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Cytotoxicity and anti-Leishmania amazonensis activity of Citrus sinensis leaf extracts


Graduate Program in Pharmaceutical Sciences, School of Pharmacy, Federal University of Rio de Janeiro, RJ, Brazil; Department of Natural Products, Farmanguinhos, Fiocruz, RJ, Brazil; Department of General Microbiology, Institute of Microbiology Paulo de Góes, Federal University of Rio de Janeiro, RJ, Brazil; Laboratory of Structural Biology and Electron Microscopy Platform Rudolf Barth, Oswaldo Cruz Institute (IOC), RJ, Brazil; Department of Botany, Institute of Biology, Federal University of Rio de Janeiro, RJ, Brazil; Department of Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro, RJ, Brazil

ABSTRACT

Context: Leishmania amazonensis is the main agent of diffuse cutaneous leishmaniasis, a disease characterized by lesional polymorphism and the commitment of skin surface. Previous reports demonstrated that the Citrus genus possess antimicrobial activity.

Objective: This study evaluated the anti-L. amazonensis activity of Citrus sinensis (L.) Osbeck (Rutaceae) extracts.

Materials and methods: Citrus sinensis dried leaves were subjected to maceration with hexane (CH), ethyl acetate (CEA), dichloromethane/ethanol (CD/Et = 1:1) or ethanol/water (CEt/W = 7:3). Leishmania amazonensis promastigotes were treated with C. sinensis extracts (1–525 μg/mL) for 120 h at 27°C. Ultrastructural alterations of treated parasites were evaluated by transmission electron microscopy. Cytotoxicity of the extracts was assessed on RAW 264.7 and J774.G8 macrophages after 48-h treatment at 37°C using the tetrazolium assay. In addition, Leishmania-infected macrophages were treated with CH and CD/Et (10–80 μg/mL).

Results: CH, CD/Et and CEA displayed antileishmanial activity with 50% inhibitory activity (IC50) of 25.91 ± 4.87, 54.23 ± 3.78 and 62.74 ± 5.04 μg/mL, respectively. Parasites treated with CD/Et (131.2 μg/mL) presented severe alterations including mitochondrial swelling, lipid body formation and intense cytoplasmic vacuolization. CH and CD/Et demonstrated cytotoxic effects similar to that of amphotericin B in the anti-amastigote assays (SI of 2.16, 1.98 and 1.35, respectively). Triterpene amyrins were the main substances in CH and CD/Et extracts. In addition, 80 μg/mL of CD/Et reduced the number of intracellular amastigotes and the percentage of infected macrophages in 63% and 36%, respectively.

Conclusion: The results presented here highlight C. sinensis as a promising source of antileishmanial agents.

Introduction

American tegumentary leishmaniasis (ATL) is a noncontagious infectious disease caused by parasites from the Leishmania genus, affecting the skin [cutaneous leishmaniasis (CL)] and mucosa [mucocutaneous leishmaniasis (MCL)] (Brazil, Ministry of Health 2010). In Brazil, L. guyanensis, L. braziliensis and L. amazonensis are the main dermotropic species. The last two species are widely distributed in the country, with cases of infection reported in all Brazilian states (Lima et al. 2017). In 2012, Brazil reported over 5000 new cases of tegumentary leishmaniasis (Carvalho et al. 2015). Leishmania amazonensis is also the aetiological agent of a much less common form of the disease known as diffuse cutaneous leishmaniasis (DCL), which is characterized by skin diffuse infiltration and the appearance of papules, tubers, infiltrated plaques and nodules (Barral et al. 1995).

Leishmaniasis treatment relies on the use of pentavalent antimonials (N-methylglucamine antimoniate and sodium stibogluconate). In addition, amphotericin B (liposomal and conventional) is used as an alternative chemotherapeutic drug in severe cases of unresponsive leishmaniasis. In uncomplicated CL cases, the use of antimony in combination with cryotherapy is often recommended (de Vries et al. 2015). Other drugs introduced for CL treatment include pentamidine and paromomycin. However, all of these drugs may lead to serious side effects, high toxicity or potentially induce parasite resistance (Berman 1998; Gontijo & Carvalho 2003; Jhingran et al. 2009).

Citrus sinensis (L.) Osbeck (Rutaceae), popularly known as sweet orange, is originally from Southern China, North Eastern India and Southern Asia (Ekwenye & Edeha 2010). It was first introduced in Brazil in the beginning of colonization. C. sinensis is a perennial tree of 5–10 m in height containing aromatic leaves, flowers and fruits (Lorenzi et al. 2006). It is widely cultivated for commercial purposes (fruit, juice, essential oil). Extracts obtained from its leaves or fruit peels have been used in folk medicine in many countries, including Brazil. In this context, this work aims to evaluate the antileishmanial activity of Citrus sinensis extracts, in order to provide an alternative to existing treatments.
medicine for the treatment of various ailments (Siddique et al. 2012). Previous studies have described diverse biological activities for different extracts and essential oils of C. sinensis, including antioestoporetic (Shalaby et al. 2011), antifungal, anti-aflatoxigenic, antioxidant (Sharma & Tripathi 2008; Singh et al. 2010) and antibacterial (Pittman et al. 2011) activities. Here, we evaluated the antileishmanial activity and toxic potential of different extracts obtained from the leaves of C. sinensis.

Materials and methods

Chemicals

The solvents used in extraction procedures were of spectroscopic grade from Tedia Brazil (Rio de Janeiro, RJ, Brazil). Dulbecco’s modified Eagle’s medium (DMEM), Schneider’s Drosophila medium, resazurin, amphotericin B and thiazyol blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from LGC Biotecnologia (São José, Cotia, Brazil).

Plant material collection and identification

Citrus sinensis leaves were collected in Nova Friburgo (State of Rio de Janeiro, Brazil) between the months of June and July 2014 from 9–10 a.m. Samples were authenticated by Dr. Rosana C. Lopes at the RFA Herbarium, Department of Botany, Federal University of Rio de Janeiro (IB/UFRJ), where a voucher specimen is deposited (n. 39990). Intact leaves of C. sinensis were dried at 40°C and kept intact under refrigeration (4°C) until extraction procedures.

Extraction procedures

Citrus sinensis dried leaves were crushed and subjected to static maceration at room temperature for 120 h with different solvents: hexane (100%), dichloromethane/ethanol (1:1), ethyl acetate (100%) or ethanol/water (7:3). After the maceration period, solvents were removed using a rotary evaporator at 40°C, giving rise to the CH, CD/Et, CEA and CEt/W extracts, respectively. The dried extracts were stored at 4°C protected from light. Stock solutions (100 mg/mL) of each extract were prepared using dimethyl sulfoxide (DMSO). Then, subsequent dilutions were made so that the final DMSO concentration did not exceed 0.5% in the biological assays.

L. amazonensis culture

Promastigote forms of L. amazonensis (10⁶ parasites/mL) were harvested at the early stationary phase, washed twice with 0.9% saline buffer and treated with CD/Et (the most promising extract from C. sinensis) at MIC and subMIC (MIC/2) concentrations. Parasites were incubated at 27°C for 24 h. Negative controls were made using untreated parasites. At the end of treatment, parasites were washed twice with 0.9% saline buffer and fixed with 0.9% glutaraldehyde solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 3.5% sucrose, pH 7.4) at 4°C for 60 min. The samples were sent to the Rudolf Barth Platform (FIOCRUZ/RJ) and processed as previously described (Rodrigues et al. 2013). Image acquisition was performed in a JEOL JEM1011 transmission electron microscope (JEOL Inc., Peabody, MA).

Cytotoxicity assay

Cytotoxicity of CH and CD/Et extracts (the most active extracts) against J774.G8 and RAW 264.7 macrophages was evaluated. Cells were maintained in polystyrene culture flasks containing DMEM culture medium supplemented with 10% FBS and cultured at 37°C and 5% CO₂. For the cytotoxic assays, 48-h cultured macrophages were washed twice with culture medium and then released from the culture flasks. A suspension of 10⁶ cells/mL was prepared, from which 100 μL was transferred to a 96-well microplate. After 24 h of incubation, cell cultures were submitted to different concentrations (15 to 260 μg/mL) of C. sinensis crude extracts and incubated for additional 48 h. The minimal cytotoxic concentration (MCC) was determined by the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (Mosmann 1983). Absorbance was measured at 570 nm in a SpectraMax M5 spectrophotometer (Molecular Devices, CA). The 50% cytotoxic concentration (CC₅₀) was calculated by logarithmic regression analysis using Microsoft Excel 2013. The cytotoxicity assays were performed in triplicate.

Analysis of C. sinensis CH and CD/Et extract by thin layer chromatography (TLC)

TLC was performed using silica gel plates and 7:3 hexane:acetate as solvent system. The purple spots in the chromatogram, incubated at 27°C for 120 h. At the end of the incubation period, resazurin at a concentration of 0.00083% was added to the plates and parasite viability was determined by measuring the absorbance at 490 and 595 nm in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA), as previously described (Rolón et al. 2006). Amphotericin B (reference drug) was used as a positive control (concentrations ranging from 0.031 to 1 μg/mL). The 50% inhibitory concentration (IC₅₀) was calculated by logarithmic regression analysis using Microsoft Excel 2013. Alternatively, the wells that showed no cellular growth had their supernatants collected, centrifuged and washed twice with 0.9% saline buffer. The resulting pellets were resuspended in fresh culture medium in the absence of the extracts and incubated at 27°C for 120 h in order to observe parasite growth. The lack of turbidity represented a leishmanicidal activity, while the presence of turbidity demonstrated that the extracts exerted a leishmanistatic effect. In addition, the lack or presence of growth was confirmed by light microscopy. The inhibitory assays were performed in triplicate.

Transmission electron microscopy (TEM)

Promastigote forms of L. amazonensis (10⁶ parasites/mL) were harvested at the early stationary phase, washed twice with 0.9% saline buffer and treated with CD/Et (the most promising extract from C. sinensis) at MIC and subMIC (MIC/2) concentrations. Parasites were incubated at 27°C for 24 h. Negative controls were made using untreated parasites. At the end of treatment, parasites were washed twice with 0.9% saline buffer and fixed with 0.9% glutaraldehyde solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 3.5% sucrose, pH 7.4) at 4°C for 60 min. The samples were sent to the Rudolf Barth Platform (FIOCRUZ/RJ) and processed as previously described (Rodrigues et al. 2013). Image acquisition was performed in a JEOL JEM1011 transmission electron microscope (JEOL Inc., Peabody, MA).
indicative of terpenoids, were visualized after reaction with vanillin-sulphuric acid reagent.

**Analysis of CH and CD/Et extract by gas chromatography-mass spectrometry (GC-MS)**

The TLC analysis showed that the main substances present in CH and CD/Et were terpenoids with low polarity and probably volatiles. Therefore, the extracts were analyzed by GC-MS to determine its components. The heating temperature was ramped from 70 °C to 305 °C at 5 °C/min, and the helium carrier gas was set to 1.5 mL/min. A 1% solution of each extract (1 μL) was injected in split mode. Mass spectra were obtained in an Agilent Technologies 6890 N system, adjusted with 5% diphenyl/95% dimethyl polysiloxane-fused silica capillary column (HP – 5 MS, 30 x 25 mm, 0.25 μm) operating in electron ionization mode at 70 eV with a scan mass range of 40–700 m/z. The identification of the extract components was made by comparing the obtained mass spectra with the apparatus Wiley library.

**Anti-intracellular amastigote activity**

Raw 264.7 macrophages were cultured in DMEM culture medium supplemented with 10% FBS at 37 °C and 5% CO₂ atmosphere. After 48-h culture, the cells were harvested, washed twice with PBS (150 mM NaCl; 20 mM phosphate buffer, pH 7.2) and allowed to adhere on coverslips placed in 24-well culture plates at the concentration of 2.0 x 10^5 cells/well. After 24-h incubation at 37 °C and 5% CO₂ atmosphere, *L. amazonensis* promastigotes were added in a ratio of 10 parasites/macroage, and cells were allowed to interact for 4 h. Then, free parasites were removed by washing the plates with PBS, and the cultures were incubated for additional 24 h in order to enable promastigotes differentiation into amastigotes. The *L. amazonensis*-infected cultures were treated with CH or CD/Et extracts (10 to 80 μg/mL) for 48 h. Alternatively, infected macrophages were treated with the reference drug amphotericin B (0.125 to 1.0 μg/mL). Finally, the coverslips were fixed and Giemsa-stained and the number of intracellular amastigotes/100 macrophages as well as the % of infected cells was determined by light microscopy. The IC₅₀ values were calculated as previously described in the antileishmanial activity assay. The anti-intracellular amastigote activity assays were performed in duplicate.

**Results and discussion**

**Inhibition of promastigote growth**

In the present study, four crude extracts obtained from *C. sinensis* were tested in order to determine the *in vitro* antileishmanial effect against *L. amazonensis*, the causative agent of cutaneous and diffuse leishmaniasis. Figure 1 shows the viability of *L. amazonensis* promastigotes after treatment with CH, CD/Et and CEA. CE/W was not active against the parasites. We observed a dose-dependent antileishmanial activity for the CH, CD/Et and CEA extracts. The MIC values for these extracts were 62.8, 131.2 and 256.2 μg/mL, respectively. It is worth mentioning that parasites treated with MIC values were no longer able to grow when they were reintroduced into fresh culture medium. This result demonstrates that CH, CD/Et and CEA extracts displayed leishmanicidal activity.

The 50% inhibitory concentrations (IC₅₀) for parasites treated with the extracts are shown in Table 1. CH and CD/Et presented the highest activity observed in the present study with IC₅₀ values of 25.91 and 54.23 μg/mL, respectively. Indeed, *C. sinensis* has been described as an interesting source of antimicrobial agents. Previous studies have shown that the *C. sinensis* essential oil was able to inhibit growth and spore germination of *Aspergillus* spp. (Sharma & Tripathi 2006) and *Penicillium* spp. (Martos et al. 2008). Mehmoold et al. (2015) reported the antibacterial activity of several *C. sinensis* crude extracts. In their study, the ethanol and methanol extracts displayed significant growth inhibition of *Escherichia coli*, *Klebsiella pneumonia* and *Serratia odorifera* (Mehmood et al. 2015). In regard to the antiprotozoal activity, Chenniappan and Kadarkarai (2010) demonstrated that the cyclohexane and methylene chloride extracts obtained from *C. sinensis* seeds were active against *Plasmodium falciparum* (IC₅₀ = 7.34 and 8.63 μg/mL, respectively). Bagavan et al. (2011) reported that the hexane and ethyl acetate extracts obtained from the plant peel displayed antiplasmodial activity at 42.13 and 25.67 μg/mL (IC₅₀ values), respectively. However, to date, reports
of *C. sinensis* extracts activity against *Leishmania* species are completely missing.

**Ultrastructure alterations**

Figure 2 shows the ultrastructure damage caused by CD/Et on *L. amazonensis* promastigotes. After 24-h treatment, parasites presenting a rounded body and cytoplasmic lipid droplets were observed (Figure 2(B,C)). Increased mitochondrial volume with loss of the matrix content followed by increased cytoplasmic vacuolization is shown in Figure 2(C,D). Flagellar pocket alterations, such as the presence of double flagella (Figure 2(B)) and vacuoles containing cytoplasmic matrix, were also noted (Figure 2(B–D)). Similar results were described in a previous study reporting the antileishmanial activity of *Arrabidaea chica* (HBK) Verlot, Bignoniaceae, hexane extract (Rodrigues et al. 2014). The lipophilic character and the similar composition (sterols, fatty acids, vitamin E, terpenes) of the extracts may explain their similar effects. However, in the present work, we observed intense lipid body formation in the promastigote cytoplasm. Mitochondrial dysfunction and lipid accumulation suggest that CD/Et treatment may lead to lipid metabolism disturbance, a mechanism of action widely described in the literature (Medina et al. 2012; Godinho et al. 2013; Stefanello et al. 2014). A clerodane diterpene obtained from the hexane extracts of *Polyalthia longifolia* var. *pendula* Linn (Annonaceae) leaves induced *L. donovani* ultrastructure alterations typical of apoptosis, including mitochondrial disturbance, cytoplasmic vacuolization, increased accumulation of lipid droplets and multivesicular bodies (Kathuria et al. 2014).

### Table 1. *In vitro* antileishmanial and cytotoxic activities of *C. sinensis* extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>PRO IC$_{50}$ (±SE)</th>
<th>AMA IC$_{50}$ (±SE)</th>
<th>J774.G8* CC$_{50}$ (±SE)</th>
<th>RAW 264.7** CC$_{50}$ (±SE)</th>
<th>SI* PRO</th>
<th>PRO AMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>25.91 (± 4.87)</td>
<td>39.78 (± 2.45)</td>
<td>35.75 (± 11.51)</td>
<td>86.17 (± 9.93)</td>
<td>1.4</td>
<td>3.32</td>
</tr>
<tr>
<td>CD/Et</td>
<td>54.23 (± 3.78)</td>
<td>51.0 (± 0.16)</td>
<td>123.79 (± 20.95)</td>
<td>101.07 (± 7.09)</td>
<td>2.3</td>
<td>1.86</td>
</tr>
<tr>
<td>CEA</td>
<td>62.74 (± 5.04)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CEt/W</td>
<td>na</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Amph. B</td>
<td>0.085 (± 0.001)</td>
<td>1.54 (± 0.42)</td>
<td>8.4 (± 0.44)</td>
<td>2.09 (± 0.69)</td>
<td>98.8</td>
<td>24.58</td>
</tr>
</tbody>
</table>

CH: *C. sinensis* hexane extract; CD/Et: *C. sinensis* dichloromethane/ethanol (1:1) extract; CEA: *C. sinensis* ethyl acetate extract; CEt/W: *C. sinensis* ethanol/water (7:3) extract; Amph. B: amphotericin B; PRO: promastigotes; AMA: amastigotes; SI: selective index; nd: not determined; na: not active; SE: standard error.
Cytotoxic effects of active extracts

The most active extracts, CH and CD/Et, were selected for cytotoxicity evaluation. CH minimal cytotoxic concentrations were 125.6 (CC50 = 35.75 µg/mL) and 125 µg/mL (CC50 = 86.17 µg/mL) against J774.G8 and RAW264.7 macrophages, respectively. CD/Et presented minimal cytotoxic concentrations of 262.5 (CC50 = 123.79 µg/mL) and 250 µg/mL (CC50 = 101.07 µg/mL), respectively. The essential oil obtained from sweet orange peel displayed cytotoxic activity at 5.51 g/mL (CC50) against HeLa cells (Liu et al. 2012). Here, the leaf extracts CH and CD/Et were less toxic for both macrophage lineages tested. Nonetheless, considering the intracellular amastigote-based SI, CH and CD/Et exhibited low selectivity. However, the cytotoxic effect was similar to the reference drug amphotericin B (SI = 2.16, 1.98 and 1.35, respectively) (Table 1). Recently, the hexane extracts of different Hypericum species (Hypericaceae), H. linoides A. St.-Hil., H. carinatum Griseb. and H. polyanthemum Klotzsch ex Reichardt were described as low cytotoxic for murine macrophages, presenting SI at 1.2, 1.8 and 4, respectively (Dagnino et al. 2015).

CH and CD/Et effects on macrophage infection

Studies evaluating the efficacy of drug candidate substances against the intracellular stage of Leishmania are necessary, since this is the infective form for vertebrate hosts. CH and CD/Et were most active when compared to the other extracts. Thus, CH and CD/Et were selected for the evaluation of anti-intracellular amastigote activity. CH (40 µg/mL) was able to reduce the number of internalized parasites as well as the percentage of infected macrophages in 36.2% and 16.6%, respectively (Figure 3(A)). On the other hand, CD/Et displayed better results. We observed a reduction in the amastigote numbers of 16, 46 and 63% when cells were treated with 10, 20 and 80 µg/mL, respectively (Figure 3(B)). In addition, the percentage of infected macrophages reduced in 33% when cells were treated with 80 µg/mL of CD/Et. Amphotericin B was able to reduce macrophage infection by 48.5% when cells were treated with 1 µg/mL (Figure 3(C)).

Phytochemical profile of CH and CD/Et

Based on the preliminary analysis by TLC, which revealed the presence of volatile terpenoids, we subjected CH and CD/Et to GC-MS. The main substances present in the extracts are listed in Table 2. Among those, the triterpene α-amyrin, the major component found in the extracts, has been previously described as an antiprotozoal agent. Mwangi et al. (2010) reported the antiprotozoal activity of α-amyrin isolated from Teclea trichocarpa Enge. (Rutaceae) against P. falciparum and L. donovani (amastigote) with IC50 values of 0.96 and 7.90 µg/mL, respectively. The authors also demonstrated that α-amyrin presented CC50 values for myoblast (L-6) cells >90 µg/mL. In a previous study, the acetylated form of β-amyrin showed antileishmanial activity against L. amazonensis amastigotes with IC50 values of 14 µg/mL (Schinor et al. 2007). Those results further support the antileishmanial potential of triterpene amyrins.

### Table 2. Chemical composition of the Citrus sinensis CH and CD/Et extracts.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CH</th>
<th>CD/Et</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Amyrin</td>
<td>13.9</td>
<td>1.09</td>
</tr>
<tr>
<td>Dihydroactinidiolide</td>
<td>17.3</td>
<td>–</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>18.4</td>
<td>–</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>18.6</td>
<td>–</td>
</tr>
<tr>
<td>β-Sinensal</td>
<td>21.0</td>
<td>–</td>
</tr>
<tr>
<td>6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-2-naphthalenol</td>
<td>21.7</td>
<td>–</td>
</tr>
<tr>
<td>Neophytadiene</td>
<td>24.0</td>
<td>2.83</td>
</tr>
<tr>
<td>Hexahydrofarnesyl acetone</td>
<td>24.2</td>
<td>1.01</td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>25.9</td>
<td>0.56</td>
</tr>
<tr>
<td>Ethyl palmitate</td>
<td>27.2</td>
<td>1.32</td>
</tr>
<tr>
<td>Phytol</td>
<td>29.3</td>
<td>1.71</td>
</tr>
<tr>
<td>Ethyl linoleate</td>
<td>30.3</td>
<td>1.61</td>
</tr>
<tr>
<td>4,8,12,16-Tetramethylheptadecan-4-olide</td>
<td>33.4</td>
<td>1.49</td>
</tr>
<tr>
<td>Eicosane</td>
<td>38.9</td>
<td>7.16</td>
</tr>
<tr>
<td>Squalene</td>
<td>43.3</td>
<td>–</td>
</tr>
<tr>
<td>Octodecane</td>
<td>44.4</td>
<td>4.83</td>
</tr>
<tr>
<td>Tocopherol</td>
<td>44.6</td>
<td>0.62</td>
</tr>
<tr>
<td>Nonacosane</td>
<td>44.8</td>
<td>–</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>45.3</td>
<td>4.24</td>
</tr>
<tr>
<td>Campesterol</td>
<td>45.7</td>
<td>0.90</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>46.1</td>
<td>1.33</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>46.9</td>
<td>6.97</td>
</tr>
<tr>
<td>Docosane</td>
<td>47.1</td>
<td>–</td>
</tr>
<tr>
<td>3-β-Lanost-8-en-3-ol</td>
<td>47.2</td>
<td>2.07</td>
</tr>
<tr>
<td>β-Amyrin</td>
<td>47.3</td>
<td>7.63</td>
</tr>
<tr>
<td>Lupen-3-one</td>
<td>47.4</td>
<td>2.35</td>
</tr>
<tr>
<td>α-Amyrin</td>
<td>47.9</td>
<td>30.5</td>
</tr>
<tr>
<td>Friedelin</td>
<td>49.6</td>
<td>4.07</td>
</tr>
<tr>
<td>Identified compounds</td>
<td>92.21</td>
<td>84.34</td>
</tr>
</tbody>
</table>

Rt: retention time; CH: C. sinensis hexane extract; CD/Et: C. sinensis dichloromethane extract.
investigation of *C. sinensis* as a promising source of antileishmial agents.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**ORCID**

Mariana M. B. Azevedo [http://orcid.org/0000-0001-8997-1206](http://orcid.org/0000-0001-8997-1206)

Alane B. Vermelho [http://orcid.org/0000-0001-5926-4172](http://orcid.org/0000-0001-5926-4172)

Igor A. Rodrigues [http://orcid.org/0000-0001-9432-3991](http://orcid.org/0000-0001-9432-3991)

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**Conclusions**

Here, we demonstrated the antileishmanial potential of *C. sinensis* extracts. The hexane (CH) and dichloromethane/ethanol (CD/Et) extracts displayed potential activity against *L. amazonensis* promastigotes and intracellular amastigotes. In addition, the extracts presented cytotoxic effect at the same level of the reference drug. The stereoisomeric α- and β-amyrins content of the extracts (over 38.13 and 33.12%, respectively) may be responsible for their antileishmanial activity. Nevertheless, further investigation will be necessary in order to establish the bioactive substance(s) found in CH and CD/Et. This study highlights the

**Figure 3.** Effect of *C. sinensis* on *L. amazonensis* intracellular amastigotes. Raw 264.7 macrophages previously infected with *L. amazonensis* promastigotes were treated with *C. sinensis* extracts, CH (A) and CD/ Et (B), or with the reference drug amphotericin B (C) for 48 h. The number of internalized amastigotes per 100 macrophages was determined by direct count under a light microscope. Each bar represents the mean ± standard error of at least two independent experiments. Statistical analysis of the differences between mean values obtained for the experimental groups was done by Student’s *t*-test. *p*-Values ≤ 0.05 (one asterisk), ≤ 0.005 (two asterisks) and ≤ 0.0001 (three asterisks) were considered significantly different from that for the control.


