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

Eutrophe Le Doux Kamto^{a,b,c,*}, Olivier Placide Noté^{b,d,*}, Mc Jesus Kinyok^e, Anke Wilhelm^a, Joséphine Ngo Mbing^b, Cyril Antheaume^f, Alex de Théodore Atchadé^b, Dieudonné Emmanuel Pegnyemb^b, Derek Tantoh Ndinteh^c, Mohamed Haddad^d, Susan L. Bonnet^a

Affiliation

^aDepartment of Chemistry, University of the Free State, 205 Nelson Mandela Avenue, Bloemfontein 9301, South Africa

^bDepartment of Organic Chemistry, Faculty of Science, University of Yaounde 1, P.O. Box 812 Yaounde, Cameroon

^cDepartment of Chemical Sciences, Faculty of Science, University of Johannesburg, Doornfontein 2028, South Africa

^dUMR 152 Pharma Dev, Université de Toulouse, IRD, UPS, France

^eDepartment of Chemistry, Higher Teachers Training College, University of Yaounde 1, Yaounde, Cameroon

^fInstitut de Science et d'Ingénierie Supramoléculaire, Laboratoire de Chimie Supra Moléculaire (Prof. Lehn), 8 allée Gaspard Monge, BP 70028, F-67083, Strasbourg Cedex, France

Dedication

¹This 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.

*Correspondence

Dr. Eutrophe Le Doux Kamto

Department of Organic Chemistry, Faculty of Science, University of Yaounde 1, P.O. Box 812 Yaounde, Cameroon,

Phone.: +237 675 515 050;

Fax: +237 222 234 496 ;

lekamto@gmail.com

Pr. Olivier Placide Noté

Department of Organic Chemistry, Faculty of Science, University of Yaounde 1, P.O. Box 812 Yaounde, Cameroon,

Phone: +237 672 700 015;

Fax: +237 222 234 496

oliviernote1@yahoo.fr

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 (^1H , ^{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 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)-[α -L-arabinofuranosyl-(1 \rightarrow 4)]- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-fucopyranosyl} polygalacic acid 28-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl ester (**1**), 3-*O*-{ α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)-[α -L-arabinofuranosyl-(1 \rightarrow 4)]- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-fucopyranosyl} polygalacic acid 28-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl ester (**2**), and 3-*O*-{ α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranosyl-(1 \rightarrow 3)- β -D-fucopyranosyl} polygalacic acid 28-*O*-[α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl ester (**3**). This is the first report of triterpenoid saponins from *P. guineense*.

Keywords: *Piperaceae*; *Piper guineense*; *Triterpenoid saponins*; *NMR*; *Guineenosides*.

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 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, antifungal, 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 C₈₆H₁₄₀O₄₆ was established from an (+)HR-ESI-MS ion peak at *m/z* 1909.8470 [M+H]⁺ (calcd for C₈₆H₁₄₁O₄₆, 1909.8694, Δ=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 ¹H 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 ¹³C-NMR spectrum showed two olefinic carbon signals at δ 124.0 and 144.0, suggesting that **1** had an olean-12-ene

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

The ^1H 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 D)], 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 ^1H 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 $^3J_{\text{H-1,H-2}}$ 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 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 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)-[α -L-arabinofuranosyl-(1 \rightarrow 4)]- α -L-rhamnopyranosyl-(1 \rightarrow 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 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl. Consequently, the structure of guineenoside A (**1**) was established as 3-*O*-{ α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)-[α -L-arabinofuranosyl-(1 \rightarrow 4)]- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-fucopyranosyl}polygalacic acid 28-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 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, $\Delta=7.7$ ppm). As previously described for **1**, acid hydrolysis of **2** also afforded a polygalactic 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 1H and ^{13}C NMR data of the aglycone part of **2** were the same as for compound **1** (polygalactic 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 polygalactic acid derivative with sugar chains linked to C-3 and C-28 through an ether and ester bond, respectively. The 1H 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 D)], 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 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 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 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)-[α -L-arabinofuranosyl-(1 \rightarrow 4)]- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-fucopyranosyl} polygalacic acid 28-*O*- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 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 C₆₄H₁₀₄O₃₀Na, 1375.6505, Δ =1.4 ppm) corresponding to the molecular formula C₆₄H₁₀₄O₃₀. Of the 64 carbon signals, 30 were assigned to the aglycone and 34 to the saccharide signals. A quick inspection of the ¹H and ¹³C 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 ¹H and ¹³C NMR spectroscopic data, as well as the HMBC et NOESY correlations. The trisaccharide was recognized as α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- β -D-xylopyranoside (Table 3).

Similarly to **1** and **2**, the fucose unit ($\delta_{\text{H}}/\delta_{\text{C}}$ 4.88/104.1) in **3** was firstly attached to C-3 of the aglycone ($\delta_{\text{H}}/\delta_{\text{C}}$ 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 Xyl I, allowed us to locate Rha I at C-2 of Xyl I. Thus, the trisaccharide α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranosyl-(1 \rightarrow 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 \rightarrow 2)- β -D-xylopyranosyl-(1 \rightarrow 3)- β -D-fucopyranosyl} polygalacic acid 28-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 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], *Crocasmia* (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. ¹H NMR (600 MHz) and ¹³C NMR (150 MHz) spectra were recorded at room temperature in methanol-d₄ 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 μ L) and column oven (40 °C). The separations were performed using a Synergi MAX-RP 100A (50x2 mm, 2.5 μ particle size) with a H₂O (+0.1 % HCOOH) (A)/acetonitrile (+0.1 % HCOOH) (B) gradient (flow rate 500 μ L/min, injection volume 5 μ L). 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 x 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 F₂₅₄, Merck) using the system solvent n-BuOH-AcOH-H₂O, 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 H₂SO₄ 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.

4.4. 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 H₂O, 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 CHCl₃-MeOH-H₂O (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 CHCl₃-MeOH-H₂O (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 CHCl₃-MeOH-H₂O (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 CHCl₃-MeOH-H₂O (70:30:5).

4.4. Acidic hydrolysis

Each saponin (2 mg) was hydrolyzed with 2 N aqueous CF₃COOH (5 mL) at 100 °C during 2 h. After cooling, the reaction mixture was diluted with H₂O (10 mL) and extracted with CH₂Cl₂ (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 CHCl₃-MeOH-H₂O (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); ^1H 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 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{86}\text{H}_{141}\text{O}_{46}$, 1909.8694).

4.5.2. *Guineenoside B*

White amorphous powder; $[\alpha]_D^{25}$ -24.9 (c 0.16, MeOH); ^1H 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 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{92}\text{H}_{151}\text{O}_{51}$, 2071.9222).

4.5.3. *Guineenoside C*

White amorphous powder; $[\alpha]_D^{25}$ -29.2 (c 0.16, MeOH); ^1H 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 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{64}\text{H}_{104}\text{O}_{30}\text{Na}$, 1375.6505).

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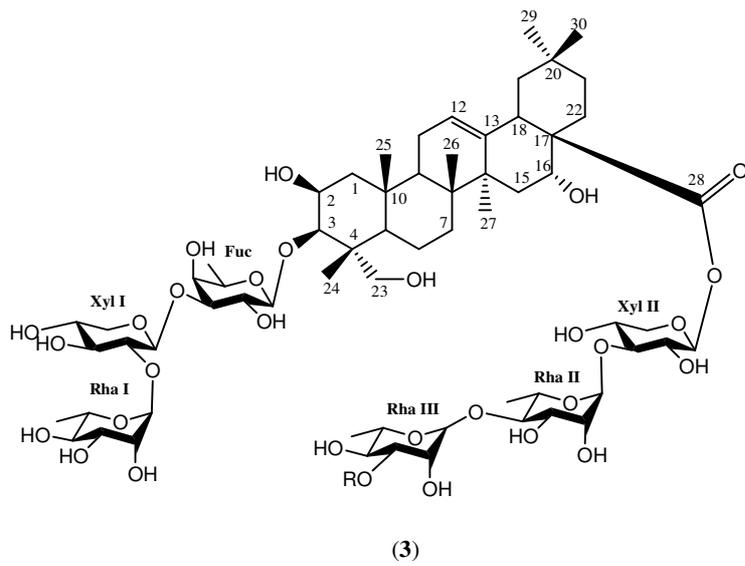
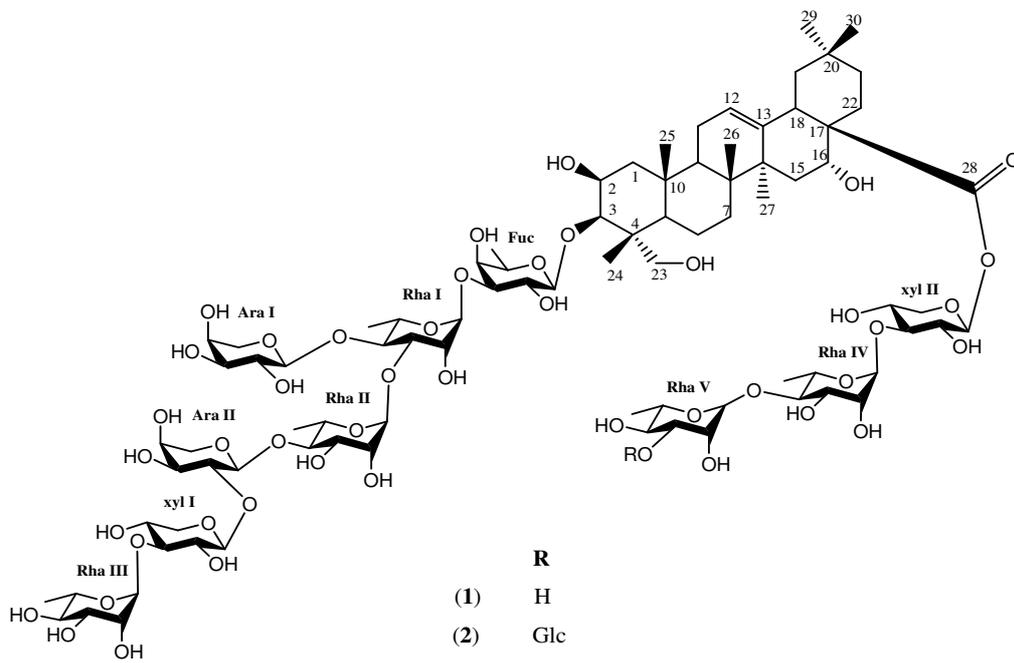


Fig. 1. Structures of compounds 1–3

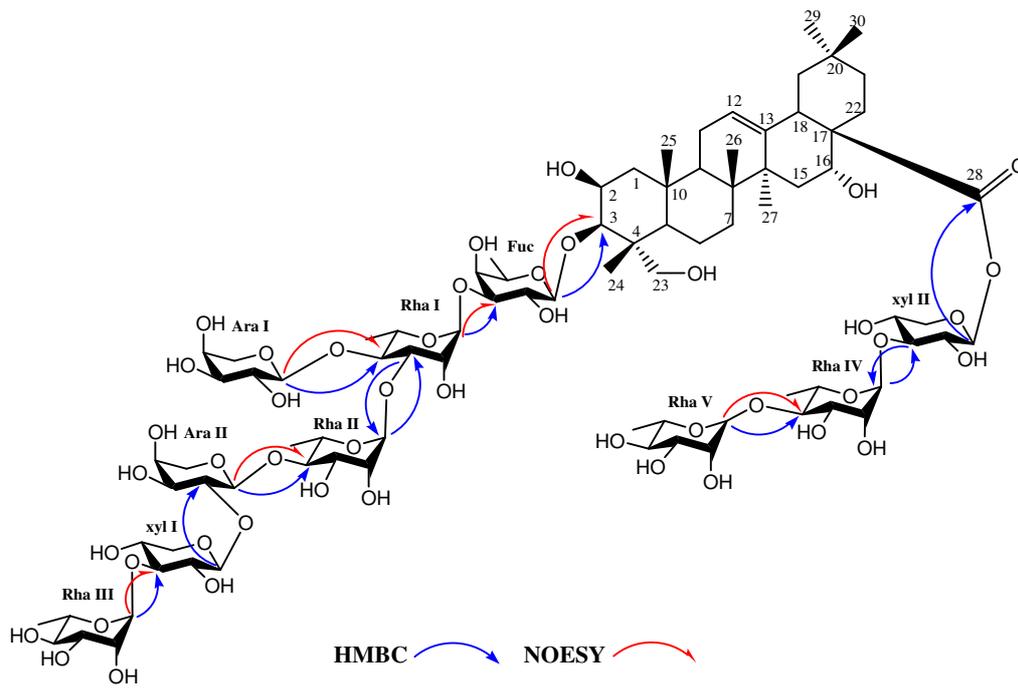


Fig. 2. Key HMBC and NOESY correlations of **1**.

Table 1. NMR spectroscopic data (600 MHz for ^1H 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 ^1H 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- <i>O</i> -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	63.8	3.28; 4.16 dd	63.7		

(4.6, 11.7)

(4.3, 11.4)

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 ¹H and 150 MHz for ¹³C) for the sugar moieties attached at C-28 of compounds **1–3** (δ in ppm and *J* in Hz)^a in methanol-d₄.

Position	1		2		3	
	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}	δ_{H} (<i>J</i> in Hz)	δ_{C}
28- <i>O</i> -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.

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

Eutrophe Le Doux Kamto^{a,b,c,*}, Olivier Placide Noté^{b,d,*}, Mc Jesus Kinyok^e, Anke Wilhelm^a, Joséphine Ngo Mbing^b, Cyril Antheaume^f, Alex de Théodore Atchadé^b, Dieudonné Emmanuel Pegnyemb^b, Derek Tantoh Ndinteh^c, Mohamed Haddad^d, Susan L. Bonnet^a

Graphical abstract



Branches of *Piper guineense*

