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Artigo

ISOLATION AND CHARACTERIZATION OF SESQUITERPENE LACTONES FROM *Calea uniflora* Less. AND THEIR LEISHMANICIDAL AND TRYPANOCIDAL ACTIVITIES

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Calea uniflora Less., commonly known in Brazil as "erva-de-lagarto" and "arnica-da-praia", is used in folk medicine to treat internal and external bruises and as anti-inflammatory. However, despite the popular use there is a lack of information in terms of isolation and biological evaluation of its secondary metabolites. Thus, the aims of this study were to identify and characterize compounds of *C. uniflora* leaves with trypanocidal and leishmanicidal potential. **Methods**: Structures of compounds were determined by HRESIMS spectra, and 1D and 2D NMR spectra. Then, they were tested for *in vitro* leishmanicidal and trypanocidal activities against *Leishmania amazonensis* and *Trypanosoma cruzi* intracellular amastigotes, respectively. **Results**: Four germacranolide-type sesquiterpene lactones were isolated and characterized from dichloromethane fraction: 2α-hydroxy-8β-2',3',5'-trihydroxy-angeloyloxycostunolide **(1)**, desacetyleupaserrin **(2)**, 2α-hydroxy-8β-3'-hydroxy-2',5'-epoxyangeloyloxycostunolide **(3)** and ovatifolin **(4)**. All compounds are described herein for the first time for *C. uniflora* and *Calea* genus. Lactones **1**, **2** and **4** demonstrated weak or no inhibition of the *T. cruzi* amastigotes, whereas compound **3** revealed a moderate effect (36.8%). Regarding the leishmanicidal activity, **4** exhibited a 59.9% inhibitory effect on the growth of *L. amazonensis*. **Conclusion**: None of the isolated compounds presented promising trypanocidal effect and **4** was the most active compound against *L. amazonensis* amastigotes.

Keywords: Asteraceae; antiprotozoal activity; *Calea uniflora*; sesquiterpene lactones.

INTRODUCTION

Calea uniflora Less., belonging to the Asteraceae family (tribe Heliantheae, subtribe Ecliptinae), is a perennial and subshrub herb with yellow inflorescences.[1] Commonly known in Brazil as "erva-de-lagarto" and "arnica-da-praia",[2,3] this species is native to South America (Uruguay, Paraguay, Argentine, and Southern Brazil). In Southern Brazil, *C. uniflora* aerial parts are widely used by local population as anti-inflammatory, for wound healing, in treatment of internal and external bruises, as antiseptic (to treat mosquito bites), for rheumatism, and to treat urinary infections and flu.[4]

Few studies have been carried out on the chemical analysis and pharmacological properties of *C. uniflora*. Earlier investigations in different parts of the plant revealed the presence of the following secondary metabolites: *p*-hydroxyacetophenones derivatives,[5] chromanones,[6] chromones, flavonoids aglycones and heterosides, chalcones and phenolic acids.[7] Regarding the biological activity, this species and/or its isolated compounds revealed leishmanicidal,[6] antinociceptive, cytotoxic,[8] anti-inflammatory,[9] trypanocidal, and leishmanicidal activities.[7]

Phytochemical studies conducted on *Calea* species have confirmed that this genus is rich in sesquiterpenes lactones, an attractive chemical class in the search for bioactive molecules. Several pharmacological effects have been reported for sesquiterpene lactones, including anticancer,[10] anti-inflammatory,[11] antidiabetic,[12] antiprotozoal,[13] and antimycobacterial activities.[14] However, it is important to emphasize that the toxicological profile of these metabolites must be carefully characterized, since the same activities that make sesquiterpene lactones useful medicines can also cause severe toxicity.[15]

During our investigation, four sesquiterpenes lactones (**1-4**) were isolated and characterized from *C. uniflora* leaves. Previous investigations reporting the leishmanicidal and trypanocidal effects of natural sesquiterpenes lactones from *Calea*[16,17] prompted us to evaluate these compounds against *Leishmania amazonensis* and *Trypanosoma cruzi* amastigotes forms.

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MATERIAL AND METHODS

General experimental procedures

Chemical structures of compounds were determined by melting point (m.p.), NMR spectroscopy and mass spectrometry. 1D and 2D NMR spectra were recorded on Bruker AVANCE spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C. NMR samples were dissolved in acetone-d_6, TMS was used as chemical shift reference and coupling constants were quoted in Hertz (Hz). High-resolution electrospray ionization mass spectra (HRESIMS) were measured on a Xevo® G2-XS Qtof mass spectrometer (Waters).

Plant material

C. uniflora leaves were collected (October 2012) in Imbituba (28º20'36" S; 48º67'21" W), Santa Catarina, Brazil, by Dr. Tamires Cardoso Lima de Carvalho. Plant identification was confirmed by Dr. John Pruski and a voucher specimen (MO-2383317 number) was deposited in New York Botanical Garden Herbarium, New York, United States.

Extraction and isolation of chemical constituents

Fresh leaves of *C. uniflora* (2.3 kg) were submitted to static maceration using 92% ethanol at room temperature for 15 days (three

extractions). After, solvent was removed at reduced pressure, giving 104 g of crude extract. Crude extract was subsequently suspended in H_2O (500 mL) and partitioned with solvents of increasing polarity (hexane, CH_2Cl_2, and EtOAc - 4 x 200 mL each), yielding hexane (26.8 g), CH_2Cl_2 (3.2 g) and EtOAc (6.2 g) fractions, respectively, as well as a residual aqueous fraction that was lyophilized to give 67.8 g of a hygroscopic solid. Dichloromethane fraction (3.2 g) was subjected to vacuum liquid chromatography (VLC) using silica gel (230-400 mesh) and eluted with hexane, CH_2Cl_2, EtOAc and MeOH, pure solvents or in binary solvent mixtures, providing twelve sub-fractions (A, B, C, D, E, F, G, H, I, J, L and M). The following binary mixtures were used: hexane-CH_2Cl_2 (7:3), hexane-CH_2Cl_2 (1:1), hexane-CH_2Cl_2 (3:7), pure CH_2Cl_2, CH_2Cl_2-EtOAc (9:1), CH_2Cl_2-EtOAc (8:2), CH_2Cl_2-EtOAc (6:4), CH_2Cl_2-EtOAc (4:6), CH_2Cl_2-EtOAc (2:8), pure EtOAc, EtOAc-MeOH (7:3), and EtOAc-MeOH (1:1). Sub-fraction H (650.0 mg) was submitted to silica gel column chromatography (CC, 240-400 mesh) and eluting with a gradient of CH_2Cl_2, EtOAc and MeOH [CH_2Cl_2-EtOAc (98:2), CH_2Cl_2-EtOAc (95:5), CH_2Cl_2-EtOAc (9:1), CH_2Cl_2-EtOAc (85:15), CH_2Cl_2-EtOAc (8:2), CH_2Cl_2-EtOAc (7:3), CH_2Cl_2-EtOAc (6:4), CH_2Cl_2-EtOAc (1:1), CH_2Cl_2-EtOAc (3:7), pure EtOAc, and EtOAc-MeOH (1:1)], furnishing 270 fractions (F1-F270). Subsequently, fractions F136-F161 and F174-F186 were combined and purified by preparative TLC [CH_2Cl_2-EtOAc (7:3)], affording 12.2 mg of the compound **1** and 70.0 mg of **2**, respectively. Sub-fraction F (660.0 mg) was also subjected to silica gel column (230-400 mesh) using hexane, EtOAc and MeOH, pure solvents or binary solvent mixtures, as phase mobile [pure hexane, hexane-EtOAc (95:5), hexane-EtOAc (9:1), hexane-EtOAc (8:2), hexane-EtOAc (7:3), hexane-EtOAc (6:4), hexane-EtOAc (1:1), hexane-EtOAc (3:7), pure EtOAc, EtOAc-MeOH (8:2), and EtOAc-MeOH (1:1)], yielding 240 fractions (F1-F240). Fractions F130-F139 were reunited and purified through preparative TLC [CH_2Cl_2-EtOAc (7:3)], providing 2.5 mg of compound **3**. Additionally, F207-F213 (22.7 mg) was purified by chromatography on a Sephadex LH-20 column using pure acetone as eluent to afford 31 fractions (F1-F31). Fractions F13-F20 were reunited, yielding 5.2 mg of compound **4**.

Physical and spectroscopic data

Compound 1: Orange oil. ^{1}H NMR (acetone-d_6, 600 MHz): δ_H 6.18 (d, 3.6, H-13a), 6.01 (ddd, 4.2, 2.8, 1.0, H-8), 5.70 (d, 3.2, H-13b), 5.26 (dd, 9.9, 8.4, H-6), 5.11 (dd, 9.7, 1.2, H-1), 5.05 (dd, 9.9, 1.5, H-5), 4.75 (ddd, 9.7, 9.7, 5.7, H-2), 4.51 (q, 6.6, H-3'), 4.35 (d, 11.2, 5a'), 4.20 (d, 11.2, H-5b'), 3.29 (dddd, 8.4, 3.6, 3.2, 1.0, H-7), 2.72 (dd, 14.4, 4.2, H-9a), 2.66 (dd, 10.9, 5.7, H-3a), 2.57 (dd, 14.4, 2.8, H-9b), 2.07 (m, H-3b), 1.80 (d, 1.5, H-15), 1.61 (d, 1.2, H-14), 1.50 (d, 6.6, H-4'); ^{13}C NMR (acetone-d_6, 150 MHz): δ_C 170.6 (C-1'), 169.7 (C-12), 143.0 (C-4), 137.6 (C-11), 135.8 (C-1), 134.5 (C-10), 130.8 (C-5), 121.7 (C-13), 80.2 (C-2'), 75.7 (C-6), 75.6 (C-8), 69.5 (C-2), 67.3 (C-5'), 59.8 (C-3'), 53.1 (C-7), 49.8 (C-3), 45.1 (C-9), 20.3 (C-14), 19.4 (C-4'), 19.0 (C-15).

Compound 3: White solid. m.p: 190.5-193.0 ºC. ^{1}H NMR (acetone-d_6, 600 MHz): δ_H 6.15 (d, 3.6, H-13a), 5.96 (ddd, 4.2, 2.6, 1.0, H-8), 5.66 (d, 3.2, H-13b), 5.23 (dd, 9.9, 8.5, H-6), 5.09 (dd, 9.6, 1.0, H-1), 5.05 (dd, 9.9, 1.4, H-5), 4.75 (m, H-2), 4.45 (q, 6.7, H-3'), 3.83 (d, 11.0, 5a'), 3.67 (d, 11.0, H-5b'), 3.24 (dddd, 8.5, 3.6, 3.2, 1.0, H-7), 2.74 (dd, 14.3, 5.2, H-9a), 2.66 (dd, 10.9, 5.8, H-3a), 2.53 (dd, 14.3, 2.6, H-9b), 2.06 (m, H-3b), 1.82 (d, 1.4, H-15), 1.67 (d, 1.0, H-14), 1.44 (d, 6.7, H-4'); ^{13}C NMR (acetone-d_6, 150 MHz): δ_C 172.8 (C-1'), 169.7 (C-12), 143.0 (C-4), 137.8 (C-11), 135.8 (C-1), 134.1 (C-10), 130.8 (C-5), 121.6 (C-13), 82.4 (C-2'), 75.8 (C-6), 75.3 (C-8), 69.6 (C-2), 66.2 (C-5'), 60.1 (C-3'), 53.4 (C-7), 49.8 (C-3), 45.2 (C-9), 20.1 (C-14), 19.6 (C-4'), 19.2 (C-15). Positive mode HRESIMS *m/z* 401.1582 $[M+Na]^+$ (calc. for $C_{20}H_{26}NaO_7$, 401.1571).

Leishmanicidal and trypanocidal screening

Trypanocidal and leishmanicidal assays of compounds **1**, **2**, **3**, and **4** were performed using the same methodology to assay phenolic derivatives previously isolated from *C. uniflora*.[7] Human monocyte cells lines THP-1 (ATCC TIB-202) were grown in RPMI-1640 medium (phenol red-free) (Sigma-Aldrich, CO. St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, USA), 12.5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 2 mM Glutamax. Cell culture was maintained at 37 °C and 5% CO_2 incubator. *L. amazonensis* MHOM/BR/77/LTB0016 promastigotes, expressing β-galactosidase, were maintained in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5% heat inactivated FBS and 2% human urine at 26 °C.

For the leishmanicidal screening against *L. amazonensis* intracellular amastigotes, THP-1 cells (3.0 x 10^4 *per* well) were cultivated in 96 well plates containing RPMI-1640 medium supplemented as described above and treated with 100 ng mL^{-1} of phorbol 12-myristate 13-acetate (PMA) for 72 h at 37 °C and 5% CO_2, to allow THP-1 cells differentiation into non-dividing macrophages.[18]

Promastigotes (4.0 x 10^6 parasites *per* mL) at 4 days of culture 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. After, THP-1 cells were incubated with a parasite/cell ratio of 10:1 at 34 °C for 3h and 5% CO_2. After this period, infected cultures were washed with PBS to remove free parasites and nonadherent cells. Then, infected cells were incubated with complete supplemented RPMI-1640 medium (180 µL) for another 24 h to permit the transformation of promastigotes into intracellular amastigotes.

Tulahuen strain *T. cruzi* expressing β-galactosidase[19] was provided by 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 x 10^4 cells *per* well) in 96-well microplates in a parasite/cell ratio of 3:1 and incubated overnight at 37 °C and 5% CO_2. The medium containing non-internalized parasites was removed and replaced by 180 µL fresh medium.

Compounds **1-4** were dissolved in 1% dimethyl sulfoxide (DMSO) and diluted from 50 µmol L^{-1} to 1.56 µmol L^{-1}. Infected cells layer was treated by addition of 20 µL of each compound followed by incubation at 34 °C and 5% CO_2 for 48 h. After treatment, cells were carefully washed with PBS and incubated for 16 h at 37 °C with 250 µL Chlorophenolred-ß-D-galactopyranoside 100 µmol L^{-1} (Sigma-Aldrich Co., St. Louis, MO, USA) (CPRG) and 0,1% Nonidet P-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. Results were expressed as percentage (%) growth inhibition of *L. amazonensis* and *T. cruzi* amastigotes forms. Amphotericin B 2 µmol L^{-1} (Bristol-Myers, Squibb) and Benznidazole 20 µmol L^{-1} (Sigma) were used as positive control for leishmanicidal and trypanocidal activities, respectively. As negative control group was utilized 1% DMSO.

RESULTS AND DISCUSSION

Phytochemical investigation of the dichloromethane fraction from *C. uniflora* leaves revealed the presence of four sesquiterpene

lactones with germacranolide skeleton: 2α-hydroxy-8β-2',3',5'-trihydroxy-angeloyloxycostunolide (**1**), desacetyleupaserrin (**2**), 2α-hydroxy-8β-3'-hydroxy-2',5'-epoxyangeloyloxycostunolide (**3**) and ovatifolin (**4**). Sesquiterpene lactones were characterized using analysis of their physical (m.p.) and spectral data (1D and 2D NMR, and HRESIMS), as well as comparison with published data.

For compounds **1** and **3**, ^{1}H and ^{13}C NMR spectral data were early described by Pearce *et al.*[20] on different solvents: DMSO-d_6, C_5D_5N and $CDCl_3$-MeOD (3:1). In this work, NMR data of these compounds are reported for the first time in acetone-d_6, and chemical structures of compounds **2** and **4** were established by comparison of their spectral data with those previously described.[21,22]

Compound **1**, named 2α-hydroxy-8β-2',3',5'-trihydroxy-angeloyloxycostunolide, was obtained as an orange oil. ^{1}H NMR spectrum of this compound presented characteristic signals of an α,β-unsaturated sesquiterpene lactone with an α-methylene-γ-lactone moiety: two doublets at δ 6.18 (1H, d, *J*=3.6 Hz, H-13a) and 5.70 (1H, d, *J*=3.2 Hz, H-13b) coupled with a hydrogen resonating at δ 3.29 (1H, dddd, *J*=8.4, 3.6, 3.2, 1.0, H-7), ascribed to H-7. Hydrogen H-7 was also coupled to a hydrogen at δ 5.26 (1H, dd, *J*=9.9, 8.4 Hz) assigned to H-6 and to another hydrogen at δ 6.01 (1H, ddd, *J*=4.2, 2.8, 1.0 Hz) corresponding to H-8. Two doublets signals resonating at δ 1.80 (3H, d, *J*=1.5 Hz, H-15) and 1.61 (3H, d, *J*=1.2 Hz, H-14) inferred the existence of methyl hydrogens, presenting characteristic long range allylic coupling and chemical shifts of methyl groups attached to a double bond. Ester side-chain presented four signals: at δ 4.35 (d, *J*=11.2, H-5a') and 4.20 (d, *J*=11.2, H-5b'), typical of geminal methylene hydrogen close to oxygen, and δ 4.51 (1H, q, *J*=6.6, H-3') coupled with a doublet at δ 1.50 (3H, d, *J*=6.6) corresponding to H-4', indicating a vicinal coupling.

Carbon chemical shift values were obtained by HSQC and HMBC correlation maps. 2D NMR spectra showed the presence of 20 carbon signals that after analysis were established as three methyl, four methylene (including one methylene sp^2 at δ 121.7 and other oxygenated at δ 67.3), seven methine (of which four were oxygenated and two were alkene carbons), and six quaternary carbons (including one sp^3 oxygenated carbon, three alkene carbons, one ester carbonyl, and one lactone carbonyl). These data are consistent with a germacranolide skeleton.

Compound **3** was obtained as a white solid and showed ^{1}H NMR signals remarkably similar to compound **1**, exhibiting significant differences only in chemical shift values of some signals of the side-chain. In **1**, hydrogen signals H-5a' and H-5b' appeared as two doublets at δ 4.35 (1H, d, *J*=11.2, H-5a') and 4.20 (1H, d, *J*=11.2, H-5b'), whereas in compound **3** these signals are more shielding, resonating at δ 3.83 (1H, d, *J*=11.0, H-5a') and 3.67 (1H, d, *J*=11.0, H-5b'). These results suggest that the vicinal diol present in compound **1** was replaced by an epoxide ring in **3** (Figure 1). Further, molecular formula of **3** was established by HRESIMS in positive mode. HRESIMS spectrum exhibited the formation of a high-abundance sodium adduct ion peak $[M+Na]^+$ at *m/z* 401.1582 (calc. for $C_{20}H_{26}NaO_7$, 401.1571), compatible with a sesquiterpene lactone of molecular formula $C_{20}H_{26}O_7$. From this deduction, chemical structure of **3** was determined as 2α-hydroxy-8β-3'-hydroxy-2',5'-epoxyangeloyloxycostunolide.

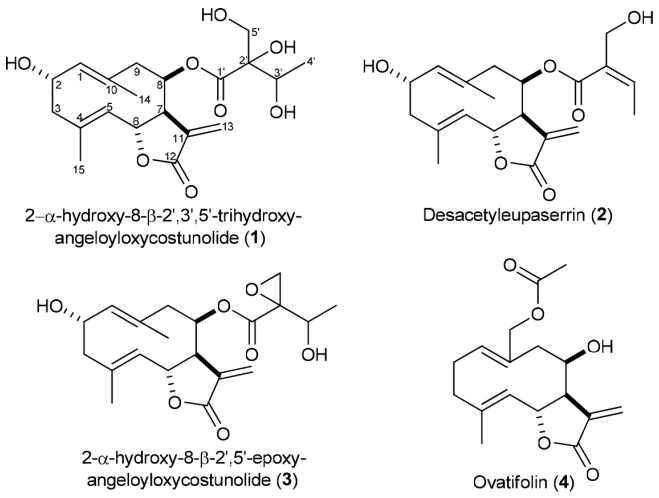

Figure 1. *Sesquiterpene lactones isolated from C. uniflora leaves*

Compounds **1**, **2**, **3,** and **4** are here described for the first time for *C. uniflora* species and *Calea* genus, but they have been identified in other plants of Asteraceae family. Desacetyleupaserrin (**2**) was previously reported in *Helianthus pumilus*,[23] *Eupatorium mikanioides*[24] and *E. glehni*,[25] 2α-hydroxy-8β-2',3',5'-trihydroxy-angeloyloxycostunolide (**1**) and 2α-hydroxy-8β-3'-hydroxy-2',5'-epoxyangeloyloxycostunolide (**3**) in *Helianthus resinosus*,[20] and ovatifolin (**4**) in *Podanthus ovatifolius, P. mitiqui*[22,26,27] and *Blainvillea latifolia* species.[28]

Results of the trypanocidal and leishmanicidal assays were expressed as percentage of growth inhibition from *T. cruzi* and *L. amazonensis* amastigotes and are summarized in Table 1. Ability of compounds to inhibit the parasites growth was evaluated at concentration of 50 μmol L^{-1}. DMSO was used as negative control at a maximum concentration of 1% and did not interfere on parasites growth in any of bioassays. Amphotericin B at a concentration of 2.0 μmol L^{-1} was used as positive control against *L. amazonensis*, producing 82.4% growth inhibition. Benznidazole 20 μmol L^{-1}, the drug of choice to treat Chagas' disease, was used as positive control against *T. cruzi*, causing an 82.7% inhibitory effect on parasitic growth.

Considering the leishmanicidal activity, compound **4** exhibited higher activity, with a growth inhibition of 59.9% on *L. amazonensis* amastigotes, followed by **2** (19.4%), **1** (14.4%), and **3** (13.4%). Regarding the trypanocidal activity, compound **3** exhibited moderate inhibitory effect on growth of *T. cruzi* amastigotes (36.8%), and compounds **1** and **2** did not showed trypanocidal activity. Although several natural sesquiterpene lactones present notable antiprotozoal

Table 1. Leishmanicidal and trypanocidal activities of isolated compounds from *C. uniflora* against *L. amazonensis* and *T. cruzi*

Tested compounds Concentration (50 μmol L^{-1})	%Growth inhibition ± SD *L. amazonensis* amastigotes	%Growth inhibition ± SD *T. cruzi* amastigotes
1	14.4 (±1.5)	No activity
2	19.4 (±2.5)	No activity
3	13.4 (±2.0)	36.8 (±1.8)
4	59.9 (±1.8)	5.0 (±1.1)
DMSO 1%	0	0
Benznidazole 20 μmol L^{-1}	-	82.7 (±0.9)
Amphotericin B 2 μmol L^{-1}	82.4 (±1.9)	-

activity, compounds isolated herein did not show promising effect against the morphological stages of *L. amazonensis* and *T. cruzi* tested strains.

CONCLUSIONS

Phytochemical analyses of *C. uniflora* leaves led to the isolation and structural determination of four germacranolide-type sesquiterpene lactones, all isolated for the first time for the *C. uniflora* species and *Calea* genus. These results are in agreement with previous chemical studies, which have confirmed that *Calea* species are rich in sesquiterpene lactones. It is important to point out that the identification of chemical constituents is truly relevant for ensuring the safety and efectiveness of popular medicine. Regarding the antiprotozoal activity, the assays revealed that ovatifolin (**4**) was the most bioactive compound against *L. amazonensis* intracellular amastigotes and none of isolated sesquiterpene lactones showed promising trypanocidal activity.

SUPPLEMENTARY MATERIAL

1D and 2D-NMR, and HRESIMS data of compounds **1** and **3** (Figures 1S-9S) are available free of charge in the supplementary material at http://quimicanova.sbq.org.br/ as PDF.

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