In vitro trypanocidal activity of the Egyptian plant Schinopsis lorentizii against trypomastigote and amastigote forms of Trypanosoma cruzi

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ABSTRACT

Chagas’ disease is a chronic illness caused by the protozoan Trypanosoma cruzi. According to estimates, approximately 16-18 million people are infected in Latin America. Plant extracts exhibit a wide variety of secondary metabolites and can play an important role in the discovery of new compounds with biological potential. The in vitro trypanocidal activity of the extracts obtained from six plant species collected in Egypt (Parkia africana, Parkia roxburgi, Lagerstroemia speciosa, Schinopsis lorentzii, Lagerstroemia indica, and Sapindus saponaria) was assayed against trypomastigote and amastigote forms of T. cruzi. The cytotoxic activity of the most active extract was also evaluated by conducting MTT assays. S. lorentzii and S. saponaria were the most active extracts against the amastigotopform;IC₅₀ values were 9.9 and 27.34 µg/mL, respectively. The S. lorentzii extract was also evaluated against the amastigote form (IC₅₀ was 111.5 µg/mL). The S. lorentzii extract did not exhibit significant cytotoxic activity. The selectivity index value indicated that this extract was highly selective for the parasite. The S. lorentzii and S. saponaria extracts exhibit trypanocidal activity, probably as a result of the presence of different constituents in their concentrations in the extracts.

INTRODUCTION

Neglected tropical diseases are a group of lethal diseases or disabling infections affecting more than a billion people worldwide. Poor populations living in rural areas and urban slums are at higher risk of infection. Therefore, people suffering from these diseases constitute an unattractive market to the private research sector (Schmidt et al., 2012). Seventeen neglected tropical diseases, including Chagas’ disease, leishmaniasis, and African trypanosomiasis affect millions of people around the world. It is estimated an annual incidence of 28,000 cases of Chagas’ disease in the region of the Americas, to 8 million people infected and approximately 12,000 deaths per year (WHO). Chagas’ disease, also known as American trypanosomiasis, is caused by the intracellular obligatory parasite Trypanosoma cruzi, which is transmitted to humans and other mammals by hematophagous insects (Rocha et al., 2007). After infection, a short-term acute phase (4-8 weeks) with patent parasitemia that often goes undiagnosed is followed by a lifelong chronic phase that appears after a long latent period known as indeterminate form. The chronic phase of Chagas disease is characterized by scarce circulating parasites. These parasites cause symptomatic chronic cardiomyopathy and/or digestive symptoms in approximately 30% of Chagas disease patients (Marin-Neto et al., 2007; Campi-Azevedo et al., 2015). Although the discovery of Chagas’ disease dates back to over one hundred years, the drugs that are currently available to treat infected individuals, Nifurtimox and benznidazole have serious drawbacks.
These medications exhibit high toxicity and limited effect against different T. cruzi isolates and stages of the disease, which makes the discovery of novel pharmaceuticals a matter of utmost importance (Soeiro 2009; Andriani et al., 2011). In this context, natural sources such as plants, which contain various secondary metabolites, can play an important role in the discovery of new substances with biological potential (Schimidt et al., 2012).

In continuation of our previous works on the antiparasitic studies of Egyptian plants (Rashed et al., 2013a,b), we now report the evaluation of the in vitro trypanocidal activity of six Egyptian plant extracts against trypanostigmate form of T. cruzi, which have not yet been described. Additionally, we have also assessed the trypanocidal activity against amastigote form of T. cruzi and cytotoxic activity against LLCMK₂ cells of the most active plant extract.

MATERIAL AND METHODS

Plant material

Parkia africana, Parkia roxburgi, Lagerstromeia speciosa, Schinopsis lorentzii, Lagerstromeia indica, and Sapindus saponaria barks were collected from the Al-Zohiriya Garden in Giza, Egypt, in May 2011.

All the plants were identified by Dr. Mohammed El-Gebaly at the Department of Botany, National Research Centre (NRC), and by Mrs. Tereea Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and director of the Orman Botanical Garden, in Giza, Egypt. A voucher specimen was deposited in the herbarium of the Al-Zohiriya Garden in Giza, Egypt.

Preparation of the extracts

Air-dried bark of the plants (280 g) was extracted with methanol/distilled water 80:20 (v/v) several times (five times), at room temperature, by the maceration method. Each extract was concentrated under reduced pressure, to afford 12 g, 14.5 g, 10.5 g, 16 g, 11.5 g, or 13 g of dry (S. lorentzii, S. speciosa, S. saponaria) extracts, respectively.

Phytochemical analysis.

Phytochemical screening of each extract was conducted according to the methods described by Yadav and Agarwala (2011).

Parasites and Life cycle

The Y strain of T. cruzi was used in the assays. This strain is characterized by thin forms and tropism for phagocytic mononuclear cells (Pereira da Silva and Nussenzweig, 1953). The Y strain of T. cruzi is routinely maintained by serial passages through BALB/c mice. The Ethics Committee for Animal Care of the University of Franca authorized all the experiments; all the experimental protocols were in accordance with the national and international accepted principles for laboratory animal use and care.

Anti-trypanostigmate assay

The in vitro trypanocidal assay was undertaken by using the trypanostigmate form of T. cruzi, obtained by culturing in LLCMK₂ cell lineage (Macaca mulatta kidney cell). The cells were cultured in RPMI-1640 medium supplemented with L-glutamine (2 mM), NaHCO₃ (10 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% inactivated fetal calf serum. The culture was kept in a 96-well microplate at 37°C, under atmosphere of 5% CO₂ and 95% humidity. Trypanostigmates obtained from the blood of infected animals (by cardiac puncture) at the parasitemic peak were added to the cell culture at a 5:1 ratio. After seven days, the culture supernatant was removed and centrifuged, and the free forms of the parasite were harvested for the tests. Each well was filled with 1x10⁶ cells, which were counted with the aid of a Neubauer chamber. The samples were dissolved in dimethyl sulfoxide (DMSO) and added to the wells to give final concentrations of 12.5, 25, 50, 100, and 200 μg/mL. The microplate was incubated at 37°C for 24 h, and the biological activity was evaluated by direct quantification of the parasites in a Neubauer chamber. RPMI 1640 medium plus DMSO was used as negative control; Benznidazole was used as positive control. All the experiments were performed in triplicate. Results are expressed as mean± SD.

Anti-amastigote assay

The anti-amastigote assays were developed in LLCMK₂ cell cultures as described by Giorgio et al., 1998. The cells were cultured in RPMI 1640 medium supplemented with glutamine (2 mM), NaHCO₃ (10 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% inactivated fetal bovine serum. The culture was kept in a 24-well microplate for 24 h, at 37 °C, in 5% CO₂ with 95% humidity. Each well was filled with 1 x 10⁶ cells, which were counted by using a Neubauer chamber. After 24 h, the trypanostigmates obtained from the cell culture were collected and centrifuged at 760 rpm for 8 min, at 12 °C. The supernatant consisted of trypanostigmates, whereas the cells constituted the sediment. After a second centrifugation (at 3000 rpm for 30 min, at 12°C), the trypanostigmates (1 x 10⁶) were added to the culture, and the microplate was incubated for 48 h. The extract was dissolved in dimethyl sulfoxide (DMSO) and added to the wells to give final concentrations of 12.5, 25, 50, 100, and 200μg/mL. After 96 h of incubation, the colorimetric assay was accomplished by the Giemsa-staining method. The infected cells were counted, and the percentage of parasitic reduction was determined by comparison with the negative control (DMSO 0.5%).

Cytotoxicity assay

The LLCMK₂ fibroblast cells were grown in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% inactivated fetal calf serum, and maintained at 37°C in 5% CO₂. A cell suspension was seeded at a concentration of 1x10⁶ cells/mL in a 96-well microplate containing RPMI 1640 medium. Thereafter, the cells were treated with S. lorentzii extract at different concentrations (12.5, 25, 50,
100, and 200(µg/mL). The plates were incubated at 37°C for 24 and 96 h. The biological activity was evaluated by using the MTT colorimetric method [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in a microplate reader at 540 nm. RPMI 1640 medium plus DMSO and RPMI 1640 medium were used as positive and negative controls, respectively. All the experiments were performed in triplicate. The percentage of cytotoxicity was determined by the formula: % cytotoxicity = [1−[(Y−N)/(N−P)]] ×100, where Y=absorbance of the well containing cells and the extract at different concentrations; N=negative control; P=positive control. The selectivity index (SI) was determined for trypomastigotes as the ratio of CC50 to IC50 values.

Statistical analysis
The statistical tests were performed with the Graphpad Prism (version 5.0) software. The data were statistically analyzed by one-way analysis of variance.

RESULTS AND DISCUSSION
The biological potential of medicinal plants as a source of new drugs deserves further exploration. The use of plant extracts or plant-derived chemicals to treat several diseases has stood the test of time. In recent years, there has been growing interest in the use of medicinal plants in both underdeveloped and developing countries—natural drugs have been reported to be safe, especially when compared to synthetic drugs (Abirami et al., 2012).

Table 1 lists the results obtained for the trypanocidal activity of the assayed plant extracts against T. cruzi trypomastigotes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% lysis ± S.D.</th>
<th>µg/mL</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>12.5</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>69.5±1.0</td>
<td>50.7±4.9</td>
<td>27.8±4.1</td>
<td>32.6±2.1</td>
<td>54.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44.4±2.5</td>
<td>28.5±1.6</td>
<td>21.6±1.6</td>
<td>10.0±3.3</td>
<td>6.1±0.9</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>46.0±3.1</td>
<td>24.8±0.9</td>
<td>21.1±2.4</td>
<td>5.8±1.8</td>
<td>200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>97.8±1.0</td>
<td>73.9±4.3</td>
<td>75.0±4.7</td>
<td>55.4±1.0</td>
<td>9.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>61.3±3.1</td>
<td>16.7±2.0</td>
<td>13.2±2.0</td>
<td>0±0</td>
<td>170.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>62.3±0.6</td>
<td>79.4±1.7</td>
<td>61.3±3.1</td>
<td>50.1±1.0</td>
<td>30.5±1.7</td>
<td>27.34</td>
<td></td>
</tr>
</tbody>
</table>

1: Parkia africana extract, 2: Parkia roxburgi extract, 3: Lagerstromiae speciosa extract, 4: Schinopsis lorentzii extract, 5: Lagerstromiae indica extract, 6: Sapindus saponaria extract. Benznidazole (positive control): IC50= 9.8 µg/mL.

The anti-Trypanosoma cruzi activity of the extracts was assessed at concentrations of 12.5, 25, 50, 100, and 200µg/mL for 24 hours. The trypanocidal activity was determined by comparing the count of trypomastigotes in each sample with the count of trypomastigotes in control groups. The S. lorentzii and the S. saponaria extracts were the most active against the Y strain of T. cruzi; IC50 was 9.9 and 27.34µg/mL, respectively. At 200 µg/mL, 100 µg/mL, and lower concentrations, the S. lorentzii extract provided 97%, 95%, and over 70% trypomastigote lysis, respectively.

Compared to the S. lorentzii and S. saponaria extracts, the P. Africana and L. indica extracts exhibited lower trypanocidal potential, with IC50 values of 54.5 and 170.8µg/mL, respectively, and 69% and 61% trypomastigote lysis, respectively. The P. Roxburgi and L. speciosa extracts did not display significant trypanocidal activity (IC50>200µg/mL).

According to Osório et al. (2007), plant extracts with IC50 < 10 µg/mL, IC50 > 10 µg/mL and < 50 µg/mL, IC50 > 50 µg/mL and < 100 µg/mL, and IC50 > 100 µg/mL can be classified as highly active, active, moderately active, and non-active with regard to their antiprotozoal activity, respectively. Based on this classification, the S. lorentzii (IC50 = 9.9 µg/mL) and the S. saponaria (IC50 = 27.3 µg/mL) extracts are highly active and active, respectively. Flavonoids and triterpenes, classes of constituents of these extracts, have been shown to exhibit trypanocidal action (Uchiyama, 2009; Izumi et al., 2011) and may account for the observed activity.

Table 2 shows data concerning the activity of the S. lorentzii extract (the most active against T. cruzi trypomastigotes) against T. cruzi amastigotes. Compared with its activity against T. cruzi trypomastigotes, the S. lorentzii extract was less active against T. cruzi amastigotes: IC50 was 111.5µg/mL, and parasite lysis was around 60% and 44% at 200 and 100 µg/mL, respectively. The lower susceptibility of amastigotes to the extract can be rationalized as follows: to gain access to the intracellular parasite (amastigote), the extract has to cross the host cell membrane and the parasitophorous vacuole membrane, whereas the extracellular trypanosome is directly exposed to the extract.

Table 2: In vitro trypanocidal activity of plant extracts against the amastigote form of T. cruzi

<table>
<thead>
<tr>
<th>Sample</th>
<th>% lysis ± S.D/concentration (µg/mL)</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. lorentzii</td>
<td>60.5±4.4</td>
<td>19.1±4.4</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>44.4±4.0</td>
<td>170.8±4.0</td>
</tr>
<tr>
<td>3</td>
<td>40.9±4.2</td>
<td>30.5±1.7</td>
</tr>
<tr>
<td>4</td>
<td>27.5±3.2</td>
<td>27.3±4.1</td>
</tr>
<tr>
<td>5</td>
<td>19.1±4.4</td>
<td>170.8±4.0</td>
</tr>
<tr>
<td>6</td>
<td>111.5</td>
<td>57</td>
</tr>
</tbody>
</table>

Positive Control: Benznidazole (IC50 = 19.1 µg/mL); Negative Control: DMSO 0.5%

MTT assays on LLCMK2 fibroblast cells allowed us to ascertain the cytotoxicity of the S. lorentzii extract; the assayed concentration range was chosen on the basis of in vitro studies conducted with T. cruzi. The cell cultures were treated with the extract at concentrations of 12.5, 25, 50, 100, and 200µg/mL for 24 and 96 hours. Cell viability was determined by the ratio between the absorbance values obtained in the treated and untreated (control) groups (Fig. 1).

The S. lorentzii extract was not significantly cytotoxic to LLCMK2 cells within the first 24 hours of the experiment: CC50 was greater than 400µg/mL, and cell viability remained around 79% at the highest tested concentration. Similarly, after 96 hours of treatment, CC50 was greater than 400 µg/mL, and the percentage
of viable cells was around 78% at the higher assayed concentrations (400 and 200 μg/mL).

Fig. 1: Effects of S. lorentzii extract on the viability of LLCMK2 fibroblast cells. Cytotoxicity was determined by the MTT assay after 24 and 96 h of treatment with the indicated concentrations. Values are expressed as mean ± S.D.

The selectivity index represents the selectivity of a compound for a parasite cell and for a normal cell line. This index indicates the potential use of a given compound in clinical trials. To compare the trypanocidal activity and the toxicity of the S. lorentzii extract, we used mammalian cells to estimate the selectivity index (SI) of this extract. According to Lenta et al. (2007), SI values greater than 10 suggest that a compound is safe for use in mammals. The S. lorentzii extract gave SI of 40, which indicated that it was highly selective for the parasite.

Table 3: Results of the phytochemical screening of the plant extracts.

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Plant Family</th>
<th>Plant part</th>
<th>Phytoconstituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parkia africana</td>
<td>Leguminoseae</td>
<td>Bark</td>
<td>Triterpenes/Sterols</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Carbohydrates/glycosides</td>
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<td></td>
<td></td>
<td></td>
<td>Flavonoids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tannins</td>
</tr>
<tr>
<td>Parkia roxburgi</td>
<td>Leguminoseae</td>
<td>Bark</td>
<td>Triterpenes/Sterols</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Carbohydrates/glycosides</td>
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<td>Flavonoids</td>
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<tr>
<td></td>
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<td>Tannins</td>
</tr>
<tr>
<td>Lagerstromeia</td>
<td>Lythraceae</td>
<td>Bark</td>
<td>Triterpenes/Sterols</td>
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<tr>
<td>speciosa</td>
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<td>Flavonoids</td>
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<td></td>
<td>Tannins</td>
</tr>
<tr>
<td>Schinopsis</td>
<td>Anacardiaceae</td>
<td>Bark</td>
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<td>lorentizii</td>
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<td></td>
<td>Tannins</td>
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<tr>
<td>Lagerstromeia</td>
<td>Lythraceae</td>
<td>Bark</td>
<td>Triterpenes/Sterols</td>
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<tr>
<td>indica</td>
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<td>Carbohydrates/glycosides</td>
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<td>Flavonoids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tannins</td>
</tr>
<tr>
<td>Sapindus</td>
<td>Sapindaceae</td>
<td>Bark</td>
<td>Triterpenes/Sterols</td>
</tr>
<tr>
<td>saponaria</td>
<td></td>
<td></td>
<td>Carbohydrates/glycosides</td>
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<td>Flavonoids</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Saponins</td>
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</table>

The phytochemical analyses of the investigated plant extracts showed the presence of triterpenes, flavonoids, tannins, and carbohydrates; the S. saponaria extract also contained saponins (Table 3). Several studies have reported on the trypanocidal activity of natural flavonoids (Takeara et al., 2003; Sulsen et al., 2007; Mai et al., 2015). The lyophilized aqueous extract from Lycnophora pinaster Mart (Asteraceae) exhibits trypanocidal action (113.62 μg/mL); chemical characterization of this extract by HPLC revealed the presence of caffeic acid, isochlorogenic acid, vitexin, isovitexin, and quercetin (Silveira et al., 2005).

Flavonoids like hispidulin and santin have significant trypanocidal and leishmanicidal activities. These flavonoids could serve as potential lead compounds for the development of more efficient drugs to treat leishmaniasis and Chagas disease (Sulsen et al., 2007).

Terpenoids are a class of natural substances with various biological applications (Roberts, 2007). Their in vitro and in vivo trypanocidal potential has been extensively studied (Duarte et al., 2002; Rosas et al., 2007; Ferreira et al., 2013).

Recently, Santos et al. (2013) have described the leishmanicidal and trypanocidal activities of two triterpenes, maytenin and pristimerin, isolated from Maytenus ilicifolia. The compounds are effective against Leishmania amazonensis and Leishmania chagasi as well as Trypanosoma cruzi. Both compounds have IC₅₀ lower than 0.3 nM against T. cruzi epimastigotes.

Oleanolic and ursolic acids are ubiquitous triterpenoids in the plant kingdom, particularly in medicinal herbs, and are an integral part of the human diet. Previous studies reported by our group have shown that these triterpenes display significant in vitro trypanocidal activity (Cunha et al., 2003, Cunha et al., 2006; Ferreira et al., 2010).

The industrial applications and properties of vegetable tannins stem from their ability to form complexes with proteins via hydrogen bonds. Tannins can inhibit microorganism growth by irreversibly deactivating enzymes (Venter et al., 2012). These compounds exhibit leishmanicidal and trypanocidal activities (Kolodziej and Kiderlen, 2005; Ogbadoyi et al., 2007).

Shuaibu et al. (2008) have reported on the in vitro anti-trypanosomal activity of Anogeissus leiocarpus and Terminalia avicennoides methanolic extracts against four Trypanosoma species strains. Hydrolyzable tannins present in these extracts display trypanocidal action (MIC = 7.5-27.5 μg/mL or 14-91 μM). Moreover, these compounds are not significantly toxic to fibroblasts.

Cejas et al. (2011) authored one of the few literature studies on the antiparasitic activity of S. lorentzii. More specifically, these authors evaluated whether a commercially available polyphenolic vegetable extract from S. lorentzii (Bioquina®) reduced coccidiosis in broiler chicks. These authors suggested that the extract may have an impact against avian coccidiosis, but they stated that further studies on the potential
value of this product as a therapeutic or prophylactic anticoxidial agent are necessary.

According to Barberis et al. (2012), the species of the genus *Schinopsis* have numerous applications, especially in animal husbandry and cattle ranching as well as logging activities. Several authors have reported that species of this genus contain phenols that can control gastrointestinal parasites in ruminants and rodents (Paolini et al., 2003; Max et al., 2005; Athanasiadou et al., 2007). Moreover, tannins present in *S. lorentzii* can also reduce helminths in birds (Marzoni et al., 2005). Thus, the tannins identified in the most active extracts of the present study could also account for the antiparasitic activity displayed by these extracts.

Given the important limitations of the currently available treatment for Chagas disease, especially the low efficacy of the medication in the chronic phase, new steps have been taken toward the search for chemotherapeutic approaches that can improve the control of this disease (Urbina, 2010).

Investigations into natural products can potentially provide positive results, particularly in the case of diseases that are difficult to treat and for which there is no cure. Determining the efficacy or optimum concentration of a tested drug is extremely important and acquiring information about its effect on parasite biology and parasite interaction with the host cell is of great value (Maya et al., 2007).

**CONCLUSION**

This work has demonstrated the trypanocidal activity of *S. lorentzii* on the two evolutive forms of *T. cruzi* for the first time. The trypanocidal activity observed for the extracts of *S. lorentzii* and *S. saponaria* can be result of different constituents and their concentrations in the extracts. The low toxicity of the *S. lorentzii* extract to mammalian cells associated with its significant trypanocidal action makes this extract a promising candidate for the discovery of new trypanocidal drugs. Further studies on the isolation, identification, and bioassays of active compounds against the trypomastigote and amastigote forms of *T. cruzi* in vivo assays are essential to elucidate structure-activity relationships, unveil the mechanisms of parasite death induced by the most promising substances, and identify their putative intracellular targets. Hopefully, such studies will culminate in the development of novel therapeutic agents to treat Chagas’ disease.

**Conflict of Interest Statement:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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