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Triterpenoid saponins from *Calliandra calothyrsus* Meisn. and their antiproliferative activity against two digestive carcinoma human cell lines

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ABSTRACT

The chemical investigation of the flowers and twigs of *Calliandra calothyrsus* (Fabaceae) led to the isolation of three new oleanane-type triterpenoid saponins, named calothyrsusosides A–C (1–3). Their structures were established by direct interpretation of their spectral data, mainly HRESIMS, 1D NMR and 2D NMR (^1H , ^1H -NMR DOSY, ^{13}C -NMR, COSY, HSQC, HMBC, HSQC-TOCSY and NOESY) and by comparison with literature data. Compounds **1** and **2** were tested for their antiproliferative activity against two digestive carcinoma human cell lines: Hep3B (hepatocellular carcinoma) and Caco-2 (epithelial colorectal adenocarcinoma). Both compounds exhibited an antiproliferative activity against the Hep3B cell line, with IC_{50} values of 6.0 and 6.5 μM , respectively, while no effect was detected against the epithelial colorectal adenocarcinoma Caco-2 ($\text{CC}_{50} > 25 \mu\text{M}$).

Keywords: *Calliandra calothyrsus*; *Fabaceae* ; *Triterpenoid saponins*; *Calothyrsusoside*; *cytotoxicity*

1. Introduction

The genus *Calliandra* (Fabaceae) is made up of about 140 species native to tropical and subtropical regions of the Americas. *Calliandra calothyrsus* Meisn. is a small leguminous tree of the Fabaceae family that is widely distributed throughout Central America and southern Mexico [1,2]. It is usually 13 to 20 feet (4 to 6 m) high but might reach 40 feet (12 m) under favorable conditions. The leaves are suitable for forage and give a higher yield than other shrub legumes, especially when grown on acid soil [3]. It has been introduced into many tropical regions where it is used in agroforestry systems for fuelwood, plantation shade, as an intercropping hedgerow and more recently as livestock forage. Acetonic extracts of the leaves of *C. calothyrsus* exhibited *in vitro* negative effects on eggs and larvae of *Haemonchus contortus* parasite of ruminants [4]. Previous phytochemical studies of *C. calothyrsus* revealed the presence of glycosylflavonoids of its leaves [5]. No previous phytochemical study has been reported on saponins from this plant, but studies on related species of this genus revealed the presence of triterpenoid saponins, consisting mainly of glycosides of oleanolic acid and echinocystic acid as aglycon with an Ara-²Ara-⁶GlcNAc-³Agly oligosaccharidic sequence commonly encountered [6-12]. In this paper, we describe the isolation and structure elucidation of three new triterpenoid saponins designated as calothyrsusosides A-C (1-3). Compounds **1** and **2** were tested for their antiproliferative effects on two digestive cancer cell lines, i.e. Hep3B (hepatocellular carcinoma) and Caco-2 (epithelial colorectal adenocarcinoma).

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a Jasco P-2000 polarimeter. NMR experiments were performed at 298 K in pyridine-d₅ and D₆MSO on a Bruker AVANCE 500MHz spectrometer equipped with a 5 mm Z-gradient TCI cryoprobe. HRESIMS spectra were recorded using a UHPLC-DAD-LTQ Orbitrap XL instrument (Thermo Fisher Scientific, UK) equipped with an

electrospray ionization source (ESI). Isolations were carried out using a medium-pressure liquid chromatography (MPLC) system [Buchi Pump manager C-615, Buchi Pump module C-605, Column Buchi, borosilikat (460 x 25 mm and 460 x 15 mm)] and column chromatography (CC) on reversed-phase RP-18 silica gel 60 (25-40 μm). Thin layer chromatography (TLC) was performed on precoated silica gel plates (60 F₂₅₄, Merck) using the system solvent *n*-BuOH-AcOH-H₂O, 60:15:25 as eluent. Vacuum-liquid chromatography (VLC) was carried out using RP-18 silica gel 60 (25-40 μm) and silica gel 60 (15-40 μm and 40-63 μm). The spray reagent for saponins was vanilin reagent (10% mixture of conc. H₂SO₄ soln. and 1% vanilin in EtOH).

2.2 Plant Material

The flowers and branches of *Calliandra calothyrsus* Meisn. were harvested at Nkolbisson, Yaoundé peripheral quarter, in Cameroon in June 2017 under the guidance of Mr. Paul Nana, botanist of the National Herbarium of Cameroon (NHC), where one specimen (52065/HNC) was deposited.

2.3. Extraction and Isolation

The methanolic extract (122 g) of the flowers of *C. calothyrsus* (750 g) was suspended in 200 mL of H₂O and partitioned successively with hexane, ethyl acetate and *n*-BuOH sat.H₂O. The *n*-BuOH-soluble fraction (23.5 g) was precipitated in 400 mL H₂O for 48h to separate the saponins from the tannins. After filtration, the *n*-BuOH soluble phase was evaporated to dryness to afford (6g) brown gum residue and dissolved in 10 mL of H₂O and then submitted to vacuum-liquid chromatography (VLC) using RP-18 silica gel 60 (25-40 μm) eluted with a gradient of MeOH-H₂O (100% H₂O to 100%MeOH). The 65-85% MeOH fraction (3.32 g) was subjected to MPLC using silica gel 60 (15-40 μm) eluted with gradient system of CHCl₃-MeOH-H₂O (70:30:5 and 65:35:10, lower phase) to give six main subfractions (B1-B6). Subfraction B6 (1.7 g) was purified by MPLC using gradient system (70:30:5 and 65:35:10, lower phase) to give ten other

subfractions Fr1 to Fr 10. Fr 7 (113 mg) and Fr 8 (150 mg) were purified by MPLC using silica gel 60 (15-40 μm) eluted with gradient system of CHCl_3 -MeOH- H_2O (70:30:5 and 65:35:10, lower phase) to give **1** (19.7 mg) and **2** (30.0 mg).

The methanolic extract (10 g) of the branches of *C. calothyrsus* (300 g) was suspended in 200 mL of H_2O and partitioned successively with hexane, ethyl acetate and *n*-BuOH sat. H_2O . The *n*-BuOH-soluble fraction (1.8 g) were dissolved in 10 mL of H_2O and then submitted to vacuum-liquid chromatography (VLC) using RP-18 silica gel 60 (25-40 μm) eluted with a gradient of MeOH- H_2O (100% H_2O →100%MeOH). The 65-85% MeOH fraction (500 mg) was subjected to MPLC using silica gel 60 (15-40 μm) eluted with gradient system of CHCl_3 -MeOH- H_2O (70:30:5 and 65:35:10, lower phase) to give eleven main subfractions (F1-F11). Subfraction F9 (20 mg) was purified by CC using reversed-phase RP-18 silica gel 60 (25-40 μm) eluted with isocratic system MeOH- H_2O (60:40) to give compound **3** (3 mg).

2.3.1. *Calothyrsusoside A (1)*

White amorphous powder; ^1H NMR ($\text{C}_5\text{D}_5\text{N}$, 500 MHz) and ^{13}C NMR ($\text{C}_5\text{D}_5\text{N}$, 125 MHz) data, see [Table 1 and 2](#); HR-ESI-MS m/z 1974.8572 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{87}\text{H}_{141}\text{NO}_{47}\text{Na}^+$, 1974.8571).

2.3.2. *Calothyrsusoside B (2)*

White amorphous powder; ^1H NMR ($\text{C}_5\text{D}_5\text{N}$, 500 MHz) and ^{13}C NMR ($\text{C}_5\text{D}_5\text{N}$, 125 MHz), see [Table 1 and 2](#); HR-ESI-MS: m/z 1812,8122 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{81}\text{H}_{131}\text{NO}_{42} \text{Na}^+$, 1812.8043).

2.3.3. *Calothyrsusoside C (3)*

White amorphous powder; ^1H NMR (D_6MSO , 500 MHz) and ^{13}C NMR (D_6MSO , 125 MHz), see [Table 1 and 2](#); HR-ESI-MS: m/z 1872.8255 $[\text{M} + \text{Na}]^+$ (calcd 1872.8254 for $\text{C}_{83}\text{H}_{135}\text{NO}_{44}\text{Na}^+$).

2.4. Acid hydrolysis and sugar analysis

Each saponin (2 mg in MeOH) was hydrolyzed with 2 ml of 2 M HCl at 85 °C during 2 h. After cooling, the solvent was removed under reduced pressure. The sugar mixture was extracted from the aqueous phase (10 ml) and washed with CH₂Cl₂ (3x 5 ml). The combined CH₂Cl₂ extracts were washed with water to give after evaporation the aglycone moiety. The sugars were first analyzed by TLC over silica gel (CHCl₃–MeOH–H₂O, 8:5:1) by comparison with standard samples. The absolute configuration of each monosaccharide was determined from GC–MS analysis of their trimethylsilylated derivatives by comparison with authentic samples using the method previously described [13]. The following sugars were detected: D-glucose, D-xylose, L-arabinose, L-rhamnose and N-acetylglucosamine.

2.5. Antiproliferative bioassays

The antiproliferative activity of compounds **1** and **2** was assessed against the human hepatocarcinoma cell line Hep3B. Hep3B cells were grown in a sterile medium DMEM supplemented with 10% of heat inactivated fetal bovine serum, L-glutamine, sodium pyruvate and non-essential amino acids, at 37°C in humidified 5% CO₂ atmosphere. Hep3B cells were seeded as 5000 cells per well in 96-well microplates. After 24h, Hep3B cells were incubated for 48h with the isolated compounds with a concentration range from 0.001 to 100 µg/mL containing 0.1% of DMSO. The antiproliferative activity was then evaluated by luminescence using the ATPlite assay (Perkin Elmer) according to the previous described method [14]. Inhibitory concentrations at 50% (IC₅₀) of cell growth were calculated with the GraphPad Prism 6.0 software, compiling 3 independent experiments.

The evaluation of the cytotoxicity of compounds **1** and **2** against the Caco-2 cell line (human epithelial cell line, Sigma-Aldrich) was done according to Mosmann method with slight modifications. Briefly, cells (1.10⁵ cells/mL) in 100 µL of complete medium, [DMEM High Glucose supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1X NEAA and antibiotics (100U/mL penicillin and 100 µg/mL streptomycin)] were seeded into each well of 96-

well plates and incubated at 37 °C in a humidified 5% CO₂ with 95% air atmosphere. After a 24 h incubation, 100 µL of medium with various compound's concentrations and appropriate controls (DMSO, 0.5%final) were added and the plates were incubated for 72 h at 37 °C. Each plate-well was then microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT solution (0.5 mg/mL in DMEM) were then added to each well. Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was removed and DMSO (100 µL) was added to dissolve the resulting formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with a microplate spectrophotometer. DMSO was used as blank and doxorubicin (purchased from Sigma Aldrich) as positive control. CC₅₀ were calculated by non-linear regression analysis processed on dose–response curves, using Table Curve 2D V5 software. CC₅₀ values represent the mean value calculated from three independent experiments.

3. Results and discussion

The methanolic extract of the flowers and branches of *C. calothyrsus* was purified by multiple chromatographic steps over silica gel and reversed phase. This purification yielded three undescribed saponins, calothyrsusoside A (**1**), calothyrsusoside B (**2**) and calothyrsusoside C (**3**) (Fig. 1). Their structures were established by extensive analysis of NMR techniques, mainly 1D NMR (¹H, ¹³C) and 2D NMR (COSY, NOESY, HSQC, HSQC-TOCSY and HMBC) experiments, HRESIMS and by comparison with the literature data.

Compound **1** was obtained as a white amorphous powder. The HRESIMS (positive-ion mode) exhibited a pseudomolecular ion peak at m/z 1974.8572 [M+Na]⁺, (calcd for C₈₇H₁₄₁NO₄₇Na 1974.8571) indicating a molecular formula of C₈₇H₁₄₁NO₄₇. Extensive analysis of 1D and 2D-NMR spectra (¹H-NMR, ¹H-NMR DOSY, ¹³C-JMOD, COSY, NOESY, HSQC, TOCSY and HMBC) indicated the presence of seven tertiary methyl groups at δ_H 0.94 (s, H-25), 1.02 (s, H-29), 1.03 (s, H-30), 1.05 (s, H-24), 1.17 (s, H-26), 1.27 (s, H-23) and 1.87 (s, H-27) showing correlations in the HSQC spectrum with their corresponding carbons at δ_C 15.5 (C-25),

33.0 (C-29), 24.4 (C-30), 16.9 (C-24), 17.2 (C-26), 27.9 (C-23) and 27.1 (C-27), respectively. Furthermore, other characteristic signals were observed such as one olefinic proton at δ_H 5.67 (1H, brs, H-12) showing HSQC correlation with δ_C 122.4 (C-12) and one quaternary carbon at δ_C 144.6 (C-13) and one oxymethine protons at δ_H 3.40 (brd, $J=13.7$ Hz, H-3), in accordance with the typical signals of an olean-12-ene skeleton. The downfield position of the axial group at C-14 (Me-27, δ_C 1.87) in the 1H NMR spectrum, implied an additional axial (α) hydroxyl group at C-16. The locations of oxygenated methines were deduced to be at C-3 and C-16 also by HMBC cross peaks at δ_H 1.27 (H-23)/ δ_C 89.9 (C-3), and COSY cross peak at δ_H 2.04 (H-15)/ δ_H 5.24 (H-16). NOESY cross peaks at δ_H 3.40 (H-3)/ δ_H 0.88 (d, $J = 12.5$ Hz, H-5), δ_H 2.04 (H-15)/ δ_H 1.17 (H-26), δ_H 4.61 (H-16)/ δ_H 2.04 (H-15), revealed the α -orientation of H-3 and β -orientation of H-16, confirming the stereochemistry of the aglycone. The aglycone moiety of **1** was thus recognized to be echinocystic acid by 1H NMR and ^{13}C NMR analyses (Table 1) using the correlations observed in COSY, NOESY, HSQC, and HMBC spectra, and was in full agreement with literature data [15-17]. The chemical shifts of C-3 (δ_C 89.9) and C-28 (δ_C 176.3) (Table 1) indicated that **1** is a bidesmosidic glycoside [18-20] of echinocystic acid with sugar chains linked to C-3 and C-28 of the aglycone through an an glycosidic bond and acylglycosidic bond, respectively. The sugar moieties of **1** were determined to be D-glucose (Glc), L-rhamnose (Rha), D-acetamidoglucose (GlcNAc), D-xylose (Xyl) and L-arabinose (Ara) by acid hydrolysis followed by GC analysis of their trimethylsilylated derivatives and comparison with authentic samples (Section 2). The 1H NMR spectrum of the sugar portion of compound **1** showed ten anomeric signals at δ_H 5.11 (d, $J=7.1$ Hz, GlcNAc H-1), 5.06 (d, $J = 6.2$ Hz, Ara I H-1), 5.04 (d, $J = 7.5$ Hz, Ara II H-1), 5.13 (d, $J = 6.7$ Hz, Ara III H-1), 4.99 (d, $J = 7.5$ Hz, Xyl I H-1), 6.07 (d, $J = 7.8$ Hz, Glc I H-1), 5.89 (brs, Rha H-1), 5.54 (d, $J = 7.5$ Hz, Xyl II H-1), 5.23 (d, $J = 7.9$ Hz, Xyl III H-1) and 5.35 (d, $J = 7.8$ Hz, Glc II H-1), which correlated with ten anomeric carbon atom resonances at δ_C 104.4, 102.2, 105.2, 102.0, 105.4, 94.8, 101.7, 103.9, 105.4 and 104.7, respectively in the HSQC spectrum (Table 2). All proton signals of the sugar moieties were identified by careful analysis of the

COSY, HSQC-TOCSY and NOESY spectra. Carbon assignment was based on HSQC and further confirmed by HMBC spectrum (Table 2). The sugar unit with anomeric signal at δ_H 5.11 ($J=7.1$ Hz) was assigned to a 2-acetamido-2-deoxy- β -D-glucose unit. The characteristic signals were at δ_H 9.28 (d, $J = 8.0$ Hz, NHAc) and at δ_H 2.35 (s, NHCO-CH₃) [15]. This was supported by the HMBC correlation observed between δ_H 4.61 (H-2) of GlcNAc and the carbonyl signal at δ_C 172.3 (NH-CO-CH₃). Data from the above experiments indicated that Glc, GlcNAc, Xyl and Ara were in their pyranose form. The anomeric proton of Xyl I, II and III was determined to have the β -orientation based on its relatively large $^3J_{H-1, H-2}$ value 7.5, 7.6 and 7.9 Hz, respectively. The remaining anomeric protons of Glc, GlcNAc were determined to have β anomeric configuration and α anomeric configuration for Ara, based on their relative large $^3J_{H-1, H-2}$ values (6.2 -7.5 Hz) in their pyranose form. The sequencing of the glycoside chains was achieved by analysis of HMBC and NOESY experiments. The cross-peak correlations observed in the HMBC correlations between H-1 of GlcNAc (δ_H 5.11 $J=7.1$ Hz) and C-3 of the aglycone (δ_C 89.9), and in the NOESY spectrum between H-1 of GlcNAc (δ_H 5.11, $J=7.1$ Hz) and H-3 of echinocystic acid (δ_H 3.40, $J=13.7$ Hz), suggested that GlcNAc is directly attached to C-3 of the aglycone. Moreover, the HMBC correlation observed between H-1 of Ara I (δ_H 5.06, $J=6.2$ Hz) and C-6 of GlcNAc (δ_C 69.0) established the connectivity between the two sugar units, which was confirmed by the NOESY correlation observed between H-6 of GlcNAc (δ_H 4.60) and H-1 of Ara I (δ_H 5.06, $J=6.2$ Hz). HMBC correlation observed between H-1 of Ara II (δ_H 5.04, $J=7.5$ Hz) and C-2 of Ara I (δ_C 79.5) allowed us to locate Ara II at C-2 of Ara I. This was supported by the NOESY correlation observed between H-1 of Ara II (δ_H 5.04, $J=7.5$ Hz) and H-2 of Ara I (δ_H 4.54). This Ara II was substituted at its C-2 by Ara III, as evidenced by the direct and reverse correlations observed in the HMBC spectrum between H-1 of Ara III (δ_H 5.13, $J=6.7$ Hz) and C-2 of Ara II (δ_C 79.8) and between H-2 of Ara II (δ_H 4.50) and C-1 of Ara III (δ_C 102.0). This was supported by the NOESY correlation observed between H-1 of Ara III (δ_H 5.13 $J=6.7$ Hz) and H-2 of Ara II (δ_H 4.50). Furthermore, HMBC correlation observed between H-1 of Xyl I (δ_H 4.99, $J = 7.5$ Hz) and C-2 of

Ara III (δ_C 79.8) allowed us to locate Xyl I at C-2 of Ara III. This was supported by the NOESY correlation observed between H-1 of Xyl I (δ_H 4.99, $J = 7.5$ Hz) and H-2 of Ara III (δ_H 4.52). The terminal position of Xyl I was evidenced by the absence of any ^{13}C NMR glycosylation shifts for this sugar moiety. Thus, the pentasaccharide β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl-(1 \rightarrow 6)-2-acetamido-2-deoxy- β -D-glucopyranosyl 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 6.07/ δ_C 94.8 (Glc I H-1/C-1) suggested that this sugar should be directly attached to C-28 of the aglycone through an acylglycosidic bond. Moreover, the HMBC correlation observed between H-1 (δ_H 6.07, $J = 7.8$ Hz) of Glc I and C-28 (δ_C 176.3) of the aglycone supported this location. The direct correlation observed in the HMBC spectrum between H-1 of Rha (δ_H 5.89) and C-2 of Glc I (δ_C 77.7) and the NOESY correlation observed between H-1 of Rha (δ_H 5.89) and H-2 of Glc I (δ_H 4.09) allowed us to locate Rha at C-2 of Glc I. Moreover, the HMBC correlation observed between H-1 of Xyl II (δ_H 5.54, $J = 7.5$ Hz) and C-4 of Rha (δ_C 77.9) permitted us to attach Xyl II at C-4 of Rha. This was supported by the NOESY correlation observed between H-1 of Xyl II (δ_H 5.54 $J = 7.5$ Hz) and H-4 of Rha (δ_H 4.07). The direct and reverse correlations observed in the HMBC spectrum between H-1 of Xyl III (δ_H 5.23, $J = 7.9$ Hz) and C-3 of Xyl II (δ_C 87.4) and between H-3 of Xyl II (δ_H 4.19) and C-1 of Xyl III (δ_C 105.4), and the NOESY correlation observed between H-1 of Xyl III (δ_H 5.23, $J = 7.9$ Hz) and H-3 of Xyl II (δ_H 4.19) allowed us to locate Xyl III at C-3 of Xyl II. The terminal position of Xyl III was evidenced by the absence of any ^{13}C NMR glycosylation shifts for this sugar moiety. Moreover, the HMBC correlation observed between H-1 (δ_H 5.35, $J = 7.6$ Hz) of Glc II and C-3 (δ_C 82.3) of Rha permitted us to attach Glc II at C-3 of Rha. Thus, the pentasaccharide β -D-xylopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 4)[- β -D-glucopyranosyl-(1 \rightarrow 3)]- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-glucopyranosyl moiety was established to be attached at C-28 of the aglycone (Fig. 2). Based on the above evidences, the structure of compound **1** was established as 3-O-[β -D-xylopyranosyl-

(1→2)- α -L-arabinopyranosyl-(1→2)- α -L-arabinopyranosyl-(1→2)- α -L-arabinopyranosyl-(1→6)]-2-acetamido-2-deoxy- β -D-glucopyranosyl] echinocystic acid 28-O-[β -D-xylopyranosyl-(1→3)- β -D-xylopyranosyl-(1→4)]- β -D-glucopyranosyl-(1→3)]- α -L-rhamnopyranosyl-(1→2)- β -D-glucopyranosyl ester.

Compound **2** was obtained as a white amorphous powder. The HRESIMS (positive-ion mode) exhibited a pseudomolecular ion peak at m/z 1812,8122 [$M + Na$]⁺ (calcd for C₈₁H₁₃₁NO₄₂Na, 1812.8043) indicating a molecular formula of C₈₁H₁₃₁NO₄₂. Analysis of the ¹H- and ¹³C-NMR spectra of **2** and comparison with compound **1** and literature data allowed the identification of echinocystic acid as aglycone (Table 1). The presence of nine monosaccharide units was suggested by the nine anomeric protons at δ_H 5.05 [d, $J = 7.8$ Hz, acetamidoglucose (GlcNAc)], 5.06 (d, $J = 4.6$ Hz, Ara I H-1), 5.02 (d, $J = 6.8$ Hz, Ara II H-1), 5.13 (d, $J = 5.1$ Hz, Ara III H-1), 4.96 (d, $J = 7.5$ Hz, Xyl I H-1), 6.12 (d, $J = 7.8$ Hz, Glc I H-1), 6.35 (brs, Rha H-1), 5.20 (d, $J = 7.5$ Hz, Xyl II H-1), and 5.14 (d, $J = 7.0$ Hz, Xyl III H-1), which correlated in the HSQC spectrum with δ_C 104.6, 102.2, 105.5, 102.2, 105.7, 94.6, 101.0, 105.8, and 105.6, respectively. Comparison of the NMR spectra of compounds **1** and **2** revealed that the sugar moiety at C-3 was identical to that of **1**. These two compounds differ slightly only by the sugar chain attached at C-28 of the aglycone, which showed the disappearance of the signals of the glucopyranosyl unit previously attached at C(3) of Rha (Table 2) (δ_C 82.3) in compound **1** as evidenced from the observation of the shielded signal (-10.2 ppm) of C-3 of Rha in compound **2** (δ_C 72.1) in comparison to C-3 of Rha (δ_C 82.3) in compound **1**. From these data and by comparison of the NMR data with literature values [6-9], the structure of **2** was elucidated as 3-O-[β -D-xylopyranosyl-(1→2)- α -L-arabinopyranosyl-(1→2)- α -L-arabinopyranosyl-(1→2)- α -L-arabinopyranosyl-(1→6)]-2-acetamido-2-deoxy- β -D-glucopyranosyl] echinocystic acid 28-O-[β -D-xylopyranosyl-(1→3)- β -D-xylopyranosyl-(1→3)- α -L-rhamnopyranosyl-(1→2)- β -D-glucopyranosyl ester.

Compound **3** was obtained as a white amorphous powder. Its molecular formula $C_{83}H_{135}NO_{44}$ was deduced from its HRESIMS in positive-ion mode which exhibited a pseudo-molecular ion peak $[M+Na]^+$ at m/z 1872.8255 (calcd 1872.8254 for $C_{83}H_{135}NO_{44}Na$). Comparison of the 1H and ^{13}C NMR signals due to the aglycone part of **3** to those of **1** and **2**, revealed that the aglycone of compound **3** was also echinocystic acid. The 1H NMR spectrum of the sugar portion of compound **3** showed nine anomeric signals at δ_H 4.29 [d, $J = 8.2$ Hz, acetamidoglucose (GlcNAc)], 4.48 (d, $J = 4.5$ Hz, Ara I H-1), 4.26 (d, $J = 7.3$ Hz, Ara II H-1), 4.42 (d, $J = 8.0$ Hz, Glc I H-1), 5.30 (d, $J = 7.6$ Hz, Glc II H-1), 4.95 (brs, Rha H-1), 4.67 (d, $J = 7.6$ Hz, Xyl I H-1), 4.39 (d, $J = 5.2$ Hz, Xyl II H-1) and 4.40 (d, $J = 7.8$ Hz, Glc II H-1), which correlated with nine anomeric carbon atom resonances at δ_C 103.8, 101.5, 105.1, 103.5, 93.7, 100.7, 103.2, 104.8 and 103.8, respectively in the HSQC spectrum (Table 2). The cross-peak correlations observed in the HMBC correlations between H-1 of GlcNAc (δ_H 4.29 $J=8.2$ Hz) and C-3 of the aglycone (δ_C 88.3) of echinocystic acid (δ_H 3.06, $J=13.6$ Hz), suggested that GlcNAc was directly attached to C-3 of the aglycone. Moreover, the HMBC correlation observed between H-1 of Ara I (δ_H 4.48, $J = 4.5$ Hz) and C-6 of GlcNAc (δ_C 68.4) established the connectivity between the two sugar units. HMBC correlation observed between H-1 of Ara II (δ_H 4.26, $J = 7.3$ Hz) and C-2 of Ara I (δ_C 79.4) allowed us to locate Ara II at C-2 of Ara I. The terminal position of Ara II was evidenced by the absence of any ^{13}C NMR glycosylation shifts for this sugar moiety. Moreover, the HMBC correlation observed between H-1 (δ_H 4.42, $J = 8.0$ Hz) of Glc I and C-3 (δ_C 77.5) of Ara I permitted us to attach Glc I at C-3 of Ara I. Thus, the tetrasaccharide α -L-arabinopyranosyl-(1 \rightarrow 2)[- β -D-glucopyranosyl-(1 \rightarrow 3)]- α -L-arabinopyranosyl-(1 \rightarrow 6)-2-acetamido-2-deoxy- β -D-glucopyranosyl moiety was established to be linked at C-3 of the aglycone (Fig 4). Moreover, comparison of the NMR spectra of compounds **1** and **3** revealed that the sugar moiety at C-28 was identical in both compounds. Thus, the tetrasaccharide β -D-xylopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 4)[β -D-glucopyranosyl-(1 \rightarrow 3)]- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -L-glucopyranosyl moiety was established to be attached at C-28 of the aglycone (Fig. 4). Based on

the above evidences, the structure of compound **3** was established as 3-O-[α -L-arabinopyranosyl-(1 \rightarrow 2)]- β -D-glucopyranosyl-(1 \rightarrow 3)]- α -L-arabinopyranosyl-(1 \rightarrow 6)-2-acetamido-2-deoxy- β -D-glucopyranosyl] echinocystic acid 28-O-[β -L-xylopyranosyl-(1 \rightarrow 3)- β -D-xylopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranosyl-(1 \rightarrow 3)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-glucopyranosyl ester.

Since compound **3** was not obtained in sufficient quantity to perform the biological tests, only compounds **1** and **2** were tested for their antiproliferative and cytotoxic effects on both human carcinoma cell lines Hep3B (hepatocellular carcinoma) and Caco-2 (epithelial colorectal adenocarcinoma), respectively (Table 3). Both compounds exhibited an antiproliferative activity against the Hep3B cell line, with IC₅₀ values of 6.0 and 6.5 μ M, respectively, which are in the range of the one of sorafenib, one of the most clinically used molecule [21]. However, none antiproliferative activity had been detected for the same compounds on the Caco-2 cell line (CC₅₀ > 25 μ M).

4. Conclusion

Three new oleanane-type triterpenoid saponins were isolated from the flowers and twigs of *Calliandra calothyrsus*. Their structures were established from spectral data, mainly HR-ESI-MS, 1D NMR, and 2D NMR and by comparison with the literature. Compounds **1** and **2** were evaluated for their antiproliferative activity against two human digestive cancer cell lines, i.e. Hep3B and Caco-2 cells. Both compounds exhibited a moderate antiproliferative activity against Hep3B cells, though in the range of the palliative molecule sorafenib. In contrast, none effect has been seen against the Caco-2 cells at the tested concentrations. As a perspective, we project to test compounds **1** and **2** against a wider range of cancer cell lines to assess their possible selectivity against hepatocarcinoma cells. This could contribute to identify specific molecular targets of this cancer type and promote the use of this plant for traditional medicine purpose. However, from a chemotaxonomic point of view, our results represent a valuable contribution to the knowledge of the chemistry of plants of the genus *Calliandra* of the Mimosaceae subfamily, some of which have

revealed the presence of oleanane-type triterpenoid saponins. These saponins present the N-acetylglucosamine directly attached to the C-3 of the aglycone as well as the sugar sequence Ara-²Ara-⁶GlcNAc-³Agly. These characteristics are consistently encountered in all the isolated saponins of the genus *Calliandra* [6-12]. Comparing our saponins with those previously isolated from the genus *Calliandra*, it appears that they share the same C-3 oligosaccharide sequence but with one and two additional sugars, respectively. This could therefore be a specificity of the saponins of the *Calliandra calothyrsus* species.

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Disclosure statement

The authors declare that there is no conflicting interest.

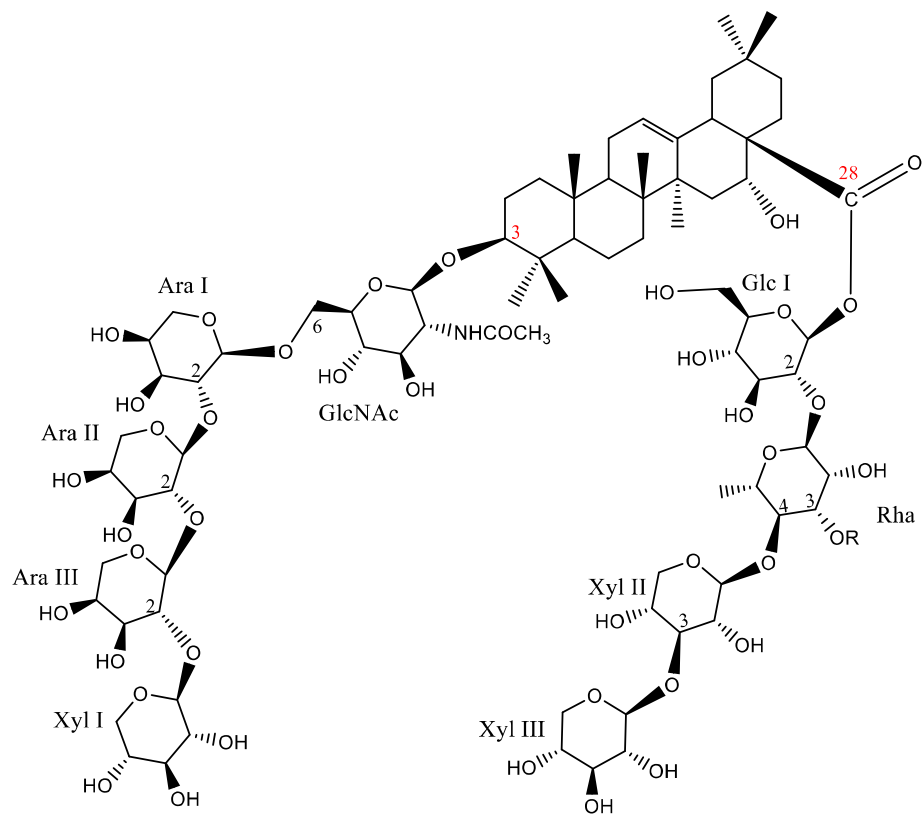
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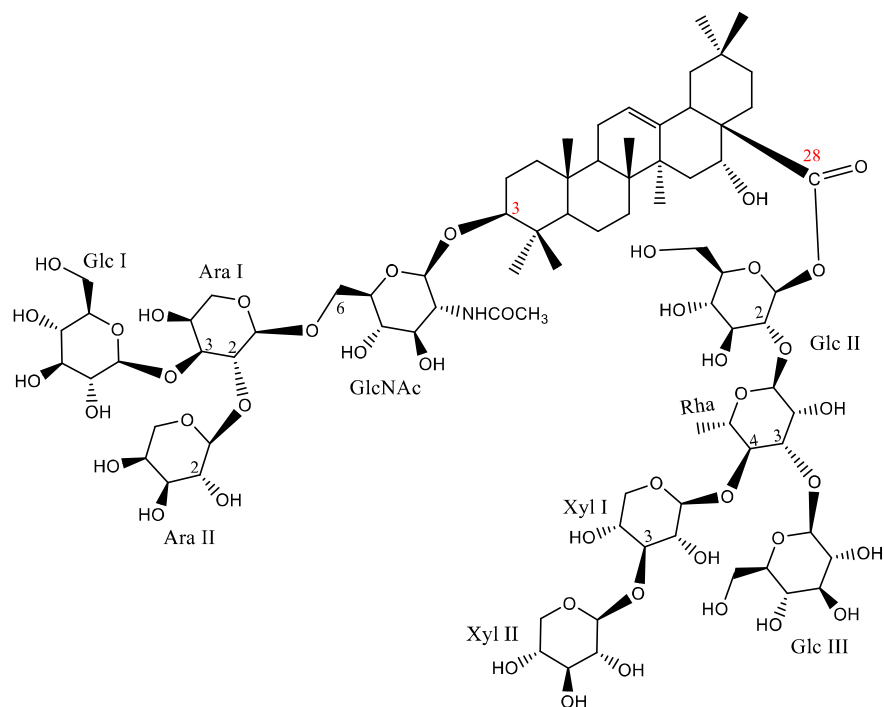
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R

1 Glc

2 H



3

Fig. 1. Chemical structures of compounds 1- 3.

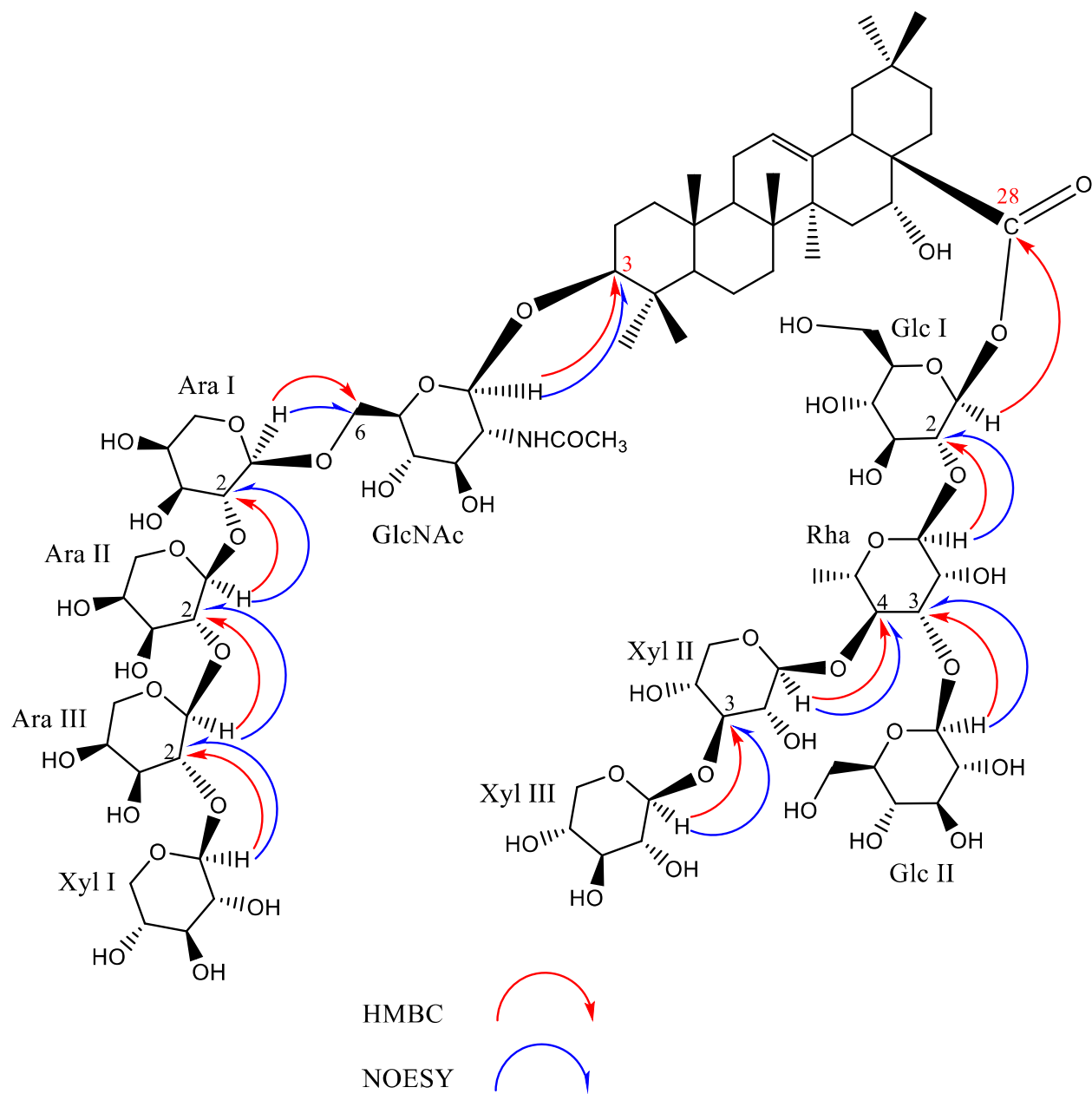


Fig. 2. Key HMBC and NOESY correlations of compound **1**

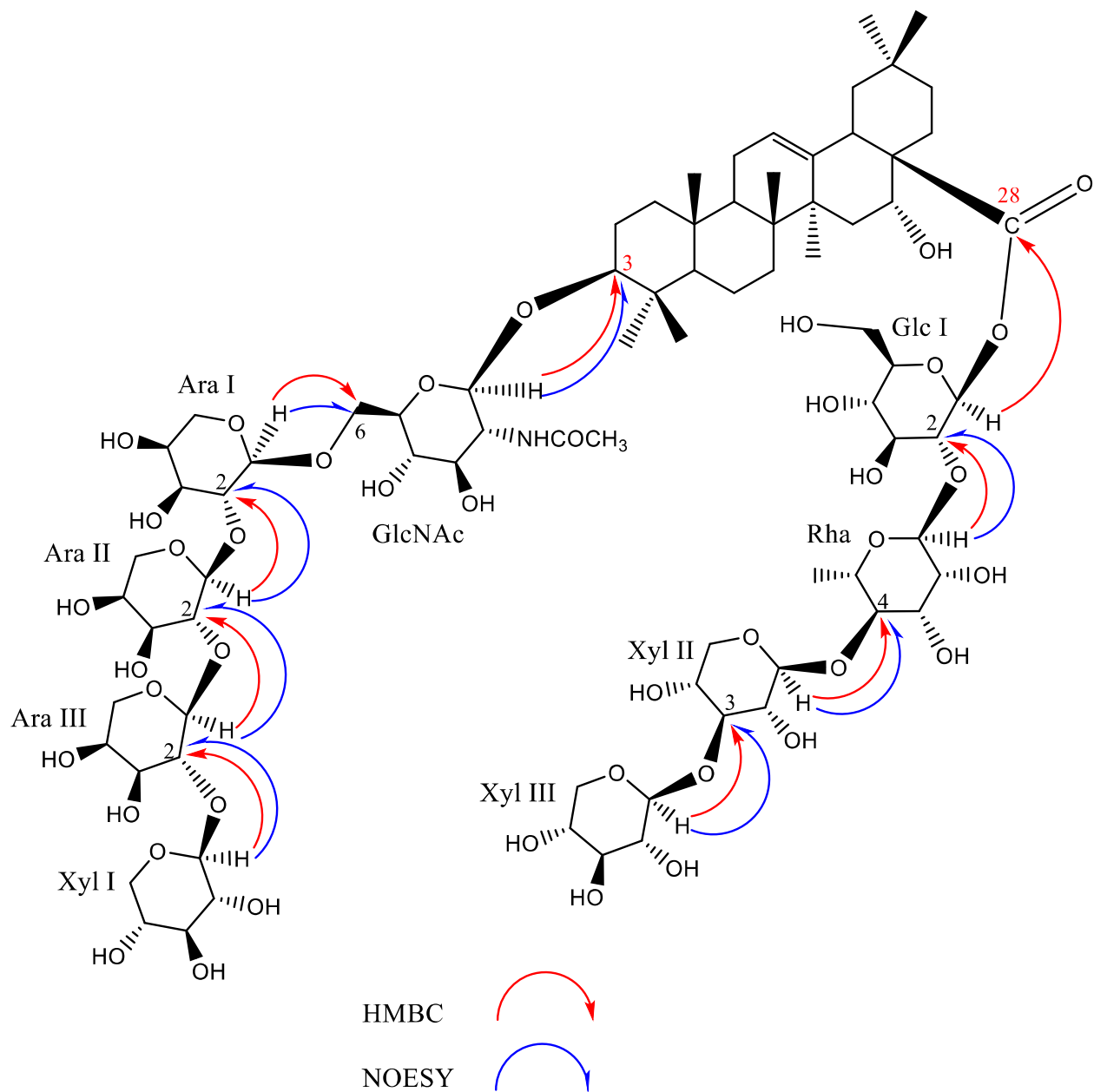


Fig. 3. Key HMBC and NOESY correlations of compound **2**.

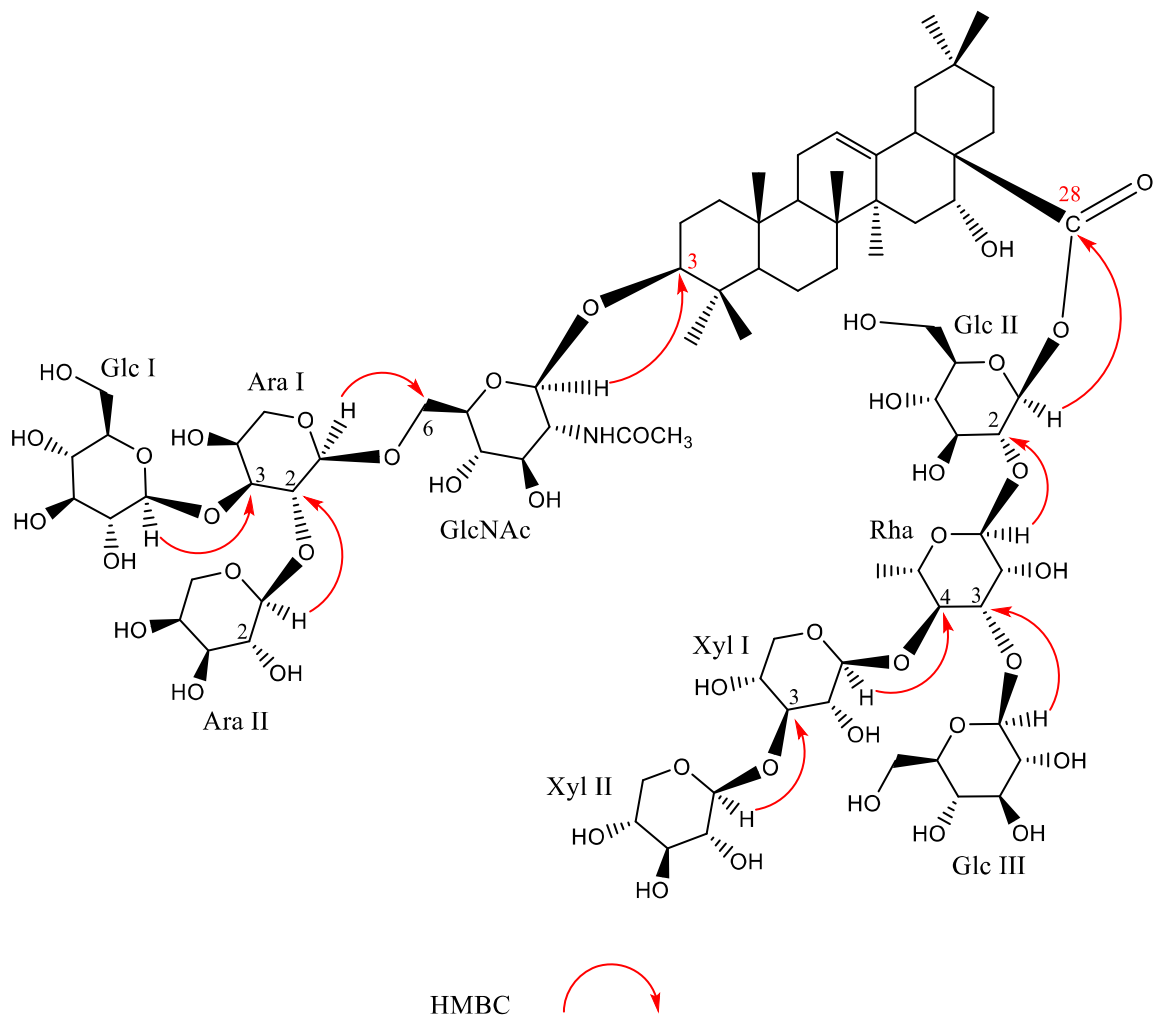


Fig. 4. Key HMBC correlations of compound 3.

Table 1. NMR spectroscopic data (500 MHz for ^1H and 125 MHz for ^{13}C) for the aglycone moieties of compounds **1-3** (δ in ppm and J in Hz)^{a)}

Pos	1		2		3	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	38.7 (CH ₂)	1.06 1.65	38.5 (CH ₂)	1.06 1.64	38.6 (CH ₂)	0.94 1.53
2	26.3 (CH ₂)	1.92 2.30	26.3(CH ₂)	1.89 2.29	26.1(CH ₂)	1.50 1.80
3	89.9 (CH)	3.40 (1H, brd, 13.7)	89.2 (CH)	3.35(1H, brd, 13.2)	88.3 (CH)	3.06 (1H, brd, 13.6)
4	39.7 (C)	-	39.9 (C)	-	39.0 (C)	-
5	55.5 (CH)	0.88 (1H, d, 12.5)	55.8 (CH)	0.86 (1H, brd, 4.8)	55.4 (CH)	0.71 m
6	18.2 (CH ₂)	1.44 1.63	18.4 (CH ₂)	1.41 1.64	18.2 (CH ₂)	1.25 1.45
7	33.3 (CH ₂)	1.02 1.68	33.1 (CH ₂)	1.01 1.69	32.9 (CH ₂)	1.30 1.41
8	39.9 (C)	-	41.9 (C)	-	39.4 (C)	-
9	46.9(CH)	1.84	47.1 (CH)	1.82	46.5 (CH)	1.53
10	36.8 (C)	-	36.7 (C)	-	36.6 (C)	-
11	23.6 (CH ₂)	2.02 2.12	23.4 (CH ₂)	2.02 2.11	22.6 (CH ₂)	1.25 1.32
12	122.4(CH)	5.67(1H, brs)	122.4(CH)	5.64(1H, brs)	122.0 (CH)	5.23 (1H, brs)
13	144.6 (C)	-	144.3 (C)	-	143.7 (C)	-
14	41.9 (C)	-	42.0 (C)	-	41.4 (C)	-
15	35.8 (CH ₂)	2.03 2.32	35.8 (CH ₂)	2.04 2.40	35.1 (CH ₂)	1.29 1.61
16	73.8 (CH)	5.24 (1H, brs)	73.8 (CH)	5.24 (1H, brs)	73.3 (CH)	4.35 (1H, brs)
17	49.1 (C)	-	49.1 (C)	-	48.3 (C)	-
18	41.2 (CH)	3.42 (1H, d, 14.3)	41.2 (CH)	3.42(1H, d, 13.2)	40.7 (CH)	2.84 (1H, d, 14.4)
19	47.3 (CH ₂)	1.38 m 2.78 m	47.2 (CH ₂)	1.35 2.77(1H, t, 13.9)	47.1 (CH ₂)	1.01(1H, brd, 11.4) 2.25 m
20	30.5 (C)	-	30.4 (C)	-	30.6 (C)	-
21	35.6 (CH ₂)	1.33 m 2.42(1H, d, 13.3)	35.6 (CH ₂)	1.30 m 2.40 m	35.5 (CH ₂)	1.10 m 1.93 m
22	31.5 (CH ₂)	2.19 2.43	31.8 (CH ₂)	2.16 2.43	31.1 (CH ₂)	1.65 1.83
23	27.9 (CH ₃)	1.27 (s)	28.1 (CH ₃)	1.25 (s)	28.0 (CH ₃)	0.90 (s)
24	16.9 (CH ₃)	1.03(s)	16.9 (CH ₃)	0.98 (s)	16.9 (CH ₃)	0.65 (s)
25	15.5 (CH ₃)	0.94 (s)	15.5 (CH ₃)	0.86 (s)	15.7 (CH ₃)	0.86 (s)
26	17.2 (CH ₃)	1.17 (s)	17.3 (CH ₃)	1.10 (s)	17.1 (CH ₃)	0.67 (s)
27	27.1 (CH ₃)	1.87 (s)	27.0 (CH ₃)	1.85 (s)	27.0 (CH ₃)	1.31 (s)
28	176.3	-	176.1	-	174.9	-
29	33.0 (CH ₃)	1.02 (s)	33.0 (CH ₃)	0.95 (s)	33.4 (CH ₃)	0.84 (s)
30	24.4 (CH ₃)	1.05 (s)	24.5 (CH ₃)	0.97 (s)	24.7 (CH ₃)	0.90 (s)

Assignments were based on the HMBC, HSQC, COSY and J mod experiments.

^{a)}Overlapped ^1H NMR signals are reported without designated multiplicity; nf: not found.

Table 2. NMR spectroscopic data (500 MHz for ^1H and 125 MHz for ^{13}C) for the sugar moieties of compounds **1-3** (δ in ppm)^{a)}

Pos.	1		2		3	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}
3-O-Sugars						
GlcNHAc						
1	104.4	5.11 (1H, d, 7.1)	104.6	5.05 (1H, d, 7.8)	103.8	4.29 (1H, d, 8.2)
2	57.1	4.61 (1H, d, 8.5)	57.3	4.59 m	56.1	3.42
3	74.9	4.05	75.1	4.44	74.1	3.14
4	71.7	4.31	72.3	4.13	71.5	3.57
5	75.4	4.12	75.4	4.07	74.3	3.29
6	69.0	4.25; 4.60 (1H, d, 8.5)	69.1	4.20 (1H, brd, 4.8); 4.61m	68.4	3.53; 3.88(1H, brd, 9.0)
NHCOCH ₃	23.3	2.35	23.2	2.28	23.8	1.77
Ara-I						
1	102.2	5.06 (1H, d, 6.2)	102.2	5.06 (1H, d, 4.6)	101.5	4.48 (1H, d, 4.5)
2	79.5	4.54	79.5	4.54	79.4	3.57
3	72.4	4.40	72.4	4.37	77.5	3.88
4	67.5	4.45	67.5	4.40	71.0	3.47
5	64.6	3.80 (1H, dd, 10.7; 13.8); 4.34	64.4	3.76(1H, t, 12.0); 4.30	63.5	3.37; 3.68
Ara-II						
1	105.2	5.04 (1H, d, 7.5)	105.5	5.02 (1H, d, 6.8)	105.1	4.26 (1H, d, 7.3)
2	79.8	4.50	80.0	4.50	74.4	3.02
3	73.5	3.97	73.8	4.13	76.5	3.17
4	68.7	4.27	68.7	4.30	69.9	3.32
5	66.6	3.49; 4.36m	66.3	4.29; 4.44(1H, brd, 10.3)	66.2	3.10(1H, d, 10.4); 3.75
Ara-III						
1	102.0	5.13 (1H, d, 6.7)	102.2	5.13 (1H, d, 5.1)		
2	79.8	4.52	80.0	4.51		
3	71.9	4.46	72.1	4.14		
4	67.1	4.50	67.0	4.30		
5	63.9	3.83(1H, dd, 10.7; 13.8); 4.36	63.7	3.78 (1H, t, 12.0); 4.33		
Xyl-I						
1	105.4	4.99 (1H, d, 7.5)	105.7	4.96 (1H, d, 7.5)		
2	74.8	4.03	75.0	4.04		
3	77.1	4.19	77.4	4.15		
4	70.4	4.18	70.6	4.18		
5	66.5	3.65m; 4.46	66.8	4.28; 4.44 (1H, brd, 10.3)		
Glc-I					103.5	4.42 (1H, d, 8.0)
					72.7	3.47
					75.6	3.31

					70.9	3.46
					76.9	3.18
					61.0	3.45; 3.63
28-O-Sugars						
Glc-I				Glc-II		
1	94.8	6.07 (1H, d, 7.8)	94.6	6.12 (1H, d, 7.8)	93.7	5.30 (1H, d, 7.6)
2	77.7	4.09	76.3	4.31	77.1	3.28
3	77.2	4.20	75.4	4.43	76.8	3.55
4	70.6	4.24	70.9	4.27	70.1	3.52
5	78.5	4.04	78.8	4.03	77.7	3.20
6	61.5	4.30(1H, d, 8.7); 4.43	61.7	4.32; 4.44(1H, brd, 10.3)	61.5	3.45 ; 3.59
Rha						
1	101.7	5.89 (1H, brs)	101.0	6.35 (1H, brs)	100.7	4.95 (1H, brs)
2	70.3	5.24	70.1	4.82	68.9	4.13
3	82.3	4.93	72.1	4.69	81.2	3.87
4	77.9	4.07	83.4	4.42	76.9	3.54
5	68.8	4.32	68.1	4.48	67.7	3.63
6	18.7	1.76 (1H, d, 6.0)	18.8	1.76 (1H, d, 6.0)	18.4	1.15 (1H, brd, 5.5)
Xyl-II				Xyl-I		
1	103.9	5.54 (1H, d, 7.5)	105.8	5.20 (1H, d, 7.5)	103.2	4.67 (1H, d, 7.6)
2	74.2	4.06	74.6	4.06	73.5	3.14
3	87.4	4.19	87.1	4.06	86.9	3.35
4	69.2	4.10	68.7	4.29	68.6	3.39
5	65.9	3.58(1H, t, 11.1); 4.23	66.4	4.22; 4.43(1H, brd, 10.3)	65.6	3.11(1H, d, 10.4) ; 3.77
Xyl-III				Xyl-II		
1	105.4	5.23 (1H, d, 7.9)	105.6	5.14 (1H, d, 7.0)	104.8	4.39 (1H, d, 5.2)
2	74.9	4.05	74.8	4.04	74.2	3.10
3	77.0	4.21	77.3	4.15	76.5	3.15
4	70.2	4.20	70.4	4.17	68.9	3.37
5	66.5	3.71(1H, brd, 8.6); 4.27	66.8	4.30; 4.43(1H, brd, 10.3)	66.1	3.09; 3.74(1H, brd, 5.5)
Glc-II				Glc-III		
1	104.7	5.35 (1H, d, 7.8)			103.8	4.40 (1H, d, 7.8)
2	74.9	4.17			74.1	3.11
3	77.2	4.21			76.5	3.31
4	71.0	4.09			70.3	3.52
5	77.5	3.98			76.8	3.16
6	62.0	4.20 (1H, d, 11.1); 4.51			61.4	3.46; 3.68

Assignments were based on the HMBC, HSQC, COSY, TOCSY, NOESY and Jmod experiments.

^{a)}Overlapped ¹H NMR signals are reported without designated multiplicity.

Table 3. Antiproliferative and cytotoxic activities of **1** and **2** on human cancer cells Hep3B and Caco-2, respectively.

Compounds	IC₅₀ (μM) on Hep3B	CC₅₀ (μM) on Caco-2
1	6.0 ±1.48	>25
2	6.5 ±1.87	>25
Sorafenib	7.6 ± 2.3	-
Doxorubicine	-	13.23

Triterpenoid saponins from *Calliandra calothyrsus* Meisn. and their antiproliferative activity against two digestive carcinoma human cell lines

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Graphical abstract

