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Cycloartane triterpenes from the leaves of *Neoboutonia macrocalyx* L.

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ABSTRACT

Neoboutonia macrocalyx is used by people in south western Uganda around Kibale National Park in the treatment of malaria. Phytochemical investigation on the leaves of this plant led to the isolation of nine cycloartane triterpenes (**1**–**9**) and one phenanthrene; 7-methoxy-2,8 dimethyl-9, 10-dihydrophenantherene-3,6 diol (**10**) along with three known compounds which included 22-de-*O*-acetyl-26-deoxyneoboutomellerone (**11**), mellerin B (**12**) and 6-hydroxystigmast-4-en-3-one (**13**). The chemical structures of the compounds were established mainly through a combination of spectroscopic techniques. The isolated compounds were evaluated for antiplasmodial activity against the chloroquine-resistant FcB1/Colombia strain of *Plasmodium falciparum* and for cytotoxicity against the KB (nasopharyngeal epidermoid carcinoma) and MRC-5 (human diploid embryonic lung) cells. Seven out of 13 compounds exhibited good antiplasmodial activity with IC_{50} of $\leqslant$5 μg/ml with two compounds exhibiting low cytotoxicity and five compounds having significant cytotoxicity.

Introduction

The Family Euphorbaceae is widely known for its biologically active compounds (Cateni et al., 2003). The chemistry of genus Neoboutonia which belongs to this family has not been extensively studied. However sterols, diterpenoids and triterpene derivatives have been isolated from some Neoboutonia species (Long et al., 2011; Tchinda et al., 2003; Zhao et al., 1998). During our survey around Kibale National Park in the South western Uganda, *Neoboutonia macrocalyx* was found to be used by the people in the treatment of malaria (Namukobe et al., 2011). The same plant is also used in Meru and Kilifi Districts of Kenya to treat headaches and fevers and the antiplasmodial activity of the stem extract has been reported (Kirira et al., 2006). Kirira et al. (2007) has also reported tigliane diterpenoids from the stem bark of the same plant.

As part of our search for biologically active compounds from Ugandan flora, we report the isolation of 9 new cycloartane triterpenes, in addition to the known neoboutomellerone triterpene, a daphnane diterpenoid and a 3-keto steroid from the ethylacetate extract of the leaves of *N. macrocalyx*.

Results and discussion

Characterization of compounds

Purification of the ethylacetate extract led to the isolation of 9 new cycloartane triterpenes (**1–9**) in addition to the known cycloartane triterpenoid; neoboutomellerone (Long et al., 2011), daphnane type diterpenoid; mellerin B (Zhao et al., 1998) and a "3-keto steroid; 6-hydroxystigmast-4-en-3-one (Esperanza et al., 2006; Della Greca et al., 1990). One dimensional ^{1}H and ^{13}C spectra along with two dimensional COSY, HMQC, HMBC, and NOESY experiments were used to elucidate the structures of the new triterpenes. Their structures were further confirmed by MS. In addition to the above experiments, the known compounds were identified by comparison of their spectroscopic data with that published in literature.

Compound **1** was obtained as yellow crystals. Its structure was characterized using spectroscopic methods. Its molecular formula was established as $C_{32}H_{46}O_6$ on the basis of its high resolution-electron

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spray ionization-mass spectrum (HR-ESI-MS) ion at m/z = 527.3350 $[M+H]^+$ in the positive mode. This formula suggested that compound **1** has 10 degrees of unsaturation. The NMR data (Table 1) showed that five of the ten sites of unsaturation came from two C—C double bonds and three carbonyl groups, suggesting that the remaining five sites of unsaturation could be a five ring skeleton. The presence of seven-membered α, β unsaturated lactone was shown by the IR band at 1693 cm^{-1} (Shen et al., 2005). The two coupled doublets at δ_H 0.98 and 0.89 (J = 4.5) indicated the presence of a cyclopropane ring (Shen et al., 2005). The IR absorption bands at 3456 and 1735 cm^{-1} implied the presence of hydroxyl (OH) and carbonyl (CO) functionalities. The IR band at 1693 cm^{-1} indicated the presence of a conjugated carbonyl (CO) group. The UV spectrum of **1** exhibited maximum absorption peaks at λ 207 and 245 nm indicating conjugated double bonds (Pretsch et al., 2000). Analysis of the ^{1}H NMR, ^{13}C-NMR, DEPT and HSQC data revealed the presence 32 non equivalent carbon atoms including of

Table 1
^{1}H NMR data of compounds **1**–**9** isolated from the leaves of *N. macrocalyx*.

	1[a] δ_H (J in Hz)	**2**[b] δ_H (J in Hz)	**3**[b] δ_H (J in Hz)	**4**[b] δ_H (J in Hz)	**5**[b] δ_H (J in Hz)	**6**[b] δ_H (J in Hz)	**7**[a] δ_H (J in Hz)	**8**[a] δ_H (J in Hz)	**9b** δ_H (J in Hz)
1	6.06, d (2.9)	6.08, d (12.6)	5.96, d (12.7)	5.96, d (12.7)	6.01, d (12.7)	6.03, d (12.6)	6.15, d (12.3)	1.34, ddd (14.4,11.4,4.2) 2.08, m	3.49, s
2	5.90, d (12.9)	5.90, d (12.5)	5.83, d (12.7)	5.81, d (12.7)	5.95, d (12.7)	5.95, d (12.6)	5.92, d (12.3)	2.46, qd (11.4, 5.3) 2.26, td (11.4, 4.7)	3.61,d (8.8)
3									3.44, d (9.7)
4	4.43, m	4.44, qd (6.8, 4.3)	4.58, dt (12.1, 6.5)	4.56, dt (12.0, 6.3)	4.64, qd (6.7, 4.6)	4.64, qd (6.7, 4.6)		5.62, ddd (17.2,10.2,8.6)	
5	2.23, m	2.22, m	2.18, m	2.15, t (4.2)	2.61, dd (4.0, 2.3)	2.6, dd (3.6, 2.3)	2.71, dd (12.1, 3.9)	2.28, m	1.88, m
6	1.83, m	1.81, m	4.45, t (4.3)	4.39, t (4.3)	3.31, dd (4.2, 2.6)	3.33, dd (3.8, 2.4)	1.86, br d (12. 0)	1.58, m	1.59, m
	0.79, m	0.79, qd (13.0, 2.4)					1.15, m	0.79, br q (12. 0)	0.79, m
7	1.41, m	1.40, m	1.49, m	1.47, m	3.03, dd (4.2, 1.7)	3.01, dd (4.2, 1.9)	1.42, m	1.21,d (12.4)	1.28, m
	1.15, m	1.11, m	1.44, m	1.39, m			1.19, m	1.02,m	1.11, m
8	1.78, dd (10.6, 6.7)	1.79, m	2.29, dd (13.0, 4.9)	2.29, dd (13.0, 4.9)	2.80, br s	2.81, br s	1.80,dd (10.9, 4.5)	1.47,dd (12.4, 3.7)	1.47, dd (12.2, 3.6)
11	2.10, m	2.06, m	2.22, dd (11.0,6.7)	2.18, t (7.5)	2.05, m	2.08, m	2.13, m	2.13, m	2.29, m
	1.49, m	1.52, m	1.38, m	1.39, m	1.40,d (16.1)	1.41, d (15.4)	1.43, m	1.15, m	1.19, m
12	1.70, m	1.68, m	1.71, m	1.72, m	1.61, m	1.61, td (13.9, 4.4)	1.69, m	1.67, br d (10. 7)	1.64, m
	1.63, m	1.68, m	1.71, m	1.72, m	1.50, dd (13.4, 4.3)	1.51, dd (13.9, 3.9)	1.65, m	1.66, br d (12. 0)	1.64, m
15	2.33, dd (7.3, 15.1)	2.30, dd (13.6, 8.0)	2.33, dd (14.1,7.9)	2.27, dd (14.1,7.9)	2.46, dd (13.2, 7.4)	2.43, dd (13.4, 7.8)	2.37,br dd (14.0, 8.0)	2.34, dd (14.1,7.9)	1.27, m
	1.29, m	1.24, ddd (14.0,12.6,3.7)	1.30, dd (14.1, 4.3)	1.28, dd (14.1, 4.2)	1.50, dd (13.6, 4.8)	1.48, dd (13.9, 4.5)	1.32, br dd (10.0, 2.8)	1.28, dd (14.1, 4.4)	1.27, m
16	5.32, m	5.02, ddd (7.8,7.3,4.3)	5.32, td (7.4, 4.3)	5.03, td (7.4, 4.3)	5.37, ddd (7.7,7.5,5.1)	5.11, ddd (7.7,7.5,5.1)	5.33, ddd (7.6,6.9,3.9)	5.32, ddd (7.9,6.9,4.4)	1.90, m
									1.20, m
16b	2.06, s	2.05, s	2.06, s	2.03, s	2.10, s	2.09, s	2.07, s	2.05, s	2.10, s
17	2.48, m	2.24, dd (10.7,7.3)	2.45, m	2.23, m	2.49, dd (10.7, 7.7)	2.23, dd (10.5, 8.3)	2.48, dd (10.7,6.9)	2.45, dd (14.1, 4.4)	1.60, m
18	1.12, s	1.10, s	1.17, s	1.16, s	1.15, s	1.15, s	1.14, s	1.14, s	0.93, s
19	0.98, m	0.98, m	1.84, d (4.0)	1.86, d (4.0)	1.99, d (4.2)	1.96, d (4.3)	1.00, m	0.47, d (4.3)	0.66, d (3.3)
	0.89, d (4.5)	0.90, m	0.98, d (4.0)	0.94, d (4.0)	0.33, d (4.2)	0.35, d (4.3)	0.98,m	0.30, d (4.3)	0.46, d (3.3)
20	2.39, m	2.52, m	2.41, m	2.53, m	2.43, m	2.56, m	2.40, m	2.40, m	1.37, m
21	0.62, d (6.7)	0.83, d (6.8)	0.63, d (6.7)	0.84, d (6.9)	0.63, d (6.6)	0.85, d (7.3)	0.63, d (6.4)	0.62, d (6.4)	0.87, d (6.3)
22	4.66, s	5.53,br s	4.66, s	5.53, s	4.66, d (5.8)	5.54, d (1.5)	4.67, br d(2.9)	4.67, br s	1.55, m
									1.12, m
22b		2.14, s		2.12, s		2.13, s			
23									2.10, m
									1.86, m
24a	6.00, s	5.99, s	6.00, s	5.95, s	6.01, s	5.99, s	6.01, s	6.01, s	4.69, s
	5.85, s	5.76, s	5.86, s	5.72, s	5.86, br s	5.75, s	5.86, s	5.85, s	4.64, s
25	2.92, m	2.87, sp (6.8)	2.91, m	2.85, m	2.91, m	2.85, m	2.91, sp (6.7)	2.91, sp (6.8)	2.21, sp (6.7)
26	1.03, d (6.7)	0.98, d (6.8)	1.03, d (6.9)	0.98, d (6.9)	1.02, d (6.8)	0.98, d (6.8)	1.03, d (6.7)	1.03, d (6.8)	1.00, d (6.7)
27	1.10, d (6.7)	1.04, d (6.8)	1.09, d (6.9)	1.04, d (6.8)	1.10, d (6.8)	1.04, d (6.7)	1.10, d (6.7)	1.09, d (6.8)	1.00, d (6.7)
28	1.30, d (6.7)	1.29, d (6.8)	1.62, d (6.6)	1.60, d (6.6)	1.60, d (6.6)	1.58, d (6.8)	4.80, s	5.02, dd (17.2, 1.4)	0.96, s
							4.33, s	4.96, dd (10.2, 1.4)	
29									0.78, s
30	0.96, s	0.90, s	1.00, s	0.94, s	0.91, s	0.86, s	0.98, s	0.97, s	0.93, s

[a] Recorded on Brucker 500 MHz.
[b] Recorded on Brucker 600 MHz.

seven methyls, seven methylenes, ten methines and eight quaternary carbons (Table 2). Among the deshielded carbons, was one ketone (δ_C 203.7), two ester carbonyls (δ_C 168.2 and 170.3), three oxygenated carbon atoms (δ_C 74.8, 77.0, 79.3) and four olefinic (δ_C 123.2, 152.4, 119.7 and 150.2) carbons. The ester functionality at C-16 (Fig. 1) was identified as an acetate from the HMBC correlation between CO (δ_C 170.3) and the methyl group at δ_H 2.06 and its location was determined by HMBC correlations between H-16 (δ_H 5.32) and CO (δ_C 170.3). The position of OH was identified at C-22 due to the up field proton signal at δ_H 4.66 and HMBC correlations between H-22 (δ_H 4.66), and the methyl at C-21 (δ_C 11.6). The relative stereo chemistry of the methyl group at H-28 was established through NOESY correlations between H-28 (δ_H 1.30), H-6 (δ_H 1.83) and H-5 (δ_H 2.23). The structure of compound **1** was established as 16-acetoxy-22α-hydroxy-29-nor-24-methylcycloart-1,24(24a)-dien-3,23-dion-3,4-lactone (Fig. 1) and was assigned a trivial name Neomacrolactone.

Most of the cycloartane triterpenes that have been described have two methyl groups on C-4 (Gao et al., 2008; Nuanyai et al., 2011; Shen et al., 2005). Although cycloartane triterpenoids with one methyl group on C-4 have been recently described (Long et al., 2011), to the best of our knowledge, this is the first time a cycloartane triterpenoid with one methyl group on a seven-membered α, β unsaturated lactone is described.

Compound **2** was isolated as a light yellow solid. The ^{1}H and ^{13}C NMR data of compound **2** were similar to those of compound **1** suggesting structural similarity of the two compounds (Tables 1 and 2). However, compound **2** had additional ^{1}H and ^{13}C NMR signals at δ_H 2.14 and δ_C 21.0 respectively for a Me-22b. This data confirmed that compound **2** is similar to compound **1** but with one more acetate group at C-22 (δ_C 77.5, δ_H 5.53). Using other methods like MS, the structure of compound **2** was confirmed to be 16,22α-diacetoxy-29-nor-24-methylcycloart-1,24(24a)-dien-3,23-dion-3,4-lactone (Fig. 1) and was assigned a trivial name 22α-acetoxyneomacrolactone.

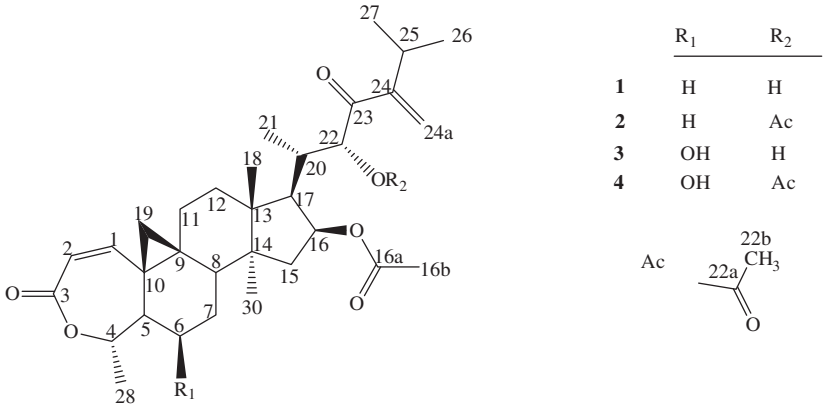
Compound **3** was isolated as a light yellow solid and its molecular formula was established as $C_{32}H_{46}O_7$ on the basis of HR-ESI-MS ion at m/z = 543.3315 $[M+H]^+$, in the positive mode. This formula suggested that compound **3** has 10 degrees of unsaturation and differed from that of compound **1** with only one more oxygen atom. Compound **3** also exhibited an oxygenated deshielded proton and carbon at δ_H 4.45 and δ_C 66.1 respectively in addition to the deshielded carbons and protons exhibited by compound **1**. The position of the OH group at C-6 was established from HMBC correlations between H-6 (δ_H 4.45), C- 8 (δ_C 40.2) and C-10 (δ_C 35.0) and COSY correlation between H-6 and H-5 (δ_H 2.18). These data established that compound **3** is similar to compound **1** but with an OH at C-6 (Fig. 1). The structure of compound **3** was elucidated as 16-acetoxy-6,22α-dihydroxy-29-nor-24-methylcycloart-1, 24(24a)-dien-3,23-dion-3,4-lactone (Fig. 1) and was assigned a trivial name 6-hydroxy neomacrolactone.

Compound **4**, isolated as a light yellow solid, had similar ^{1}H NMR with that of compound **3** suggesting that the two compounds had the same skeleton. HR-ESI-MS analysis of **4** gave an ion at m/z = 585.3427 $[M+H]^+$ in the positive mode, implying a molecular formula of C_{34} H_{48} O_8 which suggested that the structure had 11 sites of unsaturation. In the ^{1}H NMR of **4**, eight methyl signals were observed, two of which were acetates. The acetates were determined using the HMBC correlations between the ester carbonyl Carbons δ_c 170.0 and 171.0 with methyls at δ_H 2.03 and δ_H 2.12,

Table 2
^{13}C NMR data of compounds **1**–**9** isolated from the leaves of *N. macrocalyx*.

Carbon	**1**[a]	**2**[b]	**3**[b]	**4**[b]	**5**[b]	**6**[b]	**7**[a]	**8**[a]	**9**[b]
1	150.2, CH	150.5, CH	151.3, CH	152.0, CH	148.3, CH	148.6, CH	151.1, CH	28.7, CH_2	75.7, CH
2	119.7, CH	119.4, CH	118.8, CH	119.1,CH	120.3, CH	120.0, CH	119.6, CH	31.9, CH_2	72.7, CH
3	168.2, C	168.5, C	168.3, C	168.0, C	167.6, C	167.7, C	164.8, C	179.5, C	77.8, CH
4	79.3, CH	79.3, CH	79.0, CH	79.1, CH	78.5, CH	78.5, CH	159.4, C	142.7, CH	40.4, C
5	44.8, CH	44.6, CH	48.0, CH	47.8, CH	43.3, CH	43.1, CH	42.7, CH	42.3, CH	39.5, CH
6	23.4, CH_2	23.1, CH_2	66.1, CH	65.8, CH	51.9, CH	51.7, CH	26.4, CH_2	29.0, CH_2	21.0, CH_2
7	24.5, CH_2	24.3, CH_2	33.1, CH_2	32.9, CH_2	55.0, CH	54.8, CH	24.7, CH_2	24.8, CH_2	25.8, CH_2
8	46.3, CH	45.6, CH	40.2, CH	40.0, CH	38.0, CH	37.7, CH	46.9, CH	48.5, C	48.3, CH
9	30.4, C	30.4, C	29.6, C	29.5, C	30.2, C	30.2, C	31.5, C	23.6, C	20.4, C
10	37.0, C	37.0, C	35.0, C	35.0, C	35.9, C	35.5, C	35.8, C	28.0, C	29.1, C
11	28.6, CH_2	28.4, CH_2	28.5, CH_2	28.4, CH_2	28.1, CH_2	27.9, CH_2	28.5, CH_2	27.0, CH_2	26.2, CH_2
12	32.5, CH_2	32.3, CH_2	32.7, CH_2	32.6, CH_2	31.6, CH_2	31.4, CH_2	32.6, CH_2	32.9, CH_2	33.0, CH_2
13	45.7, C	45.7, C	46.0, C	46.1, C	46.2, C	46.2, C	45.7, C	45.7, C	45.3, C
14	47.2, C	47.2, C	47.2, C	46.6, C	46.2, C	46.2, C	47.3, C	47.3, C	49.0, C
15	46.8, CH_2	46.6, CH_2	47.4, CH_2	47.2, CH_2	44.4, CH_2	44.2, CH_2	47.1, CH_2	47.5, CH_2	35.9, CH_2
16	77.0, CH	76.2, CH	77.2, CH	76.3, CH	75.7, CH	75.7, CH	77.1, CH	77.4, CH	28.3, CH_2
16a	170.3, C	170.4, C	170.4, C	170.0, C	170.3,C	170.8, C	170.3, C	170.4, C	
16b	21.9, CH_3	22.8, CH_3	21.9, CH_3	21.9, CH_3	21.9, CH_3	22.0, CH_3	22.0, CH_3	21.7, CH_3	
17	50.5, CH	50.4, CH	50.5, CH	50.7, CH	48.9, CH	48.7, CH	50.6, CH	50.6, CH	52.5, CH
18	18.7, CH_3	18.4, CH_3	19.2, CH_3	19.2, CH_3	15.4, CH_3	15.1, CH_3	19.0, CH_3	19.5, CH_3	18.4, CH_3
19	32.1, CH_2	31.8, CH_2	36.3, CH_2	32.9, CH_2	24.5, CH_2	24.4, CH_2	33.0, CH_2	28.7, CH_2	29.7, CH_2
20	35.7, CH	32.3, CH	35.8, CH	32.3, CH	35.9, CH	32.3, CH	35.7, CH	35.8, CH	36.3, CH
21	11.6, CH_3	12.8, CH_3	11.6, CH_3	12.6, CH_3	12.2, CH_3	13.2, CH_3	11.6, CH_3	11.6, CH_3	18.5, CH_3
22	74.7, CH	77.5, CH	74.7, CH	77.5, CH	74.9, CH	77.5, CH	74.7, CH	74.8, CH	35.1, CH_2
22a		170.9, C		171.0, C		170.9, C			
22b		21.0, CH_3		20.9, CH_3		21.0, CH_3			
23	203.7, C	197.8, C	203.7, C	198.0, C	203.8, C	197.9, C	203.7, C	203.9, C	31.5, CH_2
24	152.4, C	153.2, C	152.5, C	154.0, C	152.6, C	153.3, C	152.5,C	152.4, C	157.0, C
24a	123.2, CH_2	121.7, CH_2	123.3, CH_2	121.0, CH_2	123.3, CH_2	121.6, CH_2	123.2, CH_2	123.2, CH_2	106.2, CH_2
25	28.7, CH	28.6, CH	28.7, CH	28.7, CH	28.8, CH	28.6, CH	28.8, CH	28.7, CH	34.0, CH
26	22.3, CH_3	22.0, CH_3	22.3, CH_3	22.2, CH_3	22.3, CH_3	22.1, CH_3	22.4, CH_3	22.3, CH_3	22.1, CH_3
27	21.7, CH_3	21.3, CH_3	21.7, CH_3	21.4, CH_3	21.7, CH_3	21.3, CH_3	21.7, CH_3	21.9, CH_3	22.2, CH_3
28	18.1, CH_3	18.0, CH_3	18.3, CH_3	18.1, CH_3	18.6, CH_3	18.5, CH_3	96.1, CH_2	114.5, CH_2	25.9, CH_3
29									14.6, CH_3
30	20.1, CH_3	20.1, CH_3	20.8, CH_3	20.7, CH_3	19.3, CH_3	19.3, CH_3	20.3, CH_3	20.6, CH_3	19.7, CH_3

[a] Recorded on Brucker 500 MHz.
[b] Recorded on Brucker 600 MHz.



	R_1	R_2
1	H	H
2	H	Ac
3	OH	H
4	OH	Ac

Fig. 1. Chemical structures of compounds **1–4** isolated from the leaves of *N.macrocalyx*.

respectively. The chemical shifts of these carbons and protons were similar to those of compound **3**. The UV spectrum of compound **4** exhibited λ_{max} at 230 nm and 251 nm. All these data confirmed that compound **4** is a diacetate triterpene which is similar to compound **3** (Fig. 1).Thus The structure of compound **4** was established as 16,22α-diacetoxy-6-hydroxy-29-nor-24-methylcycloart-1, 24(24a)-dien-3,23-dion-3,4-lactone and was assigned a trivial name 22α-acetoxy-6-hydroxyneomacrolactone.

Compounds **1–4** have the same carbon skeleton as that of a cycloartane type triterpenoid (Gao et al., 2008; Shen et al., 2005). The novelty of these compounds stems from the presence of only one methyl group on C-4 of the lactone ring of the cycloartane triterpenoid in comparison to those that have been identified before. Recently, Long et al. (2011) isolated cycloartane triterpenoids with one methyl group on the C-4 of the cyclohexenone ring. Demethylation at C-4 is one of the well known biosynthetic sequences leading to steroids in higher plants (Dewick, 2002). The single methyl group at C-4 of the lactone ring of these compounds is a result of oxidation and decarboxylation of one of the methyl groups on C-4 of most cycloartane compounds. Compounds **1–4** only differ in their side chains in terms of hydroxylation and acetylation (Fig. 1).

Compounds **5** and **6** were isolated as light yellow amorphous solids. Their ^{1}H NMR and HR-ESI-MS data demonstrated that these two compounds **5** and **6** had two protons less compared to compounds **3** and **4**, respectively. The HR-ESI-MS revealed molecular structures of $C_{32}H_{44}O_7$ and $C_{34}H_{46}O_8$ for compound **5** and **6**, respectively. In its ^{1}H NMR, compound **5** displayed one acetyl group (CH_3 at δ_H 2.09 and CO at 170.3) while compound **6** displayed two acetyl groups (CH_3CO) (Table 1). The signals at δ_H 3.31, δ_C 51.9 and δ_H 3.03, δ_C 55.0 for compound **5** and δ_H 3.33, δ_C 51.7 and δ_H 3.01, δ_C 54.8 for compound **6** indicated the presence of the epoxide ring (Long et al., 2011; Pretsch et al., 2000). The position of the epoxide ring between C-6 and C-7 was determined from COSY correlation between H-6—H-5 and H-7—H-8. The ^{1}H NMR of these compounds also displayed isolated signals for H-5 at δ_H 2.61 and H-8 at δ_H 2.80 for compound **5** , H-5 at δ_H 2.59 and H-8 at δ_H 2.81 for compound **6.** The rest of the carbons and protons were assigned from detailed analysis of ^{1}H NMR, HMBC, COSY, HSQC data and comparison with NMR data for compounds **3** and **4** (Tables 1 and 2). The UV spectrum of **5** and **6** exhibited λ_{max} at 211 nm and 233 nm respectively. The structure of compound **5** was established as 16-acetoxy-22α-hydroxy-29-nor-24-methyl-6,7-epoxycycloart-1, 24(24a)-dien-3,23-dion-3,4-lactone while that of **6** was found to be 16,22α-diacetoxy-29-nor-24-methyl-6,7-epoxy-cycloart-1,24(24a)-dien-3,23-dion-3,4-lactone (Fig. 2) and the trivial names; 6,7-epoxyneomacrolactone and 22α-acetoxy-6,7-epoxyneomacrolactone, respectively.

Compound **7**, an orange solid, exhibited the molecular formula $C_{32}H_{44}O_6$ as determined by HR-ESI-MS data. This formula suggested that compound **7** had 11 sites of unsaturation. ^{1}H NMR and ^{13}C NMR of compound **7** displayed several peaks which were similar to those of compound **1** except for the absence of a CH_3 doublet at C-28 and the presence of unsaturated methylene signals at δ_H 4.80 and 4.33 which were associated with δ_C 96.1. The position of this double bond was located through HMBC correlations of its proton signals with C-4 (δ_C 159.4) and C-5 (δ_C 42.7). Detailed ^{1}H NMR, ^{13}C-NMR, DEPT and HSQC analysis revealed that compound **7** contained six methyls, eight methylenes, nine methines and nine quartenary carbons. The acetate at C-16 (δ_C 77.1) and δ_H 5.33 was established due to the HMBC correlations between the methyl at δ_H 2.07 and δ_C 170.3. The UV spectrum of compound **7** exhibited maximum wave length at λ_{max} 226 nm. The structure of compound **7** was established as 16-acetoxy-22α-hydroxy-29-nor-24-methyl-cycloart-1,4, 24(24a)-trien-3,23-dion-3,4-lactone (Fig. 2) and was assigned a trivial name 4-methylen-neomacrolactone.

Compound **8** was obtained as a yellow gum. Its molecular formula was established as $C_{32}H_{48}O_6$ on the basis of HR-ESI-MS ion at m/z = 527.3375 $[M-H]^-$ in the negative mode and m/z = 551.3361 $[M+Na]^+$ in the positive mode. This formula suggested that compound **8** has 9 degrees of unsaturation. The UV spectrum of **8** exhibited a maximum absorption peak at λ_{max} 219 nm. Analysis of the ^{1}H NMR, ^{13}C-NMR , DEPT and HSQC data revealed the presence of six methyls, ten methylenes, eight methines and eight quartenary carbons (Table 2). Among the deshielded carbons included one ketone (δ_C 203.9), one ester carbonyl (δ_C 170.4), two oxygenated (δ_C 74.8, 77.4), four olefinic (δ_C 123.2, 152.4, 114.5 and 142.7) and one CO for the carboxylic acid group (δ_C 179.5) (Pretsch et al., 2000). The carboxyl carbon (δ_C 179.5) had HMBC correlations with H-2 at δ_H 2.46 and 2.26. The ester at C-16 was identified as acetate from the HMBC correlation between CO (δ_C 170.4) and the methyl at δ_H 2.05. The ^{1}H NMR of 8 showed typical signals of a 3,4-*seco*cycloartane triterpene including a distinguished pair of doublets at δ_H 0.30 and 0.47 (d, J = 4.3), attributed to the C-19 protons of the cyclopropane ring and two doublets of the exomethylene moiety at δ_H 5.02 and 4.96. The exomethylene protons (H-28) had HMBC correlations with C-5 (δ_C 42.3) and C-4 (δ_C 142.7). The presence of the exomethylene protons at C-24a (δ_H 6.01and 5.85) was determined from their HMBC correlations with C-24 (δ_C 152.4) and C-25 (δ_H 28.7). The rest of the carbons and protons were assigned from detailed analysis of the ^{1}H NMR, ^{13}C-NMR, and DEPT and HSQC data and also in comparison to a related compound Gardenoin H (Nuanyai et al., 2011) in which there is a difference in the side chain only.

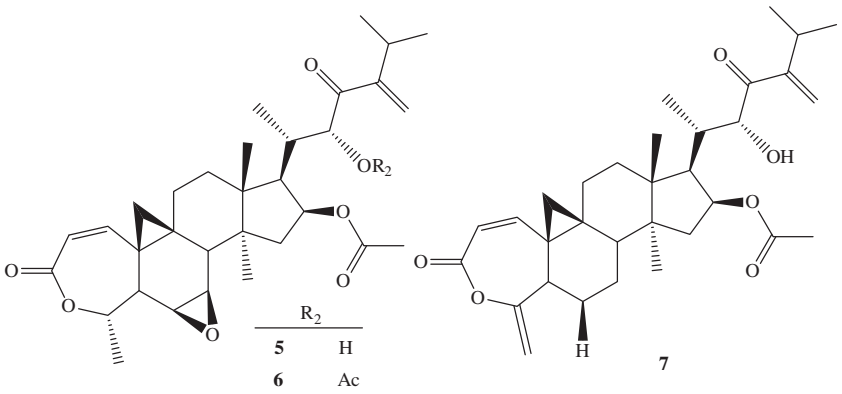


Fig. 2. Structures of compounds **5–7** isolated from the leaves of *N. macrocalyx*.

Compound **8** differed from compounds **1–7** due to the absence of the lactone ring and the presence of the carboxyl group at C-2. The structure of compound **8** was therefore established as 16-acetoxy-22α-hydroxy-29-nor-3,4-seco-24-methylcycloart-4,24(24a)-dien-23-on-3-oic acid (Fig. 3) and was assigned a trivial name Neomacroin.

Compound **9**, was isolated as a white solid and its molecular formula was established as $C_{31}H_{52}O_3$ on the basis of HR-ESI-MS ion at m/z = 471.3838 $[M-H]^-$ in the negative mode. This formula suggested that compound **9** has 6 degrees of unsaturation. The ^{1}H NMR spectrum of **9** (Table 1), exhibited a pair of proton resonances at δ_H 0.46 and 0.66 (d, J = 3.3) characteristic of a cyclopropane methylene. Detailed analysis of ^{1}H NMR, ^{13}C-NMR and HSQC spectra indicated the presence of seven methyls, ten methylenes, eight methines and six quartenary carbons (Table 2). The ^{1}H NMR also showed two singlets for the exo methylene protons at δ_H 4.69 and 4.64, three oxymethine protons at δ_H 3.49 (s), 3.61 (d, J = 8.8) and 3.44 (d, J = 9.7). These data revealed that compound **9** was a cycloartane-type triterpenoid with one double bond and three hydroxyl groups (Tao et al., 2008). The positions of the OH groups at C-1, C-2 and C-3 were established from COSY correlations between H-2—H-3, H-2—H-1. The relative configuration of compound **9** was determined by analysis of its NOESY correlations of H-28—H-3, H-1—H-19b. The hydroxyl groups at C-1, C-2, and C-3 were therefore determined to be α–, α–, β–oriented, respectively. The structure of compound **9** was elucidated as cycloartan-24(24a)-ene-1α,2α,3β–triol (Fig. 3) and was assigned a trivial name Neomacrotriol.

Compound **10**, a yellow solid, has a molecular formula of $C_{17}H_{18}O_3$ as revealed from its HR-ESI-MS data with an ion at m/z 269.1161 $[M-H]^-$. This information showed that compound **10** has 9 site of unsaturation. The ^{1}H NMR spectrum of compound **10**, exhibited three singlets for aromatic protons at δ_H 7.21, 7.17 and 6.94. The ^{1}H NMR also indicated three methyl groups, one of which was a methoxy at δ_H 3.76, and two methylenes. Detailed analysis of ^{1}H NMR, ^{13}C-NMR and HSQC spectra revealed that compound **10** was a phenantherene with three aromatic protons, two methyls, two methelenes, one methoxy and two hydroxyl groups. The location of the H-4 and H-5 protons was established through ^{1}H—^{13}C long-range HMBC correlations. H-4 had a cross peak with C-2, C-1a and C-5a at δ 123.9, 128.2 and 131.5, respectively. H-5 had cross peaks with C-4a and C-8a. H-1 had HMBC correlation with C-4a and a NOESY correlation with H-10.The methyl at C-2 was established from HMBC cross peak between Me-2 and C-2 and the NOESY correlation between Me-2 and H-1. There was a cross peak for Me-8 with C-8 and C-8a and a NOESY cross peak with H-9 and these were used to locate the position of the methyl at C-8. The methoxy group position was established at C-7 due to its NOESY correlation with Me-8. The location of the hydroxyl groups was revealed by HMBC, in which H-2a (δ 2.18), H-1 (δ 6.94), and H-4 (δ 7.20) were correlated with C-3 while H-5 (δ 7.17) was correlated with C-6. HMBC and NOESY correlations (Fig. 4) allowed positioning of all the groups. Phenanthrenes with the same skeleton have been isolated before from several plant species (Della Greca et al., 1997, 1996; Yang et al., 2006). Compound **10** was therefore established as a new dihydrophenantherene, 7-methoxy-2,8-dimethyl-9,10-dihydrophenantherene-3,6-diol and was assigned a trivial name Neonthrene.

The known compounds were identified as 22-de-*O*-acetyl-26-deoxyneoboutomellerone (**11**) (Long et al., 2011), mellerin B (**12**)

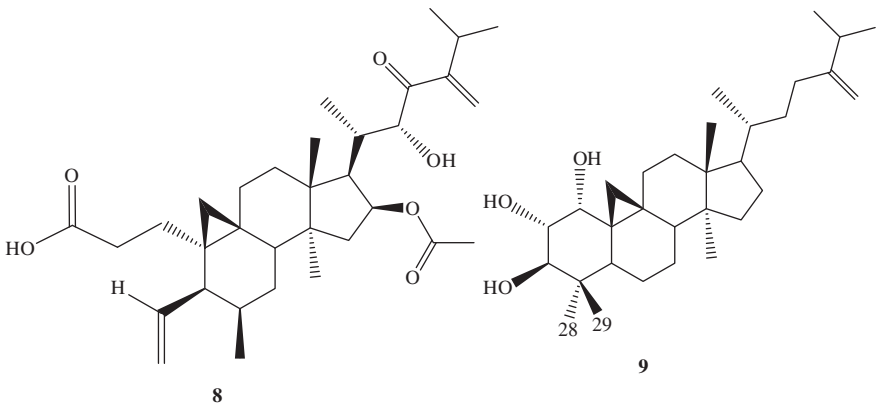


Fig. 3. Structures of compound **8** and **9** isolated from the leaves of *N. macrocalyx*.

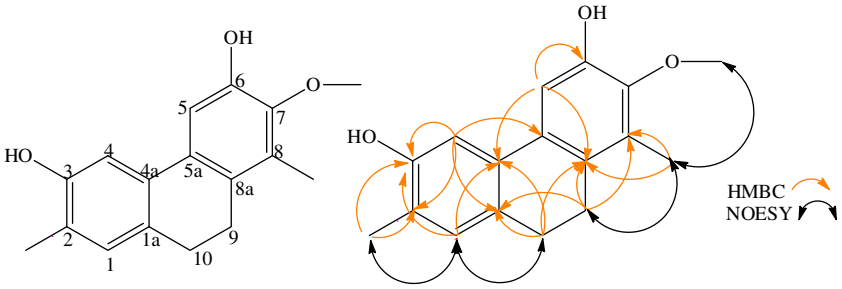


Fig. 4. Structure of 7-methoxy-2,8-dimethyl-9,10-dihydrophenantherene-3,6-diol (**10**).

Table 3
Antiplasmodial and cytotoxic activities of the compounds and extract.

Compound Reference	IC_{50} Pf FcB1 (μg/ml) (n = 3)	% Inhibition KB cells	% Inhibition MRC5 cells
1	1.1	76.0 ± 1.0	91.0 ± 1.0
2	1.4	24.0 ± 3.0	25.0 ± 4.0
3	0.8	83.0 ± 1.0	96.0 ± 1.0
4	1.6	82.0 ± 2.0	93.0 ± 1.0
5	5.1	0.0 ± 6.0	3.0 ± 7.0
6	6.4	77.0 ± 3.0	90.0 ± 1.0
7	1.0	65.0 ± 1.0	95.0 ± 1.0
8	1.7	10.0 ± 2.0	24.0 ± 9.0
9	>10	37.0 ± 1.0	32.0 ± 4.0
10	9.8	65.0 ± 3.0	71.0 ± 1.0
11	1.1	98.0 ± 1.0	99.0 ± 1.0
12	9.6	100.0 ± 1.0	100.0 ± 2.0
13	>10	30.0 ± 12.0	32.0 ± 13.0
Extract	>10	39.0 ± 4.0	24.0 ± 4.0
Chloroquine (nM)	90.0 (0.046 μg/ml)	NT	NT
Taxotere	NT	>84.0	>90.0

NT: not tested; n: number of independent assays.

(Zhao et al., 1998), 24 (S)-24-ethyl-stigmat-4-en-6β-ol-3-one (**13**) (Esperanza et al., 2006; Della Greca et al., 1990; Woldemichael et al., 2003). The compounds were identified by comparing their spectroscopic data with values reported previously in literature. These compounds have been isolated from this plant for the first time.

Antiplasmodial and cytotoxicity activity of the compounds

All the compounds, and the ethylacetate extract, were found to have activity against *Plasmodium falciparum*. Most of the compounds had IC_{50} of $\leqslant$5 μg/ml with some of them displaying significant cytotoxicity (Table 3). Compound **3** which was the most active compound with an IC_{50} of 0.8 μg/ml also exhibited a high cytotoxicity activity. Therefore the antiplasmodial activity of most of the compounds could be correlated to their cytotoxicity nature. However, there were some compounds which were very active with low cytotoxicity values. For instance compounds **2** and **8** had significant antiplasmodial activities with low cytotoxicity values (Table 3).

Generally, the presence of a cyclopropyl ring conjugated with α, β-unsaturated carbonyl moiety confers to the cycloartanes a particular activity because these two functionalities are Michael acceptors (Long et al., 2011). It was also noted that the presence of OH group at positions 6 and 22 increased the antiplasmodial activity for example in compounds **1** and **3**, while acetylation at position 22 decreased the activity of these compounds like in compounds **1** and **2**, and compounds **3** and **4**.

Conclusion

This research yielded bioactive compounds that have not been described before. The identification of antiplasmodial cycloartane triterpenoids from *N. macrocalyx* suggests that the compounds may play a role in the medicinal properties of the plant. Three of the thirteen compounds showed good antiplasmodial and low cytotoxity properties. Some of the compounds had good antiplasmodial activities but their potential as antimalarial drugs is limited because of the high cytotoxicity. The results of this study also suggest that hydroxylation at positions 6 and 22 of the cycloartanes could be responsible for the antimalarial activities of the compounds.

Experimental

General experimental procedures

Optical rotations were measured at 25 °C on an Anton Paar MCP 300/500 polarimeter. IR spectrum of compound **1** was obtained on a Perkin Elmer Spectrum BX FT-IR system and that for compound **9** was recorded on Shimadzu IR prestige 21 Fourier transform spectrophotometer with KBr pellets. UV spectra were obtained on a Perkin Elmer Lamba 5 spectrophotometer. The NMR spectra of compounds **2**–**6** and **9**–**11** were recorded on a Bruker Avance 600 MHz and those of compounds **1**, **7**, **8**, **12**, were recorded on 500 MHz , while **13** on 300 MHz (Bruker; Switzerland) NMR instruments. High resolution MS data were obtained on a LCT Premier XE (Waters) ESI-ToF mass spectrometer. Column chromatography was performed on a medium pressure column (4PSI) with silica gel (3–35 μm). Analytical and preparative HPLC separation was performed with a C18 column Sunfire (5 μm; 4.6 × 150 mm) and (5 μm; 19 × 150 mm) (Waters) respectively. The SFC analysis was performed on a Thar Waters SFC Investigator II System using a Waters 2998 photodiode array detector and a 3-Diol column (5 μm; 250 × 10 mm) equipped with a Waters Acquity ELSD 2424 detector, with CO_2–MeOH as mobile solvents in different conditions. Analytical TLC was carried out on precoated silica gel $60F_{254}$ (Merck) and the spots were visualized by heating after spraying with Ammonium Molybdate. Preparative TLC was done using TLC glass plates prepared with Silicagel $60F_{254}$.

Plant material

The plant material was collected from Kibale National Park and identified at the Botany department, Makerere University. A voucher specimen under reference UFC-380 has been deposited in Makerere University herbarium. The plant material was air dried and ground into a powder.

Extraction and isolation

The leaf powder (867 g) was extracted with 1000 ml of ethylacetate and filtered through a whatman filter paper. This process was repeated until a clear extract was obtained. The extracts from the different extractions were combined and concentrated under

reduced pressure at 40 °C to give 21 g of dried ethylacetate extract. 10 g of this extract was subjected to chromatography on silica gel (6–32μ) column with a gradient system of hexane-Teac and EtOAc–MeOH to yield 20 fractions (I—XX). Each fraction was evaluated for antiplasmodial and cytotoxic activity against KB and MRC5 cell lines and a bioactivity-guided fractionation method was used for the isolation of fractions with significant activity. Fraction XIII was purified by repeated preparative TLC with a mixture of Hexane–EtOAc (7:3) as developing solvent to give compound **1** (Neo13f), (64 mg). After preparative TLC with Hexane–EtOAc (6:3) and Supercritical chromatography (SFC) of fraction XIV, compound **2** (Neo14B-1) (65 mg) was obtained. Compound **3** (Neo17A-3) (2.2 mg) and **4** (Neo17A-2) (3.7 mg) were obtained from Prep. TLC Hexane–EtOAc (7:3) of fraction XVII and SFC analyses with the condition of CO_2–MeOH 85–15, 12 ml/min, 150 bar. Compound **5** (Neo16A-2-2) (5.6 mg), and **6** (Neo16A-1-1) (2.8 mg) were obtained from Prep. TLC with Hexane–EtOAc (7:3) solvent system of fraction XVI and SFC analyses with the condition of CO_2–MeOH, 88–12, 12 ml/min, 150 bar. Compounds **7** (Neo9-2) (2.8 mg), **8** (Neo9-5) (72 mg) and compound **13** (Neo9-7) (7.9 mg) , were isolated from fraction IX while **10** (Neo10-1) (2.6 mg) and **11** (Neo7-2) (1.5 mg) were isolated from fraction X and VII, respectively using preparative HPLC with a gradient elution 50/50 to 0/100 (solvent A: H_2O + 0.1% HCOOH, B: MeOH + 0.1% HCOOH). Compounds **9** (Neo16c-1-2) (2.6 mg) and **12** (Neo16c-1-1) (2.1 mg) were obtained from Prep. TLC with Hexane–EtOAc (7:3) solvent system of fraction XVI and SFC analyses with the condition of CO_2–MeOH, 88–12, 12 ml/min, 150 bar.

*Neomacrolactone (**1**)*

Yellow crystals; $[\alpha]_D^{25}$ +20° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; IR (KBr) ν_{max} cm^{-1}: 3456, 2960, 2917; 2849, 1735, 1693, 1462, 1378, 1291 ,1240,1024; HR-ESI-MS (positive ion mode) *m/z* 527.3350 $[M+H]^+$ (calcd for $C_{32}H_{47}O_6$, 527.3373); HR-ESI-MS (positive ion mode) *m/z* 549.3320 $[M+Na]^+$ (calcd for $C_{32}H_{46}O_6Na$, 549.3345).

*22α-Acetoxyneomacrolactone (**2**)*

Light yellow solid; $[\alpha]_D^{25}$ −10° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (positive ion mode) *m/z* 569.3474 $[M+H]^+$ (calcd for $C_{34}H_{49}O_7$, 569.3478).

*6-Hydroxyneomacolactone (**3**)*

Light yellow solid; $[\alpha]_D^{25}$ −10° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (positive ion mode) *m/z* 543.3315 $[M+H]^+$ (calcd for $C_{32}H_{47}O_7$, 543.3322).

*22α-Acetoxy-6-hydroxyneomacrolactone (**4**)*

Light yellow solid; $[\alpha]_D^{25}$ −10° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (positive ion mode) *m/z* 585.3427 $[M+H]^+$ (calcd for $C_{34}H_{49}O_8$, 585.3427).

*6,7-Epoxyneomacrolactone (**5**)*

Light yellow amorphous solid: $[\alpha]_D^{25}$ −10° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (positive ion mode) *m/z* 541.3151 $[M+H]^+$ (calcd for $C_{32}H_{45}O_7$, 541.3165).

*22α-Acetoxy-6,7-epoxyneomacrolactone (**6**)*

Light yellow amorphous solid; $[\alpha]_D^{25}$ −30° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (positive ion mode) *m/z* 583.3248 $[M+H]^+$ (calcd for $C_{34}H_{47}O_8$, 583.3271); HR-ESI-MS (positive ion mode) *m/z* 605.3092 $[M+Na]^+$ (calcd for $C_{34}H_{46}O_8Na$, 605.3090).

*4-Methylen-neomacrolactone (**7**)*

Orange solid; $[\alpha]_D^{25}$ +70° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (positive ion mode) *m/z* 525.3208 $[M+H]^+$ (calcd for $C_{32}H_{45}O_6$, 525.3216).

*Neomacroin (**8**)*

Yellow gum; $[\alpha]_D^{25}$ +70° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; IR (KBr) ν_{max} cm^{-1}: 3445, 2929, 2981, 1734, 1375, 1240, 1024. HR-ESI-MS (positive ion mode) *m/z* 551.3361 $[M+Na]^+$ (calcd for $C_{32}H_{48}O_6$, 551.3349); HR-ESI-MS (Negative ion mode) *m/z* 527.3375 $[M-H]^-$ (calcd for $C_{32}H_{47}O_6$, 527.3373).

*Neomacrotriol (**9**)*

White solid; $[\alpha]_D^{25}$ +3.50° (c 0.1, MeOH); ^1H and ^{13}C NMR, see Tables 1 and 2 respectively; HR-ESI-MS (negative ion mode) *m/z* 471.3833 $[M-H]^-$ (calcd for $C_{31}H_{52}O_3$, 471.3838).

*Neonthrene (**10**)*

Yellow solid; $[\alpha]_D^{25}$ −0.25° (c 0.1, MeOH); ^1H NMR (600 MHz, DMF): δ_H 6.94 (H-1), 7.21 (H-4), 7.17 (H-5), 2.66 (H-9), 2.66 (H-10), 3.76 (O-CH_3), 2.18 (H_{2a}-CH_3) 2.21 (H_{8b}-CH_3); ^{13}C NMR, δ_C 131.0 (C-1), 128.2 (C-1a) 123.9 (C-2), 155.9 (C-3), 110.9 (C-4), 134.1 (C-4a), 110.4 (C-5), 131.5 (C-5a), 149.9 (C-6), 146.8 (C-7), 129.7 (C-8), 128.2 (C-8a), 25.9 (C-9), 29.0 (C-10), 60.6 (O-CH_3), 16.3 (C-2a), 12.5 (C-8b); HR-ESI-MS (negative ion mode) *m/z* 269.1161 $[M-H]^-$ (calcd for $C_{32}H_{45}O_6$, 269.1178).

Antiplasmodial activity

The antiplasmodial activity was evaluated against the chloroquine-resistant FcB1/Colombia strain of *P. falciparum*. The test was performed using the method of Desjardin et al. (1979). Stock solutions of test extract and pure compounds were prepared in dimethyl sulfoxide (DMSO) at a 10 mg/ml concentration. The extract and pure compounds were diluted with culture medium to the expected concentrations and negative controls with equivalent amounts of DMSO were prepared as well. Serial dilutions of Chloroquine (in water) were used as positive controls. Asynchronous parasite cultures were then added (1% parasitemia and 1% final hematocrite), and plates were maintained for 24 h at 37 °C in a candle jar. Hypoxanthine (0.5 μCi) was subsequently added to each well, and parasites were maintained for an additional 24 h. The growth inhibition for each well was determined by comparison of the radioactivity incorporated into the treated culture (drugs/DMSO) with that in the control culture (DMSO without drug) maintained on the same plate. The IC_{50} was obtained from the drug concentration-response curve.

Cytotoxicity studies of the compounds

Cytotoxicity was done against KB (nasopharyngeal epidermoid carcinoma) cells and MRC-5 (human diploid embryonic lung cell). Samples were dissolved in DMSO to make a final concentration of 5 μg/ml. Samples were then added to a plate containing cells in a fixed volume of DMSO and incubated for 72 h. DMSO was used in the control experiments. The percentage inhibition was then calculated from the number of viable cells measured at 490 nm with

the MTS reagent with reference to the control. The cytotoxicity assays were performed according to published procedures (Tempete et al., 1995). Taxotere® was used as a control compound and experiments were performed in triplicate.

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