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Cytotoxic Oleanane-Type Saponins from the Leaves of *Albizia anthelmintica* Brongn.

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Abstract. Two new oleanane-type saponins: 3-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucopyranosyl oleanolic acid 28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl ester **(1)** and 3-*O*-*β*-D-xylopyranosyl-(1→2)-*α*-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl oleanolic acid 28-*O*-*β*-D-glucopyranosyl ester **(2)**, along with two known saponins: 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl oleanolic acid **(3)** and 3-*O*-*β*-D-glucopyranosyl-(1→2)-[*α*-L-arabinopyranosyl-(1→6)]-*β*-D-glucopyranosyl oleanolic acid **(4)** were isolated from the acetone-insoluble fraction obtained from the 80% aqueous methanol extract of *Albizia anthelmintica* Brongn. leaves. Their structures were identified using different NMR experiments including: ^{1}H NMR, ^{13}CNMR, HSQC, HMBC and ^{1}H,^{1}H COSY, together with HRESIMS/MS, as well as by acid hydrolysis. The four isolated saponins and the fractions of the extract exhibited cytotoxic activity against HepG-2 and HCT-116 cell lines. Compound **2** showed the most potent cytotoxic activity among the other tested compounds against the HepG2 cell line with an IC_{50} value of 3.60 µM. Whereas, compound **1** showed the most potent cytotoxic effect with an IC_{50} value of 4.75 µM on HCT-116 cells.

Key words. *Albizia anthelmintica*; Fabaceae; oleanane saponins; cytotoxicity; MTT.

Introduction. - The genus *Albizia* (Fabaceae) comprises about 150 species that are widely distributed in Africa and South America. Saponin glycosides with diverse pharmacological activities were isolated from different *Albizia* species [1, 2]. Several studies have reported the strong cytotoxicity of *Albizia* saponins. For instance, Adianthifolioside A-D isolated from *A. adiantifolia* showed a potent cytotoxic activity on Jurkat cells [3]. Coriarioside A from *A. coriaria* showed a strong cytotoxic activity on different colon cell lines [4]. Furthermore, grandibracteoside A-C from *A. grandibracteata* showed cytotoxicity on KB and MCF7 cell lines [5]. *A. anthelmintica* Brongn. is a spiny, medium tree native to Africa. Its bark is used widely by farmers as an anthelmintic for livestock [6]. Previous phytochemical studies have reported the isolation of few echinocystic acid saponins from the bark of *A. anthelmintica* [7], and flavonoids from the leaves [8]; however, no report has been traced regarding the saponin composition of *A. anthelmintica* leaves. To the best of our knowledge, no studies have so far been reported on the cytotoxic activity of *A. anthelmintica* saponins. Therefore, this study was undertaken to determine the cytotoxicity of *A. anthelmintica* and to isolate the compounds responsible for the bioactivity. Hepatocellular and colon carcinoma cell lines were chosen for this study because the colon and liver cancers are among the five most common and lethal cancers worldwide [9].

In the present study, bioactivity guided fractionation of the acetone-insoluble fraction obtained from the 80% aqueous methanol extract of *A. anthelmintica* leaves led to the isolation of two new oleanane-type saponins: 3-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucopyranosyl oleanolic acid 28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl ester **(1)** and 3-*O*-*β*-D-xylopyranosyl-(1→2)-*α*-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl oleanolic acid 28-*O*-*β*-D-glucopyranosyl ester **(2)**, together with two known ones: 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl oleanolic

acid **(3)** and 3-*O*-*β*-D-glucopyranosyl-(1→2)-[*α*-L-arabinopyranosyl-(1→6)]-*β*-D-glucopyranosyl oleanolic acid **(4)**, for the first time from *A. anthelmintica* leaves. The cytotoxic activity of the four isolated saponins and all the fractions of the extract were tested against HepG-2 (hepatocellular carcinoma) and HCT-116 (colon carcinoma) cell lines.

Results and Discussion. - The 80% aqueous methanol extract of *A. anthelmintica* leaves was treated with acetone to precipitate saponins. The acetone insoluble fraction was fractionated using Diaion HP-20 column chromatography to yield four main sub-fractions I-IV. Two new saponins **1** and **2**, together with two known ones **3** and **4** (*Fig. 1*) were isolated from the sub-fractions III and IV. The structures of the compounds were elucidated on the basis of ^{1}H NMR, ^{13}C NMR (*Table 1*), 2D NMR (^{1}H,^{1}H COSY, HSQC and HMBC), HRESIMS/MS (Supplementary data), as well as acid hydrolysis. The monosaccharides obtained by acid hydrolysis of **1** and **2** were identified by comparison with authentic sugar samples on TLC.

The HRESIMS of compound (**1**) displayed a molecular ion peak at *m/z* 1189.5955 $[M-H]^-$, which is consistent with a molecular formula of $C_{58}H_{93}O_{25}$. The ^{1}H-NMR spectrum (CD_3OD, 400 MHz) showed characteristic signals of oleanolic acid and five sugar moieties. Seven methyl singlets and a broad singlet of the olefinic proton H-C(12) at *δ*(H) 5.25 (1H, *brs*) suggested that **1** is a triterpenoid saponin with an aglycone of the oleanene skeleton (*Table 1*). The presence of five sugar moieties was evidenced by the presence of five anomeric protons at *δ*(H) 5.48 (1H, *d*, *J* = 7.0 Hz), 5.31 (1H, *brs*), 4.67 (1H, *d*, *J* = 7.7 Hz), and two overlapping doublet signals at *δ*(H) 4.44 and 4.46. The ^{13}C-NMR spectrum of **1** (CD_3OD, 100 MHz) showed a pattern of signals characteristic for the oleanolic acid aglycone, including seven sharp signals for the methyl carbons at *δ*(C) 28.5, 16.9, 16.0, 17.7, 26.1, 33.4 and 24.0 corresponding to C(23), C(24), C(25),

C(26), C(27), C(29) and C(30), respectively. Two signals at δ(C) 123.8 and 144.9 were typical for the olefinic carbons C(12) and C(13), respectively (*Table 1*). Furthermore, an extra methyl signal at δ(C) 18.2 (which has HMBC correlations with the sugar protons) was assigned to a methyl group of a rhamnose sugar. The signal of C(3) at δ(C) 91.5 exhibited a glycosylation shift of about +10 ppm compared to the free un-glycosylated oleanolic acid, which suggested that the oleanolic acid is glycosylated at C(3). Five anomeric carbon signals at δ(C) 95.3, 101.1, 104.5, 105.4, and 107.1 were detected. The up-field value of 95.3 suggested that one of the sugar moieties formed an ester linkage with the carboxylic group at C(28). Based on these findings, **1** was suggested to be a bisdesmosidic saponin. Comparing the proton and carbon chemical shift values of the sugars with those in literature [10-13], together with tracing the COSY, HSQC and HMBC correlations indicated that the sugar moieties were two glucopyranose moieties, a rhamnopyranose and two xylopyranose moieties (*Fig. 2*). Glycosylation shifts were observed for C(4) of the rhamnopyranose unit at δ(C) 84.4, and C(2) of the glucopyranose unit at C(3) at δ(C) 81.3 indicated the substitution at these positions with terminal sugars. Acid hydrolysis of **1** yielded glucose, rhamnose and xylose as sugar components (detected by co-TLC with authentic sugars). Extensive HMBC analysis (*Fig. 2*) confirmed that the sugar sequence at C(28) is β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside. While the sugar sequence at C(3) was determined as β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside. The structure of **1** was confirmed by HRESIMS/MS (Supplementary data), where a fragment ion at *m/z* 1057.5581 $[(M-H)-132]^-$ was detected, which accounts for the loss of a terminal xylose unit. Another fragment ion appeared at *m/z* 779.4540 $[(M-H)-132-132-146]^-$, corresponding to the loss of two terminal xylose units (132 x 2) and a rhamnose (146). A fragment ion at *m/z* 617.4028 $[(M-H)-132-132-146-162]^-$ corresponds to the sequential loss of a glucose unit.

Finally, a fragment ion at *m/z* 455.3525 was detected corresponding to the aglycone unit (oleanolic acid), which results from the loss of all sugar moieties. This fragmentation pattern confirms the structure of compound (**1**) suggested by NMR spectral data. Therefore, compound (**1**) was identified as 3-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-glucopyranosyl oleanolic acid 28-*O*-*β*-D-xylopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl ester.

The HRESIMS of compound (**2**) revealed a molecular ion peak at *m/z* 1084.5526 $[M-H]^-$, corresponding to a molecular formula of $C_{54}H_{86}O_{21}N$. The molecular ion peak indicated a tetrasaccharide saponin containing four sugar units; one acetamido-deoxy hexose, one hexose and two pentoses. Comparing the NMR data (*Table 1*) with those of compound (**1**)**,** compound (**2**) was proved to have an oleanolic acid aglycone. The ^{13}C-NMR spectrum (CD_3OD, 100 MHz) showed a pattern of signals characteristic for the oleanolic acid aglycone. Four anomeric carbon signals were observed at *δ*(C) 95.4, 101.0, 104.9, and 106.7, that correlated in the HSQC spectrum with four anomeric proton signals at *δ*(H) 5.44 (1H, *d*, *J* = 7.0 Hz), 5.28 (IH, *brs*), 4.44 (IH, *d*, *J* = 8.2 Hz) and 4.52 (IH, *d*, *J* = 7.6 Hz), respectively. Comparing the NMR data with those of related compounds in literature [11, 14-16], the sugars were confirmed to be one 2-acetamido-2-deoxy-*β*-D-glucopyranose, one glucopyranose, one *α*-L-arabinopyranose and one *β*-D-xylopyranose units. The anomeric proton (H-(C1) of the arabinose sugar appeared as broad singlet which indicated the equatorial-equatorial coupling between H(C-1) and H (C-2) of arabinose (^3J H (C-1),H (C-2) < 3 Hz for equatorial orientation). The arabinose moiety was therefore confirmed to be in the $^1C_4$ conformation [17]. Acid hydrolysis of **2** confirmed the presence of these sugars. The up-field value of C(1) of a glucopyranose unit at *δ*(C) 95.4 suggested that the glucopyranose moiety formed an ester linkage with the carboxylic group at

C(28). Hence, **2** was suggested to be a bisdesmosidic saponin. The presence of an *N*-acetamido group was suggested by the appearance of a signal at δ(H) 1.96 (3H, *s*, $NHCOCH_3$), correlating in HSQC with a carbon at δ(C) 23.2 ($NHCOCH_3$); these values are typical for protons and a carbon of the methyl group of an *N*-acetamido unit [14, 16]. Furthermore, the appearance of a carbonyl amide carbon signal at δ(C) 173.4 ($NHCOCH_3$), along with the HMBC correlation (*Fig. 3*) between the proton signal at δ(H) 1.96 ($NHCOCH_3$) and the quaternary carbon at δ(C) 173.4 ($NHCOCH_3$) suggested the presence of an *N*-acetamido group [14-16]. The attachment of the *N*-acetamido group to C(2) of the 2-deoxy-*β*-D-glucopyranose unit was established from the presence of a carbon signal at δ(C) 57.6 (typical for C(2) of a 2-deoxy-*β*-D-glucopyranose unit), which correlates in the HSQC spectrum with a proton at δ(H) 3.69 (H-C(2) of the 2-deoxy-*β*-D-glucopyranose moiety). The attachment of 2-acetamido-2-deoxy-*β*-D-glucopyranosye to C(3) of the aglycone was evident from the HMBC correlation (*Fig. 3*) between the anomeric proton (H-C(1) of the 2-deoxy-*β*-D-glucopyranose moiety (δ(H) 4.44) and the C(3) of the aglycone at δ(C) 91.1. The sequential assignments of all proton and carbon signals of the individual sugars were achieved based on the COSY, HSQC and HMBC spectral data. Glycosylation shifts were observed for C(6) (δ(C) 67.2) of the 2-deoxy-*β*-D-glucopyranose unit and C(2) (δ(C) 83.7) of the *α*-L-arabinopyranose moiety indicated the substitution at these positions. The HRESIMS/MS of **2** (Supplementary data), revealed a fragment ion at *m/z* 994.5296 $[(M-H)-90]^-$, which results from the fragmentation of a glucose unit, where the ring opens and a loss of three carbons with attached protons and oxygens occurs. A fragment ion also appeared at *m/z* 952.5217 $[(M-H)-132]^-$, indicating the loss of a terminal xylose unit. Accordingly, compound (**2**) was elucidated as 3-*O*-*β*-D-xylopyranosyl-(1→2)-*α*-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl oleanolic acid 28-*O*-*β*-D-glucopyranosyl ester.

The acetone insoluble fraction exhibited a potent cytotoxic activity on HepG2 and HCT-116 cell lines, with IC_{50} values of 1.50 µg/ml and 2.30 µg/ml, respectively. While the acetone soluble fraction showed a less potent cytotoxic activity, with IC_{50} values of 5.62 µg/ml and 18.10 µg/ml, respectively (*Table 2*). Based on the results of this study, the viability of the hepatocellular carcinoma cells (HepG2) were reduced in a dose-dependent manner after a 48 h exposure period by the tested fractions and compounds. The IC_{50} values of the four sub-fractions I, II, III, IV were >50.00 µg/ml, 5.71 µg/ml, 1.32 µg/ml and 1.48 µg/ml, respectively. The results showed that sub-fractions III and IV (rich in saponins) have the most potent cytotoxic effect among the other sub-fractions, followed by sub-fraction II (containing flavonoids), while sub-fraction I (containing mainly sugars) showed no cytotoxic activity (*Table 2*). Regarding the isolated saponins, compound **2** showed the most potent cytotoxic activity among the other tested compounds, with an IC_{50} value of 3.60 µM against the HepG2 cell line, while compounds **1** and **3** showed a moderate cytotoxicity, with IC_{50} values of 18.47 and 20.50 µM, respectively. Compound **4** showed the least cytotoxic activity with an IC_{50} value of 33.53 µM. The IC_{50} value of doxorubicin (the reference cytotoxic agent used) on HepG2 cells was 0.94 µM (*Table 2*). Similarly, the results of the present study demonstrated that the viability of the colon carcinoma (HCT-116) cells were reduced in a dose-dependent matter after a 48 h exposure period by the tested samples. The IC_{50} values were >50.00 µg/ml, 22.40 µg/ml, 10.20 µg/ml and 4.51 µg/ml, respectively for the four sub-fractions I, II, III, IV, respectively. Whereas, compound **1** showed the most potent cytotoxic activity among the other tested compounds with an IC_{50} value of 4.75 µM, while compound **2** showed a moderate cytotoxic activity (IC_{50} value of 15.11 µM) on HCT-116 cells. Compounds **3** and **4** showed the least cytotoxic activity with IC_{50} values of 46.38 and

41.53 μM, respectively. Doxorubicin produced an IC_{50} value of 0.85 μM on HCT-116 cells (*Table 2*).

3. Conclusions

Bioactivity guided fractionation of the 80% aqueous methanol extract of *A. anthelmintica* leaves led to the isolation of four oleanane type saponins, two of them (**1** and **2**) are new saponins. Compound **2** showed the most potent cytotoxic activity among the other tested compounds against the HepG2 cell line with an IC_{50} value of 3.60 μM. Whereas, compound **1** showed the most potent cytotoxic activity among the other tested compounds with an IC_{50} value of 4.75 μM on HCT-116 cells.

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The authors report no declarations of interest.

Experimental Part

General. The NMR spectra were measured by a *Bruker Ascend 400/R* spectrometer (*Burker Avance III*, Fällanden Switzerland) operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C (APT). Spectra were recorded at 25°C using CD_3OD-d_4 or DMSO-d_6 as solvents. Proton and carbon spectra were referenced internally to TMS signal using a value of 0.00 ppm. The HRESI-MS was performed on a *Bruker micrOTOF-$_Q$* quadrupole time-of-flight mass spectrometer (Bremen,

Germany). The ionization technique was a pneumatically assisted electrospray. The mass spectrometer was operated in the negative mode with the following settings: capillary voltage, 4000V; end plate offset, –500V. Heated drying gas (N_2) flow rate was 12 l/min; the drying gas temperature was 200 ^{o}C. The gas flow to the nebulizer was set at a pressure of 1.6 bar. For collision-induced dissociation measurements (MS/MS), the voltage over the collision cell varied by collision sweeping mode from 4.5 to 85 eV. Argon was used as a collision gas. A melting point apparatus (*Stuart Scientific*, Staffordshire, UK) was used to measure the melting point of the compounds, with a heating rate 1 to 10 ^{o}C per min and a temperature range from 50 up to 400 ^{o}C. The optical rotation of the compounds was measured using a *A.KRÜSS Optronic P8000* GmbH polarimeter (Hamburg, Germany), with a light source (Light Emitting Diode, LED), a filter and a maximum tube length of 220 mm. TLC was performed on precoated Silica gel 60 F254 plates (*Fluka Chemika*, Swizerland). Column chromatography was done on Diaion HP-20 polymer gel with particle size 250-850 µm (*Mitsubishi Chemical Co.*, (Tokyo, Japan) and silica gel 60 of 70–230 mesh (*Merck*, Darmstadt, Germany). Authentic sugars (glucose, xylose, arabinose, rhamnose and 2-acetamido-2-deoxy-glucose) were obtained from *Sigma-Aldrich Chemical Co*. (St. Louis, MO, USA). All solvents used for extraction and isolation were of distilled analytical grade.

Plant Material. The leaves of *A. anthelmintica* Brongn. were collected in January 2013 from the Zoo, Giza, Egypt. They were kindly authenticated by *Mrs. Therease Labib*, the taxonomy specialist in El-Orman botanical garden, Giza, Egypt. A voucher specimen (PHG-P-AA-2013) was deposited at the herbarium of the Faculty of Pharmacy, Ain shams University, Cairo, Egypt.

Extraction and Isolation. The air dried leaves (1.5 kg), were extracted by maceration with 80% methanol (3 x 18 l). The extract was evaporated till dryness under reduced pressure then freeze dried to yield 135 g of the dried extract. The total extract was dissolved in 200 ml MeOH, then treated with acetone (x 10 volume) to yield the acetone soluble fraction (60 g) and the acetone insoluble fraction (72 g). The acetone insoluble fraction (50 g) was subjected to Diaion HP-20 column chromatography (220 g, 5 x120 cm) using H_2O-MeOH gradient of decreasing polarity to give four main sub-fractions: Sub-fraction I (15.6 g, eluted with water), sub-fraction II (9.2 g, eluted with 50% methanol), sub-fraction III (14.1 g, eluted with 75% methanol), and sub-fraction IV (2.5 g, eluted with 100% methanol). Sub-fraction III (4 g) was applied to a silica gel 60 column (200 g, 5 x120 cm) (Merck, Germany) using CH_2Cl_2–MeOH gradient of increasing polarity (9:1–1:9) to yield four smaller fractions, A (0.04 g), B (0.12 g), C (0.85 g) and D (2.00 g). Sub-fraction B was further purified by preparative TLC using *n*-BuOH–H_2O–AcOH (4:1:5, upper butanol layer) as a solvent system. The edge of TLC plates was cut then sprayed with vanillin/ H_2SO_4 reagent (1% vanillin in 10% H_2SO_4 in MeOH) to detect the compounds, the separated bands were scratched, macerated in methanol, filtered then the filtrate was dried under reduced pressure followed by freeze drying to obtain compounds **1** (65 mg) and **2** (48 mg). Sub-fraction IV was subjected to preparative TLC using CH_2Cl_2–MeOH–AcOH–H_2O (7:3:1:0.1) a solvent system to give compounds **3** (35 mg) and **4** (38 mg).

3-O-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl oleanolic acid 28-O-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester (**1**). White amorphous powder. mp 230-232℃. $[\alpha]_D^{20}$ = −34.6 (c = 0.3, MeOH). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data: see *Table 1*. HRESIMS (neg.): *m/z* 1189.5955 ($[M-H]^-$, $C_{58}H_{93}O_{25}$; calc. 1189.5979).

HRESIMS/MS: *m/z* 1057.5581 [(*M*−H)−132]$^-$, 779.4540 [(*M*−H)−132−132−146]$^-$, 617.4028 [(*M*−H)−132−132−146−162]$^-$, 455.3525 [(*M*−H)−132−132−146−162−162]$^-$.

3-O-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl oleanolic acid 28-O-β-D-glucopyranosyl ester (**2**). White amorphous powder. mp 187-189 ℃. $[\alpha]_D^{20}$ = −18.5 (c = 0.3 MeOH). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data: see *Table 1*. HRESIMS (neg.): *m/z* 1084.5526 ([*M*−H]$^-$, $C_{54}H_{86}O_{21}N$; calc. 1084.5667). HRESIMS/MS: *m/z* 994.5296 [(*M*−H)−90]$^-$, 952.5217 [(*M*−H)−132]$^-$.

3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl oleanolic acid (**3**). White amorphous powder. ^{1}H NMR and ^{13}C NMR data: consistent with those reported in literature from *A. inundata* [2, 20]. HRESIMS (neg.): *m/z* 779.4409 ([*M*−H]$^-$, $C_{42}H_{67}O_{13}$; calc. 779.4563). HRESIMS/MS: *m/z* 617.3883 [(*M*−H)−162]$^-$, 455.3329 [(*M*−H)−162−162]$^-$.

3-O-β-D-glucopyranosyl-(1→2)-[α-L-arabinopyranosyl-(1→6)]-β-D-glucopyranosyl oleanolic acid (**4**). White, amorphous powder. ^{1}H NMR and ^{13}C NMR data: consistent with those reported in literature from *A. gummifera* [21]. HRESIMS (neg.): *m/z* 911.4970 ([*M*−H]$^-$, $C_{47}H_{75}O_{17}$; calc. 911.4983). HRESIMS/MS: *m/z* 749.4363 [(*M*−H)−162]$^-$, 617.3960 [(*M*−H)−162-132]$^-$, 455.3356 [(*M*−H)−162-132-162]$^-$.

Acid Hydrolysis. A solution of 5 mg of saponins **1** or **2** was added to 10 ml 0.02 N H_2SO_4, then heated at 100℃ for 4 h. The mixture was left to cool, neutralized with 0.02 N KOH and extracted with CH_2Cl_2. [22]. The sugars in the aqueous layer were detected by TLC using authentic sugars and *n*-BuOH–H_2O–AcOH (4:1:5, upper butanol layer) as a solvent system. The TLC was sprayed by vanillin/ H_2SO_4 reagent.

Cytotoxicity MTT Assay. HepG2 (Human Hepatocellular Carcinoma) and HCT-116 (Human Colon Carcinoma) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG 2 and HCT-116 cell lines were grown in Roswell Park Memorial Institute medium (RPMI-1640 medium) supplemented with 10% heat inactivated fetal bovine serum (FBS), 50 units/ml of penicillin and 50 gm/ml of streptomycin and maintained as a monolayer culture at 37°C in a humidified atmosphere containing 5% CO_2. Cytotoxicity was monitored by determining the effect of the tested samples on the cell viability using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay [23]. The cancer cells were treated for 48 h with the tested samples dissolved in DMSO, such that the DMSO concentration in the medium did not exceed 0.1%. The plates were read on an ELISA micro-plate reader using a test wavelength of 570 nm. The IC_{50} values were calculated according to the equation for Boltzman sigmoidal concentration–response curve using the nonlinear regression fitting models (Graph Pad, Prism Version 5). Doxorubicin was used as a standard cytotoxic agent.

Figure legends

Fig. 1. Chemical structures of compounds **1**-**4**

Fig. 2. HMBC correlations of compound **1**

Fig. 3. HMBC correlations of compound **2**

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Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data (CD_3OD-d_4) *of Compounds* **1** and **2**.

	1						**2**					
	aglycone			**sugar**			**aglycone**			**sugar**		
Pos.	δ(C)	δ(H)		**Pos.**	δ(C)	δ(H)	**Pos.**	δ(C)	δ(H)	**Pos.**	δ(C)	δ(H)
1	39.9	0.98/1.62 (*m*)		**Glu I at 28**			**1**	39.9	1.00/1.63 (*m*)	**Glu at 28**		
2	26.1	1.36/1.95 (*m*)		**1**	95.3	5.48 (*d*, *J* = 7.0)	**2**	26.8	1.70/1.93 (*m*)	**1**	95.4	5.44 (*d*, *J* = 7.0)
3	91.5	3.19 (*m*)		**2**	76.7	3.61 (*m*)	**3**	91.1	3.11 (*m*)	**2**	76.4	3.58 (*m*)
4	40.4			**3**	77.6	3.28 (*m*)	**4**	39.8		**3**	75.9	3.47 (*m*)
5	57.0	0.77 (*m*)		**4**	71.9	3.24 (*m*)	**5**	57.0	0.77 (*m*)	**4**	71.8	3.34 (*m*)
6	19.4	1.39/ 1.57 (*m*)		**5**	78.4	3.55 (*m*)	**6**	19.4	1.36/1.54 (*m*)	**5**	77.6	3.27 (*m*)
7	29.2	1.55/1.72 (*m*)		**6**	62.8	3.65/3.85 (*m*)	**7**	31.9	1.71/1.91 (*m*)	**6**	62.7	3.70/3.84 (*m*)
8	40.7			**Rha**			**8**	40.8		**Glu-NAc at 3**		
9	49.2	1.57 (*m*)		**1**	101.1	5.31 (*brs*)	**9**	48.1	1.62 (*m*)	**1**	104.9	4.44 (*d*, *J* = 8.2)
10	37.8			**2**	71.8	3.96 (*m*)	**10**	37.8		**2**	57.6	3.69 (*m*)
11	23.9	1.90; 1.15 (*m*)		**3**	72.3	3.85 (*m*)	**11**	23.7	1.91;1.16 (*m*)	**3**	75.9	3.48 (*m*)
12	123.8	5.25 (*brs*)		**4**	84.4	3.53 (*m*)	**12**	123.4	5.32 (*brs*)	**4**	72.0	3.34 (*m*)
13	144.9			**5**	68.8	3.76 (*m*)	**13**	144.7		**5**	76.1	3.49 (*m*)
14	42.9			**6**	18.2	1.29 (*d*, *J* = 5.3)				**6**	67.2	3.19/3.88 (*m*)
15	30.8	1.30 (*m*)		**Xyl I at 4 Rha**			**14**	42.7		**$NHCOCH_3$**		
16	24.6	1.89;1.15 (*m*)		**1**	107.1	4.46	**15**	30.7	0.90/1.29 (*m*)	CO	173.4	
17	49.6			**2**	76.1	3.24 (*m*)	**16**	24.5	1.91;1.16 (*m*)	NH		8.55 (*brs*)
18	42.7	2.83 (*m*)		**3**	78.3	3.33 (*m*)	**17**	50.2		CH_3	23.2	1.96 (*s*)
19	48.2	1.57 (*m*)		**4**	70.8	3.53 (*m*)	**18**	42.2	2.95 (*m*)	**Ara at 6 Glu-NAc**		
20	31.5			**5**	67.2	3.19/3.87 (*m*)	**19**	47.9	1.05/2.28 (*m*)	**1**	101.1	5.28 (*brs*)
21	34.9	1.22/1.36 (*m*)		**Glu II at 3**			**20**	31.3		**2**	83.7	3.57 (*m*)
22	34.0	1.42 (*m*)		**1**	105.4	4.44	**21**	36.5	1.45/1.69 (*m*)	**3**	72.2	3.85 (*m*)
23	28.5	1.09 (*s*)		**2**	81.3	3.59 (*m*)	**22**	34.3	1.42/1.52 (*m*)	**4**	68.9	3.74 (*m*)
24	16.9	0.86 (*s*)		**3**	77.8	3.29 (*m*)	**23**	28.6	0.97 (*s*)	**5**	63.1	3.62/3.84(*m*)
25	16.0	0.95 (*s*)		**4**	71.5	3.24 (*m*)	**24**	17.1	0.86 (*s*)	**Xyl at 2 Ara**		
26	17.7	0.77 (*s*)		**5**	78.2	3.21 (*m*)	**25**	16.1	0.96 (*s*)	**1**	106.7	4.52 (*d*, *J* = 7.6)
27	26.1	1.15 (*s*)		**6**	63.1	3.64/3.85 (*m*)	**26**	17.7	0.77 (*s*)	**2**	76.0	3.26 (*m*)
28	177.9			**Xyl II at 2 Glu**			**27**	27.1	1.36 (*s*)	**3**	78.0	3.35 (*m*)
29	33.4	0.90 (*s*)		**1**	104.5	4.67 (*d*, *J* = 7.7)	**28**	177.0		**4**	71.1	3.49 (*m*)
30	24.0	0.92 (*s*)		**2**	76.3	3.25 (*m*)	**29**	33.3	0.88 (*s*)	**5**	66.5	3.32/3.92(*m*)
				3	78.3	3.34 (*m*)	**30**	24.9	0.95 (*s*)			
				4	71.1	3.49 (*m*)						
				5	66.5	3.31/3.91 (*m*)						

Assignments were based on the COSY, HSQC and HMBC spectra
Overlapped signals are reported without multiplicity

Table 2. Cytotoxicity of the acetone soluble and insoluble fractions, four sub-fractions and the isolated saponins from A. anthelmintica leaves on HepG 2 and HCT-116 cell lines.

Sample	**Main fractions**		**Sub-fractions**				**Compounds**			
Cell line	**Acetone sol. fr.**	**Acetone insol. fr.**	**I**	**II**	**III**	**IV**	**1**	**2**	**3**	**4**
HepG 2	5.62	1.50	>50.00	5.71	1.32	1.48	18.47	3.60	20.50	33.53
HCT-116	18.10	2.30	>50.00	22.40	10.20	4.51	4.75	15.11	46.38	41.53

[a]) Cells were treated with the tested samples for 48 h, and the cell viability was determined by the MTT assay. IC_{50} values (µg/mL for fractions or µM for compounds) was calculated from the % cell viability on the basis of triplicate measurements.

[b]) IC_{50} values of doxorubicin were 0.94 µM and 0.85 µM on HepG 2 and HCT-116, respectively.

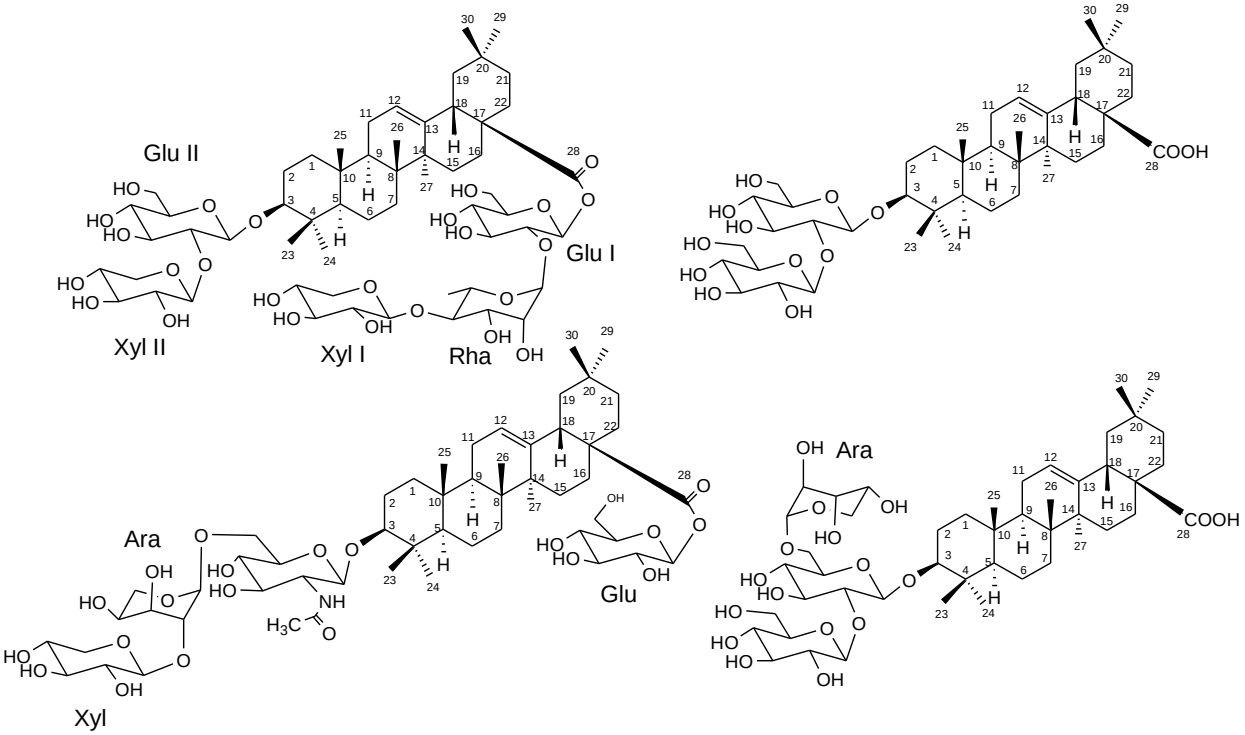

Fig. 1. *Chemical structures of compounds* **1-4**

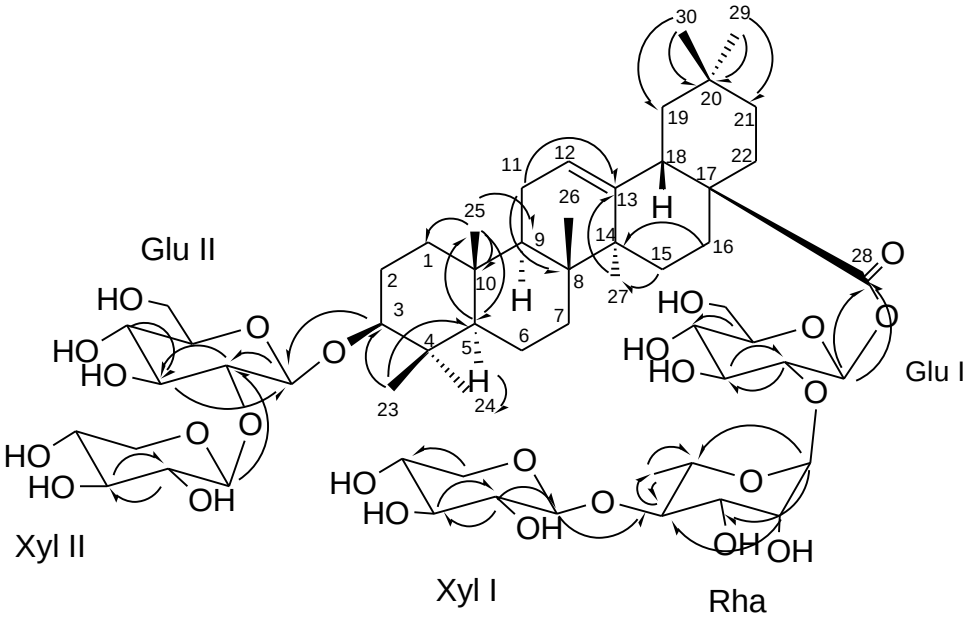

Fig. 2. *HMBC correlations of* compound **1**

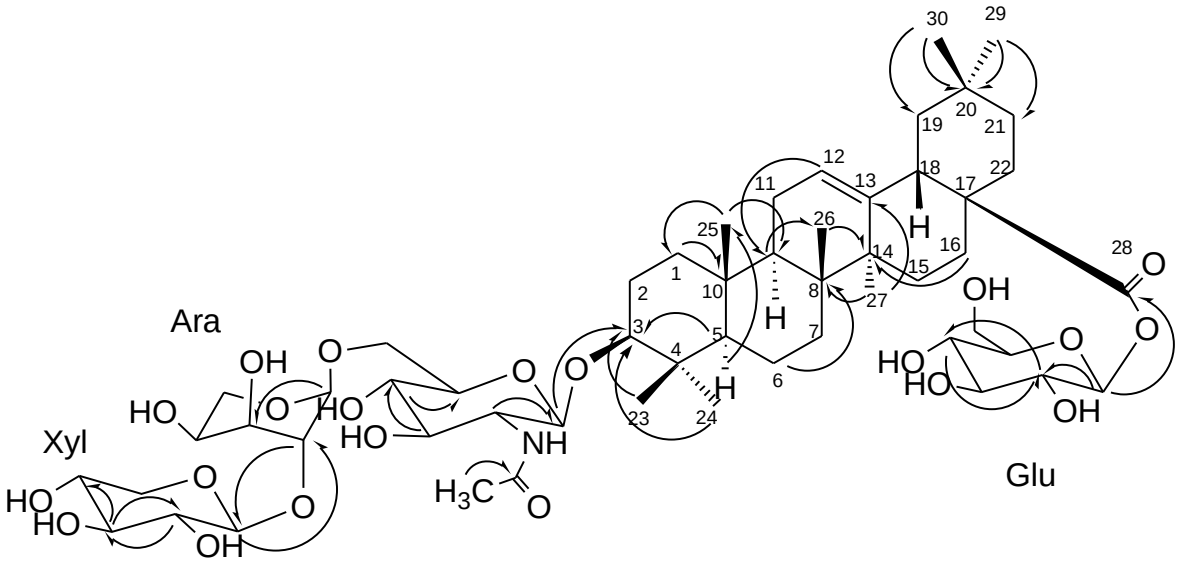

Fig. 3. *HMBC correlations of compound* **2**