

SUPPLEMENTARY MATERIAL

Brasiliensic and isobrasiliensic acids: isolation from *Calophyllum brasiliense* Cambèss. and anti-*Helicobacter pylori* activity

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Abstract

The occurrence of chromanone derivatives has been noticed as a distinctive feature of the genus *Calophyllum* (Calophyllaceae). Previous studies have demonstrated that the extract of the stem bark of *Calophyllum brasiliense* and its chromanone-rich fractions show antiulcer activity in murine gastric ulcer models. In this work, brasiliensic and isobrasiliensic acids, the two main compounds of the *n*-hexane extract of the stem bark extract of *C. brasiliense*, were isolated by flash chromatography using silica gel impregnated with silver nitrate and their structures were elucidated by NMR techniques and mass spectrometry. ¹³C NMR data is available for the first time for both compounds. Brasiliensic and isobrasiliensic acids showed good bacteriostatic activity *in vitro* against *Helicobacter pylori*, and, at least in part, they are responsible for the bacteriostatic anti-*H. pylori* activity of the *n*-hexane extract of the stem bark extract of *C. brasiliense*.

Keywords: *Calophyllum brasiliense*; chromanone; anti-*Helicobacter pylori* activity

S1. Experimental

S1.1. General experimental procedures

1D- and 2D-NMR spectra were acquired on a Bruker Avance DRX-400 spectrometer. All spectra were measured in CDCl₃ at 295 K. HRESIMS were measured on a Bruker Daltonics microTOF-Q II™ ESI-Qq-TOF mass spectrometer. Vacuum liquid chromatography (VLC) was carried out over silica gel 60 (40-63 μm, 70–230 mesh, Merck, art. 9385). Flash column chromatography (FCC) was carried out over silica gel 60 (40-63 μm, 70–230 mesh, Merck) impregnated with silver nitrate. For the impregnation process, 2 g of silver nitrate in 20 mL of MeCN:MeOH (1:5) were mixed with 18.46 g of silica gel. The mixture was dried in an oven at 75 °C for 24 h before packing. These processes were carried out protected from light or under red/yellow light. Fractions were monitored by TLC on precoated silica gel 60 F₂₅₄ plates (Merck, art. 5554) that were previously immersed in a 0.5 % methanolic solution of AgNO₃ for 5 min, dried on a rack, and then activated up to 10 min in an oven at 75°C.

Analytical HPLC separations were performed on a Shimadzu chromatograph (LC-10AD pumps, SCL-10A controller, DGU-14A degasser, SIL-10AD injector, DAD SPD-M10A detector and Class VP software, version 5.02) using a C18 Shimadzu Shim-pack column (5 μm, 4.6 x 250 mm). Semi-preparative HPLC separations were performed on a Shimadzu chromatograph (LC-6AD pumps, CBM-20A controller, DGU-20A degasser, SPD-20A detector, and LC solution software, version 1.25), using a C18 Shimadzu Shim-pack column (5 μm, 20 x 250 mm).

S1.2. Plant material

The stem bark of *C. brasiliense* was collected in June 2010 by Iberê Ferreira da Silva Júnior PhD, at the fountain of the Coxipó River (S 15°38'40.8'', W 056°03'05.6''), Cuiabá, Mato Grosso, Brazil. A voucher specimen (# 37993) was deposited at the Herbarium UFMT and was identified by Prof. Germano Guarim Neto and ratified by MSc. Harri Lorenzi of the Instituto Plantarum de Estudos da Flora, Nova Odessa, São Paulo, Brazil.

S1.3. Extraction and isolation

The stem bark of *C. brasiliense* was cleaned with a hand brush, dried at room temperature (*ca.* 25 °C) and ground in an electric mill (model TE-625 TECNAL, São

Paulo, Brazil) with sieve of mesh size of 40. The dried powdered material (90 g) was subjected to maceration with *n*-hexane at a ratio of 1:5 (w/v) for seven days, with daily stirring. The macerate was filtered and concentrated under reduced pressure at approximately 40 °C in a rotary evaporator to obtain the *n*-hexane extract (HECb). The residual solvent was eliminated in an oven at 40 °C, to yield 9.2 g (10.4%) of dry extract that was kept protected from light and stored at 4 °C.

The HECb (9.2 g) was fractionated by VLC with mixtures of increasing polarity of *n*-hexane-EtOAc as mobile phase (7:3, 1:1 and 1:9 v/v, 150 mL each) to obtain four fractions (Fr1 to Fr4), the first two being eluted in the less polar mobile phase and the remaining two fractions in the other two mobile phases. Fr3 (7.12 g) was submitted to a new VLC eluted with a stepwise gradient of *n*-hexane-EtOAc (99:1 to 83:18 v/v, 300 mL each) resulting in 17 fractions (Fr3.1 to Fr3.17). Fractions Fr3.11 and Fr3.12 (from *n*-hexane-EtOAc 89:11 and 88:12, respectively) were pooled (2.92 g) by similarity after TLC analysis, and named FrC.

A sample of FrC (150 mg) was subjected to a FCC (glass column, 2 cm diameter by 14 cm height) using silica gel 60 impregnated with silver nitrate as the stationary phase and 360 mL of *n*-hexane-EtOAc 6:4 with 1% formic acid as the mobile phase. The collected fractions (10 mL each) were pooled after TLC analysis. Fractions 9-13 and 15-16 were further purified by semi-preparative HPLC (MeCN-H₂O 83:17, isocratic mobile phase, flow rate 10 mL/min) to afford compounds **1** and **2**. The isolated compounds were submitted to analytical HPLC analysis for purity testing (MeCN-H₂O 83:17, isocratic mobile phase, flow rate 10 mL/min).

Brasiliensic acid (1): yellow gum; UV (MeOH) λ_{\max} 245, 297, 363 nm; ¹H-NMR and ¹³C-NMR, see Table S1; HRESIMS *m/z* 549.3170 [M + Na]⁺ (calcd. for C₃₂H₄₆O₆Na, 549.3192), 527.3366 [M + H]⁺ (calcd. for C₃₂H₄₇O₆, 527.3373), 515.2301, 459.2732, 393.2365, 335.1479 and 214.9162.

Isobrasiliensic acid (2): yellow gum; UV (MeOH) λ_{\max} 245, 297, 363 nm; ¹H-NMR and ¹³C-NMR, see Table S1; HRESIMS *m/z* 549.3004 [M + Na]⁺ (calcd. for C₃₂H₄₆O₆Na, 549.3192), 527.3197 [M + H]⁺ (calcd. for C₃₂H₄₇O₆, 527.3373), 459.2586 and 335.1378.

S1.4. Bacteria and growth conditions

The *Helicobacter pylori* type strain ATCC 43504 (Maryland, USA), stored at -80 °C in Skim milk media, was used in this study. The solid media used comprised of

fresh blood agar plates (Mueller Hinton agar with 5% sheep blood) with 10% fetal calf serum (FCS) and 1:500 Skirrow (*Campylobacter* supplement III, Sigma-Aldrich). The plates were incubated at 37 °C in microaerophilic conditions (candle jars) for 5 days. For bacterial suspension, Brain Heart Infusion (BHI) broth supplemented with 10% FCS and 1:500 Skirrow was used.

S1.5. Anti-Helicobacter pylori assay

The anti-*H. pylori* activity was evaluated using broth microdilution assay, according to the guidelines established by CLSI (2003). One hundred microliters of the stock solutions (20 mg/mL in 2% Tween 80) of HECb, **1** and **2** was added to each well to a final concentration ranging from 200 to 3.125 µg/mL on adding 100 µL of the bacterial suspension (6×10^8 CFU/mL or 2 McFarland scale) with final volume being 200 µL/well. Clarithromycin (200 - 3.125 µg/mL) was used as positive antibacterial control. The plates were incubated at 37 °C under microaerophilic conditions (candle jars) for 72 h. Results were expressed as minimal inhibitory concentration (MIC), which is defined as the lowest concentration that inhibits growth, judged by the lack of turbidity in the well. The turbidity was measured at 450 nm by using a spectrophotometer (Spectronic Genesys 5 Spectrophotometer, Thermo / Milton Roy, Pennsylvania, USA). The test was carried out in duplicate of three independent assays and results are shown as mean. The antibacterial activity was considered as follow: good for MIC values < 100 µg/ mL, moderate for $100 < \text{MIC} \leq 500$ µg/mL, weak for $5000 < \text{MIC} \leq 10000$ µg/mL and inactive for MIC values > 10000 µg/mL (Holetz et al. 2002).

S1.6. Determination of Minimal Bactericidal Concentration (MBC)

In order to investigate whether the drugs have bacteriostatic or bactericidal action, the MBC was determined according to the method of Mbah et al. (2012). An aliquot (10 µL) of bacterial cells from the wells containing lower than the MIC, the MIC and above the MIC were subcultured on fresh blood agar plates and incubated at 37 °C under microaerophilic conditions (candle jars) for 72 h. The assays were carried out in duplicate of two independent assays.

S2. References

[CLSI] Clinical and Laboratory Standards Institute. 2003. Approved Standard-NCCLS document M7-A6 (6 ed).

Holetz V. 2010. Potential of Cameroonian plants and derived products against microbial infections: a review. *Planta Med.* 76:1479-1491.

Mbah JA, Ngemenya MN, Abawah AL, Babiaka SB, Nubed LN, Nyongbela KD, Lemuh ND, Efange SM. 2012. Bioassay-guided discovery of antibacterial agents: *in vitro* screening of *Peperomia vulcanica*, *Peperomia fernandopoioana* and *Scleria striatinux*. *Ann Clin Microbiol Antimicrob.* 11:10.

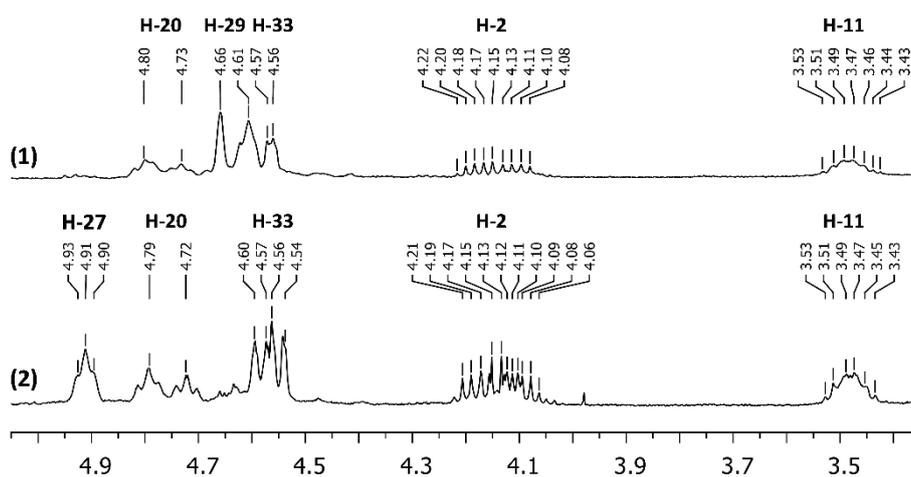


Figure S1. ^1H NMR spectra (5 - 3 ppm) of brasiliensic acid (1) and isobrasiliensic acid (2).

Table S1. ¹H and ¹³C NMR data of compounds **1** and **2** (CDCl₃, 400 MHz)

Position	Brasiliensic acid (1)	Isobrasiliensic acid (2)		
	δ_c , type	δ_H , (mult, J in Hz)	δ_c , type	δ_H , (mult, J in Hz)
2	81.3, CH	4.15 dq, J = 6.6, 10.2	80.3, CH	4.16 dq, J = 6.4, 9.9
3	43.7, CH	2.46 dq, J = 6.9, 10.2	43.7, CH	2.47 dq, J = 6.6, 10.2
4	196.3, C		196.3, C	
5	n.o.		n.o.	
6	176.7, C		177.1, C	
7	57.3, C		57.3, C	
8	195.2, C		195.2, C	
9	112.7, C		112.6, C	
10	167.8, C		167.9, C	
11	30.1, CH	3.49 m	30.0, CH	3.47 m
12	37.7, CH ₂	2.87 dd, J = 8.6, 2.0 2.71 dd, J = 8.4, 1.8	37.7, CH ₂	2.89 dd, J = 8.6 2.71 ddl, J = 6.4
13	176.7, C		176.8,	
14	34.5, CH ₂	1.72 m 1.45 m	34.5, CH ₂	1.81 m 1.49 m
15	21.0, CH ₂	1.23 m	21.01, CH ₂	1.23
16	14.2, CH ₃	0.89 t, J = 7.3	14.2, CH ₃	0.88 t, J = 7.4
17	19.3, CH ₃	1.55 m, buried	19.3, CH ₃	1.55 m, buried
18	9.3, CH ₃	1.17 d, J = 6.8	9.3, CH ₃	1.17 d, J = 6.8
19	41.8, CH ₂	2.69 m, 2.48 dd, J = 13.5, 6.9	42.1, CH ₂	2.60 m, 2.47 dd, J = 13.7, 6.9
20	117.4, CH	4.77 m	117.4, CH	4.77 m
21	135.3, C		135.3, C	
22	17.8	1.58 br s	17.7, CH ₃	1.56 br s
23	25.8	1.58 br s	25.7, CH ₃	1.57 br s
24	44.3, CH ₂ *	2.09 m*	41.5, CH ₂ *	2.12 m*
25	44.0, CH*	2.01 m*	42.1, CH*	2.03 m*
26	32.5, CH ₂ *	1.34 m*	33.5, CH ₂	1.92 m
27	32.9, CH ₂ *	1.34 m*	121.9, CH	4.91 pseudo t
28	145.7, C		132.4, C	
29	109.8, CH ₂	4.64 m	17.9, CH ₃	1.56 br s
30	22.5, CH ₃	1.67 s	25.8, CH ₃	1.67 br s
31	146.7, C		147.2, C	
32	18.0, CH ₃	1.61 s	17.8, CH ₃	1.57 br s
33	113.1, CH ₂	4.59 m	112.5, CH ₂	4.56 m

n.o., not observed,

*, tentatively assigned