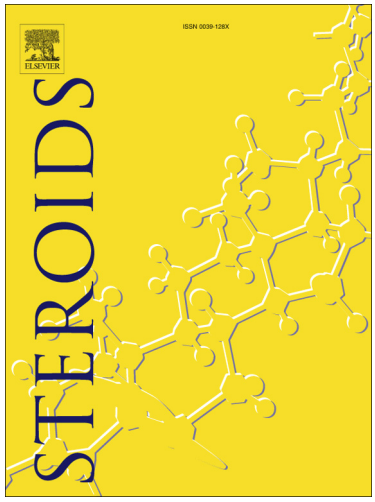

Furostanol saponins from Chinese onion induce G2/M cell-cycle arrest and apoptosis through mitochondria-mediate pathway in HepG2 cells

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Furostanol saponins from Chinese onion induce G2/M cell-cycle arrest and apoptosis through mitochondria-mediate pathway in HepG2 cells

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ABSTRACT

Phytochemical investigations on the bulbs of Chinese onion led to the isolation of three new furostanol saponins (**1**, **2**, **5**) together with seven known furostanol saponins (**3**, **4**, **6**-**10**). Their chemical structures were elucidated on the basis of spectroscopic and chemical methods, including IR, MS, NMR, and GC analyses. The anti-proliferative and anti-inflammatory activities of the isolates were evaluated. Compounds **7**–**10** showed potential anti-proliferative activities against human cancer cell lines (HepG2, A549, SPC-A-1, MGC80-3, MDA-MB-231, SW620 and CNE-1) with IC_{50} values below 30 μM. Compounds **4** and **7** could induce G2/M cell-cycle arrest and apoptosis through mitochondria-mediate pathway in HepG2 cells. Compounds **7** and **10** showed strong inhibitory effects against LPS induced NO production in RAW264.7 cells with IC_{50} values of 2.01 ± 1.40 μM and 2.49 ± 1.54 μM, respectively.

Keywords: furostanol saponins; Chinese onion; *Allium chinense*; anti-proliferative activity; anti-inflammatory activity

1. Introduction

The genus *Allium*, belongs to family Amaryllidaceae (subfamily Allioideae), comprises more than six hundred different species found throughout North America, Europe, Asia and North Africa [1]. Members of *A.* family have been cultivated in the Middle and Far East for at least five thousand years. Many *Allium* species possess characteristic pungent flavor and are prized as foodstuffs or vegetables in widespread areas of the world [2, 3].

Chinese onion (*A. chinense* G. Don), also known as oriental onion, is an ancient vegetable native to China. It is widely cultivated in Asian countries such as China (common known as "Jiaotou"), Japan (known as "Rakkyo"), Korea, Vietnam, and Indonesia, for its edible bulbs and leaves, which may be eaten raw or cooked. The flowers are also edible and may be used as a garnish on salads [4]. In China, the bulbs of Chinese onion are constantly served as sweet or sour pickles after steeped in sugar or/and brine. It also can be pickled as a pleasing seasoning or cooked with other ingredients to afford various featured Chinese cuisine [5]. In Japan, they are consumed mainly in pickles as side dishes. Chinese onion is also used as a pickled meal during Vietnamese New Year–Tet celebrations. Additionally, Chinese onion is also introduced into some similar latitude of Northern America [6].

In China, Chinese onion is also well known as a plant for both food and medicine suggested by the government. In traditional Chinese medicine, the bulbs of this plant served as one of the botanical origins of the crude drug "Xiebai", which is included in some classic prescription for treating chest pain, stenocardia, as well as heart

asthma [7]. Pharmacological studies have shown that preparations from "Xiebai" show a number of biological activities, including antiatherogenic, hypolipidemic, anti-platelet aggregation, antihypertension, antioxidant, and analgesic effects [5].

In the past decades, Chinese onion has attracted increasing attention in the field of food, nutraceuticals and phytomedicine because of its wide health benefits. It has been reported to possess a wide range of biological activities such as anti-hyperlipidemic activity [8], anti-tumor activity [9, 10], and anti-Alzheimer's disease [11, 12]. Phytochemical investigates on Chinese onion have led to the isolation of sulfur-containing compounds which responsible for its onion-like flavor [13], nitrogen-containing constituents [14], and steroidal saponins [15-19]. As an important class of natural products discovered in many advanced plants, steroidal saponins are found to possess a variety of biological activities, such as antiproliferation, hemolytic, anti-platelet aggregation, anti-inflammatory, and anti-bacterial activities [20].

In our previously study, spirostanol saponins isolated from Chinese onion exert pronounced anti-inflammatory and anti-proliferative activities [19]. In our continuing efforts to screen the bioactive constituent of Chinese onion, further phytochemical study was carried out. As a result, ten furostanol saponins including three new were isolated and identified from the bulbs of Chinese onion. Some saponins exhibit potential anti-proliferative, and anti-inflammatory activities and could induce G2/M cell-cycle arrest and apoptosis through the mitochondria-mediate pathway in HepG2 cells.

2. Experimental

2.1 General Experimental Procedures.

Optical rotations were determined with a JASCO P-1020 polarimeter (Tokyo, Japan). IR spectra (4000–450 cm^{-1}) of the saponins (as pellets in KBr) were recorded using a 100 FT-IR spectrometer (Perkin Elmer Inc., Waltham, MA). All NMR spectra were acquired on a Bruker Avance III 500 MHz digital NMR spectrometer (Bruker BioSpin GmbH, Switzerland). HR-ESI-MS data were acquired on a UPLC-Q-TOF Micro mass spectrometer equipped with an ESI source (Waters Corp., Milford, MA). Preparative HPLC was carried out on a Waters HPLC system (Waters Corp., Milford, MA), including a 2535 pump and a 2489 UV/Visible detector. The separation was performed on a 5C18-MS-II (10ID×250 mm) semi-preparative HPLC column or 5C18-AR-II (20ID×250 mm) preparative HPLC column (Cosmosil, Nacalai Tesque, Kyoto, Japan). Microplate reader (KHB-ST-360) was product of Kehua Technologies, Inc. (Shanghai, China).

2.2 Chemicals and Reagents.

Macroporous resin (D101) was purchased from Xi′an Lanxiao Resin Corporation Ltd. (Xi′an, China). Silica gel (200-300 mesh, Anhui Liangchen Silicon Material Co. Ltd., Lu′an, China) and ODS (40-60 μm, Merck KGaA, Darmstadt, Germany) were used for column chromatography. HPLC-grade methanol was the product of Oceanpak Alexative Chemical Co., Ltd. (Gothenburg, Sweden). The standard sugar reagents for GC analysis (D-glucose, D-galactose, L-arabinose, and D-xylose),

lipopolysaccharides (LPS) and 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Corp. (St. Louis, MO). Fetal bovine serum (FBS) and cell culture medium were provided by Gibco Life Technologies (Grand Island, NY). Antibodies for western blot assay (PARP, c-Caspase-3, c-Caspase-9, Bcl-2, Bax and *β*-actin) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.3 Plant Material.

Chinese Onion (122.9 Kg) were collected from Shu′an County of Jiangxia District (Wuhan, China) and were identified as *A. chinense* G. Don by Prof. X.J. He of Guangdong Pharmaceutical University. A voucher specimen (No. GDPU-NPR-2012-ACG) was deposited in the School of Pharmacy, Guangdong Pharmaceutical University (Guangzhou, China).

2.4 Extraction and Isolation.

Air dried bulbs of Chinese onion were pulverized and then extracted with 3 volumes of 60% (v/v) methanol at reflux (2 h×3). The combined filtrate was concentrated under vacuum at 55 ºC to remove the methanol and then suspended in distilled water and applied to a macroporous resin column (D101, 100×1200 mm), which was eluted with methanol/water (0:100, 30:70, 100:0, v/v) successively. The methanol/water (100:0, v/v) fraction (152.1 g) was then subjected to a silica gel chromatography (200-300 mesh, 100 × 1100 mm) and eluted with a $CHCl_3$/MeOH

gradient elution (100:0 to 0:100, v/v) to give 14 fractions (Frs. 1-14). Fr. 10 (4.19 g) was purified on an ODS column (30 × 300 mm) eluted gradient with MeOH/H_2O (20:80 to 100:0, v/v), to give seven subfractions (Subfrs. 10.1–10.7). Subfr. 10.5 was subjected to a preparative HPLC column (20 × 250 mm) which eluted with 65% (v/v) methanol in water at the flow rate of 8.0 mL/min. Compounds **1** (12.0 mg) and **5** (32.0 mg) were obtained at the retention time of 17.3 min and 35.9 min respectively. Fr.12 (9.18 g) was separated into ten subfrs. (Subfrs. 12.1–12.10) with an ODS column (30 × 300 mm) eluted gradient with MeOH/H_2O (from 10:90 to 90:10, v/v). Subfrs. 12.7 (3.63 g) was chromatographed on a preparative HPLC column (20 × 250 mm) eluted with 60% (v/v) methanol in water (flow rate: 8.0 mL/min), to afford compounds **2** (160 mg) and **3** (130 mg). Fr. 13 (8.50 g) was chromatographed on an ODS column (30×300 mm) eluted gradient with MeOH/H_2O (10:90 to 90:10, v/v) and afford ten subfrs. (Subfr. 13.1–13.10). The Subfr. 13.6 (1.56 g) was finally purified by a semi-preparative HPLC column (10×250 mm) eluted with 55% (v/v) methanol in water at the flow rate of 4.0 mL/min to afford compounds **7** (67.7 mg) and **8** (37.4 mg) at the retention times of 41.6 and 48.4 min respectively. Fr.14 (98.66 g) was separated into nine subfrs. (Subfrs. 14.1–14.9) with a flash ODS column (60.8×190 mm) eluted gradient with MeOH/H_2O (from 10:90 to 90:10, v/v). Compound **4** (10.85g) was obtained from the Subfrs. 14.2. Subfr. 14.4 (1.56 g) was further separated by ODS column (30 × 300 mm) eluted gradient with MeOH/H_2O (50:50 to 90:10, v/v) and yield seven subfrs. (Subfr. 14.4.1–14.4.7). Subfr. 14.4.5 (744 mg) was finally purified by a preparative HPLC column (20 × 250 mm) eluted

with 50% (v/v) methanol in water (Flow rate: 8.0 mL/min), to afford compound **10** (270.6 mg) at the retention time of 64.2 min. Subfr. 14.4.6 (620 mg) was also purified by preparative HPLC column (20×250 mm) eluted with 55% methanol (v/v) in water (Flow rate: 8.0 mL/min), compound **6** (15.7 mg) was obtained at the retention time of 35.5 min. Subfr. 14.5 (1.00 g) was subjected to a semi-preparative HPLC column (10×250 mm) and eluted with 55% (v/v) methanol in water (Flow rate: 4.0 mL/min), compound **9** (101.0 mg) was obtained at the retention time of 17.7 min.

26-O-β-D-glucopyranosyl 3β,22α,26-trihydroxy-25(R)-5α-furostan-6-one (**1**)**:** White amorphous powder; $[\alpha]_D^{14}$ –41.9 (*c* 0.23, MeOH); IR ν_{max} (KBr) cm^{-1}: 3426 (OH), 2937(CH), 1706 (C=O); ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data for the aglycone moiety see Table 1; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data of sugar moieties, see Table 2; HR-ESI-Q-TOF-MS (positive) m/z 633.3634 $[M + Na]^+$, calcd. for $C_{33}H_{54}O_{10}Na$. 633.3615.

26-O-β-D-glucopyranosyl 3β,22α,26-trihydroxy-25(R)-5α-furostan-6-one 3-O-β-D-glucopyranoside (**2**): White amorphous powder; $[\alpha]_D^{14}$ –38.9 (*c* 0.35, MeOH); IR ν_{max} (KBr) cm^{-1}: 3343 (OH), 2941 (CH), 1708 (C=O), 1076; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data for the aglycone moiety see Table 1; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data of sugar moieties, see Table 2; HR-ESI-Q-TOF-MS m/z 795.4170 $[M + Na]^+$, calcd. for $C_{39}H_{64}O_{15}Na$ 795.4143.

26-O-β-D-glucopyranosyl 3β,26-dihydroxy-25(R)-5α-furostan-20(22)-en-6-one (**5**): White amorphous powder; $[\alpha]_D^{14}$ –20.1 (*c* 0.50, MeOH); IR ν_{max} (KBr) cm^{-1}: 3279 (OH), 2948 (CH), 2911, 2868, 1708 (C=O), 1449, 1070; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data for the aglycone moiety see Table 1; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5) data of sugar moieties, see Table 2; HR-ESI-Q-TOF-MS m/z 615.3491 $[M + Na]^+$, calcd. for $C_{33}H_{52}O_9Na$ 615.3509.

2.5 Acid Hydrolysis and GC Analysis.

The hydrolysis and GC analysis of he isolated saponins were performed as a reported method [21]. Briefly, each saponin (1-2 mg) was dissolved in 2 M HCl (5 mL) and held at 90 °C for 2–8 h. The liberated aglycone was extracted with EtOAc (5 mL × 3) and then the aqueous residue was evaporated at 60 °C under reduced pressure. Then 600 μL C_5D_5N and 5 mg $NH_2OH{\cdot}HCl$ were added to the residue and the mixture was heated at 90 °C for 30 min. After cooling, Ac_2O (300 μL) was added to the mixtures and homogenized, followed by holding at 90 °C for 1 h. After cooling, the reaction mixture was analyzed using GC with standard aldononitrile peracetates as reference samples under the following conditions: Agilent 6820 (G1176A) Gas Chromatograph equipped with a FID detector (detection temperature 300 ºC), column: Agilent DB-5MS(30 m×0.250 mm×0.25 μm), column temperature: 150ºC (hold 2min) - 300℃ (hold 10 min) with the rate of 15℃/min, carrier gas: N_2, injection temperature: 250℃, injection volume:0.5-2 μL. As a result, the acetate

derivatives of D-glucose, D-galactose, L-arabinose, and D-xylose were detected at the following retention times: t_R (min) = 10.13 for L-arabinose, 10.23 for D-xylose, 12.10 for D-glucose and 12.33 for D-galactose.

2.6 Anti-proliferative Assay.

Human cancer cell lines, including HepG2, MDA-MB 231, A549, SPC-A-1, CNE-1, MGC80-3, and a human non-neoplastic cell line MRC-5 were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Cell culture and cell viability assay were carried out according to the reported protocol [22]. Briefly, cells were cultured in RPMI 1640 or DMEM supplemented with 10% FBS, 10 mmol/L Hepes, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg /mL gentamicin and were maintained at 37 °C in 5% CO_2. A total of 2.5×10^4 cells were placed in each well of the 96-well flat-bottom plate. After 12 h of incubation, the growth medium was replaced by media containing various concentrations of the test samples. Cell viability was determined at 48 h from the MTT absorbance at 490 nm. At least three replications for each sample were tested.

2.7 Western Blot Analysis.

HepG2 cells (2×10^5) were treated for 24 h with the indicated concentrations of compound **4** or **7**, then harvested and washed with ice-cold PBS. The cells were lysed in ice-cold RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride according to the manufacturer's instructions. The lysates were collected and centrifuged at 12,000 × g at 4 °C for 10 min to remove insoluble debris. Protein concentration in the

supernatants was determined with the BCA protein assay (Beyotime biotechnology). Aliquots of the supernatants (20 μg of protein per lane) were run on 12% SDS polyacrylamide gel. Proteins were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (0.45 μm). Membranes were blocked at room temperature for 1 h with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST). After four 5 min washing in TBST, membranes were incubated overnight with primary antibodies (1:2000 in TBST) at 4 °C. Subsequent to washing, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody at 1:10000 in TBST, and then finally the bands were detected using ECL and visualized using the FluorChem E detection instrument from ProteinSimple (Santa Clara, CA). The values shown were quantified, normalized to the internal control β-actin, and then the densitometry was performed using the Alpha View image acquisition and analysis software from ProteinSimple. Phosphorylated proteins were shown as the ratio of detected phospho-protein/total protein.

2.8 Bioassay for Inhibitory Effect on LPS-induced NO Production.

Macrophage RAW264.7 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured as previously described [19]. NO produced in the culture medium was measured by assaying the content of NO_2^- *via* the Griess reaction [23].

2.9 Statistical Analysis.

Data were presented as mean ± SD. Statistical analysis was performed with one-way ANOVA followed by Dunnett's test using GraphPad Prism software. The difference with p value < 0.05 was perceived to be statistically significant.

3. Results and discussion

3.1 Structural elucidation of the isolated steroidal saponins.

In this research, bulbs of Chinese onion were collected, dried and extracted with 60% methanol. The obtained extract was then successively chromatographed on D101 macroporous resin, silica gel, ODS C.C. and finally purified by RP-HPLC to obtain 3 new steroidal saponins (**1**, **2**, **5**,), as well as 7 known congeners (Figure 1).

Compound **1** was obtained as a white amorphous powder and showed a purple colouration with Ehrlich agent. This suggested **1** is a furostanol saponin. Its molecular formula was determined as $C_{33}H_{54}O_{10}$ by HR-ESI-MS with the ion of *m/z* 633.3634 $[M + Na]^+$ (calcd. 633.3615). The IR spectrum displayed absorption bands for hydroxyl (3426cm^{-1}) and carbonyl (1706 cm^{-1}) functionalities. Acid hydrolysis of **1** only afforded D-glucose. The ^{1}H NMR spectrum displayed two angular methyl signals at δ_H 0.75 and 0.84, two secondary methyl signals at δ_H 1.34 (d, J = 6.9 Hz, 3H) and 0.96 (d, J = 6.7 Hz, 3H), indicative of the methyl groups of the furostanol skeleton (Table 1). An anomeric proton at δ_H 4.82 (d, J = 7.8 Hz, 1H) and some overlapping signals from δ_H 3.58 to 4.93 consistent with the acid hydrolysis results revealed the presence of a D-glucose in the molecule. In the ^{13}C NMR spectrum, the quaternary carbon signal at δ_C 110.9 was assigned to C-22 of the furostanol skeleton. The carbonyl carbon signal at δ_C 210.5 was due to be located at the C-6 position.

Comparing the ^{13}C NMR data of **1** with those of the known compound chinenoside I, which was also isolated from Chinese onion, it displayed similarity except for variation at the A ring. These indicated the hydroxyl group at C-3 of **1** was free and the only one sugar moiety in the molecule linked to C-26 position of the aglycone. Furthermore, the cross-peaks observed in the HMBC spectrum between the H-1′ (δ_H 4.82) of the glucose and C-26 (δ_C 75.5) of the aglycone disclosed the D-glucose attached to C-26 of the aglycone (Fig. 2). The α-configuration of C-22 hydroxyl group of the aglycone was deduced from the semi-ketal carbon signal at δ_C 110.9 instead of δ_C 115.5 for β-configuration, and further evidenced by the ROESY correlation between H-20 (δ_H 2.22) and H-23 (δ_H 2.02).[24, 25] The 25-*R* configuration was inferred by the chemical shift difference (0.34 ppm) between equatorial proton signal (δ_H 3.94) and axial proton signal (δ_H 3.60) of H-26, a few smaller than 0.48 ppm.[25, 26] Therefore, the structure of **1** was identified to be 26-O-β-D-glucopyranosyl 3β,22α,26-trihydroxy-25(*R*)-5α-furostan-6-one.

Compound **2** was obtained as a white amorphous powder with the molecular formula of $C_{39}H_{64}O_{15}$ on the basis of HR-ESI-MS with the ion of m/z 795.4170 $[M + Na]^+$ (calcd. 795.4143). The ^1H and ^{13}C NMR signals of the aglycone moiety of **2** were almost identical to those of **1**, suggesting the aglycone of **2** to be laxogenin (Table 1). The inferred molecular formula has an additional 162 mass unit than that of **1**, corresponding to a hexose unit. Acid hydrolysis of **2** only gave D-glucose, but two anomeric protons at δ_H 5.05 (d, J = 7.6 Hz, 1H) and 4.82 (d, J = 7.8 Hz, 1H), and two anomeric carbons at δ_C 105.1 and 102.5 indicated the presence of two

glucosyl units. The downfield chemical shift of C-3 (δ_C 77.1) by +6.9 ppm indicated the glycosylation of C-3 position. The linkages of the sugar units were further verified in the HMBC experiment by the correlations between H′-1 (δ_H 5.05) of one glucose and C-3 (δ_C 77.1), and the H′′-1 of the other glucose with C-26 (δ_C 75.5) (Figure 2). Therefore, the structure of **2** was elucidated as 26-O-*β*-D-glucopyranosyl 3*β*,22*α*,26-trihydroxy-25(*R*)-5*α*-furostan-6-one 3-O-*β*-D-glucopyranoside.

Compound **5** was isolated as a white amorphous powder, which showed a purple colouration with Ehrlich agent. Its molecular formula was inferred as $C_{33}H_{54}O_{10}$ by HR-ESI-MS with the ion of m/z 615.3491 $[M+Na]^+$ (calcd. 615.3509). The IR spectrum displayed absorption bands for hydroxyl (3479 cm^{-1}) and carbonyl (1708 cm^{-1}) functionalities. Acid hydrolysis of **5** only afforded D-glucose. The ^1H NMR spectrum of **5** displayed characteristic signals for four methyls at δ_H 1.63 (s, 3H), 1.01 (d, J = 6.6 Hz, 3H), 0.74 1 (s, 3H), and 0.65 (s, 3H). An anomeric proton signal was observed at δ_H 4.84 (d, J = 7.7 Hz, 1H) suggesting the presence of a D-glucose and the *β* anomeric orientation of the glucose was inferred. In the ^{13}C NMR spectrum, four steroidal methyl groups were observed at δ_C 17.6 (Me-27), 14.5 (Me-18), 13.4 (Me-19), and 12.0 (Me-21) respectively, while the anomeric carbon of D-glucose was determined at δ_C 105.1, which giving correlations with the signals of δ_H 4.84 in the HSQC spectrum. Two olefinic carbons at δ_C 152.7and 103.7 indicated a $\Delta^{20(22)}$ unsaturated aglycone for **5**, which were consistent with the literature value for a $\Delta^{20(22)}$ furostanol saponin [16]. These were further supported by the HMBC correlations between H-21 (δ_H 1.63) and C-20 (δ_C 103.7), C-22 (δ_C 152.7), C-17 (δ_C

64.5) (Figure 2). The ^{1}H and ^{13}C NMR spectrum of **5** (Table 1, Table 2) were similar to those of chinenoside III, except for the absence of the signals for the arabinopyranosyl-(1→6)-glucopyranosyl residue linked to C-3 position of the aglycon and the significant upfield chemical shift by 6.6 ppm of the signal due to C-3 of the aglycone. These suggested the hydroxyl group at C-3 of **5** was free and the sugar moiety in the molecule linked to C-26 position of the aglycone. The difference observed in ^{1}H NMR chemical shifts of the germinal protons H-26a and H-26b (δ_{Ha} - δ_{Hb} = 0.33) supported *R* configuration of C-25 since this difference is usually larger than 0.57 ppm in 25*S* furostane-type steroidal saponins and smaller than 0.48 ppm in 25*R* compounds. Thus, the structure of **5** was determined as 26-O-*β*-D-glucopyranosyl 3*β*,26-dihydroxy-25(*R*)-5*α*-furostan-20(22)-en-6-one.

The known compounds were identified as 26-O-*β*-D-glucopyranosyl 3*β*,22,26-tridyroxy 25(*R*)-5*α*-furostan-6-one 3-O-*α*-L-arabinopyranosyl(1→6)-*β*-D-glucopyranoside (**3**) [18], 26-O-*β*-D-glucopyranosyl 3*β*,22,26-tridyroxy-25(*R*)-5*α*-furostan-6-one 3-O-*β*-D-xylopyranosyl(1→4)-[*α*-L-arabinopyranosyl (1→6)]-*β*-D-glucopyranoside (**4**) [18], 26-O-*β*-D-glucopyranosyl 3*β*,26-didyroxy-25(*R*)-5*α*-furostan-20(22)-en-6-one 3-O-*β*-D-xylopyranosyl(1→4)-[*α*-L-arabinopyranosyl (1→6)]-*β*-D-glucopyranoside (**6**) [16], (25R)-26-O-*β*-D-glucopyranosyl-5*α*-furost-3-*β*,26-didyroxy- 3-O-{O-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside} (**7**) [27], tomatoside A (**8**) [27], macorstemonoside C (**9**) [28], (25R)-26-O-*β*-D-glucopyranosyl-22-hydroxy-furostane-2*α*,3*β*,26-triol-3-O-*β*-D-glucopyranosyl (1→2)

-[β-D-glucopyranosyl (1→3)]-β-D-glucopyranosyl (1→4)-β-D- galactopyranoside (**10**), by comparing the NMR data with those reported in the literature.

3.2 Anti-proliferative activity.

All the isolated compounds were evaluated for their anti-proliferation against human hepatocellular carcinoma (HepG2), human lung cancer (A549 and SPC-A-1), human gastric adenocarcinoma (MGC80-3), human breast cancer (MDA-MB-231), human colon cancer (SW620), and human nasopharyngeal carcinoma (CNE-1) cells and human non-neoplastic cells (MRC-5). The anti-proliferative results were summarized in Table 3. Among the tested compounds, compounds **7**–**10** showed potent anti-proliferative activities against all the seven tumor cell lines, with IC_{50} values below 30 μM, except compound **7** was inactive against SW620 cells and compound **9** was inactive to MGC80-3 and MDA-MB-231 cells. Compound **7** exhibited a significant selective inhibition against cancer cells except for SW620 cells with IC_{50} values ranging from 12.14 to 26.63 μM, in comparison with the normal cells MRC-5 (IC_{50} > 100 μM). Compounds **1**–**6** bearing 6-one in the A-ring were inactive (IC_{50} > 100 μM) except compound **4** displayed mild inhibition against HepG2 and CNE-1 cells with IC_{50} values of 64.01 ± 4.54 μM and 40.99 ± 2.82 μM respectively. These results revealed that functionalization of the aglycone significantly influenced the cytotoxicity of furostanol saponins. This is similar to the results observed in the spirostanol saponins as previously reported [29]. On the other hand, the variations in the sugar moiety of furostanol saponins also could

considerably affect their cytotoxicity. This could be illustrated by, when an additional D-glucose was incorporated to the C_3-OH of the inner glucose in compound **8**, the inhibition significantly increased compared with compound **7**, especially when tested to CNE-1, MDA-MB-231, and SW620 cells. Although further SAR study focused on functionalization as well as the variation of the sugar moiety should be carried out, the current results allowed us to conclude that cytotoxicity of furostanol saponins was influenced both by the aglycone and the sugar moiety.

3.3 Effects on apoptotic and cell cycle arrest in HepG2 cells.

Apoptosis, known as a physiological cell suicide process, is a main type of cell death way that is considered an ideal way. In this study, we investigated if compound **4** and **7** could induce apoptosis on HepG2 cells. As shown in Figure 3, compound **4** (75 and 150 μM) and compound **7** (20 and 40 μM) were selected to determine whether the cytotoxicity of the cancer cells was a result of apoptosis via an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) binding assay. Flow cytometric analyses showed enhanced apoptosis of HepG2 cells treated with high concentration of compound **7**, based on the formation of a significant accumulation of early apoptosis and late apoptosis in HepG2 cells.

There is evidence that apoptosis is related to cell cycle arrest. Compounds that can induce cell cycle arrest and apoptotic cell death are generally considered to be potent anticancer drugs. In order to investigate the effect of compounds **4** and **7** on the cell

cycle phase distribution in HepG2 cells, propidium iodide (PI)/RNase Staining Buffer detection kit was used in the flow cytometric analysis (Figure 4). The results showed that treatment with compound **4** (75 or 150 μM) or compound **7** (40 μM) for 48 h increased subG$_1$ phase (a sign of fragmentation related to apoptosis) in HepG2 cells. Compound **7** promoted cell-cycle arrest in G2/M phase in HepG2 cells. There was no obvious G1, S or G2 arrest after treatment by compound **7**. These results demonstrated that compounds **4** and **7** may be responsible for inducing apoptosis of HepG2 cells.

3.4 Effects on apoptosis signaling.

To explore the mechanism by which induces apoptosis of HepG2 cell line we determined the correlative proteins of mitochondria-mediate intrinsic apoptosis including PARP, caspase-9, caspase-3, Bcl-2, and Bax. As shown in Figure 5, the activation of caspase-9 (the initiator caspases) in turn an increase in the levels of cleaved-caspase-3 (the effector caspase) and its substrates (PARP) was observed after treatment with compound **4** (10, 50, 100 μM) or compound **7** (10, 20, 50 μM) for 24 h, respectively. Treatments with compound **7** or compound **4** resulted in down-regulation of the level of Bcl-2 and up-regulation of the level of Bax. Therefore, the ratio of Bcl-2/Bax was significantly decreased in a concentration-dependent manner compared with control cells. Thus, both compounds **4** and **7** activate the caspase cascade, and increase the ratio of expression levels of Bax to Bcl-2 finally triggering the execution of apoptosis.

3.5 Anti-inflammatory activity.

Inflammation is a normal biological response to injury and infection, and involves the recruitment of the immune system to neutralize invading pathogens, repair injured tissues, and promote wound healing. Nitric oxide (NO) is a well-known endogenous cellular signaling molecule and served as an important mediator in many physiological processes. However, pharmacological studies have demonstrated that inflammation is related to overproduction of NO. During chronic or excessive activation of the immune system, NO and pro-inflammatory cytokines such as interleukin 6 and interleukin 1β are released, which are implicated as a promoter of the severity of a series of diseases such as cancer, diabetes, and stroke.

In this study, the isolated saponins were also evaluated for their inhibitory effects on LPS induced NO production in murine macrophage RAW264.7 cells. Cell viability was determined by the MTT method to define whether inhibitory activities were due to their cytotoxicity and no obvious cytotoxicity against RAW 264.7 cells was observed at the tested concentrations. As shown in Table 4, compounds **7** and **10** showed strong inhibitory effects against NO production with IC_{50} values of 2.01 ± 1.40 μM and 2.49 ± 1.54 μM respectively, followed by compound **6** (IC_{50} = 25.74 ± 0.79 μM). Compounds **1**, **3** and **4** displayed moderate inhibition with IC_{50} values ranging from 70.77 to 82.10 μM. It is interesting to note that, compared with compound **7**, an additional β-D-glucosyl at C-3 of the glucose in compound **8** induced significant reduction of the NO inhibitory activity. When another D-glucosyl

was introduced to the C-4 position of the inner glucose of compound **9** with the presence of a methoxy at the C-22 position, it is inactive. However, when a D-glucosyl was incorporated to the C-4 position of the inner glucose of compound **10** with the presence of a hydroxyl group at the C-2 position, it displayed potential inhibitory activity with an IC_{50} value of 2.49 ± 1.54 μM. These results indicated that the NO inhibitory activity of furstanol saponins influenced by the sugar chain as well as the aglycone.

In summary, Chinese onion is an ancient medicinal vegetable and widely cultivated and consumed in Asian countries. It has been reported to possess serious health benefits such as anti-hyperlipidemic activity, anti-tumor activity, and anti-Alzheimer's disease. In this study, ten furostanol saponins, including three new compounds, were isolated and identified. Some saponins exhibit potential anti-proliferative and anti-inflammatory activities. Further study suggested that some saponins could induce G2/M cell-cycle arrest and apoptosis through mitochondria-mediate pathway in HepG2 cells. Results of this study provided a basis for further development and utilization of this ancient vegetable as functional foods or natural source of chemopreventive agents for cancer.

Conflicts of interest

The authors declare that there was no competing interest.

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Supplementary data

The 1D and 2D NMR, HR-ESI-MS and IR spectra of the new compounds **1**, **2**, and **5**. This material can be found as supplementary material.

References

[1] The Angiosperm Phylogeny Group, An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III, Bot. J. Linn. Soc. 161(2) (2009) 105-121.

[2] G.R. Fenwick, A.B. Hanley, J.R. Whitaker, The genus allium-part 1, C R C Critical Reviews in Food Science and Nutrition 22(3) (1985) 199-271.

[3] E. Block, The organosulfur chemistry of the genus Allium–implications for the organic chemistry of sulfur, Angew. Chem. Int. Ed. 31(9) (1992) 1135-1178.

[4] H.D. Rabinowitch, L. Currah, Allium Crop Science: Recent Advances, CABI Pub.2002.

[5] Q. He, S. Huang, Y. Wu, W. Zhang, F. Wang, J. Cao, Q. Sheng, Z. Liang, L. Liu, W. Ou, Comparative study on the composition of free amino acids and derivatives in

the two botanical origins of an edible Chinese herb "Xiebai", i.e., *Allium chinense* G. Don and *Allium macrostemon* Bunge species, Food Res. Int. 106 (2018) 446-457.

[6] F.Z. Wang, J. Tang, Flora of China, Science Publishing House, Beijing, 1980.

[7] Chinese Pharmacopeia Commission, Pharmacopoeia of the People's Republic of China 2010, Chinese medical science and technology press, Beijing, 2015.

[8] Y.P. Lin, L. Lin, H.Y. Yeh, C.H. Chuang, S.W. Tseng, Y.H. Yen, Antihyperlipidemic activity of *Allium chinense* bulbs, J. Food Drug Anal. 24(3) (2016) 516-526.

[9] M. Baba, M. Ohmura, N. Kishi, Y. Okada, S. Shibata, J. Peng, S.S. Yao, H. Nishino, T. Okuyama, Saponins isolated from *Allium chinense* G. Don and antitumor-promoting activities of isoliquiritigenin and laxogenin from the same drug, Biol. Pharm. Bull. 23(5) (2000) 660-662.

[10] J. Peng, T. Narui, H. Suzuki, R. Ishii, H. Abuki, T. Okuyama, Anti-blood coagulation and cytotoxic effects of compounds from Chinese plants used for thrombosis-like diseases, Nat. Med. 50(5) (1996) 358-362.

[11] A. Kumar, V. Sharma, V.P. Singh, M. Kaundal, M.K. Gupta, J. Bariwal, R. Deshmukh, Herbs to curb cyclic nucleotide phosphodiesterase and their potential role in Alzheimer's disease, Mech. Ageing Dev. 149 (2015) 75-87.

[12] M. Kuroda, Y. Mimaki, A. Kameyama, Y. Sashida, T. Nikaido, Steroidal saponins from *Allium chinense* and their inhibitory activities on cyclic AMP phosphodiesterase and Na^+K^+ ATPase, Phytochemistry 40(4) (1995) 1071-1076.

[13] J. Pino, V. Fuentes, M. Correa, Volatile Constituents of Chinese Chive (*Allium tuberosum* Rottl. ex Sprengel) and Rakkyo (*Allium chinense* G. Don), J. Agric. Food Chem. 49(3) (2001) 1328-1330.

[14] T. Okuyama, K. Fujita, S. Shibata, M. Hoson, T. Kawada, M. Masaki, N. Yamate, Effect of Oriental plant drugs on platelet aggregation. V. Effects of Chinese drugs "Xiebai" and "Dasuan" on human platelet aggregation (*Allium bakeri, A. sativum*), Planta Med. 55(3) (1989) 242-244.

[15] Y. Jiang, N.L. Wang, X.S. Yao, S. Kitanaka, Steroidal saponins from the bulbs of *Allium chinense*, Studies in Plant Science, Elsevier1999, pp. 212-219.

[16] J.P. Peng, X.S. Yao, Y. Tezuka, T. Kikuchi, Furostanol glycosides from bulbs of *Allium chinense*, Phytochemistry 41(1) (1996) 283-285.

[17] J.P. Peng, X.S. Yao, Y. Tezuka, T. Kikuchi, T. Narui, New furostanol glycosides, chinenoside IV and V, from *Allium chinense*, Planta Med. 62(5) (1996) 465-468.

[18] H. Matsuura, T. Ushiroguchi, Y. Itakura, T. Fuwa, A furostanol glycoside from *Allium chinense* G. Don, Chem. Pharm. Bull. 37(5) (1989) 1390-1391.

[19] Y. Wang, C. Li, L. Xiang, W. Huang, X. He, Spirostanol saponins from Chinese onion (*Allium chinense*) exert pronounced anti-inflammatory and anti-proliferative activities, J. Funct. Foods 25 (2016) 208-219.

[20] S.G. Sparg, M.E. Light, J. van Staden, Biological activities and distribution of plant saponins, J. Ethnopharmacol. 94(2–3) (2004) 219-243.

[21] L. Xiang, Y. Wang, X. Yi, J. Feng, X. He, Furospirostanol and spirostanol saponins from the rhizome of *Tupistra chinensis* and their cytotoxic and anti-inflammatory activities, Tetrahedron 72(1) (2016) 134-141.

[22] C. Wang, Z. Zhang, Y. Wang, X. He, Cytotoxic Indole Alkaloids against Human Leukemia Cell Lines from the Toxic Plant *Peganum harmala*, Toxins 7(11) (2015) 4507-4518.

[23] K.M. Miranda, M.G. Espey, D.A. Wink, A rapid, simple spectrophotometric method for simultaneous detection of Nitrate and Nitrite, Nitric Oxide 5(1) (2001) 62-71.

[24] Y. Zhao, L.P. Kang, Y.X. Liu, Y.G. Liang, D.W. Tan, Z.Y. Yu, Y.W. Cong, B.P. Ma, Steroidal saponins from the rhizome of *Paris polyphylla* and their cytotoxic activities, Planta Med. 75(4) (2009) 356-363.

[25] K. Zou, J. Wang, M. Du, Q. Li, G. Tu, A pair of diastereoisomeric steroidal saponins from cytotoxic extracts of *Tupistra chinensis* rhizomes, Chem. Pharm. Bull. 54(10) (2006) 1440-1442.

[26] P.K. Agrawal, Assigning stereo-diversity of the 27-Me group of furostane-type steroidal saponins *via* NMR chemical shifts, Steroids 70(10) (2005) 715-724.

[27] T. Yamanaka, J.P. Vincken, P. de Waard, M. Sanders, N. Takada, H. Gruppen, Isolation, characterization, and surfactant properties of the major triterpenoid glycosides from unripe tomato fruits, J. Agric. Food Chem. 56(23) (2008) 11432-11440.

[28] H. Chen, N. Wang, X. Yao, Study on bioactive steroidal saponins of *Allium Macrostemon Bunge*, Chinese Journal of Medicinal Chemistry 15(3) (2005) 142-147.

[29] K. PerezLabrada, I. Brouard, S. Estevez, M. Marrero, F. Estevez, J. Bermejo, D. Rivera, New insights into the structure-cytotoxicity relationship of spirostan saponins and related glycosides, Biorg. Med. Chem. 20(8) (2012) 2690-2700.

Table 1 ^{1}H and ^{13}C NMR Data (500 and 125 MHz, in Pyr-d_5) for aglycone moieties of compounds **1**, **2** and **5**

	1		**2**		**5**	
No.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	37.4	1.63º	37.4	1.58º	37.2	1.63º
		1.15º		1.05º		1.16º
2	32.0	2.05º	29.7	2.09º	31.6	1.91º
		1.91º		1.56º		1.44 m
3	70.2	3.85 m	77.1	4.05º	70.2	3.84 m
4	31.4	3.31 m	27.3	2.37 m	31.4	2.31 m
		1.90º		1.72º		1.81º
5	57.2	2.28 m	56.7	2.09º	57.1	2.28º
6	210.6		209.9		210.5	
7	47.1	2.31 dd (12.7, 4.2)	47.1	2.35 dd (13.1, 4.3)	47.2	2.36 dd (13.0, 4.6)
		1.97º		1.95º		2.02 d (12.5)
8	37.7	1.90º	37.7	1.99º	37.4	1.83º
9	54.0	1.14º	54.0	1.10º	53.9	1.14º
10	41.2		41.1		41.2	
11	21.8	1.52 m	21.8	1.50 m	22.0	1.54 m
		1.28º		1.18º		1.27 m
12	40.0	1.75 m	40.0	1.73 m	39.6	1.73 m
		1.15º		1.11º		1.19º
13	41.7		41.7		44.2	
14	56.7	1.24º	56.7	1.22 m	55.0	1.05 m
15	32.3	1.67º	32.3	1.92º	34.3	1.98º
		1.36 m		1.33º		1.37º
16	81.1	4.93 m	81.1	4.92 q (7.2)	84.4	4.75 m
17	64.0	1.95º	64.0	1.95º	64.5	2.46 d (10.3)
18	16.9	0.84 s	16.8	0.82 s	14.5	0.65 s
19	13.5	0.75 s	13.3	0.62 s	13.4	0.74 s
20	40.8	2.22 dd (8.7, 4.5)	40.8	2.22 q (6.7)	103.7	
21	16.7	1.34 d (7.1)	16.7	1.33 d (6.9)	12.0	1.63 s
22	110.9		110.9		152.7	
23	37.2	2.02º	37.0	2.03º	23.8	2.20 m
		2.02º		2.03º		2.20 m
24	28.6	2.02º	28.6	2.05º	31.9	2.04º
		1.65º		1.67º		1.64º

25	34.5	1.90[o]	34.5	1.91[o]	33.6	1.93[o]
26	75.5	3.94 dd (9.3, 6.9)	75.5	3.94 dd (9.3, 7.1)	75.2	3.94 dd (9.2, 7.1)
		3.60 dd (9.7, 5.8)		3.60 dd (9.4, 6.0)		3.61 dd (9.6, 5.8)
27	17.7	0.96 d (6.6)	17.7	0.97 d (6.6)	17.6	1.01 d (6.6)

[o] Signals are overlapped.

Table 2 ^{1}H and ^{13}C NMR Data (500 and 125 MHz, in Pyr-d_5) for sugar moieties of compounds **1**, **2** and **5**

	1		**2**		**5**	
No.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
C-3:						
1			102.5	5.05 d (7.3)		
2			75.6	4.00[o]		
3			78.8	4.24[o]		
4			72.1	4.19[o]		
5			78.7	3.91[o]		
6			63.2	4.62 dd (11.6, 1.8)		
				4.27 m		
C-26:						
1	105.2	4.82 d (7.8)	105.1	4.82 d (7.8)	105.1	4.84 d (7.7)
2	75.4	4.04 t (8.2)	75.4	4.00[o]	75.4	4.05 t (8.1)
3	78.8	4.27[o]	78.8	4.24[o]	78.8	4.26[o]
4	71.9	4.23[o]	72.0	4.19[o]	71.9	4.24[o]
5	78.7	3.95[o]	78.7	3.91[o]	78.7	3.97 m
6	63.0	4.58 dd (11.9, 2.1)	63.1	4.55 dd (11.7, 2.2)	63.0	4.58 dd (11.9, 1.8)
		4.39 dd (11.7, 5.4)		4.30 m		4.39 dd (11.7, 5.4)

[o] Signals are overlapped.

Table 3 Anti-proliferative activity of compounds **1-10** [a, b]

	IC_{50} (μM)							
Compd.	HepG2	SPC-A-1	A549	CNE-1	MGC80-3	MDA-MB-231	SW620	MRC-5
4	64.01 ± 4.54	> 100	> 100	40.99 ± 2.82	> 100	> 100	> 100	> 100
7	12.14 ± 1.61	19.67 ± 1.25	16.91 ± 1.04	12.30 ± 1.95	26.63 ± 1.12	25.42 ± 3.49	> 100	> 100
8	5.44 ± 0.45	22.59 ± 3.50	11.38 ± 0.86	2.45 ± 0.19	26.93 ± 2.30	4.16 ± 1.25	12.27 ± 2.52	4.74 ± 0.52
9	6.91 ± 0.35	25.07 ± 0.99	13.70 ± 1.81	4.09 ± 0.48	> 100	> 100	1.76 ± 0.54	9.28 ± 1.44
10	3.77 ± 0.53	22.83 ± 1.38	12.06 ± 1.00	4.15 ± 1.09	19.72 ± 1.63	13.61 ± 1.69	10.68 ± 1.45	11.71 ± 2.21
Cisplatin [c]	3.48 ± 0.31	7.22 ± 0.71	4.07 ± 0.40	7.14 ± 0.77	10.22 ± 1.43	13.61 ± 1.69	10.68 ± 1.45	

[a] Values are the mean ± SD; n=3.

[b] Compounds **1**–**3**, **5** and **6** are inactive (IC_{50} > 100 μM).

[c] Cisplatin was used as positive control.

Table 4 The inhibitory effects of compounds **1-10** on LPS induced NO production in RAW264.7 cells [a]

Compd.	IC_{50} (μM)	Compd.	IC_{50} (μM)
1	82.10 ± 11.39	**7**	2.01 ± 1.40
2	> 100	**8**	> 100
3	53.22 ± 5.36	**9**	> 100
4	63.01 ± 6.79	**10**	2.49 ± 1.54
5	> 100		
6	25.74 ± 0.79	Indomethacin [b]	47.4 ± 4.5

[a] Values are the mean ± SD; n=3.

[b] Indomethacin was used as positive control

FIGURE CAPTIONS

Figure 1. Chemical structures of compounds **1**–**10**.

Figure 2. Selected key HMBC correlations for compounds **1**, **2** and **5**.

Figure 3. Effects of compounds **4** and **7** on apoptosis in HepG2 cells.

Figure 4. Effects of compounds **4** and **7** on cell cycle arrest in HepG2 cells.

Figure 5. Effects of compounds **4** and **7** on the expression of apoptosis-related proteins in HepG2 cells.

Figure 1.

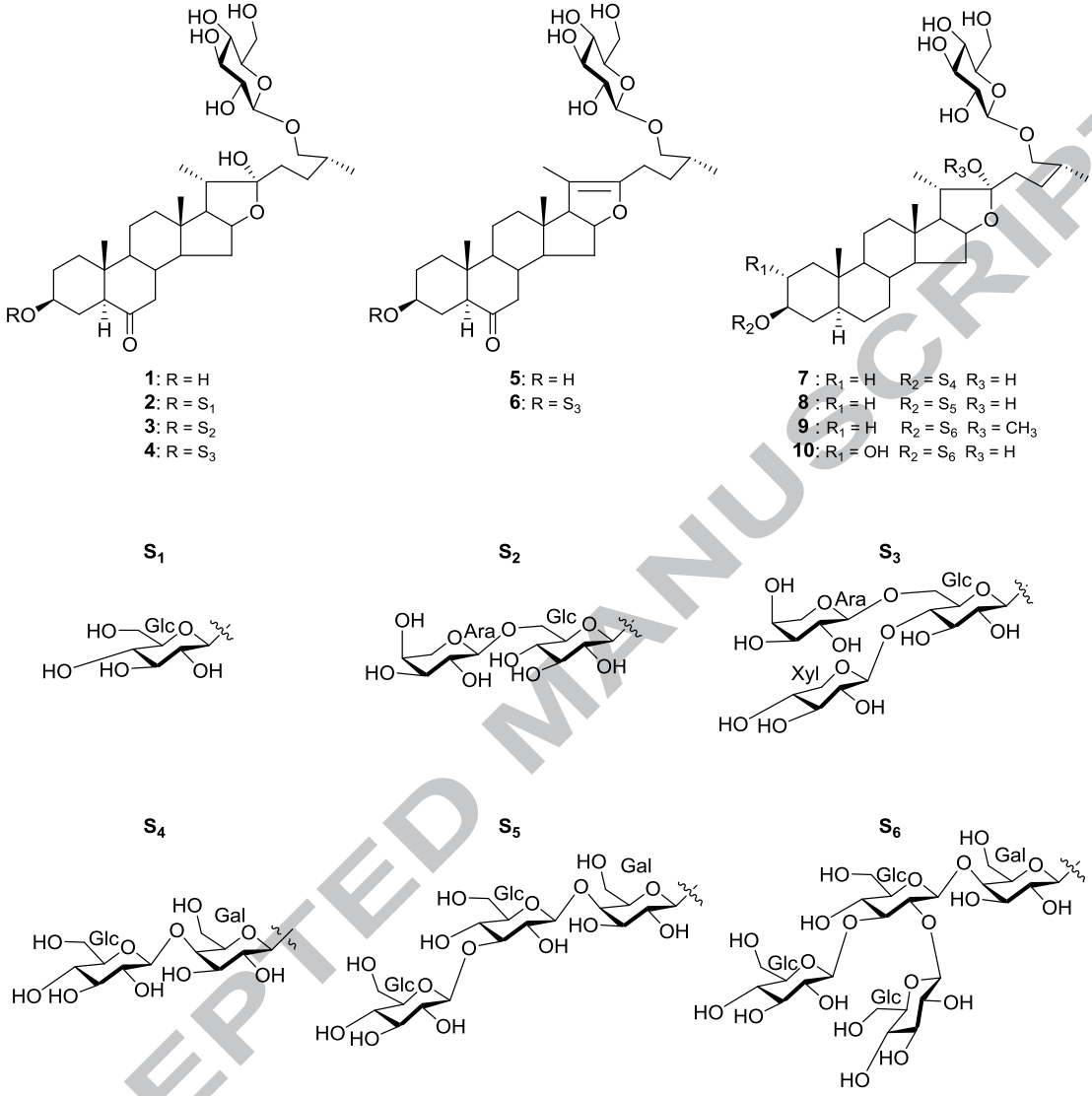


Figure 2.

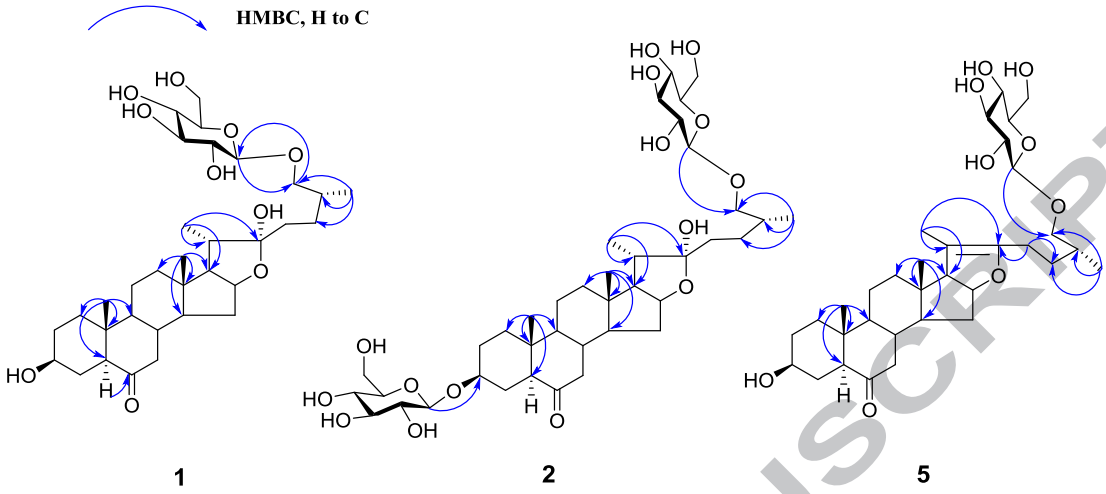


Figure 3.

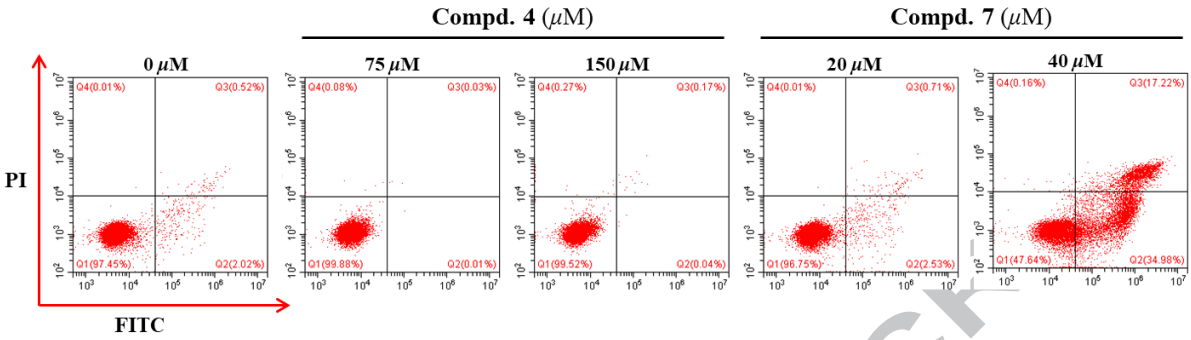


Figure 4.

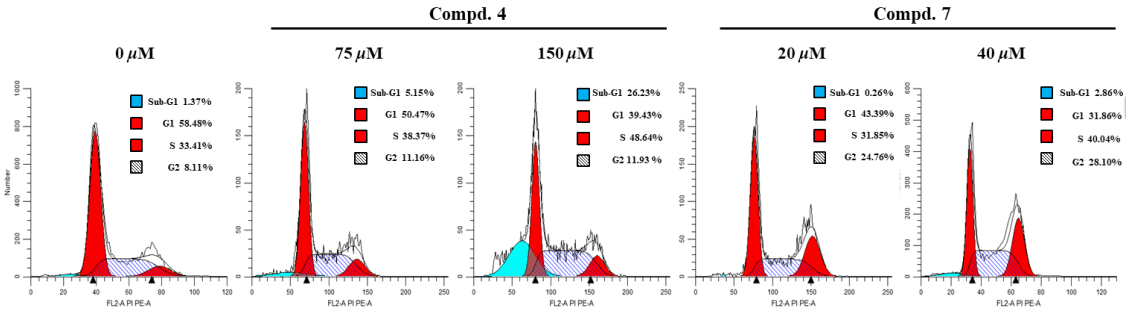
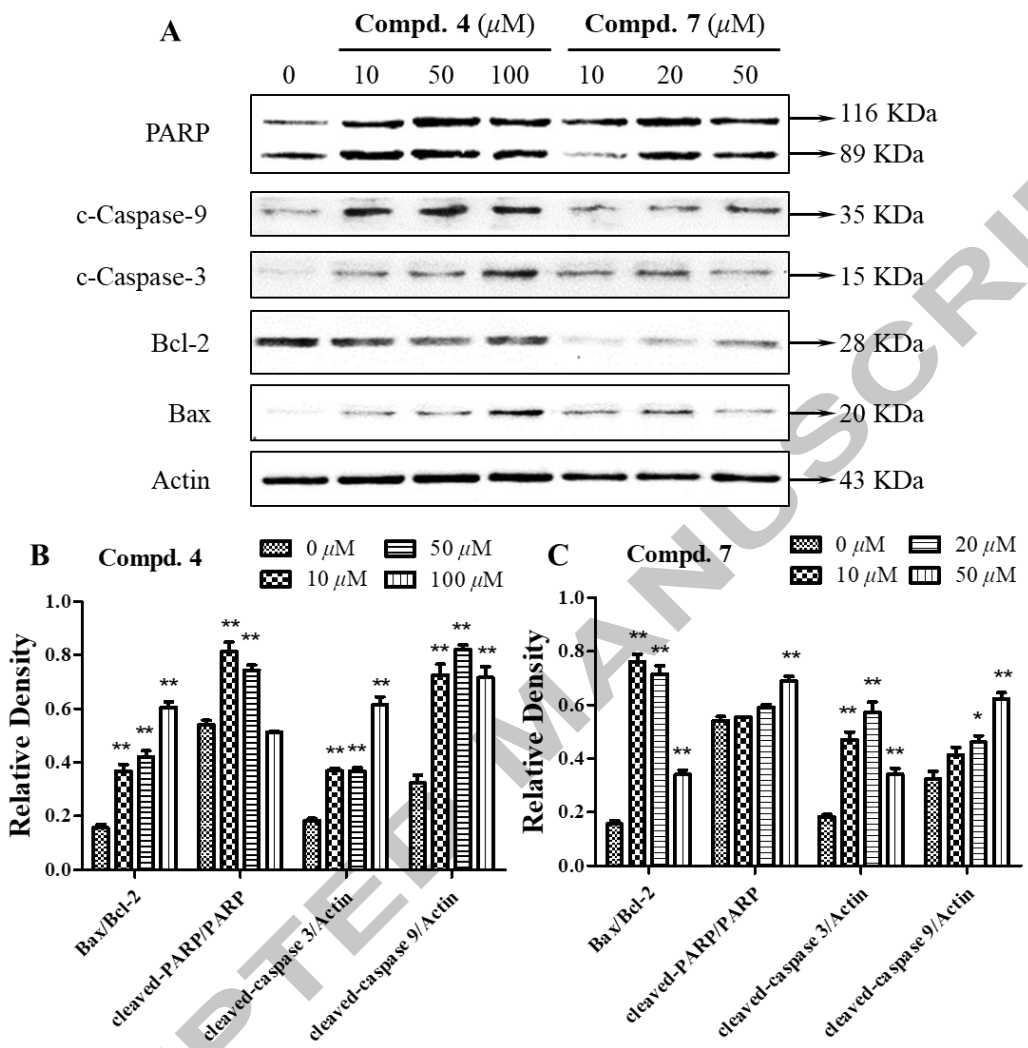
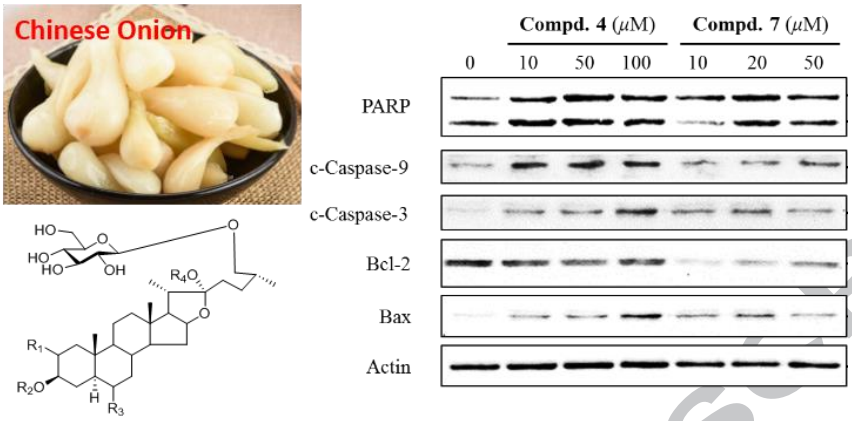


Figure 5.



Graphical abstract



Furostanol saponins from Chinese onion exert antiproliferative activity

Highlights

1. Three new furostanol saponins were isolated from the bulbs of Chinese onion.

2. Structures were elucidated through IR, 1D, 2D NMR and HR-ESI-MS.

3. Some furostanols exhibited potent antiproliferative and anti-inflammatory activities.

4. Compounds **4** and **7** could induce G2/M cell-cycle arrest and apoptosis in HepG2 cells.