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Determination of chemical structure and anti-*Trypanosoma cruzi* activity of extracts from the roots of *Lonchocarpus cultratus* (Vell.) A.M.G. Azevedo & H.C. Lima

Aline Antunes Maciel Bortoluzzi [a,1], Izabela Virginia Staffen [a,1], Fernanda Weyand Banhuk [a], Aline Griebler [a], Patricia Karoline Matos [a], Thaís Soprani Ayala [a], Edson Antonio Alves da Silva [b], Maria Helena Sarragiotto [c], Ivânia Teresinha Albrecht Schuquel [c], Tereza Cristina Marinho Jorge [a], Rafael Andrade Menolli [a,*]

[a] Center of Medical and Pharmaceutical Sciences, Western Parana State University (UNIOESTE), 2069 Universitaria St., Cascavel, PR, Brazil
[b] Center of Exact Sciences and Technology, Western Parana State University (UNIOESTE), 2069 Universitaria St., Cascavel, PR, Brazil
[c] Center of Exact Sciences, Department of Chemistry, State University of Maringa (UEM), 5790 Colombo Av., Maringa, PR, Brazil

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ABSTRACT

Trypanosoma cruzi is the agent of Chagas disease, an infection that affects around 8 million people worldwide. The search for new anti-*T. cruzi* drugs are relevant, mainly because the treatment of this disease is limited to two drugs. The objective of this study was to investigate the trypanocidal and cytotoxic activity and elucidate the chemical profile of extracts from the roots of the *Lonchocarpus cultratus*. Roots from *L. cultratus* were submitted to successive extractions with hexane, dichloromethane, and methanol, resulting in LCH, LCD, and LCM extracts, respectively. Characterization of extracts was done using ^{1}H-RMN, ^{13}C-RMN, CC and TLC. Treatment of *T. cruzi* forms (epimastigotes, trypomastigotes, and amastigotes) with crescent concentrations of LCH, LCD, and LCM was done for 72, 48, and 48 h, respectively. After this, the percentage of inhibition and IC_{50}/LC_{50} were calculated. Benznidazole was used as a positive control. Murine macrophages were treated with different concentrations of both extracts for 48 h, and after, the cellular viability was determined by the MTT method and CC_{50} was calculated. The chalcones derricin and lonchocarpine were identified in the hexane extract, and for the first time in the genus *Lonchocarpus*, the presence of a dihydrolonchocarpine derivative was observed. Other chalcones such as isocordoin and erioschalcone B were detected in the dichloromethane extract. The dichloromethane extract showed higher activity against all tested forms of *T. cruzi* than the other two extracts, with IC_{50} values of 10.98, 2.42, and 0.83 μg/mL, respectively; these values are very close to those of benznidazole. Although the dichloromethane extract presented a cytotoxic effect against mammalian cells, it showed selectivity against amastigotes. The methanolic extract showed the lowest anti-*T. cruzi* activity but was non-toxic to peritoneal murine macrophages. Thus, the genus *Lonchocarpus* had demonstrated in the past action against epimastigotes forms of *T. cruzi* but is the first time that the activity against infective forms is showed, which leading to further studies with *in vivo* tests.

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1. Introduction

American trypanosomiasis is popularly known as Chagas disease, which is caused by the protozoan *Trypanosoma cruzi* (Trypanosomatidae). *T. cruzi* is a parasite of mammals and, although its transmission through insect vectors is common, infection also occurs via contaminated food, blood transfusion, and through the placental route and breast milk, with a high number of infected people in Latin America (Rassi Jr et al., 2012). Chagas' disease has recently become a problem in developed countries, driven by blood transfusion (Angheben et al., 2015).

Despite the significant rates of morbidity and mortality, investments in research, drug production, and transmission control for trypanosomiasis are poor (Canuto et al., 2015). The treatment of trypanosomiasis is active only during the phase in which the parasite can be found in the blood (i.e., the acute phase). Two medicines are currently available: benznidazole and nifurtimox.

In this context, the research for new trypanocidal drugs is relevant, since many natural products like alkaloids, terpenes, and phenols have revealed surprising useful anti-parasitic properties and selectivity (Sülsen et al., 2006).

Several studies with plants of the genus *Lonchocarpus* (Fabaceae) report anti-parasitic activities. Magalhães et al. (1996) reported on phenolic compounds with anti-*T. cruzi* activity in *L. xuul*. Chalcones similar to isocordoin have been reported in studies of the species *L. neuroscapha* (Mello et al., 1974).

L. cultratus is native of South America and in Brazil, popularly known as embira. There is sparse phytochemical and biological activity information about *L. cultratus*, though it also presents botanical classification problems with a large number of synonyms (Silva and Tozzi, 2012). Thus, considering the potential of anti-parasitic activities demonstrated in other species of the genus *Lonchocarpus*, we performed this study to identify the chemical components and investigate the anti-*T. cruzi* and cytotoxic activity of extracts from the roots of *L. cultratus*.

2. Material and methods

2.1. Plant material

Root 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 April 2016. The voucher specimen was deposited in the Herbarium of UNIOESTE (UNOP No. 20).

2.2. Plant extract

The roots collected from *L. cultratus* were dried in a circulating air incubator (<40 °C) and ground in a knife mill. Plant material obtained (849.85 g) was subjected to successive macerations under different solvents as follow in Fig. 1. First, it was extracted in hexane, filtered, and concentrated on a rotary evaporator under reduced pressure, yielding 10.05 g of hexane extract (LCH). The remaining plant residue after filtration was subjected to the above procedure using a dichloromethane solvent in place of hexane. This method yielded 6.24 g of dichloromethane extract (LCD). After that, the process was repeated using methanol as the extracting solvent and obtaining 85.28 g of methanolic extract (LCM).

2.3. Chemical characterization of extracts

Silica gel 60 (Merck) was used for column chromatography (CC) and silica gel 60 G and 60 GF-254 (Vetec) for thin-layer chromatography (TLC) analyses. Sephadex LH-20 (GE Healthcare) was also used. Nuclear magnetic resonance (NMR) spectra were obtained using Varian (Mercury Plus, BB 300 MHz) and Bruker (500 MHz) spectrometers at the Chemistry Department of the State University of Maringá-PR (UEM), Brazil using $CDCl_3$ as the solvent and trimethylsilane (TMS) as an internal reference.

The chemical characterization of each extract (LCH, LCD, and LCM) was performed by NMR, and the extracts with more activity in the previous anti-trypanosomal test were submitted to chromatographic analysis.

The extracts LCH (500 mg) and LCD (1.80 g) were separately subjected to column chromatography (CC) on silica gel, eluted in hexane: dichloromethane in different proportions. After analysis of thin-layer chromatograms and ^1H and ^{13}C NMR of the fractions, it was confirmed that the CC of LCH made it possible to obtain 26 mg of a mixture of three substances designated as LC-1, LC-2 and LC-3, and the isolation of 9 mg of additional LC-2. From the CC performed with the LCD extract, 7 mg of a mixture of two substances was obtained: LC-4 and LC-5. The LCM extract was not studied by chromatography.

2.4. Anti-Trypanosoma cruzi activity

The anti-*T. cruzi* activity of LCH, LCD and LCM plant extracts was performed by *in vitro* assays using epimastigote, trypomastigotes, and amastigotes of the *T. cruzi* Y strain. Epimastigote forms were maintained with weekly subcultures in Liver Infusion Tryptose (LIT) medium supplemented with 10% fetal bovine serum 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 supplemented with 10% FBS and 50 μg/mL gentamycin at 37 °C in a 5% humidified CO_2 atmosphere. Benznidazole (Lafepe) was used as a positive control, and dimethyl sulfoxide (DMSO) was used as the solvent for the plant extracts.

The assays with the epimastigote forms were performed with *T. cruzi* cultures in Falcon tubes (1×10^5 parasites/ml), in the presence of each of the LCH, LCD and LCM plant extracts dissolved in DMSO (up to a maximum of 0.6%). The following controls were used in the tests: untreated (UC), consisting of *T. cruzi* cultures in the LIT medium; DMSO (DC), consisting of *T. cruzi* and DMSO cultures (solvent of the plant extracts at a concentration of 0.6%) and treated with reference drug (BZN), consisting of benznidazole (the reference drug used to treat trypanosomiasis). The final volume in each tube was 3 mL. All tests were performed in triplicate, and BZN, LCH, LCD, and LCM were used at concentrations of 1, 10, 15, 50, 100, 150, and 175 μg/mL. The growth of *T. cruzi* after 72 h of culture was verified by direct counting of protozoa in a Neubauer chamber using an Olympus Model CBA optical microscope with a 400-fold magnification.

The anti-*T. cruzi* activity of LCH, LCD and LCM extracts against trypomastigote forms was performed in 96-well plates (4×10^5 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 (LCH, LCD, and LCM), in triplicate. Viable parasites were counted in a Neu-
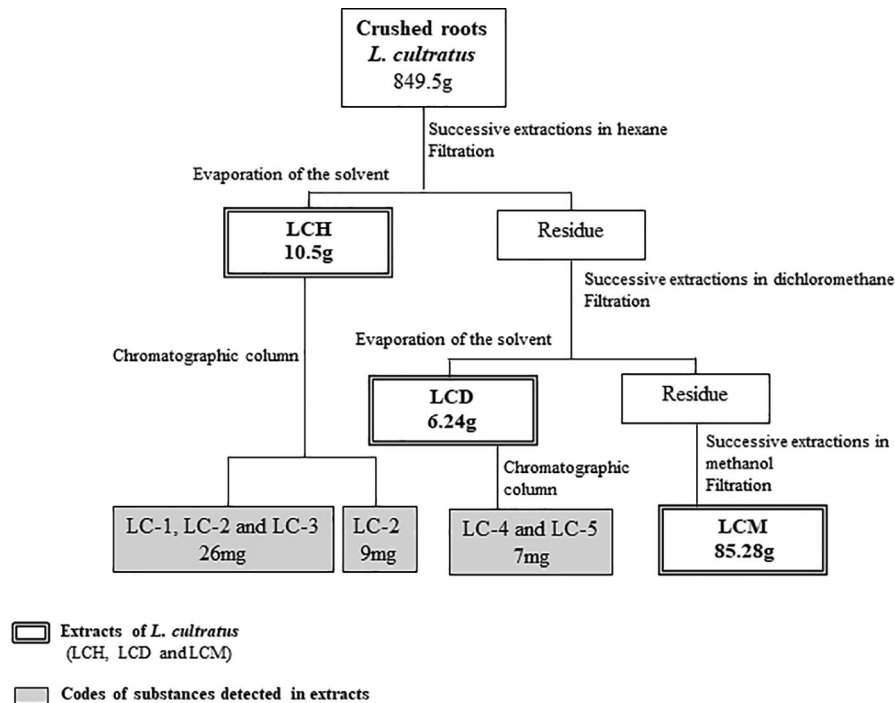
Fig. 1. Scheme of extraction of the LCH, LCD and LCM extracts from the roots of *L. cultratus*.

bauer chamber 24 and 48 h after incubation using an Olympus Model CBA optical microscope with a 400-fold magnification. Controls used here were the same used as before: untreated (UC), DMSO (DC), and treated with 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 intracellular amastigote forms, peritoneal macrophages from Balb/c mice were harvested and seeded at 2×10^5 cells/well on round glass coverslips inside the wells of a 24-well plate, in an RPMI 1640 medium supplemented with 10% inactivated FBS and allowed to adhere for 3 h at 37 °C in a 5% CO_2 atmosphere. Adhered macrophages were then infected with culture-derived trypomastigote forms using a 1:10 ratio at 37 °C for 4 h. Afterward, non-internalized parasites were removed by washing, and the infected culture was incubated for 48 h in the absence or presence of different concentrations of the extracts: LCM at 1–175 µg/L, LCH at 1–100 µg/mL and LCD at 1–15 µg/mL. For these two last extracts, only the lowest values were used because higher concentrations were found to be toxic to the macrophages (Fig. 7A-C). The cells were fixed and stained with Giemsa and the 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 with a 400-fold magnification. Controls used here were the same used as before: untreated (UC), DMSO (DC), and treated with the reference drug (BZN). BZN was used at concentrations of 1–175 µg/mL.

The trypanocidal effect of the extracts was calculated counting the number of parasites after 72 h (epimastigotes) and 48 h (trypomastigotes). To verify the action on amastigotes, the phagocytic index was used, which was calculated by multiplying the percentage of macrophages containing at least one ingested parasite by the number of amastigotes ingested by these macrophages.

The inhibitory concentration 50 (IC_{50}) was calculated for the epimastigote and amastigote forms, and the lethal concentration 50 (LC_{50}) was calculated for the trypomastigote form, based on the mean percentage reduction of parasites compared to the

untreated controls. Data were evaluated by non-linear regression in GraphPad Prism 6.0.

2.5. Cytotoxic bioassay

The toxicity of each plant extract (LCM, LCD, and LCH) was assessed in murine peritoneal macrophage cells, employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Macrophages were suspended in RPMI medium supplemented with 10% fetal bovine serum and gentamycin solution (50 µg/mL) in a CO_2 atmosphere (5%) at 37 °C, adjusted to 2×10^5 cells per well in 96-well microplates. These plates were incubated for 2 h before treatment and each well was washed with PBS. Then, each plant extract (LCM, LCD, and LCH) was dissolved in RPMI medium at concentrations of 1, 10, 15, 50, 100, 150 and 175 µg/mL and added to the plates. The plates were incubated for 48 h under the same conditions as above. After this time, 300 µL of the MTT reagent (5 mg/mL) was added to each well and an additional 3 h of incubation was carried out. Next, 150 µL of additional DMSO was added to solubilize the crystals that were formed, and this solution was read using a Biotek microplate reader, Model EPOCH (λ 550 nm). In the test, untreated control, consisting of RPMI medium, and DMSO control, consisting of RPMI medium and DMSO (solvent of plant extracts), were used. Triplicates were carried out. Cytotoxic activity was expressed as the concentration of extracts capable of killing 50% of macrophages (CC_{50}) after 48 h of treatment. The cytotoxic activity (CC_{50}) was calculated using non-linear regression in GraphPad Prism 6.0 and was related to the trypanocidal activity (IC_{50} and LC_{50}) by determining the corresponding selectivity index ($SI = CC_{50}/IC_{50}$ or LC_{50}).

2.6. Statistical analysis

The Kruskal-Wallis non-parametric test with Dunn's multiple comparisons post-test was used to compare the median number of parasites and the phagocytic index for the epimastigote, trypomastigote, and amastigote forms. The results were processed using

the software GraphPad Prism 6.0, at a level of significance of 95% ($p\text{-value} \leq 0.05$).

3. Results and discussion

3.1. Characterization of extracts

The LCH extract of *Lonchocarpus cultratus* root (10.50 g) was produced at a yield of 1.18%. The other extracts, LCD (6.24 g) and LCM (85.80 g) were obtained with yields of 0.73% and 10.03%, respectively.

Fig. 2 shows the ^{1}H NMR (500 MHz) spectra of the LCM, LCH, and LCD extracts.

The ^{1}H NMR spectrum shows that the signals obtained by LCH and LCD extracts indicate that they are constituted mainly by substances of low and medium polarity, such as those extracted by solvents used to obtain LCH (hexane) and LCD (dichloromethane). In the δ 0.8–2.0 ppm range in the spectra of the LCD and LCH extracts, there are signals corresponding to hydrogens of the –CH$_3$, –CH$_2$, and –CH groups. Signals in the region between δ 2.8 and 5.0 ppm indicate the presence of hydrogens linked to electronegative elements, such as O. In the region between δ 3.5 and 6.0 ppm, signals are corresponding to hydrogens in double-bonded carbons and the region between δ 6.0 and 7.8 ppm these are related to the presence of aromatic substances present in the constituents of the extracts. Finally, LCH signals in the range of δ 12.0–14.0 ppm indicate the presence of hydrogens in strong interactions, such as those of –OH chelated in intramolecular bridges, between the hydrogen of –OH and the carbonates of chalcones and flavonoids. The ^{1}H NMR spectrum of LCM extract showed no signals in high fields as in previous spectra (LCH and LCD), indicating the absence of chalcones. The signals of LCM were exhibited mainly in the region between 5.0 and 2.5 ppm, which are characteristic of polar hydrogen bonds to electronegative elements and in –CH, –CH$_2$, and –CH$_3$ groups in aliphatic compounds, suggesting the presence of terpenes.

Subsequently, the fractions obtained from chromatographic columns made with each LCH and LCD extract were analyzed by NMR. From LCH, a mixture of three substances was obtained, named LC-1, LC-2, and LC-3, as well as the isolation of an additional amount of LC-2. From the LCD extract, another mixture was obtained consisting of two substances named LC-4 and LC-5.

The Fig. 3 shows the chemical structures of chalcones identified in LCH and LCD extracts. The identification of LC-1, LC-2, LC-3, LC-4, and LC-5 was performed by comparing data of the respective NMR spectra with those described in the literature. From LCH, the chalcones LC-1 as derricin (C$_{21}$H$_{22}$O$_3$) (Gonçalves de Lima et al., 1975) and LC-2 as lonchocarpine (C$_{20}$H$_{18}$O$_3$) (Lima et al., 2013) were identified (Supplementary Table 1 and 2). LC-3 was identified as a dihydrolonchocarpine derivative (C$_{20}$H$_{20}$O$_3$), which had not yet been recognized in the genus *Lonchocarpus*.

In the chemical structure of LC-3, there is no double bond between Cα and Cβ, as in those of the derricin and lonchocarpine. The signals related to the hydrogens bound to these (Cα-Cβ) on LC-3 were the same as those for chalcone crotaramosmine (m; 3.23 ppm-Hα and m; 3.05 ppm-Hβ for LC-3 and 3.21 ppm-Hα and 3.01 ppm-Hβ for crotaramosmine). However, the comparison of ^{1}H and ^{13}C NMR data of crotaramosmine (Krohn et al., 2002; Rao et al., 1998) with those obtained for LC-3 showed that there is C-OH for crotaramosmine in C-4 and C-H for LC-3. The other values of the ^{1}H and ^{13}C NMR signals of crotaramosmine were the same as those of lonchocarpine, except for B-ring hydrogens and carbons, due to the presence of –OH in C-4. The value of 203.7 ppm (C=O) on the ^{13}C NMR spectrum of LC-3 was also con-

sistent with the absence of conjugation (Cα=Cβ). Then, LC-3 was identified as dihydrolonchocarpine (Supplementary Table 3).

In the LCD extract, two other chalcones were identified: LC-4 as isocordoin (C$_{20}$H$_{20}$O$_3$) (Borges-Argáez et al., 2002; Mello et al., 1974) and LC-5 as erioschalcone B (C$_{20}$H$_{22}$O$_3$) (Awouafack et al., 2008) (Supplementary Table 4).

Derricin and lonchocarpine are frequent in *Lonchocarpus*. These substances have already been isolated from the hexane fraction of *L. sericeus* barks (Fontenele et al., 2009). These chalcones have several biological activities such as cytotoxicity to leukemia cells, antiplatelet, analgesic and, reduction of activity of ATPase enzyme (da Silva Landim et al., 2019; Fontenele et al., 2009, 2001; Reyes-Chilpa et al., 2006), but any antimicrobial activity was found using these substances isolated (Cunha et al., 2003; Magalhães et al., 2007). This justifies the higher trypanocidal capacity of the LCD, as isocordoin was founded in it, a substance with known anti-*Trypanosoma* activity (Borges-Argáez et al., 2009, 2007).

The erioschalcone B present in the LCD extract was previously isolated from another species of genus *Lonchocarpus* (*L. xuul*) (Yam-Puc and Peña-Rodríguez, 2009) from its roots, using hexane and acetone as a solvent, but authors did not do any biological test.

3.2. Anti-Trypanosoma cruzi activity

Parasite counting after 72 h showed that the treatment with the LCH, LCD, and LCM extracts significantly reduced the number of epimastigotes at the highest concentrations (>100 µg/mL to LCH and LCD, and > 150 µg/mL to LCM) compared to the UC (Fig. 4A-C). However, the effects of LCH and LCD were more obvious regarding the number of protozoa, abruptly reducing the number of parasites, similar to BZN treatment (Fig. 4D).

Growth inhibition percentages were obtained to determine IC$_{50}$ values. The extracts from the roots of *L. cultratus* at the concentration of 175 µg/mL showed growth inhibition of 99.32%, 95.96%, and 36.06%, for LCD, LCH, and LCM, respectively. The BZN control showed a 97.96% growth inhibition at a concentration of 50 µg/mL. The DMSO control (DC) showed no significant difference (p-value 0.092) versus the UC group but was different compared to the groups treated with the extracts (p-value < 0.05). Thus, the solvent did not influence the anti-*Trypanosoma* activity of the extracts.

Table 1 shows the IC$_{50}$ values of the extracts. According to Osorio et al. (2007), the three extracts are classified as active against epimastigotes, i.e., an IC$_{50}$ between 10 and 50 µg/mL (LCD 10.98, LCH 15.01 and LCM 22.71). The isocordoin obtained from *Lonchocarpus xuul* showed an IC$_{50}$ of 26.7 µg/mL against *T. cruzi* epimastigotes (Borges-Argáez et al., 2009), and despite being a purified compound, it showed a higher IC$_{50}$ compared to the IC$_{50}$ of extracts such as LCD and LCH. Altogether, it indicates that more studies are necessary to establish if the action of the compounds presents in these extracts acts equally when either isolated or if the action is due to a synergistic effect of the compounds.

The trypomastigote forms showed a mortality rate against LCD, LCH, and LCM extracts of 95.84%, 88.89%, and 97.67% after 24 h of exposition and 100%, 91.17%, and 95.25% (after 48 h) at a concentration of 175 µg/mL. These percentages were very close to the one showed by Benznidazole (175 µg/mL), i.e. 70.91% (24 h) and 92.6% (48 h). All treatments with the extracts and BZN showed significant differences (Fig. 5A-D) in the mortality rate in relation to the UC at concentrations above 150 µg/mL, while the DMSO group didńt, indicating that the solvent was not responsible for the activity of the extracts.

The LC$_{50}$ values of the three extracts are shown in Table 1. LCD was very active against trypomastigotes with a value of 2.42 µg/mL, a slightly higher value than that observed with BZN (1.36 µg/mL). LCH and LCM reached values of 23.99 and 17.71 µg/mL, a
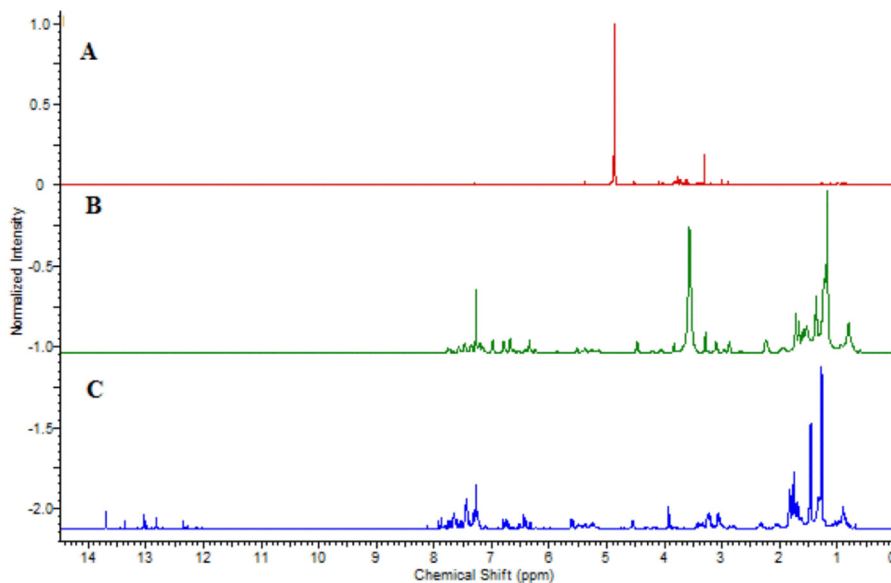
Fig. 2. ^{1}H nuclear magnetic resonance (NMR; 500 MHz) spectra of the LCH, LCD and LCM extracts from the roots of *L. cultratus*. A: methanolic extract (LCM) at CD$_3$OD as the solvent. B: dichloromethane extract (LCD) at CDCl$_3$ as the solvent. C: hexane extract (LCH) at CDCl$_3$ as the solvent. Chemical shifts are expressed in δ, ppm.
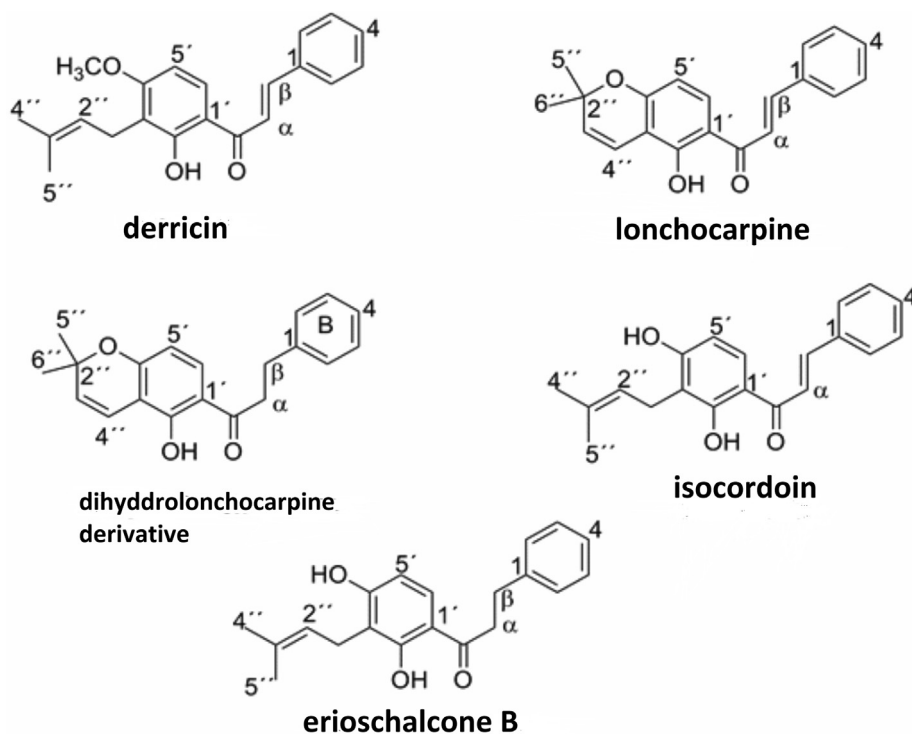

Fig. 3. Chemical structures of LC-1: derricin, LC-2: lonchocarpine, LC-3: dihydrolonchocarpine derivative, LC-4: isocordoin and LC-5: erioschalcone B.

trend that is opposite to that observed with the epimastigote form, on which the LCH extract showed greater activity than LCM. The activity against the trypomastigote form was very interesting since this is the infecting form and should be the target of the drugs.

The effects of the extracts on the intracellular form of *Trypanosoma cruzi* were evaluated by the phagocytic index (Fig. 6A-D), which showed that, at the highest concentrations of LCH and LCD, the tested substances could act internally on phagocytic cells to enhance the destruction of parasites. In macrophages treated with LCM, a tendency toward a lower phagocytic index was found,

but a statistically significant difference was not observed. The mechanism by which the extracts diminished the presence of amastigotes was not clear, but preliminary results (Supplementary Fig. 1) point to a direct effect on the amastigotes since these extracts were not able to induce the production of NO by murine macrophages. The reduction in the percentage of the amastigote form was calculated and found to be 73.99%, 59.9%, and 61% inhibition at the higher concentrations of LCD (15 µg/mL), LCH (100 µg/mL) and LCM (175 µg/mL). LCD and LCH were not tested at all concentrations since a high degree of toxicity was demonstrated in
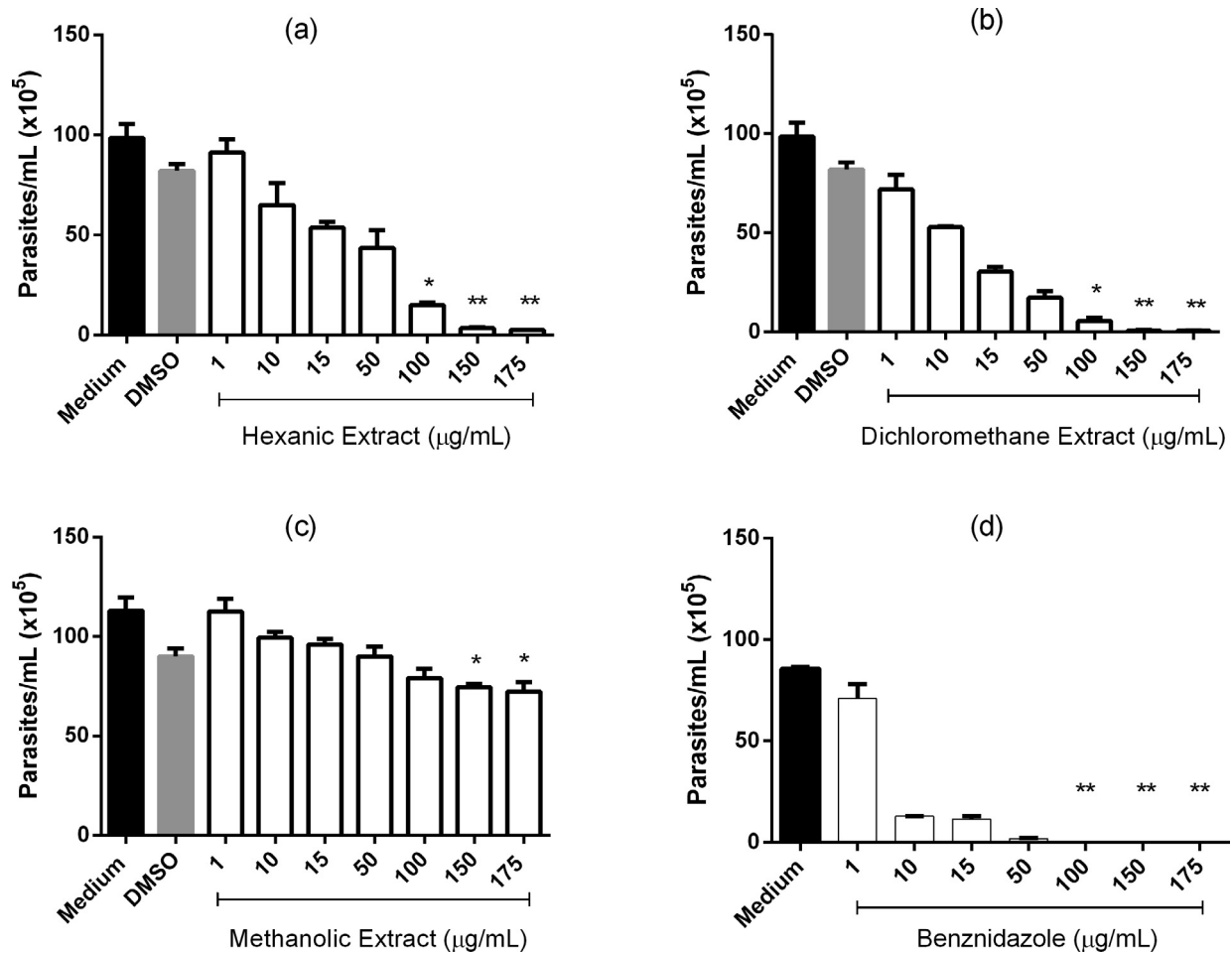
Fig. 4. Effects of LCH (hexane), LCD (dichloromethane), LCM (methanolic) extracts, and Benznidazole on *T. cruzi* epimastigotes (a, b, c, and d). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Control corresponds to the medium or medium plus DMSO in the absence of extracts.

Table 1
IC_{50} and CC_{50} data obtained from epimastigotes, trypomastigotes, amastigotes, and murine macrophages treated with LCH, LCD, and LCM extracts.

Extracts	Epimastigotes IC_{50} (μg/mL)	Trypomastigotes LC_{50} (μg/mL)	Amastigotes IC_{50} (μg/mL)	Macrophages CC_{50} (μg/mL)	SI (epi)	SI (trypo)	SI (ama)
LCH	15.01	23.99	6.89	46.68	3.11	1.95	6.78
LCD	10.98	2.42	0.83	31.14	2.84	12.87	37.52
LCM	22.71	17.71	14.54	>500	>22	>28	>34
BZN	3.13	1.36	0.61	>500	>159	>367	>819

LCH: hexane extract, LCD: dichloromethane extract, LCM: methanolic extract, and BZN: benznidazole (reference drug control). SI (selectivity index): CC_{50}/IC_{50}.

macrophages. BZN treatment led to a 94.3% reduction at 175 μg/mL. The DMSO was not different from the UC group, indicating that the solvent was not responsible for the activity of the extracts.

Table 1 shows the IC_{50} value against amastigotes. LCD was the more active at 0.83 μg/mL. LCH showed an intermediate value of 6.89 μg/mL and LCM was the least active at 14.54 μg/mL. The BZN IC_{50} value was 0.60 μg/mL.

Chemical studies on plant species of the genus *Lonchocarpus* have reported the isolation of secondary metabolites, especially in less polar extracts such as those obtained with petroleum ether, hexane, dichloromethane and chloroform (Magalhães et al., 2007). This information justifies the inhibition of *T. cruzi* observed with LCH and LCD extracts since these extracts showed a greater amount of active principles than LCM, but with more toxicity to macrophages.

Muelas-Serrano et al. (2000) demonstrated that aqueous extracts from the bark of *Mimosa tenuiflora* (Willd.) Poiret (Fabaceae Family), at a concentration of 100 μg/mL, led to weaker growth inhibition (GI) in *T. cruzi* epimastigotes (GI% = 18.28%) compared to the LCH, LCD and LCM extracts at the same concentration (GI% LCH: 81.67%, LCD: 93.57% and LCM: 29.94%), which indicates the importance of selecting the correct solvent to carry the major active compounds.

The Fabaceae family of *L. cultratus* has been studied regarding anti-protozoan effects, with some very active extracts (Ribeiro et al., 2015; Zhai et al., 1995); this justifies the search for new compounds in *Lonchocarpus* species. The chalcones of the genus *Lonchocarpus* have already demonstrated activity against trypanosome species. Salem and Werbovetz (2005) demonstrated that chalcones isolated from *Psorothamnus polydenius* (Fabaceae)
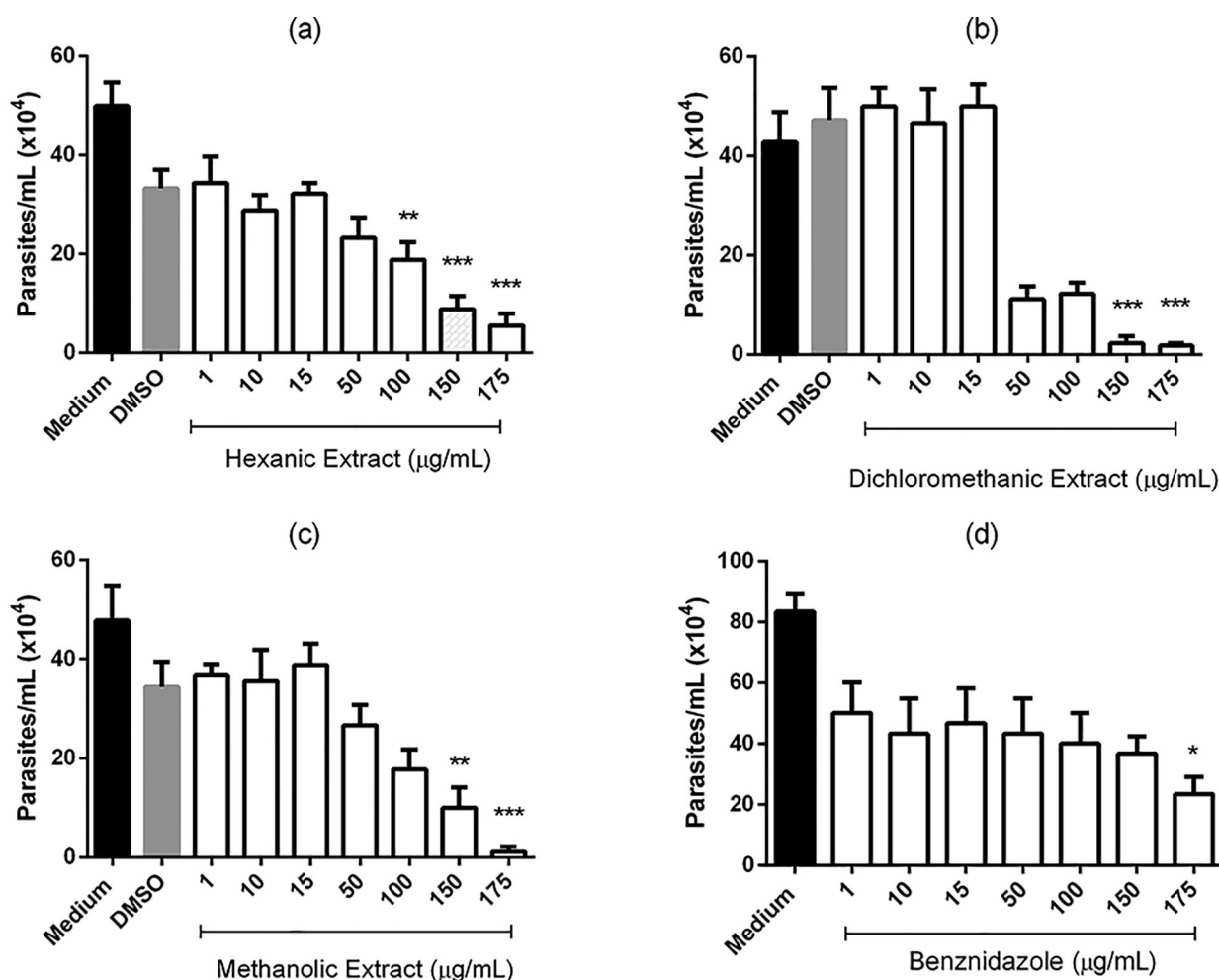
Fig. 5. Effects of LCH (hexane), LCD (dichloromethane), LCM (methanolic) extracts, and Benznidazole on *T. cruzi* trypomastigotes (a, b, c, and d). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Control corresponds to the medium or medium plus DMSO in the absence of extracts.

showed inhibitory effects (IC$_{50}$: 6.3 and 6.8 μg/mL) against *Trypanosoma brucei*, which causes trypanosomiasis popularly known as sleeping sickness. However, like the extracts obtained in the present study, toxicity to macrophages was observed (SI: 1.2 and 1.4).

Compound founded on LCD (isocordoin) had been evaluated as an isolated compound and demonstrated anti-*T. cruzi* and anti-*Leishmania* activity. IC$_{50}$ values against epimastigotes forms of *T cruzi* of isocordoin (IC$_{50}$ = 26.70 μg/mL) (Borges-Argáez et al., 2009) are higher than those obtained by the LCD (IC$_{50}$ = 10.98 μg/mL), shows that crude extract was more efficient against the protozoan *T. cruzi* than each substance alone, which could demonstrate a synergistic effect between compounds. Although there is an interest in the antiparasitic properties of chalcones of the genus *Lonchocarpus*, his ability against infective forms of *T. cruzi* (trypo and amastigotes) was not explored, once the literature does not report any study with these two forms. However, a study with trypomastigotes of *T. brucei* (dos Santos et al., 2009) showed the activity of isolonchocarpine (which was not found in *L. cultratus* extracts), with an IC$_{50}$ of 54.84 μg/mL, which is higher (LCH 23.99 and LCD 2.42 μg/mL) than that of the extracts obtained here from *L. cultratus* and with similar selectivity (isolonchocarpine 5.3, LCH 3.47 and LCD 5.10). Several activities were founded associated to the lonchocarpine; as the inhibition of the gastric ATPase

enzyme (Reyes-Chilpa et al., 2006), the inhibition of activation of microglial cells provided by the LPS (Jeong et al., 2017), and the inhibition of ROS production by astrocytes stimulating the antioxidant enzymes (Jeong et al., 2016). Isocordoin has been identified as a cytotoxic compound (da Silva Landim et al., 2019), with some antiproliferative activity, which could explain the toxicity founded in LCH and LCD extracts. The erioschalcone B present by LCD extract was previously isolated from *Eriosema glomerata* by Awouafack et al., 2008 and showed significant activity against several microorganisms, such as *Bacillus megaterium*, *Escherichia coli*, *Chlorella fusca*, and *Microbotryum violaceum*. Erioschalcone B has not been tested for anti-*T. cruzi* activity, but in this study, it was detected in an active extract (LCD).

Terpenes have been found in another species of *Lonchocarpus* (*L. muehlbergianus*) (de Oliveira et al., 2008) and the antiprotozoal ability of these metabolites has been identified in various genera of plants (Annang et al., 2016), with promising substances against *Leishmania* (Rosa et al., 2003), showing that the activity found in LCM should be explored further.

Any target on *T. cruzi* of chalcones present on *L cultratus* extracts was demonstrated, but one of the targets explored for drugs against *T. cruzi* is the inhibition of cruzain, the enzyme involved in the multiplication and intracellular differentiation of the protozoan (da Silva et al., 2016). It is critical for the development and
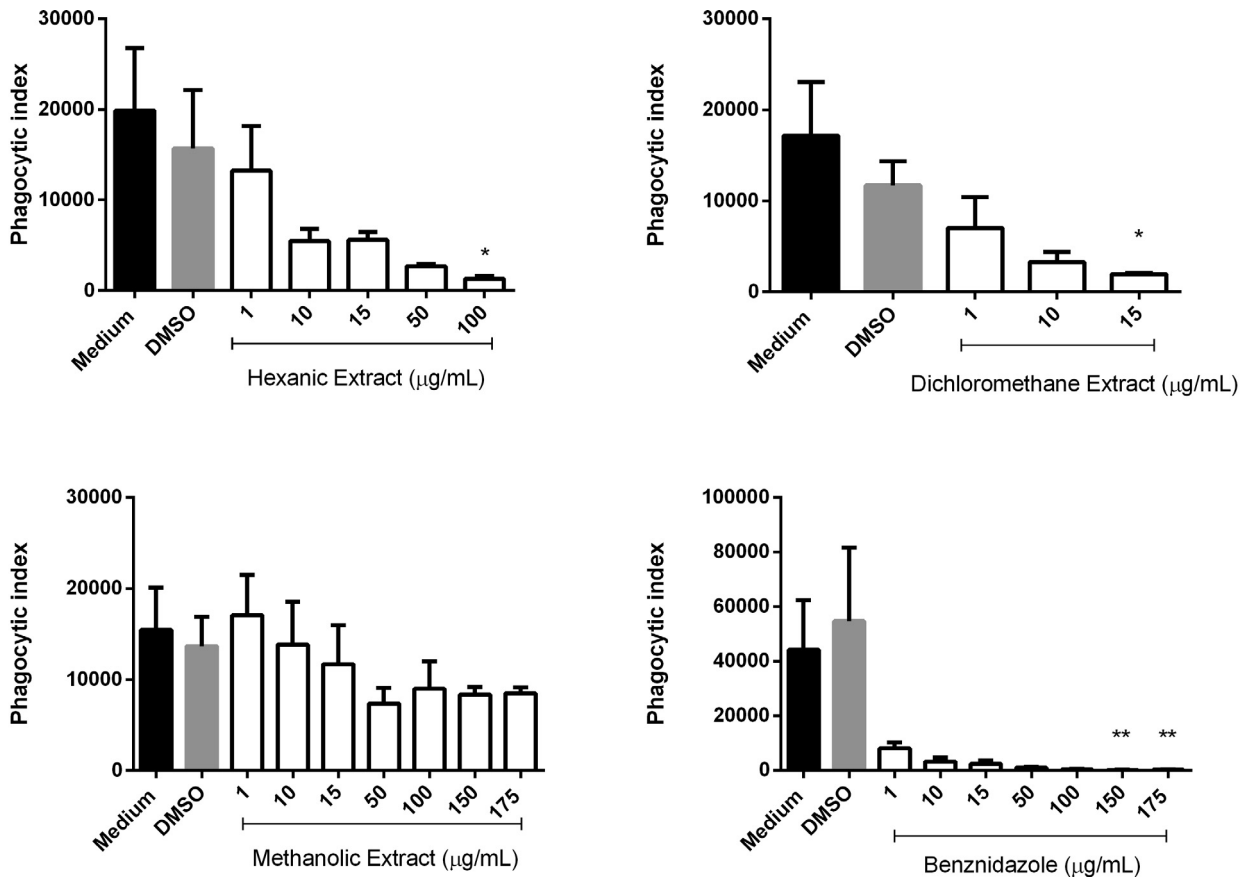
Fig. 6. Effects of LCH (hexane), LCD (dichloromethane), LCM (methanolic) extracts, and Benznidazole on *T. cruzi* amastigotes (a, b, c, and d).). The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. *Significantly different from control, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Control corresponds to the medium or medium plus DMSO in the absence of extracts.

survival of the parasite within host cells. Li et al. (1996) demonstrated the inhibition of the enzymatic function of cruzain through other chalcones, demonstrating their potential as candidates for new trypanocidal drugs. A study with licochalcone A (Zhai et al., 1995) showed that this compound interferes with mitochondrial activity in *Leishmania major,* another species of the Trypanosomatidae family, which may indicate the mechanism of action of the extracts from *L. cultratus* against *T. cruzi.*

3.3. Cytotoxic activity

To evaluate whether the LCH, LCD, and LCM extracts were cytotoxic to host cells, cytotoxic bioassays were performed with these plant extracts at the same concentration of anti-*T. cruzi* activity and the cytotoxic concentration for 50% of macrophages (CC_{50}) was calculated (Table 1).

Fig. 7 shows that the LCM extract was not cytotoxic because it did not interfere with the viability of the tested cells. These data provide the knowledge that the LCM extract was the one that best distinguished between macrophages and parasites (SI > 22, >28 and > 34, against epimastigotes, trypomastigotes, and amastigotes, respectively), although it was the extract with the lowest efficacy against *T. cruzi in vitro.* The LCH extract presented a CC_{50} of 46.68 μg/mL, which generated an SI of 3.11, 1.95, and 6.78 for the three forms of the parasite, indicating the highest toxicity between of the three extracts from *L. cultratus.* A comparison of these results with results from similar plant extracts indicates that LCH cytotoxicity is due to its components. Cunha et al. (2003)

showed that the hexane crude extract of *Lonchocarpus sericeus* had marked cytotoxicity in mammalian tumor cells (CC_{50} 17.6 μg/mL) and this cytotoxicity is due to derricin and lonchocarpin founded at crude extract.

The CC_{50} value of the LCD extract (31.14 μg/mL) showed toxicity to murine macrophages, resulting in different selectivity index (SI) according to parasite form, while LCD showed a low SI against epimastigote forms (3.11), it presented a better capacity to discern between mammalian cells and infective forms (trypo and amastigotes), with SI values of 12.87 and 37.52, respectively. BZN showed excellent selectivity, with an SI higher than 159 to all tested forms. According to the Program for Research and Training in Tropical Disease (TDR), WHO's criteria considers a compound as a drug hit for Chagas' disease with an $IC_{50} < 1$ μg/mL and a SI > 50 (Nwaka and Hudson, 2006), and despite extracts from *L. cultratus* are not purified, the LCD extract presented values against amastigote forms near of the criteria, while LCM showed no toxicity against murine macrophages and high SI, which shows that they are a promising starting point for further explorations.

4. Conclusion

Extracts from the roots of *L. cultratus* present an anti-*T. cruzi* activity and this activity is due to the presence of derricin, lonchocarpine, isocordoin, and terpenes present in these extracts, although the first three are responsible for the higher cytotoxicity found in LCH and LCD extracts.
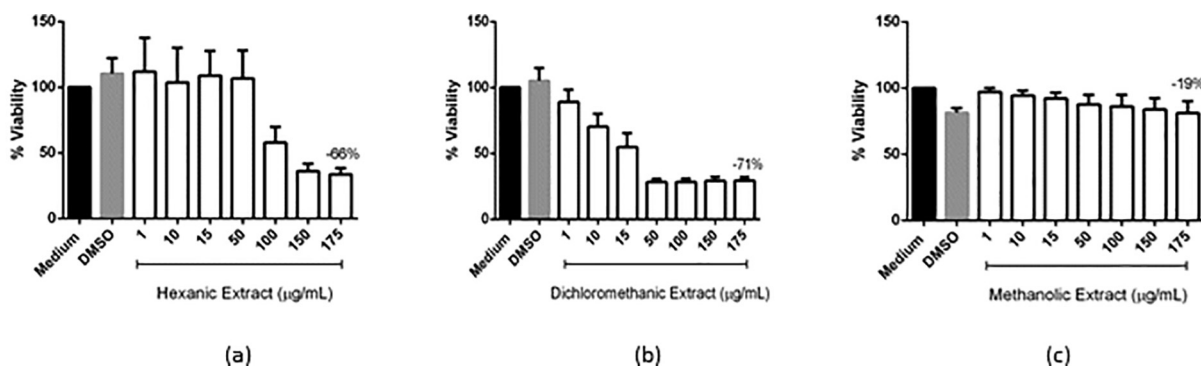
Fig. 7. Effects of LCH (hexane) (a), LCD (dichloromethane) (b), and LCM (methanolic) (c) extracts from the roots of *L. cultratus* on macrophage viability. The values shown are the mean ± SEM of three independent experiments, each performed in triplicate. Controls correspond to the medium or medium plus DMSO in the absence of extracts.

This study is preliminary but showed a potential for the development of new anti-trypanosomal drugs and suggests possible synergism between the constituents of these extracts, even though additional research should be conducted.

Ethical approval

All legal recommendations of Brazilian legislation (Law No. 11.794 Oct. 2008) for animal handling procedures for scientific research were used and this study was approved by the Animal Ethics Committee of Unioeste.

CRediT authorship contribution statement

Aline Antunes Maciel Bortoluzzi: Data curation, Investigation, Writing - original draft. **Izabela Virginia Staffen:** Data curation, Investigation, Writing - original draft. **Fernanda Weyand Banhuk:** Data curation, Investigation. **Aline Griebler:** Data curation, Investigation. **Patricia Karoline Matos:** Data curation, Investigation. **Thaís Soprani Ayala:** Formal analysis, Validation, Writing - review & editing. **Edson Antonio Alves da Silva:** Formal analysis, Validation. **Maria Helena Sarragiotto:** Formal analysis. **Ivânia Teresinha Albrecht Schuquel:** Investigation, Validation. **Tereza Cristina Marinho Jorge:** Conceptualization, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Rafael Andrade Menolli:** Conceptualization, Formal analysis, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2020.08.036.

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