Antimicrobial activity of leaf extracts and isolated constituents of Croton linearis

Antimicrobial activity of leaf extracts and isolated constituents of *Croton linearis*

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Abstract

Ethnopharmacological relevance: Leaves of *Croton linearis*, known as "rosemary", are widely used in folk medicine in Caribbean countries to treat fever and colds (associated to infections). *Aim of the study*: To evaluate the *in vitro* antimicrobial activity of extracts and fractions derived from *C. linearis* leaves. *Materials and methods*: Bioassay-guided fractionation and isolation of compounds from an ethanolic extract of *C. linearis*, using flash chromatography and semi-preparative HPLC-DAD-MS (High Performance Liquid Chromatography – Diode Array Detection – Mass Spectrometry). Isolated compounds were characterized by MS, 1D- and 2D-NMR (Nuclear Magnetic Resonance) spectroscopy. The microdilution method with resazurin, as well as direct counting with an optical microscope, were used to determine the *in vitro* antimicrobial activity against bacteria, fungi and parasites. Moreover, the cytotoxicity on human fetal lung fibroblasts (MRC-5) was evaluated. *Results*: The total extract and chloroform fraction (Cl-F) showed high activity against protozoa with IC_{50} values ranging from 1 to 26 µg/mL, but also cytotoxicity on MRC-5 and PMM (Peritoneal Murine Macrophages). Seven compounds were isolated and characterized for first time in this species: the alkaloids laudanidine, laudanosine, reticuline, corydine, glaucine and cularine and the flavonoid glycoside isorhamnetin-3-*O*-(6"-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside. Reticuline showed a weak activity against *L. infantum* (IC_{50} 148.0 ± 1.2 µM), while the flavonoid was active against *T. cruzi* (IC_{50} 35.6 ± 2.3 µM). *Conclusions*: The results show the antiprotozoal potential of the extract and some isolated constituents, which supports the use of this species in Caribbean folk medicine.

Key words: *Croton linearis*, antimicrobial, cytotoxicity, bioassay-guided isolation, isoquinoline alkaloids

1 Introduction

The World Health Organization reported that infectious diseases are responsible for one third of global mortality and that they still pose a major threat to public health in numerous countries (WHO, 2017). Their impact is more evident in developing countries due to the limited accessibility to medicines and the emergence of widespread drug resistance (Macedo et al., 2015). The increasing resistance of microorganisms to available antimicrobial agents is one of the major concerns for scientists and clinicians worldwide, and has become a global problem in the last years (Abad et al., 2012).

Over the past decade, interest in the search for antimicrobial natural products has risen (Ahmad et al., 2006). From all possible sources of natural products, plants are considered as the most promising ones, since they can be selected on the basis of their ethnopharmacological uses and obtained quite easily (Macedo et al., 2015). Nevertheless, it is important to provide scientific evidence that supports such popular uses. Indeed, in some cases the isolation of the major constituents did not prove the ethnobotanical use, but revealed other pharmacological activities (Heinrich et al., 2012).

The genus *Croton* is one of the largest of the family Euphorbiaceae (Almeida-Pereira et al., 2017, Salatino et al., 2007). The pharmacological effects reported for *Croton* species have been attributed to different types of compounds, but mainly to alkaloids, terpenoids (diterpenes, triterpenoids and steroids), proanthocyanidins, flavonoids, and other phenolic compounds (Marcelino et al., 2012).

Croton linearis Jacq. is a shrub that grows on the Southern coast of the Eastern region of Cuba and it is widely used by people to treat fever associated with infections (Roig, 1974). In other Caribbean countries, ethnobotanical reports refer to its effectiveness as insecticidal and sedative

herbal drug (Mitchell and Ahmad, 2006), to reduce pain and to treat colds (Holt-McCormack et al., 2011). It is used by Jamaican peasants as a hair wash, in baths for fever and colds, but also as a tea (Asprey and Thornton, 1955). However, there are only few phytochemical and pharmacological investigations to support these ethnobotanical reports. Several alkaloids were isolated from this species (Haynes et al., 1967, 1968; Farnsworth et al., 1969). Additionally, the insecticidal effect of a diterpene, isolated from leaves, was reported (Alexander et al., 1991) More recently, the antileishmanial activity of the essential oil of leaves was reported by our research group (García et al., 2018).

Considering the potential of *Croton* species and the interest in new antimicrobial natural products, the aim of this study is to evaluate the *in vitro* antimicrobial activity of extracts and fractions derived from *C. linearis* leaves through bioassay-guided fractionation and to isolate the compounds which could be responsible for this activity.

2 Materials and methods

2.1 Plant Material

The leaves of *C. linearis* were collected in November 2017 in the Siboney-Juticí Ecological Reserve, Santiago de Cuba, Cuba. A specimen of the plant was identified and a voucher specimen was deposited at the Herbarium of the Eastern Center of Ecosystems and Biodiversity BSC "Jorge Sierra Calzado" under number 21 659. Leaves were air-dried for 3 weeks, shaded from direct sunlight, and were grounded in a blade mill (MRC Model KM 700/Germany) until a particle size < 3 mm.

2.2 Solvents and reagents

All solvents, including *n*-hexane, chloroform, methylene chloride, ethyl acetate, isopropyl alcohol, methanol and dimethyl sulfoxide (DMSO) were purchased from Acros Organics (Geel, Belgium) or from Fisher Scientific (Leicestershire, UK) and were analytical grade. All reagents, such as formic acid (≥ 98%) and sodium hydroxide (NaOH) were purchased from Acros Organics or Sigma-Aldrich (St. Louis, MO, USA). Solvents used for HPLC, i.e. methanol and acetonitrile, were HPLC grade and were purchased from Fisher Scientific. Water was dispensed by a Milli-Q system from Millipore (Bedford, MA, USA) and was passed through a 0.22 μM membrane filter before usage. Methanol-d_4 (99.8% D) and chloroform-*d* (99.8% D) were used as solvents for NMR spectroscopy and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Extraction and bioassay-guided fractionation

A crude extract was obtained from 140 g of air-dried milled leaves of *C. linearis* by percolation with ethanol 95%. The ethanolic extract was filtered and concentrated under reduced pressure at 40 °C in a rotary evaporator (KIRKA–WERKE / Germany) yielding 13.23 g of dark green extract. The ethanolic extract was dissolved in ethanol 70% and defatted with *n*-hexane yielding

a residue of 3.98 g. Next, the extract was basified with NaOH 10% until pH > 9 and a liquid-liquid partition with chloroform, ethyl acetate, and chloroform / methanol (1:1) was performed. The yield of the four fractions was: 1.95 g for the chloroform fraction (**Cl-F**), 0.30 g for the ethyl acetate fraction (**EtOAc-F**), 4.86 g for the chloroform / methanol fraction (**ClM-F**) and 2.14 g for the residual fraction (**R-F**). **Cl-F, EtOAc-F** and **ClM-F** were concentrated under reduced pressure at 40 °C and a NP-TLC (Normal Phase Thin Layer Chromatography) analysis was carried out on NP F254 plates (20 x 20 cm) from Merck (Darmstadt, Germany), with *n*-hexane / $CHCl_3$ / CH_3OH (15:12:5) as the mobile phase. The plates were observed under UV light (254 and 366 nm) and under visible light after spraying with iodoplatinate reagent.

The ethanol extract and its subfractions were evaluated for their *in vitro* antimicrobial activity against a broad panel of pathogens (bacteria, fungi and parasites).

2.4 Antimicrobial Assay

2.4.1 Microorganism and dilutions

Leishmania infantum MHOM/MA (BE)/67, *Leishmania donovani* MHOM/ET/67/L82, *Trypanosoma cruzi* (Tulahuen CL2, *β*-galactosidase strain (nifurtimox-sensitive)), *Trypanosoma brucei brucei* Squib 427 strain (suramin-sensitive) or *Trypanosoma brucei rhodesiense* (strain STIB-900), *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC8739, *Mycobacterium tuberculosis* (strain H37Ra), *Candida albicans* ATCC B59630 (azole-resistant) and *Aspergillus fumigatus* ATCC16404 were used for *in vitro* antimicrobial activity screening. Bacteria were cultured in MHB (Mueller Hinton Broth) and maintained on TSA (Tryptone Soy Agar), while fungi were cultured in RPMI-1640 medium supplemented with MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer and glucose, and maintained on PDA (Potato Dextrose Agar). *Leishmania* spp. were maintained in the Golden Hamster (*Mesocricetus auratus*), while *T.*

cruzi strains were cultured in MRC-5_{SV2} (human lung fibroblast) cells in MEM medium, supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% inactivated fetal calf serum. *T. brucei brucei* and *T. brucei rhodesiense* were maintained in Hirumi (HMI-9) medium, supplemented with 10% inactivated fetal calf serum. The stock solutions of the samples were prepared in DMSO (100%) at 20 mg/mL. The compounds were then serially diluted (2 or 4 fold) in DMSO followed by a further (intermediate) dilution in demineralized water to ensure a final in-test DMSO concentration less than 1% to avoid toxic effects due to the solvent. The final concentration range tested was from 0.25 to 128 µg/mL.

2.4.2 Antibacterial and antifungal assay

The microdilution method with resazurin (redox indicator) in sterile 96-well microplates was performed (Cos et al., 2006). In each well, 10 µL of the aqueous compound dilutions were added together with 190 µl of bacteria inoculum (5 x 10^5 CFU/mL) or yeast inoculum (5 x 10^3 CFU/mL). Microbial growth was compared to untreated-control wells (100% cell growth) and control wells containing only the growth medium (0% cell growth). Microplates were incubated according to the microorganism: yeast (24 h at 37 °C), bacteria (17 h at 37 °C), mold (7 days at 27 °C). Then 10 µL of resazurin at 50 µg/mL per well was added and the microplates were incubated at the same temperature. After the incubation period (*S. aureus*: 15 min, *E. coli*: 45 min, *A. fumigatus*: 48 h, *C. albicans*: 24 h, *M. tuberculosis*: 7 days) fluorescence was measured (λ_{ex}= 550 nm, λ_{em}= 590 nm) in a microplate reader (TECAN GENios, Germany). Doxycycline, flucytosine, terbinafine and isoniazid were used as positive controls.

2.4.3 Antiprotozoal assay

2.4.3.1 Antileishmanial assay

Amastigotes of *L .infantum* were collected from the spleen of an infected donor hamster using three centrifugation purification steps (300 rpm, keeping the supernatants, 2200 rpm, keeping the supernatants and 3500 rpm, keeping the pellet) and spleen parasite burdens were assessed using the Stauber technique (Stauber, 1966). Primary peritoneal mouse macrophages, which were used as host cells for the *Leishmania* parasites, were collected 2 days after peritoneal stimulation with a 2% potato starch suspension. The assay was performed in 96-microwell plates. Each well contained 10 µL of the compound dilutions together with 190 µL of macrophage / parasite inoculum (3 x 10^5 cells and 3 x 10^6 parasite per well). After 5 days incubation (at 37 °C and 5% CO_2), parasite burdens (mean number of amastigotes/macrophage) were microscopically assessed after staining the cells with a 10% Giemsa solution. The results are expressed as the percent reduction in parasite burden compared to untreated control wells, calculating the IC_{50}. Miltefosine was used as positive control.

2.4.3.2 Antitrypanosomal assay

The assay was performed in 96-microwell plates. Each well contained 10 µL of compound dilutions together with 190 µL of the parasite suspension of trypomastigotes (1.5 x 10^4 parasite/well for *T. brucei brucei* and 4 x 10^3 parasite/well for *T. brucei rhodesiense*). Parasite growth was compared to untreated-infected (100% parasite growth) and uninfected controls containing only growth medium (0% growth). After 3 days incubation, parasite growth was assessed fluorimetrically after addition of 50 µl resazurin per well. After 6 h (*T. rhodesiense*) or 24 h (*T. brucei*) at 37 °C, fluorescence was measured (λ_{ex} 550 nm, λ_{em} 590 nm). The results are expressed as % reduction in parasite growth / viability compared to control wells and the IC_{50} was calculated. Suramine was used as positive control.

For the *T. cruzi* assay (Buckner et al., 1996), 190 µl of MRC-5 cell/parasite inoculum (4 $x10^3$ cells/well + 4 $x10^4$ parasites/well) was seeded together with 10 µL of the aqueous compound dilutions in sterile 96-well microtiter plates. The parasite growth was compared with untreated infected controls (100% growth) and non-infected controls (0% growth) after seven days of incubation. The parasite burden was assessed after adding chlorophenol red-*β*-D-galactopyranoside (CPRG) as colorimetric detection reagent: 50 µL/well of stock solution containing 15.2 mg of CPRG and 250 µL Nonidet in 100 mL phosphate buffered saline (PBS). Then, the change in color was measured spectrophotometrically at 540 nm after 4 h incubation at 37 °C. The results are expressed as % reduction in parasite burdens compared to control wells and the IC_{50} was calculated. Benznidazole was used as positive control (Cos et al., 2006).

2.5 Cytotoxicity assay

MRC-5_{SV2} (human fetal lung fibroblasts) cells were purchased from ATCC (American Type Culture Collection) and cultured in MEM + Earl's salts-medium, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% inactivated fetal calf serum. 190 µL of MRC-5_{SV2} inoculum (1.5 x 10^5 cells/mL) were seeded in sterile 96-well microtiter plates, containing 10 µL of the test substance at different concentrations (64, 16, 4, 1, and 0.25 µg/mL) and incubated at 37 °C and 5% CO_2 for 72 h. Cell growth was compared to untreated-control wells (100% cell growth) and control wells containing only growth medium (0% cell growth). The cell viability was assessed fluorometrically 4 h after the addition of 50 µL/well resazurin solution (2.2 µg/mL) using a microplate reader (TECAN GENios, Germany) at λ_{ex} 550 nm, λ_{em} 590 nm. The results are expressed as % reduction in cell growth / viability compared to control wells, and the IC_{50} was determined. Tamoxifen was used as positive control (Cos et al., 2006).

2.6 Isolation of constituents from active fractions

According to the first antimicrobial screening, the **Cl-F** (1.7 g) and **EtOAc-F** (250 mg) fractions were subjected to flash chromatography on a GraceResolv 80 g silica column using a Reveleris iES system (Columbia, MD, USA) and with gradient analysis, using dichloromethane (A), ethyl acetate (B) and methanol (C) as mobile phases. These solvents were applied as follows: for **Cl-F**: 0 min 100% A, 0% B, 0% C, changing linearly to 0% A and 100% B at 29.1 min; then a linear change to 25% B/75% C until the end of the run (87.5 min); for **EtOAc-F**: 0 min 100% A, 0% B, 0% C, changing linearly to 0% A and 100% B at 24.0 min; then a linear change to 25% B/75% C until the end of the run (71.8 min). The flow rate was 30 mL/min and for detection an evaporating light scattering detector (ELSD) and UV absorption at 254 and 366 nm were used. All the subfractions were joined according to their TLC profile resulting in 7 subfractions for **Cl-F** (C-1 to C-7) and 3 subfractions for **EtOAc-F** (B-1 to B-3). TLC analysis performed on all subfractions indicated the presence of alkaloids in **C-5**, **C-6** and **C-7**.

The subfractions **C-5** (60 mg), **C-6** (80 mg), **C-7** (120 mg) (because of the presence of alkaloids) and **B-1** (120 mg) (because it seemed chromatographically reasonably pure and was available in a good amount) were selected for isolation of pure compounds by semi-preparative HPLC-DAD-MS analysis (Waters (Milford, MA, USA). Therefore, the system was operated with a C_{18} Luna column (250 mm × 10.0 mm, particle size, 5 μm) from Phenomenex (Utrecht, Netherlands). Linear gradients with H_2O + 0.1% formic acid (A) and acetonitrile (B) were applied. For **subfraction C-5**: 0 to 5 min 15% B, 35 min 40% B, 40-45 min 100% B, 50-60 min 15% B and the flow rate was 3.0 mL/min. For **subfraction C-6**: 0 to 5 min 15% B, 35 min 50% B, 40 min 80% B, 45-50 min 100% B, 55-60 min 15% B and the flow rate was 3.0 mL/min. For

subfraction C-7: 0 to 5 min 10% B, 30 min 35% B, 40-45 min 100% B, 50-60 min 10% B and the flow rate was 3.0 mL/min.. For **subfraction B-1**: 0 to 5 min 20% B, 40 min 25% B, 45-50 min 100% B, 55-60 min 20% B and the flow rate was 4.75 mL/min.

The DAD spectrum was recorded from 200 to 450 nm, and mass spectra were taken in the ESI+ mode, MS scan range: *m/z* 150 to 750; $V_{capillary}$ 3.00 kV, V_{cone} 50 V, $V_{extractor}$ 3 V, $V_{RF\ Lens}$ 0.2 V, T_{source} 135 °C, $T_{desolvation}$ 400 °C, desolvation gas flow 750 L/h, cone gas flow 50 L/h. Peaks of interest were selected based on their UV spectrum and the *m/z* value. The collection of the eluate was triggered as long as the intensity of selected *m/z* values exceeded the set threshold. Seven compounds were isolated: **C-5A** (3 mg) from **subfraction C-5**; **C-6A** (1 mg) and **C-6B** (3 mg) from **subfraction C-6**; **C-7A** (4 mg), **C-7F** (3 mg) and **C-7E** (8 mg) from **subfraction C-7** and **B-1C** (7 mg) from **subfraction B-1**.

2.7 Structure elucidation

The isolated compounds were characterized by means of 1D ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR. In addition, DEPT-135, DEPT-90 and 2D NMR (COSY, HSQC and HMBC) experiments were recorded. NMR spectra were recorded on a Bruker DRX-400 instrument (Rheinstetten, Germany), equipped with either a 3 mm broadband inverse (BBI) probe or a 5 mm dual $^{1}H/^{13}C$ probe, using standard Bruker pulse sequences. NMR data processing was performed with MestReNova software version 6.1.0-6224 for Windows. The results were matched with the information derived from the mass spectra obtained with the semi-preparative HPLC-DAD-MS system.

Cularine (compound C-5A): amorphous, colorless powder (3 mg).^{1}H NMR (400 MHz, $CDCl_3$): δ 2.83 (s, 3H, NCH_3); 3.04-2.95 (m, 2H, 2H-4); 3.28-3.16 (m, 2H, H-5, H-7); 3.45-3.35 (m, 1H, H-5); 3.72-3.61 (m, 1H, H-7); 3.80 (s, 3H, 9-OCH_3); 3.88 (s, 6H, 1-OCH_3, 10-OCH_3); 4.86 (dd,

J= 12.3, 3.2 Hz, 1H, H-6a); 6.58 (s, 1H, H-8); 6.82 (s, 1H, H-11); 6.87 (d, *J*= 8.4 Hz, 1H, H-2); 6.96 (d, *J*= 8.4 Hz, 1H, H-3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 23.16 (C-4), 34.19 (C-7), 40.74 (NCH3), 46.83 (C-5), 56.35 (1-OCH3, 10-OCH3), 56.41 (9-OCH3), 57.91 (C-6a), 105.50 (C-11), 112.26 (C-2), 113.28 (C-3a), 116.76 (C-7a), 123.28 (C-3a), 125.15 (C-3), 127.98 (C-1b), 145.61 (C-1a), 145.85 (C-10), 148.21 (C-9), 148.64 (C-11a), 150.22 (C-1). ESI-MS (positive mode): *m/z* 342 $[M+H]^+$ ($C_{20}H_{23}NO_4$).

Corydine (c**ompound C-6A)**: yellow-red powder (1 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.81 (s, 3H, NCH_3); 2.86-2.83 (m, 1H, H-7); 3.06-2.99 (m, 1H, H-5); 3.17 (dd, *J*= 13.8, 3.4 Hz, 1H, H-7); 3.52-3.42 (m, 2H, H-4, H-5), 3.74 (s, 3H, 10-OCH_3); 3.92 (s, 6H, 1-OCH_3, 11-OCH_3); 6.71 (s, 1H, H-3); 6.91 (d, *J*= 8.3 Hz, 1H, H-8); 7.12 (d, *J*= 8.3 Hz, 1H, H-9); 3.74 (overlapping H-6a). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.52 (C-4), 33.37 (C-7), 40.10 (NCH_3), 51.67 (C-5) 56.23 (1-OCH_3, 11-OCH_3), 61.43 (C-6a), 62.24 (10-OCH_3), 111,14 (C-3), 111.63 (C-8), 123.10 (C-3a), 125.03 (C-9), 126.08 (C-7a), 128.14 (C-11a), 143.80 (C-1), 144.30 (C-10), 150.79 (C-2), 152.74 (C-11). ESI-MS (positive mode): *m/z* 342 $[M+H]^+$ ($C_{20}H_{23}NO_4$).

Glaucine (compound C-6B): yellow powder (3 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.87 (dd, *J*= 16.9, 3.4 Hz, 1H, H-4); 2.95 (s, 3H, NCH_3); 3.18-3.12 (m, 3H, H-5, 2H-7); 3.65 (s, 3H, 1-OCH_3); 3.89 (s, 3H, 9-OCH_3); 3.90 (s, 3H, 2-OCH_3); 3.92 (s, 3H, 10-OCH_3); 3.97 (d, *J*= 7.6 Hz, 1H, H-6a), 6.62 (s, 1H, H-3), 6.78 (s, 1H, H-8); 8.06 (s, 1H, H-11); 3.59 (overlapping, H-4), 3.66 (overlapping, H-5).^{13}C NMR (100 MHz, $CDCl_3$): δ 25.44 (C-4), 31.96 (C-7), 40.09 (NCH_3), 52.21 (C-5), 60.44 (1-OCH_3), 61.69 (C-6a) 56.06 (2-OCH_3, 9-OCH_3, 10-OCH_3), 110.37 (C-3) 111.03 (C-8), 111.73 (C-11), 120.45 (C-3a), 123.35 (C-7a), 125.89 (C-11a), 125.52 (C-1b), 127.16 (C-1a), 145.01 (C-1), 147.87 (C-9), 148.41 (C-10), 153.25 (C-2). ESI-MS (positive mode): *m/z* 356 $[M+H]^+$ ($C_{21}H_{25}NO_4$).

Laudanosine (compound C-7A): yellow powder (8 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.86 (s, 3H, NCH_3); 2.98 (dd, *J*= 17.0, 5.7 Hz, 1H, H-4); 3.12-3.04 (m, 1H, H-4); 3.41-3.31 (m, 1H, H-3); 3.46 (s, 3H, 7-OCH_3); 3.69-3.57 (m, 1H, H-3); 3.80 (s, 3H, 5'-OCH_3); 3.84 (s, 3H, 4'-OCH_3); 3.86 (s, 3H, 6-OCH_3); 4.25 (d, *J*= 8.9, 1H, H-1); 5.75 (s, 1H, H-8); 6.55 (dd, *J*= 8.1, 1.4 Hz, 1H, H-6'), 6.63 (s, 1H, H-5); 6.71 (d, *J*= 1.2 Hz, 1H, H-2'); 6.74 (d, *J*= 8.2 Hz, 1H, H-5'), 2.87/3.81 (overlapping, H-1a). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.90 (C-4), 39.83 (NCH_3), 40.73 (C-1a), 44.59 (C-3), 55.66 (7-OCH3), 56.07 (6-OCH3, 4'-OCH3), 56,13 (5'-OCH3), 65.06 (C-1), 111.17 (C-5), 111.22 (C-5'), 111.36 (C-8), 113.25 (C-2') 120.78 (C-8a), 121.30 (C-4a), 127.95 (C-1'), 147.42 (C-6), 148.38 (C-4'), 149.17 (C-3'), 149.24 (C-7). ESI-MS (positive mode): *m/z* 358 $[M+H]^+$ ($C_{21}H_{27}NO_4$).

Laudanidine (compound C-7E): yellow powder (5 mg).^{1}H NMR (400 MHz,CDCl3): δ 2.77-2.68 (m, 1H, H-1a); 2.81 (s, 3H, NCH_3); 2.95 (dd, *J*= 17.1; 5.1 Hz, 1H, H-4); 3.10-3.00 (m, 1H, H-4); 3.37-3.28 (m, 1H, H-4); 3.46 (s, 3H, 7-OCH_3), 3.60-3.57 (m, 1H, H-3); 3.65-3.61 (m, 1H, 1a), 3.84 (s, 6H, 6-OCH_3 and 4'-OCH_3); 4.22 (d, *J*= 9.1 Hz, 1H, H-1); 5.77 (s, 1H, H-8); 6.49 (d, *J*= 8.1 Hz, 1H, H-6'); 6.61 (s, 1H, H-5); 6.72 (d, *J*= 8.2 Hz, 1H, H-5'); 6.78 (s, 1H, H-2'). ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.76 (C-4), 39.72 (N-CH_3), 40.57 (C-1a), 44.69 (C-3), 55.46 (7-OCH_3), 55.91 (4'-OCH_3), 56.04 (6-OCH_3), 65.13 (C-1), 110.84 (C-5'), 110.94 (C-5), 111.18 (C-8), 116.24 (C-2'), 120.65 (C-8a), 121.47 (C-6'), 121.91 (C-4a), 128.62 (C-1'), 145.84 (C-3'), 146.03 (4'), 147.30 (C-7), 149.14 (C-6). ESI-MS (positive mode): *m/z* 344 $[M+H]^+$ ($C_{20}H_{25}NO_4$).

Reticuline (compound C-7F): yellow powder (3 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.89 (s, 3H, NCH_3); 3.11-3.00 (m, 3H, H-1a, 2H-4); 3.27 - 3.20 (m, 1H, H-3); 3.71-3.66 (m, 1H, H-3); 3.87 (s, 6H, 6-OCH_3, 4'-OCH_3); 4.45 (d, *J*= 5.7 Hz, 1H, H-1); 6.23 (s, 1H, H-8); 6.64 (dd, *J*= 8.5, 0.6 Hz, 1H, H-2'); 6.70 (s, 1H, H-6'); 6.81 (s, 1H, H-5); δ 6.91 (d, *J*= 8.1 Hz, 1H, H-3'). ^{13}C

NMR (100 MHz, $CDCl_3$): δ 23.71 (C-4), 40.94 (H-1a), 41.06 (N-CH_3), 47.14 (C-3), 56.64 (6-OCH_3, 4'-OCH_3), 66.30 (H-1), 112.71 (C-5), 113.15 (C-3') 115.78 (C-8), 117.71 (C-6'), 120.64 (8a), 122.28 (C-2'), 122.88 (4a), 128.09 (C-1'), 145.03 (C-7), 146.09 (C-5'), 147.08 (C-4'), 147.95 (C-6). ESI-MS (positive mode): *m/z* 330 $[M+H]^+$ ($C_{19}H_{23}NO_4$).

Isorhamnetin-3-*O*-(6''-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside (compound B-1C): (yellow powder (7 mg).^{1}H NMR (400 MHz, CD_3OD): δ 3.57-3.45 (m, 4H, H-2", H-3", H-4", H-5"); 3.94 (s, 3H, 5'-OCH_3); 4.30 (dd, *J*= 10.6, 4.5 Hz, 2H, 2H-6"); 5.36 (d, *J*= 7.4 Hz, 1H, H-1"); 6.08 (d, *J*= 15.9 Hz, 1H, H-8'"); 6.17 (s, 1H, H-6); 6.32 (s, 1H, H-8); 6.82 (d, *J*= 8.5 Hz, 2H, H-3'", H-5'"), 6.87 (d, *J*= 8.4 Hz, 1H, H-3'); 7.32 (d, *J*= 8.5 Hz, 2H, H-6'", H-2'"); 7.40 (d, *J*= 15.9 Hz, 1H, H-7'"); 7.59 (dd, *J*= 8.4, 1.8 Hz, 1H, H-2'); 7.89 (d, *J*= 1.9 Hz, 1H, H-6').13C NMR (100 MHz, CD_3OD): δ 57.52 (5'-OCH_3), 65.06 (C-6"), 72.69 (C-4"), 76.70 (C-2", c-5"), 78.87 (C-3"), 95.21 (C-8), 100.34 (C-6), 103.88 (C-1"), 105.46 (C-10), 115.12 (C-2'), 115.43 (C-8'"), 116.91 (C-5'), 117.63 (C-3'", C-5'"), 123.77 (C-1'), 124.73 (C-6'), 127.88 (C-1'"), 132.05 (C-2'", 6'"), 136.05 (C-3), 147.46 (C-7'"), 149.14 (C-3'), 151.74 (C-4'), 159.19 (C-9), 159.70 (C-2), 162.07 (C-4'"), 163.83 (C-5), 166.81 (C-7) 169.0 (C=O), 180.0 (C-4). ESI-MS (positive mode): *m/z* 625 $[M+H]^+$ ($C_{31}H_{28}O_{14}$).

The general scheme of the bioassay-guided fractionation is summarized in Figure 1.

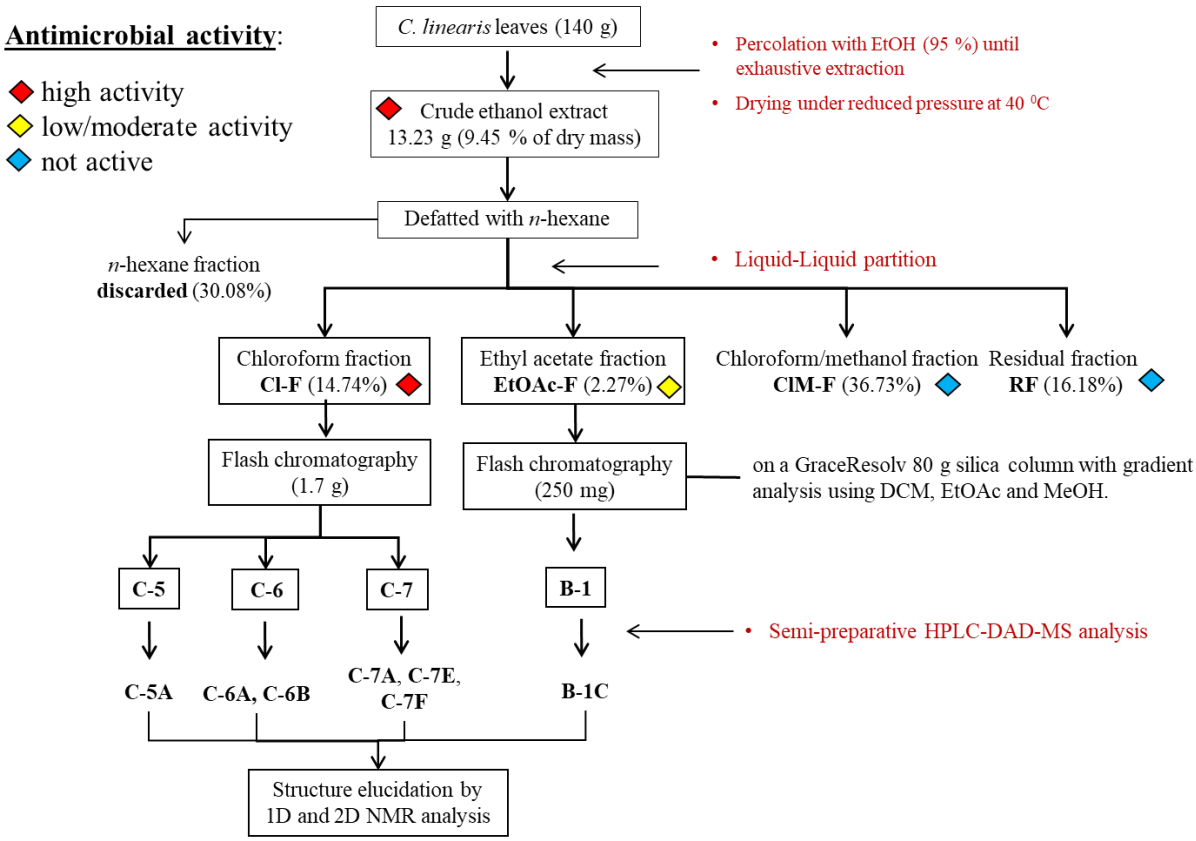


Figure 1. General scheme of the bioassay-guided fractionation performed on the ethanolic extract from *C. linearis* leaves.

3 Results and discussion

3.1. Antimicrobial activity

3.1.1 Extracts and fractions

Table 1 and 2 show the results of the *in vitro* antimicrobial bioassay performed on the total extract and fractions derived from leaves of *C. linearis*. In general, the samples were not active against *S. aureus*, *E. coli*, *C. albicans*, *A. fumigatus* and *M. tuberculosis* (H37Ra) at the tested concentrations (0.25 to 128 µg/mL). Only the **TE** (total extract) and **Cl-F** were active against *S. aureus* with IC_{50} of 62.67 and 103.6 µg/mL, respectively. It can be noted that against *S. aureus* (Table 1) and all parasites (Table 2) the IC_{50} value of the total extract (TE) was lower than the

IC_{50} values observed for the subfractions. This may be due to synergistic effects, or to loss of active constituents during defatting with *n*-hexane.

Table 1. *In vitro* antibacterial and antifungal activity of the total extract and fractions from *C. linearis* leaves.

Samples	Antimicrobial screening (IC_{50} µg/mL)				
	S. aureus	*E. coli*	*C. albicans*	*A. fumigatus*	*H37Ra*
TE	**62.67 ± 1.62**	>128	>128	>128	>128
Cl-F	**103.6 ± 2.5**	>128	>128	>128	>128
C-5	>64	>64	>64	nd	nd
C-6	>64	>64	>64	nd	nd
C-7	>64	>64	>64	nd	nd
EtOAc-F	>128	>128	>128	>128	>128
B-1	>64	>64	>64	nd	nd
ClM-F	>128	>128	>128	>128	>128
R-F	>128	>128	>128	>128	>128

TE: total extract, **Cl-F**: chloroform fraction, **EtOAc-F**: ethyl acetate fraction, **ClM-F**: chloroform/methanol fraction, **R-F**: residual fraction. *S. aureus*: *Staphylococcus aureus*; *E. coli*: *Escherichia coli*; *C. albicans*: *Candida albicans*; H37Ra: *Mycobacterium tuberculosis*. Reference compounds: Doxycycline (*S. aureus*) IC_{50} 0.03 µM; Doxycycline (*E. coli*) IC_{50} 0.04 µM; Flucytosine (*C. albicans*) IC_{50} 0.72 µM; Terbinafine (*A. fumigatus*) IC_{50} 0.50 µM and Isoniazid (H37Ra) IC_{50} 5.99 µM. nd: not determined

Table 2 *In vitro* antiprotozoal activity and cytotoxicity of the total extract and fractions from *C. linearis* leaves.

Samples	Cytotoxicity (CC_{50} µg/mL)		Antimicrobial screening (IC_{50} µg/mL)			
	MRC-5	PMM	*T. cruz*	*L. inf*	*T. bruc*	*T. rhod*
TE	7.63 ± 0.49	16	**3.08 ± 1.00**	**16.23 ± 0.55**	**1.56 ± 0.19**	**4.00 ± 1.53**
Cl-F	4.00 ± 2.69	64	**16.78 ± 0.25**	**25.40 ± 1.20**	**16.35 ± 0.67**	**16.11 ± 1.21**
C-5	nd	nd	nd	nd	nd	nd
C-6	10.11 ± 4.09	32	8.21 ± 0.95	28.26 ± 5.95	31.76 ± 0.78	nd
C-7	>64	>64	>64	>64	>64	nd
EtOAc-F	69.61 ± 0.83	>128	**85.81 ± 1.83**	>128	>128	>128
B-1	14.73 ± 6.43	>64	**13.50 ± 2.79**	>64	>64	nd
ClM-F	21.93 ± 0.90	>128	>128	>128	>128	>128
R-F	>128	>128	>128	>128	>128	>128

TE: total extract, **Cl-F**: chloroform fraction, **EtOAc-F**: ethyl acetate fraction, **ClM-F**: chloroform/methanol fraction, **R-F**: residual fraction. MRC-5: Human fetal lung fibroblasts; PMM: Peritoneal Murine Macrophages; *T. cruz.*: *Trypanosoma cruzi*; *L. inf.*: *Leishmania infantum*; *T. bruc.*: *Trypanosoma brucei*; *T. rhod*: *T. rhodesiense*. Reference compounds: Tamoxifen (MRC-5) IC_{50} 9.71 µM; IC_{50} 2.7 µM Benznidazole (*T. cruz.*) IC_{50} 1.80 µM; Miltefosine (*L .inf.*) IC_{50} 10.77 µM; Suramine (*T. bruc.*) IC_{50} 0.02 µM Suramine (*T. rhod*) IC_{50} 0.03 µM. nd: not determined

Antibacterial and antifungal activity has been reported for several *Croton* species, but generally at higher concentrations than the ones used in this study. Antibacterial activities of *n*-hexane,

dichloromethane, ethyl acetate, acetone and methanol extracts of *C. sylvaticus* leaf against *E. coli*, *Enterococcus faecalis*, *S. aureus* and *Pseudomonas aeruginosa* were evaluated showing microbial growth inhibition but at a concentration of 1.25 mg/mL; almost ten times higher than evaluated in this study. The same report mentions an acceptable antifungal activity against *Cryptococcus neoformans*, with mean minimum inhibitory concentration (MIC) of 0.07 mg/mL (Maroyi, 2017). Aqueous and alcoholic extracts of bark and leaf from *C. roxburghii* were tested against enteric pathogens causing urinary tract infections. The ethanol extract of bark exhibited inhibitory activity against a higher diversity of strains compared with aqueous extracts, but with higher MIC values from 0.356 to 0.625 mg/mL (Panda et al., 2010). Hexane and methanol extracts of *C. pullei* stems showed moderate antibacterial and antifungal activity against Gram-negative bacteria, Gram-positive bacteria and yeast species responsible for various human infections (Peixoto et al., 2013). Once again, the concentrations were higher than the ones internationally accepted as being microbiologically relevant (Cos et al., 2006) and the ones used in this study, ranging from 0.078 to 5 mg/mL and from 0.156 to 10 mg/mL for methanol and *n*-hexane extracts, respectively

In contrast, *C. linearis* extract and fractions exhibited good activity against the parasites *T. cruzi*, *L. infantum*, *T. brucei* and *T. rhodesiense*, except for the more polar fractions (**ClM-F** and **R-F**). Therefore, no phytochemical screening was performed on the latter two. The **total extract** and **Cl-F** exhibited a strong antiparasitic activity with IC_{50} values ranging from 1 to 26 µg/mL, depending on the parasite tested. *L. infantum*, causing leishmaniasis, was less sensitive with IC_{50} values of 16.23 and 25.40 µg/mL observed for **TE** and **Cl-F**, respectively. On the other hand, *T. brucei*, causing African sleeping sickness, was more sensible to **TE** (IC_{50} 1.56 µg/mL). **EtOAc-F** exhibited a weak activity against *T. cruzi* with IC_{50} 85.81 µg/mL.

Several *Croton* species are used traditionally to treat ailments caused by parasites (Salatino et al., 2007). However, there are only a few reports on the antiparasitic activity of *Croton* species in the literature. The methanolic extract of *C. macrostachys* leaves exhibited chemosuppressive activity against *Plasmodium berghei* in infected mice (Bantie et al., 2014). The essential oil from *C. cajucara* leaves and its constituent 7-hydroxycalamenene were found to inhibit *in vitro* growth of promastigote forms of *L. chagasi*, with MIC values of 250 and 15.6 µg/mL, respectively (Rodrigues et al., 2013).

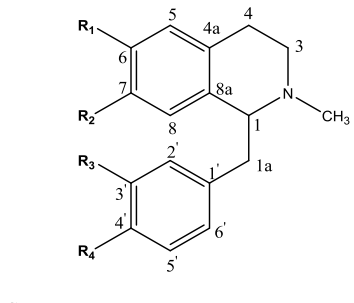
In order to evaluate the selectivity of the antimicrobial activity, the cytotoxicity on MRC-5 cell and peritoneal murine macrophages (for the *Leishmanial* model) was evaluated. Most of the samples showed cytotoxicity on MRC-5 cells with CC_{50} values below 27 µg/mL, with the exception of **R-F** (CC_{50}>128 µg/mL). In the same way, the more polar fractions (**EtOAc-F**, **ClM-F** and **R-F**) exhibited no toxicity against peritoneal murine macrophages at the highest concentration tested (CC_{50} > 128 µg/mL), while **TE** and **Cl-F** were cytotoxic with CC_{50} values of 16 and 64 µg/mL, respectively. In general, the samples classify as non-selective due to the small difference between the active and toxic concentrations expressed through the selectivity index (for MRC5 < 9 and for PMM <3).

3.2 Compounds isolated from the chloroform and ethyl acetate fraction

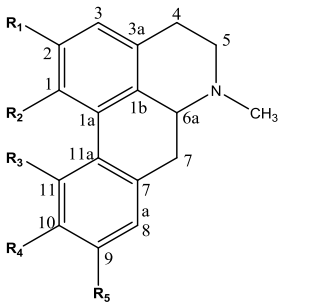
Seven compounds were isolated and characterized from leaves of *C. linearis*: six alkaloids from **Cl-F** and one flavonoid glycoside from **EtOAc-F**.

Compounds C-7A, C-7E and **C-7F** were identified as laudanosine, laudanidine and reticuline (Fig. 2), respectively, by comparison of their spectral assignments with published data (Leboeuf et al., 1982; Janssen et al., 1989; Blanchfield et al., 2013). **Compounds C-6A** and **C-6B** were identified as corydine and glaucine (Fig. 2), respectively. NMR data coincided with the data

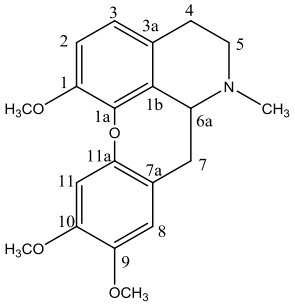
reported in literature by Rong et al. (2016) and Hassan et al. (2013). **Compound C-5A** was identified as cularine by comparison with literature data (Holland et al., 1878; Rodrigues et al., 2004). The substitution pattern of these compounds was confirmed by HSQC and HMBC (Fig. 3).



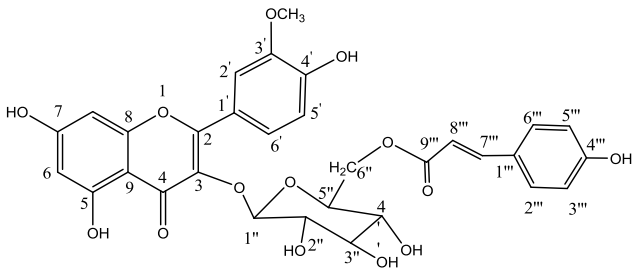
Compound	R_1	R_2	R_3	R_4
C-7A	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$
C-7E	$-OCH_3$	$-OCH_3$	-OH	$-OCH_3$
C-7F	$-OCH_3$	-OH	-OH	$-OCH_3$



Compound	R_1	R_2	R_3	R_4	R_5
C-6B	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H	$-OCH_3$
C-6A	$-OCH_3$	-OH	$-OCH_3$	$-OCH_3$	-H



Compound C-5A



Compound B-1C

Figure 2. Structures of compounds isolated from leaves of *C. linearis*.

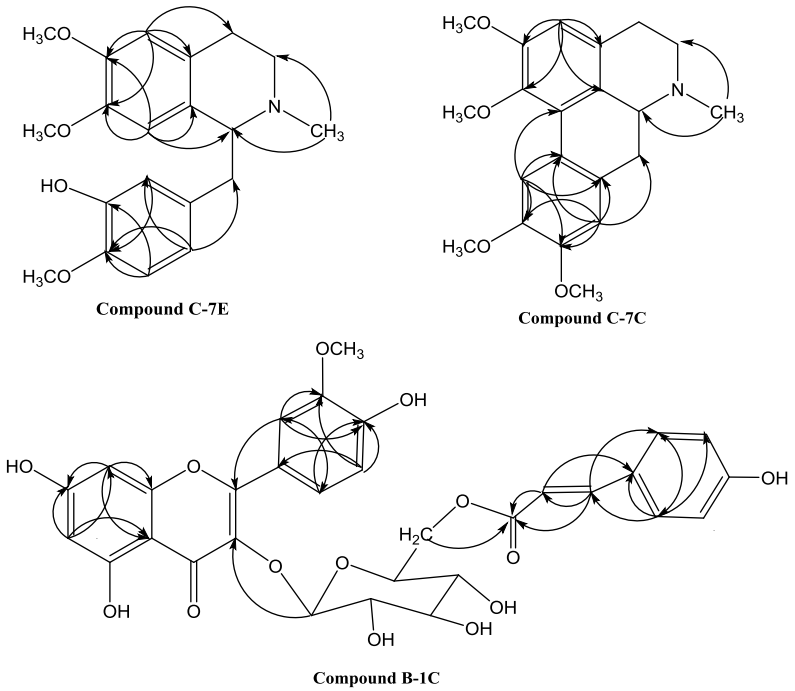


Figure 3 Correlations $^{2}J_{H\text{-}C}$ and $^{3}J_{H\text{-}C}$ observed in the HMBC spectra of compounds **C-7E**, **C-7C** and **B-1C**.

Compound B-1C was identified as isorhamnetin-3-*O*-(6"-*O*-*p*-*trans*-coumaroyl)-*β*-glucopyranoside. The substitution pattern was established by the correlations observed in the HMBC spectrum (Fig 3). The NMR spectral data were in agreement with the assignments reported by Jou et al. (2004). The mass spectrum showed 4 signals: $[M+Na]^+$ at *m/z* 647, $[M+H]^+$ at *m/z* 625, $[aglycon+H]^+$ at *m/z* 317, and $[coumaroylhexose]^+$ at *m/z* 309. The molecular formula was confirmed as $C_{31}H_{28}O_{14}$ corresponding with the base peak ion $[M+H]^+$ at *m/z* 625.

None of the seven compounds isolated and characterized from **Cl-F** and **EtOAc-F** was reported before in *C. linearis*. However, these compounds have been isolated from other *Croton* species, except for cularine (**C-5A**). Laudanosine (**C-7A**) and laudanidine (**C-7E**) were isolated from leaves of *C. hemiargyreus* (Lin et al., 2003) and from *C. celtidifolius* leaves and stems (Amaral and Barnes, 1997), respectively. Glaucine (**C-6B**) has been isolated from leaves of *C. draconoides* (Bettolo and Scarpati, 1979) and *C. lecheleri* (Milanowski et al., 2002). Pereira et

al. (1999) reported the isolation of glaucine (**C-7C**) and corydine (**C-6A**) from leaves and stems of *C. hemiargyreus*. Reticuline (**C-7F**) has been reported from leaves and stems of *C. celtidifolius* (Amaral and Barnes, 1997) and *C. hemiargyreus* (Pereira et al., 1999). The flavonol glycoside (**B-1C**) was isolated from the ethanolic extract of leaves of *C. pedicellatus* (Lopes et al., 2012).

The six alkaloids that were isolated (benzylisoquinoline, aporphine and cularine type alkaloids), as well as the alkaloids identified before in this species (aporphine and morphinandienone type) (Haynes et al., 1967, 1968; Farnsworth et al., 1969) belong biogenetically to the isoquinoline alkaloids (Robinson, 1981; Cordell, 2013).

Isoquinoline-type alkaloids are an important chemotaxonomic group of secondary metabolites found in American *Croton* species: namely *C. celtidifolius*, *C. draco*, *C. flavens*, *C. hemiargyreus*, *C. lechleri*, *C. palanostigma* and *C. salutaris* (Salatino et al., 2007; Marcelino et al., 2012). Based on the results of this study, *C. linearis* can be added to the list of plants producing isoquinoline-type alkaloids.

The isoquinoline alkaloids are well-known for their antiprotozoal activity. Many antiprotozoal isoquinolines have been isolated from various plant families such as the Annonaceae, Berberidaceae, Menispermaceae and Hernandiaceae. The phenolic benzylisoquinoline coclaurine and norarmepavine proved to inhibit the growth of *T. cruzi* epimastigotes *in vitro* with an IC_{50} of around 0.30 mM (Morello et al., 1994). On the other hand, the apomorphine alkaloids predicentrine, glaucine, and boldine inhibited *in vitro* growth of *T. cruzi* epimastigotes with IC_{50} values of 0.08, 0.09, and 0.11 mM, respectively (Osorio et al., 2008). Three major apomorphine alkaloids actinodaphnine, cassythine, and dicentrine from *Cassytha filiformis* L. (Lauraceae) showed IC_{50} values of 3-15 µM against *T. b. brucei* (Hoet et al., 2004).

From a chemical point of view, the types of alkaloids isolated in this study are in agerement with the types reported before: a benzylisoquinolinic type from subfraction C-7 and an apomorphine type from **C-6**. Subfraction **C-6** showed good activity against *T. cruzi* (IC_{50}=8.21 µg/mL), *L. infantum* (IC_{50}=28.26 µg/mL) and *T. brucei* (IC_{50}=31.76 µg/mL). Nevertheless, subfraction **C-7** was not active in the antiprotozoal assays, neither was the benzylisoquinoline alkaloid laudanidine (compound **C-7E**), isolated from this subfraction, presenting only weak activity against *L. infantum* (IC_{50}=148.0 ± 1.2 µM) (Table 2).

Due to the limited yield of some alkaloids, the antimicrobial activity could not be assessed for all isolated compounds. Nevertheless, it can be concluded that small structural differences between apomorphine and benzylisoquinoline type alkaloids result in significantly different antiprotozoal activities. The obtained results suggest that the activity observed for the total extract and the chloroform fraction could be related to the apomorphine alkaloids from subfraction **C-6** and cannot be attributed to the benzyisoquinoline alkaloids from subfraction **C-7**.

In previous reports, it was mentioned that the apomorphine alkaloids (+)-lirioferine (IC_{50} 210 µM) and (+)-*N*-methylaurotetanine (IC_{50} 395 µM) were more active against promastigotes of *L. mexicana* than the benzyisoquinoline alkaloid reticuline (IC_{50}= 518 µM) (Singh *et al*., 2014). This higher activity has been related to the greater lipophilicity of apomorphine alkaloids, as well as to their planar conformation, which allows better interaction with the biological targets (Osorio et al., 2008) The mechanism of action of some apomorphine alkaloids seems to be related to the inhibition of topoisomerase II by DNA intercalation or minor groove binding (Hoet *et al*., 2004).

From fraction **EtOAc-F**, subfraction **B-1** showed good activity against *T. cruzi* (IC_{50} 13.50 µg/mL). From this subfraction compound **B-1C** (isorhamnetin-3-*O*-(6"-*O*-*p*-*trans*-coumaroyl)-*β*-

glucopyranoside) was only active against *T. cruzi* (IC_{50} 35.6 ± 2.3 µM), but with a selectivity index <1. This result is not in agreement with the report of Grael et al. (2005) in which this compound was not active against *T. cruzi* at concentrations of 100, 250 and 500 µg/mL. However, Baldim et al. (2017) reported the activity of the aglycone isorhamnetin on *T. cruzi* (IC_{50} 9.48 µM), *T. brucei* (IC_{50} 2.81 µM) and *L. donovani* (IC_{50} 1.20 µM).

4. Conclusions

Croton linearis is used by people from the Caribbean countries to treat fever and common cold. In this study, weak antimicrobial activity against bacteria and fungi was observed; however, potent antiprotozoal activity of the ethanolic extract and chloroform fraction (Cl-F) was detected. These results may be associated to the apomorphine type alkaloids and the flavonol glycoside isolated, and can support, at least in part, the ethnomedicinal use of this species. The seven compounds (six isoquinoline alkaloids and one flavonoid) isolated and characterized in this study are reported for the first time in this species. This contributes to the scientific knowledge about *C. linearis*, and is of added value in the search for news plant-based products with antiprotozoal potential.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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