New Cytotoxic Cucurbitacins from *Wilbrandia ebracteata* Cogn.

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Abstract

Chemical investigation of the roots of *Wilbrandia ebracteata* Cogn. (Cucurbitaceae) led to the isolation of two new (**1**–**2**) and four known (**3**–**6**) cucurbitacins. Their structures were elucidated by NMR and MS and compared with related compounds. The *in vitro* cytotoxicity of isolated compounds was evaluated against RD, KB, HCT-8, and A549 cell lines showing strong activity.

Key words

Wilbrandia ebracteata · Cucurbitaceae · cucurbitacins · cytotoxicity

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Wilbrandia ebracteata Cogn., Cucurbitaceae is one of the species commonly known in Brazil as "tayuya" or "taiuiá", the native name of many species of Cucurbitaceae that have tuberous roots with a characteristic strong bitter taste. The roots have been used in indigenous medicine mainly as a laxative and for the treatment of leprosy, ulcers, rheumatism, and syphilis [1–4]. In the first Brazilian Pharmacopoea from 1929 [5], the plant drug originally included as "taiuiá" was *Cayaponya tayuya* Cong., but nowadays the most frequently used and marketed species is *Wilbrandia ebracteata* (Cong.) Cogn [6,7]. In previous studies, we reported the isolation of twelve known and six new cucurbitacins from *Wilbrandia* roots [8,9]. More recently, some of these compounds have been more deeply investigated in respect to anti-inflammatory properties and also antitumor activity [10–12]. In the present work, we describe the isolation and structure elucidation of two new cucurbitacins with unusual side chains from the roots of *W. ebracteata*, along with an additional cucurbitacin not previously reported in this species, and three known compounds of the same family. Additionally, the isolated compounds were evaluated for their *in vitro* cytotoxicity against RD, KB, HCT-8, and A549 human cancer cell lines using the MTT assay.

Compound **1** (**Fig. 1**) was obtained as a white solid with a molecular formula $C_{30}H_{44}O_6$ provided by HRMS (ESI/APCI). The analysis of the ^{1}H NMR spectrum revealed the presence of seven singlet methyl groups, together with a doublet of doublets at δ_H 5.80 (1H), typical in a cucurbitane-type skeleton. Additional signals included two doublets of doublets at δ_H 4.42 (1H) and 4.35 (1H), indicating respectively, the presence of an α-ketol in the A ring and a hydroxyl group in the D ring, which are common structural elements in many cucurbitacins. The ^{13}C NMR and DEPT spectra revealed the presence of 30 carbon atoms corresponding to ten quaternary carbons, six methines, seven methylenes, and seven methyls (**Table 1**). Among these, three ketone carbonyl signals at δc 213.0, 212.1, and 213.9 were assigned, considering the 2D NMR spectra as C-3, C-11, and C-22, respectively. Signals at δc 110.4, 120.4, 140.5, and 144.5 indicated the presence of two olefinic systems, one of them readily identified as the usual B-ring unsaturation present in most cucurbitacins. The remaining unsaturation corresponded to an isopropylidene moiety, with the typical signals at δ_H 4.70 (1H, 0.8 Hz), δ_H 4.76 (1H, 0.8 Hz), and

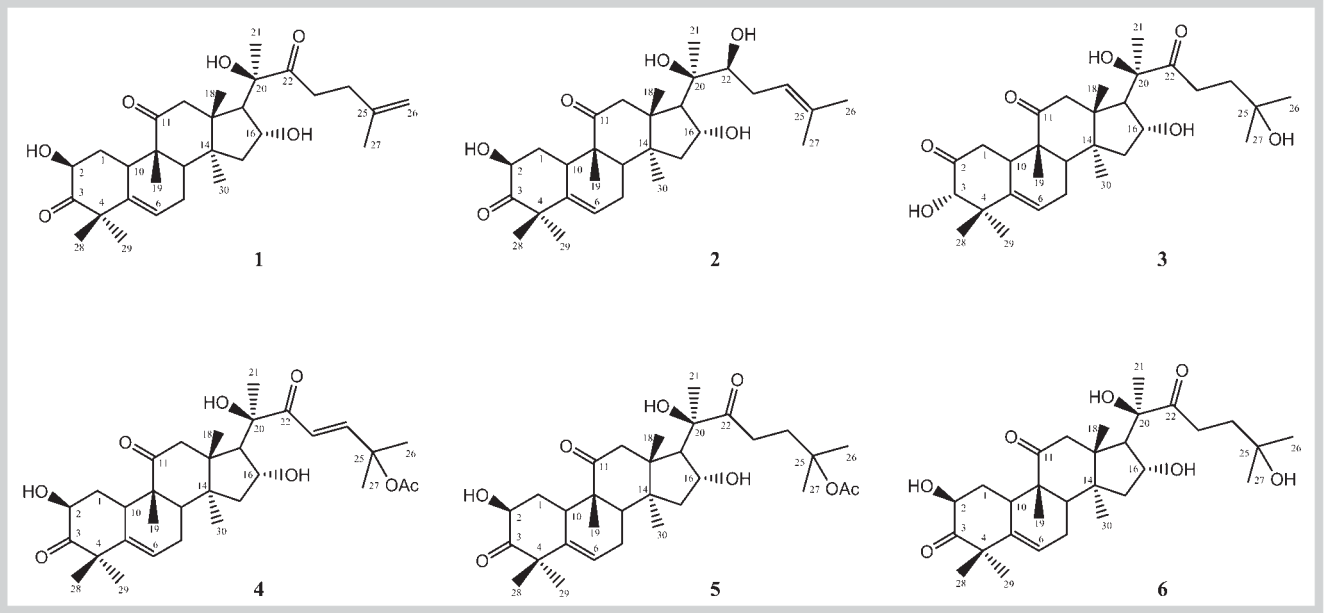

Fig. 1 Cucurbitacins from *Wilbrandia ebracteata*.

Table 1 NMR spectroscopic data (500 MHz for ^{1}H; 125 MHz for ^{13}C; $CDCl_3$) of compounds **1** and **2**.

Position	Compound 1		Compound 2	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	2.32 ddd (13, 6, 3), Ha 1.23 ddd (13, 13, 13), Hb	36.0	2.27 ddd (13, 6, 3), Ha 1.22 ddd (13, 13, 13), Hb	36.0
2	4.42 dd (13, 6)	71.6	4.40 dd (13, 6)	71.6
3	–	213.0	–	213.1
4	–	50.3	–	50.2
5	–	140.5	–	140.4
6	5.80 ddd (6, 2, 2)	120.4	5.80 ddd (6, 2, 2)	120.4
7	1.97 ddd (20, 6, 2), Ha 2.42 dddd (20, 8, 4, 2), Hb	23.9	2.03 ddd (20, 6, 2), Ha 2.43 dddd (20, 8, 4, 2), Hb	23.9
8	2.0 d (8)	42.3	1.98 d (8)	42.4
9	–	48.4	–	48.3
10	2.74 m	33.8	2.73 m	33.7
11	–	212.1	–	212.7
12	3.26 d (14.5), Ha 2.74 d (14.5), Hb	48.6	3.15 d (14.5), Ha 2.62 d (14.5), Hb	48.8
13	–	48.3	–	47.8
14	–	50.7	–	51.3
15	1.40 dd (14, 2), Ha 1.86 dd (14, 9), Hb	45.4	1.55 dd (13.3, 2), Ha 1.92 dd (13.3, 9), Hb	44.6
16	4.35 ddd (9, 7, 1)	71.6	4.60 ddd (9, 7, 1)	71.4
17	2.56 d (7)	57.6	2.36 d (7)	55.2
18	0.98 s	19.8	0.97 s	19.7
19	1.09 s	20.1	1.07 s	19.9
20	–	78.8	–	75.7
21	1.43 s	24.3	1.32 s	24.9
22	–	213.9	3.35 dd (10.7, 3.4)	82.3
23	2.91 ddd (17, 10, 5.5), Ha 2.66 ddd (17, 10, 5.5), Hb	31.6	2.31 m, Ha 2.18 m, Hb	30.7
24	2.34 m, Ha 2.34 m, Hb	34.4	5.21 m	120.6
25	–	144.5	–	135.1
26	4.70 d (0.8) 4.76 d (0.8)	110.4	1.74 s	25.8
27	1.75 s	22.6	1.65 s	18.0
28	1.35 s	29.3	1.34 s	29.4
29	1.28 s	21.2	1.28 s	21.2
30	1.38 s	18.8	1.38 s	18.7

δ_H 1.75 (s, 3H). This group was located at the terminus of the side chain by analysis of the HMBC and COSY spectra. The above-mentioned data allowed us to characterize the structure of **1** as 2*β*,16*α*,20*R*-trihydroxy-10*α*,17*α*-cucurbit-5,25-dien-3,11,22-trione, a cucurbitacin with a new side chain, which possibly was originated in the elimination of the C-25 acetyl group of dihydrocucurbitacin B during biosynthesis.

Compound **2** (**Fig. 1**) was obtained as a white solid with a molecular formula $C_{30}H_{46}O_6$ (ESI/APCI). The analysis of the ^{1}H NMR spectrum (**Table 1**) indicated a structure similar to that of compound **1** with the presence of 8 singlet methyls, and the characteristic doublet of doublets δ_H 5.80 (1H) of cucurbitane-type triterpenes. Unlike most cucurbitacins, analysis of the ^{13}C NMR spectrum showed the existence of an unusual unsaturation between C-24 and C-25 (δ_c 120.6 and 135.1) confirmed by the presence of two olefinic methyls at δ_H 1.74 and 1.64 bound to the same quaternary carbon (C-25). Moreover, the presence of a hydroxyl group was detected at C-22 (3.35, dd, 10.7, 3.4 Hz, 1H), a position normally found oxidized in this class of compounds. In order to elucidate the relative stereochemistry at C-22, we prepared the acetonide derivative (compound **7**, **Fig. 2**). The analysis of the NOESY spectrum clearly showed a correlation between the signals at δ_H 3.88 (H-22) and δ_H1.40 (s, 3H, Me-21). Assuming the typical 20*R* configuration in cucurbitane-type triterpenes, the observed correlation indicated an *S* configuration for C-22. Comparison with literature data [13] allowed the identification of compound **2** as 2*β*,16*α*,20*R*,22*S*-tetrahydroxy-10*α*,17*α*-cucurbit-5,24-dien-3,11-dione. Stuppner and colleagues [13] described the isolation of a related compound from the roots of *Picrorhiza kurrooa* (Scrophulariaceae). However, the previously reported compound was glycosidated at C-2, and the configuration at C-22 was not determined.

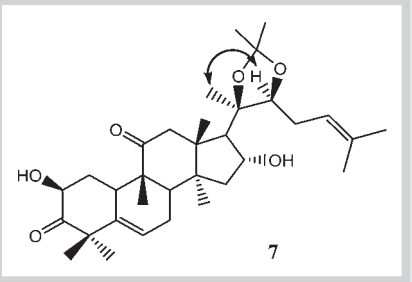

Fig. 2 NOE correlation of acetonide derived from compound **2**.

Table 2 Cytotoxicity of the isolated cucurbitacins on different human cancer cell lines.

Isolated tested compounds	Cell lines [CC_{50}[a] (μM)]			
	A549	KB	RD	HCT-8
1	3.82 ± 0.46	2.37 ± 0.29	2.06 ± 0.07	5.09 ± 0.32
2	4.81 ± 0.02	2.79 ± 0.85	2.15 ± 0.04	2.06 ± 0.64
3	19.51 ± 1.92	16.27 ± 0.69	13.50 ± 1.09	12.80 ± 1.56
4	0.03 ± 0.01	0.03 ± 0.01	0.010 ± 0.001	0.02 ± 0.01
5	12.84 ± 0.58	14.72 ± 0.36	12.74 ± 3.75	10.57 ± 0.85
6	1.45 ± 0.11	1.58 ± 0.21	1.08 ± 0.08	3.32 ± 0.02
Paclitaxel	0.054 ± 0.010	0.006 ± 0.001	0.024 ± 0.002	0.0170 ± 0.0001
Doxorubicin	1.459 ± 0.160	0.335 ± 0.005	0.79 ± 0.09	1.726 ± 0.131

Values represent the mean ± standard deviations of three independent experiments.
[a] Cytotoxicity was determined by MTT assay on each human tested cancer cell line

A detailed analysis of spectroscopic data and comparison with literature references [14] allowed the identification of compound **3** (**Fig. 1**) as 16*α*,3*α*,20*R*,25-tetrahydroxy-10*α*,17*α*-cucurbit-5-en-2,11,22-trione, namely isocucurbitacin R, and to the best of our knowledge it has not yet been reported for the genus *Wilbrandia*. Compounds **4**–**6** (**Fig. 1**) were identified on the basis of their spectroscopic data [9] and comparison with available standards as cucurbitacin B, 23, 24-dihydrocucurbitacin B and cucurbitacin R, respectively. The purity of all compounds ranged from 94.8% (**3**) – 99.3% (**4**) as determined by analytical HPLC with UV detection.

The cytotoxicities of **1**–**6** against four tumor cell lines were evaluated by MTT assay [15]. The CC_{50} (drug concentration required to reduce cell growth by 50%) value of each cucurbitacin tested was measured on the basis of cell viability, after 72 h treatment. The results (**Table 2**) showed that the new compounds **1** and **2** exhibited significant cytotoxicities against all cell lines with CC_{50} values ranging from 2.06 to 5.09 μM. Compounds **3** and **5** were less cytotoxic with CC_{50} values up to 10 μM. Compounds **4** and **6** appeared to be the most cytotoxic against all tested cell lines with

CC_{50} values comparable to those of the antitumoral drugs paclitaxel and doxorubicin. This suggested that the cytotoxicity of the cucurbitacins from *W. ebracteata* appears to be correlated mainly with structural differences present at the side chain as already shown in previous works [16,17]. However, the comparison of the observed levels of activity of these compounds, taking into account their structural similarities and differences, could not lead to safe conclusions on the specific features that influence their cytotoxicity. Even though the panel of the tested compounds is rather small, it seems that the observed cytotoxic activity cannot be correlated with the presence or absence of specific functional groups, and it is probably influenced by a combination of factors, including the overall three-dimensional structure of the molecules and the spatial orientation of their substituents. Thus, more extensive studies are needed before a clear structure-activity relationship concerning the cytotoxic activity can be reached.

Materials and Methods

Optical rotations were measured on a Perkin-Elmer model 341 polarimeter with a 1 dm cell. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer. IR spectra were obtained on a Shimadzu Prestige 2 instrument by using KBr pellets. NMR spectra were recorded on a Bruker Avance 2 500 MHz spectrometer, and the 2D NMR spectra were obtained using standard pulse sequences. High-resolution ESI (ESI-HR-MS) mass spectra were recorded on a Bruker-Daltronics MicroTOF – Q II mass spectrometer. HPLC analysis was performed on a Shimadzu SCL-10A with an UV detector SPD-10AV. Column chromatography was performed using Kieselgel 60 and RP-18 (Merck®). TLC was performed using Kieselgel 60 F_{254} (Merck® aluminum support plates). The solvents used for HPLC analyses were purchased from Tedia® (HPLC grade). All other reagents used were of analytical grade and were purchased from Nuclear®.

Roots of *W. ebracteata* were purchased from Lohmann Company Ltd., located in Nova Petrópolis, RS, Brazil, and compared with a material previously identified by Prof. Dr. Sergio A.L. Bordignon (Unilassale, Canoas, RS, Brazil). Specimens of *W. ebracteata* have been deposited at the Herbarium of the Federal University of Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brazil (ICN 95292).

Dried and powdered roots of *W. ebracteata* (1.5 kg) were extracted with CH_2Cl_2 at room temperature for 72 h. After exhaustive extraction, the extract was filtrated and taken to dryness under reduced pressure and a temperature below 45 °C generating the dichloromethane extract (9 g). This extract was subjected to vacuum liquid column chromatography (12 cm i.d. × 15 cm) on silica gel using hexane with increasing amounts of ethyl acetate (EtOAc) to afford 8 fractions: hexane/EtOAc 20% (F1 – 300 mL), hexane/EtOAc 30% (F2 – 300 mL), hexane/EtOAc 40% (F3 – 300 mL), hexane/EtOAc 50% (F4 – 500 mL), hexane/EtOAc 60% (F5 – 300 mL), hexane/EtOAc 80% (F6 – 500 mL), hexane/EtOAc 90% (F7 – 500 mL), EtOAc (F8 – 500 mL). After comparison by TLC, the fractions F3 and F4 (500 mg) were combined because of their chromatographic similarities and subjected to CC using silica gel as adsorbent (particle size 63–200 µm) and hexane/EtOAc 40% as mobile phase (2 L) providing compounds **1** (10 mg), **2** (18 mg), **4** (50 mg), and **5** (300 mg). Fraction F6 (300 mg) was subjected to CC using RP-18 as adsorbent and H_2O/acetonitrile 50% as eluent mixture (1.5 L) providing compounds **3** (15 mg) and **6** (200 mg).

*2β,16α,20R-Trihydroxy-10α,17α-cucurbit-5,25-dien-3,11,22-trione (**1**):* white amorphous solid; $[\alpha]_D^{25}$ + 38.8 (*c* 1.60, $CHCl_3$); UV (MeOH) λ_{max} 273.1 nm; IR (KBr) ν_{max} 3452, 1710, 1693, 1651, 1373, 1259, 1211, 1089, 985, 887, 736 cm^{-1}; ^1H NMR and ^{13}C NMR data: see **Table 1**; ESI-MS *m*/*z* 501.3193 $[M + H]^+$ (calcd. for $C_{30}H_{45}O_6$, 501.321).

*2β,16α,20R,22S-Tetrahydroxy-10α,17α-cucurbit-5,24-dien-3,11-dione (**2**):* white amorphous solid; $[\alpha]_D^{25}$ + 62.08 (*c* 1.78, $CHCl_3$); UV (MeOH) λ_{max} 282.6 nm; IR (KBr) ν_{max} 3445, 1712, 1693, 1681, 1643, 1384, 1222, 1062, 985, 619 cm^{-1}; ^1H NMR and ^{13}C NMR data: see **Table 1**; ESI-MS *m*/*z* 525.3200 $[M + Na]^+$ (calcd. for $C_{30}H_{46}NaO_6$, 525.3187).

Compound **2** (30 mg, 59 µM) was dissolved in anhydrous acetone (1.0 mL) and 2,2-dimethoxypropane (0.3 mL), treated with *p*-toluenesulfonic acid monohydrate (2 mg, 1.05 mmol) and left under constant stirring at room temperature for 3 h. The reaction was quenched by the addition of 25% NH_4OH and concentrated. The residue was purified by CC on silica gel using hexane/EtOAc 20% as the eluent mixture to give compound **7**.

Compound **7:** ^1H NMR (500 MHz, $CDCl_3$) δ 5.80 (dd, *J* = 3.5, 2.0 Hz, 1H, H-6), 5.12 (dd, 8.6, *J* = 6.7 Hz, 1H, H-24), 4.60 (dd, *J* = 9.6, 7.7 Hz, 1H, H-16), 4.41 (ddd, *J* = 13.0, 6.0, 4.0 Hz, 1H, H-2), 3.88 (dd, *J* = 8.8, 5.6 Hz, 1H, H-22), 3.0 (d, *J* = 15.0 Hz, 1H, H-12a), 2.70 (m, 1H, H-23a), 2.69 (d, *J* = 13.0 Hz, 1H, H-10), 2.59 (d, *J* = 15.0 Hz, 1H, H-12b), 2.40 (m, 1H, H-23b), 2.40 (m, 1H, H-7a), 2.33 (d, *J* = 7.7 Hz, 1H, H-17), 2.27 (ddd, *J* = 13.0, 6.0, 3.0 Hz, 1H, H-1a), 2.0 (dd, *J* = 20.0, 5.0 Hz, 1H, H-7b), 1.97 (d, *J* = 5.0 Hz, 1H, H-8), 1.95 (m, 1H, H-15a), 1.74 (s, 3H, H-26), 1.66 (s, 3H, H-27), 1.54 (s, 3H, H-32), 1.49 (m, 1H, H-15b), 1.43 (s, 3H, H-33), 1.40 (s, 3H, H-21), 1.35 (s, 3H, H-28), 1.33 (s, 3H, H-30), 1.27 (s, 3H, H-29), 1.22 (ddd, *J* = 13.0, 13.0, 13.0 Hz, 1H, H-1b), 1.05 (s, 3H, H-19), 0.9 (s, 3H, H-18).

Supporting Information

The spectroscopic data of compounds **3**–**6** and the description of the MTT assay method are available as Supporting Information.

Acknowledgements

The authors gratefully acknowledge the financial support of this study and also the research stipends from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, MCT, Brazil) to T.R.G., V.R.M., L.A.Z., I.T.S., M.R.T., C.M.O.S., and E.P.S., from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, MEC, Brazil) to K.L.L., and from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) and UBA (Univerdad de Buenos Aires, Argentina) to J.A.P. and F.J.D. We are also grateful to Prof. Dr. Gabriela M. Cabrera for the ESI-MS analysis.

The authors declare no conflict of interest with respect to this publication.

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received November 18, 2010
revised January 26, 2011
accepted March 13, 2011

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DOI http://dx.doi.org/10.1055/s-0030-1270962
Published online April 6, 2011
Planta Med 2011; 77: 1648–1651

ISSN 0032-0943

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