http://dx.doi.org/10.5935/0103-5053.20160186

J. Braz. Chem. Soc., Vol. 28, No. 2, 367-375, 2017.
Printed in Brazil - ©2017 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

A New Furanoheliangolide Sesquiterpene Lactone from *Calea pinnatifida* (R. Br.) Less. (Asteraceae) and Evaluation of Its Trypanocidal and Leishmanicidal Activities

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Calea pinnatifida (R. Br.) Less. is commonly known in Brazil as "cipó-cruz", "quebra-tudo" or "aruca". This species is employed in folk medicine as giardicidal, amoebicidal and to treat digestive disorders. The present paper describes the isolation and structure determination of a new furanoheliangolide sesquiterpene lactone named 11,13-dihydroxy-calaxin, in addition to seven known compounds: ethyl caffeate, vanillin, 12-hydroxy-encecalin, phytol, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid from the ethanol extract of *C. pinnatifida* leaves. Additionally, 11,13-dihydroxy-calaxin, vanillin, 12-hydroxy-encecalin, phytol, 3,4-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid and the mixture of 3,4-di-*O*-caffeoylquinic, 3,5-di-*O*-caffeoylquinic and 4,5-di-*O*-caffeoylquinic acids were assayed against the amastigote forms of *Leishmania amazonensis* and *Trypanosoma cruzi*, and the sesquiterpene lactone 11,13-dihydroxy-calaxin exhibited a promising trypanocidal and leishmanicidal activity, displaying IC_{50} values of 5.27 and 8.30 μM, respectively.

Keywords: *Calea pinnatifida*, furanoheliangolide, phenolic compounds, leishmanicidal, trypanocidal

Introduction

Leishmaniasis and Chagas' disease constitute serious health care problems, mostly in Sub-Saharan Africa, Latin America and Middle East Asia. These parasitic diseases are caused by kinetoplastid protozoan of the genus *Leishmania* and *Trypanosoma,* respectively, and are responsible for substantial global morbidity, mortality, economic adversity and huge health care costs.[1,2] The available chemotherapy for the treatment of these illnesses presents important limitations, including partial efficacy, toxicity, side effects, long-term therapies and high number of resistance cases, resulting in treatment failure.[3] Thus, there is an urgent demand for the discovery of new antiprotozoal compounds and/or the development of novel therapeutic strategies which are safer, effective, accessible and less toxic. In this context, the natural products from plants, in particular, represent a remarkable source to furnish new bioactive substances.[4,5]

Calea pinnatifida (R. Br.) Less. (Heliantheae, Asteraceae), popularly known in Brazil as "cipó-cruz", "quebra-tudo" or "aruca",[6,7] is a plant commonly utilized in popular medicine as giardicidal, amoebicidal and to treat digestive problems.[7-9] To the best of our knowledge, few chemical and biological works have been performed with this species. Previous phytochemical studies on aerial parts and leaves from *C. pinnatifida* led to the isolation and identification of one polyacetylene, one sesquiterpene lactone, fatty acid esters, steroids, benzoic acid derivatives[10,11] and chromenes.[6] Furthermore, it has also been reported antiproliferative and leishmanicidal properties for some of its extracts (dichloromethane and ethanol) or chemical constituents (sesquiterpene lactones and chromenes).[6,12]

Thus, the main goals of the present study were to explore the chemical composition from the ethanol extract of the leaves of *C. pinnatifida*, as well as to evaluate the *in vitro* trypanocidal and leishmanicidal activities of the isolated compounds against intracellular forms (amastigotes) of *Trypanosoma cruzi* and *Leishmania amazonensis.*

Experimental

General experimental procedures

Melting points were determined on a Microquímica MQAPF-301 melting point apparatus. Optical rotation was measured in MeOH using a Schmidt-Haensch

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Polartronic E polarimeter. All NMR analysis (^{1}H, ^{13}C, correlation spectroscopy (COSY), nuclear Overhauser spectroscopy (NOESY), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC)) were carried out employing Bruker Fourier 300 UltraShield, Bruker AVANCE-400 and/or Ascend 600 spectrometers, operating at 300, 400 and 600 MHz for ^{1}H; and 75, 100 and 150 MHz for ^{13}C, respectively. $CDCl_3$, acetone-d_6 or methanol-d_4 with TMS as internal standard (0.00 ppm) were used as solvents for acquisition of the NMR spectra. Chemical shifts (δ) were given in ppm and coupling constants (J) were expressed in hertz (Hz). High-resolution electrospray ionization mass spectra (HRESIMS) were measured on an Exactive™ Plus Orbitrap mass spectrometer (Thermo Fisher Scientific) and/or Xevo® G2-XS QTof mass spectrometer (Waters). Preparative high-performance liquid chromatography (HPLC) was performed on a Shimadzu HPLC system (Kyoto, Japan) equipped with two LC-10AD pumps, SCL-10ADVP system controller, SPD-10AV UV detector and manual injection system, using a C18 column Luna type (10 µm, 250 × 10 mm, Phenomenex®). Medium pressure liquid chromatographic (MPLC) separation was carried out using an FMI fluid metering lab pump, model QSY (Fluid Metering Inc., Syosset, NY). Silica gel (230-400 mesh) used for column chromatography and vacuum liquid chromatography was obtained from Vetec, Brazil; analytical TLC and silica gel RP-18 (240-400 mesh) were purchased from Silicycle; and Sephadex LH-20 was obtained from Tedia Brazil, Brazil.

Collection of plant material

C. pinnatifida (R. Br.) Less. leaves were collected at the "Costa da Lagoa", Florianópolis, Santa Catarina, Brazil, in June 2013. Botanical identification and authentication of the plant material were performed by PhD John F. Pruski, New York Botanical Garden, and specimen samples are deposited in the Missouri Botanical Garden Herbarium, St. Louis, Missouri, USA (No: MO-2383318) and in the Rio de Janeiro Botanical Garden Herbarium, Rio de Janeiro, Brazil (No: RB00906349).

Extraction and isolation of the chemical constituents

Fresh leaves from *C. pinnatifida* (2.6 kg) were cut into small irregular pieces with a scissors and submitted to maceration with ethanol 92% for 15 days at room temperature (ca. 25 ºC) until exhaustion (solvent was renewed four times). The solvent was concentrated using a rotary evaporator at 40 ºC under reduced pressure to afford 142.0 g of a dark green crude extract. Crude extract was solubilized in a minimum volume of H_2O and sequentially partitioned with hexane (5 × 500 mL), dichloromethane (4 × 500 mL), and ethyl acetate (3 × 500 mL), to yield hexane (68.5 g), dichloromethane (7.6 g) and ethyl acetate (5.9 g) phases, as well as a residual aqueous fraction that was lyophilized to give a hygroscopic solid (60.0 g).

Dichloromethane fraction (7.6 g) was subjected to vacuum liquid chromatography (VLC) on SiO_2 gel and eluted with hexane, acetone and MeOH (pures or in binaries mixtures), affording ten sub-fractions (A-J). An aliquot of sub-fraction D (500.0 mg) was purified by column chromatography (CC) on silica gel using gradient elution with hexane, $CHCl_3$ and EtOAc to give 158 fractions of 40 mL each. Fractions F69-78 (194.4 mg) and F84-89 (91.3 mg) were re-chromatographed over Sephadex LH-20 with $CHCl_3$ and acetone, respectively, affording 56.3 mg of the compound 11,13-dihydroxy-calaxin (**1**) and 17.5 mg of ethyl caffeate (**2**). Fractions F32-33, eluted with pure $CHCl_3$, yielded 7.0 mg of a compound with satisfactory purity grade, vanillin (**3**). In addition, sub-fractions B and C were reunited (222.0 mg) and submitted to CC using hexane-acetone with gradual increases in polarity to give 91 fractions of 50 mL each. Fractions F44-50 (21.3 mg), after successive preparative TLC (hexane-acetone 75:25, v/v), afforded 3.8 mg of a yellow oil, 12-hydroxy-encecalin (**4**). Yet, fractions F31-37 were combined to give 8.7 mg of the phytol (**5**).

Subsequently, a part of ethyl acetate fraction (4.9 g) was further chromatographed by VLC on silica gel and the elution was carried out using a solvent gradient (CH_2Cl_2 with increasing proportions of EtOAc, followed by EtOAc with increasing proportions of MeOH), obtaining eight sub-fractions (A-H). An aliquot of sub-fraction E (800.0 mg) was purified by Sephadex LH-20 using acetone-MeOH (1:1) as mobile phase to afford 37 fractions of 25 mL each. An aliquot of the fractions F16-19 (60 mg) was submitted to successive preparative HPLC performed under isocratic conditions at a flow rate of 1.5 mL min^{-1} with a mobile phase consisting of ACN-H_2O (20:80, v/v) containing 1% formic acid and UV detection at 325 nm, yielding the 3,4-di-*O*-caffeoylquinic acid (**6**) (6.4 mg) and 4,5-di-*O*-caffeoylquinic acid (**8**) (10.3 mg), respectively. Finally, sub-fraction C (126.5 mg) was subjected to an MPLC over reverse phase RP-18 utilizing H_2O-MeOH mixtures as mobile phase to afford 73 fractions of 25 mL each. Fractions F50-53, eluted with H_2O-MeOH (60:40), gave 22.7 mg of the 3,5-di-*O*-caffeoylquinic acid (**7**).

Spectroscopic data

11,13-Dihydroxy-calaxin (**1**)

White solid; m.p. 144.5-146.0 ºC; $[\alpha]_D^{25} = -0.733$ (MeOH;

c 0.013 g mL^{-1}; ca. 25 ºC); ^1H NMR (600 MHz, $CDCl_3$), see Table 1; ^{13}C NMR (150 MHz, $CDCl_3$), see Table 1; HRMS (pESI) *m/z*, calcd. for $C_{19}H_{23}O_8$ $[M + H]^+$: 379.1393, found: 379.1379; HRMS (nESI) *m/z*, calcd. for $C_{19}H_{21}O_8$ $[M - H]^-$: 377.1236, found: 377.1234.

Trypanocidal and leishmanicidal screening

The trypanocidal and leishmanicidal assays of the compounds **1**, **3**-**5**, **6**, **8** and the mixture of **6** + **7** + **8** were performed as previously described by Lima *et al.*[13]

Human monocytic leukemia TPH-1 cell line (ATCC TIB202) was grown in 96-well plates containing RPMI-1640 medium in absence of phenol red (Sigma-Aldrich Co., St. Louis, MO, USA) and supplemented with 10% bovine fetal serum (FBS) (Life Technologies, USA), 12.5 mM HEPES, Glutamax (2 mM) and the antibiotics penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}). The cell culture was maintained at 37 °C in a humidified 5% CO_2 atmosphere. *L. amazonensis* MHOM/BR/77/LTB0016 promastigotes, expressing β-galactosidase, were grown in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA) and supplemented with 5% heat inactivated FBS and 2% of human urine at 26 °C.

To perform the screening of the leishmanicidal activity against intracellular amastigotes of *L. amazonensis*, THP-1 cells (3.0×10^4 *per* well) were cultivated in 96-well plates using the RPMI-1640 medium supplemented as described previously, treated with 100 ng mL^{-1} of phorbol 12-myristate 13-acetate (PMA) at 37 °C in a 5% CO_2 for 72 h, to allow THP-1 cells differentiation into non-dividing macrophages.[14]

Four days culture promastigotes (4.0×10^6 parasites *per* mL) were washed with phosphate buffered saline (PBS), pH 7.4, and incubated in RPMI-1640 medium supplemented with 10% human AB^+ serum heat-inactivated at 34 °C for 1 h to parasite opsonization. THP-1 cells were incubated with a parasite:cell ratio of 10:1 for 3 h at 34 °C (CO_2 5%). After this period, non-adherent parasites were removed by one wash with PBS and infected cells were incubated with 180 µL of complete supplemented RPMI-1640 medium for another

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR data for compound **1** ($CDCl_3$)

No.	δ (mult., *J* Hz)	^1H-^1H COSY	δ (HSQC)	HMBC
1	–	–	205.4	–
2	5.59 (br s, 1H)	–	103.7	1/3/4/10
3	–	–	185.2	–
4	–	–	130.9	–
5	6.02 (dq, 1H, *J* 3.6, 1.8)	6/15	132.9	3/7
6	5.17 (ddq, 1H, *J* 4.2, 3.6, 1.8)	5/7/15	75.9	4/5/7/8/12
7	3.40 (dd, 1H, *J* 4.2, 2.2)	6/8	54.9	5/6/8/9/11/12
8	5.09 (ddd, 1H, *J* 4.4, 2.9, 2.2)	7/9a/9b	68.4	6/7/9/10/11/1'
9a	2.54 (dd, 1H, *J* 15.2, 4.4)	8/9b	43.4	1/7/8/10/14
9b	2.34 (dd, 1H, *J* 15.2, 2.9)	8/9a	43.4	1/7/10/14
10	–	–	88.4	–
11	–	–	78.1	–
12	–	–	173.4	–
13a	4.22 (d, 1H, *J* 12.7)	13b	45.5	7/8/11/12
13b	3.89 (d, 1H, *J* 12.7)	13a	45.5	7/11/12
14	1.47 (s, 3H)	–	22.2	1/8/9/10
15	2.08 (dd, 3H, *J* 1.8, 1.8)	5/6	19.8	3/4/5/7
1'	–	–	166.7	–
2'	–	–	134.9	–
3'a	6.05 (dq, 1H, *J* 2,0, 1.0)	3'b/4'	128.1	1'/2'/4'
3'b	5.65 (dq, 1H, *J* 2.0, 1.5)	3'a/4'	128.1	1'/2'/4'
4'	1.86 (dd, 3H, *J* 1.5, 1.0)	3'a/3'b	18.1	1'/2'/3'
OH	3.26 (br s, 1H)	–	–	7/11/12

^1H-^1H COSY: ^1H-^1H correlation spectroscopy; HSQC: heteronuclear single quantum correlation; HMBC: heteronuclear multiple bond correlation.

24 h to allow the transformation of promastigotes into intracellular amastigotes.

The β-galactosidase *Trypanosoma cruzi,* Tulahuen strain,[15] was kindly provided by the Laboratório de Parasitologia Molecular e Celular, Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, Brazil. Culture-derived trypomastigotes raised from infected L929 cell line were used to infect differentiated THP-1 (4.0×10^4 cells *per* well) in 96-well microplates in a parasite:cell ratio of 3:1 and incubated overnight at 37 ºC with 5% CO_2. The medium containing non-internalized parasites was removed and replaced with 180 µL of fresh medium.

Compounds **1**, **3**-**5**, **6**, **8** and the mixture of **6** + **7** + **8** were dissolved in DMSO 1% at concentrations ranging from 50 to 1.56 µM. Infected cell layer was treated by addition of 20 µL of each sample followed by incubation at 34 °C with 5% CO_2 for 48 h. After treatment, cells were carefully washed with PBS and incubated at 37 °C for 16 h with 250 µL of chlorophenol red-β-D-galactopyranoside (CPRG) (Sigma-Aldrich Co., St. Louis, MO, USA) at 100 µM and Nonidet P-40 0.1% (NP-40) (Amresco Inc, Solon, Ohio, USA). All assays were performed in triplicate. Optical density was read at 570/630 nm in an Infinite M200 TECAN, Austria. The concentration of each sample that reduced parasite viability by 50% when compared to untreated control (IC_{50}) was estimated by non-linear regression of concentration-response curves. Benznidazole (Sigma) and amphotericin B (Bristol-Myers, Squibb) were utilized as positive control groups for trypanocidal and leishmanicidal assays, respectively, and DMSO 1% was used as negative control group.

Cell toxicity assay

THP-1 cells were seeded (6.0×10^4 *per* well) in 96-well microplates and differentiated with 100 ng mL^{-1} of PMA. After 72 h of incubation at 37 ºC in a humidified 5% CO_2 atmosphere, compounds at concentration varying between 500 and 15.6 µM or DMSO 1% (negative control) were added and the cells were incubated again for 72 h at 37 ºC (CO_2 5%). The cell viability was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT) based on tetrazolium salt reduction by mitochondrial dehydrogenases, and the optical densities were read at 540 nm in a TECAN Infinite M200 microplate reader immediately after the dissolution of formazan crystals with DMSO. The CC_{50} value was defined as the concentration of each compound that reduced the absorbance of treated cells by 50% when compared with the cell control.[16]

Results and Discussion

The chromatographic fractionation of the dichloromethane and ethyl acetate fractions from the ethanol extract of *C. pinnatifida* leaves led to the isolation and structural characterization of seven known compounds: ethyl caffeate (**2**), vanillin (**3**), 12-hydroxy-encecalin (**4**), phytol (**5**), 3,4-di-*O*-caffeoylquinic acid (**6**), 3,5-di-*O*-caffeoylquinic acid (**7**) and 4,5-di-*O*-caffeoylquinic acid (**8**), as well as a new furanoheliangolide sesquiterpene lactone (**1**) (Figure 1). The structures were elucidated by detailed analysis of their physical (m.p.) and spectral data (HRESIMS, 1D and 2D NMR), as well as comparison with similar data reported in the literature.[13,17-21]

The isomeric compounds **6** and **8** were distinguished from each other based on their tandem mass spectrometry (MS/MS) fragmentation patterns and chromatographic resolution on a reversed phase packing. These isomers were identified according to a hierarchical scheme for characterizing chlorogenic acids that has been previously developed by Clifford *et al*.[19,22-24] Figure 2 shows the chromatogram of a sample containing the three isomeric dicaffeoylquinic acids (**6**, **7** and **8**).

Compound **1** was obtained from the dichloromethane fraction as a white solid with melting point = 144.5-146.0 ºC. Further, this compound appeared as a dark brown spot on TLC after spraying with anisaldehyde-sulphuric acid and heating at 100 ºC for few minutes. Its molecular formula was determined by HRESIMS in both positive and negative ionization modes. The HRESIMS spectrum in positive mode exhibited a molecular ion peak at *m/z* 379.1379 $[M + H]^+$ (calcd. for $C_{19}H_{23}O_8$: 379.1393), and the negative mode HRESIMS spectrum revealed a molecular ion peak at *m/z* 377.1234 $[M - H]^-$ (calcd. for $C_{19}H_{21}O_8$: 377.1236), both compatible with molecular formula $C_{19}H_{22}O_8$, corresponding to nine degrees of unsaturation. The ESI-MS/MS spectral data of compound **1** revealed a base ion peak with *m/z* 291 ($C_{15}H_{15}O_6$) formed by the loss of methacrylate group $[M - H - (CH_2C(CH_3)CO_2)]^-$. Moreover, there were also produced *m/z* 247 ($C_{14}H_{15}O_4$) and *m/z* 229 ($C_{14}H_{13}O_3$) fragment ions, due to the elimination of CO_2 and H_2O, respectively. A fragmentation mechanism for the sesquiterpene lactone **1** is proposed in the Figure 3.

The ^1H NMR spectrum (Table 1) of **1** displayed characteristic signals of sesquiterpene lactones common to the genus *Calea*.[25-28] Absence of the pair of doublets relative to exomethylene hydrogens provided evidence for the saturation between C-11 and C-13. The pair of doublets resonating at δ 4.22 (d, 1H, *J* 12.7 Hz, H-13a) and δ 3.89 (d, 1H, *J* 12.7 Hz, H-13b)/δ 45.5, typical of diastereotopic hydrogens attached to the carbinolic carbon, was ascribed

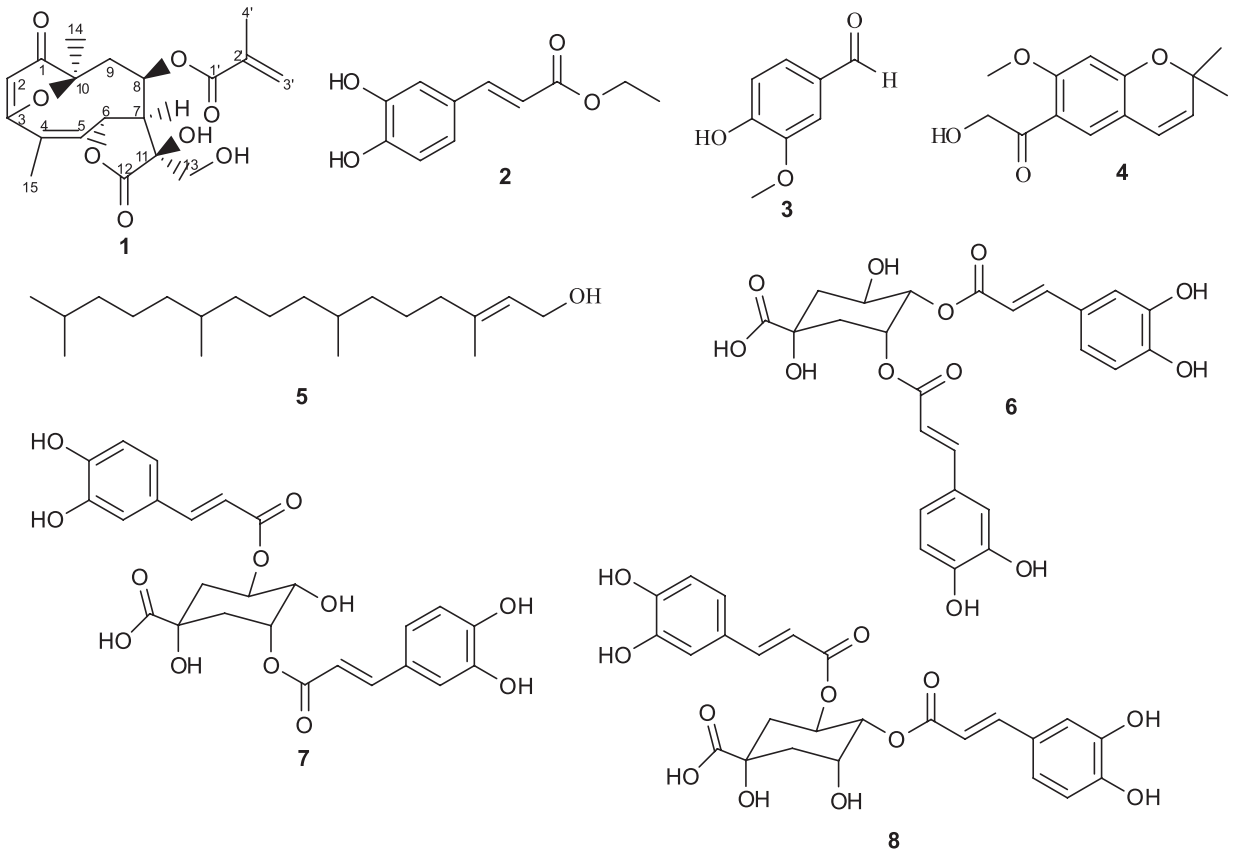

Figure 1. Chemical structures of the isolated compounds from *C. pinnatifida.*

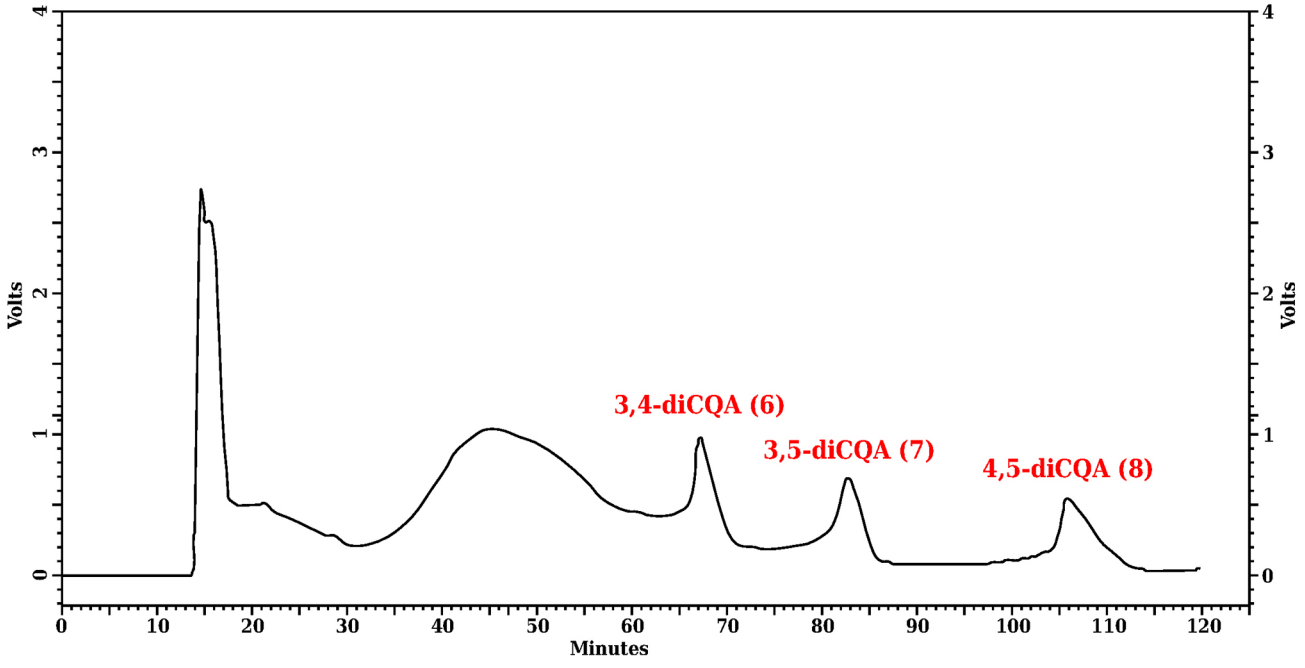

Figure 2. Chromatogram of a sample containing the isomeric dicaffeoylquinic acids **6**, **7** and **8**.

to H-13a and H-13b. A broad singlet at δ 5.59 (brs, 1H, H-2)/δ 103.7 together with a double quartet at δ 6.02 (dq, 1H, *J* 3.6, 1.8 Hz, H-5)/δ 132.9 are consistent with the presence of a sesquiterpene lactone of the furanoheliangolide type. It was also observed in the ^{1}H NMR spectrum two typical signals of oxymethinic hydrogens at δ 5.09 (ddd, 1H, *J* 4.4,

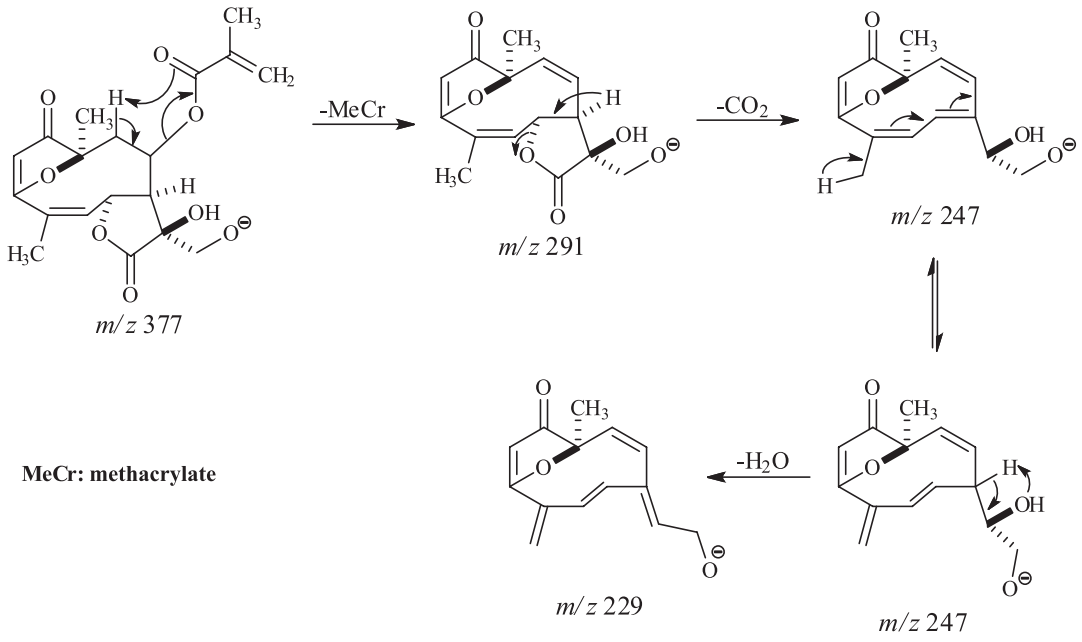

Figure 3. Proposed fragmentation mechanism (*n*ESI-MS/MS) for compound **1.**

2.9, 2.2 Hz, H-8)/δ 68.4 and δ 5.17 (ddq, 1H, *J* 4.2, 3.6, 1.8 Hz, H-6)/δ 75.9, and a methane signal at δ 3.40 (dd, 1H, *J* 4.2, 2.2 Hz, H-7)/δ 54.9. The coupling constant value ($J_{6,7}$ 4.2 Hz) observed between H-6 and H-7 corroborates the presence of a 6,12α-*trans* lactonized heliangolide with a *cis*-4,5-double bond. Additionally, the ester side chain was assigned as a methacrylate group based on three typical signals: two olefinic methylene hydrogens resonating at δ 6.05 (dq, 1H, *J* 2.0, 1.0 Hz, H-3'a) and δ 5.65 (dq, 1H, *J* 2.0, 1.5 Hz, H-3'b)/δ 128.1, and an olefinic methyl at δ 1.86 (dd, 3H, *J* 1.5, 1.0 Hz, H-4')/δ 18.1. The attachment of the ester side chain to the carbon C-8 of the furanoheliangolide skeleton was established based on HMBC correlation from the oxymethinic proton H-8 at δ 5.09 with the carbon signal at δ 166.7 (C-1'). Figure 4 displays some important ^{1}H-^{1}H COSY and HMBC correlations of compound **1**.

The chemical shift values of carbon were obtained by the HSQC and HMBC experiments. HSQC and HMBC correlation maps revealed the presence of 19 C-atom signals, including one lactone carbonyl (δ 173.4), one α,β-unsaturated ketone carbonyl (δ 205.4), one α,β-unsaturated ester carbonyl (δ 166.7), three sp^2 olefinic carbons (δ 103.7, 128.1 and 132.9), two quaternary olefinic carbons (δ 130.9 and 134.9), one oxygenated quaternary olefinic carbon (δ 185.2), three sp^3 oxygenated carbons (δ 45.5, 68.4 and 75.9), two sp^3 oxygenated quaternary carbons (δ 78.1 and 88.4), one methylene (δ 43.4), one methine (δ 54.9) and three methyl groups (δ 18.1, 19.8 and 22.2) (Table 1).

The relative configurations of stereogenic centers in compound **1** were deduced on the basis in the coupling constant values which were correlated with the dihedral angles obtained from Dreiding models and application of the Karplus curve. The relative *cis*-disposition of C(4,5) double bond associated with the coupling constant value recorded between H-6/H-7 ($J_{6,7}$ 4.2 Hz) indicated a different arrangement for H-6 and H-7. Assuming that H-7 is α-oriented, as generally occurs in sesquiterpene lactones isolated from higher plants,[29-31] it should be concluded a β-orientation for H-6. Similarly, the small coupling constant observed between H-7/H-8 ($J_{7,8}$ 2.2 Hz) suggested a β-orientation for the methacrylate side chain located at C-8 and α-orientation for the oxymethine H-8. The signal corresponding to the H-7 hydrogen has an unusually low chemical shift (δ 3.40), suggesting that this hydrogen is close to the oxygen of the furan ring. This proximity requires an α-orientation for the hydrogens H-14 of methyl group, as occurs in all furanoheliangolide sesquiterpene lactones of known stereochemistry.[32-36] The analysis of NOESY correlation map (acetone-d_6) allowed us to determine the relative configuration of C-11. Clear NOESY correlation was observed between the methinic proton H-7 (α-oriented) and the methylene hydrogens H-13a and H-13b attached to C-13 (Supplementary Information). Based on this observation, we can suggest a β-orientation for the hydroxyl group at C-11 (C–OH) and an α-orientation for the bond between the carbons C-11 and C-13 (Figure 1).

The ^{1}H and ^{13}C NMR data (Table 1) of compound **1** resembled those of sesquiterpene lactone 11,13-epoxy-calaxin previously isolated from *C. pilosa*,[18] clearly indicating that both compounds present the same skeletal arrangement. These two sesquiterpene lactones differ

only in the functional group located at the C-11 and C-13 carbons. Compound **1** possess a C-11,13-diol moiety instead of the C-11,13-epoxy moiety present in 11,13-epoxy-calaxin. The existence of a C-11,13-diol moiety was evident by the broad singlet resonating at δ 3.26 (br s, 1H, OH), clearly demonstrating the presence of at least one hydroxyl group in **1**. Moreover, this moiety was confirmed by the observed HMBC correlations between broadened OH singlet at δ 3.26 with the carbon signals at δ 54.9 (C-7), 78.1 (C-11) and 173.4 (C-12) (Figure 4). The compound **1** has been identified in the crude extract from *C. pinnatifida* by ultra-high performance liquid chromatography electrospray ionization mass spectrometry (UHPLC-ESI-MS), indicating that this compound isn't a product of ring opening of an epoxide from natural sesquiterpene lactone 11,13-epoxy-calaxin.

Collectively, these data supported the structure of a new furanoheliangolide and allowed us to name compound **1** as 11,13-dihydroxy-calaxin [(3*R*,3α*R*,4*R*,11α*S*,*Z*)-3-hydroxy-3-(hydroxymethyl)-6,10-dimethyl-2,7-dioxo-2,3,3α,4,5,6,7,11α-octahydro-6,9-epoxycyclodeca[*b*] furan-4-yl methacrylate]. This is the first report of furanoheliangolide-type sesquiterpenes in *C. pinnatifida*, however the occurrence of furanoheliangolide is common in *Calea* genus.[18,25-28,37-41]

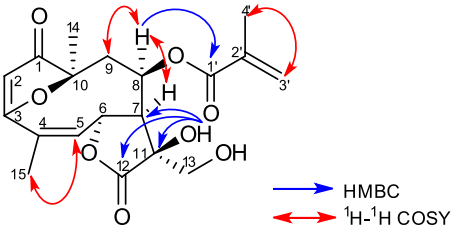

Figure 4. Selected ^{1}H-^{1}H COSY and HMBC correlations of compound **1**.

The antiprotozoal activity of compounds **1**, **3-5**, **6**, **8** and the mixture of **6 + 7 + 8** was examined against intracellular forms (amastigotes) of *T. cruzi* and *L. amazonensis*, the etiological agents of the Chagas' disease and cutaneous leishmaniasis, respectively. Compounds **2** and **7** were not examined because they have been previously tested by Lima *et al.*[13] Compound **2** exhibited a 76.06% inhibitory effect on the growth of *T. cruzi* and 24.53% on the growth of *L. amazonensis*, while compound **7** inhibited *L. amazonensis* amastigotes intracellular growth by 24.53% and exhibited no activity against *T. cruzi* amastigotes.[13]

The results of the trypanocidal and leishmanicidal assays were measured in terms of percentage of growth inhibition of *T. cruzi* and *L. amazonensis* amastigotes, and are described in Table 2. The capacity of the compounds to inhibit the parasite growth was investigated at 50 µM, and the IC_{50} (50% inhibitory concentration), CC_{50} (50% cytotoxic concentration) and SI (selectivity index = CC_{50}/IC_{50}) values were estimated only for the most bioactive compounds (%growth inhibition > 50%). DMSO 1% was utilized as negative control and did not affect the growth of the parasites. As positive controls, parasites were treated with benznidazole 20 µM and amphotericin B 2 µM, showing 82.67% of growth inhibition against *T. cruzi* and 86.88% against *L. amazonensis*, respectively.

Compounds **3-5**, **6**, **8** and the mixture of **6 + 7 + 8** displayed weak or no percentage of inhibition against intracellular amastigotes of both *T. cruzi* and *L. amazonensis*, whereas the compound **1** exhibited a strong leishmanicidal and trypanocidal effect, inhibiting the intracellular parasite growth in 81.81 and 94.30%, respectively. Furthermore, this compound displayed IC_{50} values of 5.27 ± 0.19 and 8.30 ± 0.57 µM for *T. cruzi* and *L. amazonensis*, respectively, showing a promising trypanocidal and leishmanicidal activity.

Table 2. *In vitro* leishmanicidal and trypanocidal activities of the compounds **1**, **3-5**, **6** and **8**

Compounds tested Concentration (50 µM)	%Growth inhibition ± SD *L. amazonensis* amastigotes	%Growth inhibition ± SD *T. cruzi* amastigotes
1	81.81 ± 2.12	94.30 ± 0.39
3	12.94 ± 0.45	2.36 ± 0.57
4	no activity	4.74 ± 0.82
5	no activity	2.31 ± 0.52
6	3.42 ± 0.11	1.70 ± 0.22
8	3.17 ± 0.39	1.82 ± 0.13
Mixture **6 + 7 + 8**	3.11 ± 0.45	no activity
DMSO 1%	0	0
Benznidazole 20 µM	–	82.67 ± 0.88
Amphotericin B 2 µM	86.88 ± 3.83	–

Regarding the cytotoxic potential on THP-1 cells, the sesquiterpene lactone 11,13-dihydroxy-calaxin presented a lower selectivity for the parasite cells (CC_{50} < 15.60), resulting in low selectivity index (SI value not determined). Cytotoxicity (CC_{50}) and the selectivity index (SI) has not been calculated for the other compounds because all exhibited a weak leishmanicidal and trypanocidal effect (%growth inhibition < 50%). Cytotoxicity is an essential parameter for the initial stages of the development de new drugs, once that it determines the *in vitro* selectivity for the parasites and whether the bioactive compounds cause toxic effects on mammalian host cells. Thus, the cytotoxicity tests with natural products, mainly plant species, are an important

strategy because natural products constitute a promising source of new bioactive compounds, revealing chemical scaffolds important for the discovery and development of new and selective antiprotozoal.[42,43]

Conclusions

The phytochemical investigation from *C. pinnatifida* leaves led to the isolation and characterization of a new furanoheliangolide sesquiterpene lactone in addition to seven known compounds. It is important to note that all the isolated compounds are reported here for the first time for the *C. pinnatifida* species. Moreover, the antiprotozoal assays shown that the furanoheliangolide 11,13-dihydroxy-calaxin presented a remarkable trypanocidal and leishmanicidal effect against both *T. cruzi* and *L. amazonensis* amastigotes.

Supplementary Information

1D and 2D NMR, and HRESIMS data of compound **1** (Figures S1-S13), and TLC of the compounds **6** and **8** (Figure S14) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The authors are thankful to Mr. César Simionato and PhD John Pruski for help in the plant collection and identification, respectively; MSc Rosana Casoti, PhD Fernando Batista da Costa and PhD Louis Pergaud Sandjo for mass spectrometry analysis; and CAPES/CNPq for financial support. Part of this work was performed within the Research Network Natural Products against Neglected Diseases (ResNetNPND).

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Submitted: February 26, 2016
Published online: June 21, 2016