# SUPPLEMENTARY MATERIAL

**Lipoxygenase inhibitory activity of *Cuspidaria pulchra* and isolated compounds.**

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This work evaluated the *in vitro* inhibitory activity of the crude ethanolic extract from the aerial parts of *Cuspidaria pulchra* (Cham.) L.G. Lohmann against 15-lipoxygenase (15-LOX). The bioassay guided fractionation of the *n*-butanol fraction, which displayed the highest activity, led to the isolation of three compounds: caffeoylcalleryanin (**1**), verbascoside (**2**), and 6-hydroxyluteolin-7-O-β-glucoside (**3**). Assessment of the ability of the isolated compounds to inhibit 15-LOX revealed that compounds **1**, **2**, and **3** exerted strong 15-LOX inhibitory activity; IC50 values were 1.59 ± 0.08, 1.76 ± 0.25, and 2.35 ± 0.17 µM respectively. The XTT assay showed that none of the isolated compounds seemed to be significantly toxic.

Keywords: 15-lipoxygenase inhibitory activity; *Arrabidaea*; cytotoxic activity.

#  Experimental

## General

1H and 13C NMR spectra were recorded in DMSO-*d6* or MeOD-*d4* on a Bruker AVANCE DRX 500 spectrometer, using TMS as internal standard. The analytical and preparative HPLC separation analyses were carried out on a Shimadzu LC-6AD system equipped with a degasser DGU-20A5, a UV-VIS detector SPD-20A series, a communication bus module CBM-20A, and a Rheodyne manual injector. Separations of the compounds were carried out on a SHIMADZU Shim-pack Phenyl (particle diameter 5 μm, 250 x 4.60 mm, and 250 x 20 mm) columns equipped with a pre-column of the same material. Acetonitrile used in the experiments was HPLC grade, J. T. Baker. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system, Millipore. Silica gel ODS (Fluka, 230-400 mesh) was employed during the solid phase extraction. Silica on TLC Alu foils with fluorescent indicator at 254 nm (Sigma-Aldrich) was used for thin layer chromatography.

### **Plant material**

The aerial parts of *Cuspidaria pulchra* (Cham.) L.G. Lohmann, synonym *Arrabidaea pulchra* (Cham.) Sandwith, were collected in the Brazilian Cerrado in Luiz Antonio (21°33′ - 21°37′ S and 47°45′ - 47°57′ W), state of São Paulo, in October 2008. The material was identified by Prof. V. M. M. Gimenez. A voucher specimen (SPFR12599) was deposited in the Herbarium of the Department of Biology, Laboratory of Plant Systematics, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, University of São Paulo, Brazil (Herbarium SPFR).

***Extraction and isolation***

The dry powder of the aerial parts (288.2 g) of *C. pulchra* was extracted with ethanol, at room temperature. The solution was filtered, and the solvent removed under reduced pressure, to yield 32 g of extract. Part of the ethanolic extract (20 g) was dissolved in methanol/water (2:8 v/v) and successively partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, followed by solvent removal in a rotary evaporator. Each partition yielded 2.4, 5.5, 7.9, and 3.2 g of dry material, respectively. The *n*-butanol partition was submitted to solid phase extraction using Silica gel ODS and methanol/water as eluent, to afford three fractions. Fraction 1 was submitted to preparative HPLC purification over a phenyl column, using an isocratic condition: acetonitrile/water/acetic acid (20:79.9:0.1 v/v/v), UV detection at 254 nm, and flow rate of 9 mL/min, which furnished eight fractions (Fractions 1.1 to 1.8). Fraction 1.8 afforded compound **1** (21.7 mg, tR= 50.94 min). Fraction 1.5 was purified by HPLC using acetonitrile/water/acetic acid (23:76.9:0.1 v/v/v), UV detection at 254 nm, and flow rate of 9 mL/min, which yielded compound **2** (26.3 mg, tR= 15.24 min). Fraction 1.3 was separated over phenyl column and eluted with acetonitrile/water/acetic acid (23:76.9:0.1 v/v/v), UV detection at 254 nm, and flow rate of 9 mL/min, to give five subfractions. Subfraction 4 was purified using preparative HPLC phenyl column with acetonitrile/water/acetic acid (16:83.9:0.1, v/v/v), UV detection at 254 nm, and flow rate of 9 mL/min. This step resulted in six subfractions; subfraction 5 yielded compound **3** (6.7 mg, tR= 33.96 min).

***In vitro 15-lipoxygenase inhibitory activity assay***

The ability of the target extract, fractions, and compounds to inhibit 15-LOX was evaluated using an *in vitro* enzyme assay kit (Catalog Nº 760700, Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer’s instructions, with some modifications.

The assay conducted to screen lipoxygenase inhibitors can detect and measure the hydroperoxides originating from the lipoxygenation reaction by means of a purified lipoxygenase. The extract and fractions were evaluated at concentrations of 25, 50, and 100 µg/mL; the isolated compounds were tested at 1.25, 2.5, 5, 10, 25, 40, and 80 µM, in a final volume of 210 µL. The stock solutions of the samples were dissolved in a minimum volume of DMSO and were diluted using the supplied buffer solution (Tris-HCl 0.1 M, pH 7.4). The sample solution (30 µL) was added to 70 µL of a solution of the 15-LOX enzyme in Tris-HCl buffer 0.1 M. The reaction was initiated by addition of 10 µL of substrate (arachidonic acid). Then, the 96-well plate was shaken for 5 min, which was followed by addition of 100 µL of chromogen. The plate was placed on the shaker for 5 min. 15-LOX activity was determined by measuring the absorbance at 500 nm in a multiplate reader (ELISA –Asys – UVM 340/Microwin 2000). All the determinations were carried out in triplicate. Zileuton® was used as the standard lipoxygenase inhibitor. The percent inhibition was determined using Equation (1)

$\%=\left[\frac{IA-Inhibitor}{IA}\right]×100$,(1)

where IA corresponds to the absorbance of 100% initial activity of the 15-LOX, and Inhibitor referred to the absorbance of the tested sample. IC50 was calculated from the concentration-inhibition response curve.

***XTT-based cytotoxicity assay***

The cytotoxic activity of the tested compounds was measured using the *in vitro* Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics) according to the manufacturer’s instructions. Normal human lung fibroblasts (GM07492A) and two tumor cell lines, murine melanoma (B16F10) and hepatocellular carcinoma (HepG2), were employed. The cell lines were cultured in HAM-F10 (Sigma-Aldrich) and DMEM (Sigma-Aldrich) (1:1) or only DMEM (in the case of HepG2) culture medium supplemented with 10% fetal bovine serum (Nutricell), antibiotics (streptomycin 0.01 mg/mL and penicillin 0.005 mg/mL; Sigma-Aldrich), and 2.38 mg/mL Hepes (Sigma-Aldrich) at 37ºC, with 5% CO2. For these experiments, the cells (104 cells/well) were plated onto 96-well microplates. Each well received 100 µL of HAM F10/DEMEN medium containing the desired concentration of the samples dissolved in 0.1% DMSO. The tested concentrations ranged from 6.25 to 800 µM. The negative (without treatment), solvent (0.1% DMSO), and positive (25% DMSO) controls were included. After incubation at 37ºC for 24h, the medium was removed; cells were washed with 100 μL of PBS (phosphate buffer saline) and exposed to 100 μL of HAM-F10 medium without phenol red. Then, 25 μL of XTT were added to each well. The microplates were covered and incubated at 37ºC for 17 h. The absorbance of the samples was obtained using a multiplate reader (ELISA –Asys – UVM 340/Microwin 2000) at a test wavelength of 450 nm and a reference wavelength of 620 nm. Cell viability was expressed as the percentage of untreated cells, which served as the negative control group and was designated as 100%; i.e., the results were expressed as the concentration required to reduce the cell growth by 50% compared to that for the untreated controls (CC50). The experiments were performed in triplicate.