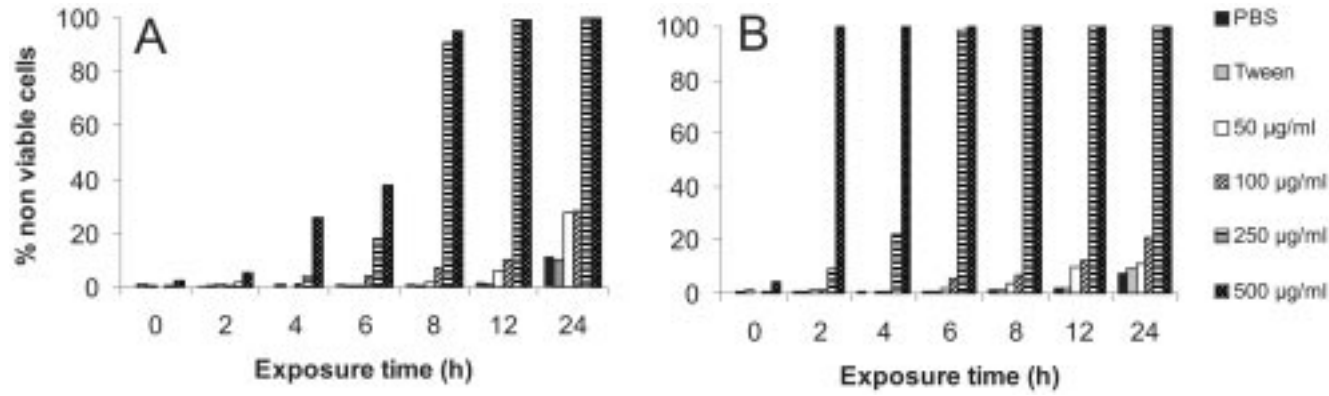
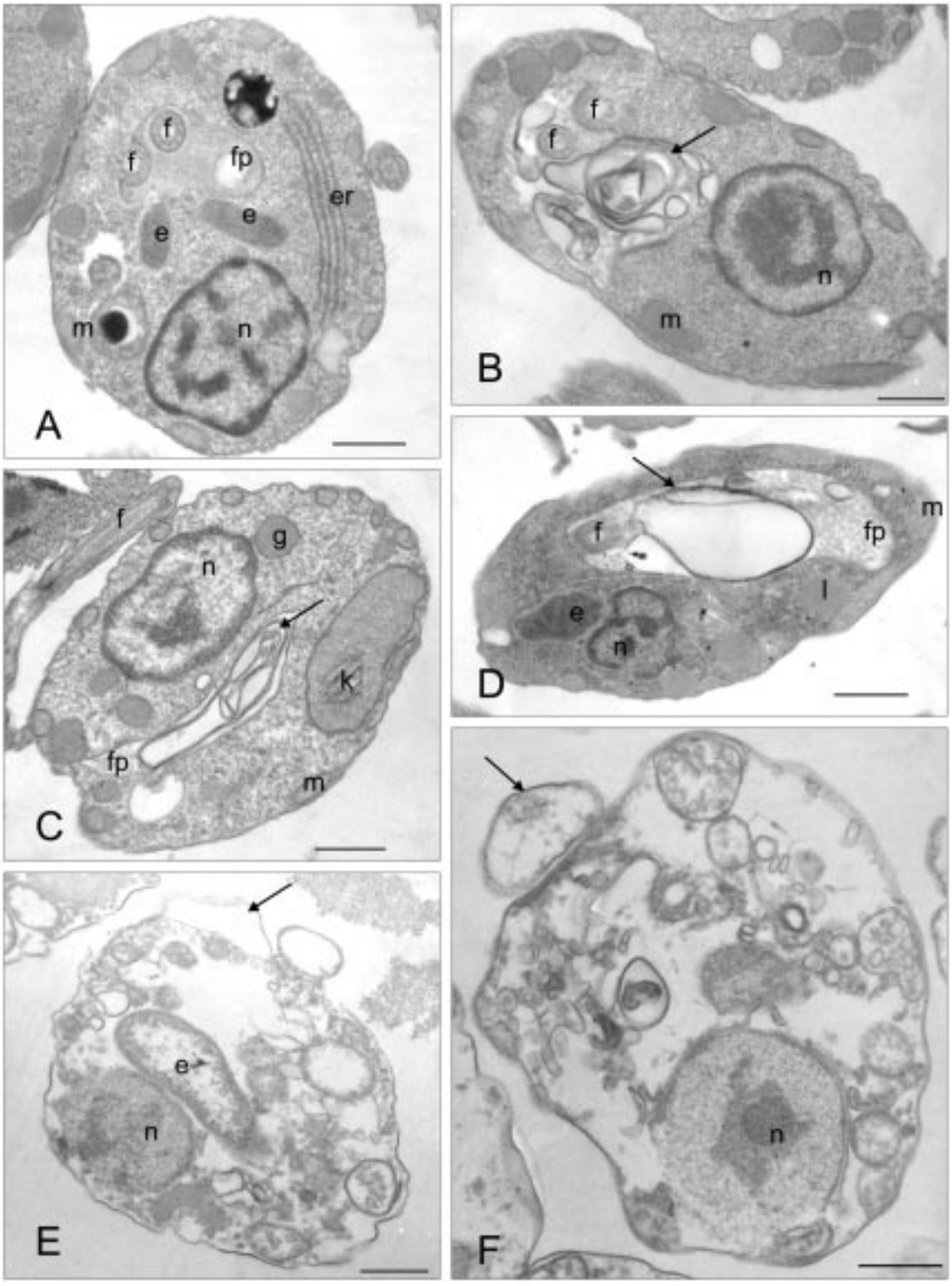


Fig. 3. Effect of the essential oil from *Cymbopogon citratus* on the viability of strains of *Crithidia deanei*: (A) endosymbiont-harbouring; (B) endosymbiont-free.



Figs 4A-F. Ultrastructural morphology of the endosymbiont-harbouring strain of *Crithidia deanei* cultured at 28°C for 48 h in the absence (**A**) or in the presence of the following compounds: the IC_{50} concentrations of essential oil from *Cymbopogon citratus* (**B** and **C**) or amphotericin B (**D**); and the IC_{90} concentrations of the essential oil from *Cymbopogon citratus* (**E**) or amphotericin B (**F**). e - endosymbiont; f - flagellum; fp - flagellar pocket; g - glycosome; k - kinetoplast; l - lipid inclusion; m - mitochondria, n - nucleus. Arrows indicate the presence of membraneous material in the flagellar pocket of treated cells at the IC_{50} concentration, and membranes detaching from the cell at the IC_{90} concentration. Scale bars: 1 µm.

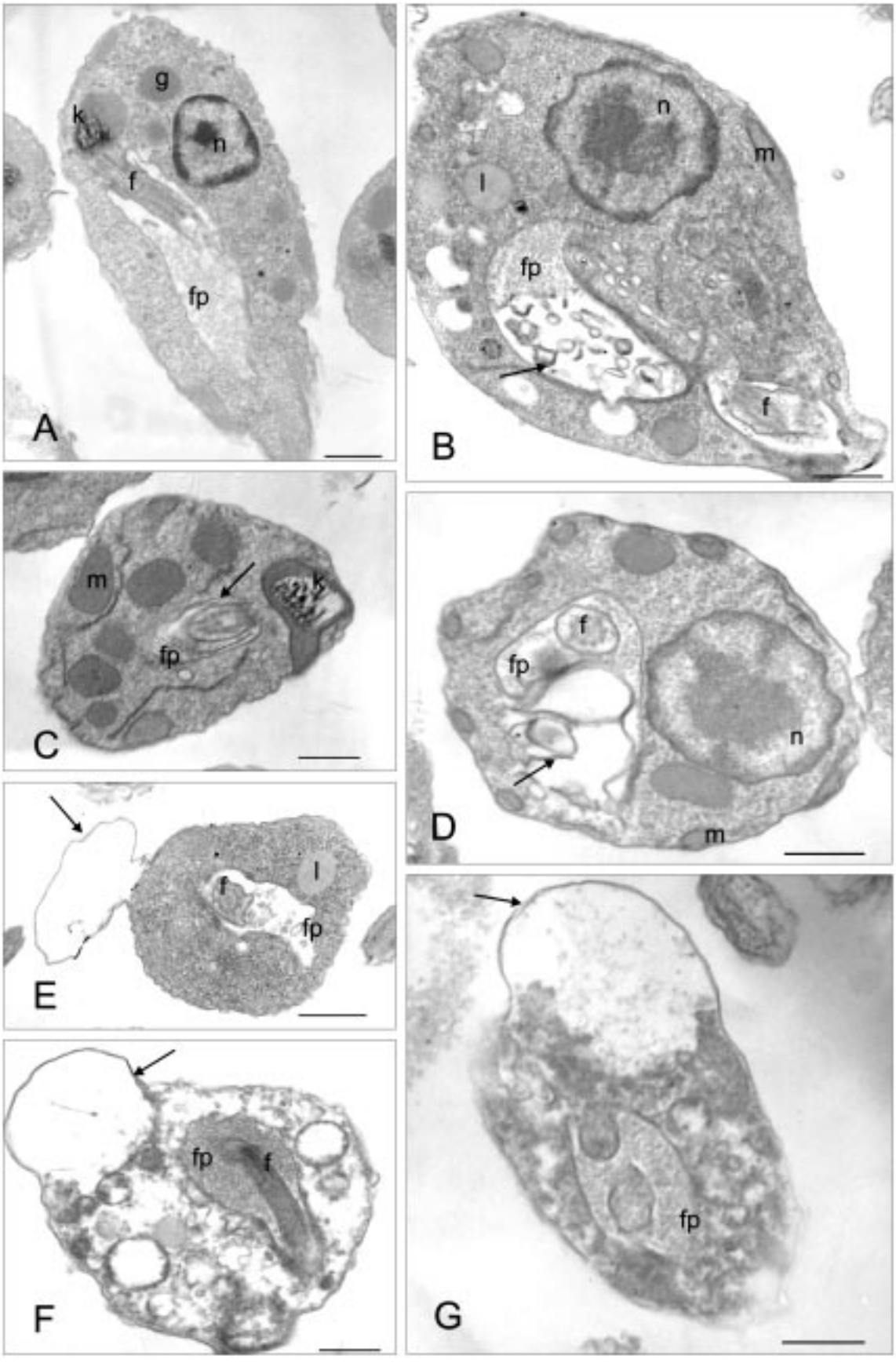

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Table 1. Minimum lectin concentration (µg/ml) required to agglutinate strains of *Crithidia deanei* in the presence of the essential oil of *Cymbopogon citratus.*

	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

of the essential oil. On the other hand, *D. biflorus* and *Canavalia ensiformis* (a D-mannose-like binding lectin) had their sugar-binding specificity decreased. Agglutination was inhibited by the respective specific monosaccharides (D-GalNAc, D-Gal, and a-D-methyl mannoside) at 0.1 M.

Ultrastructural analysis

In order to determine the ultrastructural 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, an analysis was done by means of transmission electron microscopy. For the symbiont-harbouring strain treated with the essential oil at the IC_{50} level, alterations in the flagellar pocket membrane were observed, with invaginations of this membrane and the presence of membraneous material, and an enlargement of the flagellar pocket (Figs 4B, C). When this strain was treated with the essential oil at the IC_{90} level, 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, and the endoplasmic reticulum was also well characterised (Fig. 4A). For the symbiont-free *C. deanei*, the essential oil also affected the membrane of the flagellar pocket (Figs 5B, C). Cells treated with the IC_{50} concentration showed small membrane fragments and enlargement of the flagellar pocket, with membraneous material present inside it. For cells treated with IC_{90}, cytoplasmic alterations, extensive vacuolisation, and the presence of blebs were evident (Figs 5F, G). Control cells had the nucleus located at the anterior end of the protozoa, and glycosomes were situated close to it. The kinetoplast could be observed inside the mitochondria and close to the flagellum (Fig. 5A). When symbiont-harbouring and symbiont-free strains of *C. deanei* were treated with amphotericin B, similar alterations were observed (Figs 4D, F; 5D, E,G). Blebs detaching from the cytoplasmic membrane at the IC_{50}

Figs 5A-G. Ultrastructural morphology of the endosymbiont-free strain of *Crithidia deanei* cultured at 28°C for 48 h in the absence (**A**) or in the presence of the following compounds: the IC_{50} concentrations of essential oil from *Cymbopogon citratus* (**B** and **C**) or amphotericin B (**D** and **E**); and the IC_{90} concentrations of the essential oil from *Cymbopogon citratus* (F) or amphotericin B (**G**). e - endosymbiont; f - flagellum; fp - flagellar pocket; g - glycosome; k - kinetoplast; l - lipid inclusion; m - mitochondria; n - nucleus. Arrows indicate the presence of membraneous material in the flagellar pocket of treated cells, and the presence of blebs. Scale bar: 1 µm.

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 extracted from *C. citratus* on *C. deanei* could be observed, with differences in the growth inhibition of both the endosymbiont-harbouring and symbiont-free strains. The concentration of the essential oil necessary to inhibit the endosymbiont-harbouring strain of *C. deanei* was higher than that necessary to inhibit the 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 crude hydroalcoholic extract of *C. citratus* is active against promastigote and amastigote forms of *L. amazonensis* and *T. cruzi*, with inhibition rates over 90% at 100 µg/ml.

Because *C. deanei* is a member of the family Trypanosomatidae, the 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 through 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 a natural resistance to benznidazole (IC_{50} higher than 3,840 µM). For *C. deanei* with and without endosymbionts, the IC_{50} concentrations were 841.7 and 700 µg/ml, respectively, indicating a possible resistance to this drug. Amphotericin B is a valuable drug used in the treatment of leishmaniasis. It interacts with sterols of protozoan membranes, 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 ultrastructural level, both strains of *C. deanei* treated with the essential oil of *C. citratus* and amphotericin B showed alterations in 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 membrane 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 proanthocyanidins from *Kola acuminata* (Kubata *et al.* 2005). Santos *et al.* (2006) reported alterations of the cellular membrane in *Phytomonas serpens* treated with antipain and leupeptin (cystein peptidase inhibitors), including fragmentation of the flagellar pocket.

Due to these ultrastructure alterations, the expression of membrane carbohydrate residues was determined using the lectin agglutination 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 endosymbiont-bearing and endosymbiont-free strains of *C. deanei.* They observed that the symbiont-free strain agglutinated with a wider variety of lectins than did the other strain, and that the concentration of lectins required to agglutinate the cells was lower than for the symbiont-harbouring *C. deanei*.

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 the *G. max* and *D. biflorus* lectins. For the symbiont-free strain treated with the oil, there was a 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-D-galactosamine (Shauer and Kamerling 1997). This result indicates that the essential oil may have removed the sialic acid residues and exposed 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 the symbiont-free treated cells, there was a diminution in the recognition of mannose residues, confirmed by the *C. ensiformis* lectin.

In conclusion, this study of the effect of the 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's presence in trypanosomatids treated with herbal and commercial drugs. These results can contribute to understanding 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.

Acknowledgments. This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Financiadora de Estudos e Projetos - FINEP, PRONEX/ Fundação Araucária, and Programa de Pós-graduação em Microbiologia da Universidade Estadual de Londrina.

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Received on 16th March, 2006; revised version on 10th May, 2006; accepted on 29th May, 2006