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Triterpenoid saponins from the stem bark of *Caryocar villosum*

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Abstract

Five triterpenoid saponins, caryocarosides II-22 (**3**), III-22 (**4**), II-23 (**5**), III-23 (**6**), and II-24 (**7**), have been isolated from the methanol extract of the stem bark of *Caryocar villosum*, along with two known saponins (**1**–**2**). The seven saponins are glucuronides of hederagenin (II) or bayogenin (III). Caryocaroside II-24 (**7**) is an unusual galloyl ester saponin acylated on the sugar chain attached to C-28, the 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosyl hederagenin-28-*O*-[2-*O*-galloyl-β-D-glucopyranosyl] ester. The structures of the saponins were established on the basis of extensive NMR (^{13}C, ^{1}H, COSY, TOCSY, HSQC, HMBC and ROESY) and ESI-MS studies. The cytotoxic activity of saponins **2** and **3** was evaluated *in vitro* against human keratinocytes. The DOPA-oxidase inhibition and the lipolytic activities were evaluated *ex vivo* using an explant of human adipose tissue.

Keywords: *Caryocar villosum*; Caryocaraceae; Triterpenoid saponins; Hederagenin; Bayogenin; Gallic acid; Dermocosmetic activity

1. Introduction

The genus *Caryocar* comprises 16 species and belongs to the restricted family of the Caryocaraceae. *Caryocar villosum* (Aubl.) Pers. is called pekea, pékéya, peke'a, or pikia by the local inhabitants of French Guyana, and named piquiá in Portuguese. This species is a very large tree, up to 50 m high, distributed in the forests of the region near the Amazon Mouth (Prance, 1987; Marx et al., 1997). From the pulp of the fruit, the local industry produces edible oil that is used for cookery (Marx et al., 1997). In rural households of North-East Brazil, the oils of piquiá fruit and of *C. brasiliensis* are used for their antifungal activity against dermatophytosis (Passos et al., 2003; Grenand et al., 2004). Traditionally, the fruits and stem bark of this species are used by the natives as a fish poison. This property has been attributed to the presence of saponins (Grenand et al., 2004; Alabdul Magid et al., 2006b).

We previously reported the isolation and structural elucidation of 35 new saponins called caryocarosides from the fruits of *C. glabrum* (Alabdul Magid et al., 2006a) and of *C. villosum* (Alabdul Magid et al., 2006b). In our continuing search for saponins from the *Caryocar* species, we have studied the constituents of the stem bark of *C. villosum*. We described herein the isolation and structural elucidation of five new saponins named caryocarosides II-22 (**3**), III-22 (**4**), II-23 (**5**), III-23 (**6**), and II-24 (**7**), along with two known saponins, caryocarosides II-5 (**1**) and II-7 (**2**). In addition, the lipolytic activity *ex vivo* using an explant of human adipose tissue, the inhibition of DOPA-oxidase activity and the cytotoxic activity *in vitro* against the human keratinocyte cells of saponins **2** and **3** were also evaluated.

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2. Results and discussion

The methanol extract of the stem bark of *C. villosum* (Aubl.) Pers. was subjected to VLC on RP-18 to afford a saponin fraction. This fraction was then purified using a combination of column chromatography over silica gel and RP-18, and finally by semi-prep. HPLC to give seven saponins. Acid hydrolysis of the crude saponin fraction yielded two aglycones which were identified as hederagenin (II) and bayogenin (III) by comparative TLC with authentic samples, and structural confirmation was achieved by analysis of the 1D and 2D NMR spectra of the purified saponins. The sugar composition was determined by TLC and optical rotation measurement as L-rhamnose, D-glucose, D-galactose, and D-glucuronic acid.

The two known saponins **1** and **2** were identified as 3-*O*-β-D-glucuronopyranosyl hederagenin (caryocaroside II-5) and 3-*O*-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosyl hederagenin (caryocaroside II-7) (Alabdul Magid et al., 2006a) by analysis of their ESI-MS, 1D and 2D NMR spectroscopic data.

Caryocaroside II-22 (**3**) displayed a molecular ion peak $[M + Na]^+$ at *m/z* 979.4869 in the positive HR-ESI-MS, in accordance with an empirical molecular formula of $C_{48}H_{76}O_{19}Na$. The positive ESI-MS-MS experiments of its $[M - H + 2Na]^+$ ion observed at *m/z* 1001 in the ESI-MS, gave ion fragments at *m/z* 855, 693, and 517, attributed to the successive loss of a desoxyhexose $[M - H + 2Na - 146]^+$, a hexose $[M - H + 2Na - 146 - 162]^+$ and a hexosuronic acid $[M - H + 2Na - 146 - 162 - 176]^+$. This fragmentation clearly indicated that the desoxyhexose was the terminal unit, and the hexosuronic acid was the unit directly linked to aglycone. A quick inspection of the ^1H and ^{13}C NMR spectra of the compound confirmed the presence of three monosaccharide units through the easily identifiable signals for their anomeric protons (δ_H 4.54, 4.59 and 5.08) and carbons (δ_C 105.6 (2C) and 103.8). The analysis of COSY, TOCSY, ROESY and HSQC experiments led to the identification of a β-D-glucuronic acid unit starting from the anomeric proton at δ_H 4.54 (*d*, $J = 7.9$ Hz), and characterized by a five spin system possessing large coupling constants ($J_{H1,H2}$, $J_{H2,H3}$, $J_{H3,H4}$, $J_{H4,H5} \geqslant 7.9$ Hz), a doublet axial proton H-5 ($J_{H4,H5} = 9.5$ Hz), and a carbonyl C-6 at δ_C 172.4 (Table 1). The second unit with its anomeric proton resonating at δ_H 4.59 (*d*, $J = 7.8$ Hz) was identified as a β-D-galactose, characterized by the large coupling constants $J_{H1,H2}$ and $J_{H2,H3}$ and the small coupling constant between H-3 and H-4 ($J_{H3,H4} = 3.4$ Hz) as summarized in Table 1. The third unit with its anomeric proton resonating at δ_H 5.08 (*d*, $J = 1.7$ Hz) was identified as an α-L-rhamnose with its methyl group at δ_{H6} 1.28 (*d*, $J_{H5,H6} = 6.2$ Hz) and δ_{C6} 18.0. The observed small coupling constant ($J_{H1,H2}$) of rhamnose allowed either an equatorial or axial orientation of H-1, but the chemical shift of Rha–C-5 at δ_C 70.2 indicated an α configuration for L-rhamnose (Kasai et al., 1979; Backinowsky et al., 1980). The α configuration for the rhamnose unit was also confirmed by the lack of rOe effects between H-1 with H-3 and H-5. The remaining 30 carbon signals were indicative of a triterpene moiety which was identified as hederagenin [δ_{H23} 3.27, 3.66 (*d*, $J = 11.4$ Hz), δ_{C23} 64.9, and δ_{H3} 3.68 (*dd*, $J = 9.7$, 3.3 Hz), δ_{C3} 83.2] (Mahato and Kundu, 1994; Bialy et al., 2004). Sites of attachment and sequence in the monosaccharide chain were obtained from an HMBC experiment, which provided cross-peaks between Rha–H-1/Gal–C-3 (δ_C 81.2), Gal–H-1/GlcA–C-3 (δ_C 86.8), and GlcA–H-1/C-3 (δ_C 83.2) of hederagenin. This was also confirmed by the observation of rOe correlations in the ROESY spectrum between GlcA–H-1/H-3 of hederagenin, Gal–H-1/GlcA–H-3 and Rha–H-1/Gal–H-3. On the basis of the foregoing evidence, caryocaroside II-22 (**3**) was elucidated as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosylhederagenin.

Caryocaroside III-22 (**4**) displayed a molecular ion peak $[M + Na]^+$ at *m/z* 995.4825 in the positive HR-ESI-MS, in agreement with a molecular formula of $C_{48}H_{76}O_{20}Na$ and suggesting an additional hydroxyl group by comparison with **3**. The aglycone of **4** was identified as bayogenin, characterized by its six tertiary methyl signals, a primary carbinol at position 23, and two secondary hydroxyls assigned to the 2β and 3β positions [δ_{H23} 3.25, 3.64 (*d*, $J = 11.3$ Hz), δ_{C23} 65.3, δ_{H3} 3.66 (*d*, $J = 3.3$ Hz), δ_{C3} 83.9, and δ_{H2} 4.28 (*q*, $J = 3.3$ Hz), δ_{C2} 71.5] (Mahato and Kundu, 1994; Tan et al., 1999). The ^1H NMR spectrum showed three anomeric proton signals at δ_H 4.59 (*d*, $J = 7.8$ Hz), 4.60 (*d*, $J = 7.8$ Hz) and 5.08 (*d*, $J = 1.7$ Hz). The corresponding anomeric carbon signals were located at δ_C 105.5, 105.6, and 103.8 using their HSQC correlations. Analysis of 2 D NMR experiments and comparison of the ^1H and ^{13}C NMR data of the oligosaccharide portion with those of **3**, showed that **4** contained the same trisaccharide chain linked to C-3 of the aglycone (Table 1). Thus, caryocaroside III-22 (**4**) was concluded to be 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosylbayogenin.

The positive HR-ESI-MS of caryocaroside II-23 (**5**) showed a molecular ion peak $[M + Na]^+$ at *m/z* 1141.5417, in accordance with an empirical molecular formula of $C_{54}H_{86}O_{24}Na$. The ESI-MS-MS experiment of the pseudomolecular $[M - H + 2Na]^+$ ion obtained at *m/z* 1163 in the positive ESI-MS, gave the ion fragment $[M - H + 2Na - 162]^+$ at *m/z* 1001 suggesting an additional hexose compared to **3**. Analysis of the ^1H and ^{13}C NMR spectra of **5** revealed four sugar units, three of which belonged to the sugar chain attached to the C-3 of the aglycone. This trisaccharide was elucidated as above as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranoside (Table 1). The additional hexose unit was identified as a terminal β-D-glucopyranose (δ_{C1} 95.7 and δ_{H1} 5.40, $J_{H1,H2}$, $J_{H2,H3}$, $J_{H3,H4}$, $J_{H4,H5} \geqslant 8.1$ Hz) linked to C-28 of hederagenin as deduced from the cross-peak observed in the HMBC spectrum between Glc–H-1 and C-28 (δ_C 178.1). Thus, the structure of

Table 1
^{1}H and ^{13}C NMR spectral data for the sugar moieties of compounds **3**–**7** (in CD_3OD)

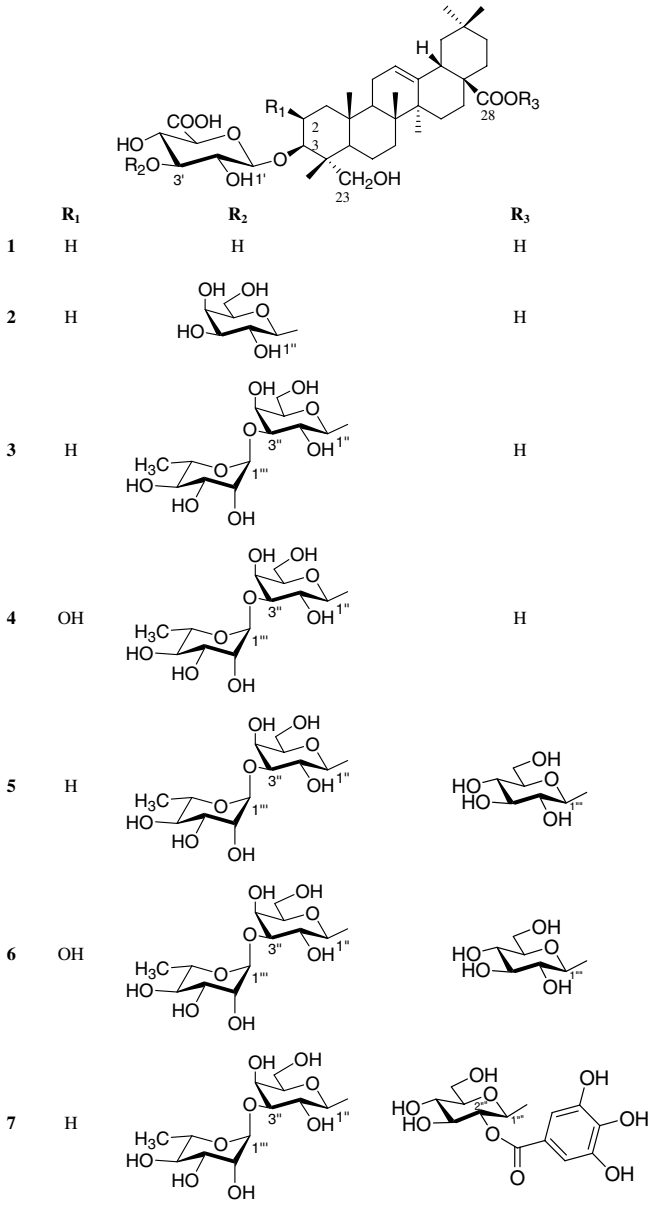
	3		**4**		**5**		**6**		**7**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
β-D-glucuronic acid (at C-3)										
1′	4.54 (*d*, 7.9)	105.6	4.59 (*d*, 7.8)	105.5	4.54 (*d*, 7.9)	105.5	4.59 (*d*, 7.8)	105.5	4.52 (*d*, 7.9)	105.6
2′	3.44 (*dd*, 9.2, 7.9)	74.6	3.55 (*dd*, 8.5, 7.7)	74.5	3.45 (*dd*, 9.3, 7.9)	74.6	3.55 (*dd*, 8.4, 7.8)	74.5	3.43 (*t*, 8)	74.6
3′	3.63 (*m*)	86.8	3.68 (*t*, 8.3)	86.5	3.62 (*t*, 9.5)	83.4	3.68 (*t*, 8.3)	86.5	3.63 (*d*, 9)	86.7
4′	3.64 (*t*, 9.2)	71.8	3.65 (*m*)	71.8	3.64 (*m*)	71.8	3.65 (*m*)	71.8	3.62 (*m*)	71.8
5′	3.87 (*d*, 9.5)	76.3	3.96 (*d*, 9.5)	76.2	3.86 (*d*, 9.5)	76.3	3.95 (*d*, 9.5)	76.2	3.86 (*m*)	77.1
6′	–	172.4	–	172.0	–	172.3	–	172.0	–	172.5
β-D-galactose (at GlcA-C-3′)										
1″	4.59 (*d*, 7.8)	105.6	4.60 (*d*, 7.8)	105.6	4.59 (*d*, 7.8)	105.6	4.60 (*d*, 7.8)	105.6	4.58 (*d*, 7.8)	105.5
2″	3.75 (*dd*, 9.8, 7.8)	72.7	3.76 (*dd*, 9.8, 7.8)	72.7	3.75 (*dd*, 9.7, 7.8)	72.6	3.76 (*dd*, 9.7, 7.8)	72.7	3.74 (*dd*, 9.8, 7.8)	72.6
3″	3.61 (*dd*, 9.8, 3.2)	81.2	3.61 (*dd*, 9.8, 3.1)	81.2	3.61 (*dd*, 9.8, 3.3)	81.2	3.61 (*dd*, 9.7, 3.1)	81.2	3.60 (*dd*, 9.8, 3.1)	81.2
4″	3.91 (*d*, 3.4)	70.1	3.91 (*d*, 3.1)	70.1	3.91 (*d*, 3.3)	70.0	3.91 (*d*, 3.1)	70.1	3.91 (*d*, 3.1)	70.1
5″	3.60 (*m*)	77.1	3.60 (*m*)	77.1	3.60 (*m*)	77.1	3.60 (*m*)	77.1	3.60 (*m*)	77.1
6″a	3.70 (*dd*, 11.5, 4.5)	62.5	3.70 (*dd*, 11.5, 4.7)	62.5	3.69 (*dd*, 11.3, 4.4)	62.5	3.70 (*dd*, 11.6, 4.7)	62.5	3.69 (*dd*, 11.4, 4.4)	62.5
6″b	3.79 (*dd,* 11.4, 7.7)		3.79 (*dd*, 11.5, 7.5)		3.79 (*dd*, 11.3, 6.4)		3.79 (*dd*, 11.6, 7.5)		3.79 (*dd*, 11.4, 5.8)	
α-L-rhamnose (at Gal-C-3″)										
1‴	5.08 (*d*, 1.7)	103.8	5.08 (*d*, 1.7)	103.8	5.08 (*d*, 1.7)	103.8	5.08 (*d*, 1.7)	103.8	5.08 (*d*, 1.4)	103.8
2‴	3.99 (*dd*, 3.4, 1.7)	72.1	3.99 (*dd*, 3.4, 1.7)	72.1	3.99 (*dd*, 3.5, 1.7)	72.0	3.99 (*dd*, 3.4, 1.7)	72.1	3.99 (*dd*, 2.9, 1.4)	72.1
3‴	3.79 (*dd*, 9.5, 3.2)	72.1	3.79 (*dd*, 9.5, 3.4)	72.1	3.79 (*dd*, 9.3, 3.5)	72.0	3.79 (*dd*, 9.5, 3.4)	72.1	3.79 (*dd*, 9.3, 3.3)	72.1
4‴	3.42 (*t*, 9.5)	74.0	3.42 (*t*, 9.5)	74.0	3.41 (*t*, 9.5)	74.0	3.42 (*t*, 9.5)	74.0	3.42 (*t*, 9.4)	74.0
5‴a	3.78 (*dq*, 9.8, 6.2)	70.2	3.78 (*m*)	70.2	3.80 (*dq*, 9.8, 6.2)	70.2	3.78 (*m*)	70.2	3.78 (*m*)	70.2
5‴b	1.28 (*d*, 6.2)	18.0	1.28 (*d*, 6.3)	18.0	1.28 (*d*, 6.2)	18.0	1.28 (*d*, 6.3)	18.0	1.28 (*d*, 6.2)	18.0
β-D-glucose (at C-28)										
1⁗					5.40 (*d*, 8.1)	95.7	5.40 (*d*, 8.1)	95.7	5.61 (*d*, 8.4)	93.6
2⁗					3.34 (*t*, 8.4)	73.9	3.34 (*t*, 8.4)	73.9	5.11 (*t*, 8.4)	73.6
3⁗					3.43 (*t*, 9.1)	78.3	3.43 (*t*, 9.1)	78.3	3.74 (*t*, 9.2)	76.1
4⁗					3.38 (*t*, 9.6)	71.1	3.38 (*t*, 9.6)	71.1	3.51 (*d*, 9.2)	71.2
5⁗					3.37 (*m*)	78.7	3.37 (*m*)	78.7	3.47 (*ddd*, 8.7, 5.1, 1.9)	78.9
6⁗a					3.70 (*dd*, 12.2, 4.5)	62.4	3.70 (*dd*, 12.2, 4.5)	62.4	3.76 (*dd*, 12.2, 5.1)	62.2
6⁗b					3.83 (*dd*, 12.2, 1.7)		3.83 (*dd*, 12.2, 1.7)		3.89 (*dd*, 12.2, 1.9)	
Gallate (at Glc–C-2⁗)										
1									–	120.8
2									7.11 (*s*)	110.6
3									–	146.5
4									–	140.2
5									–	146.5
6									7.11 (*s*)	110.6
7									–	165.4

caryocaroside II-23 (**5**) was concluded to be 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosylhederagenin 28-*O*-β-D-glucopyranosyl ester.

Caryocaroside III-23 (**6**) showed a molecular ion peak $[M + Na]^+$ at *m/z* 1157.5338 ($C_{54}H_{86}O_{25}Na$), in the positive HR-ESI-MS. Analysis of the ^1H and ^{13}C NMR spectra of this compound revealed bayogenin as the aglycone and four sugar units. Comparison of the ^1H and ^{13}C NMR values of the oligosaccharide part with those of **5**, indicated that **6** contained the same trisaccharide chain linked to C-3 and a β-D-glucopyranosyl linked to C-28 of the aglycone (Table 1). Thus, compound **6** was concluded to be 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosylbayogenin 28-*O*-β-D-glucopyranosyl ester.

Caryocaroside II-24 (**7**) showed a molecular ion peak $[M + Na]^+$ at *m/z* 1293.5515 in the positive HR-ESI-MS, in accordance with an empirical molecular formula of $C_{61}H_{90}O_{28}Na$. Its UV spectrum exhibited an absorption maximum at 280 nm indicating the presence of an aromatic acyl group. Analysis of the ^{13}C NMR spectrum indicated that **7** was a 3,28-*O*-bidesmosidic hederagenin, and contained the trisaccharide [α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosyl] linked to C-3 of hederagenin and a β-D-glucopyranosyl linked to C-28 (Table 1). In addition to the corresponding signals of **5**, the *J*-modulated ^{13}C NMR spectrum of **7** showed five quaternary carbon [δ_C 120.8, 140.2, 146.5 (2C), and 165.4] and two methine carbon signals [δ_C 110.6 (2C)] (Table 1). In the ^1H NMR spectrum, an additional signal at δ_H 7.11, integrating for 2H, was correlated in the HSQC spectrum with the methine signal at δ_C 110.6. The ^1H and ^{13}C NMR values were in accordance with a galloyl moiety (or 3,4,5-trihydroxybenzoyl) (Haslam, 1989; Mimaki et al., 2001; Reiersen et al., 2003). The location of this group at position 2 of the β-D-glucopyranose was suggested by the pronounced downfield shift of Glc–H-2 compared to the corresponding H-2 signal of **5** ($\Delta\delta + 1.77$ ppm) and the upfield shifts of both Glc–C-1 at δ 93.6 and Glc–C-3 at δ 76.1 (β-effect), relative to the corresponding values in **5** (Table 1). Further supporting information for the site of esterification was deduced from the HMBC experiment which exhibited a cross-peak between Glc–H-2 (δ_H 5.11) and the carbonyl carbon (δ 165.4) of the galloyl group. Therefore the structure of **7** was elucidated as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranosyl-(1 → 3)-β-D-glucuronopyranosylhederagenin 28-*O*-[2-*O*-galloyl-β-D-glucopyranosyl] ester. Only a few triterpenes, acylated with a galloyl group, have been described in the literature and caryocaroside II-24 (**7**) represents the second isolation of an acylated saponin containing this ester in the oligosaccharide part (Mimaki et al., 2001).

The dermocosmetic activity of saponins **2** and **3** was evaluated. No lipolytic activity was observed *ex vivo* using an explant of human adipose tissue at a dose of 100 μg/ml, and no inhibition of the DOPA-oxidase activitiy was observed at a dose of 50 μg/ml. Compounds **2** and **3** exhibited moderate cytotoxic activity *in vitro* against human keratinocyte cells with a LC_{50} of 23 (**2**), and 20.9 μM (**3**).



3. Experimental

3.1. General experimental procedures

Optical rotations were measured in MeOH or H_2O with a Perkin–Elmer 241 polarimeter. UV spectra were obtained using a Philips PU 8720 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded in CD_3OD on a Bruker Avance DRX-500 spectrometer (^1H at 500 MHz and ^{13}C at 125 MHz), and 2D-NMR experiments were performed

using standard Bruker microprograms (XWIN-NMR version 2.6 software). ESI-MS, ESI-MS-MS and HR-ESI-MS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK) with an electrospray source (eV = 35 V, 80 °C, flow of injection 5 μl/min). TLC were carried out on precoated silica gel 60 F_{254} (Merck) with $CHCl_3$–MeOH–H_2O (13:7:1), and spots were visualized by heating after spraying with 50% H_2SO_4. Column chromatography was carried out on Kieselgel 60 (63–200 μm, Merck) or LiChroprep RP-18 (40–63 μm, Merck). HPLC was performed on a Dionex apparatus equipped with an ASI-100 autosampler, a STH 585 column oven, a P580 pump, a UVD 340S diode array detector, and the Chromeleon software. A prepacked RP-18 column (Dionex, 201SP510, 10 × 250 mm, 5 μm, 90 Å) was used for semi-prep. HPLC with a binary gradient elution (solvent A: H_2O–TFA 0.0025%, solvent B: CH_3CN) and a flow rate of 3 ml min^{-1}; the chromatogram was monitored at 205, 210, and 250 nm. DMSO, sodium bromide, kojic acid, L-3,4-dihydroxyphenylalanine, caffeine, and mounting medium were purchased from Sigma, tetrazolium salt (XTT) was purchased from Interchim, K-SFM, BPE, EGF, BEM media were obtained from Gibco. All solvents and chemicals used were of analytical grade.

3.2. Plant material

The fruits of *C. villosum* were collected near Regina at Saint George de l'Oyapock station, Cayenne Island in French Guyana in May 1998. The species was identified by P. Grenand of the Botany Laboratory of the IRD Centre of Cayenne (French Guyana), and herbarium specimen (Grenand P. 3076) was deposited in the Herbarium of Guyana.

3.3. Extraction and isolation

Dried and powdered stem bark (400 g) was boiled under reflux in methanol (3.5 l) for 3 h. The methanol extract was filtered and evaporated to provide a brown residue (37.5 g; 9.3% yield). A part of this residue (32 g) was subjected to VLC over RP-18, eluting successively with 500 ml of 40%, 60% and 80% methanol in H_2O and methanol. The 80% MeOH–H_2O fractions were combined to give 0.87 g of saponin mixture (0.21% yield). The saponin mixture was purified by silica gel CC (35 g) using a gradient of $CHCl_3$–MeOH–H_2O (95:5:0–60:40:7). Frs. [70–80] eluted with $CHCl_3$–MeOH (7:3) contained 16 mg of **3**. Frs. [81–121] eluted with $CHCl_3$–MeOH (7:3) were purified on a RP-18 CC with the gradient MeOH–H_2O (4:6–9:1), and frs. eluted with MeOH–H_2O (8:2) were purified by semi-prep. HPLC with the gradient (from 37% to 42% of B) for 20 min to give 4 mg of **4** (R_t = 15.4 min), and 12 mg of **3** (R_t = 19.4). The other frs. eluted with MeOH–H_2O (8:2) were purified by semi-prep. HPLC with the binary gradient (from 40% to 43% of B) for 20 min to give 19 mg of **4** (R_t = 19.6 min), 3 mg of **2** (R_t = 20.7) and 3 mg of **1** (R_t = 24.9). Frs. [122–147] eluted with $CHCl_3$–MeOH–H_2O (70:30:5) were purified by semi-prep. HPLC with the binary gradient (from 32% to 35% of B) for 15 min to give 3 mg of **6** (R_t = 11.3 min), 16 mg of **5** (R_t = 13.3 min) and 3 mg of **7** (R_t = 15 min).

3.4. Acid hydrolysis of saponin mixture

The crude saponin mixture (100 mg) was refluxed with 15 ml of 2 N HCl for 4 h. The sapogenin was extracted with EtOAc (3 × 15 ml), and the organic layer was neutralized by washing with H_2O, and evaporated to dryness. Hederagenin and bayogenin were identified from the sapogenin residue with authentic samples by TLC in $CHCl_3$–MeOH (95:5) (R_f 0.23 and 0.13, respectively). The acid aqueous layer was neutralized with 1 N NaOH and freeze-dried. Four sugars were identified with authentic samples by TLC in MeCOEt–isoPrOH–Me_2CO–H_2O (20:10:7:6) as rhamnose, glucose, galactose and glucuronic acid. After a preparative TLC of sugar mixture (50 mg) in this solvent, the optical rotation of each purified sugar was measured to afford rhamnose (2 mg; R_f 0.57; $[\alpha]_D^{20}$ +10° (H_2O; *c* 0.1)), glucose (2.4 mg; R_f 0.34; $[\alpha]_D^{20}$ + 11° (H_2O; *c* 0.12)), galactose (2.6 mg; R_f 0.29; $[\alpha]_D^{20}$ + 45° (H_2O; *c* 0.13)), and glucuronic acid (4 mg; R_f 0.10; $[\alpha]_D^{20}$+7°; (H_2O; *c* 0.2)).

3.5. Caryocaroside II-22 (*3*)

White powder; $[\alpha]_D^{20}$ +8.5° (MeOH; *c* 1); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 1; ^1H NMR of aglycone (CD_3OD) δ 0.72 (3H, *s*, H-24), 0.82 (3H, *s*, H-26), 0.93 (3H, *s*, H-29), 0.96 (3H, *s*, H-30), 1.00 (3H, *s*, H-25), 1.19 (3H, *s*, H-27), 2.87 (1H, *dd*, *J* = 13.6 – 4.2 Hz, H-18), 3.27 (1H, *d*, *J* = 11.4 Hz, H-23a), 3.66 (1H, *d*, *J* = 11.4 Hz, H-23b), 3.68 (1H, *dd*, *J* = 9.7 – 3.3 Hz, H-3), 5.26 (1H, *t*, *J* = 3.5 Hz, H-12); ^{13}C NMR of aglycone (CD_3OD) δ 13.4 (C-24), 16.5 (C-25), 17.8 (C-26), 18.9 (C-6), 24.0 (C-16), 24.0 (C-30), 24.5 (C-11), 26.4 (C-2), 26.3 (C-27), 28.9 (C-15), 31.6 (C-20), 33.1 (C-22), 33.5 (C-7), 33.8 (C-29), 34.9 (C-21), 37.7 (C-10), 39.5 (C-1), 40.7 (C-8), 42.8 (C-18), 43.0 (C-14), 43.9 (C-4), 47.3 (C-19), 47.6 (C-17), 48.1 (C-5), 49.0 (C-9), 64.9 (C-23), 83.2 (C-3), 123.8 (C-12), 145.0 (C-13), 181.9 (C-28); ESI-MS (positive ion mode): *m/z* 1001 $[M - H + 2Na]^+$; ESI-MS-MS (1001) *m/z* 855 $[M - H + 2Na - Rha]^+$, 693 $[M - H + 2Na - Rha - Gal]^+$, 517 $[M - H + 2Na - Rha - Gal - GlcA]^+$; HR-ESI-MS (positive ion mode) *m/z* 979.4869 $[M + Na]^+$ (calcd. for $C_{48}H_{76}O_{19}Na$, 979.4879).

3.6. Caryocaroside III-22 (*4*)

White powder; $[\alpha]_D^{20}$ +9.6° (MeOH; *c* 0.42); ^1H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 1; ^1H NMR of aglycone (CD_3OD) δ 0.85 (3H, *s*, H-26), 0.93 (3H, *s*, H-29), 0.97 (6H, *s*, H-24 and H-30), 1.20 (3H, *s*, H-27), 1.30 (3H, *s*, H-25), 2.87 (1H, *dd*, *J* = 14.4–4.1 Hz, H-18), 3.25 (1H, *d*, *J* = 11.3 Hz, H-23a), 3.64 (1H, *d*,

$J = 11.3$ Hz, H-23b), 3.66 (1H, *d*, $J = 3.3$ Hz, H-3), 4.28 (1H, *q*, $J = 3.3$ Hz, H-2), 5.27 (1H, *t*, $J = 3.4$ Hz, H-12); ^{13}C NMR of aglycone (CD_3OD) δ 14.6 (C-24), 17.4 (C-25), 17.8 (C-26), 18.6 (C-6), 24.0 (C-30), 24.0 (C-16), 24.7 (C-11), 26.5 (C-27), 28.7 (C-15), 33.1 (C-22), 31.6 (C-20), 33.4 (C-7), 33.6 (C-29), 34.9 (C-21), 37.5 (C-10), 40.7 (C-8), 43.2 (C-4), 43.2 (C-14), 42.7 (C-18), 44.4 (C-1), 47.2 (C-17), 47.6 (C-19), 48.0 (C-5), 49.1 (C-9), 65.3 (C-23), 71.5 (C-2), 83.9 (C-3), 123.7 (C-12), 145.3 (C-13), 182.0 (C-28); ESI-MS (positive ion mode) *m/z* 995 $[M + Na]^+$; HR-ESI-MS (positive ion mode) *m/z* 995.4825 $[M + Na]^+$ (calcd. for $C_{48}H_{76}O_{20}Na$, 995.4828).

3.7. Caryocaroside II-23 (5)

White powder; $[\alpha]_D^{20}$ + 15.7° (MeOH; *c* 0.66); ^{1}H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 1; ^{1}H and ^{13}C NMR chemical shift values of the aglycone (CD_3OD) were identical with those described for **3** within ±0.05 and ±0.3 ppm, respectively, excepted for C-28 at δ 178.1; ESI-MS (positive ion mode) *m/z* 1163 $[M - H + 2Na]^+$, ESI-MS-MS (1163) *m/z* 1001 $[M - H + 2Na - Glc]^+$, 855 $[M - H + 2Na - Glc - Rha]^+$, 693 $[M - H + 2Na - Glc - Rha - Gal]^+$, 517 $[M - H + 2Na - Glc - Rha - Gal - GlcA]^+$; ESI-MS (negative-ion mode) *m/z* 1117 $[M - H]^-$; ESI-MS^2 (1117) *m/z* 955 $[M - H - Glc]^-$, 809 $[M - H - Glc - Rha]^-$,471 $[M - H - Glc - Rha - Gal - GlcA]^-$; HR-ESI-MS (positive ion mode) *m/z* 1141.5417 $[M + Na]^+$ (calcd. for $C_{54}H_{86}O_{24}Na$, 1141.5407).

3.8. Caryocaroside III-23 (6)

White powder; $[\alpha]_D^{20}$ +16° (MeOH; *c* 0.5); ^{1}H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 1; ^{1}H and ^{13}C NMR chemical shift values of the aglycone (CD_3OD) were identical with those described for **4** within ± 0.05 and ± 0.2 ppm, respectively, excepted for C-28 at δ 178.1; HR-ESI-MS (positive ion mode) *m/z* 1157.5338 $[M + Na]^+$ (calcd. for $C_{54}H_{86}O_{25}Na$, 1157.5356).

3.9. Caryocaroside II-24 (7)

White powder; $[\alpha]_D^{20}$+18° (MeOH; *c* 0.25); UV λ_{max}^{MeOH} nm ($\log\varepsilon$): 280 (4.1); ^{1}H and ^{13}C NMR (CD_3OD) of the glycosidic part, see Table 1; ^{1}H and ^{13}C NMR chemical shift values of the aglycone (CD_3OD) were identical with those described for **3** within ±0.05 and ±0.2 ppm, respectively; ESI-MS (positive ion mode) *m/z* 1315 $[M - H + 2Na]^+$; ESI-MS (negative ion mode) *m/z* 1269 $[M - H]^-$; HR-ESI-MS (positive ion mode) *m/z* 1293.5515 $[M + Na]^+$ (calcd. for $C_{61}H_{90}O_{28}Na$, 1293.5516).

3.10. Cytotoxicity on human keratinocyte

Human keratinocytes were grown in K-SFM supplemented with BPE and EGF in 75 cm^2 flask at 37 °C in a humidified atmosphere containing 5% CO_2. At sub-confluence, the cells were harvested and seeded at a density of 7500 cells/well in a 96 well-plate. After overnight cultivation, the culture medium was removed and replaced by saponins **2** and **3** dissolved with DMSO (1% final) in K-SFM with concentrations ranging from 0.19 to 50 μg/ml, and the cells were incubated during 48 h. After removal of the medium, 100 μl of XTT (0.2 mg/ml in medium) solution was added to each well. After 3 h incubation, the Abs_{450} value was measured with a microplate reader, and the cell viability was calculated as follows: % viability = (Sample–Blank)/(Blank) × 100.

3.11. Evaluation of the lipolytic activity

Fat tissue explants were maintained in survival medium BEM (BIO-EC'S Medium Explants). Samples were dissolved in the culture medium. On day 0, the explants were put in 100 μg/ml of medium. This treatment was repeated on day 2, day 4 and day 6. On day 2, day 4, day 6 and day 8 the culture medium from the various tubes was combined and preserved at −20 °C for analysis of lipids. After extraction of the culture medium, the lipids were separated and analysed by high performance thin layer chromatography (HPTLC). The lipolytic activity was evaluated by the analysis of the proportions of monoglycerides, diglycerides, triglycerides and free fatty acids. Caffeine at 0.1% was used as a positive control (increase lipids liberation of 300%).

3.12. Evaluation of the whitening activity

The method consisted of visualising the potential whitening effect of the test product by inhibition of the L-DOPA oxidase in human melanocytes. Epidermis pieces were immersed in 500 μl of the test compound at 5 mg/ml and 500 μl L-DOPA solution at 2 mg/ml and incubated for 5 h in a bath at 37 °C. Then the epidermis pieces were rinsed in distilled water and placed on a histological blade with a drop of the assembly liquid and covered with a plate cover-glass 22 × 22 mm. The plates were examined by optical microscopy to visualise the colour intensity of the melanocytes on the epidermal split.

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