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Cytotoxic cycloartanes from the fruits of *Caloncoba glauca*

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ABSTRACT

Two new cycloartane-type triterpenoids, glaucartanoic acids A (**1**) and B (**2**), together with five known compounds were isolated from the fruits of *Caloncoba glauca*. Their structures were elucidated by spectroscopic methods including extensive 1D and 2D NMR techniques, by chemical evidence and by comparison with literature data. The new compounds were evaluated for their *in vitro* cytotoxicity against five human cancer cell lines.

1. Introduction

The genus *Caloncoba* includes 20 species of trees and shrubs, and constitutes one of the smallest genus of the Flacourtiaceae family represented in tropical Africa (Hutchinson and Dalziel, 1954; Ziegler et al., 2002). *Caloncoba glauca* (P. Beauv.) Gilg is a tree common in the rain forests of western Cameroon (Giner-Pons et al., 1992; Hutchinson and Dalziel, 1954). Information collected directly from traditional healers indicated that the leaves are used in traditional medicines as purgative; the fruits, leaves and stem bark are used against inflammations and skin diseases. Previous phytochemical investigations of some *Caloncoba* species have led to the isolation of cyclopentanoid amino acids (Clausen et al., 2002), and friedelane, dammarane and malabaricane triterpenes (Giner et al., 1993; Giner-Pons et al., 1992; Tchuendem et al., 1996; Ziegler et al., 2002). To the best of our knowledge, there is no prior report on the chemical constituents from the fruits of *C. glauca*. This paper deals with the isolation and structural elucidation of two new cycloartanes, along with five known compounds, as well as the cytotoxic evaluation of the new compounds against the HL-60, SMMC-7721, A549, MCF-7 and SW480 human cancer cell lines.

2. Results and discussion

Repeated column chromatography over silica gel and Sephadex LH-20 of the defatted MeOH extract from the fruits of *C. glauca*, led to the isolation of two new cycloartanes (**1** and **2**) (Fig. 1) along with five known compounds.

Compound **1** was obtained as a white powder. The positive HR-ESI-MS showed a quasi-molecular ion peak at *m/z* 511.3394 $[M+Na]^+$, which in accordance with NMR data enabled the molecular formula $C_{30}H_{48}O_5$, accounting for seven degrees of unsaturation. Its IR spectrum suggested the presence of hydroxyl (3403 cm^{-1}) and carbonyl (1696 cm^{-1}) groups in the molecule. The ^1H NMR spectrum (Table 1) revealed five three-proton singlets at δ_H 1.12, 1.07, 1.04, 1.03 and 0.96, one three-proton doublet at δ_H 0.83 (J = 5.9 Hz) and a typical high-field AB doublets of the non-equivalent protons of a cyclopropane ring at δ_H 0.80 and 0.43 (each 1H, J = 4.2 Hz) indicating a cycloartane skeleton (Manoharan et al., 2005). Additionally, a signal attributed to an oxymethine proton was observed as a multiplet at δ_H 3.21. The ^{13}C NMR spectrum (Table 1) displayed signals for 30 carbon atoms which were assigned by DEPT and HSQC spectra to six methyls, eleven methylenes, five methines and eight quaternary carbons. The signals at δ_C 72.9 and 78.6 were assigned to oxygenated sp^3 carbon atoms, and the signals at δ_C 179.2 and 217.8, to a carboxylic and ketone carbonyl respectively. The methyl protons assigned to C-28 (δ_H 0.96) and C-29 (δ_H 1.03) showed long range ^1H, ^{13}C coupling (Fig. 2a) to each other, C-4 (δ_C 49.9), C-5 (δ_C 47.7) and the ketone carbon, thereby establishing a 3-oxo functionality. The carboxy group was attached to the C-14 quaternary carbon on the basis of the observed HMBC correlations of H-8 (δ_H 1.73) and H_2-15 (δ_H 2.09, 1.17) to C-14 (δ_C 62.5) and C-30 (δ_C 179.2). The oxymethine proton signal at δ_H 3.12 and the two deshielded methyl singlets at δ_H 1.12 (H_3-26) and 1.07 (H_3-27), together with the ^{13}C NMR signals at δ_C 78.6 (C-24), 72.9 (C-25), 25.9 (C-26) and 23.0 (C-27)

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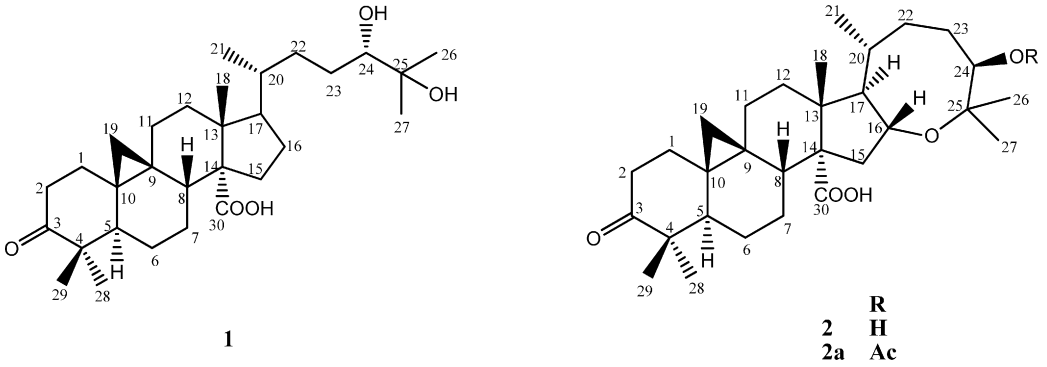

Fig. 1. Chemical structures of compounds **1**, **2** and **2a**.

were in agreement with reported data for cycloartane-24,25-diols (Grougnet et al., 2006). Consequently, compound **1** possessed a 24,25-diol in its side chain moiety. This was further confirmed by the side chain proton spin system $C_{(21)}H_3$–$C_{(20)}$H–$C_{(22)}H_2$–$C_{(23)}H_2$–$C_{(24)}$H(OH)–, established by the analysis of the ^{1}H–^{1}H COSY and HSQC data; in addition to the long-range correlations of H_3-26/H_3-27 to C-24 and C-25 observed in the HMBC spectrum. The determination of the relative stereochemistry of **1** was accomplished by analysis of the ROESY spectrum (Fig. 2b). The cross-peaks observed in this spectrum between H-8 and H-19β; H-8 and

Table 1
^{1}H and ^{13}C NMR data of compounds **1**, **2** and **2a**.

Position	**1**[b]		**2**[c]		**2a**[d]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1β	1.84 m	33.3 CH_2	1.88[a]	33.6 CH_2	2.00[a] m	32.9 CH_2
1α	1.54 m		1.55 m		1.56 m	
2β	2.66 ddd (13.9, 10.1, 5.0)	37.3 CH_2	2.72 td (13.8, 6.1 Hz)	37.7 CH_2	2.70 td (13.9, 6.4)	37.2 CH_2
2α	2.18 m		2.26 m		2.33 m	
3	–	217.8 C	–	219.3 C	–	216.4 C
4	–	49.9 C	–	50.5 C	–	49.8 C
5	1.64 dd (12.3, 3.0)	47.7 CH	1.73 brd (3.9 Hz)	48.3 CH	1.80[a]	47.2 CH
6β	1.45[a]	20.8 CH_2	1.51 m	21.5 CH_2	1.52 m	20.9 CH_2
6α	0.93 m		0.97 m		1.00 m	
7β	1.43[a]	26.7 CH_2	1.45 m	27.3 CH_2	1.42 m	26.8 CH_2
7α	1.09[a]		1.24[a]		1.27 m	
8	1.73 dd (12.5, 5.4)	45.3 CH	1.67 dd (12.3, 5.1 Hz)	47.1 CH	1.67 dd (12.2, 5.4)	46.7 CH
9	–	20.4 C	–	20.8 C	–	19.8 C
10	–	27.2 C	–	27.8 C	–	27.1 C
11β	2.22 m	28.2 CH_2	2.30 m	28.4 CH_2	2.40 m	28.0 CH_2
11α	1.27 m		1.22 m		1.23[a]	
12β	1.78 m	33.5 CH_2	1.83[a]	32.9 CH_2	1.80[a]	33.3 CH_2
12α	1.67 m		1.75[a]		1.76[a]	
13	–	47.4 C	–	48.7 C	–	49.1 C
14	–	62.5 C	–	60.8 C	–	61.4 C
15β	2.09[a]	31.4 CH_2	2.09 brd (14.1 Hz)	44.3 CH_2	1.97 m	43.1 CH_2
15α	1.17 brdd (11.4, 6.3)		1.86[a]		2.02[a]	
16β	2.07[a]	29.3 CH_2	4.36 m	77.2 CH	4.46 brt (7.1)	77.2 CH
16α	1.33 m					
17	1.46[a]	52.2 CH	1.58 brd (7.4 Hz)	60.9 CH	1.62 brd (6.7)	61.2 CH
18	1.04 s	17.8 CH_3	1.11 s	19.8 CH_3	1.13 s	18.6 CH_3
19β	0.80 d (4.2)	29.7 CH_2	0.81 d (4.0 Hz)	30.4 CH_2	0.81 d (4.0)	30.2 CH_2
19α	0.43 d (4.2)		0.56 d (4.3 Hz)		0.53 d (4.3)	
20	1.43[a]	35.3 CH	1.63 m	37.3 CH	1.65[a]	36.6 CH
21	0.83 d (5.9)	18.0 CH_3	0.90 d (6.3 Hz)	22.7 CH_3	0.95 d (5.8)	22.2 CH_3
22a	1.39 m	32.9 CH_2	1.83[a]	33.9 CH_2	1.87 m	35.8 CH_2
22b	1.24 m		1.75[a]		1.58 m	
23a	1.30 m	27.9 CH_2	1.81 m	36.5 CH_2	1.82 m	29.0 CH_2
23b	1.30 m		1.47 m		1.82 m	
24	3.21 m	78.6 CH	3.37 m	80.9 CH	4.64 dd (9.2, 3.0)	81.3 CH
25	–	72.9 C	–	78.6 C	–	77.1 C
26	1.12 s	25.9 CH_3	1.25 s	30.3 CH_3	1.23 s	29.6 CH_3
27	1.07 s	23.0 CH_3	1.19 s	18.0 CH_3	1.30 s	18.8 CH_3
28	0.96 s	21.9 CH_3	1.01 s	22.3 CH_3	1.05 s	22.1 CH_3
29	1.03 s	20.7 CH_3	1.09 s	21.1 CH_3	1.09 s	20.8 CH_3
30	–	179.2 C	–	178.2 C	–	175.8 C
24-O$\underline{C}$O$\underline{C}H_3$					–	169.9 C
24-O$\underline{C}$O$\underline{CH_3}$					2.04 s	21.3 CH_3

[a] Overlapped signals within a column.
[b] 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR, in $CDCl_3$.
[c] 400 MHz for ^{1}H and 125 MHz for ^{13}C NMR, in $CDCl_3/CD_3OD$.
[d] 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR, in $CDCl_3$.

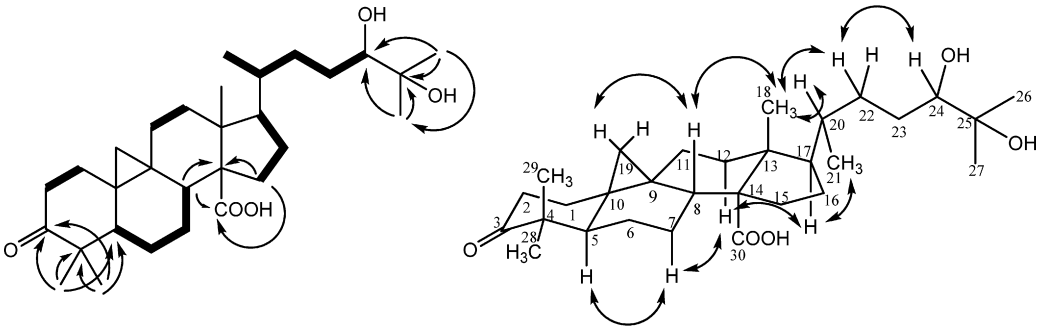

Fig. 2. (a) Selected HMBC (H → C) and ^{1}H–^{1}H COSY (▬) correlations of **1**. (b) Selected ROESY correlations of **1**.

H_3-18; H_3-18 and H-20 suggested the β-orientation of these protons. Further correlations observed between H-5 and H-7ax; H-7ax and H-12ax; H-12ax and H-17; H-17 and H_3-21 indicated their α-orientation. These were in good agreement with the relative configurations at C-5, C-8, C-9, C-10, C-13, C-14, C-17 and C-20, long ago established for the cycloartane core (Grougnet et al., 2006; Nuanyai et al., 2010; Silva et al., 1997). Consistent with this, the configuration at C-24 was determined to be *S* on the basis of the observed ROESY correlations of H-24 with H-22β; and H-22β with H_3-18. Accordingly, the structure of compound **1** was elucidated as 24(*S*),25-dihydroxy-3-oxocycloartan-30-oic acid, to which we gave the trivial name glaucartanoic acid A.

Compound **2** was obtained as a white powder. Its positive HR-ESI-MS showed a quasi-molecular ion peak at *m*/*z* 509.3254 $[M+Na]^+$, which in accordance with NMR data enabled the molecular formula $C_{30}H_{46}O_5$, accounting for eight degrees of unsaturation. The IR spectrum revealed absorption bands at 3396 and 1704 cm^{-1} corresponding to hydroxyl and carbonyl functions respectively. ^{1}H and ^{13}C NMR spectral data of **2** (Table 1) suggested a triterpene skeleton with five methyl singlets and one methyl doublet, similarly to those of **1**. Again a typical high field AB system of two non-equivalent protons of a cyclopropane ring was present, as indicated by the two doublets at δ_H 0.56 (J = 4.3 Hz) and 0.81 (J = 4.0 Hz) in the ^{1}H NMR spectrum of **2**. Although the ^{13}C NMR spectrum (Table 1) resolved only 28 carbon resonances, two other carbon signals were detected from the HMBC spectrum (Fig. 3a) which showed correlations peaks from signals at δ_H 1.01 (H_3-28), 1.09 (H_3-29), and 1.55 and 1.88 (H_2-1) to a missing carbon signal at δ_C 219.3 (C-3), and from signals at δ_H 1.67 (H-8) and 0.81/0.56 (H_2-19) to a second missing carbon signal at δ_C 27.8 (C-10). Thus, the ^{13}C NMR data of **2** and **1** were closely related, indicating that both compounds had a similar cycloartane skeleton with a 3-keto and 30-carboxy groups respectively. These functionalities and the cycloartane nucleus corresponded to seven double-bond equivalents and the remaining degree of unsaturation therefore suggested that compound **2** possessed one more ring than **1**. Detailed comparison of the ^{13}C NMR and DEPT spectra of **2** and **1** revealed that compound **2** had one less methylene than **1**, replaced by an additional oxymethine (δ_C 77.2, δ_H 4.36). The severe downfield-shift *ca.* $\Delta\delta$ 12.9 and 8.7 of the C-15 and C-17 resonances respectively of **2**, as compared to those of **1**, in addition to the HMBC correlations of the oxymethine proton at δ_H 4.36 to C-14 (δ_C 60.8) and C-20 (δ_C 37.3) allowed the C-16 position of this oxymethine. Furthermore, the downfield-shift of C-25 (δ_C 78.6) compared to that of **1** (at δ_C 72.9), together with the HMBC correlation between H-16 and C-25 indicated a cyclization of the side chain of compound **2** with the ring D through a C-16/C-25 epoxy bond. The presence of a single free hydroxyl group in **2** was further verified by its acetylation reaction at 70 °C which yielded the unique monoacetate **2a**. The new derivative **2a** had the molecular formula $C_{32}H_{48}O_6$ as determined by the HRESIMS ion peak at *m*/*z* 551.3337 $[M+Na]^+$ and NMR data, including the ^{13}C NMR spectrum which explicitly exhibited 32 carbon signals, noteworthy those of the acetyl group (δ_C 21.3 and 169.9) carbon atoms and those of the previously missed C-3 (δ_C 216.4) and C-10 (δ_C 27.1) in **2**. The stereochemistry of compound **2** was established by careful analysis of the ROESY spectrum (Fig. 3b) and the ^{13}C NMR data. The ROESY correlations of H_3-18 to H-8 and H-20; H-16 to H-20 and the absence of any cross-peak between H-16 and H-17 indicated the β-orientation of the oxymethine proton H-16. The comparison of the chemical shift of C-24 (δ_C 80.9) of **2** with that of **1** at δ_C 78.6 (24*S*), and those of Pallidiosides B and C, δ_C 78.5 (24*S*) and 80.7 (24*R*) respectively (Tigoufack et al., 2010) led us to deduce the absolute configuration of this asymmetric center as *R*. Consequently, the structure of compound **2** was elucidated as 24(*R*)-hydroxy-16α,25-epoxy-3-oxocycloartan-30-oic acid, trivially named glaucartanoic acid B.

The known compounds were identified as taraktophyllin pentaacetate (Jaroszewski et al., 1987), β-sitosterol (Tian et al., 2009), 3-*O*-acetyl-β-sitosterol (Akihisa et al., 1991), 3-*O*-β-D-glucopyranosyl-β-sitosterol (Zhang et al., 2007) and methyl β-D-glucopyranoside (Agrawal et al., 1985), by comparison of their 1D spectroscopic data and melting points with those reported in the literature.

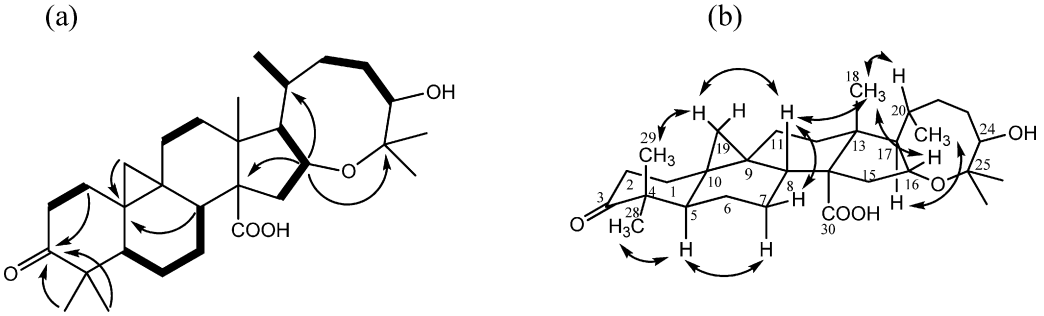

Fig. 3. (a) Selected HMBC (H → C) and ^{1}H–^{1}H COSY (▬) correlations of **2**. Selected ROESY correlations of **2**.

Table 2
Cytotoxic activities of compounds **1**, **2** and **2a** (IC_{50}, μM).

Compounds	Cell lines				
	HL-60	SMMC-7721	A549	MCF-7	SW480
1	19.00 ± 1.25^{ef}	26.50 ± 1.71^{h}	23.03 ± 0.93^{fg}	23.89 ± 1.93^{ghi}	36.42 ± 0.7^{j}
2	>40	>40	36.18 ± 2.39^{ij}	>40	>40
2a	15.67 ± 1.13^{d}	25.61 ± 4.47^{fgi}	21.41 ± 1.78^{fg}	30.38 ± 2.86^{i}	33.76 ± 3.20^{ij}
DDP	5.26 ± 0.55^{b}	18.82 ± 1.57^{ef}	16.55 ± 1.46^{de}	12.97 ± 0.96^{c}	12.61 ± 0.69^{c}
Taxol	<0.008	0.01 ± 0.00^{a}	<0.008	<0.008	<0.008

Data with similar superscript letters are not significantly different ($P < 0.05$).
All data are mean ± standard deviation of three independent experiments.

Compounds **1**, **2** and **2a** were evaluated for their cytotoxicity against human promyelocytic leukemia (HL-60), human hepatocellular carcinoma (SMMC-7721), human alveolar adenocarcinoma (A549), human breast adenocarcinoma (MCF-7) and human colon adenocarcinoma (SW480) cell lines, by the MTT method (Mosmann, 1983) using Ciplastin (DDP) and Paclitaxel (Taxol) as reference drugs (Table 2). Compounds **1** and **2a** showed various cytotoxicities with IC_{50} (μM) values ranging respectively between 18.47 and 36.42, and between 15.73 and 33.75 toward the five cancer cell lines. Compound **2** exerted very weak cytotoxic activity against the A549 cell lines with an IC_{50} value of 36.32 and showed no cytotoxic effects on the other tested cells at concentration up to 40 μM. These results suggested that the replacement of the 24-OH group by an acetoxy group in compound **2** may moderately improve its cytotoxic effect over the five cancer cell lines.

3. Experimental

3.1. General experimental procedures

Melting points were measured on a X-4 micro melting point apparatus and are uncorrected. Optical rotations were obtained with a JASCO P-1020 digital polarimeter. IR spectra were obtained with a Bruker Tensor 27 spectrophotometer with KBr pellets. The ^{1}H, ^{13}C NMR and DEPT spectra were recorded on Bruker AV-400 or DRX-500 spectrometers, while 2D NMR spectra (HSQC, HMBC, ^{1}H–^{1}H COSY and ROESY) were recorded on Bruker DRX-500 or AV-600 instruments. Chemical shifts (δ) were expressed in ppm with reference to TMS and coupling constants (J) were given in Hz. ESIMS and HRESIMS were carried out on an API Qstar time-of-flight spectrometer in positive ionization mode. Column chromatography was performed on Merck silica gel 60 (70–230 mesh) and Sephadex LH-20 (40–70 μm). TLC was carried out on precoated silica gel 60 F_{254} aluminum plates and detection accomplished by dipping into a 10% H_2SO_4 solution followed by heating. Solvents were distilled prior to use.

3.2. Plant material

The fresh fruits of *C. glauca* (P. Beauv.) Gild were collected in Bangang-Wabane village, South-west Region of Cameroon, in May 2009. The plant was authenticated by Mr. François Nana, a botanist of the National Herbarium of Cameroon (Yaoundé), where a voucher specimen (55064/HNC) was deposited.

3.3. Extraction and isolation

The air-dried and fine powdered fruits of *C. glauca* (2.3 kg) were first all defatted by maceration in *n*-hexane. The residual powder was then extracted with MeOH (3× 3 L) at room temperature for 72 h. After filtration, the solvent was removed under vacuum to give a methanolic extract (130 g). Part of it (120 g) was fractionated over a silica gel column, eluted with gradients of hexane–EtOAc (90:10, 80:20, 50:50, 30:70) and EtOAc–MeOH (100:0, 90:10, 75:25, 50:50, 0:100) to give five main fractions, A–E. Fraction A (5 g) was separated on a silica gel column eluted with hexane–EtOAc (85:15) to yield β-sitosterol (20 mg) and 3-*O*-acetyl-β-sitosterol (12 mg). Fraction B (15 g) was repeatedly subjected to column chromatography over silica gel and Sephadex LH-20 eluted with hexane–EtOAc (95:5, 90:10, 80:20: 70:30, 50:50, 0:100) and CH_2Cl_2–MeOH (1:1) respectively to afford **1** (10 mg) and **2** (16 mg). Fraction D (13 g) was chromatographed on a silica gel column eluted with hexane–EtOAc (70:30, 50:50, 30:70, 0:100) to give three sub fractions, D1–D3. D1 gave 3-*O*-β-D-glucopyranosyl-β-sitosterol (12 mg) as a precipitate washed with hexane–EtOAc (90:10). D2 (4 g) was separated on a silica gel column eluted with $CHCl_3$–MeOH (90:10, 85:15, 80:20, 75:25, 70:30, 50:50). Evaporation of $CHCl_3$ from the sub fraction obtained with $CHCl_3$–MeOH (70:30) gave a precipitate which after filtration yielded methyl β-D-glucopyranoside (20 mg). The filtrate was acetylated and on usual work-up, a solid residue was obtained. Repeated purification of this residue on silica gel and Sephadex LH-20 column chromatography afforded taraktophyllin pentaacetate (72 mg).

3.4. Glaucartanoic acid A (1)

White powder; mp 174–176 °C; $[\alpha]_D^{26.0}$ −36.5 (*c* 0.088, $CHCl_3$–MeOH, 1:1); IR (KBr) ν_{max} 3404, 2967, 2873, 1696, 1381 cm^{-1}; ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data, see Table 1; positive ESIMS *m/z* (rel. int.) 511 $[M+Na]^+$ (100), 999 $[2M+Na]^+$ (6); HRESIMS *m/z* 511.3394 $[M+Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

3.5. Glaucartanoic acid B (2)

White powder; mp 256–258 °C; $[\alpha]_D^{26.4}$ −20.3 (*c* 0.180, $CHCl_3$–MeOH, 1:1); IR (KBr) ν_{max} 3396, 2970, 2930, 1704, 1150 cm^{-1}; ^{1}H (400 MHz, $CDCl_3$–CD_3OD) and ^{13}C (125 MHz, $CDCl_3$–CD_3OD) NMR data, see Table 1; positive ESIMS *m/z* (rel. int.) 469 $[M-H_2O+H]^+$ (100), 487 $[M+H]^+$ (22), 974 $[2M+2H]^+$ (3); HRESIMS *m/z* 509.3254 $[M+Na]^+$ (calcd for $C_{30}H_{46}O_5Na$, 509.3242).

3.6. Acetylation of 2

A sample of **2** (10 mg) was treated for 4 h at 70 °C on a water bath, with Ac_2O (2 ml) in dry pyridine (1 ml), in the presence of a catalytic amount of DMAP. The reaction mixture was poured in ice–H_2O and extracted with EtOAc. The organic layer was washed respectively with 2 M HCl and 1 M $NaHCO_3$, and dried. Gel permeation over Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) gave the new derivative 24(*R*)-acetoxy-16α,25-epoxy-3-oxocycloartan-30-oic acid (**2a**). White powder; mp 276–278 °C; $[\alpha]_D^{15.2}$ −60.9 (*c* 0.097, $CHCl_3$); IR (KBr) ν_{max} 3425, 2972, 2951, 1738, 1706, 1244 cm^{-1}; ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) NMR data, see Table 1; positive ESIMS *m/z* (rel. int.) 469 $[M-AcO]^+$ (100), 529 $[M+H]^+$ (65), 551 $[M+Na]^+$ (13); HRESIMS *m/z* 551.3337 $[M+Na]^+$ (calcd for $C_{32}H_{48}O_6Na$, 551.3348).

3.7. Cytotoxic assay

Cytotoxic activity of the new compounds was investigated using five human cancer cell lines including HL-60, SMMC-7721, A549, MCF-7 and SW480. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and dispersed in replicate 96-well plates. Compounds were then added. After 48 h exposure to the compounds, cells viability were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay by measuring the absorbance at 570 nm with a microplate reader (Bio-Rad 680) (Mosmann, 1983). Each test was run in triplicate. The cytotoxic activity was shown as IC_{50}, which is the concentration of test compound (μM) to give 50% inhibition of the cell growth.

3.8. Statistical analysis

The one-way ANOVA at 95% confidence level was used for statistical analysis.

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