

## Cycloartane-type glycosides from *Astragalus brachycalyx* FISCHER and their effects on cytokine release and hemolysis



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### ABSTRACT

Two new tridesmosidic cycloartane-type triterpene glycosides (**1** and **2**) were isolated from the methanolic extract of the roots of *Astragalus brachycalyx* FISCHER (*A. brachycalyx*) along with ten (3–12) known cycloartane-type triterpene glycosides. Structures of the new compounds were established as 3-*O*- $\beta$ -*D*-xylopyranosyl-6-*O*- $\beta$ -*D*-glucopyranosyl-16-*O*- $\beta$ -*D*-glucopyranosyl-3 $\beta$ ,6 $\alpha$ ,16 $\beta$ ,24(*S*)-25-pentahydroxycycloartane (**1**), 3-*O*-[ $\alpha$ -*L*-arabino-pyranosyl-(1 $\rightarrow$ 2)- $\beta$ -*D*-xylopyranosyl]-6-*O*- $\beta$ -*D*-glucopyranosyl-16-*O*- $\beta$ -*D*-glucopyranosyl-3 $\beta$ ,6 $\alpha$ ,16 $\beta$ ,24(*S*)-25-pentahydroxycycloartane (**2**), by using 1D and 2D-NMR techniques and mass spectrometry.

*In vitro* immunomodulatory effects and hemolytic activities of the new saponins (**1** and **2**) and acetylated form of **1** (**1a**) were studied together with the BuOH and MeOH extracts of *Astragalus brachycalyx*. The results have proven that tridesmosidic *Astragalus* cycloartanes are noteworthy immunomodulatory compounds via induction of cytokine production, namely IL-2 and IFN- $\gamma$ . The test compounds also resulted slight hemolysis at very high doses substantiating a safer profile compared to the positive control QS-21.

### 1. Introduction

The genus *Astragalus* L., belonging to the family of Fabaceae with more than 3.000 species of herbs and small shrubs, is broadly distributed in Mountainous areas throughout the world, mostly in Asia. Iran alone, being the richest center of *Astragalus* habitation, shelters more than 850 species, 527 of which are endemic in the flora of Iran (Ranjbar and Karamian, 2002; Massoumi 1998). The crude drugs prepared from *Astragalus* roots are used for treating some illnesses such as leukemia, respiratory infections and diabetes in Iranian folk medicine. The phytochemical studies performed on *Astragalus* species mainly resulted in the isolation of a series of oleanane- and cycloartane-type triterpene glycosides (Bedir et al., 1998, 1999; Gülcemal et al., 2011; Horo et al., 2010; Polat et al., 2010; Savran et al., 2012). Some bioactive compounds from *Astragalus* species are reported to contain anticancer and immunostimulatory activities (Rios and Waterman, 1997; Bedir et al., 2000). Li (2000) showed that polysaccharides from *Astragalus mongholicus*, consisting of glucose and arabinose moieties, increase not only the synthesis of RNA, DNA and protein but also the specific antibody formation in mouse spleen. To discover the contributory role of immunomodulatory effects of saponins, the

lymphocyte stimulation test was performed on eight cycloartane-type saponins isolated from *Astragalus melanophurarius* (Calis et al., 1997). It was shown that the higher concentrations of the test compounds (100–200  $\mu$ g/mL) had inhibitory effect on thymidine incorporation. In another study, the effects of nineteen cycloartane-type triterpene glycosides, isolated from *Astragalus* species, were investigated on macrophage activation utilizing a transcription factor-based bioassay for the activation of Nuclear Factor Kappa B (NF- $\kappa$ B) in a human macrophage/monocyte cell line. The only compound exhibiting activity was astragaloside I, increased NF- $\kappa$ B-directed luciferase expression up to 65%. It also increased the mRNA expression of inflammatory cytokines, such as IL-1 $\beta$  and TNF- $\alpha$  (Bedir et al., 2000). Verotta et al. (2001) reported the results of their study based on the effect of cycloartane saponins obtained from *Astragalus peregrinus* as modulators of lymphocyte proliferation. The compounds showed stimulation in proliferation of mouse splenocytes, and were not cytotoxic. IL-2, a cytokine produced by activated T cells, is proven to be a powerful immunostimulatory and antineoplastic agent. Yesilada et al. (2005) reported that astragaloside VII (AST VII), a tridesmosidic glycoside of cycloastragenol, was the most remarkable compound in regards to induction of IL-2 production (%139.6). In a continuation study, Nalbantsoy et al. (2011) evaluated

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adjuvant and hemolytic effects of IL-2 stimulating *Astragalus* compounds. AST VII and Macrophyllsaponin B (MAC B) generated important specific antibody and cellular response against BSA in mice, and both compounds were proposed to be potent immunoregulatory agents to be used in vaccine formulations as adjuvants. In the case of hemolysis, AST VII showed very low (0.54%) hemolytic index at the highest treatment concentration of 500  $\mu\text{g}/\text{mL}$  (Nalbantsoy et al., 2012).

Although Iran inhabits many endemic *Astragalus* species, there is no detailed phytochemical study reported on Iranian *Astragalus* plants. Here we reported the isolation and structure elucidation of two new cycloartane-type triterpene glycosides (1–2) along with ten known cycloartane-type triterpene glycosides (3–12) from the methanolic extract of *A. brachycalyx*, an Iran-Turan endemic. Additionally, the methanol and butanol extracts of *A. brachycalyx*, and the new saponins (1 and 2) together with 1a were evaluated for *in vitro* cytokine release (IL-2, IL-17A and IFN- $\gamma$ ) from human whole blood using ELISA. Apart from this, hemolytic activities of the same samples were studied in human red blood cells.

## 2. Results and discussion

The molecular formula of 1 was determined as  $\text{C}_{47}\text{H}_{80}\text{O}_{19}$  from HRESIMS ( $m/z$  987.4962 [M + K]<sup>+</sup>) and the <sup>1</sup>H and <sup>13</sup>C NMR data. The <sup>1</sup>H NMR spectrum of 1 showed signals due to a cyclopropane methylene at  $\delta$  0.49 and 0.19 (each 1H, d,  $J = 3.9$  Hz), six tertiary methyl groups at  $\delta$  2.07, 1.52 (6H), 1.39, 1.22, 0.88, a secondary methyl group at  $\delta$  1.00 (d, 6.5) and four methine proton signals at  $\delta$  4.39 (m), 3.97 (m), 3.70 (m) and 3.53 (dd,  $J = 11.6, 4.3$  Hz), which were indicative of secondary alcoholic functions. The NMR data of the aglycone moiety of 1 were in good agreement with those reported for cycloanthogenin (Bedir et al., 1998) with glycosidation shifts for C-3 ( $\delta$  88.5), C-6 ( $\delta$  79.9) and C-16 ( $\delta$  82.4). Furthermore, the <sup>1</sup>H NMR spectrum of 1 clearly showed three anomeric proton doublets at  $\delta$  4.89 (d,  $J = 7.7$  Hz), 4.85 (d,  $J = 7.5$  Hz) and 4.73 (d,  $J = 7.7$  Hz) in the downfield region, indicative of three  $\beta$ -linked sugar units. These correlated to C-atom signals at  $\delta$  105.1, 107.4 and 106.3, respectively, in the HSQC spectrum.

The HMQC, HMBC and COSY data led to identify these sugar units as one  $\beta$ -xylopyranosyl and two  $\beta$ -glucopyranosyl units, all of which were also suggested to have a D-configuration based on the biogenetic considerations derived from *Astragalus* cycloartanes (Bedir et al., 1998, 1999; Calis et al., 1997; Gülcemal et al., 2011; Horo et al., 2010; Polat et al., 2010; Savran et al., 2012) and the optical rotation data of each isolated sugar.

The HMBC correlations between the proton signal at  $\delta$  4.85 (H-1<sub>xy1</sub>) and the carbon resonance at  $\delta$  88.5 (C-3), the proton signal at  $\delta$  4.89 (H-1<sub>glc1</sub>) and the carbon resonance at  $\delta$  79.9 (C-6) and the proton signal at  $\delta$  4.73 (H-1<sub>glc2</sub>) and the carbon resonance at  $\delta$  82.4 (C-16) allowed us to determine the linkage site of the sugar units. Thus, the new compound 1 was elucidated as 3-O- $\beta$ -D-xylopyranosyl-6-O- $\beta$ -D-glucopyranosyl-16-O- $\beta$ -D-glucopyranosyl-3 $\beta$ ,6 $\alpha$ ,16 $\beta$ ,24(S)-25-pentahydroxycycloartane.

The HRESIMS data of 2 indicated a molecular formula of  $\text{C}_{52}\text{H}_{88}\text{O}_{23}$  ( $m/z$  1103.5646 calcd.) that was deduced from the ion peak at  $m/z$  1103.5648 ( $\text{C}_{52}\text{H}_{88}\text{O}_{23}\text{Na}$ ). The <sup>1</sup>H NMR and <sup>13</sup>C chemical shifts of the aglycone moiety of 2 were superimposable with those of 1. Moreover, for the sugar portion of 2 in comparison with that of 1, the occurrence of an additional sugar unit was observed. The <sup>1</sup>H NMR showed signals for four anomeric protons at  $\delta$  5.22 (d,  $J = 6.6$  Hz), 4.91 (d,  $J = 7.7$  Hz), 4.86 (d,  $J = 6.8$  Hz) and 4.75 (d,  $J = 7.6$  Hz), which showed correlations in the HSQC spectrum with the anomeric carbon signals at  $\delta$  106.7, 105.4, 105.4 and 106.6, respectively. These data, in combination with HSQC, HMBC, COSY correlations, showed that 2 differed from 1 only by the presence of an additional  $\alpha$ -arabinopyranosyl unit. Therefore, on the basis of the HMBC correlation between the proton signal at  $\delta$  5.22 (H-1<sub>ara</sub>) and the carbon resonance at  $\delta$  83.5 (C-2<sub>xy1</sub>) the arabinose was located at C-2<sub>xy1</sub>. The D configuration of

xylose and glucose and L configuration of arabinose units were established after hydrolysis of saponin fraction P of *A. brachycalyx* and confirmation by the optical rotation data of each isolated sugar.

Consequently, compound 2 was identified as 3-O-[ $\alpha$ -L-arabinopyranosyl-(1  $\rightarrow$  2)- $\beta$ -D-xylopyranosyl]-6-O- $\beta$ -D-glucopyranosyl-16-O- $\beta$ -D-glucopyranosyl-3 $\beta$ ,6 $\alpha$ ,16 $\beta$ ,24(S)-25-pentahydroxycycloartane.

Additionally, one known cycloartane-type triterpene, cycloastragenol (3) (Bedir et al., 1999) and nine known cycloartane-type triterpene glycosides, Astragaloside IV (4) (Kitagawa et al., 1983a), cyclocanthoside E (5) (Isaev et al., 1992), astragaloside VIII (6) (Kitagawa et al., 1983b), 3-O- $\beta$ -D-xylopyranosyl-6,25-di-O- $\beta$ -D-glucopyranosyl-3 $\beta$ ,6 $\alpha$ ,16 $\beta$ ,24(S),25-pentahydroxycycloartane (7) (Gülcemal et al., 2011) cyclocephaloside I (8) (Bedir et al., 1998), astrachryoside A (9) (Gan et al., 1986), cycloaraloside D (10) (Isaev, 1991), oleifolioside A (11) and oleifolioside B (12) (Ozipek et al., 2005) were isolated.

IL-2, with a critical role in the immune system by assisting immature T cells to differentiate into regulatory T cells, effector T cells or memory T cells, helps the body to fight against infectious diseases directly or indirectly (Liao et al., 2011). IFN $\gamma$ , known as type II interferon, is another vital cytokine with immunostimulatory potential. It has been confirmed that IFN $\gamma$  can play a crucial role in immune response against some intracellular pathogens in diseases such as Chagas, and it coordinates a dissimilar array of cellular programs through transcriptional regulation of genes that are immunologically relevant (Ben-Asouli et al., 2002; Schroder et al., 2004). One of the most important properties of IFN $\gamma$  is on the innate and adaptive immune response against tumors and pathogens (Lin and Young, 2013). IL-17A, originally identified as a transcript from a rodent T-cell hybridoma by Rouvier et al. (1993), is the chief member of the family named IL-17A cytokine group, and one of its essential roles is induction and facilitation of pro-inflammatory responses (Aggarwal and Gurney, 2002). IL-17A inhibitors are under investigation to treat autoimmune diseases like rheumatoid arthritis, psoriasis and inflammatory bowel disease. This group of cytokine inhibitors can lead to an aberrant airway neutrophil response to allergen stimuli (Yang et al., 2008). The role of IL-17A for Th17 cells in vitiligo pathogenesis treatment has also been confirmed (Zhou et al., 2015). Concisely, IL-2, IFN $\gamma$  and IL-17A are essential cytokines with different functions for the immune system, and developing new molecules modulating them will have a potential use in therapy.

Previous studies suggest that the specific molecules turning on the cytokine gene expression is more beneficial than injectable therapeutic doses of cytokines signifying the importance of the discovery of potent immunostimulatory agents (Gillis and Williams, 1998; Oda et al., 2003; Pandey et al., 2016; Hu et al., 2016).

*Astragalus* derived preparations are known for their stimulatory effects on cellular and humoral immune responses. Previous studies undoubtedly pointed to induction of cytokines such as interleukins and interferons for their immunostimulatory actions (Yang et al., 2005; Nalbantsoy et al., 2011, 2012). Not only crude *Astragalus* preparations but also its purified polysaccharides and saponins were demonstrated immunomodulatory activity in line with previous studies (Behboudi et al., 1999; Yang et al., 2008; Nalbantsoy et al., 2012; Lai et al., 2014). Thus *Astragalus* species and their components deserve detailed investigation for their immunomodulatory properties.

In this study, the effects of two newly established cycloartane-type saponins (compounds 1 and 2) along with compound 1a (acetylated form of compound 1), the methanol and butanol extracts of *A. brachycalyx* were studied on the human whole blood stimulated with PMA plus ionomycin for IL-2, IFN- $\gamma$  and IL-17A cytokines' release at 3 and 6  $\mu\text{g}$  concentrations, (Fig. 3). AST VII and QS-21 saponins were used as positive controls. At 3  $\mu\text{g}$  concentration, AST VII increased the level of IL-2 ( $1263.2 \pm 0.89$  pg/mL ( $\times 30$ ),  $P < 0.001$ ) whereas it did not show any effect on IFN- $\gamma$  ( $283.6 \pm 0.12$  pg/mL ( $\times 20$ ),  $P > 0.05$ ) and IL-17A ( $783.7 \pm 0.04$  pg/mL ( $\times 20$ ),  $P > 0.05$ ) production. However, AST VII increased the concentration of IL-2 ( $1217.65 \pm 0.03$  pg/mL ( $\times 30$ ),  $P < 0.001$ ) and IL-17A ( $317.66 \pm 0.32$  pg/mL ( $\times 20$ ),

$P < 0.05$ ) at 6  $\mu\text{g}$  with no effect on IFN- $\gamma$  production ( $823.67 \pm 0.19 \text{ pg/mL}$  ( $\times 20$ ),  $P > 0.05$ ). QS-21 exhibited substantial effect on IFN- $\gamma$  ( $395.85 \pm 0.32 \text{ pg/mL}$ ,  $P < 0.001$ ) and IL-2 ( $1217.02 \pm 1.07 \text{ pg/mL}$  ( $\times 30$ ),  $P < 0.01$ ) release at 3  $\mu\text{g}$  concentration. Besides, QS-21 exhibited more potency towards IL-2 and IFN- $\gamma$  stimulation ( $1416.94 \pm 0.41$  ( $\times 30$ ) and  $399.20 \pm 0.29$  ( $\times 20$ ),  $\text{pg/mL}$ , respectively) ( $P < 0.001$ ) at 6  $\mu\text{g}$  concentration, whereas it did not show any effect on IL-17A concentration.

The results showed that the concentration of IL-2 was significantly increased by **1** (6  $\mu\text{g}$ ), **2** (6  $\mu\text{g}$ ), the MeOH extract (6  $\mu\text{g}$ ) and slightly with **1a** (3  $\mu\text{g}$ ) compared to the control group ( $P < 0.01$ ,  $P < 0.001$  or  $P < 0.05$ ). The IFN- $\gamma$  concentration was stimulated considerably at 3  $\mu\text{g}$  dose of compound **2** ( $390.33 \pm 0.02 \text{ pg/mL}$  ( $\times 20$ ),  $P < 0.001$ ) possessing tridesmosidic framework with 4 sugar moieties compared to those of **1** and **1a** with trisaccharidic nature ( $P < 0.01$  or  $P < 0.001$ ). The MeOH extract showed a slight effect on IFN- $\gamma$  induction at 6  $\mu\text{g}$  ( $P < 0.05$ ). Moreover, the concentration of IL-17A was not effected by any of the tested molecules ( $P > 0.05$ ) in comparison to the control group.

Administration of **1a**, the fully acetylated form of **1**, resulted in altered levels of cytokine release compared to **1** at 3 and 6  $\mu\text{g}$  concentrations. Compound **1a** showed slightly higher IL-2 induction ( $1165.48 \pm 0.08 \text{ pg/mL}$  ( $\times 30$ ),  $P < 0.05$ ) than compound **1** ( $1136.47 \pm 0.01 \text{ pg/mL}$  ( $\times 30$ ),  $P > 0.05$ ) at 3  $\mu\text{g}$ , whereas **1** ( $1168.56 \pm 0.04 \text{ pg/mL}$  ( $\times 30$ ),  $P < 0.05$ ) increased the level of IL-2 in comparison to **1a** ( $1116.84 \pm 0.2 \text{ pg/mL}$  ( $\times 30$ ),  $P > 0.05$ ) at 6  $\mu\text{g}$  concentration (Fig. 3). These results imply that more hydrophobic forms of tridesmosidic *Astragalus* saponins still put forth IL-2 induction at lower doses (3  $\mu\text{g}$ ) signifying their potential use in oil based adjuvant systems. In modern vaccines, oil-in-water (o/w) emulsions are used as adjuvants safely and successfully. The most remarkable o/w emulsions used clinically are MF59 and AS03. Both adjuvant systems include squalene at ~2.5% (v/v) in the final vaccine formulation (Fox, 2009).

Basically, the immune response is categorized in two groups based on the cytokine profile induced: Th1 or Th2. The Th1 profile is characterized by the initial production of IFN $\gamma$  and IL-12 with an effector phase of T lymphocytes producing IL-2 and IFN $\gamma$  and mediating cytotoxic activity (CTL). In contrast, Th2 responses are influenced by IL-4 with predominantly IL-5 and IL-10 producing T cells in the effector phase that drive antibody production (Nalbantsoy et al., 2011, 2012, Yang et al., 2005). In this paper, the presented saponins were found predominantly effective on Th1 as associated with induction of IL-2 and IFN $\gamma$ . Hu et al. (2016) tested the role of 5 different saponins on altering levels of IL-4, IL-10, IL-2, and IFN- $\gamma$ . Their results revealed increments in IFN- $\gamma$  concentrations together with upregulation of IL-2 expression, validating our immunomodulatory findings.

Saponins cause hemolysis because of their well-known effects: cell membrane swelling due to pore formation, partial entry of extracellular  $\text{Ca}^{2+}$  and ceramide formation (Bissinger et al., 2014). In this study, *Astragalus brachycalyx* extracts and isolates were also investigated for their hemolytic activities to prove that observed immunostimulatory activities are not originating from such action. As shown in Table 3, QS-21 showed a notably strong hemolytic activity in all tested concentration (5000, 500, 250, 125, 50  $\mu\text{g/mL}$  ( $P < 0.01$ ) and 25, 12.5, 2.5  $\mu\text{g/mL}$  ( $P < 0.05$ ). Moreover, AST VII exhibited slight hemolysis for 5000  $\mu\text{g/mL}$  concentration ( $P < 0.05$ ), while there was no hemolytic activity for the doses of 250, 125, 50, 25, 12.5 and 2.5  $\mu\text{g/mL}$  ( $P > 0.05$ ). As it is shown in Fig. 1, acetylated derivative **1a** (500  $\mu\text{g/mL}$ ) demonstrated a slight hemolytic activity ( $P < 0.05$ ), whereas **1** did not cause any hemolysis at all tested concentrations. According to the above mentioned results, one could state that the tridesmosidic saponins of *Astragalus* genus are safer adjuvant candidates compared to the well-investigated QS-21 that is a strong hemolytic agent.

Consequently, our findings surely warrant further studies especially focusing on the mechanism of action and in vivo potential of the described saponins.

### 3. Experimental

#### 3.1. General

Optical rotations were measured on a JASCO DIP 1000 polarimeter. The  $^1\text{H}$ ,  $^{13}\text{C}$  NMR spectra as well as 2D-NMR experiments (COSY, HSQC and HMBC) were obtained using 400 MHz for  $^1\text{H}$  NMR. 100 MHz for  $^{13}\text{C}$  NMR were measured on a Varian AS 400 Mercury spectrometer for compound **1a** and compound **2**, and standard Bruker program on a UltraShield Plus 500 MHz (Bruker) (NMR Unite at the College of Pharmacy, Prince Sattam bin Abdulaziz University) spectrometer operating at 500 MHz for proton and 125 MHz for carbon for compound **1**. The chemical shift values are reported in  $\delta$  (ppm) relative to the residual solvent peak, and the coupling constants ( $J$ ) are reported in Hertz (Hz) and MS spectra were measured on a LC/MS High resolution Time of Flight (TOF) Agilent 1200/6530 instrument; CC, silica gel 60 (Merck); GPC (General Permeation Chromatography, Sephadex LH-20). All 2DNMR spectra were acquired in Pyridine- $d_5$  (99.50%, SigmaAldrich) standard pulse sequences and phase cycling were used for COSY, HSQC and HMBC spectra.

#### 3.2. Plant material

The root and stem parts of *Astragalus brachycalyx* FISCHER was collected from Sardasht City, Landi Sheykh Mountain, altitude of 2400 m, West Azerbaijan Province, Iran, in August 2013, and identified by Dr. Ali-Asghar Massoumi (botanist) in Research Institute of Forests and Rangelands. A voucher specimen (Tehran 19536) was deposited at Herbarium Research and The Application Center in Research Institute of Forests and Rangelands, Tehran, Iran.

#### 3.3. Extraction and isolation

Air-dried and powdered roots of *A. brachycalyx* (900 g) was extracted with MeOH ( $3 \times 2.5 \text{ L}$ ) for 5 days under reflux. After filtration, the solvent was removed by rotary evaporation affording a crude extract of 98 g. The extract was dissolved in water (700 mL) and successively partitioned with *n*-hexane ( $4 \times 300 \text{ mL}$ ),  $\text{CH}_2\text{Cl}_2$  ( $4 \times 300 \text{ mL}$ ), EtOAc ( $4 \times 300 \text{ mL}$ ), and *n*-BuOH ( $4 \times 300 \text{ mL}$ ). After evaporation the *n*-BuOH extract (20.71 g) was applied to Vacuum Liquid Chromatography (VLC) using reversed-phase silica (Lichroprep RP-18, 25–40  $\mu\text{m}$ , 600 g) employing  $\text{H}_2\text{O}$ -MeOH (90:10; 80:20; 60:40; 50:50; 40:60; 20:80) and MeOH to give 23 main fractions (Fr. A-W). Fraction M (5.61 g) was applied to an open column chromatography using normal-phase silica gel ( $23 \times 1200 \text{ mm}$ , 600 g) as stationary phase. The column was started and continued with  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  mixtures (90:10:1; 85:15:1.5; 80:20:2; 75:25:2.5; 70:30:3; 61:32:7; 64:50:10) to provide 3259 fractions. Fractions M-800-970 (56 mg) were combined and fractionated over reversed-phase material (Lichroprep RP-18, 25–40  $\mu\text{m}$ ,  $20 \times 130 \text{ mm}$ , 15 g) eluting with MeOH- $\text{H}_2\text{O}$  (50:50) mixture to afford compounds **3** (8.4 mg) and **4** (11 mg). Fractions M-1200-1450 (379.9 mg) were combined and further separated over normal phase silica gel ( $23 \times 450 \text{ mm}$ , 60.6 g) using a step-gradient of EtOAc-MeOH- $\text{H}_2\text{O}$  (100:10:5; 100:15:7.5; 100:17.5:13.5; 100:20:15; 100:25:20) to give 489 subfractions. Subfractions 130–168 (38.1 mg) were combined and subjected to column chromatography by using reversed-phase material (Lichroprep RP-18, 25–40  $\mu\text{m}$ ,  $20 \times 100 \text{ mm}$ , 10.3 g) and isocratic MeOH- $\text{H}_2\text{O}$  mobile phase system (5:5) to give **5** (9.5 mg). Subfractions 171–179 (25 mg) were combined and chromatographed over normal phase silica gel ( $23 \times 450 \text{ mm}$ , 70 g) employing with  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (90:10:1; 80:20:1; 80:20:2; 70:30:3; 61:32:7) mixtures and methanol to afford compounds **2** (18 mg) and **6** (4.4 mg). Subfractions 279–302 (90 mg) were combined and subjected to column chromatography by using normal-phase silica gel ( $20 \times 260 \text{ mm}$ , 22.2 g) eluting with  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  solvent system (85:15:1.5; 80:20:2; 75:25:2.5; 70:30:3; 61:32:7; 64:50:10) to

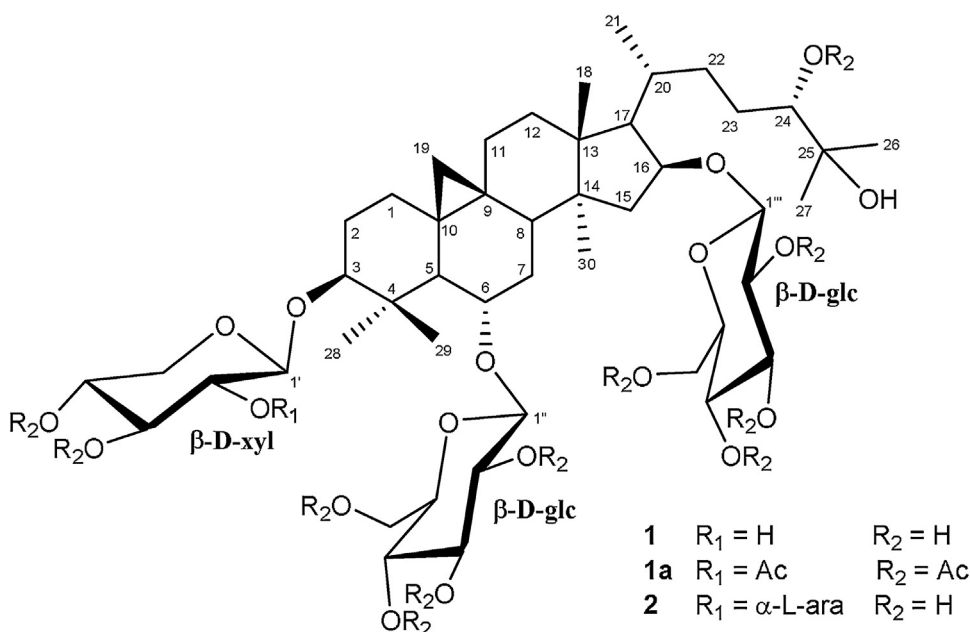


Fig. 1. Structures of compounds 1, 1a and 2.

yield a mixture including two compounds (80 mg). As the open column chromatography was not successful to separate these two metabolites due to their almost identical polarity, the mixture was acetylated using acetic anhydride and pyridine. Prior to acetylation, the mixture was analyzed by <sup>1</sup>H-NMR to make sure that no acetyl group was present for both compounds. After acetylation, the mixture was applied to column chromatography by using normal-phase silica gel (20 × 260 mm, 22.2 g) eluting with CHCl<sub>3</sub>-CH<sub>3</sub>COCH<sub>3</sub> (80:20; 70:30; 60:40; 50:50; 40:60) to yield **1a** (65 mg) and **7a** (5 mg). Compounds **1a** and **7a** were hydrolyzed with 0.5 N NaOH. After neutralization, reaction mixtures were applied to silica gel column (30 × 1000 mm, 48 g) with the solvent system CHCl<sub>3</sub>-CH<sub>3</sub>COCH<sub>3</sub> (60:40; 50:50; 40:60; 30:70) affording **1** (48 mg) and **7** (2.5 mg). The spectra and spectral data of **1a** were provided to support structural determination of **1** (see Supplementary file). Fractions M-2687-2900 (1.0 g) were combined and rechromatographed over an open column chromatography using silica gel (35 × 120 mm, 350 g) as stationary phase. Elution was performed with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (90:10:1; 80:20:1; 80:20:2; 70:30:3; 61:32:7) to give compounds **8** (25.5 mg) and **9** (40.8 mg). The main fraction O (2.70 g) was fractionated over a silica gel column (30 × 1000 mm, 390 g). Elution was carried out with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (85:15:1.5; 80:20:2; 75:25:2.5; 70:30:3) to yield 1110 fractions. Fractions O-90-110 (307 mg) were subjected to column chromatography by using normal-phase silica gel (35 × 120 mm, 150 g) eluting with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O solvent system (90:10:1; 80:20:2; 70:30:3; 61:32:7) to yield **10** (40.3). Fractions O-138-210 (100 mg) were applied to silica gel column (35 × 120 mm, 50 g) with the solvent system CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (90:10:1; 80:20:2; 70:30:3; 61:32:7) yielding **11** (9.2). Fractions N-900-943 (107 mg) were subjected to column chromatography by using normal-phase silica gel (35 × 120 mm, 55 g) eluting with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O solvent system (90:10:1; 80:20:2; 70:30:3; 61:32:7) to yield **12** (6.2).

### 3.3.1. 3-O-β-D-xylopyranosyl-6,16-di-O-β-D-glucopyranosyl-3β,6α,16β,24(S)-25 pentahydroxycycloartane (**1**)

Amorphous white solid; C<sub>47</sub>H<sub>80</sub>O<sub>19</sub>; [α]<sub>D</sub><sup>23</sup> + 14.6 (c 0.1 MeOH); IR ν<sub>max</sub><sup>KBr</sup> cm<sup>-1</sup>: 3435 (> OH), 2930 (> CH); for <sup>1</sup>H and <sup>13</sup>C NMR (Pyridine-d<sub>5</sub>, 500 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; HRESIMS m/z 987.4962 [M + K]<sup>+</sup> (calcd. for C<sub>47</sub>H<sub>80</sub>O<sub>19</sub>K, 987.4960) (positive mode).

Table 1

<sup>13</sup>C and <sup>1</sup>H NMR data (J in Hz) of the aglycone moieties of **1** and **2** (400 MHz for **2**, and 500 MHz for **1**, δ ppm, in Pyridine-d<sub>5</sub>).

C/H	1		2	
	δ <sub>C</sub>	δ <sub>H</sub> (J in Hz)	δ <sub>C</sub>	δ <sub>H</sub> (J in Hz)
1	32.1	1.58, m; 1.23, m	32.4	1.61, m; 1.31, m
2	30.0	2.36, m; 1.96, m	30.2	2.29, m; 1.96, m
3	88.5	3.53, dd (11.6, 4.3)	88.1	3.48, dd (11.4, 4.0)
4	42.4	–	42.8	–
5	52.3	1.90, d (9.1)	52.5	1.90, d (8.0)
6	79.9	3.70, m	79.4	3.74, m
7	34.6	2.36, m; 1.75, m	34.6	2.31, m; 1.87, m
8	46.2	1.76, m	45.5	1.83, m
9	21.0	–	21.5	–
10	28.5	–	28.7	–
11	26.0	1.84, m; 1.30, m	26.3	1.85, m; 1.30, m
12	34.2	2.36 (2H), m	33.0	2.29 (2H), m
13	45.5	–	45.9	–
14	46.7	–	47.0	–
15	47.3	2.44, dd (13.7, 8.0); 2.11, m	47.4	2.46, dd (13.9, 7.5); 2.13, m
16	82.4	4.39, m	82.6	4.43, dd (6.8, 1.6)
17	57.2	1.87, m	57.6	1.91, m
18	18.8	1.22, s	18.8	1.25, s
19	29.1	0.49, d (3.9)	28.5	0.55, d (4.0)
		0.19, d (3.9)		0.22, d (4.0)
20	29.4	2.36, m	29.8	2.40, m
21	17.4	1.00, d (6.5)	17.7	1.04, d (6.4)
22	33.4	2.26 m; 1.62, m	33.7	2.28, m; 1.64, m
23	28.6	2.06 m; 1.83, m	29.0	2.13 m; 1.88, m
24	77.8	3.97, m	78.3	3.98, m
25	72.8	–	72.9	–
26	25.8	1.52, s	26.0	1.54, s
27	25.8	1.52, s	26.0	1.54, s
28	28.5	2.07, s	28.3	1.94, s
29	16.4	1.39, s	17.7	1.38, s
30	19.9	0.88, s	20.0	0.97, s

### 3.3.2. 3-O-β-D-xylopyranosyl-6,16-di-O-β-D-glucopyranosyl-3β,6α,16β,24(S)-25 pentahydroxycycloartane dodecaacetate (**1a**)

Amorphous white solid; C<sub>73</sub>H<sub>106</sub>O<sub>32</sub>; [α]<sub>D</sub><sup>23</sup> + 5.0 (c 0.2 MeOH); IR ν<sub>max</sub><sup>KBr</sup> cm<sup>-1</sup>: 3435 (> OH), 2930 (> CH), 1680 (C=O); for <sup>1</sup>H and <sup>13</sup>C NMR (CDCl<sub>3</sub>, 400 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; HRESIMS m/z 1533.6456 [M + K]<sup>+</sup> (calcd. for C<sub>73</sub>H<sub>106</sub>O<sub>32</sub> K 1533.6454) (positive mode).

**Table 2**

<sup>13</sup>C and <sup>1</sup>H NMR data (*J* in Hz) of the sugar portions of **1** and **2** (400 MHz for **2**, and 500 MHz for **1**, δ ppm, in Pyridine-d<sub>5</sub>).

C/H	1		2	
	δ <sub>C</sub>	δ <sub>H</sub> ( <i>J</i> in Hz)	δ <sub>C</sub>	δ <sub>H</sub> ( <i>J</i> in Hz)
	β-D-Xyl (at C-3)		β-D-Xyl (at C-3)	
1	107.4	4.85, d (7.5)	105.4	4.86, d (6.8)
2	75.3	4.05, t (8.0)	83.5	4.12, t (7.6)
3	79.0	4.24, m	77.2	4.20, t (7.6)
4	71.6	4.14, m	71.0	4.19, m
5	66.7	4.35, dd (11.1, 5.0); 3.69, t (10.6)	66.5	3.60, m 4.29, m
	β-D-Glc (at C-6) α-L-Ara (at C-2 of Xyl)			
1	105.1	4.89, d (7.7)	106.7	5.22, d (6.6)
2	75.4	4.00, dd (8.5, 7.5)	73.7	4.61, dd (7.2, 4.8)
3	78.2	4.24, t (9.0)	74.3	4.23, dd (7.2, 2.5)
4	71.0	4.23, t (9.6)	69.1	4.31, m
5	77.9	3.97, m	67.1	4.44, dd (12.0, 3.2); 3.82, d (11.4)
6	62.8	4.61, dd (11.0, 3.5); 4.36, m	–	–
	β-D-Glc (at C-16) β-D-Glc (at C-6)			
1	106.3	4.73, d (7.7)	105.4	4.91, d (7.7)
2	75.4	4.00, dd (8.5, 7.5)	75.8	4.05, t (8.4)
3	78.2	4.15, t (9.0)	79.2	4.24, m
4	71.7	4.14, t (9.6)	72.1	4.16, t (7.6)
5	77.9	3.89, t (6.3)	78.0	3.97, t (7.6)
6	62.6	4.56, dd (11.0, 3.0); 4.35, dd (11.0, 5.0)	63.3	4.60, dd (7.6, 2.2); 4.37, m
			β-D-Glc (at C-16)	
1			106.6	4.75, d (7.6)
2			75.7	3.99, dd (7.6, 4.8)
3			78.6	4.17, t (6.8)
4			72.0	4.14, t (6.8)
5			78.2	3.90, m
6			62.9	4.55 dd (11.2, 1.6); 4.37, m

### 3.3.3. 3-O-[α-L-arabinopyranosyl-(1 → 2)-β-D-xylopyranosyl]-6-O-β-D-glucopyranosyl-16-O-β-D-glucopyranosyl-3β,6α,16β,24(S)-25-pentahydroxycycloartane (**2**)

Amorphous white solid; C<sub>52</sub>H<sub>88</sub>O<sub>23</sub> [α]<sub>D</sub><sup>23</sup> +15.0 (c 0.2 MeOH); IR ν<sub>max</sub><sup>KBr</sup> cm<sup>-1</sup>: 3445 (>OH), 2920 (>CH); for <sup>1</sup>H and <sup>13</sup>C NMR (Pyridine-d<sub>5</sub>, 400 MHz) data of the aglycone moiety and the sugar portion see Tables 1 and 2, respectively; HRESIMS *m/z* 1103.5648 [M + Na]<sup>+</sup> (calcd. for C<sub>52</sub>H<sub>88</sub>O<sub>23</sub>Na, 1103.5646) (positive mode). The structure of compound **2** and key HMBCs (H → C) is shown in Fig. 2.

### 3.4. Acid hydrolysis

The crude saponin mixture of fraction P (1 g) was heated at 60 °C with 1:1 0.5 N HCl-dioxane (3 mL) for 2 h, and then evaporated in vacuo. The solution was partitioned with EtOAc, and the H<sub>2</sub>O layer was neutralized with 0.5 M NaOH. After gaining the hydrolysed mixture, three monosaccharides were purified utilizing normal-phase silica gel as stationary phase (35 × 120 mm, 55 g) eluting with CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O solvent system (95:5:0.5, 80:20:1, 80:20:2; 70:30:3; 61:32:7). After purification, the obtained sugar units were identified by comparison with authentic samples using TLC in CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (60:40:10) system and their identity was confirmed after preparative TLC in the same solvent. The optical rotation of each purified sugar was measured to afford L-arabinose ([α]<sub>D</sub><sup>20</sup> +70.0, c 0.1, H<sub>2</sub>O), D-xylose ([α]<sub>D</sub><sup>20</sup> +22.0, c 0.1, H<sub>2</sub>O) and D-glucose ([α]<sub>D</sub><sup>20</sup> +13.0, c 0.1, H<sub>2</sub>O).

**Table 3**

Hemolytic activities of butanolic-, methanolic extracts and compounds **1**, **1a** and **2** from *A. brachycalyx*<sup>a,b,c</sup>.

Group	Absorbance value	Hemolytic activity	Hemolytic percentage (%)
Saline	0.110 ± 0.0009	0.000 ± 0.002	0.00 ± 0.23
Distilled water	0.638 ± 0.004	1.00 ± 0.001 **	100.03 ± 0.10**
AST VII (μg/mL)			
5000	0.203 ± 0.0008	0.16 ± 0.01*	16.54 ± 1.89*
500	0.155 ± 0.002	0.085 ± 0.004	8.51 ± 0.43
250	0.124 ± 0.004	0.027 ± 0.0010	2.71 ± 0.10
125	0.123 ± 0.006	0.023 ± 0.0016	2.39 ± 0.16
50	0.121 ± 0.002	0.014 ± 0.005	1.47 ± 0.50
25	0.111 ± 0.005	0.014 ± 0.001	1.41 ± 0.12
12.5	0.111 ± 0.001	0.018 ± 0.002	0.18 ± 0.28
2.5	0.873 ± 0.002	-0.04 ± 0.004	-4.35 ± 0.47
QS-21 (μg/mL)			
5000	2.573 ± 0.002	4.66 ± 0.005**	466 ± 0.50**
500	0.854 ± 0.009	1.65 ± 0.008 **	165 ± 0.82**
250	0.873 ± 0.005	1.35 ± 0.001 **	135 ± 1.34 **
125	0.984 ± 0.003	1.44 ± 0.002 **	144 ± 0.21**
50	0.785 ± 0.003	1.40 ± 0.008 **	140 ± 0.89**
25	0.514 ± 0.001	1.27 ± 0.02*	127.87 ± 2.41*
12.5	0.342 ± 0.005	0.76 ± 0.01*	76.58 ± 1.24*
2.5	0.155 ± 0.001	-0.08 ± 0.004*	-8.880 ± 0.43*
Compound 1 (μg/mL)			
5000	0.802 ± 0.000	1.30 ± 0.001***	130.91 ± 1.18***
500	0.160 ± 0.004	0.09 ± 0.010*	9.38 ± 1.00*
250	0.156 ± 0.003	0.08 ± 0.008	8.63 ± 0.82
125	0.135 ± 0.001	0.047 ± 0.002	4.71 ± 0.28
50	0.131 ± 0.003	0.01 ± 0.007	1.88 ± 0.75
25	0.118 ± 0.004	0.015 ± 0.001	1.50 ± 0.10
12.5	0.116 ± 0.001	0.011 ± 0.003	1.12 ± 0.39
2.5	0.103 ± 0.003	-0.134 ± 0.007	-1.33 ± 0.78
Compound 1a (μg/mL)			
5000	2.154 ± 0.001	3.84 ± 0.002**	384.98 ± 0.28**
500	0.152 ± 0.002	0.08 ± 0.004	8.01 ± 0.47
250	0.126 ± 0.002	0.030 ± 0.005	3.09 ± 0.54
125	0.126 ± 0.002	0.030 ± 0.005	3.09 ± 0.54
50	0.121 ± 0.002	0.02 ± 0.005	2.01 ± 0.50
25	0.111 ± 0.002	0.001 ± 0.006	0.12 ± 0.68
12.5	0.103 ± 0.003	-0.126 ± 0.008	-1.26 ± 0.89
2.5	0.103 ± 0.003	-0.013 ± 0.007	-1.32 ± 0.78
Compound 2 (μg/mL)			
5000	0.194 ± 0.002	0.15 ± 0.006*	15.95 ± 0.66*
500	0.158 ± 0.003	0.09 ± 0.007	9.13 ± 0.78
250	0.148 ± 0.000	0.07 ± 0.001	7.18 ± 0.10
125	0.137 ± 0.009	0.05 ± 0.002	5.16 ± 0.21
50	0.118 ± 0.004	0.01 ± 0.009	1.51 ± 0.09
25	0.110 ± 0.008	-0.0008 ± 0.001	-0.75 ± 0.18
12.5	0.0733 ± 0.004	-0.07 ± 0.01	-7.01 ± 1.09
2.5	0.089 ± 0.001	-0.040 ± 0.006	-3.82 ± 0.61
δ <sub>H</sub> ( <i>J</i> in Hz)			
5000	0.193 ± 0.003	0.15 ± 0.007*	15.76 ± 0.76*
500	0.133 ± 0.002	0.04 ± 0.006	4.27 ± 0.68
250	0.126 ± 0.001	0.03 ± 0.002	3.20 ± 0.21
125	0.118 ± 0.001	0.01 ± 0.003	1.43 ± 0.32
50	0.115 ± 0.008	0.008 ± 0.001	0.87 ± 0.18
25	0.109 ± 0.001	-0.002 ± 0.003	-0.26 ± 0.32
12.5	0.099 ± 0.004	-0.02 ± 0.001	-2.03 ± 0.10
2.5	0.089 ± 0.001	-0.04 ± 0.003	-4.05 ± 0.32
Butanol Extract (μg/mL)			
5000	0.487 ± 0.008	0.68 ± 0.018**	71.41 ± 1.89**
500	0.142 ± 0.002	0.61 ± 0.004	6.10 ± 0.47
250	0.131 ± 0.002	0.03 ± 0.005	3.89 ± 0.50
125	0.122 ± 0.001	0.02 ± 0.003	2.19 ± 0.37
50	0.120 ± 0.009	0.01 ± 0.002	1.94 ± 0.21
25	0.102 ± 0.002	-0.01 ± 0.004	-1.46 ± 0.47
12.5	0.093 ± 0.004	-0.03 ± 0.001	-3.16 ± 0.10
2.5	0.088 ± 0.001	-0.04 ± 0.002	-4.11 ± 0.28

<sup>a</sup> \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

<sup>b</sup> Hemolytic percents of saline and distilled water were included as minimal and maximal hemolytic control.

<sup>c</sup> All values represent the mean ± standard deviation (n ¼ 3 test).

### 3.5. Biological evaluation

#### 3.5.1. Measurement of the effects on cytokine biosynthesis

Heparinized peripheral human whole blood from healthy volunteers were stimulated with 50 ng PMA (Sigma, St. Lois, MO, U.S.A.) plus 400 ng ionomycin (Sigma, St. Lois, MO, U.S.A.) for IL-2, IFN- $\gamma$  and IL-17A and incubated in the presence of test samples or reference compounds QS-21 and AST VII for 48 h. The cultured supernatants were collected and the concentration of the cytokines produced by macrophages or lymphocytes were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (eBioscience, Vienne, Austria). The assays were performed according to the manufacturer's recommendations. The average absorbance readings of the samples were then compared with the concentrations of the standard curve, and the samples concentration of each cytokine was calculated. The results of IL-2, IFN- $\gamma$  and IL-17A were expressed as picograms per milliliter. The minimum detection limits were 9.1 pg/mL, 0.99 pg/mL, and 0.5 pg/mL for IL-2, IFN- $\gamma$  and IL-17A, respectively. Diluted supernatants were used for all assays and each assay was performed in triplicate.

#### 3.5.2. Peripheral whole-blood cultures

Human whole blood (containing 20 U/mL heparine) was suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in a 1:10 ratio. PMA (50 ng/mL) and ionomycin (400 ng/mL) were added to the whole blood for stimulation of immune system cells. The samples were dissolved as stock solution in DMSO at concentration of 5 mg/mL and DMSO was used as negative control. An amount of 1 mL of the heparinized blood which was stimulated by PMA (50 ng/mL) and ionomycin (400 ng/mL) was transferred into each well of 24-well plate and incubated at 37 °C for 48 h in the absence or presence of 3 or 6  $\mu$ g/mL concentrations of samples or QS-21 and AST VII as reference compound. The diluted culture supernatants were then mixed with sample diluent at 1:2 ratio and were assayed using the specific ELISA method for IL-2, IFN- $\gamma$  and IL-17A (Lenarczyk et al., 2000; Nalbantsoy et al., 2011). The protocol was approved by the Human Ethics Committee of Ege University, and all of the used procedures conformed to the protocol was approved by the Human Ethics Committee of Ege University, and all of the procedures

used conformed to the declaration of Helsinki. The subjects were informed about the procedures and signed the informed consent forms (approval number 15-11/19).

#### 3.5.3. Hemolytic activity assay

Hemolytic activity of the molecules was measured according to Nalbantsoy et al. (2011) with some modifications. Red blood cells obtained from human healthy volunteers. Blood was collected with BD Vacutainer TM (NH 143 I. U., Belliver Industrial Estate, Plymouth, UK). An amount of 7 mL of blood was washed three times using sterile saline solution (0.89%, w/v NaCl, pyrogen free) and then it was centrifuged at  $2000 \times g$  for 5 min. The final cell suspension was prepared by diluting the pellet to 0.5% using saline solution. A volume of 0.01 mL of the cell suspension was mixed in U button 96-well microplate with 0.05 mL diluents containing 2.5, 12.5, 25, 50, 125, 250, 500 and 5000  $\mu$ g/mL concentrations of molecules in saline solutions. The mixtures were incubated for 30 min at 37 °C and centrifuged at  $800 \times g$  for 10 min. The free hemoglobin in the supernatants was measured spectrophotometrically at 412 nm. Saline solution and distilled water were used as minimal and maximal hemolytic controls. The hemolytic percent developed by the saline control was subtracted from all groups. Each experiment included triplicates at each concentration.

#### 3.5.4. Statistical analysis

The data were expressed as mean  $\pm$  standard deviation (S.D.) and examined for their statistical significance of difference with Student *t*-test, One-way ANOVA and the post hoc test Tamhane's and dunnet (depends on homogeneity and normality of the absorbance values) using SPSS 16.0. P-values less than 0.05, 0.01 and 0.001 were considered to be statistically significant.

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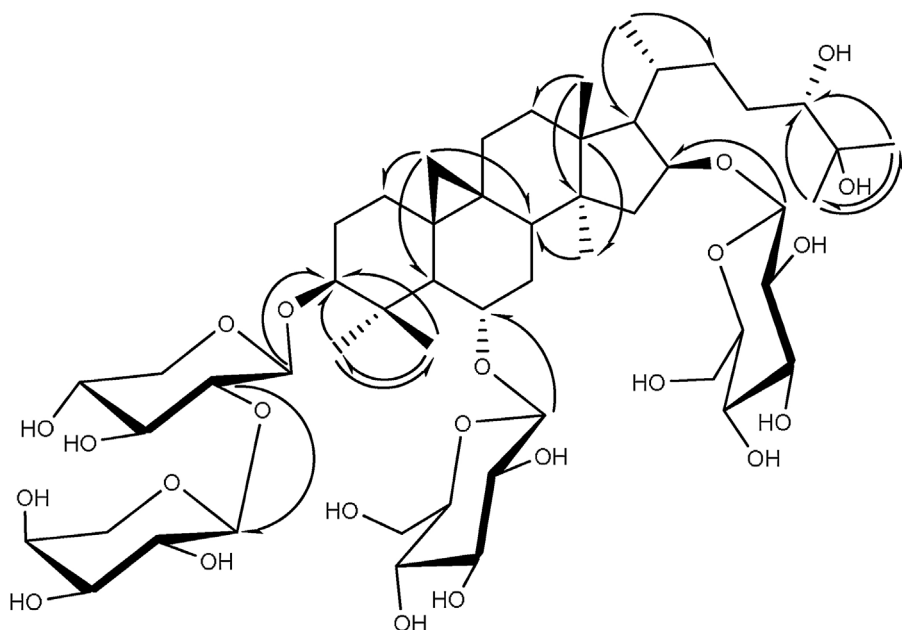


Fig. 2. Key long-range correlations [HMBCs (H  $\rightarrow$  C)] of 2.

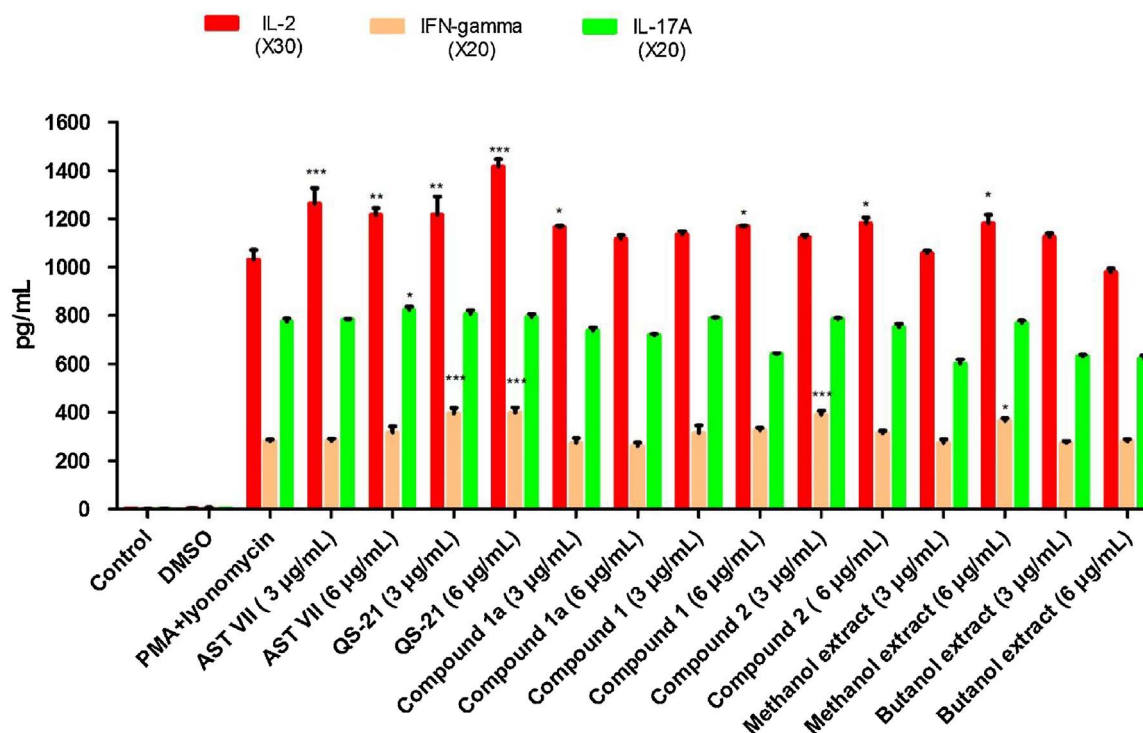


Fig. 3. Effect of the extracts and new compounds on various cytokines' production in whole blood samples after PMA plus ionomycin stimulation. The concentration of the cytokines was measured by ELISA kits as described in the text, the values are presented as mean  $\pm$  standard deviation (S.D) (n = 5). Significant differences with PMA plus ionomycin groups were designated as \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytol.2017.05.028>.

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