Phytochemistry xxx (2017) 1–9

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*

Hilal Top [a], Nazli Boke Sarikahya [a], Ayse Nalbantsoy [b], Suheyla Kirmizigul [a, *]

[a] Department of Chemistry, Faculty of Science, Ege University, 35100, Bornova, Izmir, Turkey
[b] Department of Bioengineering, Faculty of Engineering, Ege University, 35100, Bornova, Izmir, Turkey

ARTICLE INFO

Article history:
Received 28 November 2016
Received in revised form
7 February 2017
Accepted 8 February 2017
Available online xxx

Keywords:
Cephalaria balansae
Caprifoliaceae
Triterpene glycoside
Cytotoxic activity
Hemolytic activity
Immunomodulatory activity

ABSTRACT

Phytochemical investigations on n-butanol extract of *Cephalaria balansae* Raus. (Caprifoliaceae) led to the isolation of four previously undescribed triterpenoid saponins based on hederagenin type aglycone, namely, balansoides A-D, along with ten known compounds. Their structures were proposed based on 1D and 2D NMR spectroscopic data, HRESIMS analysis and chemical evidence as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin, 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester, 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-galactopyranosyl-(1 → 6)-β-D-glucopyranosyl ester and 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester. The four saponins were evaluated for their potential cytotoxic activity against HEK-293, A-549, HeLa cells and for hemolytic properties on human blood cells. Balansoide A displayed significant inhibitory effects on cancerous A-549 and HeLa cells, and non-cancerous HEK-293 cells with IC_{50} values of 12, 15 and 8 μM, respectively. Balansoides A-D together with *n*-butanol extract exhibited considerable hemolysis in human erythrocyte cells. Immunomodulatory properties of balansoides A-D were also evaluated in activated whole blood cells by PMA plus ionomycin. While balansoides A-C increased IL-1β concentration with values of 1004.47, 991.57 and 966.50 pg/ml, only balansoide B augmented a slight IFN-γ secretion with value of 5219.14 pg/ml. None of the compounds changed IL-2 levels significantly.

1. Introduction

Saponins constitute a structurally highly diverse class of natural plant defense compounds. They are widely distributed in higher plants but are also found in some bacteria and some lower animal sources, like e.g. marine invertebrates (Netala et al., 2015). They are responsible for most of the observed biological effects such as antifungal, insecticidal, anthelmintic, cytotoxic, anti-inflammatory, immunostimulant (Francis et al., 2002; Lacaille-Dubois and Wagner, 1996; Kräutler et al., 2008; Sparg et al., 2004). Especially in recent years, researches on saponins have been directed on the immunotherapy methods *in vivo* and *in vitro* conditions (Nalbantsoy et al., 2012; Rajput et al., 2007). The presence of saponins has been reported in more than 100 families of plants (Hostettmann and Marston, 1995). The genus *Cephalaria* (Schrad. ex Roem. & Schult.) (Caprifoliaceae), which is one of these plants, has been used in traditional medicine for many years due to its antimicrobial, antifungal, cytotoxic, antioxidant, antidiabetic and hypothermic activities (Mbhele et al., 2015; Mustafayeva et al., 2010; Pasi et al., 2009; Podolak et al., 2010; Sarikahya et al., 2011) Many hederagenin type saponins have been isolated from *Cephalaria* species especially by our research group and other scientists (Godevac et al., 2010; Gulcemal et al., 2014; Kirmizigul et al., 1995; Pasi et al., 2009; Sarikahya et al., 2011; Sarikahya and Kirmizigul, 2012) As well as saponins, *Cephalaria* taxa involve some specialised metabolites such as iridoids, flavonoids, alkaloids, and lignans (Mojab et al., 2003; Movsumov et al., 2009; Mustafayeva et al., 2010; Sarikahya and Kirmizigul, 2010).

Preliminary TLC analysis of the *n*-BuOH extracts of *C. balansae* Raus. (Davis, 1972) suggest that it contains numerous triterpene

* Corresponding author.
E-mail address: suheyla.kirmizigul@ege.edu.tr (S. Kirmizigul).

http://dx.doi.org/10.1016/j.phytochem.2017.02.015
0031-9422/

glycosides which prompts us to make a phytochemical examination of this fraction. As a result, four previously undescribed hederagenin type triterpene glycosides (**1**–**4**) named balansoides A-D together with ten known natural compounds were isolated from *C. balansae*. The structures of these compounds were identified by chemical methods including acidic and alkaline hydrolysis, sillylation and extensive spectroscopic analysis, along with 1D 2D NMR and HRESIMS data. The four previously undescribed triterpenoid saponins which were purified were tested for their cytotoxicity against HeLa (human cervix adenocarcinoma), A-549 (human alveolar adenocarcinoma), and a normal cell line HEK-293 (human embryonic kidney cells) by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)] method. Balansoides A-D and the *n*-butanol extract were evaluated for their hemolytic activity in human erythrocyte cells. Immunomodulatory properties of previously undescribed compounds were also tested in activated whole blood cells by PMA plus ionomycin.

2. Results and discussion

The *n*-butanol extract of the aerial parts of *Cephalaria balansae* was submitted to vacuum liquid chromatography (VLC) and fractionated by repeated medium pressure liquid chromatography (MPLC) and open column chromatography (CC) applications over normal and RP-18 silica gel, yielding four previously undescribed triterpenoid saponins called balansoides A-D (**1**–**4**) (see Fig. 1) and ten known ones. Eight compounds (**5**–**12**) were determined as known hederagenin type triterpene glycosides which can be classified as monodesmosidic and bisdesmosidic. The monodesmosidic ones were elucidated as α-hederin (**5**) (Aliev and Movsumov, 1976) and sapindoside B (**6**) (Chirva et al., 1969). The bisdesmosidic glycosides were 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester (**7**) (Kawai et al., 1988), decaisoside D (**8**), decaisoside E (**9**) (Kong et al., 1993), elmalienoside B (**10**), elmalienoside C (**11**) (Sarikahya and Kirmizigul, 2012) and dipsacoside B (**12**) (Mukhamedziev et al., 1971). The other two known compounds, **13** and **14**, were determined as oleanoic acid (Lin et al., 1998) and 3-*O*-β-D-sitosterolglycoside (Chirva et al., 1970), respectively by comparison of their physical and spectroscopic data with those reported in the literature. All isolated compounds were identified by comparison of the 1D, 2D NMR (1H, 13C, APT, COSY, HSQC, HMBC) and HRESIMS techniques and chemical methods with reference data. All compounds were isolated as amorphous powders and each monosaccharide was confirmed by extensive 2D NMR data and GC-MS analysis which compared them with authentic samples (Sarikahya and Kirmizigul, 2010).

Compound **1** (balansoide A) (see Fig. 1) has a molecular formula of $C_{52}H_{84}O_{20}Na$, determined by HRESIMS (*m/z* 1051.5399 $[M + Na]^+$). The IR spectrum of **1** indicated the presence of hydroxyl (3387 cm^{-1}) and carbonyl (1696 cm^{-1}) groups in the molecule. The 1H NMR spectrum of **1** showed characteristic singlets due to six quaternary methyl groups at δ_H 1.07, 0.85 (x 3), 0.70 and 0.57, a hydroxymethyl group at δ_H 3.10, 3.32 (m) and an olefinic proton at δ_H 5.13 (br s). The 13C NMR spectrum also revealed the signals for six quaternary carbons at δ_C 33.3, 26.0, 23.9, 17.4, 16.0 and 13.5; an oxygen-bearing methine carbon at δ_C 79.8, a hydroxymethyl group at δ_C 62.8, a set of olefinic carbons at δ_C 144.5 and 121.8 and one carbonyl carbon at δ_C 179.3 confirmed that the aglycone of **1** is a hederagenin (Sharma et al., 2013) (see Tables 1 and 2). The C-3 oxymethine carbon and C-28 carbonyl carbon were observed at δ_C 79.8 and 179.3, respectively, which suggests that compound **1** is a 3-monodesmoside of hederagenin. For the sugar moieties, the 1H NMR spectrum of **1** displayed four anomeric proton signals at δ_H 4.30 (d, $J = 6.4$ Hz), 4.30 (d, $J = 6.4$ Hz), 5.13 (br s), and 5.30 (br s),

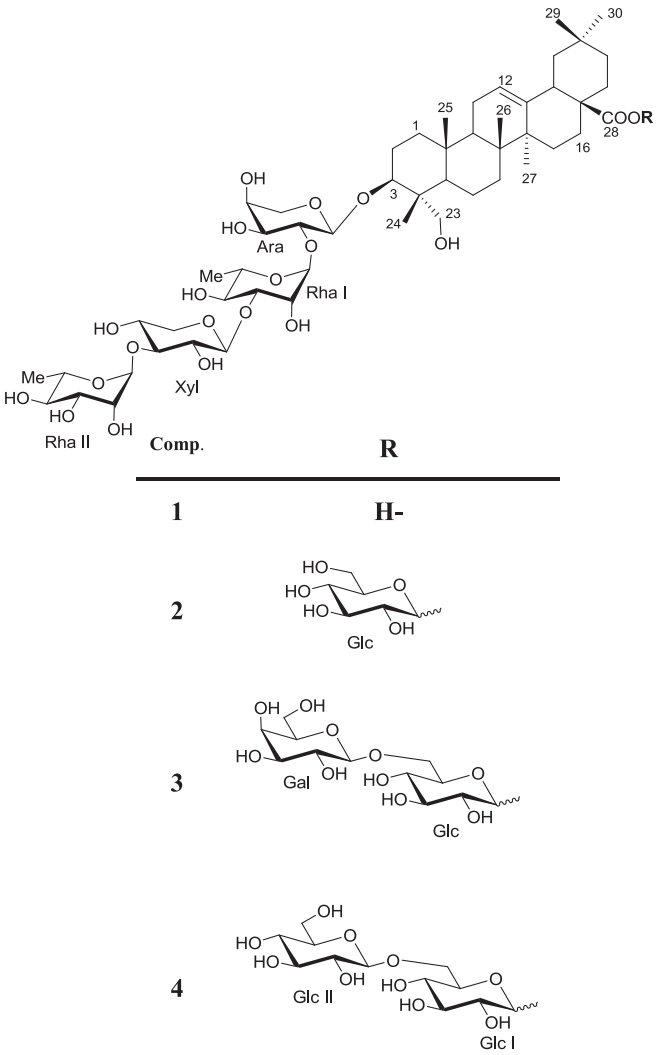

Fig. 1. Structures of compounds **1**–**4**.

giving in the HSQC spectrum cross-peaks with four anomeric carbon signals at δ_C 103.6, 105.9, 100.7 and 100.0, respectively. All proton signals for the sugar moieties were associated with COSY and HMQC spectra. While the acid hydrolysis of compound **1** gave L-arabinose, L-rhamnose, D-xylose, and hederagenin, alkaline hydrolysis showed no effect on compound **1**. The sugars obtained by aqueous acid hydrolysis of **1** were identified by GC-MS analysis which compared them with authentic samples (Sarikahya and Kirmizigul, 2010). These results were also confirmed by the HMBC data. The arabinose moiety was shown to be attached at C-3 of the aglycone by an observed HMBC correlation of δ_H/δ_C 4.30 (d, $J = 6.4$ Hz, Ara H-1)/79.8 (Agly C-3). On the other hand, long-range correlations between the H-1 proton of L-rhamnose I at δ_H 5.30 and the C-2 carbon of L-arabinose at δ_C 74.0, the H-1 proton of D-xylose at δ_H 4.30 and the C-3 carbon of L-rhamnose I at δ_C 81.5 and the H-1 proton of L-rhamnose II at δ_H 5.13 and the C-3 carbon of D-xylose at δ_C 80.8 showed the linkaging points of the sugar molecules to one another. On the basis of the above results, the structure of compound **1** was elucidated as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin.

Balansoide B (**2**) exhibited in the HRESIMS (positive-ion mode),

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015

Table 1
1H NMR data for compounds **1**–**4**[a,b].

Position	1	2	3	4
3	3.48, m	3.47, m	3.49, m	3.48, m
5	1.21, m	1.16, m	1.17, m	1.18, m
9	1.48, m	1.46, m	1.47, m	1.48, m
12	5.13, brs	5.13, brs	5.13, brs	5.14, brs
23	3.10, 3.32, m	3.08, 3.28, m	3.08, 3.30, m	3.11, 3.34, m
24	0.57, s	0.55, s	0.55, s	0.55, s
25	0.85, s	0.82, s	0.84, s	0.82, s
26	0.70, s	0.65, s	0.65, s	0.66, s
27	1.07, s	1.05, s	1.06, s	1.06, s
29	0.85, s	0.84, s	0.84, s	0.83, s
30	0.85, s	0.83, s	0.84, s	0.83, s
	Ara at C-3	**Ara at C-3**	**Ara at C-3**	**Ara at C-3**
1	4.30, d (6.4)	4.28, d (7.2)	4.29, d (7.2)	4.29, d (7.2)
2	3.50, m	3.48, m	3.50, m	3.50, m
3	3.50, m	3.55, m	3.48, m	3.48, m
4	3.56, m	3.55, m	3.56, m	3.56, m
5	3.30, 3.65, m	3.29, 3.65, m	3.30, 3.64, m	3.32, 3.66, m
	Rha I	**Rha I**	**Rha I**	**Rha I**
1	5.30, brs	5.13, brs	5.13, brs	5.14, brs
2	3.81, m	3.79, m	3.78, m	3.80, m
3	3.60, m	3.59, m	3.61, m	3.60, m
4	3.48, m	3.46, m	3.48, m	3.47, m
5	3.78, m	3.78, m	3.78, m	3.80, m
6	1.07, brs	1.06, d (6.6)	1.07, d (6.0)	1.08, d (6.0)
	Xyl	**Xyl**	**Xyl**	**Xyl**
1	4.30, d (6.4)	4.29, d (7.2)	4.29, d (7.2)	4.29, d (7.2)
2	3.18, m	3.19, m	3.16, m	3.18, m
3	3.32, m	3.32, m	3.32, m	3.32, m
4	3.32, m	3.29, m	3.32, m	3.32, m
5	3.07, 3.69 m	3.09, 3.67 m	3.06, 3.68, m	3.09, 3.69, m
	Rha II	**Rha II**	**Rha II**	**Rha II**
1	5.13, brs	5.00, brs	5.00, brs	5.00, brs
2	3.67, m	3.65, m	3.66, m	3.67, m
3	3.16, m	3.17, m	3.16, m	3.20, m
4	3.40, m	3.36, m	3.38, m	3.40, m
5	3.86, m	3.87, m	3.86, m	3.88, m
6	1.07, brs	1.06, d (6.6)	1.07, d (6.0)	1.07, d (6.0)
		Glc at C-28	**Glc at C-28**	**Glc I at C-28**
1		5.20, d (7.8)	5.20, d (8.0)	5.20, d (7.2)
2		3.07, m	3.10, m	3.16, m
3		3.18, m	3.30, m	3.10, m
4		3.13, m	3.20, m	3.18, m
5		3.09, m	3.21, m	3.08, m
6		3.42, 3.59, m	3.50, 3.92, m	3.59, 3.91, m
			Gal	**Glc II**
1			4.44, d (7.2)	4.18, d (8.4)
2			3.79, m	2.29, m
3			3.08, m	3.34, m
4			3.26, m	3.04, m
5			3.43, m	3.01, m
6			3.40, 3.60, m	3.42, 3.62, m

[a] 1H NMR data (δ) were measured in DMSO-d_6 at 400 and 600 MHz.
[b] Coupling constants (*J*) in Hz are given in parentheses.

a quasimolecular-ion peak at *m/z* 1213.5929 $[M + Na]^+$, is consistent with the molecular formula of $C_{58}H_{94}O_{25}$. The 1H and 13C NMR signals of the aglycone of **2**, assigned by extensive 2D NMR spectroscopic analyses, were almost superimposable with those of **1**. Compared to **1**, the 1H and 13C NMR signals indicated an extra glycosylation at C-28, supported by HMBC correlations at δ_H 5.20 (d, J = 7.8 Hz, Glc)/with δ_C 175.6 (C-28). Thus, the structure of **2** was determined to be 3,28-bisdesmosidic hederagenin saponin. The HSQC spectrum of **2** displayed five cross peaks of δ_H/δ_C 4.28 (d, J = 7.2 Hz)/103.7, 4.29 (d, J = 7.2 Hz)/105.9, 5.00 (br s)/100.7, 5.13 (br s)/100.0, and 5.20 (d, J = 7.8 Hz)/94.5 indicating the presence of five sugar moieties. Units of two α-L-rhamnopyranosyl, one β-D-glucopyranosy, one β-D-xylopyranosyl and one α-L-arabinopyranosyl were identified by extensive 2D NMR spectral analysis and GC-MS analysis. The correlations which were observed in the HMBC spectrum between the anomeric proton signals and aglycone carbons at δ_H 4.28 (Ara) and δ_C 79.8 (C-3 of aglycone), δ_H 5.20 (Glc) and δ_C 175.6 (C-28 of aglycone) confirmed the linkage points of the sugar moieties to the aglycone. Correlations between the H-1 proton of Rha I at δ_H 5.13 and the C-2 carbon of Ara at δ_C 73.9, the H-1 proton of Xyl at δ_H 4.29 and the C-3 carbon of Rha I at δ_C 81.5 and the H-1 proton of Rha II at δ_H 5.00 and the C-3 carbon of Xyl at δ_C 80.7 revealed the linkage points between monosaccharide units. Accordingly, the structure of **2** was characterized as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl ester.

The molecular formula of balansoide C (**3**) was determined as $C_{64}H_{104}O_{30}$ according to the $[M + Na]^+$ peak at *m/z* 1375.6455 in the HRESIMS. The 1H and 13C NMR spectral features of **3**, assigned by extensive 2D NMR analyses, were quite similar to those of **2** except that 3 had one more galactose unit. The sugar part of **3** was found to consist of six monosaccharide residues, identified as L-arabinose (δ_C 103.7/δ_H 4.29, d, J = 7.2 Hz), L-rhamnose I (δ_C 99.9/δ_H 5.13, br s), D-xylose (δ_C 105.8/δ_H 4.29, d, J = 7.2 Hz), L-rhamnose II (δ_C 100.7/δ_H 5.00, br s), D-glucose (δ_C 94.6/δ_H 5.20, d, J = 8.0 Hz) and D-galactose (δ_C 101.5/δ_H 4.44, d, J = 7.2 Hz) from its NMR spectroscopic data. In the HMBC spectrum, long-range correlations between δ_H 4.29 (H-1 of Ara) and δ_C 79.9 (C-3 of aglycone), δ_H 5.13 (H-1 of Rha I) and δ_C 73.9 (C-2 of Ara), δ_H 4.29 (H-1 of Xyl) and δ_C 81.4 (C-3 of Rha I), δ_H 5.00 (H-1 of Rha II) and δ_C 80.8 (C-3 of Xyl), δ_H 5.20 (H-1 of Glc) and δ_C 175.8 (C-28 of aglycone), δ_H 4.44 (H-1 of Gal) and δ_C 68.5 (C-6 of Glc) showed the linkage points of the monosaccharides to one another and to the aglycone. The identity of each monosaccaride was determined by an acidic hydrolysis using the GC-MS techniques which compared them with authentic sugar samples, the COSY spectral data and literature findings (Sarikahya and Kirmizigul, 2010). The alkaline hydrolysis of compound **3** yielded the compound **1** (balansoide A). Consequently, compound **3** was determined to be 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-galactopyranosyl-(1 → 6)-β-D-glucopyranosyl ester.

Compound **4** showed the molecular formula of $C_{64}H_{104}O_{30}$ on the basis of the HRESIMS data at *m/z* $[M + Na]^+$ = 1375.6444. Comparison of the 1H and 13C NMR data of **4** (see Tables 1 and 2) with those of **3** showed considerable structural similarity except for the presence of one glucose moiety instead of a galactose. In the HMBC spectrum, the C-3 (79.9) and the C-28 (175.7) carbons of the aglycone showed long-range correlations with the H-1 of the arabinose at δ_H 4.29 and the H-1 proton of glucose I at δ_H 5.20, respectively. On the other hand, long-range correlations between the H-1 proton of Rha II at δ_H 5.00 and the C-3 carbon of Xyl at δ_C 80.7 and the H-1 proton of Xyl at δ_H 4.29 and the C-3 carbon of Rha I at δ_C 81.5 and the H-1 proton of Rha I at δ_H 5.14 and the C-2 carbon of Ara at δ_C 73.9 and the H-1 proton of Glc II at δ_H 4.18 and the C-6 carbon of Glc I at δ_C 68.2 showed the link between the sugar moieties to one another. Accordingly, the structure of **4** was formulated as 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester, namely, balansoide D.

Cytotoxicity assay of the previously undescribed compounds (**1**–**4**) were evaluated using the MTT method against HEK-293, A-549 and HeLa cells. According to these evaluations, only balansoide A (**1**) displayed significant inhibitory effects on cancerous A-549 and HeLa cells, and non-cancerous HEK-293 cell with IC_{50} values of 12, 15 and 8 μM whereas IC_{50} values of doxorubicin were determined as 7, 10 and 12 μM, respectively (see Table 3 and Fig. 2). Despite the few number of tested compounds, the results indicated that the monodesmosidic type saponins exhibit significant activity

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015

Table 2
13C NMR data for compounds **1**–**4**[a,b,c].

Position	1	2	3	4	Position	1	2	3	4
	Aglycon					Sugars			
						Ara at C-3	**Ara at C-3**	**Ara at C-3**	**Ara at C-3**
1	38.7	38.7	38.9	38.7	1	103.6	103.7	103.7	103.7
2	25.9	25.9	25.9	25.9	2	74.0	73.9	73.9	73.9
3	79.8	79.8	79.9	79.9	3	73.8	73.9	73.8	73.8
4	42.7	42.9	42.8	42.7	4	68.7	68.7	68.7	68.4
5	46.6	46.6	46.7	46.6	5	65.5	65.5	65.5	65.6
6	17.6	17.5	17.8	17.5		**Rha I**	**Rha I**	**Rha I**	**Rha I**
7	32.6	32.2	32.4	32.2	1	100.0	100.0	99.9	100.0
8	nd	39.3	39.4	39.4	2	70.3	70.3	70.3	70.4
9	47.6	47.6	47.6	47.6	3	81.5	81.5	81.4	81.5
10	36.4	36.4	36.4	36.4	4	71.0	71.0	71.0	71.1
11	23.3	23.4	23.4	23.4	5	68.3	68.3	68.3	68.7
12	121.8	122.1	122.1	122.1	6	18.2	18.3	18.2	18.3
13	144.5	143.9	143.9	144.0		**Xyl**	**Xyl**	**Xyl**	**Xyl**
14	41.8	41.8	41.8	41.8	1	105.9	105.9	105.8	105.9
15	27.7	27.6	27.7	27.7	2	75.0	74.9	74.9	75.0
16	23.2	22.9	23.0	22.9	3	80.8	80.7	80.8	80.7
17	45.9	46.4	46.4	46.0	4	68.3	68.2	68.3	68.3
18	41.4	41.2	41.2	41.2	5	66.3	66.3	66.3	66.3
19	46.3	46.0	46.0	46.4		**Rha II**	**Rha II**	**Rha II**	**Rha II**
20	30.9	30.7	30.7	30.7	1	100.7	100.7	100.7	100.8
21	33.9	33.7	33.7	33.7	2	71.0	71.0	71.0	71.2
22	32.4	32.0	32.2	32.1	3	72.6	72.5	72.5	72.7
23	62.8	62.8	62.8	62.8	4	71.3	71.2	71.3	71.0
24	13.5	13.6	13.6	13.6	5	68.5	68.4	68.5	68.5
25	16.0	16.1	16.1	16.1	6	18.3	18.2	18.3	18.2
26	17.4	17.1	17.2	17.2			**Glc at C-28**	**Glc at C-28**	**Glc I at C-28**
27	26.0	26.0	26.0	26.0	1		94.5	94.6	94.5
28	179.3	175.6	175.8	175.7	2		72.8	71.0	72.5
29	33.3	33.2	33.2	30.2	3		77.2	76.8	76.9
30	23.9	23.8	23.8	23.9	4		69.9	69.6	69.7
					5		78.2	76.9	77.2
					6		61.1	68.5	68.2
								Gal	**Glc II**
					1			101.5	103.5
					2			71.6	73.8
					3			72.8	77.1
					4			67.8	70.3
					5			74.8	77.3
					6			61.8	61.4

[a] ^{13}C NMR data (δ) were measured in DMSO-d_6 at 100 and 150 MHz.
[b] The assignments are based on APT, COSY, NOESY, HSQC and HMBC experiments.
[c] nd not determined.

against HEK-293, A-549 and HeLa cells whereas the bisdesmosidic types did not demonstrate remarkable cytotoxicity up to the highest tested concentration. The literature findings on cytotoxic effect of monodesmosidic saponins are also parallel to cytotoxicity results of balansoide A (Podolak et al., 2010). Moreover, the cytotoxic activity results suggested that the balansoide A has a comparable potent effect of doxorubicin on cancer cells, indicating that it can be used in cancer treatment as a chemotherapeutic agent against A-549 and HeLa cells.

Previous studies indicating the physiological, immunological and pharmacological properties of saponins have generated significant clinical interest in these substances. Structure-activity relationships were recognized by comparison of the functional groups of each saponin and of the branched sugar chains attached to aglycone (Oda et al., 2000; Rajput et al., 2007). In this study, the analyses of IL-2, IFN-γ and IL-1β cytokine production in stimulated whole blood by activation with PMA plus ionomycin were determined by ELISA. Cytokines are proteins which represent a vital role in the human immune response. The utilities of cytokines are various and contain roles in normal T-cell-mediated immunity, the inflammatory response, autoimmunity, and cancer and allergy treatments (Borish and Rosenwasser, 1996). Compared to the PMA plus ionomicin induced whole blood control group (683.04 pg/ml), the results show that balansoides A-C enhanced considerable IL-1β secretion with values of 1004.47, 991.57 and 966.50 pg/ml, which is closely linked to the innate immune response through inflammasome-driven host resistance with infection (see Fig. 3). It is clear that there is an inverse ratio with a slight variation between the number of sugars and the IL-1β secretion. For example, while balansoide C, which has two more sugar units than balansoide A, stimulated IL-1β releasing a value of 966.50 pg/ml, balansoide A, which is a monodesmosidic saponin, induced with a value of 1004.47 pg/ml IL-1β secretion.

Table 3
Cytotoxicity of compound **1** from *C. balansae*.[a,b,c]

Sample ID	Cell Lines IC_{50} (μM)		
	HEK-293	A549	HeLa
Doxorubicin	12 ± 0.38	7 ± 0.07	10 ± 0.02
Balansoide A (1)	8 ± 0.18	12 ± 0.72	15 ± 2.04

[a] HEK-293 (non-cancerous kidney cells), A-549 (lung cells) and HeLa (cervical cells).
[b] Doxorubicin was used as positive control.
[c] The values are means ± standard errors of the experiment carried out in triplicates.

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015

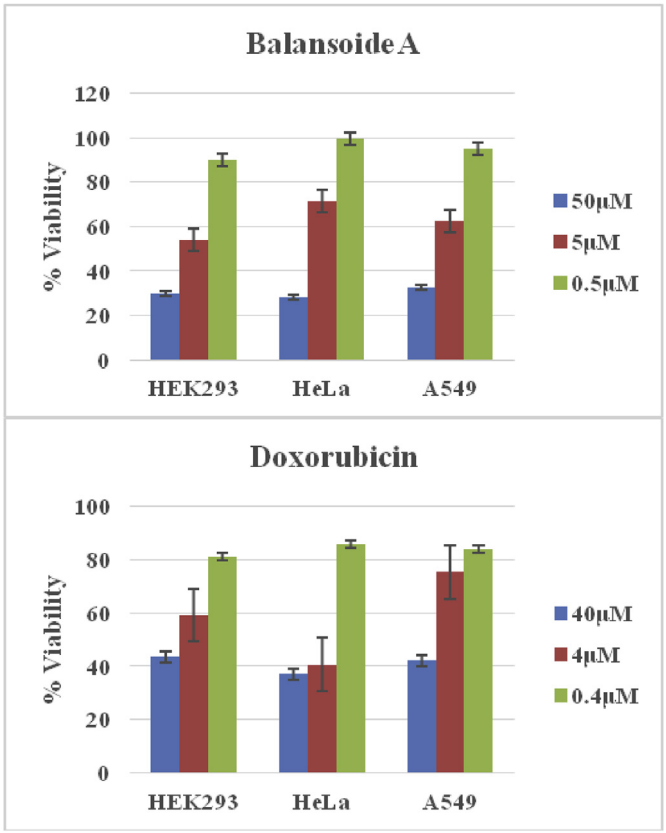

Fig. 2. Cytotoxic activity of balansoide A (**1**) from *C. balansae*.

In light of these evaluations, the IL-1β effect on the immune system through inflammasome-mediated signaling can lead to various therapeutic benefits such as the utilization of saponins as vaccine adjuvants (Dinarello, 2009; LaRock et al., 2016). A variety of immunostimulants act as vaccine adjuvants by activation of the innate immune system which has a critical role in early host defense against invading pathogens. For example, alum is a well-known inflammasome-activating adjuvant and it is the only adjuvant approved for using in human vaccines (Coffman et al., 2010). The results in our studies highlight the similarities between balansoides A-C and saponin QS-21, which was recently described as an inflammasome-activating saponin vaccine adjuvant (Marty-Roix et al., 2016). These findings indicated that hederagenin type saponins have the potential to stimulate innate immune response, thus providing an opportunity of them being used as a vaccine adjuvant system. On the other hand, only balansoide B displayed a slight IFN-γ secretion with value of 5219.14 pg/ml (see Fig. 3). None of the compounds changed IL-2 levels significantly (see Fig. 3).

Our study shows that the hemolytic activity depends on the number of sugar units in the chains (Voutquenne et al., 2008). While balansoides A, B, D and the *n*-butanol extract showed strong hemolytic activity on human eritrocytes ($p < 0.001$), balansoide C exhibited a reduced amount of hemolytic activity (see Table 4). These hemolytic activities obviously demonstrated that the monodesmosidic saponins (compound **1**) generated the strongest activity among the tested ones, resembling the QS-21 effect. The results emphasized the remarkable similarities of balansoide A with *Quillaja saponaria* saponin QS-21, which is a well-known and extensively studied saponin. This result can be attributed to the similar aglycone and sugar structures of QS-21 (see Sup. Info. Fig. 2S) and compound **1**. Moreover, these findings also confirmed that increasing the number of sugar units in the chains was a result of the lower rate hemolysis.

In conclusion, the biological activities of the four previously undescribed compounds (balansoides, **1**–**4**) out of fourteen saponins isolated from *C. balansae* provided a concrete example for the structure activity relationships. While only monodesmosidic glycosides exhibited a significant cytotoxicity, enhanced IL-1β secretion of monodesmosidic and bisdesmosidic saponins indicated the regulating effect of innate immune response properties. The hemolytic activity results showed remarkable similarities with previous studies as above-mentioned, indicating a correlation between the number of sugar moieties and hemolysis. Additionally, further studies on this subject should be conducted to investigate possible modes of action regarding active compounds.

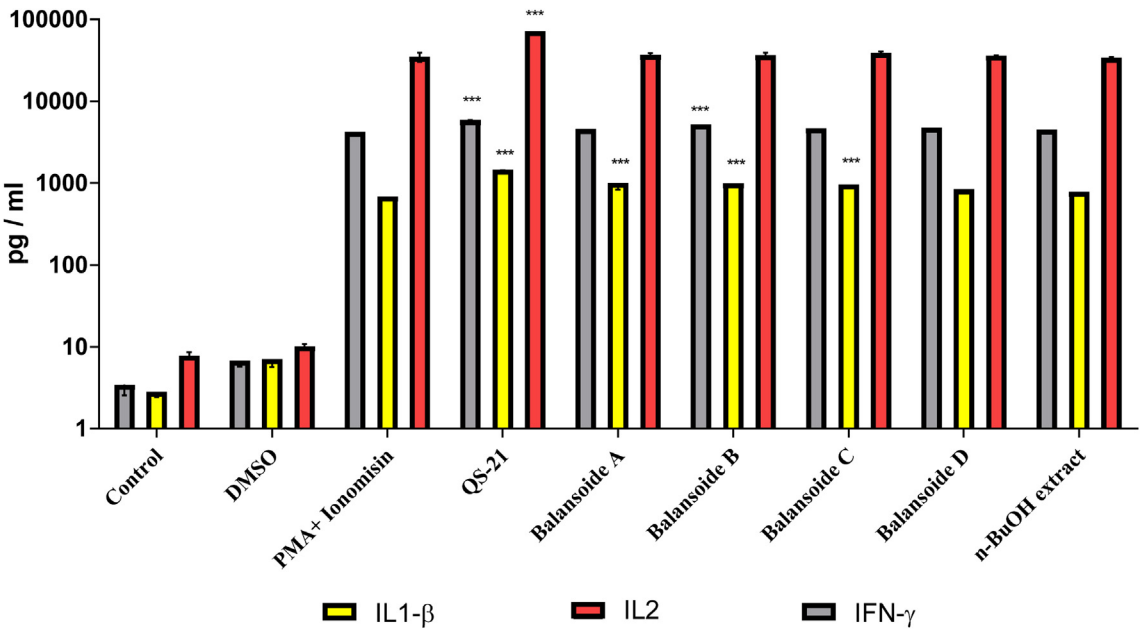

Fig. 3. Immunomodulatory activities of *n*-butanol extract and balansoides A-D (**1**–**4**) from *C. balansae*.

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015

Table 4
Hemolytic activities of *n*-butanol extract and compounds **1**–**4** from *C. balansae*.[a,b,c]

Group (μg/ml)	Absorbance value	Hemolytic percentage (%)
Saline	0.16 ± 0.01	0.00 ± 0.60
Distillated water	1.14 ± 0.04	100.00 ± 3.96***
QS-21		
500	1.00 ± 0.03	85.77 ± 2.84***
250	0.75 ± 0.01	60.04 ± 1.07***
125	0.70 ± 0.01	55.27 ± 1.17***
Balansoide A (1)		
500	0.93 ± 0.10	82.81 ± 2.42***
250	0.77 ± 0.02	61.22 ± 2.48***
125	0.71 ± 0.04	52.50 ± 1.19***
Balansoide B (2)		
500	0.56 ± 0.25	53.78 ± 2.74***
250	0.34 ± 0.20	46.38 ± 2.65***
125	0.42 ± 0.29	28.99 ± 4.31***
Balansoide C (3)		
500	0.25 ± 0.12	17.54 ± 4.29**
250	0.22 ± 0.04	14.97 ± 0.61**
125	0.23 ± 0.12	9.14 ± 1.66**
Balansoide D (4)		
500	0.24 ± 0.16	26.38 ± 2.49***
250	0.24 ± 0.14	21.64 ± 3.06**
125	0.31 ± 0.19	14.56 ± 3.57***
***n*-Butanol Extract**		
500	0.72 ± 0.05	55.68 ± 4.19***
250	0.51 ± 0.04	35.56 ± 4.27***
125	0.41 ± 0.03	24.92 ± 3.38***

[a] *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
[b] Hemolytic percents of saline and distilled water were included as minimal and maximal hemolytic control.
[c] All values represent the mean ± standard deviation (n = 3 test).

3. Materials and methods

3.1. General

Optical rotations and FTIR spectra were recorded on a Rudolph Research Analytical Autopol I automatic polarimeter and on an ATI Mattson Genesis Series Fourier transform infrared spectrophotometer, respectively. High resolution electrospray ionization mass spectra (HRESIMS) and the 1D and 2D NMR spectra were recorded on a Bruker LC micro-Q-TOF instrument and on Varian AS 400 MHz and 600 MHz spectrometers in DMSO-d_6 with TMS as an internal standard, respectively. Medium Pressure Liquid Chromatography (MPLC) was carried out using a Buchi system (Buchi C-605 pumps, coupled to a UV detector) with Buchi glass columns (26/920). Lichroprep RP-18 (25–40 μm, Merck 9303) and silica gel 60 (0.063–0.200 mm, Merck 7734) were used for both column chromatography (CC) and MPLC studies. Thin layer chromatography (TLC) was performed on F_{254} (Merck 5554) and RP-18 F_{254s} (Merck 5560) pre-coated aluminum sheets. They were detected after developing with suitable solvent systems spraying with a 20% H_2SO_4 solution followed by heating at 120 °C for 3 min. A class II laminar flow cabinet (Thermo Scientific, Germany) inverted microscope (Olympus, Japan), a CO_2 incubator, a 96 well spectrophotometer (Thermo Scientific, Germany) and a Nuve centrifuge (Turkey) were used in biological activity tests.

3.2. Plant material

Cephalaria balansae Raus. was collected from Antalya-Elmali, Finike highway 22 km, at about 1250 m altitude in July 2012 (36° 32′ 45.2″ N, 29° 59′ 11.1″ E). It was identified by H. Sumbul and R. S. Gokturk (Department of Biology, Faculty of Art and Science, Akdeniz University). A voucher specimen has been deposited (No: R.S. Gokturk 7525) at the Herbarium Research and Application Centre of Akdeniz University.

3.3. Extraction and isolation

The aerial part of the plant material (1.8 kg) was extracted with MeOH (4L × 5) at room temperature for overnight. The MeOH extract was concentrated under reduced pressure (~40 °C) and the gave dark residue (185 g) which was extracted with (200 ml × 5) the *n*-BuOH:H_2O (1:1) solvent system. The dried *n*-BuOH phase was extracted with *n*-hexane (100 ml × 13) to distinguish apolar parts. The *n*-BuOH extract (100 g) was subjected to vacuum liquid chromatography (VLC) using reversed-phase silica gel employing MeOH-H_2O solvent system with a gradient from 0% to 100% MeOH to give 11 main fractions. The 80% MeOH fraction (20.0 g) of RP-VLC was exposed to MPLC-1 over silica gel using a suitable column (26 × 920 mm), programme (max. pressure: 40 bar, flow rate: 23 ml/min) and solvent system ($CHCl_3$-MeOH-H_2O 9:1–61:32:7). Eleven sub-fractions were derived. Compound **1** (65 mg) and known compound **5** (500 mg) were obtained from 9th and 3rd fractions, respectively. The combined 5th - 6th fractions (0.65 g) of MPLC-1 were exposed to a silica gel CC eluted with $CHCl_3$-MeOH-H_2O (80:20:2) to give compound **6** (252 mg). The 60% MeOH fraction of RP-VLC was exposed to MPLC-2 over silica gel using a suitable column (26 × 920 mm), programme (max. pressure: 40 bar, flow rate: 25–27 ml/min) and solvent system ($CHCl_3$-MeOH-H_2O 90:10:1–61:32:7 + %5 MeOH). After this application, eight sub-fractions were derived. Compounds **7** (86.5 mg) and **8** (50 mg) were purified by another silica gel CC with the solvent system $CHCl_3$-MeOH-H_2O (70:30:3) from the third sub-fraction. Sub-fraction eight of MPLC-2 was chromatographed over silica gel CC with the $CHCl_3$-MeOH-H_2O (70:30:3–61:32:7) solvent system to obtain compound **3** (140 mg) and compound **9** (40 mg). Sub-fraction 5 was further chromatographed with a RP CC with MeOH-H_2O solvent system from 1:5 to 2:1 to yield compound **4** (20 mg). Sub-fractions six and seven of MPLC-2 were combined and loaded on silica gel CC with the solvent system $CHCl_3$-MeOH-H_2O (90:10:1–70:30:3) to yield seven sub-fractions. Compound **2** (28 mg) and compound **10** (65 mg) were obtained from another silica gel CC ($CHCl_3$-MeOH-H_2O 70:30:3–61:32:7) of the combined 2nd-4th sub-fractions. Compounds **11** (65 mg) and **12** (65 mg) were also obtained from combined 6th and 7th fractions by CC on a silica gel using $CHCl_3$-MeOH-H_2O (61:32:7) solvent system. Fraction 10% MeOH (18 g) of RP-VLC was then chromatographed by MPLC-3 over silica gel using a suitable column (26 × 920 mm), programme (max. pressure: 40 bar, flow rate: 26 ml/min) and $CHCl_3$-MeOH-H_2O solvent system, (90:10:0.5–70:30:3) which resulted in seven sub-fractions. Compounds **13** (22 mg) and **14** (20 mg) were also purified from 5th and 6th fractions, respectively.

3.4. Alkaline hydrolysis

Previously undescribed compounds **2**–**4** (~5 mg each) were refluxed with 5% KOH in water solution at 100 °C for 2 h. The reaction mixtures were neutralized with 5% HCl in water solution and then concentrated to dryness. The residues were extracted with *n*-BuOH-H_2O (1: 1) and all of the organic layers were evaporated to dryness (Calis et al., 1996). The *n*-BuOH fractions afforded the same prosapogenin (**1a**), which was analyzed by 1H NMR and HRESIMS methods. Alkaline hydrolysis of **2**, **3** and **4** afforded prosapogenin **1a,** which was also isolated in this study namely balansoide A (**1**).

3.5. 3-O-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin (prosapogenin ***1a***)

A white, amorphous powder (2 mg); 1H NMR (DMSO-d_6, 600 MHz); sugars: δ_H 5.12 (1H, br s, H-1 of Rha I), 5.01 (1H, br s, H-1

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015

of Rha II), 4.29 (1H, d, $J = 7.8$ Hz, H-1 of Xyl), 4.30 (1H, d, $J = 4.8$ Hz, H-1 of Ara); positive-ion HRESIMS *m/z* 1051.5446 $[M + Na]^+$ (calcd for $C_{52}H_{84}O_{20}Na$, 1051.5448).

3.6. Acid hydrolysis

The identification of the monosaccharide units of the glycosides was performed by GC-MS analysis with authentic samples (Sarikahya and Kirmizigul, 2010). Each compound (~5 mg) was hydrolyzed with 1 N HCl (2 ml) for 6 h at 90 °C. After extraction with $CHCl_3$ (3 × 5 ml), the aqueous layer was evaporated to dryness and kept under hi-vac for 24 h. Furthermore, the residue of monosaccharides was dissolved in anhydrous pyridine (1 ml), and then 1 ml of HMDS-TMCS (hexamethyldisilazane−trimethylchlorosilane, 3:1) was added, and the mixture was stirred at 70 °C for 1 h. The mixture was concentrated under a N_2 stream and dissolved in *n*-hexane for GC-MS analyses. Similarly, a mixture containing standard monosaccharide units was silylated by the same procedure. The retention times of the standard monosaccharides L-arabinose, L-rhamnose, D-xylose, D-galactose and D-glucose were found to be 5.59, 5.69, 6.57, 8.03 and 8.38 min, respectively. The monosaccharide moieties of the samples were determined as L-arabinose, L-rhamnose and D-xylose giving the peaks at 5.58, 5.69 and 6.56 min for **1**, L-arabinose, L-rhamnose, D-xylose and D-glucose giving the peaks at 5.58, 5.69, 6.56 and 8.37 min for **2**, 5.58, 5.69, 6.55 and 8.36 min for **3** and 5.58, 5.69, 6.56 and 8.37 min for **4**, respectively. D-Galactose was found only for **3**, giving a peak at 8.01 min.

3.7. 3-O-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin (**1**)

A white, amorphous powder (65 mg); $[\alpha]_D^{25}$−9.5 (*c* 2.1, MeOH); IR (KBr) ν_{max} 3387, 2945, 1696, 1452, 1363, 1227, 1045 cm^{-1}; 1H NMR (DMSO-d_6, 400 MHz) and 13C NMR (DMSO-d_6, 100 MHz): see Tables 1 and 2, respectively; positive-ion HRESIMS *m/z* 1051.5399 $[M + Na]^+$ (calcd for $C_{52}H_{84}O_{20}Na$, 1051.5448).

3.8. 3-O-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-glucopyranosyl ester (**2**)

A white, amorphous powder (28 mg); $[\alpha]_D^{25}$−15.0 (*c* 0.4, MeOH); IR (KBr) ν_{max} 3385, 2932, 1733, 1599, 1463, 1364, 1288, 1050 cm^{-1}; 1H NMR (DMSO-d_6, 600 MHz) and 13C NMR (DMSO-d_6, 150 MHz): see Tables 1 and 2, respectively; positive-ion HRESIMS *m/z* 1213.5929 $[M + Na]^+$ (calcd for $C_{58}H_{94}O_{25}Na$, 1213.5976).

3.9. 3-O-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-galactopyranosyl-(1 → 6)- β-D-glucopyranosyl ester (**3**)

A white, amorphous powder (140 mg); $[\alpha]_D^{25}$−20.0 (*c* 1.5, MeOH); IR (KBr) ν_{max} 3407, 2945, 1737, 1659, 1451, 1364, 1254, 1046 cm^{-1}; 1H NMR (DMSO-d_6, 400 MHz) and 13C NMR (DMSO-d_6, 100 MHz): see Tables 1 and 2, respectively; positive-ion HRESIMS *m/z* 1375.6455 $[M + Na]^+$ (calcd for $C_{64}H_{104}O_{30}Na$, 1375.6505).

3.10. 3-O-α-L-rhamnopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**4**)

A white, amorphous powder (20 mg); $[\alpha]_D^{25}$−10.0 (*c* 1.0, MeOH); IR (KBr) ν_{max} 3395, 2925, 1691, 1560, 1441, 1380, 1035 cm^{-1}; 1H NMR (DMSO-d_6, 600 MHz) and 13C NMR (DMSO-d_6, 150 MHz): see Tables 1 and 2, respectively; positive-ion HRESIMS *m/z* 1375.6444 $[M + Na]^+$ (calcd for $C_{64}H_{104}O_{30}Na$, 1375.6505).

3.11. In vitro bioactivity assays

3.11.1. Cytotoxicity assay

HeLa, A-549, and a normal cell line HEK-293 were used for testing cytotoxicity. All cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The cell lines were maintained in Dulbecco's modified Eagle's medium F12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco, NY, U.S.A.). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The cells were sub cultured twice a week, and the cells in the exponential growth phase were used in the experiments.

Cytotoxicity of extract and compounds was determined by using a modified MTT assay, which detects the activity of mitochondrial reductase of viable cells (Mosman and Coffman,1989; Nalbantsoy et al., 2011). The assay principle is based on the cleavage of MTT that forms formazan crystals by cellular succinate-dehydrogenases in viable cells. The addition of DMSO to wells helps formazan crystals to be dissolved. For this purpose, all cell lines were cultivated for 24 h in 96-well microplates with an initial concentration of 1×10^5 cells/well in a humidified atmosphere of 5% CO_2, at 37 °C. Then, the cultured cells were treated with different dilutions of extract or compounds followed by incubation for 48 h at 37 °C. Doxorubicine (Sigma, St. Lois, MO, U.S.A.) was used as a positive control. The optical density of the dissolved material was measured at 570 nm with UV−vis spectrophotometer (Thermo Multiskan Spectrum). The viability (%) was determined by the following formula:

%viable cells = [(absorbance of treated cells) − (absorbance of blank)]/[(absorbance of control) − (absorbance of blank)] × 100.

The morphological studies of the cells were performed with an inverted microscope compared with the control group 48 h after treatment.

The mean IC_{50} is the concentration of an agent that reduces cell growth by 50% under the experimental conditions and it is the average from at least three independent measurements that will be reproducible and statistically significant. The IC_{50} values were reported at ±95% confidence intervals (±95% CI). This analysis was performed with Graph Pad Prism 5 (San Diego, CA, U.S.A.).

3.11.2. Hemolytic activity assay

Hemolytic activity of the molecules was measured according to Nalbantsoy et al. (2011) with some modifications. Red blood cells were obtained from healthy human 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 a sterile saline solution (0.89%, w/v NaCl, pyrogen free) and then it was centrifuged at 2000/5 *g*. 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 125, 250 and 500 μg/ml concentrations of molecules in saline solutions. QS-21 was used as a control saponin compound. The mixtures were incubated for 30 min at 37 °C and centrifuged at 800/10 *g*. 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

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015

was developed by the saline control, subtracted from all groups. Each experiment was included triplicates at each concentration. The data were expressed as mean ± standard deviation (S.D.) and examined for their statistical significance of difference with Student t-test, ANOVA and the post hoc test (dunnet referring to homogeneity and normality of the absorbance using SPSS 16.0). *P*-values of less than 0.05, 0.01 and 0.001 were considered to be statistically significant.

3.11.3. Immunomodulatory assay: measurement of the effects on cytokine production

Heparinized peripheral human whole blood obtained 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-γ and IL-1β and incubated in the presence of test samples or reference compound QS-21 for 48 h. The cultured supernatants were collected and the concentration of the cytokines produced by macrophages or lymphocytes was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (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 concentrations of each cytokine were calculated. The results of IL-2, IFN-γ and IL-1β were expressed as picograms per milliliter. The minimum detection limits were 9.1 pg/ml, 0.99 pg/ml, and 0.3 pg/ml for IL-2, IFN-γ and IL-1β, respectively. Diluted supernatants were used for all assays and each assay was performed in triplicate.

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 in the absence or presence of 3 μg/ml concentrations of samples or QS-21 as the reference compound. The culture supernatants were then mixed with PBS/0.05% thiomersal at 1:2 ratio and were assayed using the specific ELISA method for IL-2, IFN-γ and IL-1β. The experimental protocol was described in a previous report (Lenarczyk et al., 2000; Nalbantsoy et al., 2011; Yesilada et al., 2005). 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: E.215344).

Acknowledgements

The authors gratefully acknowledge to the financial support of the Research Grant Office of Ege University (2016/Fen/038) and thank Prof. Dr. H. Sumbul and Prof. Dr. R. S. Gokturk for the collection and identification of the plant. H. Top would also like to thank TEV for master scholarship. TUBITAK and EBILTEM are gratefully acknowledged for providing NMR (600 MHz), HRESIMS and NMR (400 MHz) spectra, respectively. We also would like to thank Ege University, Center for Drug Research and Development and Pharmacokinetic Applications for GC-MS analysis. We also thank AREL (Ege University, School of Medicine, Research and Education Laboratory) for the permission to use their laboratory for biological activity test.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2017.02.015.

References

Aliev, A.M., Movsumov, I.S., 1976. Triterpenoid glycosides from *Cephalaria kotchyi* flowers. Khim. Prir. Soedin. 2, 264–265.
Borish, L., Rosenwasser, L., 1996. Update on cytokines. J. Allergy Clin. Immunol. 97, 719–730.
Calis, I., Zor, M., Saracoglu, I., Isimer, A., Rüegger, H., 1996. Four novel cycloartane glycosides from *Astragalus oleifolius*. J. Nat. Prod. 59, 1019–1023.
Chirva, V., Kintya, P.K., Kretsu, L.G., 1970. β-D-Glucopyranoside of β-sitosterol from *Phaseolus vulgaris*. Khim. Prir. Soedin. 6, 491–492.
Chirva, V., Kintya, P.K., Sosnovskii, V.A., 1969. Triterpene glycosides of *Sapindus mukorossi*. Khim. Prir. Soedin. 5, 450–451.
Coffman, L.R., Sher, A., Seder, R.A., 2010. Vaccine adjuvants: putting innate immunity to work. Immunity 33, 492–503.
Davis, P.H., 1972. Flora of Turkey and the East Aegean Islands, vol. 4. University of Edinburgh Press, Edinburgh,, pp. 585–592.
Dinarello, C.A., 2009. Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. 27, 519–550.
Francis, G., Kerem, Z., Makkar, H.P.S., Becker, K., 2002. The biological action of saponins in animal systems: a review. Br. J. Nutr. 88, 587–605.
Godevac, D., Menkovic, N., Vujisic, L., Tesevic, V., Vajs, V., Milosavljevic, S., 2010. A new triterpenoid saponin from the aerial parts of *Cephalaria ambrosioides*. Nat. Prod. Res. 12, 1307–1312.
Gulcemal, D., Masullo, M., Senol, S.G., Alankus-Caliskan, O., Piacente, S., 2014. Saponins from *Cephalaria aristata* C. Koch. Rec. Nat. Prod. 3, 228–233.
Hostettmann, K., Marston, A., 1995. Saponins. Cambridge University Press, Cambridge, New York, pp. 59–96.
Kawai, H., Kuroyanagi, M., Umehara, K., Ueno, A., Satake, M., 1988. Studies on the saponins of *Lonicera japonica* thunb. Chem. Pharm. Bull. 36, 4769–4775.
Kirmizigul, S., Anil, H., Rose, M.E., 1995. Triterpenoid glycosides from *Cephalaria transsylvanica*. Phytochemistry 39, 1171–1174.
Kräutler, B., Sahu, N.P., Banerjee, S., Mondal, N.B., Mandal, D., 2008. Progress in the Chemistry of Organic Natural Products. Springer Wien, New York, pp. 37–43.
Kong, J., Li, X., Wei, B., Yang, C., 1993. Triterpenoid glycosides from *Decaisnea fargesii*. Phytochemistry 33, 427–430.
Lacaille-Dubois, M.A., Wagner, H., 1996. A review of the biological and pharmacological activities of saponins. Phytomedicine 2, 363–386.
LaRock, C.N., Todd, J., LaRock, D.L., Olson, J., O'Donoghue, A.J., Robertson, A.A.B., Cooper, M.A., Hoffman, H.M., Nizet, V., 2016. IL-1β is an innate immune sensor of microbial proteolysis. Sci. Immunol. 1, eaah3539.
Lenarczyk, A., Helsloot, J., Farmer, K., Peters, L., Sturgess, A., Kirkham, B., 2000. Antigen-induced IL-17 response in the peripheral blood mononuclear cells (PBMC) of healthy controls. Clin. Exp. Immunol. 122, 41–48.
Lin, H.C., Ding, H.Y., Wu, Y.C., 1998. Two novel compounds from *Paeonia suffructicosa*. J. Nat. Prod. 61, 343–346.
Marty-Roix, R., Vladimer, G.I., Pouliot, K., Weng, D., Buglione-Corbett, R., West, K., MacMicking, J.D., Chee, J.D., Wang, S., Lu, S., Lien, E., 2016. Identification of QS-21 as an inflammasome-activating molecular component of saponin adjuvants. J. Biol. Chem. 291, 1123–1136.
Mbhele, N., Balogun, F.O., Kazeem, M.I., Ashafa, T., 2015. In vitro studies on the antimicrobial, antioxidant and antidiabetic potential of *Cephalaria gigantea*. Bangladesh J. Pharmacol. 10, 214–221.
Mojab, F., Kamalinejad, M., Ghaderi, N., Vahidipour, H.R., 2003. Phytochemical screening of some species of Iranian plants. Iran. J. Pharm. Res. 2, 77–82.
Mosmann, T.R., Coffman, R.L., 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7, 145–173.
Movsumov, I.S., Garaev, E.A., Isaev, M.I., 2009. Flavonoids from *Cephalaria grossheimii*. Chem. Nat. Compd. 45, 422–423.
Mukhamedziev, M.M., Alimbaeva, P.K., Gorovits, T.T., Abubakirov, N.K., 1971. Structure of the triterpenoid glycoside from *Dipsacus azureus*, Dipsacoside B. Khim. Prir. Soedin. 7, 153–158.
Mustafayeva, K., Giorgio, C.D., Elias, R., Kerimov, Y., Ollivier, E., De Meo, M., 2010. DNA-Damaging, mutagenic, and clastogenic activities of gentiopicroside isolated from *Cephalaria kotschyi* roots. J. Nat. Prod. 73, 99–103.
Nalbantsoy, A., Nesil, T., Erden, S., Calis, I., Bedir, E., 2011. Adjuvant effects of *Astragalus* saponins Macrophyllosaponin B and Astragaloside VII. J. Ethnopharmacol. 134, 897–903.
Nalbantsoy, A., Nesil, T., Yilmaz-Dilsiz, O., Aksu, G., Khan, S., Bedir, E., 2012. Evaluation of the immunomodulatory properties *in mice* and *in vitro* anti-inflammatory activity of cycloartane type saponins from *Astragalus* species. J. Ethnopharmacol. 139, 574–581.
Netala, V.R., Ghosh, S.B., Bobbu, P., Anitha, D., Tartte, V., 2015. Triterpenoid saponins: a review on biosynthesis, applications and mechanism of their action. Int. J. Pharm. Pharm. Sci. 7, 24–28.
Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., Yoshikawa, M., 2000. Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. Biol. Chem. 381, 67–74.

Pasi, S., Pratsinis, H., Skaltsounis, A.L., Chinou, I.B., 2009. Biologically active triterpenoids from *Cephalaria ambrosioides*. Planta Med. 75, 163–167.
Podolak, I., Galanty, A., Sobolewska, D., 2010. Saponins as cytotoxic agents: a review. Phytochem. Rev. 9, 425–474.
Rajput, Z.I., Hu, S., Xiao, C., Arijo, A.G., 2007. Review: adjuvant effects of saponins on animal immune. J. Zhejiang Univ. Sci. B 8, 153–161.
Sarikahya, N.B., Kirmizigul, S., 2012. Antimicrobially active hederagenin glycosides from *Cephalaria elmaliensis*. Planta Med. 78, 828–833.
Sarikahya, N.B., Pekmez, M., Arda, N., Kayce, P., Karabay-Yavasoglu, N.U., Kirmizigul, S., 2011. Isolation and characterization of biologically active glycosides from endemic *Cephalaria* species in Anatolia. Phytochem. Lett. 4, 415–420.
Sarikahya, N.B., Kirmizigul, S., 2010. Antimicrobial triterpenoid glycosides from *Cephalaria scoparia*. J. Nat. Prod. 73, 825–830.
Sharma, A., Sati, S.C., Sati, O.P., Sati, M.D., Kothiyal, S.K., 2013. Triterpenoid saponins from the pericarps of *Sapindus mukorossi*. HPC J. Chem. 2013, 1–5.
Sparg, S.G., Light, M.E., Staden, J.V., 2004. Biological activities and distribution of plant saponins. J. Ethnopharmacol. 94, 219–243.
Voutquenne, L., Lavaud, C., Massiot, G., Men-Olivier, L., 2008. Structure-activity relationships of hemolytic saponins. Pharm. Biol. 40, 253–262.
Yesilada, E., Bedir, E., Calis, I., Takaishi, Y., Ohmoto, Y., 2005. Effects of triterpene saponins from Astragalus species on in vitro cytokine release. J. Ethnopharmacol. 96, 71–77.

Please cite this article in press as: Top, H., et al., Immunomodulatory, hemolytic properties and cytotoxic activity potent of triterpenoid saponins from *Cephalaria balansae*, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.02.015