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Phenylpropanoids from *Croton velutinus* with cytotoxic, trypanocidal and anti-inflammatory activities

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ABSTRACT

This current study presents the phytochemical analysis of *Croton velutinus*, describing phenylpropanoids obtained from this species. The fractionation of the roots hexane extract led to the isolation of four new phenylpropanoids derivatives, velutines A–D (**1–4**) and three known (**5–7**). Their structures were established based on spectroscopic (1D-2D NMR; HRMS and IR) analysis. Cytotoxic, trypanocidal and anti-inflammatory activities of compounds **1–7** were evaluated. Only compounds **2** and **5** showed cytotoxic activity against cancer cell lines (B16F10, HL-60, HCT116, MCF-7 and HepG2), with IC_{50} values ranging from 6.8 to 18.3 μM and 11.1 to 18.3 μM, respectively. Compounds **2** and **5** also showed trypanocidal activity against bloodstream trypomastigotes with EC_{50} values of 9.0 and 9.58 μM, respectively. Finally, the anti-inflammatory potential of these compounds was evaluated on cultures of activated macrophages. All compounds exhibited concentration-dependent suppressive activity on the production of nitrite and IL-1β by macrophages stimulated with LPS and IFN-γ. These results indicate phenylpropanoids esters (**2** and **5**) from *C. velutinus* as promising cytotoxic, trypanocidal and anti-inflammatory candidates that warrants further studies.

1. Introduction

Croton, the second largest genus of the Euphorbiaceae family, is represented by approximately 1300 species, among which 68 species were reported in the Caatinga biome, where 21 are considered endemic [1–3]. Previous phytochemical studies described diterpenes and alkaloids as the main secondary metabolites found in this genus. Sesquiterpenes and monoterpenes were also reported, mostly as essential oil constituents [4].

Anti-inflammatory activity of compounds obtained from plants of the genus *Croton* have been previously described using bioguided assays [5], as well as inhibition of macrophage activation and cyclooxygenase activity assays [6,7]. The use of *Croton* species in folk medicine due to an anticancer activity have been validated by the demonstration of cytotoxicity potential of compounds obtained from this genus in several cancer cell lines [8–10]. In addition, *Croton* species also exhibit trypanocidal activity against all the evolutive forms of *Trypanosoma cruzi*, possibly by targeting the trypanotihione reductase pathway [11]. Interestingly, all the biological activities cited above have been described to compounds from the phenylpropanoid class [12–14].

Croton velutinus Baill. can be found in several areas of the Brazilian northeast region, mainly in rupestrian fields and places considered Cerrado of altitude. Cezar (2016) reported the identification of flavonoids, sesquiterpenes and one diterpene, as well as inhibition of angiotensin-I-converting enzyme (ACE), anticholinesterase, antioxidant and larvicidal activities of methanolic and hexane extracts from this species [15].

In search for bioactive compounds found in species from semi-arid Brazil region, this current work describes the occurrence of new phenylpropanoid derivatives in *C. velutinus*, unusual in the *Croton* genus, with cytotoxic, trypanocidal and anti-inflammatory activity.

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2. Experimental

2.1. General experimental procedures

Optical rotations were measured at 25 °C in $CHCl_3$ on a Jasco P-2000 polarimeter (Easton MD, USA). FTIR spectra were acquired on a Bruker Vertex 70 spectrometer (Bruker, Billerica, MA, USA). 1D and 2D NMR experiments were performed using a Bruker AVANCE III HD (400 MHz and 100 MHz for ^1H and ^{13}C) NMR spectrometer. $CDCl_3$ was used as a reference for the residual non deuterated solvent signals. MicrOTOF II (Bruker Daltonics, Billerica, MA, USA) with an electrospray ion (ESI) source, was used to perform the ESI-TOF-MS analysis. Isolation and purification of the chemical constituents were conducted using several chromatographic methods, including column chromatography (CC), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC). Semipreparative chromatographic analyses were performed using an HPLC system (Shimadzu, Kyoto, Japan) equipped with an LC-6AD binary solvent pump, a Rheodyne injector, an SPD-M10A diode array detector, and an SCL-10A system controller. A semipreparative reversed phase (RP) C_{18} column (Venusil XBP; 10 mm × 250 mm × 10 μm) was used. Silica gel (Silicycle, particle size of 0.040–0.063 mm) was used in CC. Commercial silica gel (Whatman) plates were used in TLC in layers with a thickness of 0.25 mm on an aluminum support (20 × 20 cm). The substances were analyzed by using ultraviolet radiation at wavelengths of 254 and 366 nm (Boitton brand apparatus) and by impregnating the plates in glass chambers saturated with iodine vapor.

2.2. Plant material

The specimens of *C. velutinus* were harvested in March 2016 at the city of Morro do Chapéu, Bahia, Brazil (11°33′00″S, 41°09′22″W). Access registration in the National Management System of Genetic Patrimony and Associated Traditional Knowledge (SISGEN) was encoded as A22E9B0. The botanical material was identified by Prof. M. L. S. G. and deposited at the Herbarium Alexandre Leal Costa (ALCB), Institute of Biology, Federal University of Bahia with the voucher number 123491.

2.3. Extraction and isolation

The roots of *C. velutinus* were oven-dried at 32 °C for 72 h, followed by grinding and maceration with organic solvents. The dried and pulverized material (272 g) was macerated three times with hexane (4 L/7 days) followed by the same procedure with MeOH (4 L). The corresponding solutions were then concentrated under reduced pressure until complete dryness in order to obtain the hexanic (4.5 g) and methanolic (43.5 g) extract. The hexanic extract (4.5 g) was subjected to column chromatography (CC) with silica gel as stationary phase. As mobile phase, it was used the solvents hexane and ethyl acetate, pure or in binary mixture on increasing degree of polarity. The 28 fractions obtained were analyzed by thin layer chromatography (TLC) and reunited in 14 groups according to their respective retention factors (RFs). The fractions correspondent to the groups 5 (95.3 mg), 7 (10.1 mg) and 10 (112.2 mg) were submitted to NMR analysis and identified as compounds **1**, **5** and **2**, respectively.

The fraction group 13 (15.2 mg) was chromatographed on HPLC-DAD using the following mobile phase: MeOH:HCOOH 0,1% (60:40); isocratic flow of 3 mL/min; run time: 95 min; a semipreparative reversed-phase (RP) C_{18} column (Venusil XBP; 10 mm × 250 mm × 10 μm); wavelength 220 and 254 nm, allowing the isolation of compounds **3** (t_R = 63.02 min, 3.8 mg), **4** (t_R = 89.96 min, 1.1 mg), **6** (t_R = 57.17 min, 3.5 mg) and **7** (t_R = 81.03 min, 1.2 mg).

*(E)-4-(1-Propenyl) phenyl benzoate (**1**)*. Amorphous solid; $[\alpha]_D^{25}$ + 5 (*c*. 0,1, $CHCl_3$). HRESIMS $[M + H]^+$, 239.1073 (calc. For $C_{16}H_{15}O_2$,*m/z* 239.1067). IR ν_{max}: 2955, 2924, 2851, 1730, 1269 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) see Tables 1 and 2.

*(E)-4-(1-epoxy-7,8-propen) phenyl benzoate (**2**)*. Amorphous solid; $[\alpha]_D^{25}$ + 7 (*c*. 0,1, $CHCl_3$). HRESIMS $[M + H]^+$, 255.1023 (calc. For $C_{16}H_{15}O_3$,*m/z* 255.1016). IR ν_{max}: 2958, 2925, 1736, 1601, 1265 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) see Tables 1 and 2.

*Erithro-4-(propan-7,8-diol) phenyl benzoate (**3**)*. Colorless oil; $[\alpha]_D^{25}$ + 15 (*c*. 0,1, $CHCl_3$). HRESIMS $[M + H]^+$, 273.1115 (calc. For $C_{16}H_{17}O_4$,*m/z* 273.1121). IR ν_{max}: 3391, 2923, 1734, 1268, 1204 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) see Tables 1 and 2.

*Threo-4-(propan-7,8-diol) phenyl benzoate (**4**)*. Colorless oil; $[\alpha]_D^{25}$ − 18 (*c*. 0,1, $CHCl_3$). HRESIMS $[M + H]^+$, 273.1120 (calc. For $C_{16}H_{17}O_4$, m/z 273.1121). IR ν_{max}: 3392, 2919, 1735, 1266, 1205 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) see Tables 1 and 2.

2.4. Drugs

Dexamethasone (Sigma-Aldrich, St. Louis, MO), a synthetic glucocorticoid, was used as a positive control in the anti-inflammatory activity screening assay. Gentian violet (Synth, São Paulo, SP, Brazil) was used as positive control in the cytotoxicity assays. Benznidazole (LAFEPE) was used as a reference anti-*T. cruzi* drug. Doxorubicin (doxorubicin hydrochloride, Laboratory IMA S.A.I.C., Buenos Aires, Argentina) was used as a reference anticancer drug. All compounds were dissolved in dimethyl sulfoxide (DMSO; PanReac, Barcelona, Spain) and diluted in cell culture medium for use in the assays. The final concentration of DMSO was less than 1% in all experiments.

2.5. Cells and parasites

MCF7 (human breast carcinoma), HCT116 (human colon carcinoma), HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukemia), B16F10 (murine melanoma), J774 (murine macrophages) and MRC-5 (human lung fibroblast), cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in complete medium with appropriate supplements as recommended by ATCC. All cell lines were tested for mycoplasma using the Mycoplasma Stain Kit (Sigma-Aldrich) to validate the use of cells free from contamination.

The *T. cruzi* Y strain (DTU II) was used in this study. Tissue culture trypomastigotes were obtained from the supernatants of 5 to 6-day-old infected LLC-MK2 cells maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; GIBCO) and 50 μg/mL of gentamicin (Life, Carlsbad, CA) at 37 °C in a 5% humidified CO_2 atmosphere.

2.6. Cytotoxic activity assay

Cell viability was evaluated by the Alamar blue method as previously described [16,17]. Cells were inserted in 96-well plates for all experiments (7×10^4 cells/mL for B16F10, HCT116, HepG2, MCF-7 and MRC5 cells; 3×10^5 cells/mL for HL-60 cells). After 24 h, compounds were added to each well and incubated for 72 h. Four hours before the end of incubation, 20 μL of a stock solution (0.312 mg/mL) of the Alamar blue (Resazurin, Sigma-Aldrich) was added to each well. Absorbance at 570 nm and 600 nm was measured using the SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA). To determine the inhibitory concentration 50% (IC_{50}), nonlinear regression was used. In addition, a cytotoxicity assay was done with J774 macrophages in the presence of test compounds plus LPS (500 ng/mL; Sigma-Aldrich) and INF-γ (5 ng/mL; Sigma-Aldrich), using the same method described above.

2.7. Cytotoxicity for trypomastigotes

Bloodstream trypomastigotes (4×10^5 cells/well) were seeded into

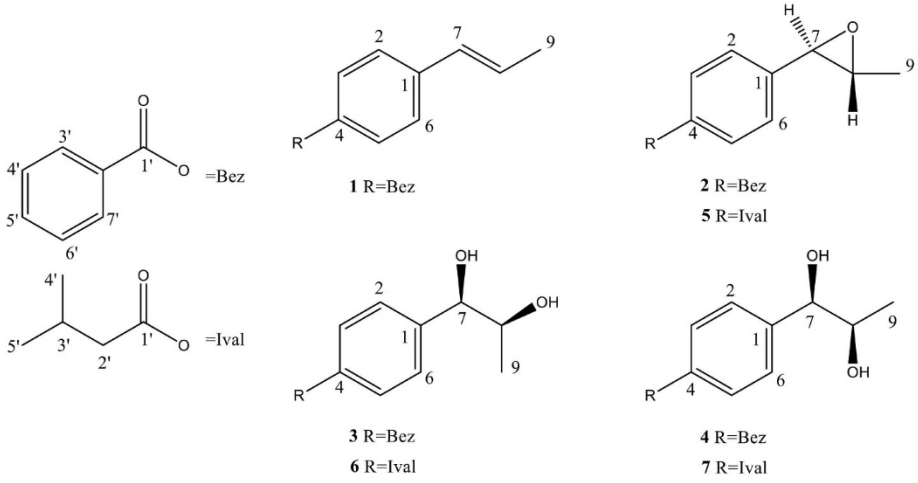

Fig. 1. Compounds isolated from *C. velutinus*.

Table 1
^{1}H NMR data at 400 MHz ($CDCl_3$) for compounds **1** to **7** (*J* in Hz).

No.	1	2	3	4	No	5	6	7
	δ_H	δ_H	δ_H	δ_H		δ_H	δ_H	δ_H
1	–	–	–	–	1	–	–	–
2/6	7.38 d (8.6)	7.32 d (8.6)	7.43 d (8.5)	7.42 d (8.5)	2/6	7.27 d (8.5)	7.36 d (8.5)	7.35 d (8.5)
3/5	7.15 d (8.6)	7.19 d (8.6)	7.21 d (8.5)	7.22 d (8.5)	3/5	7.05 d (8.5)	7.06 d (8.5)	7.06 d (8.5)
4	–	–	–	–	4	–	–	–
7	6.44 dd (15.8; 1.6)	3.61 d (1.9)	4.70 d (4.3)	4.43 d (7.2)	7	3.57 d (1.8)	4.66 d (4.2)	4.36 d (7.0)
8	6.24 dq (15.8; 6.5)	3.03 dq (5.1; 1.9)	4.02 dq (6.4; 4.3)	3.88 br quint (6.4)	8	3.01 dq (5.1; 1.9)	3.98 m	3.82 m
9	1.91 d (6.5; 1.6)	1.46 d (5.1)	1.11 d (6.4)	1.11 d (6.4)	9	1.45 d (5.1)	1.08 d (6.3)	1.06 d (6.3)
1′	–	–	–	–	1′	–	–	–
2′	–	–	–	–	2′	2.43 d (7.2)	2.43 d (7.2)	2.43 d (7.1)
3′/7′	8.21 dd (8.5; 1.0)	8.19 dd (8.0; 1.3)	8.20 dd (8.4; 1.1)	8.20 d (7.4)	3′	2.26 n (6.8)	2.23 n (6.7)	2.23 n (6.7)
4′/6′	7.52 br t (8.5)	7.50 br t (8.0)	7.51 br t (7.5)	7.51 br t (7.4)	4′/5′	1.06 d (6.7)	1.05 d (6.7)	1.05 d (6.7)
5′	7.65 br t (7.5)	7.61 br t (7.5)	7.64 br t (7.5)	7.64 br t (7.8)				

96-well plates and the compounds were added at eight concentrations ranging from 0.39 to 50 μM, in triplicate. Plates were incubated for 24 h at 37 °C and 5% of CO_2. Aliquots of each well were collected and the number of viable parasites, based on parasite integrity and motility, was assessed in a Neubauer chamber and compared to untreated cultures. Three independent experiments were performed. To determine the effective concentration 50% (EC_{50}), we used nonlinear regression.

2.8. Quantification of nitrite and IL-1β in macrophage cultures

In order to determine the amount of nitric oxide and the cytokine IL-1β, J774 macrophages at 1×10^5 per well were cultured in DMEM supplemented with a 10% fetal bovine serum (FBS) and 50 μg/mL of gentamicin for 24 h at 37 °C in an atmosphere containing 5% of CO_2. The cells were stimulated with LPS (500 ng/mL) + IFN-γ (5 ng/mL) and

Table 2
^{13}C NMR data at 100 MHz ($CDCl_3$) for compounds **1** to **7**.

No.	mult	1	2	3	4	No	mult	5	6	7
		δ_C	δ_C	δ_C	δ_C			δ_C	δ_C	δ_C
1	C	135.9	135.6	138.2	138.7	1	C	135.2	138.0	138.7
2/6	CH	126.8	126.8	127.9	127.9	2/6	CH	126.5	127.8	128.0
3/5	CH	121.7	121.9	121.8	121.7	3/5	CH	121.6	121.6	121.8
4	C	149.8	150.8	150.7	150.6	4	C	150.4	150.4	150.6
7	CH	130.2	59.3	77.2	78.8	7	CH	59.0	77.2	79.0
8	CH	126.1	59.2	71.4	72.1	8	CH	58.9	71.3	72.3
9	CH_3	18.6	18.0	17.4	18.7	9	CH_3	17.8	17.4	18.9
1′	C	165.3	165.2	165.3	165.2	1′	C	171.4	171.8	171.7
2′	C	129.7	129.6	129.5	129.3	2′	CH_2	43.2	43.4	43.5
3′/7′	CH	130.3	130.3	130.3	130.1	3′	CH	25.8	26.0	26.0
4′/6′	CH	128.6	128.7	128.7	128.5	4′/5′	CH_3	22.3	22.5	22.5
5′	CH	133.6	133.7	133.8	133.6					

Table 3
Cytotoxicity and anti-*T. cruzi* activity of compounds.

Compounds	IC_{50} (μM)						EC_{50} (μM)
	B16F10[a]	HL-60[a]	HCT116[a]	MCF-7[a]	HepG2[a]	MRC5[a]	*T. cruzi*[b]
1	> 50	> 50	> 50	> 50	> 50	> 50	> 50
2	14.4 (± 0.5)	9.8 (± 2.6)	12.9 (± 1.8)	6.8 (± 1.59)	16.7 (± 0.7)	16.3 (± 1.1)	9 (± 0.59)
3	> 50	> 50	> 50	> 50	> 50	> 50	> 50
4	> 50	> 50	> 50	> 50	> 50	> 50	> 50
5	13.8 (± 01)	11.4 (± 3.6)	13.2 (± 1.0)	11.1 (± 1.4)	18.3 (± 1.8)	19.4 (± 1.7)	9.58 (± 0.1)
6	> 50	> 50	> 50	> 50	> 50	> 50	> 50
7	> 50	> 50	> 50	> 50	> 50	> 50	> 50
Doxorubicin	0.6 (± 0.1)	0.47 (± 0.1)	0.2 (± 0.04)	1.39 (± 0.05)	0.1 (± 0.01)	0.96 (± 0.02)	–
Benznidazole	–	–	–	–	–	–	12.5 (± 0.6)
GV	–	–	–	–	–	–	–

Values represent the mean ± S.D. of 3 determinations obtained in independent experiments performed. EC_{50} = effective concentration at 50%. GV = gentian violet. IC_{50} = inhibitory concentration at 50%. S.D. = Standard deviation.
[a] Determined 72 h after incubation with compounds.
[b] Determined 24 h after incubation with compounds.

Table 4
Compounds from *C. velutinus* have low cytotoxicity against J774 macrophages and inhibit the production of nitric oxide and IL-1β by stimulated macrophages.

Compounds	CC_{50}(μM)	EC_{50}(μM)[a]	EC_{50}(μM)[b]
1	> 100	10.6 (± 1.3)	19.7 (± 2.4)
2	10.1 (± 1.4)	4.3 (± 0.6)	3.68 (± 0.2)
3	> 100	14.5 (± 0.7)	29.6 (± 2.1)
4	> 100	42.9 (± 2.6)	64.8 (± 3.5)
5	26.3 (± 0.7)	1.7 (± 0.4)	1.6 (± 0.1)
6	> 100	32.2 (± 3.4)	49 (± 1.3)
7	> 100	30.4 (± 1.9)	46.3 (± 6.5)
Dexa	–	2.9 (± 0.7)	0.55(± 0.2)
GV	0.8 (± 0.1)	–	–

Values represent the mean ± S.D. of 3 determinations obtained in independent experiments performed. CC_{50} = cytotoxicity concentration at 50%. EC_{50}[a] = effective concentration at 50% in the NO assay. EC_{50}[b] = effective concentration at 50% in the IL-1β assay. Dexa = Dexamethasone. GV = gentian violet. S.D. = Standard deviation.

treated with vehicle, dexamethasone (10 μM), or compounds **1–7** at different concentrations. The supernatants were collected 24 h after the treatment and stored at −80 °C for subsequent quantification. Nitric oxide production was estimated by the determination of nitrite levels, performed according to the Griess method [18] whereas the quantification of IL-1β was performed using the enzyme-linked immunosorbent assay (ELISA) DuoSet kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. To determine the effective concentration 50% (EC_{50}), we used nonlinear regression.

3. Results and discussion

The fractionation of the hexanic extract from *C. velutinus* roots was performed by column chromatography and semipreparative HPLC, leading to the isolation of three known and four new phenylpropanoid derivatives (See Fig. 1).

Compound **1** was isolated as a colorless oil. Its mass was defined by HRESIMS as *m/z* 239.1073 $[M + H]^+$ (calc. For $C_{16}H_{15}O_2$, m/z 239.1067), with a molecular formula of $C_{16}H_{14}O_2$, indicating the presence of ten degrees of unsaturation. The ^1H NMR spectrum has presented the following chemical shift assignments δ_H 8.21 (2H, dd, J = 8.5 and 1.0 Hz, H-3′ and H-7′), δ_H 7.65 (1H, br t, J = 7.5 Hz, H-5′), δ_H 7.52 (2H, br t, J = 8.5 Hz, H-4′ and H-6′) and δ_H 7.38 (2H, d, J = 8.6 Hz, H-2 and H-6), δ_H 7.15 (2H, dl, J = 8.6 Hz, H-3 and H-5) suggesting the presence of mono and disubstituted aromatic rings respectively. The chemical shifts at δ_H 6.44 (1H, dd, J = 15.8 and 1.6 Hz, H-7), δ_H 6.24 (1H, dq, J = 15.8 and 6.5 Hz, H-8) and δ_H 1.91 (3H, dd, J = 6.5 and 1.6 Hz, CH_3–9) corresponded to a propene in a *trans*-configuration. Regarding the ^{13}C NMR spectrum, the carbon signal δ_C 165.1 (C-1′) associated with the respective molecular formula has indicated the presence of a carbonyl ester group. HMBC experiment has provided the correlation between δ_H 8.21 (2H, dd, J = 8.5 and 1.0 Hz, H-3′ and H-7′) to δ_C 165.1 (C-1′), therefore confirming the insertion of the ester group in the monosubstituted aromatic ring. Moreover, the correlation of δ_H 7.38 (2H, d, J = 8.6 Hz, H-2 and H-6) to δ_C 133.5 (C-7) has indicated the presence of a propene group in the disubstituted aromatic ring. Further ^1H and ^{13}C resonance assignments may be found on Tables 1 and 2. Therefore, the compound **1** was assigned as (*E*)-1-(7,8-Propenyl) phenyl benzoate. This compound was synthetized by Díaz-Álvarez et al. 2012 but this is its first report as a natural product [19].

Compound **2** was isolated as a colorless oil. Its mass was defined by HRESIMS as *m/z* 255.1023 $[M + H]^+$ (calc. For $C_{16}H_{15}O_3$, m/z 255.1016), with a molecular formula of $C_{16}H_{14}O_3$, indicating the presence of ten degrees of unsaturation. The ^1H NMR spectrum has presented similar chemical shifts to compound **1** concerning the presence of mono and disubstituted aromatic rings. Besides these ^1H NMR signals, other chemical shifts such as δ_H 3.61 (1H, d, J = 1.9 Hz, H-7), δ_H 3.03 (1H, dq, J = 5.1 and 1.9 Hz, H-8) and δ_H 1.46 (3H, d, J = 5.1 Hz, CH_3–9) were correspondent to an epoxy-propane system with a *trans* relative configuration [20]. HMBC experiment has provided the correlation between δ_H 7.32 (2H, d, J = 8.6 Hz, H-2 and H-6) and δ_H 1.46 (3H, d, J = 5.1 Hz, CH_3–9) to δ_C 59.3 (C-7), therefore confirming the epoxy-propane system. Regarding the optic rotation, compound **2** was correlated to scientific literature data [20]. Complete ^1H and ^{13}C chemical shift assignment may be found on Table 1. Hence, the compound **2** was assigned as (*E*)-1-(7,8-epoxypropen) phenyl benzoate.

Compounds **3** and **4** were isolated as a colorless oil. Their molecular formulae were defined by HRESIMS as $C_{16}H_{16}O_4$ at *m/z* 273.1115 $[M + H]^+$ and $C_{16}H_{16}O_4$ at *m/z* 273.1120 $[M + H]^+$ respectively (calc. For $C_{16}H_{17}O_4$, m/z 273.1121), suggesting the presence of isomers with nine degrees of unsaturation. The ^1H NMR signals correspondent to compounds **3** and **4** were very similar, with typical chemical shifts of mono and disubstituted aromatic rings, also presented on **1** and **2**. Besides the presence of the ring systems, other chemical shifts corresponding to a propane-diol system may be assigned, where the difference between the two structures were on the relative configuration as *erythro and threo*. Compound **3** has presented the following chemical shifts δ_H 4.70 (1H, d, J = 4.3 Hz, H-7), δ_H 4.02 (1H, dq, J = 6.4 e 4.3 Hz, H-8) and δ_H 1.11 (3H, d, J = 6.4 Hz, CH_3–9), stablishing its relative configuration as the *erythro* isomer. Since compound **4** has presented the chemical shifts assignment δ_H 4.43 (1H, d, J = 7.2 Hz, H-7), δ_H 3.88 (1H, br quint, J = 6.4 Hz, H-8) and δ_H 1.11 (3H, d,

J = 6.4 Hz, CH_3–9), it was established as the *threo* isomer. In a previous research, Balboul B., et al. (1996) identified phenylpropanoid derivatives from *Narvalina domingensis* with a propane-diol system, correlating J values of ~5 Hz to *erythro* configuration and ~ 7 Hz for *threo* configuration [21]. Additional ^{13}C NMR data may be found on Table 2. Regarding the optic rotation, compounds **3** and **4** were correlated to the scientific literature data [21], and thus, **3** was assigned as *erithro*-1-(propan-7,8-diol) phenyl benzoate and **4** as *threo*-1-(propan-7,8-diol) phenyl benzoate.

According to the physical and spectroscopic data compared to published values, the known compounds were identified as sellovicine B (**5**), erythro-4-hydroxyphenylpropan-7,8-diol 4-isovalerate (**6**) and threo-4-hydroxyphenylpropan-7,8-diol 4-isovalerate (**7**) [20,21].

We next focused on evaluating the biological activity of the isolated compounds. All compounds were first evaluated against five different cancer cells line (B16F10, HL-60, HCT116, MCF-7 and HepG2) and in a noncancer cell line (MRC5), using a colorimetric method, the AlamarBlue assay. As reveled in Table 3, compound **2** presented IC_{50} values ranging from 6.8 to 16.7 μM for MCF-7 and HepG2 respectively. Compound **5** presented IC_{50} values ranging from 11.1 to 18.3 μM for MCF-7 and HepG2, respectively. Doxorubicin, a reference anticancer compound, presented IC_{50} values ranging from 0.1 to 1.39 μM for HepG2 and MCF-7 respectively. The other compounds did not present cytotoxic activity in the concentrations tested. Regarding cytotoxicity in nontumor cells, **2**, **5** and doxorubicin presented IC_{50} values of 16.3, 19.4 and 0.96 μM respectively, showing a moderate selective profile against most of cancer cells lines (Table 3). Interestingly, the data suggest a greater potency of compounds **2** and **5** compared to phenylpropanoids with cytotoxic activity already described [22–24].

The anti-*T. cruzi* activity of compounds was also investigated against the infective form of the parasite (trypomastigotes forms). As shown in Table 3, only compounds **2** and **5** possesses trypanocidal activity, with EC_{50} values of 9 and 9.58 μM respectively. Under the same conditions, the reference drug benznidazole presented an EC_{50} value of 12.5 μM. These results reinforce the antiparasitic potential of the genus Croton previously described [11,25,26].

Previous to the anti-inflammatory screening assay, we determined the effects of compounds **1–7** on the cell viability of J774 macrophages. As revealed in Table 4, compounds **1**, **3**, **4**, **6** and **7** did not present any cytotoxic effect at the highest concentration evaluated (100 μM). Compounds **2** and **5** presented cytotoxic effects, with CC_{50} values of 10.1 and 26.3 μM, respectively. Under the same conditions, gentian violet, the reference drug, presented a CC_{50} value of 0.8 μM. Therefore, non-cytotoxic concentrations were used in the subsequent experiments. The immunomodulatory activity of the compounds was evaluated of nitric oxide and IL-1β production by J774 cells stimulated with LPS and IFN-γ (Table 4). All compounds decreased the nitrite production, with EC_{50} values ranging from 1.7 and 42.9 μM, and compounds **2** and **5** were the most potent (Table 4). Under the same conditions, dexamethasone showed an EC_{50} value of 2.9 μM. Compounds that reduce production of nitric oxide (NO) by inflammatory cells, such as macrophages, have a potential anti-inflammatory activity [27]. This correlation is possible due the fact that NO is considered an important pro-inflammatory mediator involved on the pathogenesis of inflammation [28]. Corroborating its anti-inflammatory potential, compounds **1–7**, decreased the production of the inflammatory cytokine IL-1β by activated macrophages (Table 4). From the concentration-response studies, it was possible to demonstrate that compounds **1**, **2**, **3**, **4**, **5**, **6** and **7** have EC_{50} values of 19.7, 3.68, 29.6, 64.8, 1.6, 49 and 46.3 μM, respectively, of IL-1β reduction. Therefore, it is possible to conclude that compounds **2** and **5** have the best anti-inflammatory profiles. Under the same conditions, dexamethasone showed an EC_{50} value of 0.55 μM. IL-1β is another fundamental mediator related to the pro-inflammatory response and its decrease on inflammatory conditions has been used as therapeutic strategy towards important chronic inflammatory diseases such as rheumatoid arthritis [29].

Structure activity relationship of compounds **1–7** and their biological activities evaluated in this study indicate that the compounds with improved pharmacological activity (**2** and **5**) have the epoxy-propane system. It is possible that the highly strained three-membered ring might act as an electrophilic center to be attacked by cellular nucleophilic systems [30]. Some reports indicate that substances with the epoxide group as a pharmacophore have several biological activities, including cytotoxic and anti-inflammatory activities [31,32].

4. Conclusions

This work evaluated the cytotoxicity, trypanocidal and anti-inflammatory activities of seven phenylpropanoids, among which four of them are newly described (**1–4**). The phenylpropanoid derivatives **2** and **5** showed the best profiles, being able to inhibit proliferation of a panel of cancer cell lines, lyse the infective form of *T. cruzi* and decrease the production of pro-inflammatory mediators. Thus, the present work reinforces the value of chemical and pharmacological investigations of *Croton* genus, in particular *C. velutinus*, in the search for new anticancer, trypanocidal and anti-inflammatory agents.

Notes

The authors declare no competing financial interest.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

NMR and HRESIMS data of compound **1–7** are available as Supporting Information.

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