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Glycosides of polygalacic acid from the stem barks of *Piper guineense* Schum and Thonn

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This paper is dedicated to the late Pr. Jan Hendrik van der WESTHUIZEN (February 13, 1953–October 25, 2015) for his outstanding contributions to Science, Engineering and Technology.

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ABSTRACT

In a continuation of our study on constituents of *P. guineense* now focusing on the search for saponins, phytochemical investigation of the *n*-BuOH fraction of *P. guineense* stem bark led to the isolation of three previously undescribed triterpenoid saponins, named guineenosides A–C (**1**–**3**). Their structures were established on the basis of extensive analysis of 1D and 2D NMR (^{1}H, ^{13}C NMR, DEPT, COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY and HMBC) and HRESIMS experiments, and by chemical evidence as 3-*O*-{α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 3)-β-D-fucopyranosyl} polygalacic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl ester (**1**), 3-*O*-{α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 3)-β-D-fucopyranosyl} polygalacic acid 28-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl ester (**2**), and 3-*O*-{α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 3)-β-D-fucopyranosyl} polygalacic acid 28-*O*-[α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl ester (**3**). This is the first report of triterpenoid saponins from *P. guineense*.

1. Introduction

Piper, with approximately 2000 species, is the nominate genus of the Piperaceae family [1]. In Africa, *Piper* genus occurs from East of Guinea to Ethiopia and South of Angola to Mozambique including some main species as *P. betle, P. capense, P. cubeba, P. emirnense, P. nigrum, P. umbellatum, P. sylvestre, P. subeltatum, and P. guineense* [2], four of which, *P. capense, P. nigrum, P. umbellatum*, and *P. guineense,* have been, so far, listed in Cameroon [3]. *P. guineense* Schum and Thonn commonly known as the "Ashanti" or "Guinea pepper" is reported to be the most familiar medicinal Piperaceae in Africa [4]. It is used for the treatment of diarrhoea, chronic syphilis, rheumatism, stomach ache, female infertility, coughs, and wounds disorders [5–9]. In our previous contribution, we reported the isolation and structural characterization of cyclitol and quinic acid derivatives from this plant [10], beside amide alkaloids, lignans, terpenes and flavanoids which constitute the major classes of chemical compounds largely explored and isolated from *Piper* species [10–14]. However, the only triterpenoid saponins so far reported from the genus were obtained in *P. auritum* [15].

Saponins are highly polar compounds formed from glycosylated terpenoids of various structural moieties. Structurally, they have one or more hydrophilic glycoside sugars (glycone) attached to either a

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lipophilic steroid or triterpenoid called the aglycone or sapogenin [16]. These compounds are known to exhibit a wide range of biological properties such as hemolytic, anti-inflammatory, antibacterial, anti-fungal, antiviral, insecticidal, anticancer, cytotoxic, anti-diabetic, and molluscicidal, among others [17–22] and are increasingly being exploited in food, cosmetics and pharmaceutical sectors for their health benefits [23].

In a continuation of our study on constituents of *P. guineense*, focusing now on the search for saponins, the *n*-BuOH fraction of the stem bark of this species was examined.

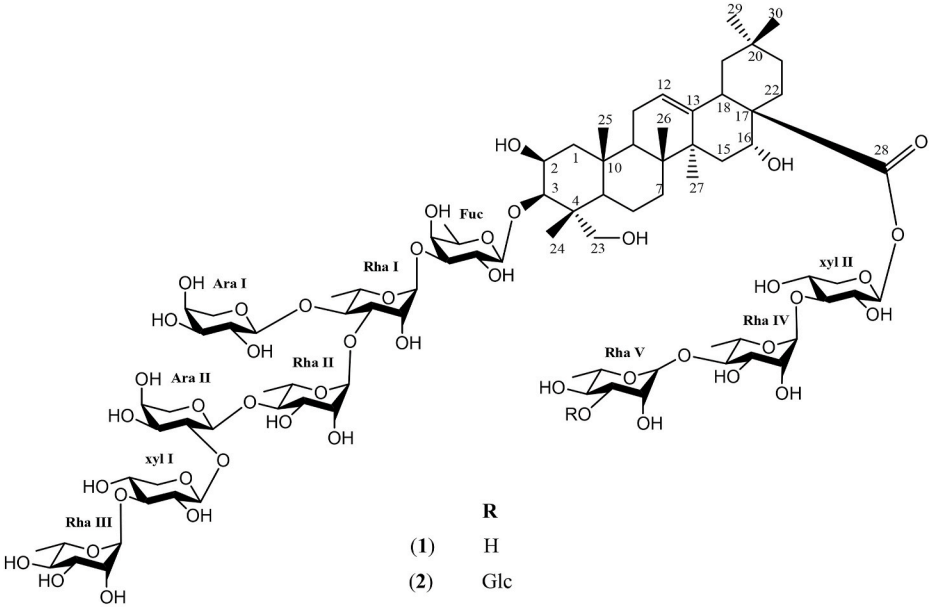
Herein, we report the isolation and structural characterization of three previously undescribed oleanane-type saponins, named guineenosides A–C (**1**–**3**), having polygalacic acid as aglycone. It is worthy to note that this is the first report on triterpenoid saponins from *P. guineense*.

2. Results and discussion

Air dried and powdered stem barks of *P. guineense* (1.6 kg) were extracted twice for 72 h at room temperature with MeOH (5 L). A dark reddish extract (35 g) was obtained after evaporation of the solvent in vacuum at 40 °C. The extract (10 g) was suspended in water (100 mL), and successively extracted with EtOAc (3 x 100 mL) and water sat. n-BuOH (3 x 100 mL) to give EtOAc (2.9 g), n-BuOH (2.8 g), and aqueous (3.5 g) fractions.

The n-BuOH fraction was repeatedly subjected to Diaion HP-20SS resin and silica gel column chromatographic purification steps to afford three new triterpenoid saponins, named guineenosides A–C (**1**–**3**) (Fig. 1).

Guineenoside A (**1**) was obtained as a white, amorphous powder. Its molecular formula of $C_{86}H_{140}O_{46}$ was established from an (+)HR-ESI-MS ion peak at *m/z* 1909.8470 $[M+H]^+$ (calcd for $C_{86}H_{141}O_{46}$, 1909.8694, $\Delta = 11$ ppm). Upon acid hydrolysis with 2.0 M HCl, **1** gave a polygalacic acid unit, identified with an authentic sample, together with arabinose (Ara), fucose (Fuc), rhamnose (Rha), and xylose (Xyl), which were identified by co-TLC with authentic samples. The ^{1}H NMR spectrum of **1** showed six angular methyl groups as singlets at δ 0.89, 0.96, 1.03, 1.26, 1.33, and 1.34 (each 3H, s), two diastereotopic protons for a hydroxymethylene unit at δ 3.52 (d, $J = 10.3$ Hz), and 4.56 (d, $J = 10.3$ Hz), three oxygenated methines at δ 3.63 (m), 4.23 (m), and 4.49 (br s), and one olefinic proton at δ 5.39 (br s). Its ^{13}C NMR spectrum showed two olefinic carbon signals at δ 124.0 and 144.0, suggesting that 1 had



(**3**)

Fig. 1. Structures of compounds **1**— **3**.

an olean-12-ene skeleton. Thus, 1D (^{1}H, ^{13}C NMR, DEPT) and 2D (COSY, TOCSY, HSQC, NOESY, HSQC-TOCSY, and HMBC) NMR techniques permitted the unambiguous assignment of all ^{1}H and ^{13}C NMR signals of the aglycone of **1**, which was thus recognized to be 2β,3β,16α,23-tetrahydroxyolean-12-ene-28-oic acid (polygalacic acid) by comparison of its ^{1}H and ^{13}C NMR signals with those reported in the literature (Fig. 1, Table 1) [24–30].

The ^{1}H NMR spectrum of **1** showed ten anomeric protons at δ 4.51 [d, $J = 7.7$ Hz, xylose (Xyl I)], 4.56 [d, $J = 7.7$ Hz, arabinose (Ara II)], 4.86 [br s, rhamnose (Rha V)], 4.89 [br s, fucose (Fuc)], 5.02 [br s, arabinose (Ara I)], 5.16 [br s, rhamnose (Rha III)], 5.26 [br s, rhamnose (Rha I)], 5.35 [br s, rhamnose (Rha IV)], 5.42 [d, $J = 6.3$ Hz, xylose (Xyl II)], and 5.60 [br s, rhamnose (Rha II)], which correlated with ten anomeric carbon atom signals at δ 105.2, 106.9, 98.4, 104.1, 104.2, 102.6, 101.4, 101.3, 95.5, and 100.9, respectively, in the HSQC spectrum. The ^{1}H and ^{13}C NMR data (Tables 2 and 3) of the monosaccharide residues were assigned starting, either from the readily identifiable anomeric proton of each pentosyl unit, or from the CH_3-proton doublet of each 6-deoxyhexosyl unit, by means of COSY, TOCSY, HSQC-TOCSY, HSQC, NOESY and HMBC spectra.

Substitutions at C-3 and C-28 of polygalacic acid were evidenced by the observed glycosylation-induced shifts of C-3 at δ 82.5, and of C-28 at δ 174.1. All of these data established that **1** was a 3,28-bidesmosidic polygalacic acid derivative, sugar chains being linked to C-3 and C-28 through an ether and ester bond, respectively [9,24,26–30].

Data indicated that the ten sugar residues were in their pyranose form. The β-anomeric configurations for the fucose and xylose units, and the α-anomeric configuration for the arabinose unit were determined by their large $^3J_{H\text{-}1,H\text{-}2}$ coupling constants and ^{13}C NMR data, while the α-Rha glycosidic bond was established by the chemical shift of its C-5 at δ_C 70.3–69.1 [29,31,32].

The absolute configuration of these sugar residues was determined to be D for Fuc and Xyl, and L for Ara and Rha by GC analysis [33]. The sequencing of the glycoside chains was achieved by analysis of HMBC and NOESY experiments.

For the sugar chain attached at C-3 of the aglycone, the cross peak

Table 1
NMR spectroscopic data (600 MHz for ^{1}H and 150 MHz for ^{13}C) for the aglycone moieties of compounds **1**–**3** (δ in ppm and J in Hz)[a] in methanol-d_4.

Position	1		2		3	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	1.16; 2.04	47.3	1.18; 2.03	47.2	1.15; 2.03	47.3
2	4.23	72.3	4.23	72.2	4.23	72.4
3	3.63 dd (4.0, 10.8)	82.5	3.63 m	82.4	3.64 m	82.6
4	–	44.2	–	44.2	–	44.3
5	1.35	48.8	1.33	48.8	1.36	49.2
6	1.67; nd	19.5	1.66; nd	19.4	1.06; 1.65	19.4
7	1.29; nd	30.8	1.28; nd	30.8	1.30; 2.29	31.5
8	–	40.0	–	39.9	–	40.0
9	1.67	48.7	1.65	48.7	1.69	48.9
10	–	37.4	–	37.4	–	37.5
11	1.98; 2.11	24.6	1.98; 2.11	24.6	1.98; 2.12	24.8
12	5.39 br s	124.0	5.39 br s	124.1	5.36 br s	124.3
13	–	144.0	–	143.9	–	144.2
14	–	43.5	–	43.4	–	43.9
15	1.45; 1.79	36.2	1.45; 1.78	36.1	1.48; 1.79	37.5
16	4.49	74.8	4.48	74.7	4.49	74.8
17	–	50.3	–	50.2	–	49.7
18	3.00	42.3	3.00	42.3	3.02	43.0
19	1.06; 2.29	47.9	1.06; 2.28	47.8	1.08; 2.29	48.4
20	–	31.4	–	31.3	–	31.7
21	1.17; 1.92	36.5	1.18; 1.91	36.4	1.17; 1.92	37.5
22	1.74; 1.91	32.1	1.78; 1.90	31.6	1.75; 1.92	33.4
23	3.52; 4.56	65.7	3.53; 4.56	65.7	3.52; 3.74	65.8
24	1.26 s	16.4	1.25 s	16.4	1.28 s	16.5
25	1.33 s	18.1	1.33 s	18.0	1.36 s	18.5
26	1.03 s	19.0	1.03 s	19.0	1.06 s	19.0
27	1.34 s	27.4	1.34 s	27.4	1.35 s	26.4
28	–	174.1	–	177.2	–	178.1
29	0.89 s	33.4	0.88 s	33.4	0.89 s	33.6
30	0.96 s	25.0	0.95 s	25.1	0.96 s	24.1

Assignments based on the HMBC, HSQC, COSY, TOCSY, NOESY, and DEPT experiments. nd, not determined. [a)]Overlapped proton NMR signals are reported without designated multiplicity.

Table 2
NMR spectroscopic data (600 MHz for ^{1}H and 150 MHz for ^{13}C) for the sugar moieties attached at C-3 of compounds **1**–**3** (δ in ppm and J in Hz)[a] in methanol-d_4.

Position	1		2		3	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
3-*O*-Sugars	Fuc		Fuc		Fuc	
1	4.89 br s	104.1	4.88 br s	104.0	4.88 br s	104.1
2	3.94	72.1	3.96	72.2	3.94	72.2
3	3.89	81.2	3.89	81.0	3.92	83.6
4	3.39	74.6	3.39	74.6	4.07	72.2
5	3.82	70.9	3.83	70.8	3.82	70.4
6	1.24 d (6.3)	18.4	1.24 d (6.2)	18.4	1.23 d (6.2)	18.3
	Rha I		Rha I		Xyl I	
1	5.26 d (1.8)	101.4	5.25 br s	101.3	4.51 d (7.5)	107.3
2	4.01	72.1	4.01	72.2	3.46	78.0
3	3.79	77.5	3.80	77.6	3.75	72.5
4	3.89	81.2	3.89	81.0	3.93	66.9
5	3.83	70.3	3.82	70.3	3.30; 4.12 dd (4.8, 11.7)	63.8
6	1.27 d (6.2)	18.6	1.28 d (6.0)	18.6	Rha I	
	Rha II		Rha II		5.59 d (1.8)	100.9
1	5.60 d (1.7)	100.9	5.58 br s	100.8	3.90	69.9
2	3.87	72.2	3.91	72.2	3.72	72.2
3	4.35	72.3	4.34	72.0	3.61	74.4
4	3.60	83.8	3.59	83.7	3.78	70.4
5	3.79	69.4	3.80	69.4	1.31 d (6.3)	18.3
6	1.31 d (6.3)	18.0	1.31 d (6.2)	18.0		
	Ara I		Ara I			
1	5.02 br s	104.2	5.02 br s	104.1		
2	4.06	72.1	4.06	72.2		
3	3.76	71.9	3.76	72.0		
4	3.56	71.0	3.55	70.9		
5	3.36 d (9.7); nd	66.9	3.33 d (10.7); nd	66.8		
	Xyl I		Xyl I			
1	4.51 d (7.7)	105.2	4.52 d (7.7)	105.1		
2	3.35	75.6	3.34	75.3		
3	3.45	84.3	3.43	84.3		
4	3.89	69.9	3.88	69.8		
5	3.20 t (10.9); 3.91	67.0	3.20 t (10.9); 3.91	66.9		
	Rha III		Rha III			
1	5.16 d (1.7)	102.6	5.15 br s	102.5		
2	3.97	72.3	3.96	72.3		
3	3.65	71.1	3.73	71.4		
4	3.40	74.1	3.41	74.0		
5	4.01	70.2	3.99	70.1		
6	1.28 d (6.1)	18.0	1.26 d (6.2)	18.1		
	Ara II		Ara II			
1	4.56 d (7.7)	106.9	4.56 d (7.6)	106.8		
2	3.48	77.9	3.49	77.9		
3	3.76	71.9	3.79	71.9		
4	3.72	71.7	3.74	71.6		
5	3.28; 4.15 dd (4.6, 11.7)	63.8	3.28; 4.16 dd (4.3, 11.4)	63.7		

Assignments based on the HMBC, HSQC, COSY, TOCSY, NOESY, and DEPT experiments. nd, not determined. [a)]Overlapped proton NMR signals are reported without designated multiplicity.

Table 3
NMR spectroscopic data (600 MHz for ^{1}H and 150 MHz for ^{13}C) for the sugar moieties attached at C-28 of compounds **1**–**3** (δ in ppm and J in Hz)[a] in methanol-d_4.

Position	1		2		3	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
28-*O*-						
Sugars	Xyl II		Xyl II		Xyl II	
1	5.42 d (6.3)	95.5	5.43 d (5.7)	95.4	5.46 d (6.6)	95.6
2	3.58	76.4	3.59	76.4	3.60	76.5
3	3.56	77.4	3.57	77.4	3.58	77.7
4	4.47	68.8	4.47	68.8	4.50	68.8
5	3.32; 3.91	66.6	3.32; 3.91	66.5	3.32; 3.93	66.9
	Rha IV		Rha IV		Rha II	
1	5.35 d (1.7)	101.3	5.34 br s	101.2	5.39 d (1.7)	101.4
2	3.93	72.1	3.94	72.0	3.93	72.5
3	3.83	72.2	3.84	72.3	3.82	72.5
4	3.60	83.8	3.59	83.7	3.60	84.4
5	3.79	69.1	3.79	69.1	3.80	69.1
6	1.33 d (6.3)	18.1	1.31 d (6.3)	18.1	1.31 d (6.3)	18.1
	Rha V		Rha V		Rha III	
1	4.86 d (1.7)	98.4	4.84 br s	98.3	4.84 br s	98.6
2	3.76	71.9	3.76	72.0	3.78	72.4
3	3.94	72.4	3.59	83.7	3.93	72.5
4	3.40	74.1	3.40	73.9	3.44	74.2
5	3.83	70.5	3.82	70.4	3.82	70.4
6	1.24 d (6.3)	18.0	1.25 d (6.2)	18.0	1.23 d (6.2)	18.3
			Glc			
1			4.46 d (7.4)	105.3		
2			3.29	75.4		
3			3.78	77.8		
4			3.56	71.0		
5			3.39	78.1		
6			3.70; 3.81	62.2		

Assignments based on the HMBC, HSQC, COSY, TOCSY, NOESY, and DEPT experiments. [a]Overlapped proton NMR signals are reported without designated multiplicity.

correlations observed in the HMBC spectrum between H-1 (δ_H 4.89) of Fuc and C-3 (δ_C 82.5) of the aglycone, and in the NOESY spectrum between H-1 (δ_H 4.89) of Fuc and H-3 (δ_H 3.63) of polygalacic acid, suggested that Fuc was directly attached to C-3 of the aglycone. Moreover, the HMBC correlation observed between H-1 (δ_H 5.26) of Rha I and C-3 (δ_C 81.2) of Fuc established the connectivity between the two sugar units. This was confirmed by the NOESY correlation observed between H-3 (δ_H 3.89) of Fuc and H-1 (δ_H 5.26) of Rha I. In addition, the HMBC correlation observed between H-1 (δ_H 5.02) of Ara I and C-4 (δ_C 81.2) of Rha I allowed us to locate Ara I at C-4 of Rha I. This was supported by the NOESY correlation observed between H-1 (δ_H 5.02) of Ara I and H-4 (δ_H 3.89) of Rha I. On the other hand, the direct and reverse HMBC correlations observed between H-1 (δ_H 5.60) of Rha II and C-3 (δ_C 77.5) of Rha I and between H-3 (δ_H 3.79) of Rha I and C-1 (δ_C 100.9) of Rha II allowed us to locate Rha II at C-3 of Rha I. Furthermore, the HMBC correlation observed between H-1 (δ_H 4.56) of Ara II and C-4 (δ_C 83.8) of Rha II allowed us to locate Ara II at C-4 of Rha II. This was supported by the NOESY correlation observed between H-1 (δ_H 4.56) of Ara II and H-4 (δ_H 3.60) of Rha II. In addition, the correlation observed in the HMBC spectrum between H-1 (δ_H 4.51) of Xyl I and C-2 (δ_C 77.9) of Ara II, allowed us to locate Xyl I at C-2 of Ara II. In the same way, the HMBC correlation observed between H-1 (δ_H 5.16) of Rha III and C-3 (δ_C 84.3) of Xyl I, and the NOESY correlation observed between H-1 (δ_H 5.16) of Rha III and H-3 (δ_H 3.45) of Xyl I allowed us to locate Rha III at C-3 of Xyl I. Thus, the heptasaccharide α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 3)-β-D-fucopyranosyl moiety was established to be linked at C-3 of the aglycone (Fig. 2).

For the sugar chain attached at C-28 of the aglycone, the cross peak observed in the HSQC spectrum at δ_H/δ_C 5.42/95.5 (Xyl II H-1/C-1) suggested that this sugar should be directly attached to C-28 of the aglycone through an ester bond. Moreover, the HMBC correlation observed between H-1 (δ_H 5.42) of Xyl II and C-28 (δ_C 174.1) of the aglycone established the connectivity between both units. In addition, the direct and reverse correlations observed in the HMBC spectrum between H-1 (δ_H 5.35) of Rha IV and C-3 (δ_C 77.4) of Xyl II and between H-3 (δ_H 3.56) of Xyl II and C-1 (δ_C 101.3) of Rha IV allowed us to locate Rha IV at C-3 of Xyl II. Moreover, the HMBC correlation observed between H-1 (δ_H 4.86) of Rha V and C-4 (δ_C 83.8) of Rha IV, and the NOESY correlation observed between H-1 (δ_H 4.86) of Rha V and H-4 (δ_H 3.60) of Rha IV allowed us to locate Rha V at C-4 of Rha IV. Thus, the trisaccharide moiety attached at C-28 of the aglycone was determined to be α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl. Consequently, the structure of guineenoside A (**1**) was established as 3-*O*-{α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1

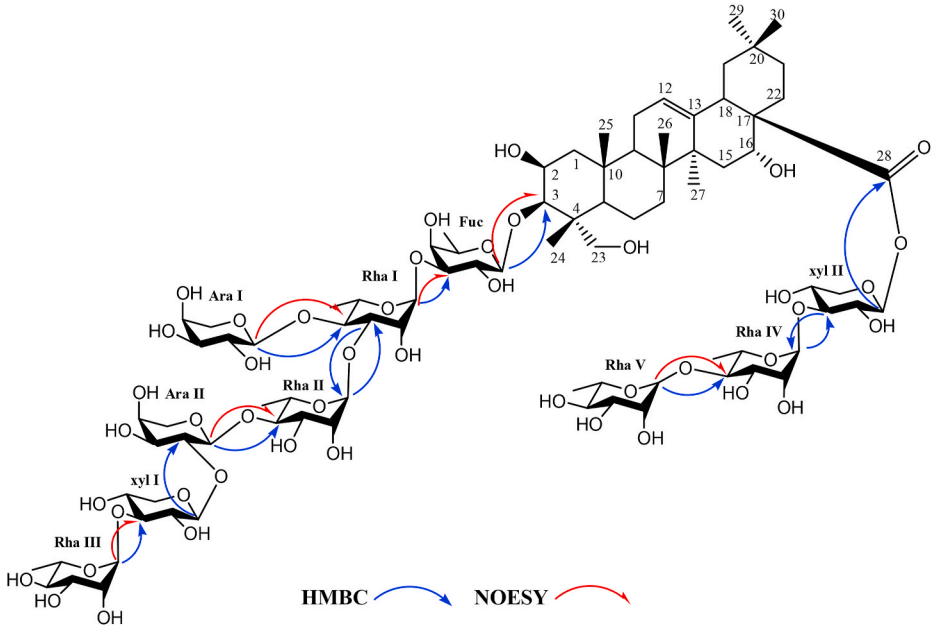


Fig. 2. Key HMBC and NOESY correlations of **1**.

→ 2)-α-L-arabinopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 3)-β-D-fucopyranosyl}polygalacic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl ester.

Guineenoside B (**2**) was obtained as a white, amorphous powder. Its molecular formula of $C_{92}H_{150}O_{51}$ was established from an (+)HR-ESI-MS ion peak at *m/z* 2071.9383 $[M+H]^+$ (calcd for $C_{92}H_{151}O_{51}$, 2071.9222, Δ = 7.7 ppm). As previously described for **1**, acid hydrolysis of **2** also afforded a polygalacic acid unit, together with D-fucose, D-glucose, D-xylose, L-arabinose and L-rhamnose sugars which were identified by co-TLC with authentic samples and GC analysis (Experimental Section). The ^{1}H and ^{13}C NMR data of the aglycone part of **2** were the same as for compound **1** (polygalacic acid) (Fig. 1, Table 1). The observation of glycosylation shifts at δ 82.4 (C-3 of aglycone), and 177.2 (C-28 of aglycone) in the ^{13}C NMR spectrum of **2** suggested that it should be also a 3,28-bidesmosidic polygalacic acid derivative with sugar chains linked to C-3 and C-28 through an ether and ester bond, respectively. The ^{1}H NMR spectrum of **2** showed eleven anomeric protons at δ 4.46 [d, *J* = 7.4 Hz, glucose (Glc)], 4.52 [d, *J* = 7.7 Hz, xylose (Xyl I)], 4.56 [d, *J* = 7.6 Hz, arabinose (Ara II)], 4.84 [br s, rhamnose (Rha V)], 4.88 [br s, fucose (Fuc)], 5.02 [br s, arabinose (Ara I)], 5.15 [br s, rhamnose (Rha III)], 5.25 [d, *J* = 1.5 Hz, rhamnose (Rha I)], 5.34 [br s, rhamnose (Rha IV)], 5.43 [d, *J* = 6.3 Hz, xylose (Xyl II)], and 5.58 [br s, rhamnose (Rha II)], which correlated with eleven anomeric carbon atom signals at δ 105.3, 106.8, 105.1, 98.3, 104.0, 104.1, 102.5, 101.3, 101.2, 95.4, and 100.8, respectively, in the HSQC spectrum (Tables 2 and 3).

Detailed comparison of NMR data of **2** (1D and 2D NMR analyses) with those of **1**, indicated identical sugar chain at C-3, and the presence of additional signals corresponding to one β-D-glucose unit in the C-28 ester oligosaccharide chain. In comparison with compound **1**, the hexose unit was glycosidically linked at C-3 (δ_C 83.7) of the Rha V as evidenced by HMBC and NOESY correlations between H-1 (δ_H 4.46) of Glc and C-3 (δ_C 83.7) of Rha V and between H-1 (δ_H 4.46) of Glc and H-3 (δ_H 3.59) of Rha V, respectively. This was further confirmed by the mass spectrum of **2** which displayed 162 mass units more than that of **1** accounting for the mass of one glucopyranosyl moiety. Hence, the tetrasaccharide moiety attached at C-28 of the aglycone of **2** was determined to be β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl. On the basis of these data, the structure of guineenoside B (**2**) was assigned as 3-*O*-{α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-[α-L-arabinofuranosyl-(1 → 4)]-α-L-rhamnopyranosyl-(1 → 3)-β-D-fucopyranosyl} polygalacic acid 28-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl ester.

Guineenoside C (**3**) was obtained as a white, amorphous powder. The (+)HR-ESI-MS exhibited a Sodium adduct ion peak at *m/z* 1375.6485 $[M+Na]^+$ (calcd for $C_{64}H_{104}O_{30}Na$, 1375.6505, Δ = 1.4 ppm) corresponding to the molecular formula $C_{64}H_{104}O_{30}$. Of the 64 carbon signals, 30 were assigned to the aglycone and 34 to the saccharide signals. A quick inspection of the ^{1}H and ^{13}C NMR spectra of **3** confirmed the aglycone to be 3,28-bidesmosidic polygalacic acid derivative as previously described for **1** and **2** (Fig. 1, Table 1).

Extensive NMR analysis of the sugar region showed signals for six anomeric protons δ 4.51 [d, *J* = 7.5 Hz, xylose (Xyl I)], 4.84 [d, *J* = 1.8 Hz, rhamnose (Rha III)], 4.88 [d, *J* = 7.8 Hz, fucose (Fuc)], 5.39 [d, *J* = 2.3 Hz, rhamnose (Rha II)], 5.46 [d, *J* = 6.6 Hz, xylose (Xyl II)], and 5.59 [d, *J* = 1.8 Hz, rhamnose (Rha I)], which correlated, in the HSQC spectrum, with six anomeric carbon signals at δ 107.3, 98.6, 104.1, 101.4, 95.6, and 100.9, respectively (Tables 2 and 3). The structure of the sugar chain attached at C-28 of the aglycone was determined to be the same as that of **1** by comparison of their ^{1}H and ^{13}C NMR spectroscopic data, as well as the HMBC et NOESY correlations. The trisaccharide was recognized as α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranoside (Table 3).

Similarly to **1** and **2**, the fucose unit (δ_H/δ_C 4.88/104.1) in **3** was firstly attached to C-3 of the aglycone (δ_H/δ_C 3.64/82.6) as evidenced by HMBC and NOESY correlations (Fig. 2). Then, the H-3 (δ_H 3.92) of Fuc showed a HMBC correlation to C-1 of Xyl I (δ_C 107.3), establishing the sequence from the anomeric position of the xylose unit to the C-3 of fucose. Moreover, the correlation observed in the HMBC spectrum between H-1 (δ_H 5.59) of Rha I and C-2 (δ_C 78.0) of Xly I, allowed us to locate Rha I at C-2 of Xyl I. Thus, the trisaccharide α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 3)-β-D-fucopyranosyl moiety was established to be linked at C-3 of the aglycone (Fig. 1). Accordingly, the structure of guineenoside C (**3**) was elucidated as 3-*O*-{α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranosyl-(1 → 3)-β-D-fucopyranosyl} polygalacic acid 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl ester.

3. Conclusion

To the best of our knowledge, this study is the first report on the characterization of saponins from *P. guineense*, having polygalacic acid as aglycone. Glycosides of polygalacic acid have also been reported from other genera such as *Aster, Bellis* and *Solidago* (Asteraceae) [9,24–30,34,35], *Crocosmia* (Iridaceae) [36,37], *Microsechium* (Cucurbitaceae) [38], and *Teucrium* (Lamiaceae) [39]. The characteristic of these saponins is the length of the oligosaccharide attached to C-3 of the aglycone with often, up to two or three sugars, and up to three or six sugars on the oligosaccharide chain at C-28, meanwhile guineenosides A–C which shared the same aglycone, possess longer glycosidic chain consisting of three to seven sugar moieties at C-3, and shorter oligosaccharide chain at C-28 consisting of three or four sugars units. On the other hand, the only saponins so far reported from another *Piper* species are glycosides of machaerinic acid and erythrodiol [15], possessing only two sugar moieties at C-3 of their aglycone, which are C-28 free carboxylic acid and acylated, respectively in comparison to guineenosides A–C. These findings may represent a valuable contribution to the knowledge of saponins from *Piper* genus.

4. Experimental

4.1. General methods

Optical rotations were measured on a Jasco P-2000 polarimeter. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded at room temperature in methanol-d_4 using a Bruker 600 MHz spectrometer. Chemical shifts are given in δ (ppm) value relative to TMS as internal standard. High resolution mass spectra were obtained through a Spectrometer (QTOF Bruker, Germany) equipped with a HRESI source. The spectrometer operates in positive mode (mass range: 100–3000, with a scan rate of 1.00 Hz) with automatic gain control to provide high-accuracy mass measurements within 0.40 ppm deviation using Na Formate as calibrant. The following parameters were used for experiments: spray voltage of 4.5 kV, capillary temperature of 200 °C. Nitrogen was used as sheath gas (10 L/min). The spectrometer was attached to an Ultimate 3000 (Thermo Fisher, Germany) UHPLC system consisting of LC-pump, Diode Array Detector (DAD) (λ: 190–600 nm), auto sampler (injection volume 10 μL) and column oven (40 °C). The separations were performed using a Synergi MAX-RP 100A (50 × 2 mm, 2.5μ particle size) with a H_2O (+0.1% HCOOH) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 500 μL/min, injection volume 5 μL). Samples were analyzed using a gradient program as follows: 95% A isocratic for 1.5 min, linear gradient to 100% B over 6 min, after 100% B isocratic for 2 min, the system returned to its initial condition (90% A) within 1 min, and was equilibrated for 1 min. GC analysis was carried out on a Termoquest gas chromatograph using a DB-1701 capillary column (30 m × 0.25 mm, i.d.) (J & W Scientific); detection, FID; detector temperature, 250 °C; injection temperature, 230 °C; initial temperature was maintained at 80 °C for 5 min and then raised to 270 °C at the rate of 15 °C/min; carrier gas, He. Thin layer chromatography (TLC)

was performed on precoated silica gel plates (60 F_{254}, Merck) using the system solvent n-BuOH-AcOH-H_2O, 65:15:25 as eluent. Water saturated n-BuOH was prepared by mixing n-butanol and distilled water (4:1, v/v) to separating funnel, shaking vigorously, standing for layering and then the lower layer (water) was separated from the upper layer, being the water saturated n-butanol. The spots were observed after spray with Komarowsky reagent, a (5:1) mixture of *p*-hydroxybenzaldehyde (2% in MeOH) and (Ethanolic H_2SO_4 50%). Column chromatography (CC) was carried out using silica gel 60 (15–40 μm and 40–63 μm).

4.2. Plant material

The stem barks of *Piper guineense* were harvested at Sok-Ekelle in the Centre region of Cameroon in January 2008 and was identified by Mr. Victor Nana, botanist of the National Herbarium of Cameroon (NHC), where a voucher specimen (11547/SRF/CAM) documenting the collection was deposited.

4.3. Extraction and isolation

Air dried and powdered stem barks of *P. guineense* (1.6 kg) were extracted twice for 72 h at room temperature with MeOH (5 L). A dark reddish extract (35 g) was obtained after evaporation of the solvent in vacuum at 40 °C. The extract (10 g) was suspended in water (100 mL), and successively extracted with EtOAc (3 x 100 mL) and water sat. n-BuOH (3 x 100 mL) to give EtOAc (2.9 g), n-BuOH (2.8 g), and aqueous (3.5 g) fractions. Part of the n-BuOH fraction (1 g) was taken in a minimum amount of water (10 mL) and then submitted to column chromatography (CC) using Diaion HP-20SS resin, eluting with H_2O, 30% MeOH, 50% MeOH, 70% MeOH, and MeOH, successively to yield six fractions (B1—B6) after TLC monitoring. Fraction B5 (437.5 mg) was submitted to CC using silica gel (15–40 μm), eluted with $CHCl_3$–MeOH–H_2O (70:30:5) to give four main subfractions (B51—B54). Subfraction B52 (202.8 mg) was further purified over silica gel (40–63 μm) with $CHCl_3$–MeOH–H_2O (65:30:5) to afford compound **1** (12.3 mg), while compound **2** (10.7 mg) was obtained from subfraction B53 (100.1 mg) by using silica gel column eluted with $CHCl_3$–MeOH–H_2O (65:35:5). Moreover, compound **3** (7.8 mg) was purified from fraction B4 (82.2 mg) by CC over silica gel (15–40 μm), eluted with $CHCl_3$–MeOH–H_2O (70:30:5).

4.4. Acidic hydrolysis

Each saponin (2 mg) was hydrolyzed with 2 N aqueous CF_3COOH (5 mL) at 100 °C during 2 h. After cooling, the reaction mixture was diluted with H_2O (10 mL) and extracted with CH_2Cl_2 (3 x 5 mL). Thereafter, the aqueous phase was repeatedly evaporated to dryness with MeOH. The reported sugars residues (Arabinose, fucose, glucose, rhamnose and xylose) were identified by comparison with standard sugars on TLC in $CHCl_3$–MeOH–H_2O (8:5:1) and their Retention factors were TLC *Rf* (glucose) 0.30, *Rf* (xylose) 0.45, *Rf* (fucose) 0.49 *Rf* (rhamnose) 0.50 and *Rf* (arabinose) 0.56. The absolute configuration of the aforementioned monosaccharides was determined to be D for Fuc, Glc and Xyl, and L for Ara and Rha by GC analysis using the method previously described [33].

4.5. Data of the isolated saponins

4.5.1. Guineenoside A

White amorphous powder; $[\alpha]_D^{25}$ −27.8 (c 0.16, MeOH); ^{1}H NMR (600 MHz, CD_3OD), see Tables 1–3; and ^{13}C NMR (150 MHz, CD_3OD), see Tables 1–3; Positive HRESIMS *m/z*: 1909.8470 $[M+H]^+$ (calcd for $C_{86}H_{141}O_{46}$, 1909.8694).

4.5.2. Guineenoside B

White amorphous powder; $[\alpha]_D^{25}$ −24.9 (c 0.16, MeOH); ^{1}H NMR (600 MHz, CD_3OD), see Tables 1–3; ^{13}C NMR (150 MHz, CD_3OD), see Tables 1–3; Positive HRESIMS *m/z*: 2071.9383 $[M+H]^+$ (calcd for $C_{92}H_{151}O_{51}$, 2071.9222).

4.5.3. Guineenoside C

White amorphous powder; $[\alpha]_D^{25}$ −29.2 (c 0.16, MeOH); ^{1}H NMR (600 MHz, CD_3OD), see Tables 1–3; ^{13}C NMR (150 MHz, CD_3OD), see Tables 1–3; Positive HRESIMS *m/z*: 1375.6485 $[M+Na]^+$ (calcd for $C_{64}H_{104}O_{30}Na$, 1375.6505).

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2021.108374.

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