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Bioactive flavonoids and saponins from *Climacoptera obtusifolia*

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Abstract

Two bidesmosidic saponins were isolated from *Climacoptera obtusifolia* (Chenopodiaceae) and their structures were determined as gypsogenin 3-*O*-[β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranoside]-28-*O*-{β-D-glucopyranosyl} ester (**1**) and hederagenin 3-*O*-[β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranoside]-28-*O*-[β-D-glucopyranosyl} ester (**2**), by spectroscopic methods. Two known compounds, isorhamnetin 3-*O*-β-D-glucopyranoside (**3**), and isorhamnetin 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (**4**) were also isolated for the first time from this plant. Compounds **1**–**4** were tested in various immunomodulatory assays. Compound **2** suppressed (92%) the reactive oxygen species (ROS) production on mononuclear cells in luminol-based chemiluminescence (CL) assay at a higher concentration (50 μg/mL). Compounds **3** and **4** demonstrated a strong inhibition on ROS production in the oxidative burst activity of whole blood, neutrophils, and mononuclear cells. Additionally compounds **3** and **4** also suppressed PHA T-cell proliferation with no cytotoxic effects.

Keywords: *Climacoptera obtusifolia*; Chenopodiaceae; Gypsogenin; Hederagenin; Bidesmosidic saponins; Flavonoids; Reactive oxygen species; T-cell proliferation

1. Introduction

About 14 species of *Climacoptera* (Chenopodiaceae) are found in Kazakhstan (Pavlov, 1961). Several species of this genus have shown to contain complex mixtures of triterpenoid glycosides (Annaev and Abubakirov, 1984; Annaev et al., 1983a,b,c; Eskalieva et al., 2004) and flavonoid glycosides (Baeva and Zapesochnaya, 1980). Plants of the genus *Climacoptera* are known for antifungal activity (Sokolov, 1986). As part of our current interest in the medicinal plants of Kazakhstan, we investigated the chemical constituents of the aerial parts of *Climacoptera obtusifolia* (Schrenk.) Botsch. for the first time. The water-soluble constituents of MeOH extract were isolated by the use of Diaion HP-20, ODS, polyamide and silica gel column chromatography. Compounds **1** and **2** were identified as new saponins, while flavonoid glycosides **3** and **4** were identified as known constituents, isolated for the first time from this plant. In this paper, we also report the immunomodulatory activities of the isolated compounds. Compounds **1**–**4** were screened over a wide range of concentrations (3.1–50 μg/mL) for their possible effects on the oxidative burst of whole blood and isolated phagocytic cells (neutrophils and mononuclear cells) using a luminol-based chemiluminescence (CL) assay (Hadjimitova et al., 2004). The results of various assays employed in the study showed compound **2** to have inhibitory activity at higher concentrations, while compounds **3** and **4** have a potential suppressive effect against oxidative burst activity and phytohemagglutinin (PHA) stimulated T-cell proliferation. All the tested compounds (**1**–**4**) were found to be non-cytotoxic on 3T3 cells.

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2. Results and discussion

The air-dried aerial parts of *C. obtusifolia* were extracted with 80% methanol–H_2O and the extract was successively partitioned with hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The butanol-soluble fraction was subjected to Diaion HP-20, ODS and polyamide column chromatography, followed by normal phase silica gel column chromatography to afford compounds **1** and **2**, and while compounds **3** and **4** were purified by the use of C_{18} recycling HPLC.

Compound **1** was isolated as a colorless gum. Its FAB-MS (−ve) exhibited the $[M-H]^-$ ion at *m/z* 925, consistent with the formula $C_{47}H_{74}O_{18}$. Its IR absorptions implied the presence of an aldehydic (1725 cm^{-1}) and ester (1730 cm^{-1}) functionalities. The presence of two hexose and one pentose sugars were inferred from the fragment ions at *m/z* 763 $[M-H-162]^-$, and 469 $[M-2\times162-132]^-$ in the FAB-MS (−ve).

The ^1H NMR spectrum of **1** (Table 1) showed six tertiary methyl groups (δ 0.79, 0.90, 0.92, 0.98, 1.08, and 1.16), an olefinic broad singlet (δ 5.25, H-12), and an oxymethine proton at δ 3.93, *dd*, $J = 11.7$ and 3.8 Hz, H-3). These signals indicated a pentacyclic skeleton in **1**. The ^1H NMR spectrum further showed a double doublets at δ 2.83 ($J = 13.1$ and 2.7 Hz) attributed to H-18, a characteristic signal of the oleanane-type skeleton. A singlet for aldehydic proton was resonated at δ 9.39 in the ^1H NMR spectrum.

The ^{13}C NMR spectrum (Table 2) of compound **1** showed the signals corresponding to 47 carbons, resolved into 6 methyl, 13 methylene, 20 methine, and 8 quaternary carbons. Among them, 17 signals were assigned to the oligosaccharide moiety. The ^{13}C NMR spectrum of **1** showed olefinic carbon signals at δ_C 123.5 (C-12) and 144 (C-13). The chemical shifts of C-3 (δ 82.8) and C-28 (δ 178.0) indicated a bisdesmosidic glycoside (Hostettmann and Marston, 1995; Jayasinghe et al., 2003). The ^1H- and ^{13}C NMR spectra of **1** exhibited three anomeric protons resonated as doublets at δ 4.20 ($J = 7.6$ Hz), 4.52 ($J = 7.3$ Hz), and 5.35 ($J = 8.0$ Hz) which corresponded to the carbon signals at δ 104.2, 105.4, and 95.4, respectively. The sugars were identified as two units of glucose, and one unit of xylose by paper chromatography and a detailed study of DEPT, 1D TOCSY, COSY, HMQC, and HMBC spectra. The β-anomeric configurations of the glucose and xylose units were deduced by their $^3J_{H1,H2}$ coupling constants (7.3–8.0 Hz) (Beier et al., 1980).

The sequence of the glycon part connected to the C-3 of the aglycon was deduced from the HMBC correlations of anomeric H-1′ (δ 4.20) of glucose moiety (Fig. 1) with carbon signal at δ 82.8 (C-3′) and 10.3 (C-24), indicating the attachment of β-glucose at C-3. The anomeric H-1″ (δ 4.52) of xylose moiety exhibited HMBC correlation with δ 86.2 (C-3′) of the glucose moiety, indicating connectivity between C-1″ and C-3′. Subsequently the xylose sugar substituted at C-3′ of β-D-glucopyranoside was also inferred from the analysis of chemical shifts data in the literature (Jayasinghe et al., 1995; Jimenez et al., 1989). The presence of second bisdesmosidic moiety at C-28 was deduced by the HMBC interactions of anomeric H-1‴ of glucose (δ 5.35) with C-28 carbonyl (δ_C 178.0). The C-23 aldehydic proton (δ 9.39) showed HMBC interactions (Fig. 1) with carbon signals at δ 10.3 (C-24), 56.2 (C-4), and 82.8 (C-3). The ^1H NMR subspectra of individual monosaccharide units were obtained by using selective irradiation of the easily identifiable anomeric proton signals, as well as irradiations of other non-overlapping proton signals in a series of 1D TOCSY and 2D COSY experiments. Acid hydrolysis of compound **1** afforded aglycon; gypsogenin (Nie et al., 1989; Tori et al., 1974) and sugars D-glucose, and D-xylose. On the basis of the above evidences, the structure of compound **1** was elucidated as gypsogenin 3-*O*-[β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranoside]-28-*O*-{β-D-glucopyranosyl} ester.

Table 1
^1H NMR spectral data for compounds **1**–**2** (400 MHz in CD_3OD)

Position	**1**	**2**
3	3.93 (*dd*, $J = 11.7$, 3.8 Hz)	3.63 (*dd*, $J = 11.8$, 4.6 Hz)
12	5.25 (*br.s*)	5.24 (*br.s*)
18	2.83 (*dd*, $J = 13.1$, 2.7 Hz)	2.82 (*dd*, $J = 10.2$, 3.0 Hz)
23	9.39 (*s*)	3.26 (*d*, $J = 11.8$ Hz)
		3.62 (*d*, $J = 11.8$ Hz)
24	1.08 (*s*)	0.68 (*s*)
25	0.98 (*s*)	0.96 (*s*)
26	0.79 (*s*)	0.78 (*s*)
27	1.16 (*s*)	1.15 (*s*)
29	0.90 (*s*)	0.91 (*s*)
30	0.92 (*s*)	0.92 (*s*)
Glu-I		
1′	4.20 (*d*, $J = 7.6$ Hz)	4.46 (*d*, $J = 7.8$ Hz)
2′	3.28*	3.32*
3′	3.48 (*t*, $J = 7.8$ Hz)	3.56 (*t*, $J = 7.6$ Hz)
4′	3.37*	3.47*
5′	3.34*	3.34 m
6′	3.78 (*br d*, $J = 11.7$ Hz)	3.80 (*br d*, $J = 11.8$ Hz)
	3.64 (*dd*, $J = 11.5$, 2.4 Hz)	3.66*
Xyl		
1″	4.52 (*d*, $J = 7.3$ Hz)	4.53 (*d*, $J = 6.9$ Hz)
2″	3.40*	3.39*
3″	3.33*	3.42*
4″	3.49 m	3.43*
5″	3.87 (*dd*, $J = 10.7$, 5.1 Hz)	3.90 (*dd*, $J = 11.2$, 5.2 Hz)
	3.20 (*t*, $J = 10.7$ Hz)	3.21 (*t*, $J = 11.2$ Hz)
Glu-II		
1‴	5.35 (*d*, $J = 8.0$ Hz)	5.36 (*d*, $J = 8.1$ Hz)
2‴	3.39*	3.32*
3‴	3.41*	3.74*
4‴	3.39*	3.39*
5‴	3.34*	3.41*
6‴	3.78 (*br d*, $J = 11.7$ Hz)	3.80 (*br d*, $J = 11.8$ Hz)
	3.64 (*dd*, $J = 11.5$, 2.4 Hz)	3.66*

* Determined by H–H and HMQC spectra, coupling constants could not be measured due to the overlay of the signals.

Compound **2** was found to have a formula $C_{47}H_{76}O_{18}$ by FAB-MS (*m/z* 927 $[M-H]^-$). The FAB-MS also displayed peaks at *m/z* 927 $[M-H]^-$, 765 $[M-H-162]^-$, 603 $[M-H-162-132]^-$, and 471 $[M-2\times162-132]^-$, representing the aglycon part of the molecule.

Table 2
^{13}C NMR Chemical shifts data for compounds **1** and **2** (CD_3OD; δ: ppm; J: Hz)

Atom	1	2	Atom	1	2
	δ C	δ C		δ C	δ C
1	39.2 (CH_2)	39.6 (CH_2)	Glc I-1′	104.2	104.6
2	24.5 (CH_2)	26.1 (CH_2)	2′	74.9	74.8
3	82.8 (CH)	82.3 (CH)	3′	86.2	86.5
4	56.2 (C)	43.8 (C)	4′	71.1	71.1
5	48.8 (CH)	48.2 (CH)	5′	78.3	78.6
6	21.3 (CH_2)	18.9 (CH_2)	6′	62.4	62.5
7	33.1 (CH_2)	33.4 (CH_2)	Xyl-1″	105.4	105.4
8	41.0 (C)	40.7 (C)	2″	74.5	74.9
9	48.0 (CH)	48.2 (CH)	3″	77.5	77.5
10	37.0 (C)	37.7 (C)	4″	70.9	71.0
11	24.0 (CH_2)	24.6 (CH_2)	5″	67.1	67.1
12	123.5 (CH)	123.8 (CH)	Glc II-1‴	95.4	95.7
13	144.8 (C)	144.4 (C)	2‴	73.9	74.8
14	43.0 (C)	43.0 (C)	3‴	78.3	78.3
15	28.8 (CH_2)	28.9 (CH_2)	4‴	71.1	72.1
16	25.5 (CH_2)	24.0 (CH_2)	5‴	78.6	78.6
17	48.0 (C)	48.0 (C)	6‴	62.4	62.5
18	42.6 (CH)	42.6 (CH)			
19	47.1 (CH_2)	47.2 (CH_2)			
20	31.5 (C)	31.5 (C)			
21	34.8 (CH_2)	34.9 (CH_2)			
22	33.2 (CH_2)	33.1 (CH_2)			
23	209.0 (CH)	65.0 (CH_2)			
24	10.3 (CH_3)	13.3 (CH_3)			
25	16.1 (CH_3)	16.5 (CH_3)			
26	17.7 (CH_3)	17.8 (CH_3)			
27	26.3 (CH_3)	26.3 (CH_3)			
28	178.0 (C)	178.1 (C)			
29	33.4 (CH_3)	33.4 (CH_3)			
30	23.9 (CH_3)	23.9 (CH_3)			

The comparison of the ^1H- and ^{13}C NMR spectra of **2** with those of **1** clearly indicated that the saponin **2** was distinctly similar to **1**, with a bisdesmosidic glycoside skeleton (see Table 1). The spectral data of **2** lacked the aldehydic signals. An increase of two a.m.u. in FABMS indicated that aldehydic group was replaced by a hydroxy methylene group. The position of the oxygenated methylene carbon (δ_C 65.0) was established at C-23 position from HMBC crosspeaks of CH_3-24 (δ 0.68), and H-3 (δ 3.63), with C-23 (δ 65.0). Acid hydrolysis of **2** afforded aglycon; hederagenin (Jayasinghe et al., 1995; Tori et al., 1974), D-glucose and D-xylose. Hence compound **2** was determined to be a new saponin, hederagenin 3-*O*-[β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranoside]-28-*O*-{β-D-glucopyranosyl} ester.

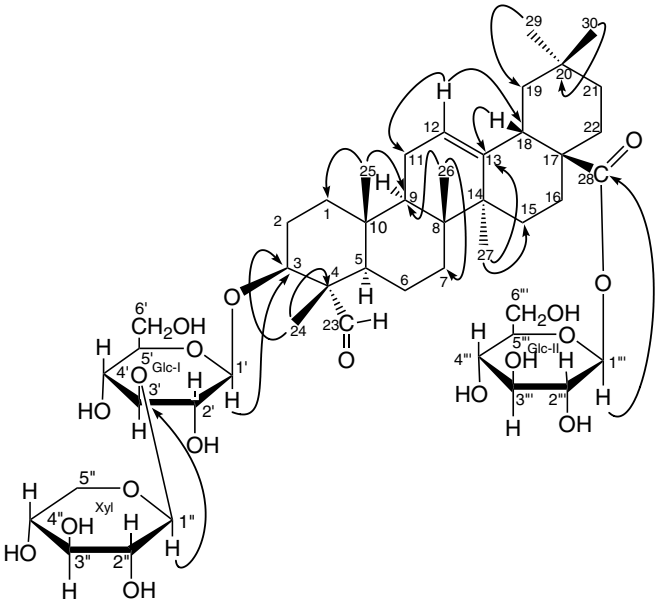

Fig. 1. Key HMBC correlations of compound **1**.

Known compounds **3** and **4** were isolated for the first time from this genus (Rahman and Ilyas, 1962; Horhammer et al., 1966).

When the two saponins **1** and **2** were screened for their immunomodulatory properties. Compound **1** did not show any significant effect on the tested system. However compound **2** showed same ($P \leqslant 0.005$) stimulatory activities with mononuclear cells (29%), at the lower concentration (3.1 μg/mL) tested (Fig. 2c). Meanwhile at the higher concentrations of 25 and 50 μg/mL, compound **2** inhibited mononuclear cells ROS activity (29% and 92%, respectively). Compounds **3** and **4** found to have potential in suppressing phagocytosis activity of whole blood, neutrophils and mononuclear cells in a dose dependent manner (Fig. 2). Compound **3** strongly suppressed the neutrophils, activity up to 73% at 3.1 μg/mL, compared to compound **4** (59.7%) (Fig. 2b). These results are in agreement with the observation of Chen et al. (2002), who showed that compound **3** at a high concentration can suppress superoxide generation, induced by phorbol 12-myristate 13-acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (fMLP), in a dose dependant manner. However, the system we employed (serum opsonized luminol dependant-chemiluminescence) can detect varieties of free radicals, such as superoxide, H_2O_2, OH, and HOCl (McNally and Bell, 1996).

Similarly, compound **3** exhibited a strong suppressive effect on the PHA stimulated T-cell proliferation with an IC_{50} of 26.2 ± 2 μg/mL, while a moderate effect was observed in case of compound **4**. Compounds **1** and **2** did not show any effect on T-cell proliferation (Fig. 3). In conclusion, compound **2** showed a significant inhibitory activity (92%) at a higher concentration (100 μg/mL) with mononuclear cells, while a stimulatory effect was observed at a lower concentration (3.1 μg/mL). Compounds **3** and **4** were also found to possess significant inhibitory activity on the innate and T-cell proliferation immune response. When compounds **1**–**4** were evaluated for their cytotoxicity on Balb/c 3T3 cells, no toxic effect was observed after 48 h of incubation (Table 3 and Chart 1).

3. Experimental

3.1. General experimental procedures

The melting points were recorded on a YANACO apparatus. Optical rotations were measured on a digital polar-

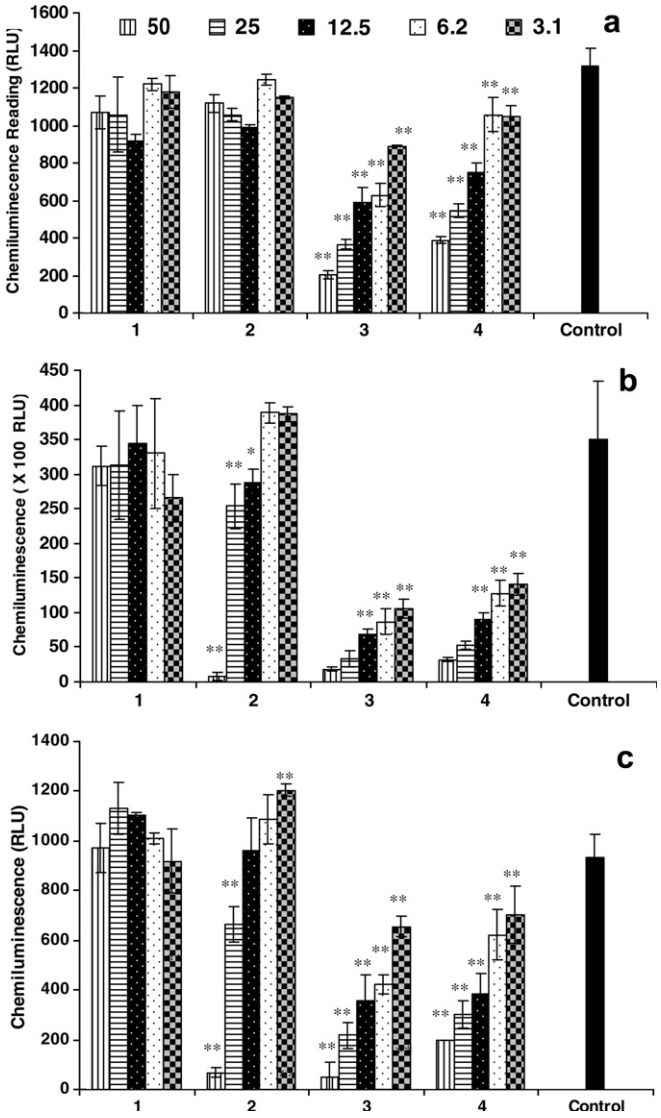

Fig. 2. Chemiluminescence effect of compounds **1**–**4** on oxidative burst using whole blood (a), neutrophils (b) and mononuclear cells (c). Various concentrations of compounds **1**–**4** were incubated with whole blood (a), or isolated polymorphoneutrophils (b) mononuclear cells (MNCs) (c) for 30 min. The compounds activity was compared with the untreated samples (control) in the chemiluminescence (CL) assay. Each plot and error bar represents readings ± SD of three repeats.

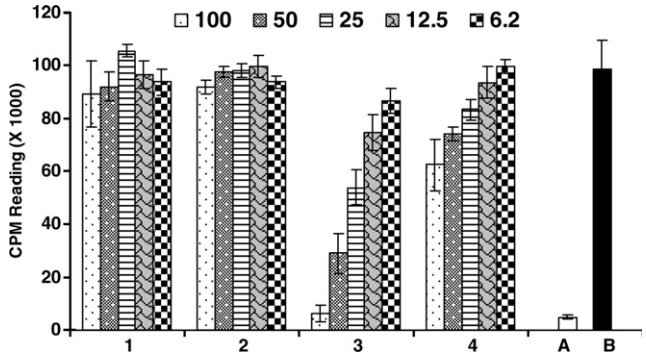

Fig. 3. Effect of compounds on phytohemagglutinin (PHA) T-cell proliferation. The bar graph represents effects of various concentrations of the test compounds **1**–**4** after 72 h incubation with peripheral blood mononuclear cells at 37 °C. Effect of compounds on T-cell proliferation response is compared with non-proliferated (A) and proliferated (B) cells. Each bar represents the mean value of triplicate reading ± SD.

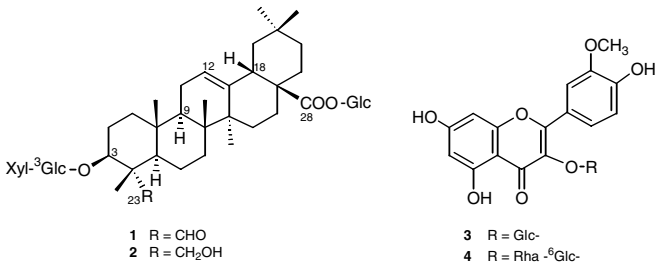

Chart 1. Structures of the compounds **1**–**4**.

Table 3
Compounds IC_{50} value from the MTT cytotoxicity studies

Compound	IC_{50} (μg/mL)
	48 h MTT Results
1	70.1 ± 2.8
2	53.4 ± 6.1
3	>100
4	>100

Serial compounds dilutions (0.195–100 μg/mL) were incubated with Swiss Balb/c 3T3 cells for 48 h and cells viability was evaluated by MTT reduction to the blue colored formazan in living cells. All values were means of 3 replicates.

imeter JASCO DIP-360 in methanol. Infrared spectra were obtained on Vector 22, Bruker spectrophotometer on KBr pellets.

FAB Mass spectra were recorded on Varian MAT 312 mass spectrometer. Accurate mass measurements were carried out with FAB source using glycerol as matrix and high-resolution-fast-atom bombardment mass spectra (HRFAB-MS) were recorded with a Jeol HX 110 mass spectrometer; in *m/z* (rel. %). The ^{1}H NMR, ^{13}C NMR, 1D TOCSY, HMQC, and HMBC spectra were recorded on Bruker AV-400 spectrometer operating at 400 (^{1}H NMR) and 100 (^{13}C NMR) MHz. The chemical shifts values were reported in δ (ppm), referenced with respect to the residual solvent signal of CD_3OD and coupling constants (*J*) were measured in Hz. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (DC-Alugram 60 UV_{254} of E. Merck), by using ceric sulphate spraying reagent. Paper chromatography (PC) was performed on Whatman 3MM (46 × 57 cm) by using *o*-toluidin spraying reagent. Column chromatography was performed using Diaion HP-20 (Mitsubishi Chem. Ind., Tokyo, Japan), ODS C-18 (63-212 μm, Wako Pure Chemical Industries Ltd., Japan), polyamide-6 DF (Riedel-De Haen AG) and silica gel (E. Merck, 230-400 μm mesh). Chemiluminescence readings were recorded with luminometer (Luminoskan RS Labsystem, Finland). T-Cell proliferation level was recorded by using liquid scintillation counter (Beckman Coulter LS 6500, USA). For Cytotoxicity, O.D. reading was taken with the micro plate readers

(SpectraMax PLUS384, Molecular Devices, CA, USA). All reagents used were of analytical grades.

3.2. Plant material

The aerial parts of *C. obtusifolia* (Schrenk.) Botsch., used in this study, were collected from the Almaty region of Kazakhstan in August 2003, and identified by Mr. Anatoli Aleshkovskii. A voucher specimen (2269a) was deposited at the Department of Botany, Al-Farabi Kazakh National University, Almaty, Kazakhstan.

3.3. Extraction and isolation

Dried and crushed aerial parts of *C. obtusifolia* (3 kg) were macerated in 80% methanol–H_2O (6 L × 3) at room temperature. The extract was filtered and concentrated under reduced pressure. The concentrated extract (126 g) was dissolved in water (1 L) and then successively extracted with hexane (2 L × 4), chloroform (2.5 L × 3), ethyl acetate (2 L × 2), and *n*-butanol (2 L × 3). Butanolic extract (31 g) was fractionated by CC on Diaion HP-20 and eluted with the mixtures of H_2O–MeOH to obtain various subfractions. A subfraction, eluted with 25–50% MeOH–H_2O, was subjected to ODS chromatography using gradients of H_2O–MeOH (100% H_2O, 10% MeOH–H_2O, 20% MeOH–H_2O, 30% MeOH–H_2O, 50% MeOH–H_2O, 100% MeOH) to afford 10 fractions (F_1–F_{10}). Fractions F_5–F_9 (10 g) were combined and subjected to Polyamide column chromatography with MeOH/H_2O as eluting solvents in a gradient manner. The final purification was carried out by column chromatography (silica gel) by using $CHCl_3$/MeOH/H_2O (8:2.8:0.2) as eluting solvents. This yielded new compounds **1** (30 mg, 10^{-3} %), and **2** (26 mg, 8.6×10^{-4} %). Fractions F_2–F_3 (1.5 g) were combined and purified by the use of C_{18} recycling HPLC, (MeOH/H_2O, 1:1) as eluting solvent. This yielded known compounds **3** (10 mg, 3.3×10^{-4} %) and **4** (13 mg, 4.3×10^{-4} %).

3.3.1. Acid hydrolysis of *1* and *2*

Compounds **1** and **2** (3 mg each) were refluxed with 5% HCl at 100 °C for 3 h. The products of acid hydrolysis were adjusted to pH 6 by $NaHCO_3$ and extracted with EtOAc. From the EtOAc part, aglycons were isolated and identified by spectroscopic methods and over m.p. The aqueous part was identified by paper chromatography by using standard sugars in the solvent system EtOAc/AcOH/H_2O (5:3:2), the results of which indicated the presence of D-glucose and D-xylose.

3.3.2. Gypsogenin 3-O-[β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranoside]-28-O-{β-D-glucopyranosyl} ester (*1*)

Colorless gummy material; $[\alpha]_D^{25}$: −191° (*c* 0.018, MeOH); IR ν_{max}^{KBr} cm^{-1} : 3440–3780 (OH), 2928 (CH), 1725 (CHO), 1730 (C═O, ester); ^1H NMR (400 MHz, CD_3OD): spectroscopic data, see Table 1; For ^{13}C NMR (100 MHz, CD_3OD): spectroscopic data, see Table 2; FAB-MS (−ve): *m/z* 925 $[M-H]^-$, 763 $[M-H-162]^-$, 469 $[M-2\times162-132]^-$; HRFAB-MS *m/z* 925.4793 $[M-H]^-$ (calcd. for $C_{47}H_{74}O_{18}$–H, 925.4797).

3.3.3. Hederagenin 3-O-[β-D-xylopyranosyl-(1 → 3)-β-D-glucopyranoside]-28-O-{β-D-glucopyranosyl} ester (*2*)

Colorless gummy material; $[\alpha]_D^{25}$ − 180° (*c* 0.02, MeOH), IR ν_{max}^{KBr} cm^{-1}: 3420–3880 (OH), 2925 (CH), 1728 (C═O, ester); ^1H NMR (400 MHz, CD_3OD): spectroscopic data, see Table 1; For ^{13}C NMR (100 MHz, CD_3OD) spectral data, see Table 2; FAB-MS (−ve): *m/z* 927 $[M-H]^-$, 765 $[M-H-162]^-$, 603 $[M-H-162-132]^-$, 471 $[M-2\times162-132]^-$; HRFAB-MS *m/z* 927.4961 $[M-H]^-$ (calcd. for $C_{47}H_{76}O_{18}$–H, 927.4953).

3.3.4. Isorhamnetin 3-O-β-D-glucopyranoside (*3*)

Yellow powder, M.p. 243–245 °C, $[\alpha]_D^{25}$: −20.5° (*c* 0.2, MeOH); ^1H NMR (400 MHz, CD_3OD): δ 6.16 (*d*, *J* = 1.8 Hz, H-6), 6.38 (*d*, *J* = 1.8 Hz, H-8), 6.79 (*d*,*J* = 8.2 Hz, H-5′), 7.55 (*dd*, *J* = 8.2, 1.8 Hz, H-6′), 7.91 (*d*, *J* = 1.8 Hz, H-2′), 3.93 (*s*, –OCH_3), 5.38 (*d*, *J* = 7.1 Hz, H-1″); FAB-MS (−ve): *m/z* 477 $[M-H]^-$, 316 $[M-H-162]^-$; HRFAB-MS *m/z* 477.1028 $[M-H]^-$ (calcd. for $C_{22}H_{22}O_{12}$ –H, 477.1033).

3.3.5. Isorhamnetin 3-O-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (narcissin) (*4*)

Yellow powder, M.p. 169–171 °C, $[\alpha]_D^{25}$: −38.7° (*c* 0.3, MeOH). ^1H NMR (400 MHz, CD_3OD): δ 6.18 (*d*, *J* = 1.8 Hz, H-6), 6.37 (*d*, *J* = 1.8 Hz, H-8), 6.82 (*d*, *J* = 8.3 Hz, H-5′), 7.59 (*dd*, *J* = 8.3, 1.9 Hz, H-6′), 7.93 (*d*, *J* = 1.8 Hz, H-2′), 3.93 (*s*, –OCH_3), 4.5 (*s*, H-1‴), 5.23 (*d*, *J* = 7.5 Hz, H-1″), 1.10 (*s*, 3H, rhamnose CH_3); FAB-MS (−ve): *m/z* 625 $[M-H]^-$, 478 $[M-146]^-$, 316 $[M-H-162-146]^-$; HRFAB-MS *m/z* 623.1621 $[M-H]^-$ (calcd. for $C_{28}H_{32}O_{16}$-H, 623.1612).

3.4. Biological assays

3.4.1. Chemiluminescence assay

Luminol-enhanced chemiluminescence assay was performed, as described by Helfand et al. (1982). Briefly, whole blood (diluted 1:200), neutrophils (1×10^7) or monocytes (1×10^6), suspended in Hank's balance salt solution with calcium and magnesium ($HBSS^{++}$), were incubated with 50 μL of compounds concentrations (1.6–50 μg/mL) for 30 min. To each well, 50 μL (20 mg/mL) zymosan (Sigma Chemical Co. USA), followed by 50 μL (7×10^5 M) luminol (G-9382 Sigma Chemical Co.) and then $HBSS^{++}$ was added to adjust the final volume to 0.2 mL. $HBSS^{++}$ alone was used as a control. Chemiluminescence peaks were recorded with the Luminometer (Luminoskan RS Labsystem, Finland).

3.4.2. T-Cell proliferation assay

Cell proliferation was evaluated by standard thymidine incorporation assay following a method reported by Nielsen

et al. (1998). Briefly, cells were cultured at a concentration of 5×10^5 cells/mL in a 96-well round bottom tissue culture plate (Nalge Nunc. Inter.). Cells were stimulated with 5 μg/mL of PHA (Sigma Chemical Co., USA). Various concentrations of compounds were added to obtain final concentrations of 6.2, 12.5, 25, 50, and 100 μg/mL, each in triplicate. Plates were incubated for 72 h at 37 °C in 5% CO_2 incubator. Cultures were pulsed later with 0.5 μCi/well tritiated thymidine (Amersham Pharmacia Biotech, Sweden), and further incubated for 18 h. Cells were harvested and the tritiated thymidine incorporation was measured by a liquid scintillation counter (LS 6500, Beckman Coulter, USA). Results were expressed as mean count per minute (CPM).

3.4.3. Cytotoxicity evaluation

The experiment was performed according to method reported earlier (Dariusz et al., 1993) with some modification. Swiss Balb/c 3T3 cells (3×10^4 cells/mL) were cultured in a 96-well plate for overnight. The supernatant was removed and 50 μL of serially diluted compounds (0.195–100 μg/mL), 150 μL Dulbecco's Modified Eagle's Medium (DMEM), penicillin [100 units/mL] and streptomycin (100 μg/mL) were added to each well. After 48 h of incubation, the culture medium was carefully removed, and 50 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL) was added to each well. The plates were incubated at 37 °C for 4 h. After the MTT solution was aspirated and cells were washed with phosphate buffer saline (PBS), 100 μL of DMSO was added to dissolve the blue insoluble MTT formazan produced by mitochondrial dehydrogenase. The plate was agitated at room temperature for 15 min and then read at 540 nm using microplate readers (SpectraMax PLUS384, Molecular Devices, USA). The percentage of viable cells was calculated as the relative ratio of optical densities (OD).

3.4.4. Statistical analysis

All data are reported as mean ± SD of the mean and the student *t*-test was used to determine the difference between test- and control preparations significance was attributed to probability values $P \leqslant 0.05$. The IC_{50} values were calculated using Excel based program.

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References

Annaev, C., Abubakirov, N.K., 1984. Triterpene glycosides of *Climacoptera transoxana*. 4. Structures of Kopteroside-G and kopteroside-H. Khim. Prir. Soedin., 60–64.

Annaev, C., Isamukhamedova, M., Abubakirov, N.K., 1983a. Triterpene glycosides of *Climacoptera transoxana* 1. Khim. Prir. Soedin., 202–205.

Annaev, T., Isamuchamedova, M., Abubakirov, N.K., 1983b. Triterpene glycosides of *Climacoptera transoxana*. 2. Structure of the Kopterosid-D. Khim. Prir. Soedin., 453–456.

Annaev, T., Jsaev, M.I., Abubakirov, N.K., 1983c. Triterpene glycosides of *Climasoptera transoxana*. 3. Structures of the Kopterosides-E and Kopterosides-F. Khim. Prir. Soedin., 596–601.

Baeva, R.T., Zapesochnaya, G.G., 1980. Flavonoids of *Climacoptera transoxana*. Khim. Prir. Soedin., 839.

Beier, R.C., Mundy, B.P., Strober, G.A., 1980. Assignment of anomeric configuration and identification of carbohydrate residues by ^{13}C NMR. Can. J. Chem. 58, 2800–2804.

Chen, G., Lu, H., Wang, C., Yamashita, K., Manabe, M., Meng, Z., Xu, S., Kodama, H., 2002. Effect of five flavonoid compounds isolated from leaves of *Diospyros kaki* on stimulus induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. Clin. Chim. Acta 326, 169–175.

Dariusz, S., Sarah, J.S., Richard, H.C., Michael, B., 1993. An improved MTT assay. J. Immunol. Meth. 157, 203–207.

Eskalieva, B.K., Ahmed, A., Burasheva, G.Sh., Abilov, Zh.A., Ahmad, V.U., 2004. Biologically active compounds from *Climacoptera*. Khim. Prir. Soedin. 40, 87–88.

Hadjimitova, V., Traykov, T., Bakalova, R., Petrova, V., Lambev, I., Ohba, H., Ishikawa, M., Baba, Y., 2004. Chemiluminescent analysis of the antioxidant and immunomodulation effects of several psychotropic drugs on peritoneal macrophages. Luminescence 19, 319–321.

Hostettmann, K., Marston, A., 1995. Saponins. Cambridge University Press, Great Britain, pp. 2–29.

Horhammer, L., Wagner, H., Arndt, H.G., Farkas, L., 1966. Isolieruing und syntheses zweier Flavonol-glykoside von *Cereus grandiflorus*. Mill. Chem. Ber. 99, 1384.

Helfand, S.L., Werkmeister, J., Roder, J.C., 1982. Chemiluminescence response of human natural killer cells. The relationships between target cell binding, chemiluminescence and cytolysis. J. Exp. Med. 156, 492–505.

Jayasinghe, L., Shimada, H., Hara, N., Fujimoto, Y., 1995. Hederagenin glycosides from *Pometa eximia*. Phytochemistry 40, 891–897.

Jayasinghe, L., Hara, N., Fujimoto, Y., 2003. Bidesmosidic saponins from the fruits of *Diploclisia glaucescens*. Phytochemistry 62, 563–567.

Jimenez, C., Villaverde, M.C., Riguera, R., Castedo, L., Stermitz, F., 1989. Triterpene glycosides from *Mussatia* species. Phytochemistry 28, 2773–2776.

McNally, J.A., Bell, A.L., 1996. Myeloperoxidase-based chemiluminescence of polymorphonuclear leukocytes and monocytes. J. Biolumin. Chemilumin. 11, 99–106.

Nie, R., Tanaka, T., Miyakoshi, M., Kasai, R., Morita, T., Zhou, J., Tanaka, O., 1989. A triterpenoid saponin from *Thladiantha hookeri* var pentadactyla. Phytochemistry 28, 1711–1715.

Nielsen, M., Gerwien, J., Nielsen, M., Geisler, C., Ropke, C., Svejgaard, A., Odum, N., 1998. MHC class II ligation induces CD58 (LFA-3)-mediated adhesion in human T-cells. Exp. Clin. Immunogenet. 15, 61–68.

Pavlov, N.V., 1961. Flora of Kazakhstan, vol. 3. Nauka, Alma-Ata, pp. 274–280 (in Russian).

Rahman, W., Ilyas, M., 1962. Flavonoids from *Argemone mexicana* Linn. J. Org. Chem. 27, 153.

Sokolov, P.D., 1986. Plant resources of the USSR: flowering plants, their chemical composition and use; family Chenopodiaceae. Leningrad, 356–361 (in Russian).

Tori, K., Seo, S., Shimaoka, A., Tomita, Y., 1974. Carbon-13 NMR spectra of olean-12-enes. Full signal assignment including quaternary carbon signals assigned by use of indirect ^{13}C, ^{1}H spin couplings. Tetrahed. Lett., 4227.