

Antitrypanosomal Activity of a Semi-Purified Subfraction Rich in Labdane Sesquiterpenes, Obtained from Flowers of *Anthemis tinctoria*, against *Trypanosoma cruzi*

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Received September 27th, 2010; revised December 15th, 2010; accepted December 29th, 2010.

ABSTRACT

In Brazil and several other Latin American countries, Chagas' disease still constitutes a serious medical and social problem, and there is a need to develop new, more-potent drugs with fewer side effects to effectively treat this disease. We investigated the antitrypanosomal effect of a crude extract, fractions, and a semi-purified subfraction rich in a mixture of isomeric labdane sesquiterpenes, obtained from flowers of *Anthemis tinctoria*, against *Trypanosoma cruzi*. In epimastigote forms, the aqueous crude extract, dichloromethane fraction, and semi-purified subfraction showed a dose-dependent inhibitory activity, with IC_{50} of 2.3 $\mu\text{g/ml}$, 1.8 $\mu\text{g/ml}$, and 0.2 $\mu\text{g/ml}$, respectively. In the interaction index, the semi-purified subfraction showed a reduction in both the percentage of infected LLCMK₂ cells and the mean number of amastigotes per infected cell. The cytotoxicity evaluation demonstrated that the cytotoxic concentrations of the semi-purified subfraction were higher for LLCMK₂ cells than for the protozoans, with a selectivity index of 35.0. Epimastigote forms treated with the semi-purified subfraction showed ultrastructural and morphological alterations such as rounding of the cells and bleb formation in the flagellum and cytoplasmic membrane. These results show that the flowers from *A. tinctoria* may be a source of new drugs with antiprotozoal activity. However, additional *in vitro* and *in vivo* studies are needed to validate the use of *A. tinctoria* in the treatment of Chagas' disease.

Keywords: Antiprotozoan Activity, Medicinal Plants, *Trypanosoma cruzi*, Ultrastructure Alterations

1. Introduction

About 65% - 80% of the population in developing countries essentially depends on plants for primary health care. Some 25% of all modern medicines are derived directly or indirectly from plants [1]. Many plants from Brazilian ecosystems such as the savanna, and the Atlantic and Amazon forests are used in traditional medicine [2]. Also, many exotic plants that were introduced into Brazil and incorporated into traditional medicine display curative properties [3]. Various studies have demonstrated a strong correlation between popular use and experimentally demonstrated pharmacological activity. Many plant extracts and essential oils have been shown to exert *in vitro* and *in vivo* activity, which justifies research on plants used in traditional medicine [4].

Plants produce a variety of compounds with antimicrobial properties, which have led to the development of new drugs for treatment of infectious diseases [5].

The family Compositae is one of the most species-rich among the flowering plants. *Anthemis* L. is the second-largest genus in this family with approximately 25 000 species, widely distributed in subtropical and temperate areas. Species of *Anthemis* are widely used in the pharmaceutical, cosmetic and food industries. *Anthemis tinctoria* L. is a perennial herb cultivated in Mediterranean countries, and several secondary metabolites have been identified in this species, such as volatile oils, triterpenes, polyacetylenes, and flavonoids [6]. In traditional medicine, this plant is used to treat liver problems and jaundice [7]. Its flowers have well-known antiseptic and medicinal properties, derived from flavonoids as well as es-

essential oils [8]. In Europe, extracts, dyes, teas, and ointments are used as anti-inflammatory, antibacterial, antispasmodic, and sedative agents [9]. The antimicrobial activity of essential oils and extracts from different species of *Anthemis* has been studied previously [10,11].

Trypanosoma cruzi is the etiologic agent of Chagas' disease, which in Brazil and several other Latin American countries constitutes a serious social and medical problem [12]. Transmission to vertebrates occurs through feces of hemipteran insects contaminated with metacyclic trypomastigotes, the infective stage of the parasite [13]. The acute phase of Chagas' disease is frequently asymptomatic, and the chronic phase usually develops 10 to 20 years after the infection, affecting about 10% to 30% of infected individuals [14].

In spite of the impressive progress in the understanding of the biology of *T. cruzi*, the drugs available (nifurtimox and benznidazole) are active on the acute stage of Chagas' disease, with about 80% effectiveness; but have limited utility against the established chronic disease [15]. The side effects of both compounds can be quite severe [16]. Therefore, trypanocidal drugs with less-serious side effects are necessary. In this context, plants are a reservoir of chemical and biological diversity that has led to the development of hundreds of pharmaceutical drugs [17]. The objective of the present study was to investigate the activity of the extracts, fractions, and a sesquiterpene-rich semi-purified subfraction, obtained from flowers of *A. tinctoria*, against *T. cruzi*.

2. Materials and Methods

2.1. General Experimental Procedures

The NMR spectra were obtained in VARIAN GEMINI 300 (7.05 T) spectrometers, using deuterated solvent, TMS as the internal standard and a constant temperature of 298 K. Sephadex LH-20; Silica gel 60 (70 - 230 and 230 - 400 mesh); TLC: silica gel plates F₂₅₄ (0.25 mm thickness).

2.2. Collections of the Plant

Flowers of *Anthemis tinctoria* were collected in November 2004 at the "Profª. Irenice Silva" Garden of Medicinal Plants of the State University of Maringá. The plant was identified through authentic comparison by Dr. Cirino Correia Júnior, and a voucher specimen (No. HUM 1133) is deposited at the Herbarium of the State University of Maringá, Paraná, Brazil.

2.3. Separation of the Components

Dried flowers of *A. tinctoria* (130 g) were extracted with ethanol:water (9:1 v/v) by maceration for 8 days at room

temperature. The solvent was removed in a rotating evaporator, to give an aqueous extract and a dark-green residue. The aqueous extract was lyophilised (21.0 g) and the water-insoluble residue was diluted with ethyl-acetate, yielding the ethyl-acetate extract (4.71 g). The aqueous and ethyl-acetate extracts were assayed against the epimastigote form of *T. cruzi*. The aqueous extract (13 g) - was submitted to vacuum-column chromatography (32 g silica gel) and eluted with hexane, dichloromethane, ethyl acetate, methanol, and methanol/water (9:1 v/v). Each fraction (hexane, F1; dichloromethane, F2; ethyl acetate, F3; methanol, F4; and methanol/water 9:1, F5) was assayed for antitrypanosomal activity. The dichloromethane fraction (500 µg), which showed the highest inhibitory activity, was chromatographed by column chromatography in Sephadex-LH-20 and eluted with chloroform/methanol (1:1 v/v). This process yielded 8 subfractions, denominated F2a, F2b, F2c, F2d, F2e, F2f, F2g, and F2h. Subfraction F2g (140 µg) was chromatographed in Sephadex phase with movable phase, chloroform/methanol (1:1 v/v), yielding the subfraction (11 µg). The subfraction was identified as a mixture of isomeric labdane sesquiterpenes by analyses of spectral data of ¹H and ¹³C (Chart 1).

2.4. Parasites

Epimastigote forms of *T. cruzi* Y strain were cultured in LIT (Liver Infusion Triptone broth) [18] with 10% fetal bovine serum (SFB) (Gibco Invitrogen Corporation, New York, USA) at 28°C for 96 h. Amastigote and trypomastigote forms were grown in monolayers of LLCMK₂ cells in DMEM medium (Gibco Invitrogen) with 10% SFB in 5% CO₂ at 37°C.

2.5. Antiproliferative Activity against Epimastigote Forms

The aqueous and ethyl-acetate crude extracts, fractions (hexane, dichloromethane, ethyl acetate, methanol, and methanol/water) and the semi-purified subfraction were dissolved in dimethyl sulphoxide (DMSO, Sigma Chemical Co., St. Louis, Missouri, USA) at a final concentration not exceeding 1% [19] and assayed against the epimastigote form of *T. cruzi*. The experiments were performed on 24-well polystyrene plates containing 1 ml of diluted compound at different concentrations (from 1.0 to 1,000 µg/ml). The starting inoculum consisted of 10⁶ parasites in logarithmic growth phase per well. The cells were incubated at 28°C and the growth was determined by counting the parasites with a Neubauer hemocytometer (Improved Double Neubauer Ruling) every 24 h over a 7-day period. Benznidazole (N-benzyl-2-nitro-1-imidazolacetamide, Roche Pharmaceuticals, Rio de Janeiro,

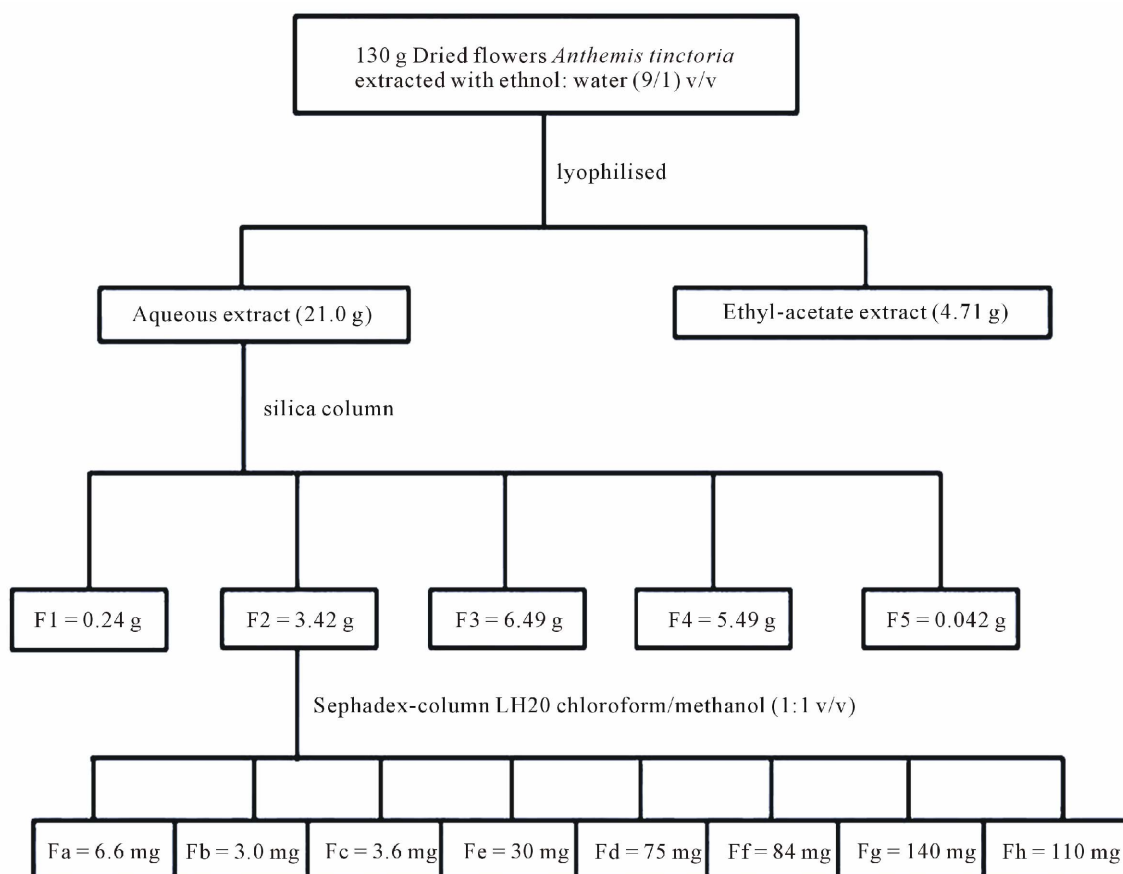


Chart 1. Fractionation scheme of sesquiterpene from aqueous extract of flowers of *Anthemis tinctoria*.

Brazil) was used as a reference drug. The assays were performed in duplicate on separate occasions.

2.6. Growth Inhibition Assay of Mammalian-Stage Amastigote Form of *T. cruzi*

LLCMK₂ cells (kidney cells from Mulatto monkey) were plated on glass coverslips (diameter 13 mm) into 24-multiwell tissue culture plates and maintained in a humidified 5% CO₂ atmosphere at 37°C. The cells were infected with trypomastigote forms at a parasite/cell ratio of 10:1. After 24 h, non-adhered parasites were washed out, and fresh DMEM medium, and the semi-purified subfraction containing sesquiterpene in various concentrations, was added. The cells were incubated in a humidified 5% CO₂ atmosphere at 37°C for 96 h. The coverslips were washed 3 times with PBS, fixed with methanol and stained with Giemsa. Next, the coverslips were mounted on glass slides using Entellan (Merck), and the percentage of LLCMK₂ cells with internalised parasites (1) and the number of internalised parasites per LLCMK₂ cell (2) were determined by counting at least

200 cells per sample under a microscope (Olympus CX 31). The product of 1 and 2 determined the survival index.

2.7. Cytotoxicity Activity

LLCMK₂ cells were seeded onto 24-well microtitre plates at a concentration of 2.0×10^5 cells/ml and allowed to proliferate for 48 h in DMEM containing 5% FBS at 37°C with 5% CO₂ to form a cell monolayer. Different concentrations of the semi-purified subfraction containing sesquiterpene were applied to the monolayer and incubated at 37°C with 5% CO₂ for 72 h. Next, the cells were treated with 10% trichloroacetic acid at 4°C for one hour, gently washed in tap water, and allowed to dry at room temperature. A solution of 0.4% sulforhodamine B (in 1% acetic acid) was added to each well, and the plate was kept protected from light for 30 min at 4°C. The wells were washed four times with 1% acetic acid, and 150 µl of 10 mM Tris-base was added and homogenised for 15 min. The absorbance was read at 530 nm in a microplate spectrophotometer (Bio Tek-Power Wave XS). The CC₅₀ (concentration that lysed 50% of cells) of the subfraction was calculated.

2.8. Evaluation of Morphological Alterations by Scanning Electron Microscopy

Epimastigote forms treated with IC₅₀ (0.2 µg/ml) or IC₉₀ (1.0 µg/ml) of the semi-purified sesquiterpene subfraction for 96 h were collected by centrifugation, washed in PBS and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, containing 1.0 mM CaCl₂ at 4°C. After fixation, small drops of the sample were placed on a specimen support with poly-L-lysine. Subsequently, the samples were dehydrated in graded ethanol, critical-point dried in CO₂, sputter-coated with gold and observed in a SHIMADZU SS-550 Scanning electron microscope.

2.9. Evaluation of the Ultrastructural Alterations in *T. cruzi* by Transmission Electron Microscopy

After treatment with IC₅₀ or IC₉₀ of the semi-purified sesquiterpene subfraction for 96 h, epimastigote forms were washed in 0.01 M phosphate-buffered saline and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The cells were postfixed in a solution containing 1% OsO₄ and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, dehydrated in acetone, and embedded in Epon. Thin sections were collected on a copper grid (300 mesh), stained with uranyl acetate and lead citrate, and observed in a Zeiss 900 transmission electron microscope.

2.10. Statistical Analysis

Statistical analysis was performed with the program GraphPad Prism 4 (GraphPad Software, San Diego, California, USA). One-Way Anova was applied, and a p-value less than 0.05 was regarded as significant.

3. Results and Discussion

In the attempt to develop new drugs for treatment of infectious diseases, studies are carried out with compounds of both natural and synthetic origin. Many studies have demonstrated that crude extracts, fractions, and compounds isolated from medicinal plants exhibit antiprotozoal activity [20-26]. In this study, we evaluated the antitrypanosomal activity of the crude extract, fractions, and a subfraction rich in a mixture of isomeric labdane sesquiterpenes, obtained from flowers of *A. tinctoria*, against epimastigote, and amastigote forms of *T. cruzi*.

The analyses of the ¹H and ¹³C NMR spectrum of the semi-purified subfraction showed a signal of a mixture of isomers of cross-conjugated terpenoid ketones derived from labdane sesquiterpenes that are frequently found in the family Asteraceae [27]. Three types of classes of

secondary metabolites have been detected in *Anthemis*: polyacetylenes [28], flavonoids [29], and sesquiterpene lactones [30]. Previous chemical studies of species of *Anthemis* have shown the presence of sesquiterpene lactones [31-37]. The three major types of sesquiterpene lactones are germacrolides, eudesmanolides, and guaianolides [38]. Recently it was demonstrated that anthecularin, a sesquiterpene lactone isolated from *Anthemis auriculata*, shows antimalarial activity, and also antitrypanosomal activity against *Trypanosoma brucei* [39].

The hydroalcoholic crude extracts, fractions, and semi-purified subfraction obtained from *A. tinctoria* flowers were used in order to investigate the antiprotozoal activity of this plant against *T. cruzi*. A progressive increase in the antitrypanosomal effect was observed in the course of the purification process. **Figure 1(a)** shows the percentage of growth inhibition of the epimastigote form treated with the aqueous phase of the crude extract for 96 h of incubation at 28°C. This extract showed a dose-dependent inhibitory activity of 77.4% and 91.9% at 5 and 10 µg/ml, respectively. In the same concentrations, the ethyl-acetate extract showed an inhibitory activity of 31.7% and 76.8%. The 50% inhibitory concentration (IC₅₀) of the crude extract aqueous phase was 2.3 µg/ml. On the basis of this finding, the crude aqueous extract was fractionated on silica gel into five fractions: hexane (F1), dichloromethane (F2), ethyl-acetate (F3), methanol (F4), and methanol:water (F5). The F2 fraction showed an IC₅₀ of 1.8 µg/ml (**Figure 1(b)**), and the F3 fraction an IC₅₀ of 5.0 µg/ml (data not shown). The other fractions (F1, F4, and F5) showed lower inhibitory activity, with IC₅₀ of 26.8, 23.6, and 145 µg/ml, respectively. Subsequently, the F2 fraction was submitted to the Sephadex column, yielding 8 subfractions: F2a, F2b, F2c, F2d, F2e, F2f, F2g, and F2h. **Figure 1(c)** illustrates the inhibitory activity of the F2g subfraction, with a percentage of inhibition of growth of the epimastigote form above 90% for all concentrations used (1 - 1000 µg/ml). The F2g subfraction, which was rich in a mixture of isomeric labdane sesquiterpenes, showed an IC₅₀ at 0.2 µg/ml. In comparison, Luize *et al.* [40] investigated the *in vitro* antiproliferative effect of eupomatenoid-5, a sesquiterpene isolated from leaves of *Piper regnellii* var. *pallecens*, a plant of the same family (Compositae) as *A. tinctoria*, against *T. cruzi*. Our results obtained with a mixture of sesquiterpenes also showed that this mixture was more active than Benzimidazole.

The effect of the semi-purified subfraction obtained from flowers of *A. tinctoria* on the interaction of *T. cruzi* with LLCMK₂ cells was evaluated. The treatment of LLCMK₂ cells infected with the amastigote form showed that the semi-purified subfraction had a dose-dependent

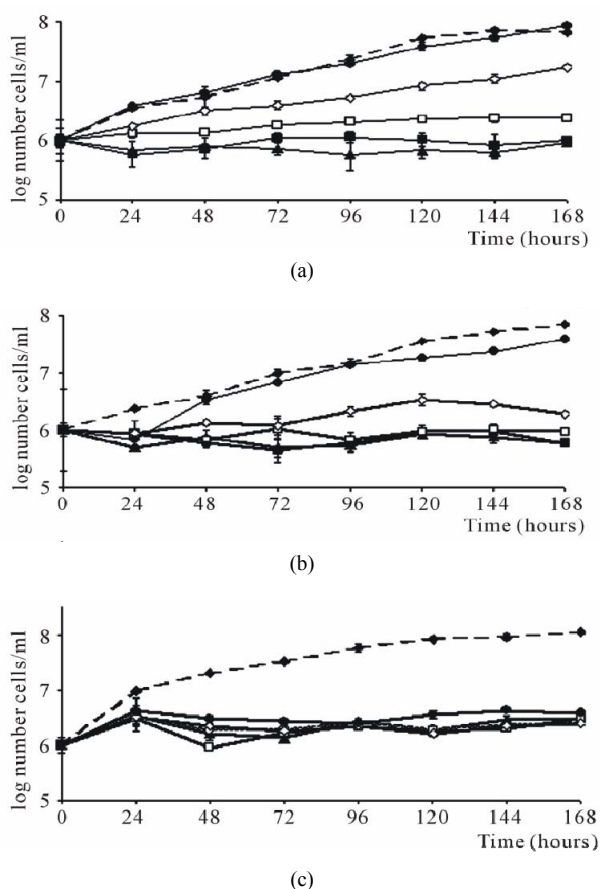


Figure 1. Effects of crude extract aqueous phase (a), dichloromethane fraction (b) and semi-purified subfraction (c) obtained from flowers of *Anthemis tinctoria*, on the growth of *Trypanosoma cruzi* epimastigote form. ♦ Control; ■ 1000 µg/ml; ▲ 100 µg/ml; ○ 50 µg/ml; □ 10 µg/ml; ◇ 5 µg/ml; ● 1 µg/ml.

antitrypanosomal activity (Figure 2), leading to considerable reduction in both the percentage of infected cells and the mean number of parasites per infected cell. After 96 h of incubation, the percentage of LLCMK₂ cells with internalised parasites was higher for the control than for cells treated with the semi-purified subfraction. At that time, the control showed a mean of 34.1 amastigotes per cell, with 40.6% of cells infected. Cells treated with 1.0 µg/ml showed a mean of 18.1 internalised amastigotes per cell, with 22.7% of cells infected. Treatment of the cells with 5.0 µg/ml resulted in only 4.7% infected cells and 4.2 parasites per cell.

Important criteria for the investigation of compounds with activity against *T. cruzi* are both their therapeutic potential, and the lack of a cytotoxic effect on mammalian cells. We evaluated the cytotoxicity in LLCMK₂ cells treated with the crude extract aqueous phase, di-

chloromethane fraction, and the semi-purified subfraction (Figure 3). The 50% cytotoxicity concentration (CC₅₀) in LLCMK₂ cells treated with the crude extract aqueous phase, dichloromethane fraction, and semi-purified subfraction were 17.3 µg/ml, 4.0 µg/ml, and 7.0 µg/ml, respectively. The best selectivity index (SI) ratio (CC₅₀ for LLCMK₂ cells/IC₅₀ for antiprotozoa) was obtained by the semi-purified subfraction, with SI of 35.0. The dichloromethane fraction and crude extract showed SI of 2.2 and 7.5, respectively (Table 1).

Morphological alterations in epimastigote forms treated

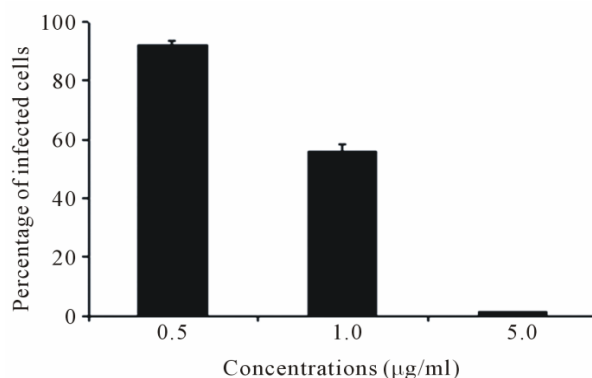


Figure 2. Growth inhibition assay of mammalian-stage amastigote form of *Trypanosoma cruzi* treated with a semi-purified subfraction obtained from *Anthemis tinctoria*.

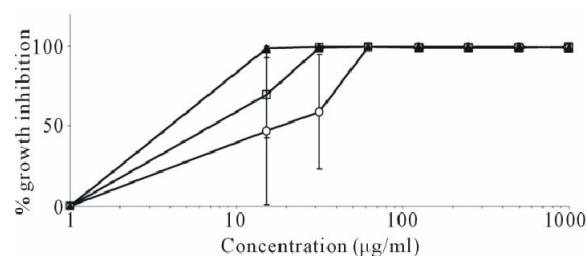


Figure 3. Cytotoxicity activity on LLCMK₂ cells. (○) aqueous extract, (▲) dichloromethane fraction; (□) semi-purified subfraction obtained from flowers of *Anthemis tinctoria*.

Table 1. Cytotoxicity, growth inhibition and selectivity index of the crude extract aqueous phase, dichloromethane fraction, and semi-purified subfraction obtained of *Anthemis tinctoria*.

Drugs	CC ₅₀	IC ₅₀	IS
	(µg/ml)	(µg/ml)	
Crude extract aqueous phase	17.3	2.3	7.5
Dichloromethane fraction	4.0	1.8	2.2
Semi-purified subfraction	7.0	0.2	35.0

IS = CC₅₀/IC₅₀

with the semi-purified subfraction were observed by scanning electron microscopy. Epimastigote forms in the presence of the semi-purified subfraction at IC_{50} (0.2 $\mu\text{g/ml}$) showed distortions of the parasite cell body, and the cell shape was severely affected. Increase of the cell volume and rounding of the cell body were observed (Figures 4(b), (c), and (d)). At IC_{90} (1.0 $\mu\text{g/ml}$), alterations were more evident (Figures 4 (e) and (f)). Figure 4(a) shows the characteristic elongated shape of an untreated protozoan, or treated only with 1% DMSO (control), with a terminal flagellum that emerges from the flagellar pocket and remains tightly attached to the cell body along its length, typical of the epimastigote form.

Ultrastructural alterations of the epimastigote form treated with the semi-purified subfraction were observed by transmission electron microscopy. Untreated *T. cruzi* or epimastigotes treated with 1% DMSO showed the typical ultrastructure (Figure 5(a)). Epimastigotes treated with the semi-purified sesquiterpene-rich subfraction at concentrations of IC_{50} (Figures 5(b), (c), and (d)) and IC_{90} (Figures 5(e) and (f)) showed ultrastructural alterations. One important change took place in the membrane lining the cell body and flagellum, with portions of the membrane detached from the body, forming blebs at the membrane flagellar (Figures 5(b) and (d)). The formation of blebs was also observed in *T. cruzi* treated with com-

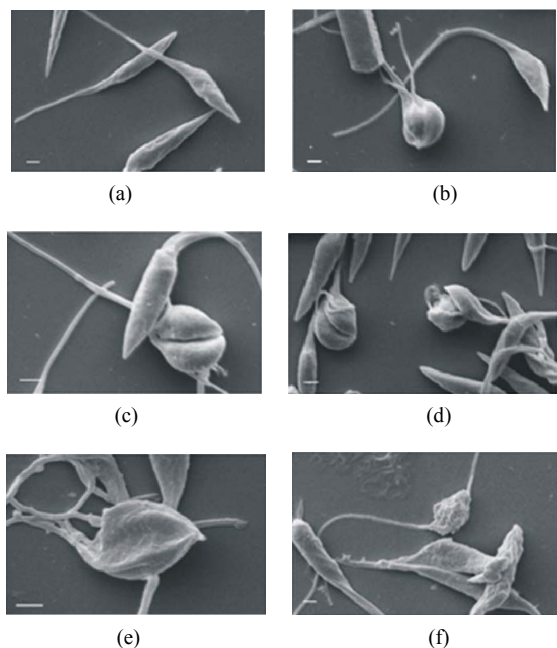


Figure 4. Morphological alterations of *Trypanosoma cruzi* epimastigote form cultured at 28°C for 96 h, observed by scanning electron microscopy, in the absence (a) and presence of the semi-purified subfraction at concentrations of IC_{50} (b), (c), and (d) and IC_{90} (e) and (f). Bar = 1 μm .

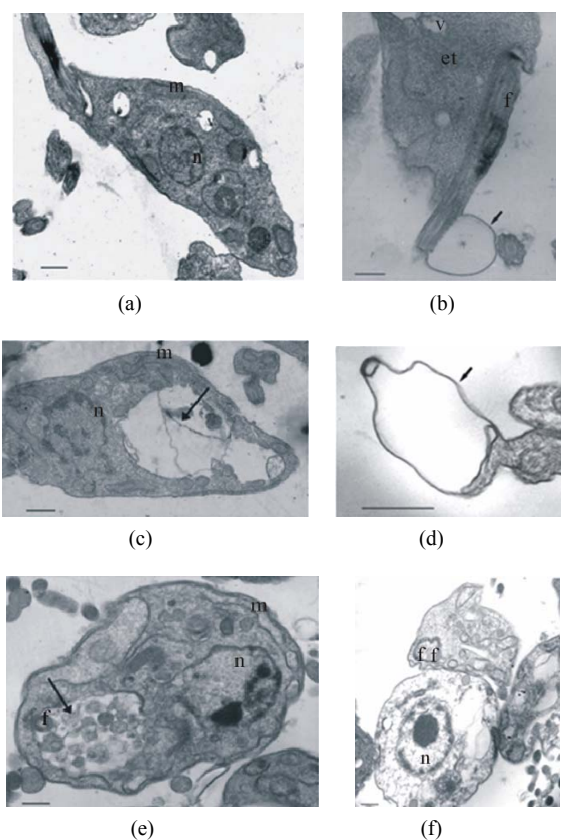


Figure 5. Ultrastructural alterations of *Trypanosoma cruzi* epimastigote forms cultured at 28°C for 96 h, observed by transmission electron microscopy, in the absence (a) and presence of semi-purified subfraction at concentrations of IC_{50} (b), (c) and (d) and IC_{90} (e) and (f) er-endoplasmic reticulum; f-flagellum; m-mitochondrion; n-nucleus; v-vacuole. Bar = 1 μm .

pounds ER27856 and BPQ-OH (3-biphenyl-4yl)-3-hydroxy quinuclidine BPQ-OH) [41]. These compounds are inhibitors of squalene synthase, the enzyme involved in the biosynthesis of ergosterol. The change in the chemical composition of the membrane alters the ratio of phospholipids and sterols, affecting the stability of the membranes, and especially the integrity of the cell body [42].

This may explain the morphological changes observed in epimastigotes treated with the sesquiterpene-rich subfraction of the flower of *A. tinctoria*. Pedrosa *et al.* [43] also showed the presence of blebs in cell and flagellar-pocket membranes in *Crithidia deanei* treated with essential oil from *Cymbopogon citratus*.

4. Conclusions

In conclusion, this study showed that extracts, fractions, and a semi-purified subfraction containing sesquiterpene obtained from flowers of *A. tinctoria* have potential anti-

proliferative activity *in vitro* against *T. cruzi*. Therefore, natural products may be a source of new drugs with anti-protozoal activity that could be used to treat parasitic infectious diseases such as Chagas' disease.

5. Acknowledgements

This study was supported through grants from DECIT /SCTIE/MS and MCT by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Programa de Núcleos de Excelência (PRONEX/Fundação Araucária), and Programa de Pós-graduação em Ciências Farmacêuticas da Universidade Estadual de Maringá.

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