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Acetylenic fatty acids from *Porcelia macrocarpa* (Annonaceae) against trypomastigotes of *Trypanosoma cruzi*: Effect of octadec-9-ynoic acid in plasma membrane electric potential

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ABSTRACT

Porcelia macrocarpa (Warm.) R. E. Fries (Annonaceae) is an endemic plant in Brazil where its tasty pulp has been eaten fresh. The hexane extract from its flowers was subjected to chromatographic procedures to afford four acetylene derivatives identified as octadec-9-ynoic (stearolic acid – **1**), (11*E*)-octadec-11-en-9-ynoic (santalbic acid – **2**), 8-hydroxyoctadec-9,11-diynoic (**3**) and 8-hydroxyoctadec-17-en-9,11-diynoic (isanolic acid – **4**) acids by NMR and HRESIMS. Among tested compounds against trypomastigote forms of *T. cruzi*, octadec-9-ynoic acid (**1**) displayed higher potential with IC_{50} = 27.6 μM and a selectivity index (SI) higher than 7. Compounds **2** and **3** showed IC_{50} of approximately 60 μM while compound **4** was inactive. The lethal action of the compound **1** was investigated using spectrofluorometric techniques to detect ROS content, plasma membrane permeability and plasma membrane potential by flow cytometry. Compound **1** showed no alteration in the production of ROS of treated trypomastigotes and no alteration of the plasma membrane permeability was observed as detected by the fluorescent probe SYTOX-green after 120 min of incubation. However, by using the potential-sensitive fluorescent probe $DiSBAC_2(3)$, compound **1** caused depolarization of the plasma membrane potential when compared to untreated parasites. Our results demonstrated the anti-*T. cruzi* effects of compounds **1–3** isolated from flowers of *P. macrocarpa* and indicated that the lethal effect of compound **1** in *T. cruzi* could be associated to the plasma membrane disturbance of the parasite.

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1. Introduction

Porcelia macrocarpa (Warm.) R. E. Fries (Annonaceae) is an endemic plant in Brazil where its tasty pulp has been eaten fresh [1]. Chemical composition from different parts of *P. macrocarpa* was previously conducted in which amides/lignanamides [2], alkaloids [3,4], flavonoids [5], terpenoids/steroids [5,6], amino-acids [7], and acetylenic acetogenins [8] were isolated. Additionally, the anti-*Trypanosoma cruzi* activity of acetylenic derivatives from seeds of *P. macrocarpa* has previously been reported [9].

T. cruzi is the hemoflagellate agent of Chagas disease (CD), which is recognized by World Health Organization as a neglected disease, affecting 8 million of people in America [10,11]. Pecoul and cols. [12] estimate that in the next five years, 200,000 people living with Chagas disease will die from heart disease and related complications. Furthermore there are only two available drugs for the treatment, benznidazole and nifurtimox, which exhibit reduced efficacy and severe side effects [11,13]. In this context, the search for new chemotherapeutic alternatives to treat CD is urgent and natural products are a promising toll approach to identify lead structure, providing prototypes and related active derivatives. In the present work, the chemical analysis and trypanocidal evaluation of four acetylenic fatty acids isolated from the *n*-hexane extract from flowers of *P. macrocarpa* against the trypomastigote forms of the etiologic agent of CD was carried out for the first time.

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2. Material and methods

2.1. General experimental procedures

Silica gel 60 (Merck, 63–210 mesh) and Sephadex LH-20 (Amersham Biosciences) were used for the column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical and thin-layer chromatography (TLC) (0.25 mm). NMR spectra were recorded on a Bruker Ultrashield 300 – Avance III spectrometer, operating at 300 and 75 MHz to the ^{1}H and ^{13}C nucleus, respectively. $CDCl_3$ (Aldrich) was used as solvent and TMS (Aldrich) as internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (J) in Hz. High resolution electrospray ionization mass spectra (HRESIMS) were obtained in a Bruker Daltonics MicrOTOF-Q II™ ESI-Qq-TOF (negative ionization mode). Optical rotations were measured on a JASCO DIP-370 digital polarimeter (Na filter, λ = 588 nm).

2.2. Plant material

P. macrocarpa flowers were collected in the Atlantic Forest area at the *Instituto de Botânica de São Paulo* (coordinates 23°38′33.8″S, 46°37′17.5″W), Brazil, in October 2015. Its voucher specimen, registered SP76791, has been deposited at the Herbarium of the same institution.

2.3. Extraction and isolation

Flowers of *P. macrocarpa* (322 g) were dried (40 °C for 120 h), powdered and exhaustively extracted with *n*-hexane. After removal of the solvent by evaporation under reduced pressure, 3.45 g of crude extract was obtained. Then, part of the obtained extract (2.5 g) was chromatographed on a silica gel column, eluted with *n*-hexane, EtOAc and MeOH in an increasing polarity gradient to give 60 fractions (50 mL each). These fractions were combined based on their TLC pattern to produce ten groups (A–J). After analysis by NMR, groups H (90 mg) and I (92 mg) were pooled together and subjected to fractionation over Sephadex LH-20 eluted with *n*-hexane:CH_2Cl_2 (1:4) and CH_2Cl_2:acetone (3:2 and 1:4) as reported in the literature [14]. This procedure afforded 60 fractions (10 mL each) which were grouped in five groups (HI-1 to HI-5) after TLC analysis. Finally, groups HI-2 (81 mg) and HI-3 (72 mg) were pooled together and purified by prep. TLC (silica gel/$AgNO_3$ 15%) eluted with *n*-hexane:EtOAc 8:2 to afford pure compounds **1** (34 mg), **2** (10 mg), **3** (42 mg) and **4** (7 mg).

2.3.1. Octadec-9-ynoic acid (stearolic acid, **1**)

Amorphous solid; HR-ESI-MS $[M-H]^-$ m/z 279.2336 (calc. to $C_{18}H_{31}O_2$: 279.2324). ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 2.36 (m, H-2), 1.63 (m, H-3), 1.27–1.47 (m, H-4 to H-7 and H-12 to H-17), 2.14 (t, J = 6.5 Hz, H-8 and H-11), 0.88 (t, J = 6.0 Hz, H-18). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 174.3 (C-1), 34.2 (C-2), 24.7 (C-3), 28.6 (C-4), 28.8 (C-5), 28.9 (C-6), 29.2 (C-7), 18.8 (C-8), 80.1* (C-9), 80.4* (C-10), 18.8 (C-11), 29.2 (C-12), 29.0 (C-13), 29.1 (C-14), 29.1 (C-15), 31.8 (C-16), 22.7 (C-17), 14.1 (C-18) (*signals might be interchanged).

2.3.2. (11E)-Octadec-11-en-9-ynoic acid (santalbic acid, **2**)

Amorphous solid; HR-ESI-MS $[M-H]^-$ m/z 277.2190 (calc. to $C_{18}H_{29}O_2$: 277.2168). ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 2.28 (t, J = 7.5 Hz, H-2 and H-8), 1.61 (m, H-3), 1.26 (m, H-4), 1.30 (m, H-5), 1.33 (m, H-6), 1.35 (m, H-7), 5.41 (d, J = 15.8 Hz, H-11), 6.03 (dt, J = 15.8 and 7.0 Hz, H-12), 1.37 (m, H-14), 1.52 (m, H-15), 1.27 (m, H-16), 1.32 (m, H-17), 0.88 (t, J = 6.0 Hz, H-18). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 176.0 (C-1), 34.1 (C-2), 24.8 (C-3), 28.6 (C-4), 28.7 (C-5), 28.7 (C-6), 28.7 (C-7), 19.2 (C-8), 88.5 (C-9), 79.2 (C-10), 109.7 (C-11), 143.3 (C-12), 32.8 (C-13), 28.7 (C-14), 28.9 (C-15), 31.5 (C-16), 22.5 (C-17), 14.0 (C-18).

2.3.3. 8-Hydroxyoctadec-9,11-diynoic acid (**3**)

Amorphous solid; $[\alpha]_D^{25}$ + 4.20 (c 2.30, MeOH); HR-ESI-MS $[M-H]^-$ m/z 291.1973 (calc. to $C_{18}H_{27}O_3$: 291.1960). ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 2.35 (t, J = 7.4 Hz, H-2), 1.44 (m, H-3), 1.36 (m, H-4), 1.36 (m, H-5), 1.67 (m, H-6), 1.67 (m, H-7), 4.41 (t, J = 6.5 Hz, H-8), 2.28 (t, J = 7.0 Hz, H-13), 1.35 (m, H-14), 1.53 (m, H-15), 1.29 (m, H-16), 1.29 (m, H-17), 0.89 (t, J = 6.5 Hz, H-18). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 179.8 (C-1), 34.0 (C-2), 24.8 (C-3), 28.8 (C-4), 28.9 (C-5), 24.6 (C-6), 37.5 (C-7), 62.8 (C-8), 76.4 (C-9), 64.3 (C-10), 69.8 (C-11), 81.8 (C-12), 19.3 (C-13), 28.5 (C-14), 28.1 (C-15), 31.3 (C-16), 22.5 (C-17), 14.0 (C-18).

2.3.4 8-Hydroxyoctadec-17-en-9,11-diynoic acid (isanolic acid, **4**)

Amorphous solid; $[\alpha]_D^{25}$ + 20.8 (c 2.13, MeOH); HR-ESI-MS $[M-H]^-$ m/z 289.1817 (calc. to $C_{18}H_{25}O_3$: 289.1804). ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 2.28 (m, H-2), 1.35–1.45 (m, H-3 to H-6), 1.70 (m, H-7), 4.41 (br s, H-8), 2.29 (t, J = 6.5 Hz, H-13), 1.50 (m, H-14 and H-15), 2.05 (q, J = 6.6 Hz, H-16), 5.78 (ddt, J = 16.9, 10.3, 6.6 Hz, H-17), 4.98 (m, H-18). ^{13}C NMR (75 MHz, $CDCl_3$), δ/ppm: 173.8 (C-1), 34.1 (C-2), 24.8 (C-3), 28.9 (C-4), 28.8 (C-5), 24.8 (C-6), 37.5 (C-7), 62.9 (C-8), 76.5 (C-9), 64.5 (C-10), 69.9 (C-11), 81.5 (C-12), 19.1 (C-13), 28.0 (C-14), 27.6 (C-15), 33.1 (C-16), 138.3 (C-17), 114.8 (C-18).

2.4. Ethics statement

BALB/c mice were obtained by the animal breeding facility at the Adolfo Lutz Institute-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. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing them with RPMI-1640 medium supplemented with 10% FBS and were maintained 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. Anti-trypomastigote activity

To determine the 50% inhibitory concentration (IC_{50}) against *T. cruzi*, trypomastigotes were obtained from LLC-MK2 cultures previously infected, seeded at 1×10^6 cells/well in 96-well plates and incubated with serial dilutions of tested compounds **1**–**4** (150–1.71 μM), during 24 h at 37 °C in a 5% CO_2-humidified incubator, with benznidazole as the 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 [15].

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