Bioorganic Chemistry 89 (2019) 103001

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Antitrypanosomal activity and effect in plasma membrane permeability of (−)-bornyl *p*-coumarate isolated from *Piper cernuum* (Piperaceae)

Thiago R. Morais[a], Thais A. Costa-Silva[b], Daiane D. Ferreira[c], Bianca J. Novais[a], Ana Claudia T. Torrecilhas[a], Andre G. Tempone[c], João Henrique G. Lago[b,*]

[a] Institute of Environmental, Chemical and Pharmaceutical Sciences, Universidade Federal de São Paulo, 09972-270 São Paulo, Brazil
[b] Center of Natural Sciences and Humanities, Universidade Federal do ABC, 09210-580 Santo Andre, São Paulo, Brazil
[c] Centre for Parasitology and Mycology, Instituto Adolfo Lutz, 01246-000 São Paulo, SP, Brazil

ARTICLE INFO

Keywords:
Trypanosoma cruzi
Piper cernuum
(−)-Bornyl *p*-coumarate
Plasma membrane permeability

ABSTRACT

This work describes the isolation of six metabolites from leaves and branches of *Piper cernuum* (Piperaceae): (−)-cubebin (**1**), (−)-hinokinin (**2**), (−)-kusunokinin (**3**), *trans*-dehydroagarofuran (**4**), 11-hydroxi-4,5-secoeudesmane-4,5-dione (**5**), and (−)-bornyl *p*-coumarate (**6**). Antitrypanosomal activity and toxicity of purified compounds were performed *in vitro* against trypomastigote forms of *Trypanosoma cruzi* and NCTC cells, respectively. Compounds **2**, **3** and **5** showed moderate activities with IC_{50} values of 33.1, 31.8 and 45.9 μM, respectively, while compounds **1** and **4** were inactive (IC_{50} > 100 μM). On the other hand, compound **6** displayed an IC_{50} value of 2.1 μM, a selectivity index (SI) of 18 and induced a considerable interference in the plasma membrane permeability (87%) in trypomastigotes of *T. cruzi*. Additionally, the lethal effect of compound **6** in *T. cruzi* could be associated to the plasma membrane permeability. Finally, experiments using scanning electron microscopy (SEM) confirmed the obtained results in which was possible to observe total alteration parasites topography after treatment with compound **6** in comparison to untreated parasites. These data indicated that the lethal action of compound **6** is directly related to structural disruption of the membrane.

1. Introduction

Species from *Piper* genus are widely distributed in tropical and subtropical regions of the world [1]. Regarding the phytochemical aspects, the genus *Piper* has been described to accumulate different classes of natural products such as alkaloids, amides, terpenoids, benzoic acids, and lignoids with antiparasitic activity, especially against *Trypanosoma cruzi* [2–14]. Despite this potential, there is no information concerning the antitrypanosomal activity of compounds isolated from *Piper cernuum*. Almost seven million people worldwide are estimated to be infected with *T. cruzi* parasites. Today, there is only one drug, benznidazole, available for the therapy of Chagas Disease (CD) in Brazil with reduced effectiveness [15]. Considering the restricted therapeutic arsenal and the elevated toxicity of the current drugs, the need for new treatments is essential. In continuation of our work aiming towards the identification of bioactive compounds, especially those with antiparasitic activity from Brazilian flora [16–18], in the present work was evaluated the activity of *n*-hexane extracts from leaves and branches from *P. cernuum* against trypomastigote forms of *T. cruzi*. Thus, as both extracts displayed activity, they were subjected to a bioactivity-guided fractionation approach to afford six compounds: (−)-cubebin (**1**), (−)-hinokinin (**2**), (−)-kusunokinin (**3**), *trans*-dehydroagarofuran (**4**), 11-hydroxy-4,5-secoeudesmane-4,5-dione (**5**), and (−)-bornyl *p*-coumarate (**6**). Furthermore the investigation of the action of the most active compound in the plasma membrane of the parasite was investigated with SYTOX green probe and by Scanning Electron Microscopy (SEM).

2. Material and methods

2.1. General experimental procedures

Starting materials and reagents were purchased from Sigma-Aldrich. Solvents were of synthetic or analytical grade. Silica gel (Merck, 230–400 mesh) was used for the column chromatographic (CC) separation while silica gel 60 PF_{254} (Merck) was used for analytical and preparative TLC. IR spectra were obtained as KBr pellets in a Perkin-Elmer Infrared Spectrometer model 1750. ^{1}H and ^{13}C NMR spectra of the compounds were recorded at 300 and 75 MHz, respectively, using a Bruker Ultrashield 300 Avance III spectrometer. Spectra were obtained

* Corresponding author.
E-mail address: joao.lago@ufabc.edu.br (J.H.G. Lago).

https://doi.org/10.1016/j.bioorg.2019.103001
Received 13 November 2018; Received in revised form 13 May 2019; Accepted 19 May 2019
Available online 20 May 2019

using $CDCl_3$ (Aldrich) as solvent and TMS as internal standard. Chemical shifts (δ) were reported in parts per million (ppm) and the coupling constant (*J*) in Hz. Optical rotations were measured in a digital polarimeter JASCO DIP-370 (Na filter, λ = 588 nm). HRESIMS and LREIMS were recorded on a Bruker Daltonics MicroTOF QII and on a MS-QP-5050A (70 eV) mass spectrometers, respectively.

2.2. Plant material

Leaves and branches of *P. cernuum* were collected from a single tree at the Atlantic Forest area in Cubatão city, São Paulo State, Brazil, in October 2014. Botanical identification was carried out by MSc. Guilherme A. Antar. A voucher specimen (A823) has been deposited in the herbarium of *Instituto Florestal de Sao Paulo*, SP, Brazil.

2.3. Extraction and isolation

Dried and powdered leaves (63 g) and branches (25 g) of *P. cernuum* were exhaustively extracted with *n*-hexane at room temperature using an accelerated solvent extractor system (Dionex ASE-350). After elimination of the solvent under reduced pressure were obtained 1.26 g and 0.51 g of leaves and branches extracts, respectively. After evaluation of the antitrypanosomal activity, it was observed that both extracts displayed activity (death of 100% of trypomastigote forms of *T. cruzi* at 200 μg/mL) and were individually subjected to bioactivity-guided fractionation procedures. Part of active extract from leaves (1.16 g) was subjected to fractionation over SiO_2 eluted with increasing amounts of EtOAc in *n*-hexane to afford 11 groups (A – K), in which B, G, H and I displayed antitrypanosomal activity. Group B (254 mg) was chromatographed over SiO_2 using *n*-hexane:EtOAc 9:1 as eluent to afford compound **4** (24 mg). Group G (232 mg) was chromatographed using SiO_2 and $CHCl_3$:MeOH 99:1 as eluent to give compounds **2** (16 mg) and **5** (3 mg). Compounds **3** (25 mg) and **1** (12 mg) were isolated, respectively, from groups H (144 mg) and I (59 mg) by column chromatography over SiO_2 eluted with $CHCl_3$:MeOH 99:1. Part of active extract from branches (0.42 g) was subjected to fractionation over SiO_2 eluted with increasing amounts of EtOAc in *n*-hexane to afford nine groups (A′–I′), in which B' and C' displayed antitrypanosomal activity. Group B' and C' were pooled together and the obtained material (B'/C', 102 mg) was chromatographed over SiO_2 using increasing amounts of EtOAc in *n*-hexane as eluents to afford five groups (B′/C′-1 to B′/C′-5). As the bioactivity was detected in group B′/C′-3, it was purified by prep. SiO_2 TLC (*n*-hexane/EtOAc, 8:2) to afford compound **6** (27 mg).

2.3.1. (−)-Cubebin (1)

White amorphous solid. $[\alpha]_D^{25}$ – 8.1 (*c* 0.45, $CHCl_3$). IR (KBr) ν_{max} 3437, 2924, 1486, 1035, 924 cm^{-1}. HRESIMS *m/z*: 379.1156 $[M + Na]^+$ (calculated for $C_{20}H_{20}O_6Na$, 379.1157). ^1H NMR (300 MHz, $CDCl_3$), *δ*/ppm: 6.63 (1H, d, *J* = 7.5 Hz, H-5′), 6.61 (1H, d, *J* = 8.5 Hz, H-3), 6.51 (1H, d, *J* = 1.5 Hz, H-2′), 6.49 (1H, dd, *J* = 7.5 and 1.5 Hz, H-6′), 6.45 (1H, d, *J* = 1.5 Hz, H-2), 6.44 (1H, dd, *J* = 8.5 and 1.5 Hz, H-6), 5.85 (2H, s, O<u>C</u>H_2O), 5.20 (1H, br s, H-9), 3.93 (1H, dd, *J* = 8.5 and 7.0 Hz, H-9′b), 3.73 (1H, dd, *J* = 8.5 and 8.0 Hz, H-9′a), 2.60 (1H, dd, *J* = 13.5 and 7.5 Hz, H-7b), 2.52 (1H, m, H-7′b), 2.50 (1H, m, H-7′a), 2.37 (1H, m, H-7a), 2.07 (1H, m, H-8), 2.05 (1H, m, H-8′). ^{13}C NMR (75 MHz, $CDCl_3$), *δ*/ppm: 147.6 (C-5), 147.5 (C-3′), 145.9 (C-4′ and C-4), 134.1 (C-1′), 133.3 (C-1), 121.8 (C-6′), 121.4 (C-2), 109.2 (C-2′), 108.9 (C-6), 108.1 (C-3), 108.0 (C-5′), 103.4 (C-9), 100.8 (O<u>C</u>H_2O), 72.2 (C-9′), 53.0 (C-8), 45.9 (C-8′), 39.2 (C-7′), 38.4 (C-7).

2.3.2. (−)-Hinokinin (2)

White amorphous solid. $[\alpha]_D^{25}$ − 29.8 (*c* 0.95, $CHCl_3$). IR (KBr) ν_{max} 2923, 1767, 1489, 1441, 1241, 1038, 928 cm^{-1}. HRESIMS *m/z*: 355.1188 $[M + H]^+$ (calculated for $C_{20}H_{19}O_6$, 355.1182). ^1H NMR (300 MHz, $CDCl_3$), *δ*/ppm: 6.66 (1H, d, *J* = 8.0 Hz, H-3), 6.63 (1H, d, *J* = 8.0 Hz, H-5′), 6.56 (1H, d, *J* = 2.0 Hz, H-2′), 6.53 (1H, dd, *J* = 8.0 and 2.0, H-2), 6.39 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6), 6.37 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6′), 5.86 (2H, s, O<u>C</u>H_2O), 4.06 (1H, dd, *J* = 9.0 and 7.0 Hz, H-9′a), 3.86 (1H, dd, *J* = 9.0 and 7.5 Hz, H-9′b), 2.91 (1H, dd, *J* = 14.0 and 5.0 Hz, H-7a), 2.77 (1H, dd, *J* = 14.0 and 7.5 Hz, H-7b), 2.52 (1H, m, H-7′a), 2.46 (1H, ddd, *J* = 8.0, 7.5 and 5.0 Hz, H-8), 2.39 (2H, m, H-7′b and H-8′). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 178.4 (C-9), 147.8 (C-5), 147.7 (C-3′), 146.4 (C-4), 146.3 (C-4′), 131.6 (C-1), 131.3 (C-1′), 122.2 (C-2), 121.5 (C-6′), 109.4 (C-5′), 108.8 (C-3), 108.3 (C-6), 108.2 (C-2′), 101.0 (O<u>C</u>H_2O), 71.1 (C-9′), 46.5 (C-8), 41.3 (C-8′), 38.4 (C-7′), 34.8 (C-7).

2.3.3. (−)-Kusunokinin (3)

White amorphous solid. $[\alpha]_D^{25}$ − 26.1 (*c* 0.99, $CHCl_3$). IR (KBr) ν_{max} 2940, 1767, 1515, 1490, 1443, 1240, 1027, 929 cm^{-1}. HRESIMS *m/z*: 371.1493 $[M + H]^+$ (calculated for $C_{21}H_{23}O_6$, 371.1494). ^1H NMR (300 MHz, $CDCl_3$), *δ*/ppm: 6.71 (1H, d, *J* = 8.0 Hz, H-5′),6.65 (1H, d, *J* = 8.0 Hz, H-3), 6.54 (1H, d, *J* = 2.0 Hz, H-6), 6.53 (1H, dd, *J* = 8.0 and 2.0 Hz, H-2), 6.51 (1H, dd, *J* = 8.0 and 2.0 Hz, H-6′), 6.42 (1H, d, *J* = 2.0 Hz, H-2′), 5.88 (2H, s, O<u>C</u>H_2O), 4.07 (1H, dd, *J* = 9.0 and 7.0 Hz, H-9′a), 3.88 (3H, s, 4′-OMe), 3.87 (3H, s, 3′-OMe), 3.81 (1H, dd, *J* = 9.0 and 7.0 Hz, H-9′b), 2.90 (1H, dd, *J* = 14.4 and 5.5 Hz, H-7a), 2.79 (1H, dd, *J* = 14.4 and 7.0 Hz, H-7b), 2.55 (1H, dd, *J* = 12.5 and 5.5 Hz, H-7′a), 2.47 (1H, ddd, *J* = 8.7; 7.0 and 5.5 Hz, H-8), 2.46 (1H, d, *J* = 12.5 Hz, H-7′b), 2.44 (1H, ddt, *J* = 8.7; 5.5 and 7.0 Hz, H-8′). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 178.4 (C-9), 149.1 (C-3′), 148.0 (C-4′), 147.9 (C-5), 146.5 (C-4), 131.5 (C-1′), 131.4 (C-1), 122.3 (C-2), 120.6 (C-6′), 111.8 (C-2′), 111.4 (C-5′), 109.5 (C-6), 108.1 (C-3), 101.0 (O<u>C</u>H_2O), 71.2 (C-9′), 55.9 (3′-OMe), 55.8 (4′-OMe), 46.5 (C-8), 41.2 (C-8′), 38.3 (C-7′), 34.8 (C-7).

2.3.4. Trans-dehydroagarofuran (4)

Colourless syrup. IR (KBr) ν_{max} 2927, 1464, 884, 1016, 962, 737 cm^{-1}. HRESIMS *m/z*: 223.2058 $[M+H]^+$ (calculated for $C_{15}H_{27}O$, 223.2062). ^1H NMR (300 MHz, $CDCl_3$), δ/ppm: 1.89 (2H, dd, *J* = 12.0 and 3.8 Hz, H-1), 1.87 (1H, m, H-7), 1.68 (2H, m, H-9), 1.57 (2H, m, H-2), 1.48 (1H, m, H-4), 1.44 (2H, m, H-6), 1.33 (3H, s, H-13), 1.25 (5H, m, H-8 and H-12), 1.19 (2H, m, H-3), 1.07 (3H, s, H-14), 1.01 (3H, d, *J* = 7.8 Hz, H-15). ^{13}C NMR (75 MHz, $CDCl_3$), *δ*/ppm: 87.8 (C-5), 80.9 (C-11), 44.5 (C-7), 40.4 (C-4), 38.4 (C-6 and C-10), 38.0 (C-9), 37.4 (C-1), 30.5 (C-12), 29.2 (C-3), 24.9 (C-8), 23.5 (C-13), 22.8 (C-14), 17.7 (C-15), 17.0 (C-2).

2.3.5. 11-Hydroxy-4,5-secoeudesmane-4,5-dione (5)

Colourless syrup. IR (KBr) ν_{max} 3451, 2940, 1706, 1375, 1268, 1028, 735 cm^{-1}. HRESIMS *m/z*: 277.1778 $[M+Na]^+$ (calculated for $C_{15}H_{26}O_3Na$, 277.1780). ^1H NMR (300 MHz, $CDCl_3$), *δ*/ppm: 2.42 (2H, m, H-3), 2.40 (2H, m, H-6), 2.12 (3H, s, H-15), 1.73 (2H, m, H-8), 1.72 (1H, m, H-7), 1.65 (2H, m, H-1), 1.49 (2H, m, H-9), 1.42 (2H, m, H-2), 1.21 (6H, s, H-12 and H-13), 1.01 (3H, s, H-14). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 215.9 (C-5), 208.5 (C-4), 72.0 (C-11), 50.0 (C-7), 47.8 (C-10), 43.5 (C-3), 39.8 (C-6), 38.3 (C-1), 36.3 (C-9), 29.7 (C-15), 27.4 (C-13), 27.2 (C-12), 21.7 (C-14), 21.5 (C-8), 17.9 (C-2).

2.3.6. (−)-Bornyl p-coumarate (6)

White amorphous solid. $[\alpha]_D^{25}$ − 26.7 ($CHCl_3$, *c* 0.22). IR (KBr) ν_{max} 3348, 2956, 1681, 1605, 1513, 1443, 1248, 1169, 1037, 831 cm^{-1}. HRESIMS *m/z*: 299.1654 $[M-H]^-$ (calculated for $C_{19}H_{23}O_3$, 299.1647). LREIMS (70 eV) *m/z* (rel. int.): 300 $[M]^+$ (8), 164 (12), 147 (1 0 0), 119 (18), 91 (20). ^1H NMR (300 MHz, $CDCl_3$), *δ*/ppm: 7.62 (1H, d, *J* = 15.8 Hz, H-7), 7.43 (2H, d, *J* = 8.3 Hz, H-2 and H-6), 6.86 (2H, d, *J* = 8.3 Hz, H-3 and H-5), 6.33 (1H, d, *J* = 15.8 Hz, H-8), 5.00 (1H, ddd, *J* = 9.6; 3.0 and 1.7 Hz, H-2′), 2.42 (1H, m, H-3′a), 2.03 (1H, m, H-6′b), 1.75 (1H, m, H-5′a), 1.71 (1H, m, H-5′b), 1.70 (1H, t, *J* = 4.2 Hz, H-4′), 1.35 (1H, m, H-6′a), 1.05 (1H, dd, *J* = 13.6 and 3.6 Hz, H-3′b), 0.93 (3H, s, H-8′), 0.89 (3H, s, H-9′), 0.87 (3H, s, H-10′). ^{13}C NMR (75 MHz, $CDCl_3$), *δ*/ppm: 167.8 (C-9), 157.6 (C-4), 144.0 (C-7), 129.9

(C-2 and C-6), 127.3 (C-1), 116.2 (C-8), 115.8 (C-3 and C-5), 80.0 (C-2′), 48.9 (C-1′), 47.8 (C-7′), 44.9 (C-4′), 36.8 (C-3′), 28.0 (C-6′), 27.2 (C-5′), 19.7 (C-9′), 18.8 (C-8′), 13.5 (C-10′).

2.4. Ethics statement

BALB/c mice were obtained by the animal breeding facility at the Instituto Adolfo Lutz - SP, Brazil. The animals were maintained in sterilized cages under a controlled environment and received water and food *ad libitum*. Animal procedures were performed with the approval of the Research Ethics Commission (project number CEUA IAL/Pasteur 02/2011), in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.5. Parasites and mammalian cell maintenance

Trypomastigotes of *T. cruzi* (Y strain) were maintained in Rhesus monkey kidney cells (LLC-MK2-ATCC CCL 7), cultivated in RPMI-1640 medium supplemented with 2% FBS at 37 °C in a 5% CO_2-humidified incubator. The murine conjunctive cells (NCTC clone 929, ATCC) and LLC-MK2 were maintained in RPMI-1640 supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

2.6. Evaluation of in vitro antitrypanosomal activity

To determine the 50% inhibitory concentration (IC_{50}) against *T. cruzi*, compounds **1–6** were previously dissolved in DMSO (concentration of DMSO used in the well did not exceed 0.5% of the final volume) serially diluted (two-fold) (150–1.71 μM) in RPMI-1640 medium − 2% SBF. Trypomastigotes obtained from LLC-MK2 cultures previously infected, were seeded at 1×10^6 cells/well in 96-well plates and incubated with the serial dilutions of tested compounds, during 24 h at 37 °C in a 5% CO_2-humidified incubator. Benznidazole was used as standard drug. The trypomastigote viability was determined by the resazurin assay (0.011% in PBS). The optical density was determined in FilterMax F5 (Molecular Devices) at 570 nm [19].

2.7. Cytotoxicity in mammalian cells

NCTC cells-clone L929 (6×10^4 cells/well) were seeded and incubated with compounds **1–6** (200–1.56 μM), previously dissolved as described above, for 48 h at 37 °C in a 5% CO_2 incubator. The cytotoxic concentration (CC_{50}) was determined by MTT assay [20]. The optical density was determined in FilterMax F5 (Molecular Devices) at 570 nm. The selectivity index (SI) was determined using the following equation: CC_{50} against NCTC cells/IC_{50} against parasites [20].

2.8. Hemolytic activity

The hemolytic activity of compound **6** was evaluated in BALB/c erythrocytes [21]. A 3% suspension of mouse erythrocytes was incubated for 2 h with compound **6** (150 to 1.17 μM) in 96-well U-shape microplate at 25 °C. After determined time the supernatant was read at 570 nm in a spectrophotometer FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices (USA). Ultrapure distilled water was used as a positive control (100% hemolysis) and phosphate-buffered saline (PBS) as a negative control (0% hemolysis). DMSO was also used at 0.5% as internal control.

2.9. SYTOX green assay for cell membrane permeability

Trypomastigotes of *T. cruzi* (2×10^6/well) obtained from LLCMK2 cell infection as described, were washed and incubated in the dark with 1 μM SYTOX Green probe (Molecular Probes) in HANKS' balanced salts solution (HBSS; Sigma-Aldrich) supplemented with 10 mM D-Glucose (HBSS + Glu) as described [17]. Compound **6** was added (t = 0 min) at IC_{50} value (2.1 μM) and fluorescence was measured every 20 min for up to 120 min. The maximum permeabilization was obtained with 0.5% Triton X-100 (TX-100). Fluorescence intensity was determined using a fluorimetric microplate reader (FilterMax F5 Multi-Mode Microplate Reader-Molecular Devices) with excitation and emission wavelengths of 485 and 520 nm, respectively. The following internal controls were used in the evaluation: (i) the background fluorescence of the compound at the respective wavelengths, (ii) the possible interference of DMSO. Samples were tested in duplicate.

2.10. Scanning electron microscopy (SEM)

Trypomastigotes of *T. cruzi* (2×10^6/well) obtained from LLCMK2 cell infection as described, were washed with HBSS + Glu and incubated with compound **6** (at IC_{50} value) by 60 min. Subsequently, parasites were washed twice in PBS at pH 7.2 and adhered on cover slips previously coated with 0.1% aqueous poly-L-lysine (Sigma) for 30 min at room temperature. The trypomastigotes were ressuspended and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, containing 0.146 M sucrose and 5 mM $CaCl_2$ for 1 h at room temperature. Sample preparation for analyses was done as previously described [22]. The images were analyzed and photographed using a Scanning Electron Microscope (Quanta -FEG).

2.11. Statistical analysis

The data obtained represent the mean and standard deviation of duplicate samples from two independent assays. IC_{50} and CC_{50} values were calculated using a sigmoid dose-response curves in Graph–Pad Prism 5.0 software (GraphPadSoftware, San Diego, CA, USA), and the 95% intervals are included in parentheses.

3. Results and discussion

Antitrypanosomal activity of the *n*-hexane extracts obtained from leaves and branches of *P. cernuum* was evaluated *in vitro* against trypomastigote forms and 100% of death of parasites at 200 μg/mL was observed. Bioactivity-guided fractionation of both extracts afforded compounds **1–5** from leaves and **6** from branches (Fig. 1). ^{1}H NMR spectra of compounds **1–3** displayed signals of aromatic hydrogens at range δ 6.4–6.7 as well as double-doublets at approximately δ 3.6 and 4.0 ($J \sim 8$ Hz) assigned to H-9′ from dibenzobutyrolactone lignans. In the spectra of these three compounds were observed singlets at δ 5.9, characteristic of methylenedioxyl units while in the case of compound **3** was detected two peaks additional singlets at δ 3.81 and 3.87, indicating the presence of two methoxyl groups. In the ^{1}H NMR spectrum of compound **1** was also observed one doublets at δ 5.15 ($J = 1.5$ Hz), indicative of the occurrence of a lactol unity at C-9. ^{13}C and DEPT 135° NMR spectrum of **1–3** displayed characteristic signals of aromatic carbon atoms at δ 108–148 (C-1 to C-6 and C-1′ to C-6′), methylenedioxy group at δ 101 and, in the case of compound **3**, two methoxyl groups at δ 55.9 and 55.8. The presence of hemiketal carbon C-9 in compound **1** was confirmed by the presence of one peak at δ 103.4 in concordance with literature data to α-epimer [23]. In the case of compounds **2** and **3** were detected two signals attributed to carbonyl carbon C-9 at δ 178.4. HRESIMS spectra of showed *quasi*-molecular ion peaks at *m/z* 379.1156 $[M + Na]^+$ (compound **1**), 355.1188 $[M+H]^+$ (compound **2**) and 371.1493 $[M+H]^+$ (compound **3**). These results allowed the identification of **1–3** as cubebin, hinokinin and kusunokinin, respectively. Finally, the negative values of optical specific rotation determined to these compounds indicated the occurrence of (−)-enantiomers, similarly to cubebin previously isolated from *P. cernuum* [23], and other related derivatives isolated from *Aristolochia lagesiana* and *A. pubescens* [24].

^{13}C and DEPT 135° NMR spectra of compounds **4** and **5** displayed 15 signals each, indicating occurrence of two sesquiterpenoids. In the case

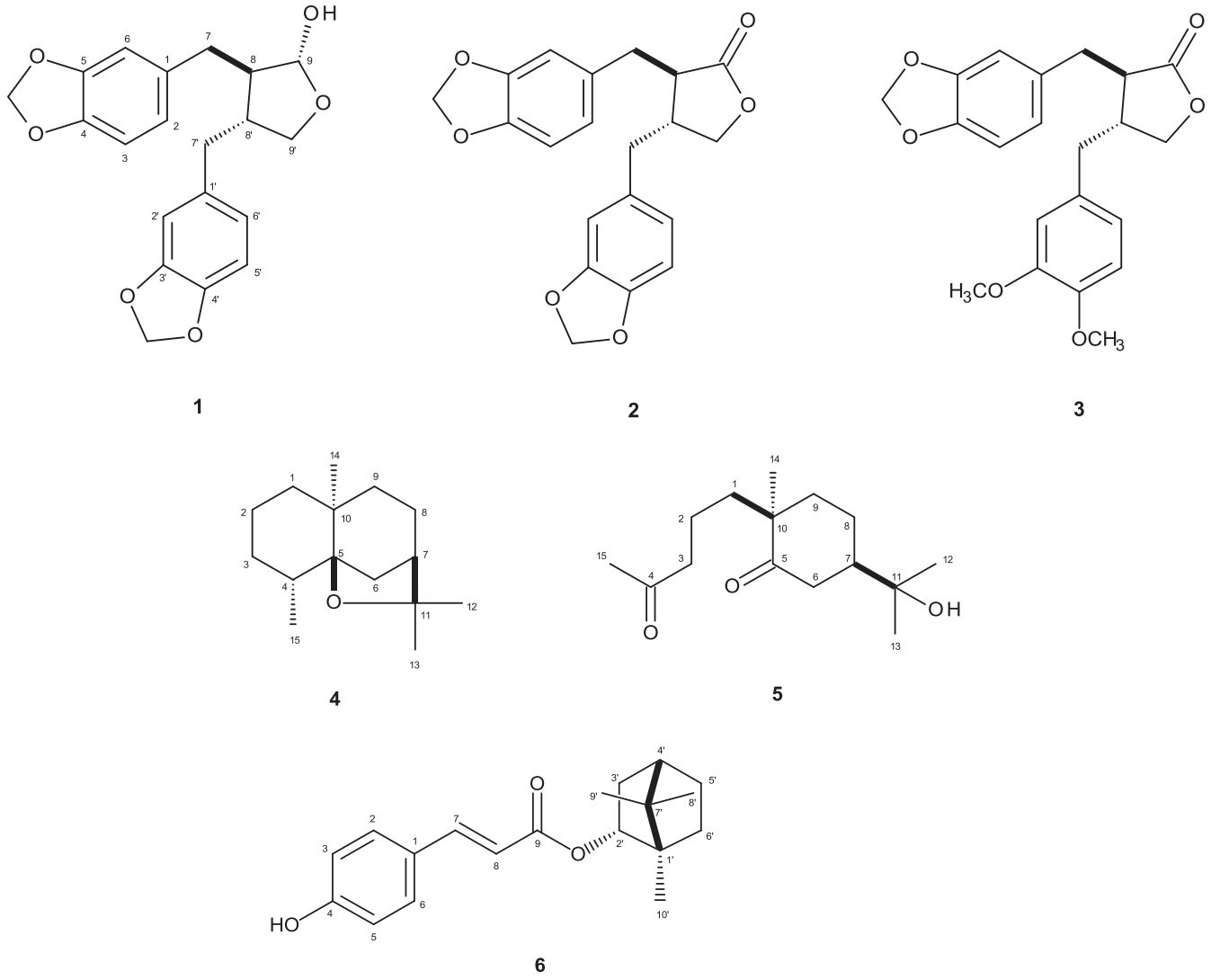

Fig. 1. Structures of compounds **1–6** isolated from *P. cernuum*.

of compound **4** were observed two carbinolic quaternary carbons at δ 87.8 (C-5) and δ 80.9 (C-11). These peaks associated to the presence of four methyl groups C-12 to C-15 respectively at δ 30.5, 23.5, 22.8 and 17.7, suggested the presence of an eudesmane skeleton. Analysis of the HMBC spectra associated to HRESIMS data, which showed the $[M+H]^+$ *quasi*-molecular ion peak at *m/z* 223.2058, allowed the identification of **4** as *trans*-dehydroagarofuran [25]. Similarly, in the ^{13}C NMR spectrum of compound **5** were observed four methyl groups C-12 to C-15 rekspectively at δ 27.2, 27.4, 21.7 and 29.7. However, this spectrum showed one carbinolic carbon at δ 72.0 (C-11) and two carbonyl carbons at δ 208.5 (C-4) and 215.9 (C-5). Analysis of the HMBC spectrum associated to HRESIMS data, which showed the $[M + Na]^+$ *quasi*-molecular ion peak at *m/z* 277.1778, allowed the identification of **5** as 11-hydroxy-4,5-secoeudesmane-4,5-dione [26]. ^1H NMR spectrum of compound **6** showed two coupled aromatic hydrogens at δ 6.86 (J = 8.3 Hz, H-3/H-5) and 7.43 (J = 8.3 Hz, H-2/H-6) as well as two doublets at δ 6.33 (J = 15.8 Hz, H-8) and 7.62 (J = 15.8 Hz, H-9) a characteristic feature of a *p*-coumaroyl unit. Additional peaks were observed at δ 5.00 (ddd, J = 9.6, 3.0 and 1.7 Hz), assigned to carbinolic hydrogen H-2′ and at δ 0.87, 0.89 and 0.93, attributed to three methyl hydrogens H-10′, H-9′ and H-8′, respectively. ^{13}C NMR of compound **6** showed 17 peaks being those at δ 167.8 (C-9), 116.2 (C-8), 144.0 (C-7) and at range δ 116 – 158 (C-1 to C-6) assigned to *p*-coumaroyl unit, confirmed by the detection of base peak ion at *m/z* 147 in the LREIMS spectrum. Remaining 10 signals were attributed to a monoterpene moiety. Since were observed signals from a carbinolic carbon at δ 80.0 (C-2′) and three methyl at δ 18.8 (C-8′), 19.7 (C-9′) and 13.5 (C-10′), was proposed the occurrence of a 2-borneol fragment. Analysis of the HMBC spectrum associated to HRESIMS data, which showed the $[M-H]^-$ at *m/z* 299.1654, allowed the identification of compound **6** as bornyl *p*-coumarate. Finally, the optical rotation $[\alpha]_D^{25}$ − 26.7° ($CHCl_3$, *c* 0.22), indicates the (−)-enantiomer, in concordance with literature [27]. This is the first report of the occurrence of compounds **2–6** in *P. cernuum*.

Compounds **1–6** were incubated 24 h with trypomastigotes forms of *T. cruzi* and the cell viability was determined by the resazurin assay. As showed in Table 1, hinokinin (**2**) and kusunokinin (**3**) showed activity against trypomastigotes with IC_{50} values of 33.1 ± 2.8 and 31.8 ± 4.1 μM, respectively, while the related dibenzobutyrolactone lignan cubebin (**1**) was inactive (IC_{50} > 100 μM). These data suggests that the effectiveness of compounds **2** and **3** could be related to the presence of the carbonyl group at C-9 instead of the hydroxyl group, as observed to compound **1**. Sesquiterpene **5** exhibited reduced activity against trypomastigotes forms with IC_{50} value of 45.9 ± 9.1 μM, while **5** showed to be inactive. Among all tested natural products, compound **6** was the most effective with an IC_{50} value of 2.1 ± 0.4 μM. Benznidazole was used as a positive control and resulted in an IC_{50} value of 16.2 ± 0.4 μM. Considering the toxicity against NCTC cells,

Table 1
Antitrypanosomal activity (trypomastigotes of *T. cruzi*) and cytotoxicity (NCTC cells) of compounds **1–6** isolated from *P. cernuum*.

Compounds	IC_{50} (μM) (± SD)	CC_{50} (μM) (± SD)	SI
1	> 100	66.2 ± 5.8	–
2	33.1 ± 2.8	75.4 ± 8.1	2.3
3	31.8 ± 4.1	67.3 ± 3.8	2.1
4	> 100	> 200	–
5	45.9 ± 9.1	> 200	> 4.3
6	2.1 ± 0.4	38.2 ± 2.4	18.2
Benznidazole	16.2 ± 0.4	> 200	> 12.3

IC_{50} – 50% inhibitory concentration, CC_{50} – 50% cytotoxic concentration, SI – selectivity index, ± S.D. standard deviation.

compounds **4** and **5** did not display cytotoxicity (CC_{50} > 200 μM) while compounds **1 – 3** exhibited CC_{50} values of 66.2 ± 5.8, 75.4 ± 8.1 and 67.3 ± 3.8 μM, respectively. The sigmoid dose response curves of the active compounds could be observed in the Fig. 2A and B. Benznidazole was used as standard drug and showed no cytotoxicity at the highest tested concentration (200 μM). Despite the cytotoxicity (CC_{50} = 38.2 ± 2.4 μM) observed to compound **6,** the selectivity index (SI) was determined as 18.2 and the compound not showed hemolytic activity at the highest tested concentration (Fig. 3). Therefore, this compound could be considered an interesting model to development of continuous studies concerning the identification of bioactive compounds based on natural products.

Antitrypanosomal activity of compounds **2** and **3** were previously reported and, in the case of hinokinin (**3**), it was also observed an *in vivo* efficacy using an acute phase murine model of *T. cruzi* [28,29]. However, no information about the biological activity of compounds **4–6** was found in the literature. Although intracellular amastigotes is considered the most relevant stage of the parasite, the discovery of active compounds against the extracellular trypomastigotes has been considered of great importance [30,15], especially to studies aiming the determination of the lethal mechanisms of active compounds. Based on this aspect, plasma membrane studies using the fluorescent probe SYTOX green demonstrated that compound **6** induced a considerable interference in the permeability (87%) of *T. cruzi* trypomastigotes when compared to the untreated parasites (Fig. 4). The plasma membrane regulates the transport of nutrients, pH homeostasis, and homeostasis of other ions, and alterations in plasma membrane permeability can alter its fluidity and cellular morphology leading in parasite death [31]. In order to confirm the previous studies on plasma membrane permeability, a scanning electron microscopy (SEM) was performed. This micromorphological investigation has been used to analyze the surface topology of cells, but also tissues and organs. SEM uses a narrow electron beam to collect high-resolution, high-magnification images of backscattered electrons emitted from sample surfaces. Due to

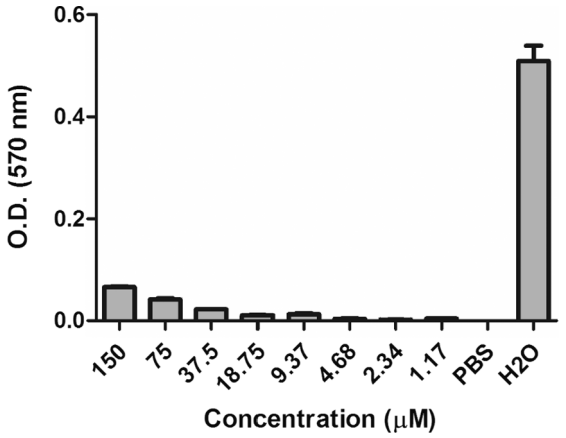

Fig. 3. Hemolytic activity of the compound **6** (150 to 1.17 μM) incubated with a suspension of mouse erythrocytes (3%) for 2 h. Ultrapure distilled water was used as a positive control (100% hemolysis) and phosphate-buffered saline (PBS) as a negative control (0% hemolysis). Supernatant was read at 570 nm in a spectrophotometer (FilterMax F5 Multi-Mode Microplate Reader, Molecular Devices - USA).

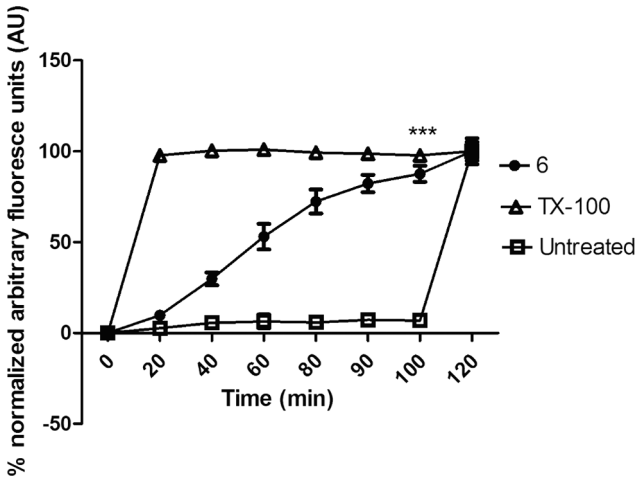

Fig. 4. Effect of compound **6** on plasma membrane permeabilization of trypomastigotes of *T. cruzi*. SYTOX green dye (1 μM) fluorescence was spectrofluorimetrically measured every 20 min. Minimum (non-treatment) and maximum permeabilization (TX-100) were obtained. Normalized fluorescence units were obtained by calculating the mean percentages of untreated (0%) and TX-100%-treated (100%) parasites. *** < 0.001.

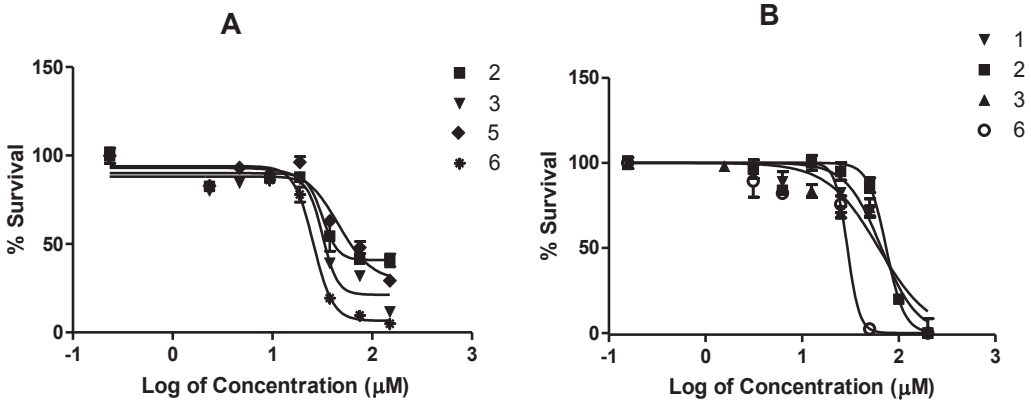

Fig. 2. (A) Determination of the dose-response curves of the active compounds **2**, **3**, **5** and **6** against trypomastigotes of *T. cruzi*. (B) Determination of the dose-response curves of the active of the compounds **1–3** and **6** against NCTC cells – clone L929. Dose response curves were obtained in GraphPad Prism 5.0 software.

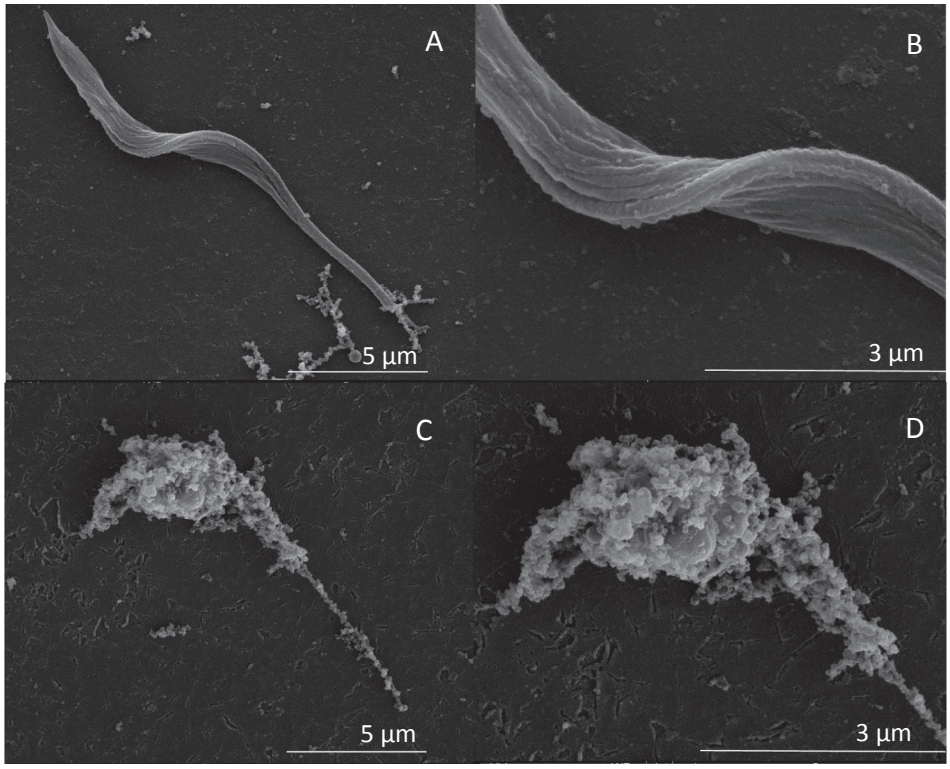

Fig. 5. Scanning electron microscopy (SEM) of trypomastigotes of *T. cruzi.* **A** and **B** show control cells (parasites without treatment) and **C** and **D** show the effect of compound **6** at the IC_{50} value in the trypomastigotes of *T. cruzi* Magnification: (A) 17,095×; (B) 50,000×; (C) 17,000×; (D) 30,000×.

the narrowness of the excitation beam, the resultant images have a high depth-of-field that can be used to understand parasites topography [32,33]. In Fig. 5 it is possible to observe the disruption of plasma membrane of the parasites, culminating in the complete destruction of cells after treatment for 60 min with **6** at the IC_{50}.

4. Conclusions

Bioactivity-guided fractionation of extracts from leaves and twigs of *P. cernuum* afforded to the isolation of six compounds bioactive compounds **1–6**. This is the first report of the compounds **2–6** in *P. cernuum*. Among the tested compounds, bornyl *p*-coumarate (**6**) was the most effective with an IC_{50} value of 2.1 ± 0.4 µM, SI > 18 and induced a considerable interference in the plasma membrane permeability (87%) of trypomastigotes. Additionally, studies using SEM confirmed the total alteration of the parasites topography after 60 min of incubation, confirming that the lethal action of compound **6** is directly related to structural disruption of the membrane.

Acknowledgements

This work was funded by grants and fellowships provided by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP – projects 2018/07885-1 and 2018/10279-6). We thank CEME (*Centro de Microscopia Eletrônica*, EPM-UNIFESP) for scanning electron microscopy experiments. We also thank CNPq scientific research award to B.J.N, J.H.G.L and A.G.T. and CAPES to fellow to T.R.M. and T.A.C.S. This publication is part of the activities of the Research Network Natural Products against Neglected Diseases (ResNetNPND): http://www.uni-muenster.de/ResNetNPND/.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103001.

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