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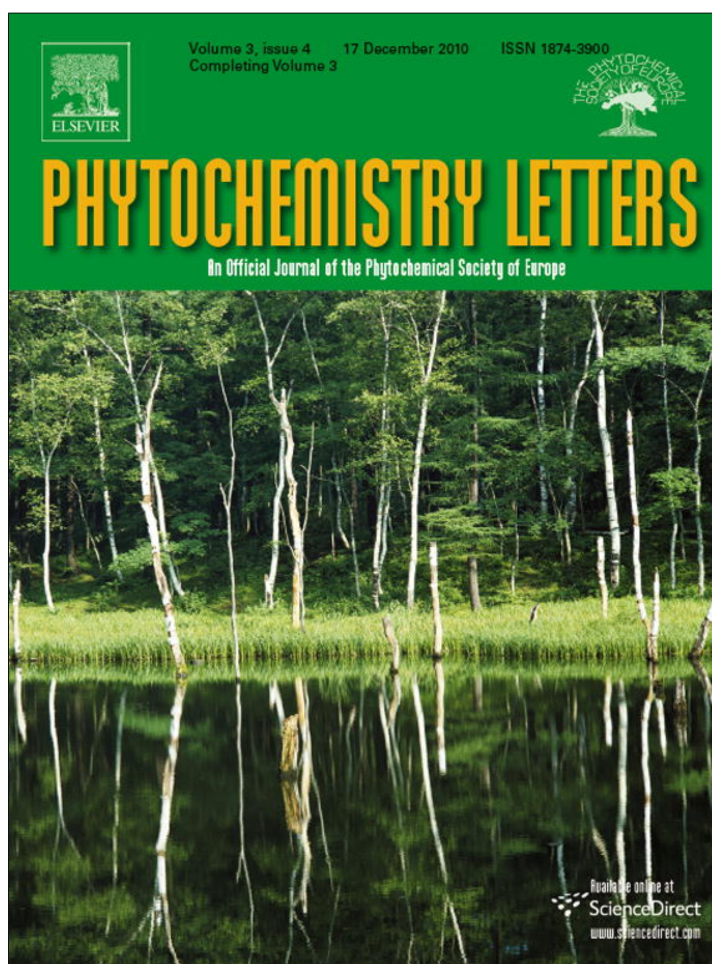

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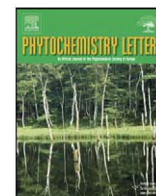

Secondary metabolites from *Calotropis procera* (Aiton)

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Three new metabolites, 5-hydroxy-3,7-dimethoxyflavone-4′-*O*-β-glucopyranoside (**1**), 2β,19-epoxy-3β,14β-dihydroxy-19-methoxy-5α-card-20(22)-enolide (**4**) and β-anhydroepidigitoxigenin-3β-*O*-glucopyranoside (**5**), along with two known compounds, uzarigenine (**2**) and β-anhydroepidigitoxigenin (**3**), were isolated from *Calotropis procera* (Asclepiadaceae). The structure elucidation was accomplished mainly by nuclear magnetic resonance (NMR) spectroscopic and mass spectrometric methods. To examine putative antimicrobial or cytotoxic activities, various bioassays were performed. Uzarigenine (**2**) demonstrated moderate cytotoxicity.

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1. Introduction

Cardiac glycosides are a class of secondary metabolites that are traditionally used to increase cardiac contractile force in patients with congestive heart failure and cardiac arrhythmias (Abarquez, 2001). Throughout history, cardiac glycoside-containing plants or their extracts have been used as arrow poisons, emetics, diuretics, and heart tonics (Kinne-Saffran and Kinne, 2002).

Calotropis procera (Aiton) W. T. Aiton (Asclepiadaceae) is a traditional medicinal plant growing wild from West Africa to South East Asia. The milky juice of the plant is used in India as a purgative, and the flowers are used as a digestive, a stomachic and a tonic and have an anti-asthmatic effect. The root bark is useful in treating skin diseases, enlargement of the abdominal viscera, intestinal worms and ascites (Khan and Malik, 1989). Further, the root of *C. procera* is used as a carminative in the treatment of dyspepsia (Kumar and Arya, 2006). The aqueous extract of the latex inhibits cellular infiltration and protects against the development of neoplastic changes in the transgenic mouse model of hepatocellular carcinoma (Choedon et al., 2006). The chloroform extract of the root exhibits protective activity against carbon tetrachloride-induced liver damage (Basu et al., 1992).

According to literature reports *C. procera* contains cardiac glycosides (Elgamal et al., 1999; Hanna et al., 1999, 2002), triterpenes (Bhutani et al., 1992, Khan et al., 1988), sterols (Khan and Malik, 1989) and flavonoids (Mossa et al., 1991). The broad pharmacological profile of *C. procera* could be interesting for the pharmaceutical industry to develope new drugs (Silva et al., 2010). Therefore, the present investigation of constituents of vegetative stems is part of an extended effort to determine the secondary metabolites of *C. procera* and to study their pharmacological activities. The isolated metabolites were evaluated for their in vitro cytotoxic activity against the human cancer cell lines HT29 and HepG2 and the mouse fibroblast cell line NIH-3T3 as well as antimicrobial activity.

2. Results and discussion

2.1. Isolation

The ethanol extract of vegetative stems of *C. procera* was fractionated by flash column chromatography; resulting fractions were eluted with benzene then with chloroform containing increasing proportions of methanol. Compound **1** was obtained from the chloroform fraction by further purification on medium-pressure liquid chromatographs (MPLC) using chloroform/methanol as an eluent. Elution of the silica gel column with chloroform/methanol resulted in a semi-solid fraction, which was purified on Sephadex LH-20, flash chromatography, and MPLC to yield pure compounds **2**–**5** (see Section 3 for details).

2.2. Structure elucidation

The molecular formula of compound **1** ($C_{23}H_{24}O_{11}$) was deduced from HRESIMS m/z ($[M+H]^+$) 477.13995. Fragment ion

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m/*z* [M+H-162]⁺ indicated the loss of a hexose moiety. The occurrence of signals of an AB spin system of H-6 (δ 6.73) and H-8 (δ 6.85) and an AA′XX′ spin system of a *p*-substituted phenyl ring at δ 7.26 (H-3′/5′) and δ 8.00 (H-2′/6′) in the aromatic part of the ^{1}H NMR and ^{1}H,^{1}H-COSY spectra of compound **1** suggested the presence of a flavonoid structure of the aglycon. Typical ^{1}H NMR signals of two methoxy groups at δ 3.84 and 3.99 correlated in the HMBC spectrum to the ^{13}C NMR signals at δ 134.4 (C-3) and 160.8 (C-7), respectively, suggesting that one methoxyl group is located at C-3 and the other at C-7. Another HMBC cross-peak of H-8 (δ 6.85) confirmed the assignment of C-7. From these data, compound **1** was preliminary identified as a 3,7-dimethoxykaempferol glycoside. The aglycon has been reported from many plants, including *Flourensia cernua* (Asteraceae) (Dillon et al., 1976) and *Ericameria diffusa* (Compositae) (Urbatsch et al., 1976).

Signals of a hydroxymethylene group of H-6″ (δ 4.00; δ 3.72) and the doublet of H-1″ at δ 5.05 (J = 7.7 Hz) and further signals of H-2 to H-5 at δ 3.4–3.5 indicated a hexose unit, which is β-configured at the anomeric center. The assignment of the ^{1}H and ^{13}C NMR signals of the glucose unit was supported by ^{1}H,^{1}H-COSY and HSQC data. The ^{13}C chemical shifts of the hexose carbon atoms were consistent with a β-glucopyranose (Agrawal, 1992). The position of the glucose moiety at C-4′ (δ 162.2) was established by a HMBC key correlation with H-1″. Therefore, compound **1** was identified as 5-hydroxy-3,7-dimethoxyflavone-4′-*O*-β-glucopyranoside, which is a new natural product. ^{13}C NMR data of a compound, which were interpreted as **1** (Pan et al., 1993), are inconsistent with the spectral characteristics of the structure herein described (Fig. 1).

On the basis of mass spectrometric and 1D and 2D NMR spectroscopic data, compound **2** was identified as uzarigenine (Elgamal et al., 1999) and compound **3** as β-anhydroepidigitoxigenin, which previously was isolated from the roots of *Nerium oleander* (Mostaqul Huq et al., 1999).

Compound **4** was obtained as a white powder. The HRESIMS data (*m*/*z* 419.24455 [M+1]⁺), which corresponded to the molecular formula $C_{24}H_{34}O_6$, and the ^{1}H and ^{13}C NMR data (Table 1), indicated an isomer of the previously isolated (19*S*)-3β,19-epoxy-2α,14β-dihydroxy-19-methoxy-5α-card-20(22)-enolide (**6**) (Hanna et al., 2002). The ^{13}C chemical shift values of the two compounds were very similar, except those of C-2 (**4**: δ 71.0; 6: δ 67.1), C-3 (**4**: δ 67.5; 6: δ 71.8) and the acetal carbon C-19 (**4**: δ 105.7; **6**: 101.3), suggesting a structural difference at C-2 and/or C-3. A cross-signal in the HMBC spectrum of **4** indicated a three-bond distance between H-2 (δ 3.77) and C-19, which is explained by the oxygen at C-2 being involved in the acetal bridge overarching ring A. The coupling constant $^{3}J_{H-2-H-3}$ = 5.1 Hz suggested the equatorial orientation of H-3. After complete assignment of ^{1}H and ^{13}C NMR chemical shifts by means of ^{1}H,^{1}H-COSY, HSQC, and HMBC spectra, the relative stereochemistry of compound **4** was inferred from a 2D ROESY spectrum. ROESY correlations of H-5 (δ 0.96) with H-3 (δ 4.00) indicated the axial α-orientation of these protons, while ROESY correlations of H-19 (δ 4.60) with H-8β (δ 2.00), H-11β (δ 1.28) and H-6β (δ 1.57) revealed that these protons are β-oriented (Fig. 2). Based on the above mentioned finding, compound **4** was identified as 2β,19-epoxy-3β,14β-dihydroxy-19-methoxy-5α-card-20(22) enolide.

HRESIMS of compound **5** showed a molecular ion peak of *m*/*z* 519.29764 ([M+H]⁺), corresponding to the molecular formula of $C_{29}H_{42}O_8$, and suggesting eight unsaturation equivalents in the molecule. The ^{1}H NMR spectrum showed two doublets of doublets (J = 17.8, 1.8 Hz) at δ 4.85 and 4.94 for H-21a and H-21b,

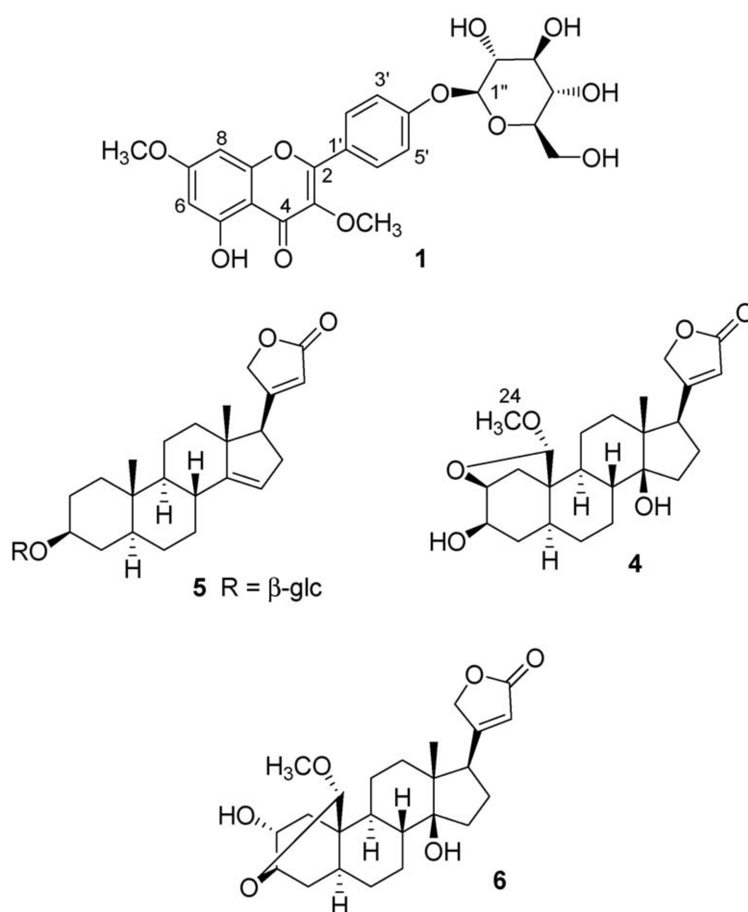

Fig. 1. Structures of new compounds **1**, **4** and **5**. Compound **6** was used as a reference for structural analysis.

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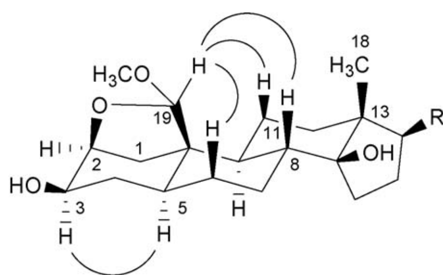

Fig. 2. Selected ROESY correlations of compound **4**.

respectively, and a doublet at δ 5.95 (*J* = 1.8 Hz), assignable to H-22 of an α,β-unsaturated-γ-lactone ring of cardenolide (Siddiqui et al., 1997). The signal of another olefinic proton at δ 5.30 (*J* = 1.8 Hz) was assigned to H-15. The two methyl signals of C-18 and C-19 appeared at δ 0.84 and 0.87, respectively. The doublet at δ 4.40 was attributed to the proton at the anomeric center of a hexose unit, and the value of the coupling constant $^3J_{H-1''-H-2''}$ = 7.8 Hz reflects the β-configuration of H-1″. In addition, coupling constants between 8.0 and 9.0 Hz (Table 1) indicated that H-2″–H-5″ are also in axial orientation, which is consistent with a glucose unit. ^{1}H-^{1}H-COSY and HSQC cross-signals were used to assign ^{1}H and ^{13}C chemical shifts of the β-glucose. HMBC key correlation of H-1′ with the C-3 (δ 79.2) revealed the connectivity of the glucose to C-3 of the β-anhydroepidigitoxigenin moiety. The full ^{1}H and ^{13}C signal assignments were established by APT, ^{1}H,^{1}H-COSY, HMQC and HMBC, which together with the MS data lead to the structure of compound **5** as β-anhydroepidigitoxigenin-3β-*O*-glucopyranoside.

2.3. Bioassays

Metabolites **1**–**5** were evaluated for in vitro cytotoxic activity against the human cancer cell lines HT29 and HepG2 and the mouse fibroblast cell line NIH-3T3. Uzarigenine (**2**) reduced the metabolic activity of HT29 and HepG2 cells at a concentration of 50 μM by 59% and 35%, respectively, while no inhibition of the metabolic activity of NIH-3T3 cells was observed. In addition, compound **1** (50 μM) caused a decrease of the metabolic activity of NIH-3T3 and HepG2 cells by 18% and 10%, respectively, whereas compounds **2**, **3** and **5** displayed no activity. Antimicrobial assays revealed no significant activities in tested compounds **1**–**5**.

3. Experimental

3.1. General experimental procedures

The optical rotation was measured on a JASCO 1030 Polarimeter (*JASCO GmbH*, Groß-Umstadt, Germany). NMR spectra (^{1}H NMR, ^{13}C NMR, APT, ^{1}H,^{1}H-COSY, HMBC, and HSQC) were measured on a

Table 1
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **4** and **5** in MeOH-d_4.

		4		**5**	
		δ_H (*J* in Hz)	δ_C[a]	δ_H (*J* in Hz)	δ_C[a]
1	α	1.55 m	36.7	1.02 dt (13.7, 3.7)	38.3
	β	1.58		1.82 td (13.0, 3.7)	
2	α	3.76 ddd (5.0, 3.8, 2.3)	71.0	1.90 dd (13.0, 2.9)	30.4
	β			1.55 dd (13.0, 3.6)	
3	α	4.00 dddd (9.2, 5.0, 4.9, 1.2)	67.5	3.72 m	79.2
4	α	2.22 m	30.4	1.75 m	35.5
	β	1.54 m		1.33 m	
5	α	0.94 m	43.5	1.12 m	45.5
6	α	1.61 m	32.6	1.39 m	29.6
	β	1.57 m		1.39 m	
7	α	0.97 m	27.7	1.34 m	31.2
	β	2.01 m		1.97 dd (12.5, 2.9)	
8	β	2.00 m	43.1	2.10 br t (11.1)	36.6
9	α	1.12 ddd (13.5,12.3, 3.0)	47.0	0.75 dd (11.1, 2.9)	55.3
10			39.0	–	37.0
11	α	1.56 m	22.9	1.72 m	22.9
	β	1.28 dd (13.2, 3.5)		1.38 m	
12	α	1.45 ddd (13.5, 13.2, 3.5)	41.1	1.39 m	42.2
	β	1.54 m		1.95 m	
13			51.0		49.7
14			86.3		155.5
15	α	2.10 m	32.7	5.30 d (2.0)	117.9
	β	1.75 dd (13.3, 3.4)			
16	α	2.14 m	28.1	2.53 m	34.5
	β	1.87 m		2.47 m	
17	α	2.83 dd (9.4, 5.4)	52.1	2.87 t (9.2)	54.0
18	β	0.90 s	16.6	0.84 s	18.6
19	β	4.58 s	105.7	0.87 s	12.4
20			178.4		174.2
21	a	4.91 dd (18.4, 1.8)	75.4	4.85 dd (17.8, 1.8)	75.4
	b	5.03 dd (18.4, 1.8)		4.94 dd (17.8, 1.8)	
22		5.90 d (1.6)	117.8	5.95 d (1.5)	116.5
23			177.3		176.8
24		3.37 s	55.5		
1′				4.40 d (7.8)	102.3
2′				3.14 dd (7.8, 9.0)	75.2
3′				3.35 dd (9.0, 9.0)	78.1
4′				3.29 dd (8.0, 9.0)	71.7
5′				3.27 ddd (8.0, 5.4, 2.0)	77.9
6′	a			3.65 dd (12.0, 5.4)	62.8
	b			3.86 dd (12.0, 2.0)	

[a] Assignments were based on HSQC, and HMBC experiments.

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Bruker DRX 500 spectrometer (*Bruker Biospin*, Rheinstetten, Germany) using an inverse-detection probe (5 mm). Operating frequencies were 500.13 MHz for acquiring ^{1}H NMR and 125.75 MHz for ^{13}C NMR spectra. Samples were measured at 300 K in CD_3OD with tetramethyl silane as an internal standard. Liquid chromatography electrospray ionization mass spectra (positive mode) were measured with an Orbitrap XL (*Thermo-Fisher*, San Jose, CA, USA) equipped with an electrospray (ESI) source and coupled to a HP1100 HPLC chromatograph (*Hewlett Packard*, Avondale, PA, USA). Full-scan spectra were recorded from *m/z* 100 to 1000 at a resolution of 30,000. MPLC was performed on a silica gel column (silica gel 60, 15–40 μm, 250 × 15 mm, flow rate: 10 ml/min, *Merck*, Darmstadt, Germany). Column chromatography (CC): silica gel 60 (40–63 μm, *Merck*, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (*Merck*) and visualized by 5% alcoholic sulfuric acid solution. HPLC was performed on an Agilent series HP 1100 (binary pump G1312A; auto sampler G1313A; diode array detector G1315B, 200–700 nm).

3.2. Plant material

C. procera (Aiton) W. T. Aiton. (Asclepiadaceae) vegetative stems were collected at Elariech (Sina, Egypt) in May 2006 and identified by Dr. M. El-Gibaly (Plant Taxonomy Unit, National Research Centre, Cairo, Egypt). A voucher specimen is deposited at the Herbarium of the National Research Centre, CP.05.006MG, Cairo, Egypt.

3.3. Extraction and isolation

The freshly ground stems of *C. procera* (20 kg) were exhaustively extracted at room temperature with 70% ethanol. The solvent was evaporated under vacuum at 45 °C and the residue (500 g) was fractionated by flash column chromatography using silica gel (40–63 μm). Benzene, followed by chloroform and chloroform containing an increasing proportion of methanol, was used as a mobile phase. Chromatographic fractions were analyzed for natural products by TLC. Elution with chloroform gave rise to 2.0 g of semi-solid residue after the solvent was evaporated. The residue was subjected to MPLC and eluted with benzene followed by chloroform to afford 0.1 g of crude compound **1**; this was re-chromatographed by MPLC using chloroform containing an increasing proportion of methanol to afford pure compound **1** (3.3 mg). Elution of the flash chromatography column with chloroform–methanol (9:2) gave rise to 5.0 g of a semi-solid residue, which was re-chromatographed on Sephadex LH-20 using methanol as an eluent. Fractions 15–20 (1.0 g) were subjected to flash chromatography using chloroform containing an increasing proportion of methanol for elution to afford a crude mixture of compounds **2**–**4** (200 mg). The mixture was purified by MPLC with gradient elution (chloroform: methanol 0–50% methanol in 60 min) to give uzarigenin (**2**) (4.0 mg), β-anhydroepidigitoxigenin (3) (20.0 mg) and 2α,19-epoxy-3β,14β-dihydroxy-19-methoxy-5α-card-20(22)-enolide (**4**) (4.2 mg). Fractions 25–27 (0.3 g) were re-chromatographed by MPLC using chloroform-methanol (9:1) as an eluent to afford β-anhydroepidigitoxigenin-3β-*O*-β-glucopyranoside (**5**) (8.0 mg). The purity of compounds **2**–**4** was examined by HPLC (LiChrosphere 100 RP-18, 250 mm × 4 mm; 5 μm; H_2O–MeOH (0.1% trifluoroacetic acid) gradient from 10% to 95% MeOH in 35 min; flow rate 0.8 ml min^{1}).

3.4. Bioassays

3.4.1. Cytotoxicity assays

The sensitivity of cell lines NIH-3T3, HepG2 and HT-29 to the isolated compound was evaluated by monitoring metabolic activity using the CellTiter-Blue®Cell Viability Assay (*Promega*, Mannheim, Germany). The cultivation of the cell lines and the cytotoxicity assays were performed as described by Shaker et al. (in press).

3.4.2. Antimicrobial assays

Antimicrobial assays were performed using *Bacillus subtilis* (DSM 347), *Erwinia amylovora* (DSM 50901), *Escherichia coli* K12 (DSM 498), *Pseudomonas fluorescence* (NCIMB 10586), *Propionibacterium acnes* (DSM 1897), *Pseudomonas aeruginosa* (DSM 50071), *Pseudomonas syringae* pv. aptata (DSM 50252), *Ralstonia solanacearum* (DSM 9544), *Staphylococcus epidermidis* (DSM 20044), *Staphylococcus lentus* (DSM 6672), *Xanthomonas campestris* (DSM 2405), and yeast *Candida glabrata* (DSM 6425). The antibacterial assays were performed as recently described by Shaker et al. (in press).

3.5. Analytical data

3.5.1. 5-Hydroxy-3,7-dimethoxyflavone-4′-O-β-D-glucopyranoside (1)

Yellow amorphous solid. HRESIMS: *m/z* 477.13995 $[M+H]^{+}$, $C_{23}H_{25}O_{11}^{+}$ (calculated for 477.13914), *m/z* 315.08714 $[M+H-162]^{+}$, $C_{17}H_{15}O_6$, aglycone (calculated for 315.08631); UV: λ_{max} (nm) 215, 276, 329; $[\alpha]^{23}_{D}$: −69.6 (0.05 c, MeOH); ^{1}H NMR (CD_3OD, 500 MHz): δ 8.00 (2H, d, *J* = 8.2 Hz, H-2′/H-6′), 7.26 (2H, d, *J* = 8.2 Hz, H-3′/H-5′), 6.85 (1H, s, H-8), 6.73 (1H, s, H-6), 5.05 (1H, d, *J* = 7.7 Hz, H-1″), 4.0 (1H, m, H-6″a), 3.99 (3H, s, 7-OCH_3), 3.84 (3H, s, 3-OCH_3), 3.72 (1H, dd, *J* = 12.1, 5.6 Hz, H-6″b), 3.52 (1H, m, H-5″), 3.51 (1H, m, H-3″), 3.50 (1H, m, H-2″), 3.42 (1H, m, H-4″); ^{13}C NMR (CD_3OD, 125 MHz): δ 184.4 (s, C-4), 165.9 (s, C-5), 162.2 (s, C-4′), 160.8 (s, C-7), 154.8 (s, C-9), 153.6 (s, C-2), 134.4 (s, C-3), 129.4 (d, C-2′/C-6′), 126.0 (s, C-1′), 118.2 (d, C-3′/C-5′), 106.7 (s, C-10), 104.9 (d, C-6), 101.7 (d, C-1″), 92.4 (d, H-8), 78.4 (d, C-5″), 78.0 (d, C-3″), 74.8 (d, C-2″), 71.3 (d, C-4″), 62.5 (t, C-6″), 61.1 (q, 3-OCH_3), 57.3 (q, 7-OCH_3).

3.5.2. 2β,19-Epoxy-3β,14β-dihydroxy-19-methoxy-5α-card-20(22)-enolide (4)

White solid; $[\alpha]^{23}_{D}$: +29.5 (0.08c, MeOH); HRESIMS: *m/z* 419.24455 $[M+1]^{+}$, $C_{24}H_{35}O_6^{+}$ (calculated for 419.24372); UV: λ_{max} (nm) 223, 264; ^{1}H and ^{13}C NMR data: see Table 2.

3.5.3. β-Anhydroepidigitoxigenin-3β-O-β-glucopyranoside (5)

White solid; $[\alpha]^{23}_{D}$: −13.5 (0.62c, MeOH); HREIMS: *m/z* 519.29764 $[M+1]^{+}$, $C_{29}H_{43}O_8^{+}$ (calculated for 519.29524); UV: λ_{max} (nm) 208 sh, 222; ^{1}H and ^{13}C NMR data: see Table 2.

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