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ORIGINAL PAPER

Croton cajucara crude extract and isolated terpenes: activity on *Trypanosoma cruzi*

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Abstract *Croton cajucara* is a plant found in the Amazon region and is known for its medicinal properties. The effects of the methanolic extract of the stem bark of *C. cajucara* (MCC) and of the isolated terpenes, *trans*-dehydrocrotonin (*t*-DCTN) and acetyl aleuritolic acid (AAA), were investigated using four isolates of *Trypanosoma cruzi*. In assays with trypomastigotes, the extract was more active than the isolated compounds, presenting IC_{50} in the range of 10 to 50 μg/mL. The trypanocidal effect of MCC, AAA and benznidazole was significantly higher in the GLT291 and C45 strains, which were recently isolated from wild animals. MCC and AAA caused a dose-dependent inhibition of epimastigote proliferation. In assays using intracellular amastigotes, AAA and MCC reduced the percent of infection and the endocytic index after 96 h of treatment, at concentrations that were non-toxic to the host cells. MCC inhibited the trypanothione reductase pathway in both epimastigotes and trypomastigotes of all the subpopulations. The absence of AAA activity on the trypanothione reductase pathway in epimastigotes of Dm28c suggests heterogeneity of the biochemical profile between this clone and the three strains. Epimastigotes and trypomastigotes (GLT291) were treated for 24 h with MCC or AAA, and both induced alterations of the plasma membrane, while AAA-treated epimastigotes also displayed mitochondrial damage.

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Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, represents an important health problem in Latin America. The major route of transmission for this disease is through triatomine vector-associated infections followed by blood transfusion and congenital routes of infection (Carlier 2007). Less common modes of transmission also include laboratory accidents (Herwaldt 2001), organ transplantation (Atclas et al. 2008) and the ingestion of infected food or contaminated insects (Steindel et al. 2008). Recently, Chagas disease has also been recognised as an opportunistic disease in HIV-infected individuals (Vaidian et al. 2004) and is now being reported throughout the world due to international immigration (Schmunis 2007). Introduced in the 1960s and 1970s, the nitroderivatives, nifurtimox and benznidazole are the most commonly used drugs for the treatment of Chagas disease. While these drugs are effective for acute infections, the data regarding their use and efficacy during the chronic phase are still controversial.

This controversy is primarily due to the undesirable side effects that frequently force the abandonment of treatment and poor indices of apparent cure from the disease (Soeiro and De Castro 2009).

In the last decades, there has been a growing interest in the therapeutic use of natural products for the treatment of Chagas disease (Coura and De Castro 2002). Our laboratory has been involved in the investigation of possible trypanocidal effects of natural products such as propolis (Salomão et al. 2004, 2009) and vegetal extracts (Menna-Barreto et al. 2008). In this context, terpenes are a widely studied class of compounds and are found in several species of plants, mediating antagonistic and beneficial interactions among organisms (Uchiyama et al. 2005; Leite et al. 2006). *Croton cajucara* Benth, locally called "sacaca", is a plant found in the Amazon region. This plant has a history of safe use in folk medicine in the form of a tea for ailments including diarrhoea, diabetes, gastrointestinal disorders and inflammation of the liver (Di Stasi et al. 1989; Hiruma-Lima et al. 1999; Maciel et al. 2000). The sub-chronic treatment of mice with a methanol extract derived from *C. cajucara* caused no mutagenicity in somatic or germ cells (Santos et al. 2006).

Phytochemical analysis of bark extracts from *C. cajucara* revealed the presence of several clerodane-type diterpenes such as *trans*-dehydrocrotonin, *trans*-crotonin and others, as well as the triterpene acetyl aleuritolic acid (Maciel et al. 2000, 2003a, b, 2007). The compound 19-*nor*-clerodane *trans*-dehydrocrotonin (*t*-DCTN) is the major component of bark extracts from adult *C. cajucara* (Maciel et al. 1998, 2003a, b) and has been directly correlated with the therapeutic effects popularly attributed to this plant. Pharmacological studies examining *t*-DCTN have shown that the compound possesses anti-inflammatory, analgesic, antitumor, antiulcer, hypoglycaemic, hypolipidemic and antioxidant effects (Carvalho et al. 1996; Souza-Brito et al. 1998; Grynberg et al. 1999; Silva et al. 2001; Tieppo et al. 2006; Perazzo et al. 2007). In addition, *t*-DCTN has also been shown to have antimutagenic activity (Âgner et al. 1999) and did not induce clastogenic, anticlastogenic, apoptotic and cytotoxic activities (Poersch et al. 2007), suggesting that the consumption of this phytotherapeutic agent by the population may be safe and hence may serve as a stimulus to the pharmaceutical industry for the development of *C. cajucara*-derived products. The pentacyclic triterpene acetyl aleuritolic acid (AAA), which is the major component of the stem bark from 18-month-old plants, displays antitumoral, antiulcerogenic, antispasmodic, anti-inflammatory and antinociceptive activities (Maciel et al. 2000; Perazzo et al. 2007). AAA was also described as a bactericidal and antifilarial agent (Peres et al. 1997; Nyasse et al. 2006) at concentrations that were shown to be non-toxic to mammalian cells (Mathabe et al. 2008).

In the present study, we aimed at evaluating the trypanocidal activity of the crude extract and isolated terpenes from *C. cajucara* using several different *T. cruzi* populations.

Materials and methods

Crude extract and isolation of terpenes Plant material was collected in Jacundá, State of Pará (Amazon region of Brazil), and identified by Nelson A. Rosa. A voucher specimen (no. 247) has been deposited in the Herbarium of the Museu Paraense Emílio Goeldi (Belém, Brazil). Extraction and isolation of compounds were carried out as previously described (Maciel et al. 1998, 2003a, b). Briefly, powdered stem bark was extracted with hexane for 24 h and with methanol for another 24-h period. After evaporation of the solvent, the hexane extract was filtered through a silica gel column, which gave three fractions: the first eluted with hexane (fraction A), the second with dichloromethane (fraction B) and the third with methanol (fraction C). Fractions B and C were also eluted in silica gel using mixtures of hexane–dichloromethane–methanol of increasing polarity giving *t*-DCTN and AAA (Fig. 1), in addition to other components. The methanol extract (MCC) was also submitted to column chromatography and elution with mixtures of hexane–ethyl acetate of increasing polarity, giving *t*-DCTN and AAA. The purity of *t*-DCTN and AAA was confirmed by spectroscopic methods using samples from the isolated terpenoids.

Stock solutions were prepared in dimethylsulfoxide (Merck, Darmstadt, Germany) at concentrations of 100 mg/mL for MCC, 15.7 mg/mL (50 mM) for *t*-DCTN and 24.9 mg/mL (50 mM) for AAA.

Parasites Strains of *T. cruzi* genotype TCI (Zingales et al. 2009), isolated from wild animals with natural infection, were maintained by cryopreservation at the Laboratório de Biologia de Tripanosomatídeos (IOC), being used were C45 (TCI) from *Philander frenatus* (Pinho et al. 2000) and GLT291 (TCI) from *Leontopithecus rosalia* (Lisboa et al.

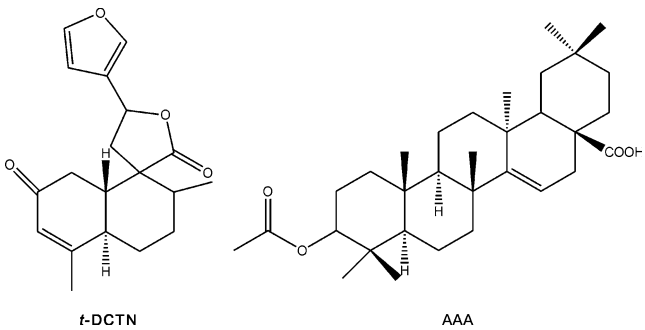

Fig. 1 Chemical structures of the terpenes isolated from *C. cajucara*: *t*-DCTN and AAA

2000). The Y strain (Silva and Nussenzweig 1953) and the Dm28c clone (Contreras et al. 1985) were used as reference. Epimastigote forms were maintained in LIT medium supplemented with 10% foetal calf serum (FCS). Vero cells were infected with metacyclic trypomastigotes from 15-day-old epimastigote cultures (Contreras et al. 1985). After 6–7 days, the supernatant containing released trypomastigotes was used to infect new Vero cultures (1×10^6 parasites/25 cm^2), which were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% FCS and 2% L-glutamine (RPMIS).

Effect on epimastigote proliferation Five-day-old cultures of epimastigotes were collected, centrifuged at 165×*g* for 1 min (Excelsa 2, Fanen, Brazil) (De Castro et al. 1992) and re-suspended in LIT to a concentration of 1×10^7 parasites/mL. This suspension (500 μL) was added to the same volume of the MCC or AAA solutions, previously prepared in LIT at twice the desired respective concentration. The bioassays were performed in 24-well plates incubated at 28°C for 4 days. The solvent final concentration never exceeded 3%, which has no deleterious effect on the parasites. Cell counting was carried out daily using a Neubauer chamber to determine the IC_{50} values, corresponding to the concentration that would cause 50% inhibition of parasite proliferation. Untreated and benznidazole-treated (25 to 500 μg/mL) parasites were used as controls.

Effect on trypomastigote lysis The supernatant of 5-day-old cultures of infected Vero cells was collected, centrifuged at 2,054×*g* for 15 min and the pellet re-suspended in RPMIS (Silva et al. 2007) to a trypomastigote concentration of 1×10^7 parasites/mL. This suspension (100 μL) was added to the same volume of MCC, AAA or *t*-DCTN, previously prepared at twice the desired respective concentration in the same medium. The bioassays were performed in 96-well plates incubated at 37°C in an atmosphere of 5% CO_2 and air for 24 h. Cell counting was carried out using a Neubauer chamber, and the IC_{50}/24 h, corresponding to the concentration that leads to 50% parasite lysis, was calculated. Untreated and benznidazole-treated parasites (0.2 to 80 μg/mL) were used as controls.

Effect on infected macrophages Peritoneal macrophages were obtained from Swiss mice, plated in 24-well plates (3×10^5 cells per well) for 24 h and then infected with trypomastigotes (4:1 parasite/host cell) in RPMIS (De Castro et al. 1992). After 1 h of incubation, the cultures were washed to remove non-internalised parasites and treated with MCC, AAA or *t*-DCTN prepared in serial dilutions of RPMIS. Fresh medium (with or without the extract or the terpenes) was replaced every 24 h. At specified intervals, the cultures were fixed in Bouin's solution, stained with Giemsa (Merck Darmstadt, Germany) and counted, using the parameters of percent of infection and the endocytic index (EI; number of parasites/100 cells; Silva et al. 2007). The IC_{50} values of each parameter were calculated. To determine the possible toxic effects of the extract and the terpenes on the mammalian cells, uninfected macrophages were incubated at 37°C with the compounds, and cell viability was measured by the MTT colorimetric assay (Mossman 1983). The absorbance was measured at 490 nm with a spectrophotometer (VERSAmax Tunable, Molecular Devices, USA), and the LC_{50} parameter, corresponding to the concentration that leads to 50% of viability loss, was measured.

Trypanothione reductase activity Epimastigotes and trypomastigotes (2×10^9 parasites) were re-suspended in 40 mM HEPES buffer containing 1 mM EDTA and lysed in a cavitation pump at 1,800 psi for 45 min (Y; Dm28c) and 2,000 psi for 2 h (C45; GLT291) and centrifuged at 12,500×*g* for 15 min (Sorvall Biofuge Stratos). The resulting supernatant, the soluble fraction (SF), was treated with the extract or the terpenes for 5 min at 37°C. The SF protein content was determined as previously described (Johnstone and Thorpe 1982). Trypanothione reductase activity was evaluated spectrophotometrically by measuring NADPH consumption at 340 nm (Girault et al. 2001). The assay mixture contained 40 mM HEPES, pH 7.5, 1 mM EDTA, 100 μM NADPH, SF (1 mg/mL protein) plus MCC or AAA. NADPH consumption was followed for 5 min at 37°C. Controls were run without NADPH and in the presence of an excess (500 μM) of oxidised glutathione as previously described (Krauth-Siegel and Schöneck 1995; Castro-Pinto et al. 2004).

Ultrastructural analysis Epimastigotes and trypomastigotes (5×10^6 cells/mL) were treated for 24 h with MCC or AAA, at concentrations corresponding to their respective IC_{50}/24-h values. Treated and control parasites were centrifuged (165×*g* for 10 min) and washed three times in PBS. For scanning electron microscopy, the parasites were adhered to poly-L-lysine-coated cover slips, fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature for 40 min and post-fixed with a solution of 1% OsO_4, 0.8% potassium ferricyanide and 2.5 mM $CaCl_2$ in the same buffer for 30 min. The cells were dehydrated in an ascending acetone series and dried via the critical point method with CO_2 (CPD 030, Balzers, Switzerland). The samples were mounted with silver sellotape on aluminium stubs that were coated with a 20-nm-thick gold layer and examined using a 940 DSM Zeiss microscope (Oberkochen, Germany). For transmission electron microscopy (TEM), after washing in PBS the parasites were fixed, post-fixed and dehydrated as described above and embedded in epoxy resin. Ultrathin sections (Leica Ultracuts, UCT, Vienna, Austria) were stained with uranyl acetate and

lead citrate and then examined using an EM10C Zeiss microscope (Oberkochen, Germany).

Statistical analysis The Mann–Whitney test was used for comparison of trypanocidal activity among the different experimental groups. Statistical significance for trypanothione reductase activity was determined using the Student's *t* test. Differences were considered to be significant when $p<0.05$.

Results and discussion

The genus *Croton* is comprised of approximately 900 species, with many representatives in the flora of the northern and northeastern regions of Brazil. In the Amazon region, *C. cajucara* represents a well-known resource for the empirical treatment of several illnesses, stimulating the scientific investigation of its potential medicinal properties (Maciel et al. 2000). Due to its resource potential, there has also been a popular mobilisation for preservation, with maintenance of the local biodiversity. The chemical characterisation of *C. cajucara* stem bark extract identified *t*-DCTN and AAA, and also of other terpenes such as *trans*-crotonin, *cis*-cajucarin B, *trans*-cajucarin B, cajucarin A, cajucarinolide, isosacacarin, α-copaene and ciperene, phenolic compounds, isoquinolinic alkaloids magnoflorine and *N*,*N*-dimethyl-lindicarpine (Maciel et al. 1998, 2003a, b, 2007) in addition to the non-polar compounds 2-methyloctadecanoic, octadecanoic and nonanedioic acids (Carvalhaes et al. 1999).

The present study reports, for the first time, the activity of *C. cajucara* extracts (MCC), *t*-DCTN and AAA against *T. cruzi*. We examined parasite subpopulations of two genotypes: TCI, comprising clone Dm28c and the strains GLT291 and C45 from wild reservoirs, and TCII, Y strain. The GLT291 and C45 strains were recently isolated and being less submitted to the selective process induced by successive passages in laboratory, preserving their original characteristics (Veloso et al. 2005).

In assays using cell culture-derived trypomastigotes, the comparison of the 1-day IC_{50} values illustrated that the MCC extract (10.7 to 49.4 μg/mL) was more active ($p<0.05$) than both terpenes (78.1 to 598.6 μg/mL; Fig. 2, Table 1). The

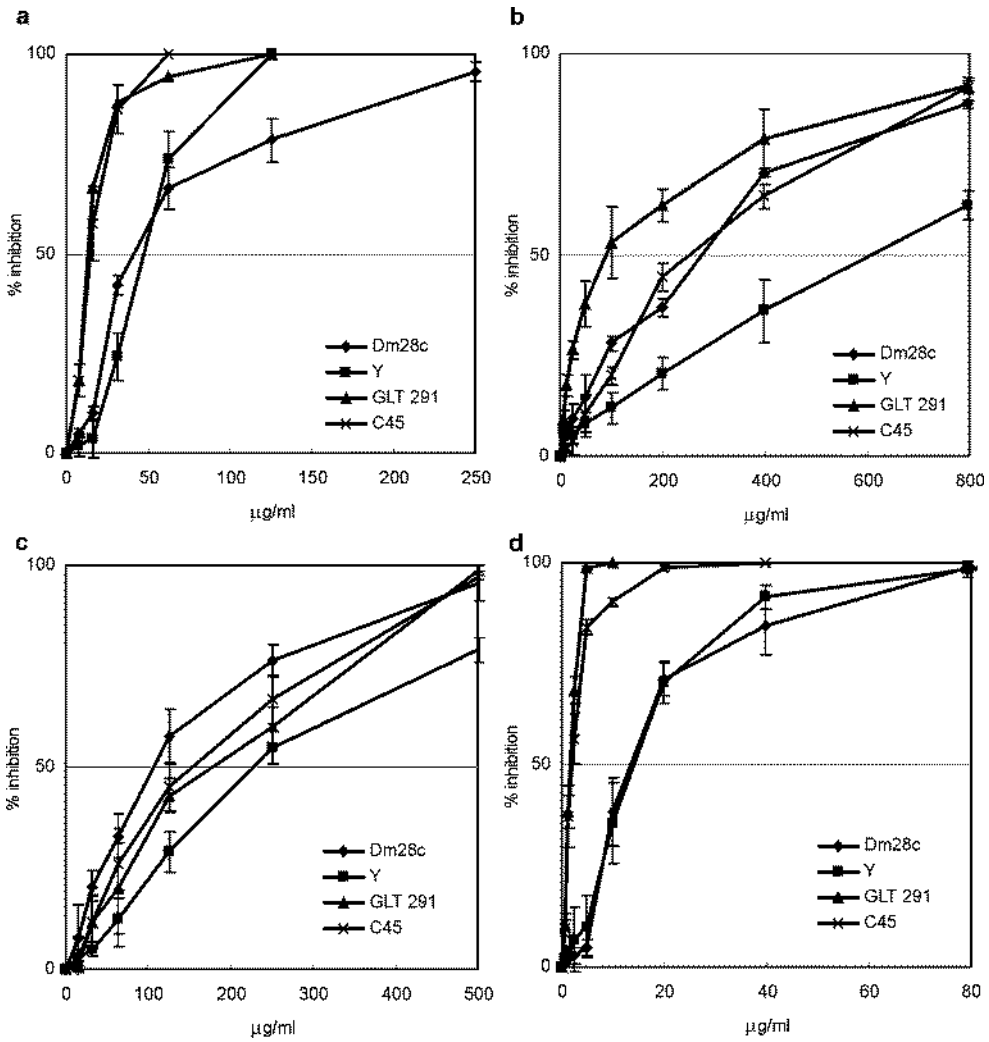

Fig. 2 Lysis of trypomastigotes of *T. cruzi* by **a** MCC, **b** AAA, **c** *t*-DCTN, and **d** benznidazole

Table 1 IC_{50} of the trypanosomicidal effect on trypomastigotes of *T. cruzi* after 1 day of treatment

	IC_{50}/1day[a] (μg/mL)			
	MCC	AAA	*t*-DCTN	Benznidazole
Dm28C	41.2±2.7	274.5±11.0	109.5±7.5[b]	13.2±0.9
Y	49.4±5.6	598.6±80.9	187.2±18.1	15.1±1.6
GLT291	10.7±1.7[b]	78.1±9.2[b]	181.9±14.5	1.7±0.2[b]
C45	11.7±2.4[b]	244.9±28.6[b]	144.4±12.4	2.3±0.2[b]

MCC methanolic extract of the stem bark of *C. cajucara*, *AAA* acetyl aleuritolic acid, *t*-DCTN *trans*-dehydrocrotonin

[a] Mean±SD of at least three independent experiments

[b] Indicates higher trypanocidal activity ($p<0.05$) when comparing the four *T. cruzi* isolates for the extract or the compounds analysed

effects of MCC and AAA were significantly higher on GLT291 and C45, while for *t*-DCTN, the highest activity was observed using the clone Dm28c (Table 1). For trypomastigotes, the Y strain was the most resistant to MCC, AAA and *t*-DCTN. Benznidazole was used as a positive control, and the 1-day IC_{50} values were 13.2±0.9, 15.1±1.6, 1.7±0.2 and 2.3±0.2 μg/mL, for Dm28c, Y, GLT291 and C45, respectively. The data regarding the in vitro susceptibility to the standard drug of the C45 and GLT291 strains are relevant from an epidemiologic perspective, considering the possibility of transmission to humans. Although the wild strains are from the same genotype as Dm28c, they were tenfold more susceptible to the standard drug than the clone. Heterogeneity within the TCI genotype has previously been demonstrated in relation to genetic characteristics (Herrera et al. 2007; Brito et al. 2008) and to the biological behaviour (Garzon et al. 2005; Lisboa et al. 2007). However, further studies need to be conducted to address this issue because in vivo resistance of TCI strains to benznidazole has been reported (Andrade et al. 1985; Toledo et al. 2003) and could have a great impact on the outcome of Chagas disease treatment.

Table 2 illustrates the IC_{50} values for the respective compounds effects after 4 days on the proliferation of epimastigotes and intracellular amastigotes and on the percent of macrophage infection. For epimastigotes, the comparison of the IC_{50} values showed that MCC was more active than AAA ($p<0.05$), as was also observed in the experiments with trypomastigotes (Table 1). This result could be explained by the complexity and/or synergism between different components of the extract. MCC is rich in AAA and *t*-DCTN but also contains other classes of components (sesquiterpenes, terpenes, isoquinolinic alkaloids, phenolic compounds and fatty acids) (Maciel et al. 2000, 2003a, b, 2007; Souza et al. 2006). Another possibility for the higher activity seen in MCC is its polarity (Maciel et al. 1998), a characteristic that was associated with the trypanocidal activity of other vegetal extracts (Campos et al. 2005; Grael et al. 2005). Interestingly, it was observed for the replicative forms, i.e. epimastigotes and intracellular amastigotes, that the Dm28c clone was more susceptible to AAA ($p<0.05$) and less susceptible to MCC ($p<0.05$) when compared to the other strains (Table 2). It was also shown that intracellular amastigotes were more susceptible than epimastigotes to the extract or terpenes. Differences in drug susceptibilities between different forms of *T. cruzi* have already been reported (Schlemper et al. 1977; De Castro et al. 1992; Salomão et al. 2009) and are likely related to differences in stage-specific metabolism.

The treatment of infected cultures revealed that MCC and the terpenes reduced both percent of infection and the EI after 4 days of treatment, in concentrations that were non-toxic for the host cells. The LC_{50} value for macrophages when examining MCC and t-DCTN was higher than 100 μg/mL and for AAA the values were higher than 50 μg/mL. It was previously reported that MCC does not contain toxic diterpene phorbol esters, present in other *Croton* species (Weber and Hecker 1978). It was also reported that the plant does not induce mutagenicity in somatic and germ cells of experimental mice (Santos et al. 2006). Additionally, *t*-DCTN in a murine model displayed no genotoxic or cytotoxic effects (Âgner et al. 1999), and AAA showed no in vitro toxicity to mammalian cells (Mathabe et al. 2008). Due to the small amount of *t*-DCTN available, this terpene was evaluated only on the Dm28c clone, the most susceptible in assays with trypomastigotes. These results showed IC_{50} 4-day values of 75.8±2.3 μg/mL for the percent infection and 17.5±1.8 μg/mL for EI (Fig. 3). Furthermore, different from the results obtained in experiments of their direct effect on both epimastigotes and

Table 2 IC_{50} (microgram per millilitre) of the effect on the proliferation of epimastigotes and intracellular amastigotes of *T. cruzi* and on infected macrophages after 4 days of treatment

	Dm28c	Y	GLT291	C45
Epimastigotes				
MCC	166.6±16.1[a]	109.1±11.5	103.5±8.8	119.2±31.6
AAA	341.9±15.4[a]	846.5±110.8	773.9±94.9	694.7±74.7
Intracellular amastigotes[b]				
MCC	69.7±3.3[a]	19.5±9.5	19.5±2.6	25.8±5.8
AAA	4.6±0.1[a]	20.0±6.0	33.7±4.2	28.7±8.5
% Infection				
MCC	82.9±5.2	24.4±9.2	34.0±9.0	32.5±1.5
AAA	24.4±4.9[a]	40.5±1.6	28.5±5.0	37.1±6.7

MCC methanolic extract of the stem bark of *C. cajucara*, *AAA* acetyl aleuritolic acid

[a] Indicates statistical differences ($p<0.05$) between Dm28c and the other three subpopulations, being the clone less susceptible to MCC and more susceptible to AAA

[b] IC_{50} values based on the endocytic index

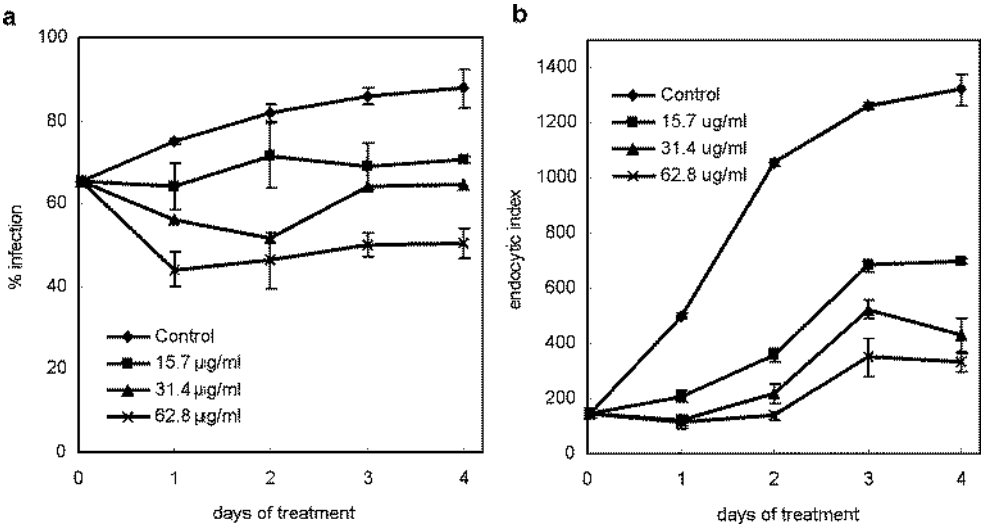

Fig. 3 Effect of *t*-DCTN on peritoneal macrophages infected with the clone Dm28c of *T. cruzi*: **a** percent of infection, **b** endocytic index. The cultures were infected with a 10:1 ratio (parasite/host cell), and after 1 h of incubation, the terpene was added as described in the "Materials and Methods" section

trypomastigotes of the clone Dm28c, AAA was more active than MCC and *t*-DCTN in assays of infected macrophages.

While AAA was less active than MCC on both the Dm28c clone epimastigotes and trypomastigotes in experiments with infected macrophages, the terpene was 15-fold more active on intracellular parasites after 4 days of treatment. AAA was also more active than MCC and *t*-DCTN against intracellular amastigotes in assays examining all other *T. cruzi* strains. This fact could be related to the higher liposolubility of this pentacyclic triterpene, leading to a higher ability of crossing the host cell membrane. The trypanocidal activity of triterpenes has previously been investigated (Abe et al. 2002; Duarte et al. 2002; Rosas et al. 2007), and structure activity relationship studies revealed that the presence of the carboxyl group at C-17 is required for activity against *T. cruzi* (Cunha et al. 2006; Leite et al. 2006).

Compounds from natural sources have been investigated as trypanothione reductase selective inhibitors in several previous studies (Bond et al. 1999; Gallo et al. 2008). Trypanosomatids possess a unique thiol metabolism based on trypanothione, which plays a crucial role in regulating the redox balance and also in the defence against oxidative stress in a pathway not shared by the human host (reviewed in Müller et al. 2003). In the present study, we investigated the effect of MCC and AAA on the trypanothione reductase pathway of *T. cruzi*. For epimastigotes, while MCC was able to inhibit the enzyme in all of the subpopulations, AAA was effective on Y, GLT291 and C45, but not on Dm28c (Fig. 4a). This result could be related to a greater thiol concentration in the soluble fraction of the specific clone, indicating it may need a higher terpene concentration for an effect similar to that displayed by the other strains. For trypomastigotes, both MCC and AAA significantly inhibited enzyme activity ($p<0.01$) in all of the strains when compared to the untreated control (Fig. 4b), and such an effect was more pronounced than that observed in epimastigotes. In fact, it was previously shown that the thiol content varies considerably between different subpopulations (Repetto et al. 1996) and that it is generally higher in epimastigotes than in the cell culture-derived trypomastigote

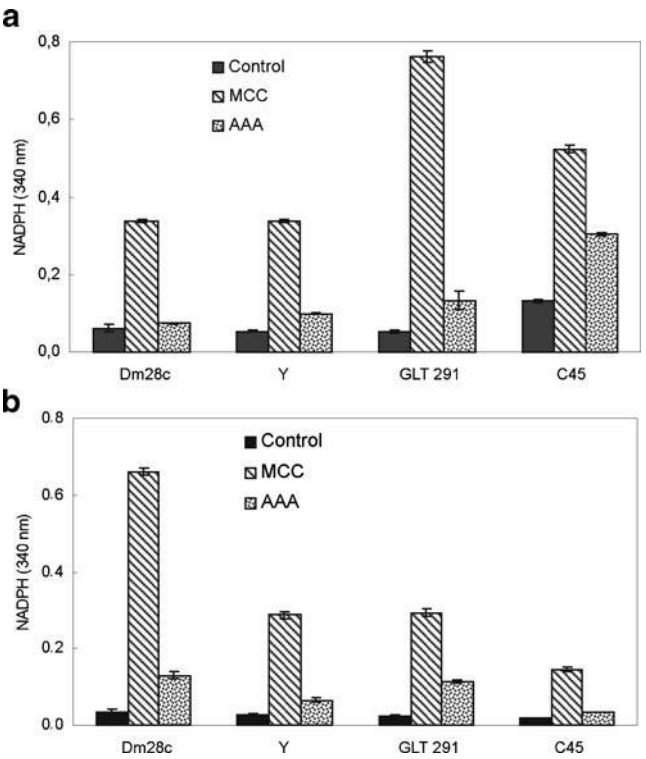

Fig. 4 Effect of MCC and AAA on the activity of trypanothione reductase: **a** epimastigotes, **b** trypomastigotes. The soluble fractions were obtained by lysis in a cavitation pump and differential centrifugations, and the enzyme activity was determined by NADPH consumption at 340 nm as described in the "Materials and Methods" section

and amastigote forms (Maya et al. 1997), leading to a lower susceptibility of the epimastigote form to compounds that interfere with the trypanothione reductase pathway. Furthermore, this heterogeneous inhibition profile among TCI populations (Dm28c, GLT291 and C45) is probably not related to differences in the genetic constitution of the enzyme because there were low polymorphism rates in the gene that codifies this enzyme in parasites of this genotype (Rojas et al. 2007). The high amino acid conservation in the active site of this enzyme suggests that the development of new drugs could work not only for parasites of the same genotype, but also for all *T. cruzi* strains and possibly also for other species of trypanosomatids (Machado and Ayala 2002; Castro-Pinto et al. 2008). The inhibitory activity of MCC on the trypanothione reductase activity of both evolutive forms of *T. cruzi* could be, at least in part, associated to the presence of AAA. However, we observed that even in the absence of AAA activity in epimastigotes of the Dm28c clone, an inhibitory effect was determined for the extract. Therefore, it would be interesting to examine the effect on the enzyme pathway for other components of MCC, such as the classes of compounds cited above. Another aspect to be investigated is the target of the extract and the compounds being evaluated. For example, it could be examined if the targets might display an effect on the recombinant enzyme of *T. cruzi* and then to also verify if they inhibit the glutathione reductase present in mammalian cells.

The ultrastructural analysis of the GLT291 strain of *T. cruzi* after treatment with MCC or AAA was monitored by transmission (TEM) and scanning electron microscopy (SEM). In epimastigotes, alterations at the plasma membrane and also shortening and rounding of the parasite's body and the presence of membrane aggregates attached to the parasites are probably originated from lysed organisms, since we used drug concentration corresponding to the IC_{50} for MCC (Fig. 5b, c) and for AAA (Fig. 5d, e). Membrane

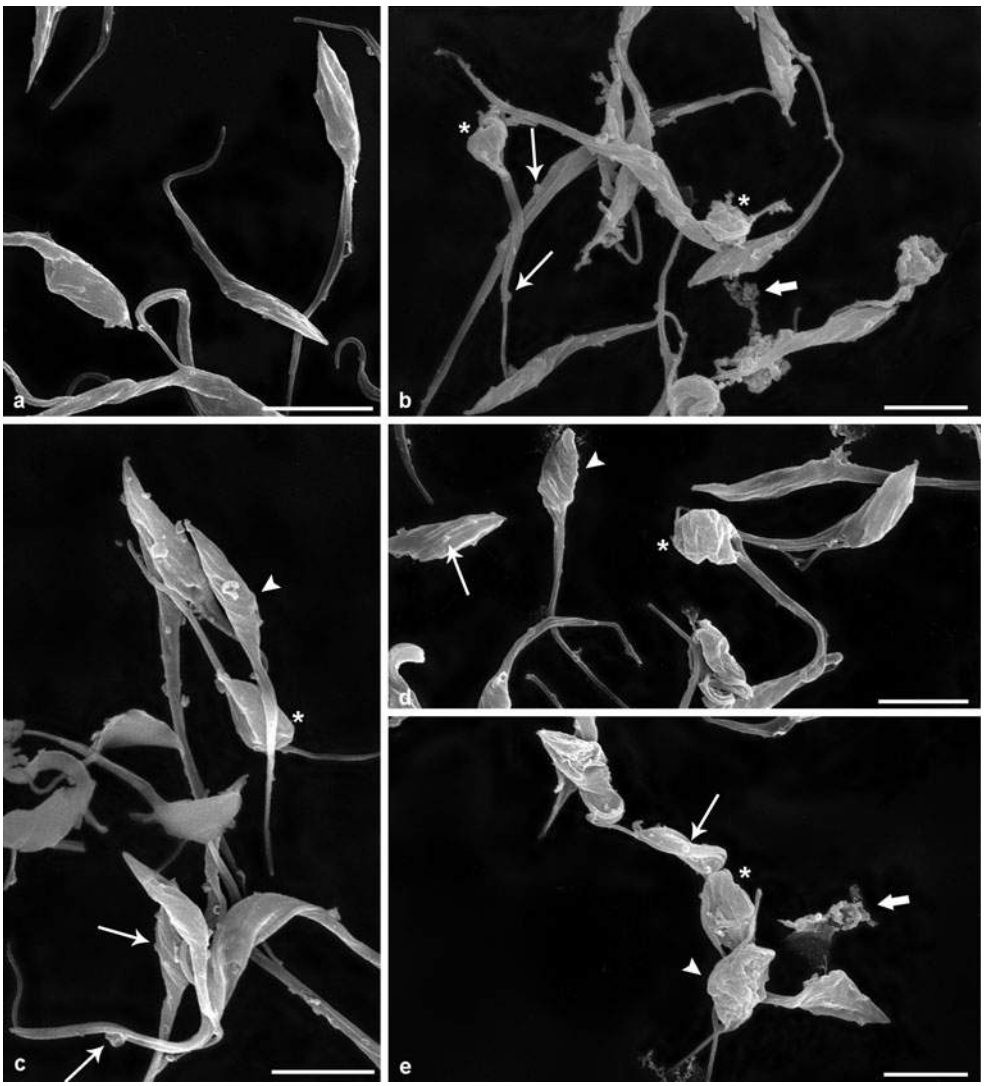

Fig. 5 Scanning electron microscopy of *T. cruzi* epimastigotes treated for 24 h. **a** Untreated parasites displaying their characteristic morphology; **b**, **c** 195.7 μg/mL MCC and **d**, **e** 488.2 μg/mL AAA led to alterations at the plasma membrane with formation of blebs (*thin arrow*) and projections (*large arrow*) beside the packaging of the structure, and also contortion (*arrowheads*), shortening and rounding (*asterisks*) of the parasite's body. *Bars*=5 μm

alterations in the body and flagellar membrane were confirmed by TEM analysis, which also revealed an intense cytoplasmatic vacuolisation and structural disorganisation of treated parasites. Alterations in the kinetoplast–mitochondrion system, with both swelling and loss of cristae (Fig. 6c), and plasma membrane blebbing were observed (Fig. 6d). The mitochondrial alterations induced by AAA could be related to the inhibitory activity on trypanothione reductase, determined by biochemical assays. Using immunofluorescence and immunoelectron microscopy, Meziane-Cherif and coworkers (1994) localised this enzyme in *T. cruzi* in the mitochondrion near the kDNA region, showing it removing ROS in a non-enzymatic form. Further studies using flow cytometry of treated parasites labelled with propidium iodide, acridine orange and rhodamine 123 are needed to further investigate the effect of AAA on the plasma membrane, reservosomes and mitochondrion. The treatment of epimastigotes with MCC led to morphological disorganisation in the Golgi complex (data not shown), and such an effect could compromise its diverse functions, including the transport of proteins to be sent to secretory vacuoles and lysosomes, and also on their glycosylation.

MET analysis of trypomastigotes treated with 10.7 μg/mL MCC or 78.1 μg/mL AAA revealed similar morphological alterations, with intense vacuolisation and material extraction (Fig. 7). In treated trypomastigotes, MCC and AAA led also

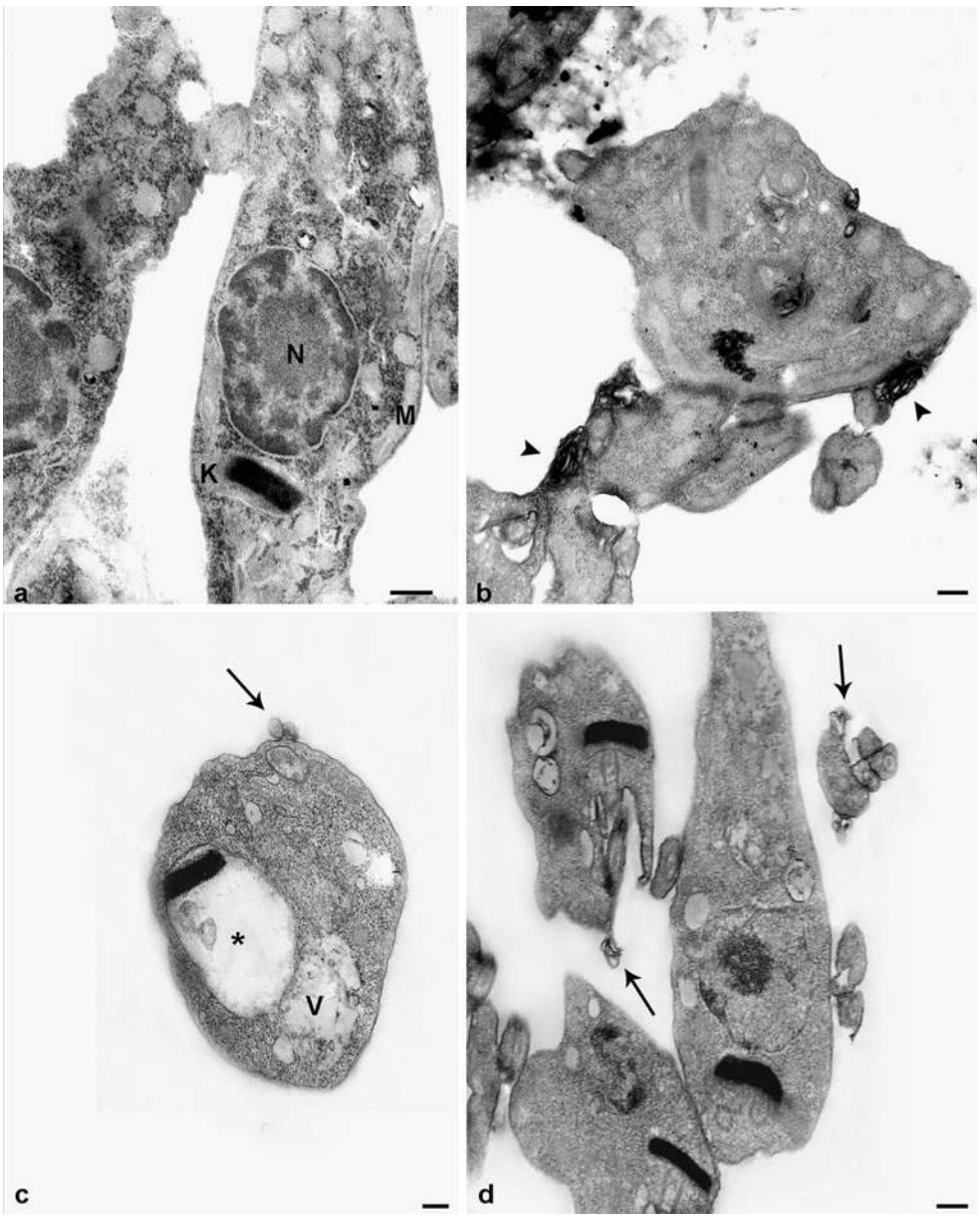

Fig. 6 Transmission electron microscopy of *T. cruzi* epimastigotes treated for 24 h. **a** Untreated parasite displaying the typical elongated body and normal morphology of mitochondrion (*M*), nucleus (*N*) and kinetoplast (*K*); **b** 195.7 μg/mL MCC led to packaging of plasma membrane (*arrowhead*); **c**, **d** 488.2 μg/mL AAA cytoplasmic disorganisation with intense vacuolisation (*V*), mitochondrial alterations (*asterisk*) and blebbing of the plasma membrane (*thin arrow*). *Bars*=0.2 μm

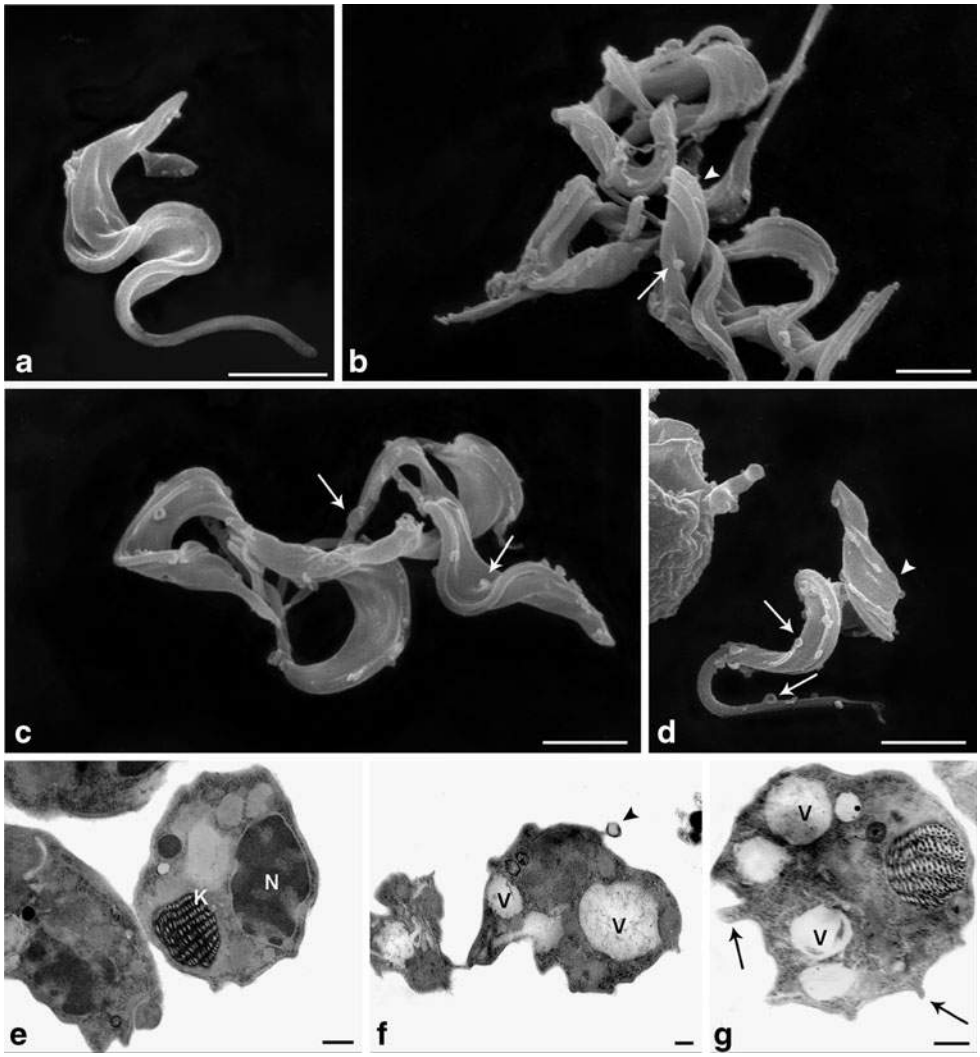

Fig. 7 Scanning and transmission and electron microscopy of *T. cruzi* trypomastigotes treated for 24 h: **a** untreated parasite by SEM presenting its normal morphology; **b**, **c** 10.7 μg/mL MCC and **d** 78.1 μg/mL AAA led to plasma membrane alterations including projection and blebs formation (*thin arrow*) and contortion of the parasite's body (*arrowhead*); **e** control parasites by TEM with normal morphology of the nucleus (*N*) and kinetoplast (*K*); **f** 10.7 μg/mL MCC showing cytoplasmatic disorganisation with intense vacuolisation and membrane packaging (*arrowhead*); **g** 78.1 μg/mL AAA (also caused intense vacuolisation (*V*) and membrane blebbing (*thin arrow*). *Bars*=2 μm (**a–d**), 0.2 μm (**e–g**)

to membrane alterations (Fig. 7b–d, f, g), with the most preserved structures being the kinetoplast and the nucleus (data not shown). In both forms of *T. cruzi*, MCC or AAA induced alterations in the plasma membrane with a loss of typical morphology. Sanchez and co-workers (2006) reported the formation of blebs on the surface of epimastigotes after treatment with the diterpene 5-epi-icetexone, isolated from *Salvia gilliessi*. They associated this alteration with cytoskeleton disorganisation. The membrane shrinkage and formation of projections have been correlated to alterations in the cytoskeleton organisation and/or in the connection plasmalemma-cytoskeleton (Vannier-Santos and De Castro 2009), and the observed alterations could indicate destabilisation of cytoskeleton components or microtubule-associated proteins.

The results obtained with MCC on all the evolutive forms of *T. cruzi* and AAA on intracellular amastigotes encourage the continuation of this study. This continuation should also include an analysis of other components present in *C. cajucara* such as the clerodanes *trans*-crotonin and isocajucarin, and also the isoquinolinic-type alkaloids, as well as compounds derived from *t*-DCTN.

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