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# Saponins from the roots of *Chenopodium bonus-henricus* L.

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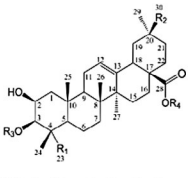
## ABSTRACT

Two new glycosides of phytolaccagenin and 2$\beta$-hydroxyoleanoic acid, namely bonushenricoside A (**3**) and bonushenricoside B (**5**) together with four known saponins, respectively compounds 3-O-L-$\alpha$-arabinopyranosyl-bayogenin-28-O-$\beta$-glucopyranosyl ester (**1**), 3-O-$\beta$-glucuronopyranosyl-2$\beta$-hydroxygypsogenin-28-O-$\beta$-glucopyranosyl ester (**2**), 3-O-$\beta$-glucuronopyranosyl-bayogenin-28-O-$\beta$-glucopyranosyl ester (**4**) and 3-O-$\beta$-glucuronopyranosyl-medicagenic acid-28-$\beta$-xylopyranosyl(1→4)-$\alpha$-rhamnopyranosyl(1→2)-$\alpha$-arabinopyranosyl ester (**6**) were isolated from the roots of *Chenopodium bonus-henricus* L. The structures of the compounds were determined by means of spectroscopic methods (1D and 2D NMR, IR and HRMS). The MeOH extract and compounds were tested for cytotoxic activity on five leukemic cell lines (HL-60, SKW-3, Jurkat E6-1, BV-173 and K-562). In addition, the ability of metanolic extract and saponins to modulate the interleukin-2 production in PHA/PMA stimulated Jurkat E6-1 cells was investigated as well.



1. $R_1 = CH_2OH$ $R_2 = CH_3$ $R_3 = Ara$ $R_4 = Glc$
2. $R_1 = CHO$ $R_2 = CH_3$ $R_3 = GluA$ $R_4 = Glc$
3. $R_1 = CH_2OH$ $R_2 = COOCH_3$ $R_3 = Ara$ $R_4 = Glc$
4. $R_1 = CH_2OH$ $R_2 = CH_3$ $R_3 = GluA$ $R_4 = Glc$
5. $R_1 = CH_3$ $R_2 = CH_3$ $R_3 = GluA$ $R_4 = Glc$
6. $R_1 = COOH$ $R_2 = CH_3$ $R_3 = GluA$ $R_4 = Xyl(1\rightarrow4)Rham(1\rightarrow2)Ara$

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*Chenopodium bonus-henricus* L.; triterpene saponins; cytotoxicity; interleukin-2

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## 1. Introduction

The genus *Chenopodium* (Amaranthaceae) includes about 150 species native mainly to subtropical and temperate regions (Uotila and Tan 1997). The most widely recognised *Chenopodium* species for their medicinal and nutrient properties are *C. ambrosioides* and *C. quinoa* (Podolak et al. 2016; Shah and Khan 2017). *Chenopodium bonus-henricus* L. is a perennial herbaceous plant and is spread in the mountainous regions of Bulgaria (Grozeva 2011). Its leaves and flowering tops are used as a vegetable in the same manner as spinach in some European countries (Cheschmedzhiev et al. 1999; Toensmeier 2007). In Bulgarian folk medicine the roots are known as 'chuven' and have been applied externally to treat skin inflammations, wounds and boils. The infusion of the drug has been also used as a mild laxative. In Bulgarian food industry the aqueous extract of the roots has been employed in production of halva (Kokanova-Nedialkova et al. 2015). Recently phytochemical investigation of the aerial parts of *C. bonus-henricus* L. led to the isolation of nine flavonoid glycosides of patuletin, spinacetin and 6-methoxykaempferol (Kokanova-Nedialkova et al. 2017). From the roots of title plant the ecdysteroids 20-hydroxyecdysone and polypodine B (Báthory et al. 1982) as well as the flavonol glycosides 6-methoxykaempferol 3-O-[*β*-apiofuranosyl(1→2)]-*β*-glucopyranosyl(1→6)-*β*-glucopyranoside, spinacetin 3-O-[*β*-apiofuranosyl(1→2)]-*β*-glucopyranosyl(1→6)-*β*-glucopyranoside and spinacetin 3-O-gentiobioside (Kokanova-Nedialkova et al. 2015) have been isolated. The flavonoids isolated from title plant have exerted hepatoprotective and antioxidant activities comparable to those of silymarin and silybine in *in vitro* model of $CCl_4$ induced liver damage. They also possessed DPPH and ABTS radical-scavenging activity and significantly inhibited the lipid peroxidation in a linoleic acid system by the ferric thiocyanate method (Kokanova-Nedialkova et al. 2015, 2017; Kokanova-Nedialkova and Nedialkov 2017). The present study deals with the isolation and structural elucidation of two new and four known triterpene saponins. The cytotoxicity of the MeOH extract and isolated compounds were evaluated on HL-60, SKW-3, Jurkat E6-1, BV-173 and K-562 tumor cell lines. In addition, the ability of the metanolic extract and saponins to modulate the interleukin-2 production in PHA/PMA stimulated Jurkat E6-1 cells was investigated as well.

## 2. Results and discussion

An extensive chromatographic procedure of the combined MeOH and aq. MeOH extracts from the roots of *C. bonus-henricus* led to the isolation and structural identification of six saponins **1–6** (Figure 1). The structures of the compounds were elucidated by means of spectral (MS, IR and NMR) methods and hydrolysis. The signals in the $^{1}H$ and $^{13}C$ NMR spectra were unambiguously assigned using 2D NMR techniques. The known compounds were identified as 3-O-*α*-arabinopyranosyl-bayogenin-28-O-*β*-glucopyranosyl ester **1**, 3-O-*β*-glucuronopyranosyl-2*β*-hydroxygypsogenin-28-O-*β*-glucopyranosyl ester **2**, 3-O-*β*-glucuronopyranosyl-bayogenin-28-O-*β*-glucopyranosylester **4** and 3-O-*β*-glucuronopyranosyl-medicagenic acid-28-*β*-xylopyranosyl(1→4)-*α*-rhamnopyranosyl(1→2)-*α*-arabinopyranosyl ester **6**, respectively by comparing their spectroscopic data with those reported in the literature. Compound **1** was initially found in *Medicago arabica* (Bialy et al. 2004). Compounds **2** and **4** have been previously isolated respectively from *Spinacia oleracea* (Mithöfer et al. 1999) and *Meliosma lanceolata* (Abe et al. 1996), respectively while **6** was found in *Medicago sativa* (Oleszek et al. 1990).

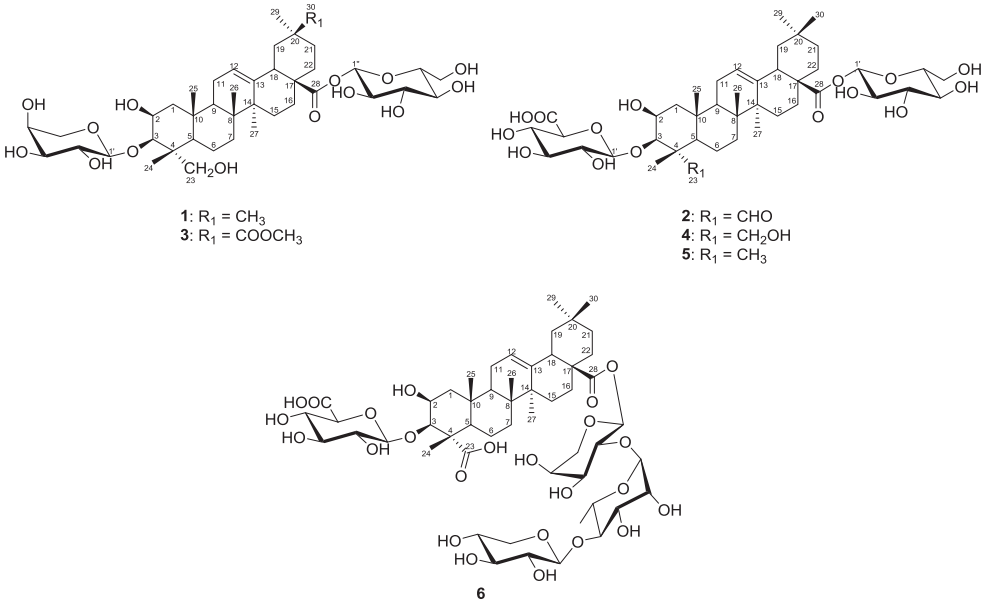


**Figure 1.** Structures of saponins **1–6**, isolated from the roots of *C. bonus-henricus* L.

Compound **3** was obtained as optically active colourless crystals with m.p. 226–227 °C and $[\alpha]_D^{20}$ +73.8 (c 0.100, MeOH). The IR spectrum showed absorption bands for hydroxyl groups (3368 $cm^{-1}$), C–H bonds (2923 $cm^{-1}$), an ester group (1718 $cm^{-1}$), carbonyl (1654 $cm^{-1}$) and C–O bonds (1050 $cm^{-1}$). The molecular formula of **3** was established as $C_{42}H_{66}O_{16}$ by means of HR-ESI-MS, showing a formyl adduct ion $[M + HCOO]^-$ at *m/z* 871.4330.

The $^{13}C$ NMR spectrum (Table S1) displayed 42 carbon resonances with two carbonyl carbons appeared at $\delta_C$ 176.4 (C-28) and 177.3 (C-30) and two olefin carbon resonances located at $\delta_C$ 124.1 (C-12) and 144.1 (C-13). Three resonances for carbons bearing oxygen were observed at $\delta_C$ 71.3 (C-2), 83.0 (C-3) and 65.6 (C-23) in addition to one methoxy and 11 oxygenated carbon resonances of one hexose and one pentose sugars. HSQC spectrum displayed signals corresponding to carbons, classified into 6 methyls, 13 methylenes, 14 methines and 9 quaternary carbons. The $^1H$ NMR spectrum (Table S1) revealed the presence of five tertiary methyl singlets at $\delta_H$ 1.33 (H-24), 1.60 (H-25), 1.17 (H-26), 1.23 (H-27), 1.18 (H-29), an O-methyl proton at $\delta_H$ 3.60 and an olefinic triplet $\delta_H$ 5.58 (H-12). The $^1H$ NMR spectrum also showed two hydroxymethine signals at $\delta_H$ 4.80 (H-2) and 4.34 (H-3) correlated by HSQC with the carbon signals at $\delta_C$ 71.3 and 83.0. The *β*-orientation of the hydroxyls at C-2 and C-3 was deduced from the coupling constant ($J$ = 3.8 Hz) of H-2 and H-3. The HMBC correlations of $\delta_H$ 1.33 (H-24) to the signal due to a hydroxymethyl carbon at $\delta_C$ 65.6 (C-23) was used to place a hydroxymethyl group at C-4. Detailed analysis of the NMR spectra pointed out phytolaccagenin (2*β*,3*β*,23-trihydroxyolean-12-en-28,30-dioic acid 30-methyl ester) as aglycone (Spengel and Schaffner 1993; He et al. 2011). The $^1H$ NMR spectrum showed two anomeric sugar proton signals at $\delta_H$ 5.03 (7.7 Hz) and 6.31 (8.2 Hz) indicating a *α*- and *β*-orientation, respectively of the glycosidic linkage. According to the proton and the carbon resonances the sugar units were identified as *α*-arabinopyranose and *β*-glucopyranose (Bialy et al. 2004). The HMBC correlations of anomeric proton signal $\delta_H$ 5.03 (H-1') of arabinose

moiety with the carbon signal at $\delta_C$ 83.0 (C-3) indicated the attachment of arabinose at C-3 of the aglycone. The presence of a second monosaccharide moiety at C-28 was deduced from the HMBC interactions of anomeric proton of glucose $\delta_H$ 6.31 (H-1″) with the signal of $\delta_C$ 176.4 (C-28). The presence of L-arabinose and D-glucose was authenticated by alkaline and acid hydrolysis and synthesis of their tolylthiocarbamoyl-thiazolidine derivatives (Tanaka et al. 2007; Nedialkov et al. 2012). Thus, the structure of **3** was established as 2*β*,3*β*,23-trihydroxyolean-12-en-28,30-dioic acid 30-methyl ester 3-O-*α*-L-arabinopyranosyl-28-O-*β*-D-glucopyranoside, named Bonushenricoside A. This compound is structurally similar to esculentoside S that was isolated from *Phytolacca acinosa* (Spengel and Schaffner 1993).

Compound **5** was obtained as a colourless amorphous powder with $[\alpha]_D^{20}$: +17.9 (c 0.100, MeOH). The IR spectrum indicated the presence of hydroxyl groups (3383 $cm^{-1}$), C–H bonds (2937 $cm^{-1}$), an ester group (1729 $cm^{-1}$), carbonyl (1653 $cm^{-1}$) and C–O bonds (1063 $cm^{-1}$). The molecular formula of **5** was determined as $C_{42}H_{66}O_{15}$ by means of HR-ESI-MS, showing a $[M - H]^-$ ion at *m/z* 809.4328. The $^1$H NMR spectrum (Table S1) showed two hydroxymethine signals at $\delta_H$ 4.73 (H-2) and 3.43 (H-3) correlated by HSQC with the carbon signals at $\delta_C$ 71.1 and 90.0. The *β*-orientation of the hydroxyls at C-2 and C-3 was deduced from the coupling constant ($J$ = 3.6 Hz) of H-3. The $^1$H NMR of **5** also revealed the presence of seven tertiary methyl singlets at $\delta_H$ 1.36 (H-23), 1.43 (H-24), 1.51 (H-25), 1.16 (H-26), 1.30 (H-27), 0.92 (H-29), 0.89 (H-30) and an olefinic proton signal resonating at $\delta_H$ 5.43 (H-12). The last correlated in the HSQC spectrum with a carbon signal at $\delta_C$ 123.5 (C-12) and together with the signal of a quaternary carbon at $\delta_C$ 144.6 (C-13) indicated a typical $\Delta^{12}$ pentacyclic triterpene derivative (De Tommasi et al. 1998). The $^{13}$C NMR spectrum (Table S1) displayed 42 carbon resonances with one carbonyl carbon appeared at $\delta_C$ 176.9 (C-28), two olefin carbon resonances located at $\delta_C$ 123.5 (C-12) and 144.6 (C-13) and two resonances for carbons bearing oxygen at $\delta_C$ 71.1 (C-2), 90.0 (C-3). The NMR spectra clearly pointed out 2*β*,3*β*-dihydroxyolean-12-en-28-oic acid as the aglycone. In addition, the $^1$H NMR spectrum showed two anomeric sugar proton signals at $\delta_H$ 5.12 (7.7 Hz) and 6.36 (8.0 Hz) indicating a *β*-orientation of the glycosidic linkage of glucose and glucuronic acid. The former signal gave a cross-peak with C-3 ($\delta_C$ 107.4) of the aglycone, while the latter correlated with the signal of C-28 ($\delta_C$ 96.3). The presence of D-glucuronic acid and D-glucose was authenticated by alkaline and enzymatic hydrolysis and synthesis of their tolylthiocarbamoyl-thiazolidine derivatives (Tanaka et al. 2007; Nedialkov et al. 2012). Thus, the structure of **5** was established as 2*β*,3*β*-dihydroxyolean-12-en-28-oic acid 3-O-*β*-D-glucuronopyranosyl-28-O-*β*-D-glucopyranoside, named Bonushenricoside B.

The methanolic extract of the plant, as well as compounds **1–6** were tested for cytotoxic activity in a panel of human leukemic cell lines, representative for some important types of human haematological malignancies, namely HL-60 (acute promyelocyte leukemia), SKW-3 and Jurkat E6-1 (T-cell leukemias), BV-173 and K-562 (chronic myeloid leukemias). The extract displayed concentration-dependent cytotoxicity with $IC_{50}$ values ranging 124.5–258 µg/ml. The juxtaposition of the MTT-bioassay data unambiguously indicated that the aforementioned cytotoxicity of the extract resides chiefly in compounds **1**, **5** and **6**, whereas the other tested agents inhibited the growth of cultured malignant cells at substantially higher concentrations with $IC_{50}$ values close to or ever exceeding 500 µM (Table S2). Further, we sought to assess the ability of the tested compounds and the total methanolic extract to modulate the mitogen-induced production of IL-2 in Jurkat E6-1 (a well-established model of human T-cell activation) following sub-cytotoxic exposure. As evident from the data presented in Table

S3, the tested saponins stimulated the PHA/PMA-triggered release of IL-2 in Jurkat E6-1 human T cells by approximately 30–60% vs. the effects of the solvent alone. In this experimental system compound **1** was the most potent of the series. Interleukine-2 is a lymphokine with a well-appreciated role in the immune system. It stimulates clonal expansion, proliferation and differentiation of native T-cells, activates the T-cells and natural killer-mediated cytotoxicity, and thus is involved in immune responses (Hoyer et al. 2008; Olejniczak and Kasprzak 2008).

## 3. Experimental

### 3.1. General experimental procedures

General experimental procedures are in the Supplementary material.

### 3.2. Plant material

The roots of *Chenopodium bonus-henricus* L. were collected from Beglica, Western Rhodopes, Bulgaria in September 2011 and were identified by P. T. Nedialkov. A voucher specimen (No. SOM-Co-169848) was deposited at the National Herbarium, Bulgarian Academy of Sciences, Sofia, Bulgaria.

### 3.3. Extraction and isolation

The roots of *C. bonus-henricus* were dried in the shade and a powdered plant material (295 g) was extracted subsequently with MeOH (3 L), 80% aq. MeOH (5 L) and $H_2O$ (1 L) by ultrasonic-assisted extraction. After filtration, the extracts were combined and the solvent was evaporated under reduced pressure to give 149 g white-yellow residue. The resulting residue was dissolved in 200 mL $H_2O$ and then subjected to CC over Diaion HP-20 (45 × 4 cm) with $H_2O$ (2 L) and 90% aq. MeOH (4 L) as eluants to obtain water and aq. methanol fractions. The MeOH fraction (56 g) was purified using silica gel, MCI gel, RP-18 to afford pure pure (**1**) (53 mg), (**2**) (64 mg), (**3**) (86 mg), (**4**) (41 mg). (**5**) (127 mg) and (**6**) (145 mg) (See a supplementary material).

#### 3.3.1. Bonushenricoside A (3) (3-O-α-L-arabinopyranosyl-phytolaccagenin-28-O-β-D-glucopyranosyl ester)

White crystals, m.p. 226–227 °C; $[\alpha]_D^{20}$ +73.8 (*c* 0.100, MeOH); IR (neat) $\upsilon_{max}$: 3368, 2923, 1718, 1654, 1050 $cm^{-1}$; HR-ESI-MS: found *m/z* 871.4330 $[M + HCOO]^-$, calcd. for $C_{43}H_{67}O_{18}$ *m/z* 871.4322; $^1H$ NMR (pyridine-$d_6$, 600 MHz): (Table S1); $^{13}C$ NMR (pyridine-$d_6$, 150 MHz): (Table S1).

#### 3.3.2. Bonushenricoside B (5) (3-O-β-D-glucuronopyranosyl-2β-hydroxyoleanoic acid-28-O-β-D-glucopyranosyl ester)

White amorphous powder; $[\alpha]_D^{20}$: +17.9 (*c* 0.100, MeOH); IR (neat) $\upsilon_{max}$: 3383, 2937, 1729, 1653, 1063 $cm^{-1}$; HR-ESI-MS: found *m/z* 809.4328 $[M - H]^-$, calcd. for $C_{42}H_{65}O_{15}$ *m/z* 809.4329; $^1H$ NMR (pyridine-$d_6$, 600 MHz): (Table S1); $^{13}C$ NMR (pyridine-$d_6$, 150 MHz): (Table S1).

### 3.4. Alkaline, acid and enzymatic hydrolysis

Compounds **3** and **5** (each 10 mg) were separately refluxed with 2 mL 5% aq. KOH for 2 h. The reaction mixtures were filtered through Amberlite IRC-86 resin and washed with $H_2O$. The eluate was filtered through a Diaion HP-20SS column and subsequently eluted with $H_2O$ and MeOH. The water portions were evaporated to dryness and analyzed for sugars by HPLC according the method of Tanaka et al. (2007) with some modifications (Nedialkov et al. 2012). The occurrence of D-glucose ($t_R$ value of the tolylthiocarbamoyl-thiazolidine derivative was 18.7 min) was found in both water residues. The methanol fraction from alkaline hydrolysis of **3** was refluxed with 4 mL of a mixture of 0.57 M $H_2SO_4$-EtOH (1:1) for 2 h. The reaction mixture was filtered through Amberlite IRC-86 resin, washed with $H_2O$ and then filtered through Diaion HP-20SS, followed by subsequent elution with $H_2O$ and MeOH. The water eluate showed presence of L-arabinose with $t_R$ of its tolylthiocarbamoyl-thiazolidine derivative at 21.2 min. While the methanol fraction from alkaline hydrolysis of **5** was dissolved in 1 mL water and 50,000 units of $\beta$-glucuronidase were added. The mixture was left for 48 h at room temperature then was filtered through Diaion HP-20SS, followed by subsequent elution with $H_2O$ and MeOH. The water portion was evaporated to dryness and analyzed for sugars by HPLC. The presence of D-glucuronic acid with $t_R$ of its tolylthiocarbamoyl-thiazolidine derivative at 19.6 min was established.

### 3.5. Cell lines and cytotoxicity assay

The cell lines HL-60 (acute myeloid leukemia), BV-173 (chronic myeloid leukemia), K-562 (chronic myeloid leukemia) and SKW-3 (T-cell leukemia) were purchased form DSMZ (Germany), and Jurkat E6-1 (clone of the Jurkat-FHCRC cell line, a derivative of the Jurkat T-cell leukemia cell line) was supplied by ATCC (USA). Cells were cultured routinely in a controlled environment: 37 °C in a 5% $CO_2$ humidified atmosphere and were maintained in RPMI-1640 medium, supplemented with 2 mM L-glutamine and 10% fetal calf serum. The cellular viability was assessed using the MTT-dye reduction assay, as described by Mosmann, with slight modifications (Mosmann 1983; Konstantinov et al. 1999). Cisplatin was used as a positive control. In addition, $IC_{50}$ values were derived from the concentration-response curves.

### 3.6. Modulation of IL-2 production assay

The IL-2 release study was conducted using Jurkat cells (E6-1 clone), a leukemic T-cell line known to produce IL-2 upon mitogen stimulation (Gillis and Watson 1980; Manger et al. 1986; McIntyre et al. 1994). Jurkat E6-1 cells were seeded in 24 well microplates ($10^6$ cells/well), in triplicate, stimulated with 20 ng/mL 12-O-tetradecanoylphorbol 13-acetate (PMA) and 10 µg/mL phytohemaglutinin (PHA) for IL-2 release and incubated either in the presence of test compounds **1–6** (at 25 µM) or the solvent DMSO for 24 h. The cultured supernatants were obtained and the concentration of the released IL-2 was directly determined by a commercially available ELISA kit (Diaclone, USA), according to the instructions of the manufacturer and quantified as ng/mL using a freshly prepared standard curve. The results were evaluated as percentage stimulation and interpreted as previously described (Yeşilada et al.

1997, 2001). Stimulation between 70 and 100% was considered high, between 40 and 69% moderate, and between 20 and 39% low. Isoprinosine was used as a positive control.

## 4. Conclusion

Phytochemical investigation of the roots of *C. bonus-henricus* led to the isolation and structural elucidation of two new glycosides of phytolaccagenin and 2*β*-hydroxyoleanoic acid, namely bonushenricoside A and bonushenricoside B together with four known saponins. The MeOH extract and compounds were tested for cytotoxicity on five leukemic cell lines (HL-60, SKW-3, Jurkat E6-1, BV-173 and K-562) and their $IC_{50}$ values showed moderate or marginal activity. In addition, the methanolic extract and saponins showed moderate stimulatory effects on interleukin-2 production in PHA/PMA stimulated Jurkat E6-1 cells.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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