Original Article

Anti-Trypanosoma cruzi activity, cytotoxicity and, chemical characterization of extracts from seeds of Lonchocarpus cultratus

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Abstract

Introduction: Trypanosoma cruzi is the agent of Chagas’ disease and affects approximately 6-8 million people worldwide. The search for new anti-T. cruzi drugs are relevant because only two drugs exist actually. The objective of this study was to investigate the effect of the extracts from the seeds of Lonchocarpus cultratus on T. cruzi, its cytotoxicity as well as to elucidate its chemical profile.

Methodology: The characterization of the extracts was done using ¹H-RMN. T. cruzi forms were treated with increasing concentrations of the extracts and after, the percentage of inhibition and IC₅₀ or LC₅₀ were calculated. Murine peritoneal macrophages were treated with different concentrations of the extracts to evaluate the cellular viability. The hemotoxicity was accessed by verifying the levels of hemolysis caused by the extracts on human red blood cells.

Results: Chalcones isocordoin and lonchocarpin were detected in the dichloromethane extract, and chalcone lonchocarpin was detected in the hexane extract. The dichloromethane extract showed higher activity against all the forms of T. cruzi, its cytotoxicity as well as to elucidate its chemical profile.

Conclusions: L. cultratus extracts have the potential to be explored for the development of new anti-trypanosomal drugs. This study was the first to demonstrate the action of extracts from the genus Lonchocarpus on infecting forms of T. cruzi.

Key words: Chagas’ disease; chalcones; trypanocidal activity; cytotoxicity; hemolytic activity.

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Introduction

American trypanosomiasis or Chagas' disease is a significant disease for the public health of several countries in the Americas, especially those in tropical regions with low-income [1]. Currently, there are approximately seven million people who have been diagnosed with Chagas’ disease and approximately ten thousand deaths annually related to this pathology [2]. In Brazil, more than twenty-three thousand deaths due to Chagas’ disease were recorded between 2009 and 2013 [3].

There are cases of Chagas’ disease in high-income countries, such as Canada, Spain, and the United States, even though they are considered nonendemic regions. It is estimated that in the USA, there are more than three hundred thousand cases, and on the European continent, the number of cases reaches approximately one hundred thousand [4]. The principal cause of the cases in these countries is the presence of migrants from endemic countries [5].

One of the goals of Chagas’ disease treatment is to prevent injuries in essential organs such as the heart,
liver, and stomach [6]. Despite the severity of clinical conditions, there are only two drugs available for treatment: nifurtimox and benznidazole (BZN). These substances are useful in patients in the acute and chronic (indeterminate) phase of the disease. However, the symptoms are usually nonspecific in the acute phase, and most patients only seek medical assistance when the infection is chronic with irreversible effects on the affected organs [7]. The use of nifurtimox and BZN in the treatment of the chronic phase is questionable because, in addition to the intensity of the side effects, they show low therapeutic effectiveness [8,9]. Considering these aspects, the development of new drugs against the agent that causes this disease, Trypanosoma cruzi, is necessary.

Medicinal plants are usually used in folk medicine to treat parasitic diseases. Several studies corroborate the therapeutic importance of natural products in American trypanosomiasis [10,11]. Substances of several classes, such as quinones, flavonoids, alkaloids, and terpenes, have anti-T. cruzi activity and have the potential to be new drugs for Chagas’ disease treatment [12].

The plants of the genus Lonchocarpus stand out for having several biological activities [13]. Among them, the species Lonchocarpus cultratus (Vell.) A.M.G. Azevedo and H.C. Lima has been studied to some extent. Its secondary metabolites have not been completely characterized, although some of its constituents, such as alkaloids [14], triterpenes, chalcones [15] and flavones [16], have already been identified.

Thus, considering the problem of Chagas' disease treatment, as well as the biological activities of the genus Lonchocarpus [17], this study aimed to identify the components and investigate trypanocidal activity and the cytotoxicity of extracts from seeds of L. cultratus.

**Methodology**

**Plant samples**

Seed samples were collected from a specimen of L. cultratus located on the banks of the Lake of the Municipal Park of Cascavel-PR, Brazil (S 24.96308°, and O 53.43674°) in May 2017. The specimen was confirmed as a species of L. cultratus by a systematic biologist, who took photographs of the vegetative and diagnostic characteristics of the specimen, in addition to collecting samples for the construction of the exsiccata. The analysis of the photographs followed the analytical key determined by Silva and Tozzi, 2012 [18], making it possible to identify the specimen as an L. cultratus. The voucher samples were deposited in the Herbarium of UNIOESTE (UNOP No. 20).

**Plant extract**

The seeds collected from L. cultratus were dried in a circulating air incubator (< 40°C) and ground in a knife mill. The plant material obtained by this process (313.26 g) was subjected to successive macerations in hexane (Sigma-Aldrich, St. Louis, USA) and was filtered and concentrated in a rotary evaporator under reduced pressure, yielding 95.94 g of hexane extract (LHS). The remaining plant residue after the filtration was subjected to the same procedure using the dichloromethane (Sigma-Aldrich, St. Louis, USA) solvent instead of hexane. This method yielded 8.17 g of dichloromethane extract (LDS). After that, the process was repeated using methanol (Sigma-Aldrich, St. Louis, USA) as the extracting solvent, which resulted in 16.45 g of methanolic extract (LMS). Figure 1 shows the experimental scheme of the study.

**Chemical characterization of the extracts**

Nuclear magnetic resonance (NMR) spectra were obtained using Varian (Mercury Plus, BB 300 MHz) and Bruker (500 MHz) spectrometers using CDCl3 as the solvent and trimethylsilyl (TMS) (Sigma-Aldrich, St. Louis, USA) as an internal reference. The chemical characterization of each extract (LHS, LDS, and LMS) was performed by ¹H NMR, comparing the ¹H signals with those described in the literature.

**Anti-Trypanosoma cruzi activity in vitro**

The anti-T. cruzi activity of LHS, LDS, and LMS were determined by in vitro assays using epimastigotes, trypomastigotes, and amastigotes forms of the T. cruzi
Y strain. The epimastigotes forms were maintained with weekly subcultures in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) (Laborclin, Curitiba, Brazil) and kept in an incubator at 28°C. Tissue culture tryptomastigotes were obtained from the supernatants of 5- to 6-day-old infected Vero cells maintained in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% FBS and 50 µg/mL gentamycin (NovaFarma, Anápolis, Brazil) at 37°C in a 5% CO₂ atmosphere. As a positive control, we used benznidazole (Lafepe, Recife, Brazil), identified as BZN in the results section, and because dimethyl sulfoxide (DMSO) (Neon Comercial, Suzano, Brazil) was the solvent of the extracts, it was analyzed whether DMSO alone, as the control of the solvent, had any effect per se on the parasites.

To perform the assay using the epimastigotes forms, *T. cruzi* was cultured in polypropylene tubes. Parasites at a density of 1 × 10⁵ forms/mL were then treated using LHS, LDS, and LMS extracts at concentrations of 1, 10, 15, 50, 100, 150, and 175 µg/mL, dissolved in DMSO (up to a maximum of 2% DMSO). The following controls were used in the tests: untreated (UC), consisting of *T. cruzi* cultures in the LIT medium; DMSO, consisting of *T. cruzi* and DMSO; and treatment with the standard drug to treat trypanosomiasis, BZN, at a concentration of 50 µg/mL. The final volume in each tube was 3 mL. All tests were performed in triplicate. After 72 hours of culture, the growth of *T. cruzi* was verified by directly counting the parasites in a Neubauer chamber using an optical microscope (Olympus Model CBA) at 400X.

The anti-*T. cruzi* activity of LHS, LDS, and LMS extracts against tryptomastigote forms was determined in 96-well plates (4 × 10⁵ parasites/well) in RPMI-1640 medium supplemented with 10% FBS and 50 µg/mL gentamycin (Novafarma, Anápolis, Brazil) at 37°C in a 5% CO₂ atmosphere. An inverted microscope (Olympus Model CBA) at 400X was used to count the parasites in a Neubauer chamber after 24 and 48 hours of incubation using an optical microscope at 400X. The following controls were used: untreated (UC), DMSO (DC), and treatment with the reference drug (BZN). The results were determined using nonlinear regression on GraphPad Prism 6.0.

**Cytotoxic bioassay**

The toxicity of each extract (LMS, LDS, and LHS) was assessed in BALB/c mice peritoneal macrophages employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco, Solon, USA) assay according to Reilly et al., 1998 [19]. All legal recommendations of Brazilian legislation (Law No. 11.794 Oct. 2008) for animal handling procedures for scientific research were used, and the Animal Ethics Committee of Western Parana State University approved this study under the number 47/17 in August 2017.

Peritoneal macrophages were collected by infusing in the donors' peritoneal cavity 8–10 mL of chilled PBS. The macrophages were suspended in RPMI medium supplemented with 10% FBS and gentamycin solution (50 µg/mL), adjusted to 2×10⁵ cells per well on a 96-well microplate and incubated in a CO₂ atmosphere (5%) at 37°C. After 3 hours of incubation in the humidified incubator, non-adherent cells were removed by washing twice with warm PBS. Each one of the extracts was dissolved in RPMI medium at a concentration of 1, 10, 15, 50, 100, 150, and 175 µg/mL.
and was added to the wells. The plates were incubated for 48 hours under the same conditions as described above. After this time, 300 μL of MTT reagent (5 mg/mL) was added to each well, followed by an additional three hours of incubation. Next, 150 μL of DMSO was added to solubilize the formazan crystals that formed, and the plates were read in a microplate reader (Biotek Model EPOCH, λ 550 nm). In the test, the UC and DC controls were used.

The cytotoxic activity (CC50) was expressed as the concentration of the extracts able to kill 50% of the macrophages after 48 hours of treatment. The CC50 was calculated using nonlinear regression in GraphPrism 6.0, and the results were related to the trypanocidal activities (IC50 and LC50) by determining their corresponding selectivity index (SI=CC50/IC50 or LC50).

Hemolytic activity

All legal recommendations for use of human samples were used, and the Human Ethics Committee of Western Parana State University approved this use under the number 1.134.714. The cytotoxic activity of the extracts and benzimidazole on human red blood cells was determined as previously described [20] with some modifications. Briefly, samples of the three extracts, at concentrations of 1, 10, 15, 50, 100, 150, and 175 μg/mL, and BZN were dissolved in DMSO (up to 2%) and added to tubes containing 4% erythrocytes. The DC group consisted of 2% DMSO and 4% erythrocytes. The UC group consisted of 4% erythrocytes, and the positive control (PC) was 100% hemolysis (2% acetic acid and 4% erythrocytes). Human erythrocytes, collected from a healthy individual, were washed three times with PBS, pH 7.4, by centrifugation at 1500 rpm/minutes for ten minutes. The supernatant was collected, and the absorbance was determined using a spectrophotometer at a 450-nm wavelength. The test was conducted in triplicate. The percentages of intact erythrocytes, as well as the percentage of red blood cell hemolysis, were calculated according to the following formulas:

\[
\text{% Intact erythrocytes} = 1 - \left( \frac{\text{Abs samples} - \text{Abs DC group}}{\text{Abs PC group} - \text{Abs UC group}} \right) \times 100
\]

\[
\text{% Hemolysis} = 100 - (\text{% intact erythrocytes})
\]

The results were expressed as the mean percent of intact erythrocytes compared to nontreated control tubes.

Evaluation of the effects of the extracts on NO secretion

Peritoneal macrophages were collected as previously described on subsection Cytotoxicity bioassay. Then, cells were incubated for 48h in the presence of the LDS, LHS, and LMS (1, 10, 15, 50, 100, 150, and 175 μg/mL), Lipopolysaccharide (LPS - 50 ng/mL) (positive control), or only medium (negative control). NO secretion was indirectly assessed by measuring nitrite concentrations in the culture medium using Griess reaction [21] with modifications. The isolated supernatants were mixed with equal volumes of Griess reagent and incubated at 25°C for 10 minutes. Absorbance was measured at 550 nm in a microplate reader. The nitrite concentration was calculated from a standard NaNO2 curve (5–100 μM). Results were expressed as μmol per 2 × 10^5 cells.

Figure 2. 1H nuclear magnetic resonance (NMR; 500 MHz) spectra of the LHS, LDS, and LMS extracts from the seeds of L. cultratus. A: hexane extract (LHS) as the solvent at CDCl3. B: dichloromethane extract (LDS) as the solvent at CDCl3. C: methanolic extract (LMS) as the solvent at CD3OD. Chemical shifts are expressed in δ ppm.
**Statistical analysis**

The Kruskal-Wallis nonparametric test with a Dunn’s multiple comparisons posttest was used to compare the median number of parasites in the epimastigotes and trypomastigotes forms and the phagocytic index for the amastigotes forms. For the hemolytic test, the analysis was done using two-way ANOVA with a Tukey’s multiple comparisons posttest. The results were processed with GraphPrism 6.0 software at a level of significance of 95% (P ≤ 0.05).

**Results**

**Characterization of extracts**

The hexane extract (LHS) of *L. cultratus* seeds (95.94 g) was produced at a yield of 30.63%. The other extracts, dichloromethane (LDS) (8.17 g) and methanolic (LMS) (16.45 g) were obtained at yields of 2.61% and 5.25%, respectively. Figure 2 shows the $^1$H NMR (500 MHz) spectra of the LHS, LDS, and LMS extracts. The $^1$H NMR spectra show that the LHS and LDS extracts are constituted mainly by substances of low and medium polarity, such as those extracted by the solvents used to obtain LHS (hexane) and LDS (dichloromethane). LHS and LDS show a signal at $\delta$ 13.68 ppm, which indicates the presence of hydrogens in strong interactions, such as those of -OH in intramolecular bridges between hydrogen and C=O. Signals in this region are related to a chelated -OH, typical of chalcones present in species of the *Lonchocarpus* genus. Signals present between $\delta$ 7.42-7.90 ppm are indicative of hydrogen linked to aromatic substances present in chalcones. Two signals exist at $\delta$ 7.57 and 7.88 ppm and are indicative of hydrogens linked to double-linked carbons that are connected to aromatic rings and C=O. Supplementary Table 1 shows the signals obtained from the $^1$H NMR of LDS and LHS, and the identification of lonchocarpin was performed by comparing data of the respective NMR spectra with those described in the literature [22,23]. The $^1$H NMR spectrum of LDS shows the presence of a signal at $\delta$13.81, indicative of a hydroxyl group chelated to C=O. In the low field of spectra, signals are seen at $\delta$ 7.44 and 7.43 ppm and are indicative of aromatic hydrogens (H2/H6 and H3/H5) from the “B” ring of chalcones, and at $\delta$ 7.60 and 7.90 ppm, hydrogens are linked to carbons $\alpha$ and $\beta$ from double-linked and conjugated to the aromatic ring and C=O. Supplementary Table 2 shows the $^1$H NMR signals of LDS that, compared with the literature, establish the presence of isocordoin [24,25].

The LMS $^1$H NMR spectrum shows no signal in the high field regions, as was seen in the previous spectra, indicating the absence of the chalcones seen in the other two extracts (from LHS and LDS). The LMS signals appear mainly in the region between $\delta$ 5.50-0.80 ppm. These signals are characteristic of bonds of polar hydrogen linked to carbons linked to electronegative elements and in groups -CH, –CH2, and –CH3 of aliphatic compounds, suggesting the presence of steroids and terpenes. The signal at $\delta$ 7.25 ppm suggests the presence of an aromatic ring.

**Anti-*Trypanosoma cruzi* activity**

The dichloromethane (LDS) and hexane (LHS) extracts promoted a decrease in the number of epimastigotes after 72 hours of treatment at concentrations over 100 μg/mL compared to the UC (Figure 3A and B). On the other hand, the methanolic extract (LMS) did not affect parasite growth, with all concentrations showing rates similar to the UC (Figure 3C).

The growth inhibition percentages were obtained to determine the IC$_{50}$. The extracts from seeds of *L. cultratus* showed inhibition of 92.30%, 68.64%, and 13.70% by LDS, LHS, and LMS, respectively, at a concentration of 175 μg/mL. The BZN control showed 97.96% inhibition at a concentration of 50 μg/mL.

Table 1 shows the IC$_{50}$ values reached by the extracts, according to Osorio et al., 2007 [26]. One of the extracts, LDS, was classified as very active against the epimastigotes forms, i.e., showing an IC$_{50}$ < 10 μg/mL (LDS reached 4.8). The extract LHS was classified as active, 10 < IC$_{50}$ < 50 μg/mL (LDS reached 8.8). The extract LMS was classified as inactive, with an IC$_{50}$ > 100 μg/mL. The positive control (BZN) reached an IC$_{50}$ of 3.1 μg/mL.

*L. cultratus* showed a mortality rate of 65.9%, 41.0%, and 40.3% after 24 hours and 95.3%, 41.6%, and 59.4% after 48 hours (LDS, LHS, and LMS, respectively) against the trypomastigote forms at the concentration of 175 μg/mL, showing that the activity of LDS was very close to that of BZN (175 μg/mL), which reached 70.91% (24 hours) and 92.6% (48 hours). All treatments with extracts showed significant differences (Figure 4A-C) in the mortality rate to the UC group at concentrations above 150 μg/mL, while the DMSO group did not, which demonstrates that the solvent is not responsible for the activity of the extracts.

The LC$_{50}$, after 24 h, from the three extracts are shown in Table 1 and shows that LDS is very active against trypomastigotes forms with a value of 18.7 μg/mL, a value twice higher than the one observed for the BZN control (7.3 μg/mL).
Figure 3. Effects of LDS (dichloromethane), LHS (hexane), and LMS (methanolic) extracts from the seeds of *L. cultratus* on *T. cruzi* epimastigotes (a, b and c). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, *P* < 0.05; **P* < 0.01; ***P* < 0.001. The control corresponds to the medium or the medium-plus DMSO in the absence of extracts. BZN corresponds to the standard drug used as the positive control at the concentration of 50 µg/mL.

Figure 4. Effects of LDS (dichloromethane), LHS (hexane), and LMS (methanolic) extracts from the seeds of *L. cultratus* on *T. cruzi* trypomastigotes (a, b and c). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, *P* < 0.05 and **P* < 0.01. The control corresponds to the medium or the medium-plus DMSO in the absence of extracts. BZN corresponds to the standard drug used as the positive control at the concentration of 50 µg/mL.

Figure 5. Effects of LDS (dichloromethane), LHS (hexane), and LMS (methanolic) extracts from the seeds of *L. cultratus* on *T. cruzi* amastigotes (a, b and c). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, *P* < 0.05. The control corresponds to the medium or the medium-plus DMSO in the absence of extracts. BZN corresponds to the standard drug used as the positive control at the concentration of 50 µg/mL.
The hexane (LHS) and methanolic (LMS) extracts reached values of 15.1 and 26.1 µg/mL, which confirms what was observed in the epimastigotes forms, in which the LHS extract showed higher activity than LMS, which was inactive on the first form tested. The activity on trypanosomes forms is interesting, as this is the infectious form and should be the target of drugs.

The activity of the three extracts was also tested against intracellular forms of *T. cruzi*, and LDS had a performance similar to BZN, with an IC50 of 0.96 (BZN 0.61 µg/mL) and inhibition of growth of 73.15% at a concentration of 175 µg/mL. LHS and LMS had inhibition of 47.79 and 24.56% on amastigotes growth at 175 µg/mL, however showing different IC50 (32.44 and 80.37 µg/mL, respectively). All treatments with the extracts showed significant differences in the mortality rate to the UC group at the higher concentrations (15 µg/mL to LDS and 175 µg/mL of LHS and LMS) (Figure 5A-C); this is due to the toxicity of LDS to macrophages at concentrations above 50 µg/mL. The mechanism by which the extracts diminished the presence of amastigotes was not clear but results from the measurement of NO from cultures of macrophages treated with LDS, LHS and LMS point to a direct effect on the amastigotes since these extracts were not able to induce the secretion of NO by murine macrophages (Supplementary Figure 1).

**Cytotoxic activity**

To evaluate whether the LDS, LHS, and LMS extracts were cytotoxic to host cells, cytotoxic bioassays were performed with these plant extracts at the same concentration at which they had anti-*T. cruzi* activity and the cytotoxic concentration for 50% of the macrophages (CC50) was calculated (Table 1).

The LHS and LMS extracts were not toxic to the macrophages, as shown in Figure 6B and 6C, maintaining the viability of the cells even at the highest concentration. These data suggest that LHS is the extract that best-distinguished macrophages from trypomastigote and epimastigote forms and, although LHS and LMS had a low efficacy against amastigote forms, both were able to distinguish these forms from mammalian cells.

The CC50 value of the LDS extract (12.8 µg/mL) was toxic to murine macrophages, resulting in a low SI against epimastigotes (2.7) and trypomastigotes (1.56) but with a better capacity to distinguish amastigotes, with an SI of 13.3. The LDS extract had a CC50 of >300 µg/mL, which generated an SI of >11.2, >19.9, and >9.26 against the three forms of the parasite (epimastigotes, trypomastigotes, and amastigotes, respectively), showing it to be the most selective compound. BZN showed good selectivity, with an SI of 31.4, 13.5, and 161.3, similar to what was found by other authors [27,28].

**Hemotoxicity test**

The toxicity of the LDS, LHS, and LMS extracts and the reference drug BZN were against human erythrocytes.

**Table 1.** IC50 and CC50 data obtained from epimastigotes, trypomastigotes, amastigotes, and murine macrophages treated with LHS, LDS, and LMS extracts.

| Extracts | Epimastigotes (72 h) IC50 (µg/mL) | Trypomastigotes (24 h) LC50 (µg/mL) | Amastigotes (48 h) IC50 (µg/mL) | Macrophages CC50 (µg/mL) | SI (epi) | SI (trypo) | SI (ama) |
|----------|-------------------------------|---------------------------------|-----------------------------|--------------------------|---------|-----------|---------|
| LHS      | 26.7                          | 15.1                            | 32.44                       | >300                     | >11.2   | >19.9     | >9.26   |
| LDS      | 4.8                           | 18.7                            | 0.96                        | 12.8                     | 2.7     | 0.70      | 13.3    |
| LMS      | inactive                      | 26.1                            | 80.37                       | >300                     | -       | >11.5     | >3.73   |
| BZN      | 3.13                          | 7.3                             | 0.61                        | 98.4                     | 31.4    | 13.5      | 161.3   |

LHS: hexane extract, LDS: dichloromethane extract, LMS: methanolic extract and BZN: benznidazole (reference drug control). SI (selectivity index): CC50/IC50
amastigote forms of genus. A characteristic of this species of the other authors [16,29], who showed that this chalcone is, as demonstrated in the past by L. cultratus parts of. Recently, a study found [25] isocordoin in the aerial once one chalcone received the name lonchocarpin. chalcones, which are common in this genus [13,22,23], dichloromethane and hexane showed the presence of activity was demonstrated only against the species against protozoa, but, in the case of T. cruzi Lonchocarpus solvents. An extensive revision regarding the genus potential of the three extracts obtained by different Discussion

The extracts from L. cultratus obtained with dichloromethane and hexane showed the presence of chalcones, which are common in this genus [13,22,23], once one chalcone received the name lonchocarpin. Recently, a study found [25] isocordoin in the aerial parts of L. cultratus, as demonstrated in the past by other authors [16,29], who showed that this chalcone is a characteristic of this species of the Lonchocarpus genus.

This is the first study showing the activity of the genus Lonchocarpus against trypomastigote and amastigote forms of T. cruzi, which demonstrated the potential of the three extracts obtained by different solvents. An extensive revision regarding the genus Lonchocarpus [13] showed the activity of various species against protozoa, but, in the case of T. cruzi, the activity was demonstrated only against the epimastigotes forms. The flavonoids are present in several species of the genus Lonchocarpus, and lonchocarpin, which is a polyphenolic, was found in different species, including L. floribundus, L. sericeus, L. neuroscapha and L. xuul [16,29–31]. It demonstrated antimicrobial activity against bacteria and fungi [28]

erythrocytes. The evaluation criterion was the percentage of erythrocytes that remained intact after contact with the substances (Figure 7).

The percentage of intact erythrocytes in UC and DC were 100%, demonstrating that DMSO at 2% is not hemolytic. The PC control constituted with the hemolytic solution had no intact erythrocytes. The data in Figure 7 show that as the concentration of the LDS and LHS extracts increased, the percentage of intact erythrocytes decreased. The percentages of intact erythrocytes after treatment with the LMS extract and BZN showed small variations, indicating that they did not produce hemolysis at the concentrations studied.

The LDS and LHS extracts in concentrations higher than 50 μg/mL had a significantly higher hemolysis percentage than LMS, and the drug used in the treatment of Chagas’ disease, BZN, did not show a difference at any concentration. At 175 μg/mL, the LDS and LHS extract had 25.90% and 14.45% hemolysis, respectively, while amphotericin B, an antibiotic widely used in the treatment of severe infections, produced 100% hemolysis at a concentration of 100 μg/mL [20]. The data obtained by treating human erythrocytes with the extracts are related to the cytotoxicity on macrophages and the anti-T. cruzi activity, in which LDS was more toxic and more active than the other extracts of L. cultratus seeds.

but did not demonstrate this ability against T. cruzi. A study that explored a hexane extract from L. sericeus [32] did not find antimicrobial activity from this extract, which contained derricin and lonchocarpin.

Isocordoin obtained from Lonchocarpus xuul showed an IC50 of 26.7 μg/mL against T. cruzi epimastigotes [17], and despite being a purified compound, it showed less antiprotozoal activity than LDS and was similar to the LHS, which indicates that extracts from L. cultratus could be explored for antiprotozoal activity.

A study [33] demonstrated that chalcones isocordoin and 2',4'-dihydroxy-3'-(γ, γ-dimethallyl)-dihydrochalcone isolated from L. xuul roots are responsible for the anti-T. cruzi activity of this species. Thus, the activity of the LDS extract is presumed to be related to the presence of isocordoin detected in the 1H NMR spectra. On the other hand, another species of the genus Lonchocarpus, L. nicou, demonstrated leishmanicidal activity from a methanolic extract. However, its isolated compounds are not similar to those found here; they were shown to be stilbenes with potent action (IC50 5.5 and 3.9 μg/mL) [32]. So, comparing the effects of LHS and LDS against the epimastigotes forms of T. cruzi with the literature, the results showed better activity from the extracts than the isolated compounds, which demonstrates the synergetic effects of the components on the parasite.

Table 1 shows an inaccurate value of CC50 (> 300 μg/mL) for the LHS and LMS extracts. To obtain an exact number, it would be necessary to have used concentrations higher than those utilized, but the model
used to calculate was able to indicate a CC50 greater than 300 μg/mL, so it is plausible to admit this value as the cytotoxic concentration of both extracts since the cytotoxicity percentage demonstrated by two extracts in higher concentration was very low (19% - LMS and 21% - LHS).

The hexane extract of Lonchocarpus sericeus had higher toxicity than its separate components (derricin and lonchocarpin) in mammalian tumor cells, and isocordoin isolated from the hexane extract of Lonchocarpus xuul showed toxicity in vitro (CC₅₀ = 2.79 μg/mL) [32,33]. A study [25] confirmed this action on tumor cells with a hexane fraction of L. cultratus extract, which contained isocordoin, showing an intense activity against various human cell lines but without selectivity against normal human tissue. These data demonstrated that the toxicity observed in the LDS extract is due to the presence of isocordoin. Some studies [32,34] have demonstrated that lonchocarpin has low toxicity, which was confirmed here by the nontoxicity of the LHS extract.

Many compounds from different sources are responsible for hemolysis in vitro. Among these compounds are substances derived from plants [35], heavy metals [36], and pharmaceuticals [37]. The direct hemolytic effect of different toxic agents is due to a variety of nonspecific mechanisms. For example, surfactants cause hemolysis through the dissolution of the erythrocyte plasma membrane, which ruptures due to an increase in fragility or due to osmotic lysis caused by the increase in permeability of the plasma membrane [38]. On the other hand, reduced xenobiotic compounds, such as phenols, can promote hemolysis through oxidation of hemoglobin, forming methemoglobin [39]. Bilirubin promotes the loss of lipids in the erythrocyte plasma membrane, with exposure of the residues of phosphatidylserine [40]. Acetic acid, used in the present study as a positive control for the evaluation of hemolytic action, produces changes in the erythrocyte membrane, causing rupture and release of characteristic hemoglobin pigments.

A study [32] evaluated the hemolytic capacity of extracts from L. sericeus roots and some isolated metabolites (derricin and lonchocarpin) at a concentration of 250 μg/mL, which showed no hemolytic activity. Thus, it is evident that some of the other components present in the LDS and LHS extracts caused the hemolytic action since lonchocarpin was not able to do so.

Conclusions
L. cultratus extracts show anti-Trypanosoma cruzi activity, among which the LDS was the most active extract, compared to LHS and LMS, and this pronounced effect of LDS on T. cruzi is accompanied by higher toxicity on the cells (macrophages and erythrocytes).

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Annex – Supplementary Items

Supplementary Table 1. RMN $^1$H data of lonchocarpin and the LDS and LHS extracts from *Lonchocarpus cultratus*.

| Structure | Lonchocarpin | LDS | LHS |
|-----------|--------------|-----|-----|
| $\delta^1$H (J Hz) | $\delta^1$H (J Hz) | $\delta^1$H (J Hz) |
| 1         | -            | -   | -   |
| 2,6       | 7.62 (m)     | 7.65 (m) | 7.65 (m) |
| 3,5       | 7.40 (m)     | 7.40 (m) | 7.42 |
| 4         | 7.40 (m)     | 7.40 (m) | 7.42 |
| $\alpha$  | 7.54 (d, 15.5) | 7.57 (d, J) | 7.57 (d, J) |
| $\beta$   | 7.85 (d, 15.5) | 7.88 (d, J) | 7.88 (d, J) |
| C=O       | -            | -   | -   |
| 1'        | -            | -   | -   |
| 2'        | -            | -   | -   |
| 3'        | -            | -   | -   |
| 4'        | -            | -   | -   |
| 5'        | 6.37 (d, 8.9) | 6.39 (d, J) | 6.39 (d, J) |
| 6'        | 7.70 (d, 8.9) | 7.74 (d, J) | 7.73 (d, J) |
| 1''       | -            | -   | -   |
| 2''       | -            | -   | -   |
| 3''       | 5.57 (d, 10)  | 5.61 (d, J) | 5.60 (d, J) |
| 4''       | 6.74 (d, 10)  | 6.76 (d, J) | 6.76 (d, J) |
| 5''       | 1.45 (s)     | 1.48 | 1.47 |
| 6''       | 1.45 (s)     | 1.48 | 1.47 |
| OCH$_3$   | -            | -   | -   |
| 2'-OH     | 13.67 (s)    | 13.68 (s) | 13.68 (s) |

Lonchocarpin structure was obtained from literature [22,23]; LDS and LHS (500 MHz/ CDCl$_3$); LHS: hexane extract and LDS: dichloromethane extract from *L. cultratus*.

Supplementary Table 2. RMN $^1$H data of isocordoin and the LDS extract from *Lonchocarpus cultratus*.

| Structure | Isocordoin | LDS |
|-----------|------------|-----|
| $\delta^1$H (J Hz) | $\delta^1$H (J Hz) |
| 1         | -          | -   |
| 2,6       | 7.43 m     | -   |
| 3,5       | 7.43 m     | -   |
| 4         | 7.43 m     | -   |
| $\alpha$  | 7.60 d (15.4) | 7.60 d |
| $\beta$   | 7.89 d (15.4) | 7.90 d |
| C=O       | -          | -   |
| 1'        | -          | -   |
| 2'        | -          | -   |
| 3'        | -          | -   |
| 4'        | -          | -   |
| 5'        | 6.44 d (8.9) | 6.39 d |
| 6'        | 7.74 d (8.9) | 7.75 d |
| 1''       | 3.49 d (7.0) | 3.51 d |
| 2''       | 5.31 t (7.0) | 5.34 t |
| 3''       | -          | -   |
| 4''       | 1.85 s     | 1.85 s |
| 5''       | 1.78 s     | 1.78 s |
| 6''       | -          | -   |
| OCH$_3$   | -          | -   |
| 2'-OH     | 13.76 s    | 13.81 s |

Isocordoin structure was obtained from literature [23]; LDS (500 MHz/ CDCl$_3$); LDS: dichloromethane extract from *L. cultratus*. 
**Supplementary Figure 1.** Production of Nitric oxide (NO) by murine peritoneal macrophages treated with LDS (dichloromethane) (A), LHS (hexane) (B), and LMS (methanolic) (C) extracts from the seeds of *L. cultratus*. The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. The controls correspond to the medium, the medium-plus DMSO, and lipopolysaccharide (LPS) in the absence of extracts.