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Natural and semi-Synthetic Clerodanes of *Croton cajucara* and their Cytotoxic Effects against Ehrlich Carcinoma and Human K562 Leukemia Cells

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O diterpeno clerodano *trans*-desidrocrotonina (**1**), constituinte majoritário de *Croton cajucara*, tem sua ocorrência correlacionada com o uso dessa planta na medicina popular. Investigações fitoquímicas levaram ao isolamento dos metabólitos **1**, cajucarinolida (**6**), isocajucarinolida (**7**), *trans*-crotonina (**2**), *trans*-cajucarina B (**3**), *cis*-cajucarina B (**4**), *trans*-cajucarina A (**5**), *N*-metiltirosina, ácido vanílico e ácido 4-hidróxi-benzóico. **6** e **7** foram sintetizados em bons rendimentos através da oxidação de **1** com oxigênio-singlete. Todos os clerodanos foram ensaiados frente a células da leucemia humana K562 e do carcinoma de Ehrlich. Os efeitos inibitórios do crescimento celular foram dependentes da dose para os ensaios com o carcinoma de Ehrlich com IC$_{50}$ = 166 µM (**1**), 164 µM (**2**), 65 µM (**6**) e 10 µM (**7**). Além disso, atividade citotóxica moderada foi observada contra as células da leucemia K562 com IC$_{50}$ = 38 µM (**3**), 33 µM (**5**), 36 µM (**6**) e 43 µM (**7**). Os **6** e **7** semi-sintéticos mostraram resultados semelhantes quando comparados com os correspondentes clerodanos naturais.

The clerodane-type diterpene, *trans*-dehydrocrotonin (**1**) the major component of *Croton cajucara* has shown striking correlation with its therapeutic use in traditional folk medicine. Phytochemical investigations led to the isolation of the metabolites **1**, cajucarinolide (**6**), isocajucarinolide (**7**), *trans*-crotonin (**2**), *trans*-cajucarin B (**3**), *cis*-cajucarin B (**4**), *trans*-cajucarin A (**5**), *N*-methyltyrosine, vanillic acid and 4-hydroxy-benzoic acid. **6** and **7** were synthesized in good yield by regiospecific oxidation of **1** using singlet-oxygen. All clerodanes were studied for their cytotoxic effects against human K562 leukemia and Ehrlich carcinoma cells. Ehrlich carcinoma assays with IC$_{50}$ = 166 µM (**1**), 164 µM (**2**), 65 µM (**6**) and 10 µM (**7**) related to cell growth inhibitory effects were dose dependent. Furthermore, moderate cytotoxic activity against K562 leukemia cells was observed with IC$_{50}$ = 38 µM (**3**), 33 µM (**5**), 36 µM (**6**) and 43 µM (**7**). The semi-synthetic **2**, **6** and **7** showed similar results when compared to the corresponding natural clerodanes.

Keywords: *Croton cajucara, trans*-dehydrocrotonin, cajucarinolide, isocajucarinolide, cytotoxic activity

Introduction

According to traditional folk medicine, clerodane *trans*-dehydrocrotonin (**1**) possesses striking correlation with therapeutic use of *Croton cajucara* and included among its biologic properties are antiinflammatory and antinociceptive,[1-3] antioestrogenic,[4] antigenotoxic[5,6] and antiulcerogenic[1,7] activities. In addition, hypoglycemic,[8] antiatherogenic and hypolipidaemic effects[9] were also recorded. Clerodanes *trans*-crotonin (**2**), *trans*-cajucarin B (**3**), *cis*-cajucarin B (**4**), *trans*-cajucarin A (**5**), cajucarinolide (**6**) and isocajucarinolide (**7**) were previously isolated as minor compounds of the stem bark of *Croton cajucara*.[10,11] The cajucarinolides (**6** and **7**)

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proved to possess antiinflammatory activity[11] and **2** showed antiinflammatory, antinociceptive and antiulcerogenic effects.[1,2]

Through generations, the use of *Croton* species in the Brazilian Amazon culture is believed to provide health benefits to its users.[1,12,13] However, during the 1990s, several cases of toxic hepatitis were reported by public hospitals of Belém city (capital of Pará state) located in the Amazon region. This disease resulted from the abusive use, e.g., extended treatment and high doses of this plant. In spite of the warning about the toxicity effects of its leaves towards hepatitis, many people still use them to reduce weight, catching this disease as a result. In the early years of this new century, a Brazilian TV program called 'Globo Reporter', warned about the toxicity of

Croton cajucara, emphasizing that it could cause hepatitis. This folk medicine observation may be correlated to the extended use required for losing weight and slimming, for *C. cajucara* toxicological effects were not observed by Farias *et al.*[14] They have found an absence of acute toxicity of a hydroalcoholic extract obtained from its leaves. This observation does not agree with Kubo *et al.*[15] who had found cytotoxic effects of the methanol extract of the stem bark of *C. cajucara* in hepatocytes of mice *in vitro*. Reinforcing the *C. cajucara* traditional use of the major constituent of its stem bark, **1**, was not genotoxic to mice.[5] The folk toxic warning about the use of *C. cajucara* combined with our early phytopharmacological results prompted us to examine the possible cytotoxicity of **1-7** on the growth of ascitic Ehrlich carcinoma cells
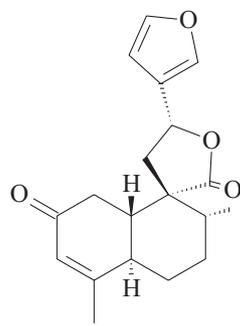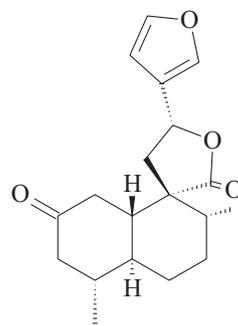
1   **2**
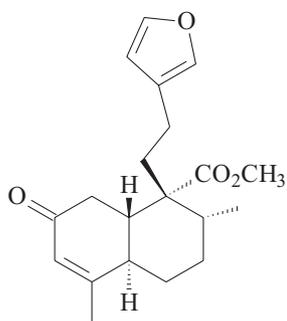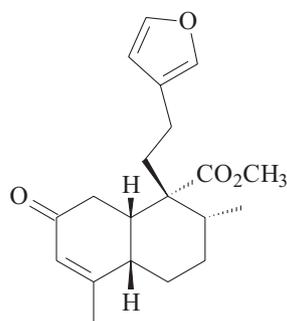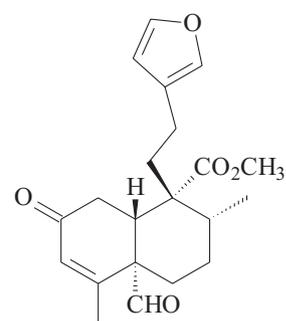
3   **4**   **5**
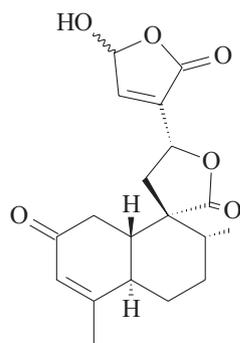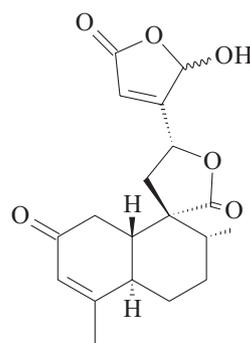
6   **7**

and human K562 leukemia cells. We also expanded the stem bark chemical investigations, obtaining two phenolic compounds and an amino acid, which have not been reported yet as constituents of this plant. The semi-synthetic **6** and **7** were synthesized by regiospecific oxidation of **1**, affording a new tool in the program of chemical transformation of the target molecule **1**.

Experimental

Plant material

The stem bark of *Croton cajucara* was collected in Jacundá, Pará state (Amazon region-Brazil) and identified by Nelson A. Rosa. A voucher specimen (No. 247) was deposited in the Museu Paraense Emílio Goeldi Herbarium (Belém-Brazil).

General chemical procedures

Melting points were determined with a Kofler (Jasco DIP-370) apparatus and were not corrected. Optical activities were measured in $CHCl_3$ on a Perkin-Elmer 341 polarimeter. IR spectra (KBr and $CHCl_3$) were taken on a Perkin-Elmer FT-16PC spectrophotometer and UV spectra (MeOH) on a GBC UV/VIS 911A CG instrument. The $^{13}C(^1H)$/DEPT135 spectra were recorded on Varian-Gemini and Bruker-Advance spectrometers (300 MHz for ^1H and 75 MHz for ^{13}C). Mass spectra were run on a CG/MS Finnigan-4000 and VG Auto Spec Q instruments at 70 eV. TLC was carried out on 0.25 mm layers of silica gel PF 254 (Merck). Classical column chromatography was performed with silica gel 60 (70-230 mesh).

Extraction and isolation of the constituents

The dried and powdered stem barks (6 kg) of native mature plants were extracted in a Soxhlet apparatus with hexane and then methanol (MeOH) each for 48 h. The respective extracts were concentrated in a vacuum, at 40 °C, affording residues of 471.8 and 202.0 g, respectively. For chemical isolation, the obtained extracts were submitted to chromatographic fractionation. The hexane extract was chromatographed on a silica gel column with hexane affording fraction A; CH_2Cl_2 (fraction B) and then, MeOH (fraction C). Fraction B was chromatographed using mixtures of hexane/CH_2Cl_2 affording the new fractions FB1-6 [hexane/CH_2Cl_2 (9:3)], FB7-16 (6:4), FB17-20 (5:5), FB21-24 (1:4) and FB25-28 (0:1) and then with hexane/EtOAc affording fractions FB29,30 (9:1), FB31,32 (7:3), FB33-36 (1:1) and FB37-

40 (0:1). Fractions FB7-36 gave a solid material which was crystallized from hexane/Me_2CO (1:1) affording 37.2 g of **1** [colourless crystals; mp 139-140 °C; literature[15,16] 138.5-140.5 °C; $[\alpha]_D$ +10.6° ($CHCl_3$, *c* 0.6). The mother liquor residue from FB9-24 was chromatographed on silica gel [hexane/EtOAc (7:3, 1 L) and (1:1, 2 L)] to yield an amorphous solid material and an oily residue. The solid material was crystallized from hexane/Me_2CO (1:1) affording 0.151 g of **2** [colorless crystals; mp 130-132 °C, literature[15] 131-132 °C; $[\alpha]_D$ +1.5° ($CHCl_3$, *c* 0.8). The oily residue was subjected to preparative TLC using hexane/EtOAc (8:2) as solvent (eluted four times; R_f 0.4) to yield 0.308 g of **3** [colourless oil; $[\alpha]_D$ –10.2° ($CHCl_3$, *c* 1.6)] and 0.064 g of **4** [colourless oil; $[\alpha]_D$ –13.1° ($CHCl_3$, *c* 1.7)].

Fraction C was chromatographed with mixtures of hexane/EtOAc (8:2 to 0:1) to yield 22.3 g of **1**, which was obtained from fractions FC3 (1:1 of hexane/EtOAc), FC4 (1:3) and FC5 (0:1). The remaining material of these fractions gave a solid material which, after crystallization, [$CHCl_3$/Me_2CO (9:2)] afforded 0.020 g of **6** (colorless needles; mp 202- 204 °C, literature[11] 202-203 °oC) and 0.006 g of **7** (colorless needles; mp 204-205 °C, literature[11] 205-206 °C).

The MeOH extract was chromatographed with mixtures of hexane/EtOAc affording fr_1 (7:3), fr_2 (6:4), fr_3 (5:5), fr_4 (1:3) and fr_5 (0:1) and then with mixtures of EtOAc/MeOH to obtain two fractions [fr_6 (1:1) and fr_7 (0:1)]. The fractions fr_1-fr_7 produced 26.3g of **1**. The remaining material from fractions fr_1 and fr_2 afforded 0.051g of a phenolic acids mixture (vanillic acid and 4-hydroxy-benzoic acid). This mixture was subjected to preparative TLC using hexane/EtOAc (6:4) as eluting solvent (it was eluted five times, R_f 0.5) to yield 0.037 g of vanillic acid (white needles, mp 209-210 °C) and 0.005 g of 4-hydroxy-benzoic acid (white needles, mp 214-215 °C). The remaining material from fractions fr_3 to fr_5 was chromatographed with mixtures of hexane/EtOAc (7:3 to 0:1) affording 0.062 g of **5**, 0.062 g of **6** [crystalline material which was crystallized from hexane/Me_2CO (1:1)] and 0.143 g of *N*-methyltyrosine [white powder; mp 240-241 °C; crystallization from MeOH/H_2O/HCl (8:1.5:0.5); its TLC analysis was performed using *n*-BuOH/Me_2CO/HOAc/H_2O (2.0:3.5:3.5:1.0), detection performed with Ninhydrin reagent, R_f 0.4].

Oxidation of 1 using singlet oxygen

Using the regioselective oxidation method previously described for furanic compounds,[17] a solution of **1** (0.3 g) in dichloromethane (30 mL) containing polystyrene-bound

rose bengal catalyst (2 mL) was stirred at –78 °C under oxygen atmosphere and irradiated with a 500 W Tungsten incandescent lamp. Aliquots of the reaction mixture were periodically analyzed by TLC until reaction appeared to be completed (6 h). The reaction mixture was allowed to warm to room temperature and filtered through a pad of cotton, and the solvent was evaporated under a vacuum. The synthetic material after evaporation was submitted to chromatography on a silica gel column eluted with a mixture of hexane/EtOAc (1:1) and then MeOH to obtain the semi-synthetic derivatives **6** and **7**. The methanolic fraction afforded 0.2 g (65%) of colorless needles, mp 204-205 °C corresponding to **7** isomeric with **6** (20% of colorless needles, mp 202-204 °C, literature[11] 202-203 °C). The semi-synthetic derivatives **6** and **7** were purified by similar techniques to those used for the natural cajucarinolides.

Spectroscopic data of the isolated and semi-synthetic compounds

The characterization of the natural **1-7** was previously performed using spectroscopic methods such as IR, UV, MS and ^{1}H and ^{13}C NMR.[1,10,18] The semi-synthetic derivatives **6** and **7** were compared with authentic samples and shown to be identical. Aromatic acids (mixture of vanillic acid and 4-hydroxy-benzoic acid) and its derivatives **1** and **2**, and also *N*-methyltyrosine were characterized by spectroscopic methods such as IR, UV, MS and NMR.

Assays

The assays against human K562 leukemia and murine Ehrlich carcinoma were performed in three independent experiments. The cells were grown on RPMI-1640 medium supplemented with 5 % fetal bovine serum, 2 mmol L^{-1} glutamine, 100 µg mL^{-1} streptomycin, and 100 U mL^{-1} penicillin, and were incubated at 37 °C under 5% CO_2 atmosphere. In these experiments, cells were seeded in quadruplicate onto 96-wells plates (2×10^4 cell mL^{-1} for K562, and 5×10^5 cell mL^{-1} for Ehrlich) with the tested compounds dissolved in DMSO (final DMSO concentration in culture of 0.2% v/v) at various concentrations. The cultures were incubated for 96 h (for K562) and 48 h (for Ehrlich). Upon incubation, MTT was added and after three hours, formazan was dissolved in 100 mL acidified isopropanol. Tumor cell growths were quantified by the ability of living cells to reduce the yellow dye MTT to a purple formazan product.[19] The absorbance was measured at 550 nm using an Elisa microplate reader, and for each tested compound the concentration required to reduce the absorbance by 50% (IC$_{50}$) in comparison to control cells, was determined.

Results and Discussion

Hexane and methanol extracts afforded the major constituent 19-*nor*-clerodane **1** (1.4%), and the minor components **2** (0.002%), **3** (0.005%), **4** (0.001%), **5** (0.001%), **6** (0.0003%) and **7** (0.0001%).[1,10,18] In addition, the common metabolites 4-hydroxy-3-methoxy-benzoic acid (vanillic acid) and 4-hydroxy-benzoic acid [0.005% in the ratio 87:13 (RMN), respectively] and an amino acid 2-methylamino-3-(4-hydroxyphenyl)-propanoic acid (0.014%, *N*-methyltyrosine) were isolated. The structures of vanillic acid, 4-hydroxy-benzoic acid and *N*-methyltyrosine were identified by spectroscopic analysis (including 300 MHz ^{1}H and ^{13}C NMR and MS experiments) and by chemical transformation of the vanillic acid, with diazomethane giving the two methylated derivative esters (**8** and **9**). ^{1}H and ^{13}C NMR (DMSO/DCl) data of *N*-methyltyrosine were in accordance with the authentic sample of 2-amino-3-(4-hydroxyphenyl)-propanoic acid (tyrosine) obtained from commercial sources. The different observed peaks were assigned to the *N*-Me group of *N*-methyltyrosine.

Two of the aromatic acids vanillic and 4-hydroxy-benzoic acid have shown remarkable antioxidant activity in other species.[20,21] Based on such results, *C. cajucara* could be expected to possess antioxidant properties. Reinforcing this suggestion, several kaempferol metabolites have proved to be antioxidant agents[22-24] and *C. cajucara* leaves also contain two of them, e.g. kaempferol 3,4',7-trimethyl ether and 3,7-dimethyl ether.[1]

Within the research program for the major biological constituents of **1,** we are improving its chemical transformation, which means preparing derivative compounds for screening as pharmacological agents.[1] In the present work, using the method of regioselective oxidation[17] the **7**-derivative was synthesized in good yields (67%) by synthetic transformation of the furan moiety of **1**, with singlet oxygen generated from molecular oxygen by irradiating a polymer-bound rose bengal catalyst in dichloromethane solution at –78 °C (in the presence of a hindered base, such as diisopropylethylamine). In the absence of a hindered base, the reaction of **1** with singlet oxygen yielded an isomeric mixture of **6** and **7**, which was purified by TLC chromatography. The structures of both **6** and **7**-derivatives were determined by spectroscopy, including 2D-NMR experiments (COSY 45, HSQC, HMBC) and the observed data corresponded to those of the natural isolated compounds.[10,11]

CH₃O – CO₂R²

R¹O

Vanillic acid   R¹ = R² = H
8   R¹ = H, R² = CH₃
9   R¹ = R² = CH₃

CO₂H

NHR

HO

N-methyltyrosine   R = CH₃
Tyrosine   R = H

CO₂H

HO

4-Hydroxy-benzoic acid
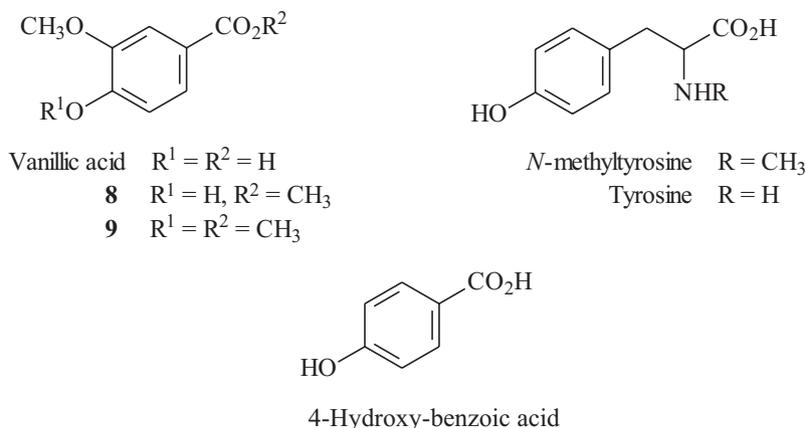
Pharmacological profiling

In order to identify the antiproliferative action of *Croton cajucara* constituents we studied the effects of natural clerodanes **1**, **3**, **4**, **5**, **6** and **7** and also the semi-synthetic **6** and **7**-derivatives against ascitic Ehrlich carcinoma and human leukemia K562 cells. The experiments were performed using the MTT assay,[19] with quercetin and vincristine as positive controls for Ehrlich and leukemia K562, respectively.

In vitro tests using Ehrlich carcinoma cells, natural **6**, **7**, **1** and **2**, showed a significant cytotoxicity with dose dependent responses over a 48 h culture period. The IC_{50} values were 166 µM for **1**, 164 µM for **2**, 65 µM for **6**, and 10 µM for **7**, compared to quercetin with an IC_{50} = 44 µM (Table 1). The semi-synthetic cajucarinolide derivatives (**6** and **7**) showed similar antiproliferative activities compared to natural **6** and **7**. The remaining natural clerodanes **3**, **4** and **5** do not show cytotoxic activity against Ehrlich carcinoma cells.

Natural clerodanes **1**-**7** and also the semi-synthetic **2**, **6** and **7** were also tested for their cytotoxic effects against human K562 leukemia cell line using the Mossman assay[19] and 48 h of cell culture. Among the natural clerodanes assayed, only **3**, **4**, **6** and **7** (natural and semi-synthetic) showed concentration-dependent growth inhibiting activities on cultured K562 leukemia cells. The IC_{50} values were 33 µM (**3**), 38 µM (**5**), 36 µM (**6**) and 43 µM (**7**). The cajucarinolide-derivatives indicated similar significant antiproliferative activity. Figure 1 shows the comparative cytotoxic effects of the tested clerodanes against both Ehrlich carcinoma and human K562 leukemia cells. The phenolic acids were also assayed against Ehrlich and K562 leukemia cells, but were inactive, under the same experimental conditions.

3 structure has only one α,β-insaturated ketone and its cytotoxicity on K562 cells was similar to that observed for **6**, which has this moiety in addition to the conjugated

Table 1. IC_{50} values of metabolites from *Croton cajucara* against Ehrlich carcinoma and human K562 leukemia cells

Metabolites	Ehrlich carcinoma		K562 cells	
	IC_{50}/(µM)	IC_{50}/(µg mL⁻¹)	IC_{50}/(µM)	IC_{50}/(µg mL⁻¹)
1	166	52.2	n.a.	n.a.
2	164	51.8	n.a.	n.a.
3	n.a.	n.a.	33	11.1
4	n.a.	n.a.	n.a.	n.a.
5	n.a.	n.a.	38	12.6
6	65	22.4	36	12.6
7	10	3.5	43	14.9
Quercetin	44	13.3	-	-
Vicristine	-	-	0.060	0.049

n.a. = not active.

lactone carbonyl in the hydroxybutenolide ring. The similar cytotoxic effects on K562 cells observed for **3**, **5**, **6** and **7**, suggested no significant contribution of hydroxybutenolide ring. Meanwhile, **4**, the diastereoisomer of **3**, lacked efficacy indicating the importance of the decaline system stereochemistry. Further, **1** and **2** did not present significant cytotoxic effect against K562 cultured cells until 50 µM. This result was in accordance with the lower cytotoxic activity of **1** against promyelocytic HL60 cells with IC_{50} = 300 µM, after 24 h, and 180 µM, after 96 h of culture.[26]

According to Wattenberg[25] compounds containing an α,β-unsaturated carbonyl moiety have been shown to bind to receptors that induce increased activities of phase II enzymes responsible for metabolizing xenobiotic agents. Thus, the hydroxybutenolide moiety present in **6** and **7**, probably led to increase of activities in the Ehrlich carcinoma assay. Taking into account the cytotoxic effect of **7** (10 µM), we suggest that its higher antiproliferative action on growth Ehrlich cells compared to **6** (65 µM) is related to the stereoposition of the hydroxyl group in the hydroxybutenolide moiety (**7** with OH at position 16 and its isomeric stereoisomer **6** with OH at position 15). Further, the clerodanes **3**, **5** and **6** showed comparable effects against human K562 leukemia cells.
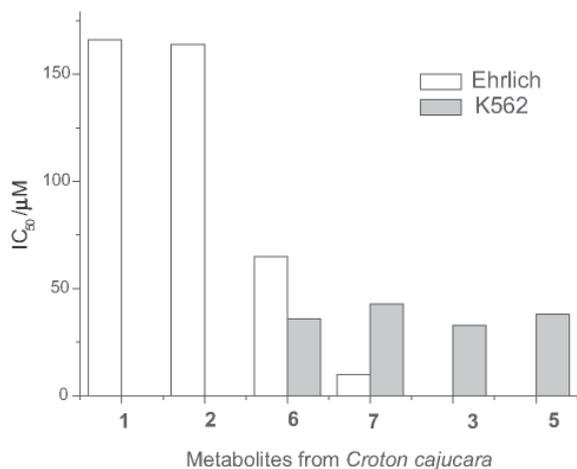
Figure 1. Graphic of the IC$_{50}$ values of *Croton cajucara* metabolites against Ehrlich carinoma and human K562 leukemia cells.

Conclusions

The further phytopharmacological studies of *Croton cajucara* have demonstrated that the therapeutic potential of this plant is undeniable. The tested natural bioactive clerodanes **1-7** and also the semi-synthetic derivatives of **1** had their purity confirmed by NMR analysis. The clerodanes **1**, **2**, **6** and **7** showed weak to moderate cytotoxic effect against Ehrlich carcinoma cells and moderate efficacy against human K562 leukemia cells was observed for the clerodanes **3**, **5**, **6** and **7**. Only the hydroxybutenolide clerodanes **6** and **7** (natural and semi-synthetic) showed antiproliferative effect on both Ehrlich and K562 cells.

Synthetic transformation of **1**, with satisfactory yields by simple and low cost methodologies, afforded **6**, which was isolated as minor natural constituents of *C. cajucara*. Further new phytochemical investigation yielded two aromatic acids (vanillic acid and 4-hydroxy-benzoic acid) and an amino acid (*N*-methyltyrosine).

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