*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 $^1$H-RMN. *T. cruzi* forms were treated with increasing concentrations of the extracts and after, the percentage of inhibition and $IC_{50}$ or $LC_{50}$ 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* compared to the other two extracts, but the hexane showed the best selectivity index. The cytotoxicity observed in murine macrophages was confirmed in human erythrocytes, with dichloromethane extract having the highest toxicity. The methanolic extract showed the lowest anti-*T. cruzi* activity but was nontoxic to peritoneal murine macrophages and red blood cells.
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.

## 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 completed 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 $CDCl_3$ as the solvent and trimethylsilane (TMS) (Sigma-Aldrich, St. Louis, USA) as an internal reference. The chemical characterization of each extract (LHS, LDS, and LMS) was performed by ^{1}H NMR, comparing the ^{1}H 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*

**Figure 1.** Scheme of extraction of the LHS, LDS, and LMS extracts from the seeds of L. cultratus.
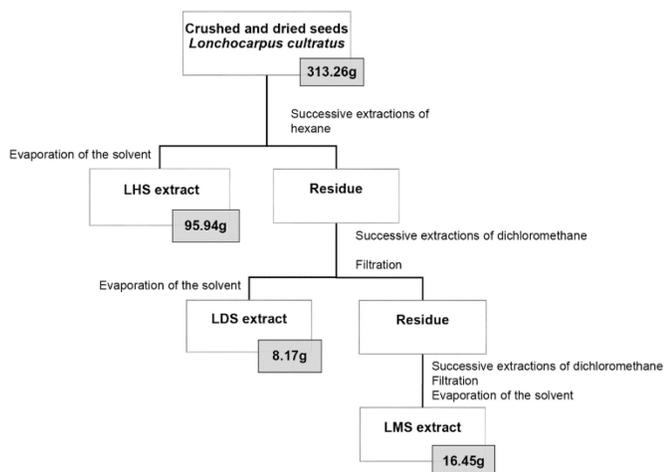
Y strain. The epimastigotes forms were maintained with weekly subcultures in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) (Laborclin, Curitiba, Brazil) and kept in an incubator at 28°C. Tissue culture trypomastigotes were obtained from the supernatants of 5- to 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% $CO_2$ 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 $1 \times 10^5$ 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 trypomastigote forms was determined in 96-well plates ($4 \times 10^5$ parasites/well) in RPMI medium supplemented with 10% FBS and 50 µg/mL of gentamycin in the absence or presence of different concentrations (1–175 µg/mL) of the plant extracts (LHS, LDS, and LMS). This study was performed in triplicate. Viable parasites were counted in a Neubauer chamber after 24 and 48 hours of incubation using an optical microscope at 400x. The controls were: untreated (UC), DMSO (DC), and treatment with the reference drug (BZN). The final volume in each well was 300 µL. BZN was used at concentrations between 1 and 175 µg/mL.

To evaluate the effects of *L. cultratus* extracts on the intracellular form of the parasite (amastigotes), peritoneal macrophages from BALB/c mice were harvested and seeded on round glass coverslips inside the wells of a 24-well plate at a density of $2 \times 10^5$ cells/well in RPMI 1640 medium supplemented with 10% inactivated FBS. The cells were allowed to adhere for three hours at 37°C in a 5% $CO_2$ atmosphere. Adhered macrophages were then infected with culture-derived trypomastigote forms at a ratio of 1:10 at 37°C for four hours. Afterward, no interiorized parasites were removed, and the infected culture was incubated for 48 hours in the absence or presence of the extracts at different concentrations. The following concentrations were used: LMS and LHS at 1–175 µg/mL and LDS at 1-15 µg/mL. For this last extract, only the lowest concentrations were used because of the toxicity presented at higher concentrations, as demonstrated in the subsection *Cytotoxic activity* in the Results section.. The cells were fixed and stained with Giemsa, and parasite infection was determined by direct counting of the total number of intracellular amastigotes per 200 cells in duplicate using an Olympus Model CBA optical microscope at 400x. The following controls were used: untreated (UC), DMSO (DC), and treatment with the reference drug (BZN). BZN was used at concentrations between 1 and 175 µg/mL.

The inhibitory concentration 50 ($IC_{50}$) was calculated for the epimastigotes and amastigotes forms, and the lethal concentration 50 ($LC_{50}$) was calculated for the trypomastigotes form, from the mean percentage reduction of parasites compared to that in the untreated controls. 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 $2 \times 10^5$ cells per well on a 96-well microplate and incubated in a $CO_2$ 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, $\lambda$ 550 nm). In the test, the UC and DC controls were used.

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

*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 benznidazole 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 r/minutes for 10 minutes. After that, the suspension of erythrocytes was incubated at 37°C for one hour with extracts and controls. Then, the cells were pelleted 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:

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

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

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 $NaNO_2$ curve (5–100 µM). Results were expressed as µmol per $2 \times 10^5$ cells.

**Figure 2.** [1]H 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 $\delta$ ppm.
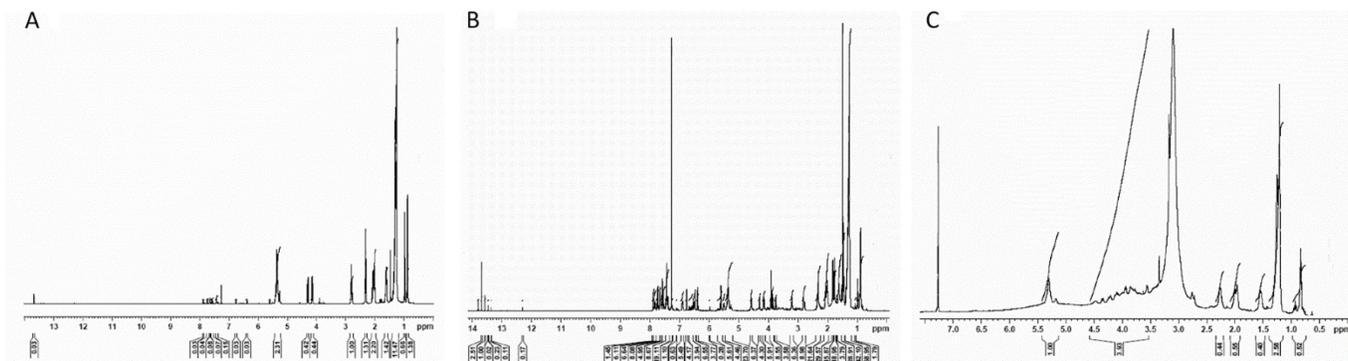
*Statistical analysis*

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 δ 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 δ 7.42-7.90 ppm are indicative of hydrogen linked to aromatic substances present in chalcones. Two signals exist at δ 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 δ13.81, indicative of a hydroxyl group chelated to C=O. In the low field of spectra, signals are seen at δ 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 δ 7.60 and 7.90 ppm, hydrogens are linked to carbons α and β 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 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 δ 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 δ 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, with a value of 26.7. However, LMS was 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.
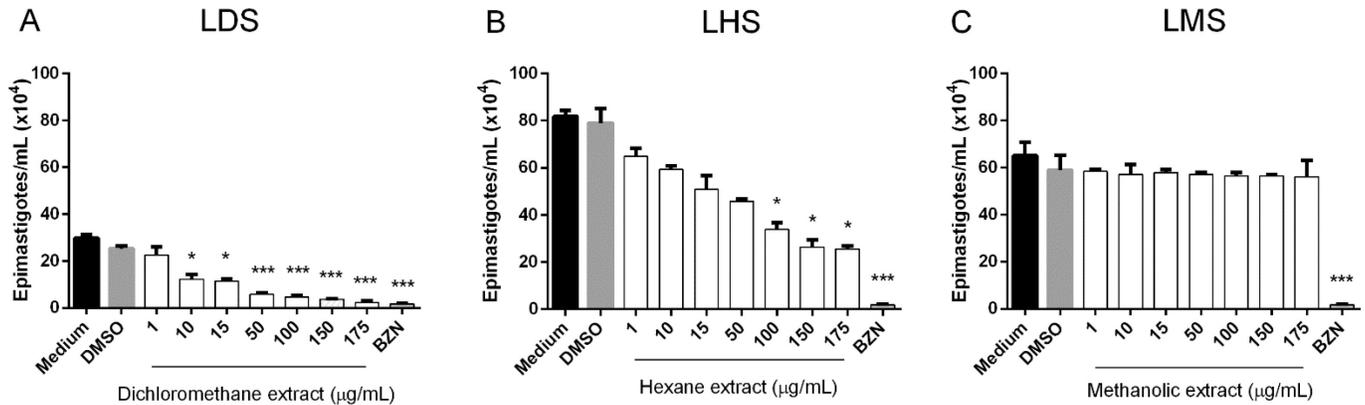


**Figure 5.** Effects of LDS (dichloromethane), LHS (hexane), and LMS (methanolic) extracts from the seeds of *L. cultratus* on *T. cruzi* amastigotes (a, b and c). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, *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.
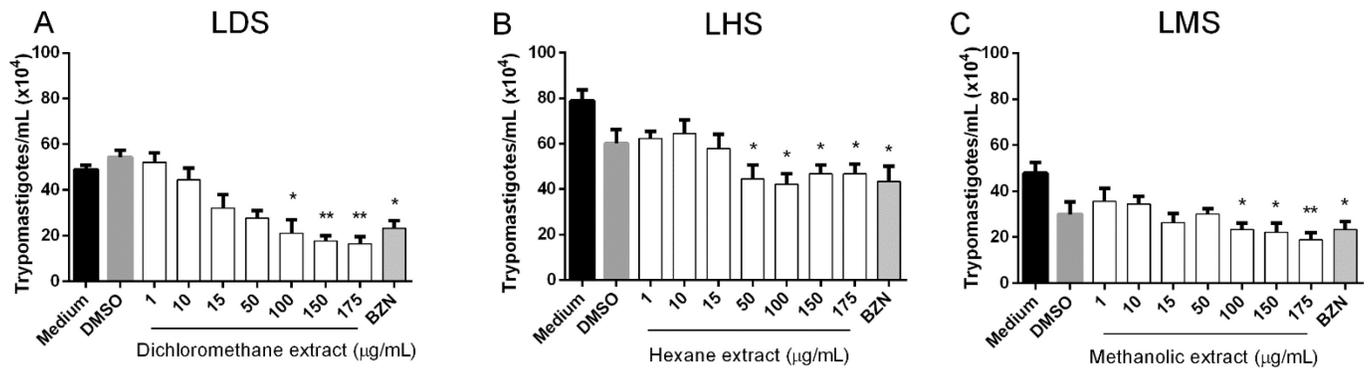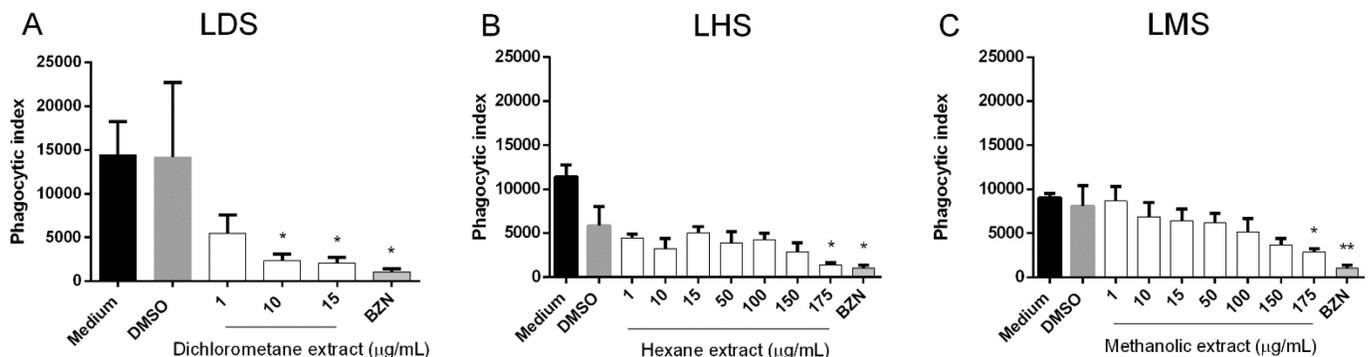
The hexane (LHS) and methanolic (LMS) extracts reached values of 15.1 and 26.1 µg/mL, which confirms what was observed in the epimastigotes forms, in which the LHS extract showed higher activity than LMS, which was inactive on the first form tested. The activity on trypomastigotes forms is interesting, 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 IC$_{50}$ 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 IC$_{50}$ (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 (CC$_{50}$) 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 epimastigotes forms and, although LHS and LMS had a low efficacy against amastigote

**Figure 6.** Effects of LDS (dichloromethane), LHS (hexane), and LMS (methanolic) extracts from the seeds of *L. cultratus* on macrophage viability (a, b and c). The effects of BZN on viability are shown in (d). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. The controls corresponding to the medium or the medium-plus DMSO in the absence of extracts.
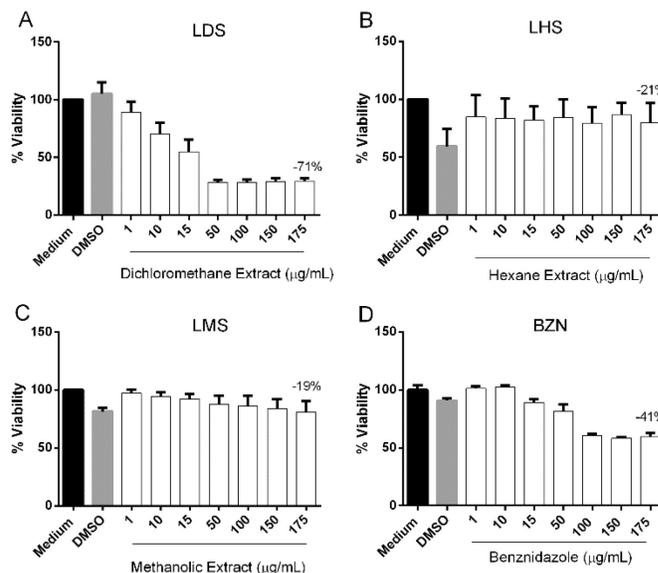


forms, both were able to distinguish these forms from mammalian cells.

The CC$_{50}$ 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 LHS extract had a CC$_{50}$ 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

**Table 1.** IC$_{50}$ and CC$_{50}$ data obtained from epimastigotes, trypomastigotes, amastigotes, and murine macrophages treated with LHS, LDS, and LMS extracts.

| Extracts | Epimastigotes (72 h) IC$_{50}$ (µg/mL) | Trypomastigotes (24 h) LC$_{50}$ (µg/mL) | Amastigotes (48 h) IC$_{50}$ (µg/mL) | Macrophages CC$_{50}$ (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): CC$_{50}$/IC$_{50}$

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.

## 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]

**Figure 7.** Effects of LDS (dichloromethane), LHS (hexane), and LMS (methanolic) extract from the seeds of *L. cultratus* and BZN (benznidazole) on erythrocyte integrity. The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different means of LHS and LDS to LMS and BZN, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.
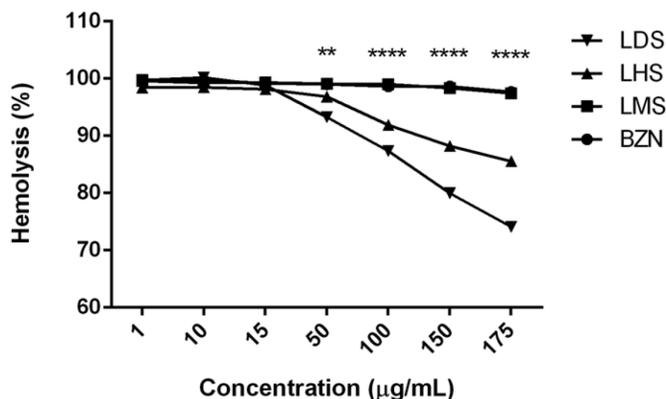


but did not demonstrate this ability against *T. cruzi*. A study that explored a hexane extract from *L. sericeus* [32] did not find antimicrobial activity from this extract, which contained derricin and lonchocarpin.

Isocordoin obtained from *Lonchocarpus xuul* showed an IC$_{50}$ 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 - dimethylallyl)-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 ${}^{1}$H 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 (IC$_{50}$ 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 synergic effects of the components on the parasite.

Table 1 shows an inaccurate value of CC$_{50}$ (> 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* ($CC_{50}$ 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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## References

1. Drugs for Neglected Diseases initiative (2020) Chagas disease. Avaliable: https://dndi.org/diseases/chagas/facts/. Accessed: 05 Mar 2020.
2. World Health Organization (2018) Chagas disease (American trypanosomiasis). Available: https://www.who.int/en/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis). Accessed: 12 February 2020.
3. Dias JCP, Cláudio LDG, Lima MM, Albajar-Viñas P, Albuquerque e Silva R, Alves RV, da Costa VM (2016) Changes in the paradigm of clinical and therapeutic management of Chagas' disease: progress and perspectives in the pursuit of comprehensive health. Epidemiol Health Serv 25: 1–10.
4. Traina M, Meymandi S, Bradfield JS (2016) Heart failure secondary to Chagas disease: an emerging problem in non-endemic areas. Curr Heart Fail Rep 13: 295–301.
5. Angheben A, Boix L, Buonfrate D, Gobbi F, Bisoffi Z, Pupella S, Gandini G, Aprili G (2015) Chagas disease and transfusion medicine: A perspective from non-endemic countries. Blood Transfus 13: 540–550.
6. Scarim CB, Jornada DH, Chelucci RC, de Almeida L, dos Santos JL, Chin CM (2018) Current advances in drug discovery for Chagas disease. Eur J Med Chem 155: 824–838.
7. Bern C (2015) Chagas' disease. N Engl J Med 373: 456–466.
8. Castro JA, DeMecca MM, Bartel LC (2006) Toxic side effects of drugs used to treat Chagas' disease (American Trypanosomiasis). Hum Exp Toxicol 25: 471–479.
9. Morillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi Jr. A, Rosas F, Villena E, Quiroz R, Bonilla R, Britto C, Guhl F, Velazquez E, Bonilla L, Meeks B, Rao-Melacini P, Pogue J, Mattos A, Lazdins J, Rassi A, Connolly SJ, Yusuf S (2015) Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. N Engl J Med 373: 1295–1306.
10. Bezerra WS, Meneguetti DUDO, Camargo LMA (2012) The pursuit of drugs for Chagas disease treatment (American trypanosomiasis): 103 years of neglect. Saúde 38: 9–20.
11. Sangenito LS, da Silva Santos V, d'Avila-Levy CM, Branquinha MH, Souza dos Santos AL, de Oliveira SSC (2019) Leishmaniasis and Chagas disease – neglected tropical diseases: treatment updates. Curr Top Med Chem 19: 174–177.

12. Saúde-Guimarães DA, Faria AR (2007) Natural compounds with anti-*Trypanossoma cruzi* activity. Braz J Pharmacogn 17: 455–465.

13. Cassidy CE, Setzer WN (2012) *Lonchocarpus* polyphenolics and their biological activities. Nat Prod J 1: 75–104.

14. Magalhães AF, Santos CC, Magalhães EG, Nogueira MA (2002) Detection of polyhydroxyalkaloids in *Lonchocarpus* extracts by GC-MS of acetylated derivatives. Phytochem Anal 13: 215–221.

15. Méllo JF, Lima OG, Albuquerque MM, Marini-Bettòlo GB, Lyra FD, Silva EC (1974) O and C prenylated chalcones with antineoplastic and antibiotic activities isolated from *Lonchocarpus neuroscapha* Benth. Rev Inst Antibiot 14: 39–50.

16. Menichini F, Delle Monache F, Marini Bettolo G (1982) Flavonoids and rotenoids from Tephrosieae and related tribes of Leguminosae. Planta Med 45: 243–244.

17. Borges-Argáez R, Balnbury L, Flowers A, Giménez-Turba A, Ruiz G, Watermanb PG, Peña-Rodríguez LM (2007) Cytotoxic and antiprotozoal activity of flavonoids from *Lonchocarpus* spp. Phytomedicine 14: 530–533.

18. Silva, MJ, Tozzi, AMGA (2012) Taxonomic revision of *Lonchocarpus s. str.* (*Leguminosae, Papilionoideae*) from Brazil. Acta Bot Bras 26: 357-377.

19. Reilly TP, Bellevue FH, Woster PM, Svensson CK (1998) Comparison of the *in vitro* cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochem Pharmacol 55: 803–810.

20. Paris AP, Persel C, Serafin CF, Simão RCG, Gandra RF (2016) Susceptibility of *Candida albicans* isolated from blood to *Wickerhamomyces anomalous* mycocins. Curr Microbiol 73: 878–884.

21. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite and 15N nitrate in biological fluids. Anal Biochem 126: 131–138.

22. Lima NM, Andrade JIA, Lima KCS, Dos Santos FN, Barison A, Salomé KS, Matsuura T, Nunez CV (2013) Chemical profile and biological activities of *Deguelia duckeana* A.M.G. Azevedo (Fabaceae). Nat Prod Res 27: 425–432.

23. Borges-Argáez R, Peña-Rodríguez LM, Waterman PG (2002) Flavonoids from two *Lonchocarpus* species of the Yucatan peninsula. Phytochemistry 60: 533–540.

24. Caamal-Fuentes EE, Peraza-Sánchez SR, Torres-Tapia LW, Moo-Puc RE (2015) Isolation and identification of cytotoxic compounds from *Aeschynomene fascicularis*, a mayan medicinal plant. Molecules 20: 13563–13574.

25. da Silva Landim EMBM, Ruiz ALTG, de Carvalho JE, Pomini AM, Pastorini LH, Santin SMO (2019) Antiproliferative activity and chemical constituents of *Lonchocarpus cultratus* (Fabaceae). Nat Prod Res. In press.

26. Osorio E, Arango GJ, Jiménez N, Alzate F, Ruiz G, Gutiérrez D, Paco MA, Giménez A, Robledo S (2007) Antiprotozoal and cytotoxic activities *in vitro* of Colombian Annonaceae. J Ethnopharmacol 111: 630–635.

27. Meira CS, Barbosa-Filho JM, Lanfredi-Rangel A, Guimarães ET, Moreira DRM, Soares MBP (2016) Antiparasitic evaluation of betulinic acid derivatives reveals effective and selective anti-*Trypanosoma cruzi* inhibitors. Exp Parasitol 166: 108–115.

28. Pizzo C, Faral-Tello P, Salinas G, Fló M, Robello C, Wipfe P, Mahler SG (2012) Selenosemicarbazones as potent cruzipain inhibitors and their antiparasitic properties against *Trypanosoma cruzi*. Med Chem Commun 3: 362.

29. Gonçalves de Lima O, de Méllo JF, de Barros Coêlho JS, de Andrade Lyra FD, Machado de Albuquerque M, Marini-Bettólo GB, Delle Monache G, Delle Monache F (1975) New prenylated chalcones from *Lonchocarpus neuroscapha* Benth. (*Cordoa piaca*). Farmaco Sci 30: 326–342.

30. Fontenele JB, Leal LKAM, Ferreira MAD, Silveira ER, Viana GSB (2005) Antiplatelet effect of lonchocarpin and derricin isolated from *Lonchocarpus sericeus*. Pharm Biol 43: 726–731.

31. Magalhães AF, Tozzi AMGA, Magalhães EG, Sannomiya M, Soriano MDPC, Perez MAF (2007) Flavonoids of *Lonchocarpus montanus* A.M.G. Azevedo and biological activity. An Acad Bras Cienc 79: 351–367.

32. Cunha GM de A, Fontenele JB, Nobre Júnior H V., de Sousa FCM, Silveira ER, Nogueira NAP, Moraes MO, Viana GSB, Costa-Lotufo LV (2003) Cytotoxic activity of chalcones isolated from *Lonchocarpus sericeus* (Pocr.) Kunth. Phyther Res 17: 155–159.

33. Borges-Argáez R, Vela-Catzín T, Yam-Puc A, Chan-Bacab MJ, Moo-Puc RE, Cáceres-Farfán M (2009) Antiprotozoal and cytotoxic studies on some isocordoin derivatives. Planta Med 75: 1336–1338.

34. Jeong YH, Park JS, Kim DH, Kang JL, Kim HS (2017) Anti-inflammatory mechanism of lonchocarpine in LPS- or poly(I:C)-induced neuroinflammation. Pharmacol Res 119: 431–442.

35. Gandhi VM, Cherian KM (2000) Red cell haemolysis test as an *in vitro* approach for the assessment of toxicity of karanja oil. Toxicol Vitr 14: 513–516.

36. Ribarov SR, Benov LC (1981) Relationship between the hemolytic action of heavy metals and lipid peroxidation. Biochim Biophys Acta 640: 721–726.

37. Yamamoto T, Tsurumaki Y, Takei M, Hosaka M, Oomori Y (2001) *In vitro* method for prediction of the phototoxic potentials of fluoroquinolones. Toxicol Vitr 15: 721–727.

38. Aparicio RM, García-Celma MJ, Pilar Vinardell M, Mitjans M (2005) *In vitro* studies of the hemolytic activity of microemulsions in human erythrocytes. J Pharm Biomed Anal 39: 1063–1067.

39. Bukowska B, Kowalska S (2004) Phenol and catechol induce prehemolytic and hemolytic changes in human erythrocytes. Toxicol Lett 152: 73–84.

40. Brito MA, Silva RFM, Brites D (2002) Bilirubin induces loss of membrane lipids and exposure of phosphatidylserine in human erythrocytes. Cell Biol andToxicology 18: 181–192.

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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*.

|  | Lonchocarpin | LDS | LHS |
|---|---|---|---|
|  | δ $^1$H (*J* Hz) | δ $^1$H (*J* Hz) | δ $^1$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 |
| α | 7,54 (d, 15,5) | 7,57 (d, *J*) | 7,57 (d, *J*) |
| β | 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*.

|  | Isocordoin | LDS |
|---|---|---|
|  | δ $^1$H (*J* Hz) | δ $^1$H (*J* Hz) |
| 1 | - | - |
| 2,6 | 7,43 m | 7,44 |
| 3,5 | 7,43 m | 7,43 |
| 4 | 7,43 m | 7,43 |
| α | 7,60 d (15,4) | 7,60 d |
| β | 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.