

Article

## Two New Oleanane-Type Triterpenoids from *Platycodi Radix* and Anti-proliferative Activity in HSC-T6 Cells

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**Abstract:** Two new oleanane-type triterpenoids, named platycodonoids A and B (**1**, **2**), together with five known saponins, including platycodin D (**3**), deapioplatycodin D (**4**), 3-*O*- $\beta$ -D-glucopyranosyl polygalacic acid (**5**), 3-*O*- $\beta$ -D-glucopyranosyl platycodigenin (**6**) and polygalacin D (**7**), were isolated from the roots of *Platycodon grandiflorum*. On the basis of spectral data and chemical evidence, the structures of the new compounds were elucidated as 2 $\beta$ ,3 $\beta$ ,23,24-tetrahydroxy-28-nor-olean-12-en-16-one (**1**) and 2 $\beta$ ,3 $\beta$ ,23,24-tetrahydroxy-28-nor-olean-12-en-16-one-3-*O*- $\beta$ -D-glucopyranoside (**2**). Compounds **1**–**7** were evaluated for their *in vitro* anti-proliferative activity against the HSC-T6 cell line.

**Keywords:** *Platycodon grandiflorum*; oleanane-type triterpenoid; triterpenoid saponin

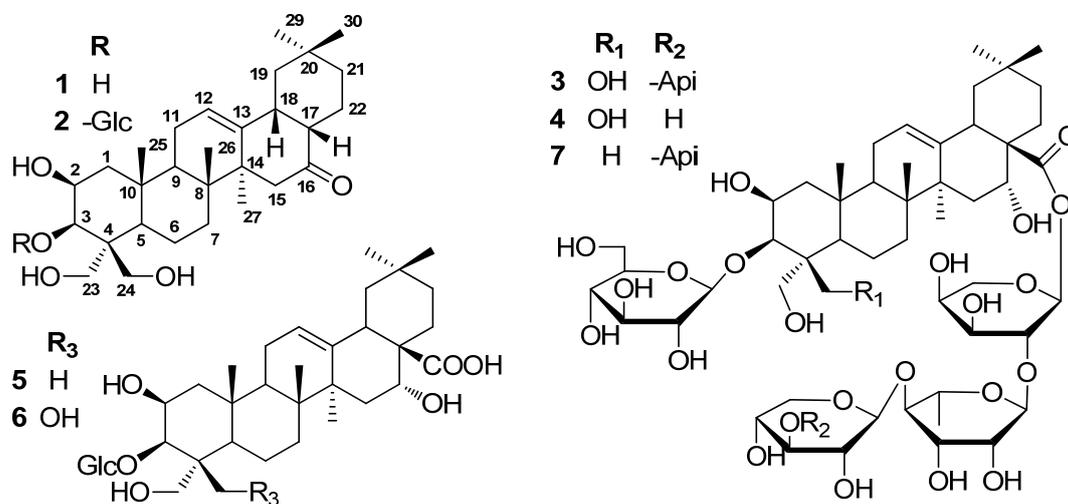
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### 1. Introduction

*Platycodon grandiflorum* A. DC. is a perennial plant in the family Campanulaceae which grows widely in East Asia. *Platycodi Radix*, the root of *P. grandiflorum*, has been used in traditional Oriental medicine as an expectorant for pulmonary disease and a remedy for respiratory disorders [1,2]. *Platycodi Radix* has recently been reported to exhibit many pharmacological activities, including anti-cancer properties [3–8], atopic dermatitis-like skin lesions treating [9–11], anti-skin photoaging effects [12],

antiobesity and glucose metabolism regulation [13–17], anti-atherosclerotic [18], and anti-hyperlipidemic [19] activities. Chemical investigation of *Platycodi Radix* revealed that triterpenoid saponins were the main chemical components, and more than 55 triterpenoid saponins have been isolated from *Platycodi Radix* to date [20,21]. Based on the structures of the aglycones, the triterpenoid saponins are classified into three types: the platycodigenin type, platycogenic acid A lactone type and polygalacic acid type [22]. It has been previously demonstrated that *Platycodi Radix* showed protective effects against acute ethanol, acetaminophen-, carbon tetrachloride-, thioacetamide and cholestasis-induced hepatotoxicity or hepatic injury in mice and inhibited the progress of hepatic fibrosis in rats [23–30]. In our preliminary pharmacological study, the 70% EtOH extract of *Platycodi Radix* was also found to exhibit significant protective activities against liver fibrosis in rats. In a continued effort to search for possible hepatoprotective component from this herb, an investigation of *Platycodi Radix* was undertaken, and this has led to the isolation of two new triterpenoids [an aglycone and its saponin, named platycodonoids A (**1**) and B (**2**) (Figure 1)], which possess a rare 28-nor-oleanane-type with a C-16 keto group in the aglycone structure. Besides, five known saponins were isolated and identified as platycodin D (**3**) [31], deapio-platycodin D (**4**) [21], 3-*O*- $\beta$ -D-glucopyranosyl polygalacic acid (**5**) [32], 3-*O*- $\beta$ -D-glucopyranosyl platycodigenin (**6**) [33] and polygalacin D (**7**) [32,34], by comparison of their IR, NMR and MS data with literature values. To the best of our knowledge, it was first time triterpenoids such a platycodonoids A and B having a 28-nor-oleanane-type skeleton are reported from the genus *Platycodon*.

**Figure 1.** Structures of compounds 1–7.



## 2. Results and Discussion

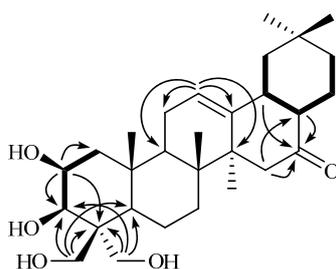
The air-dried roots of *P. Grandiflorum* were extracted three times with 70% EtOH under reflux. The combined extract was chromatographed over a macroporous adsorbing resin column and partitioned as described in the Experimental section. After repeated column chromatography, two new compounds **1** and **2** and five known saponins **3–7** were isolated and identified.

Compound **1** was obtained as white amorphous powder. Its molecular formula was assigned to be  $C_{29}H_{46}O_5$  based on the HRESIMS spectrum ( $m/z$  497.3243  $[M+Na]^+$ , calcd. for  $C_{29}H_{46}O_5Na$ , 497.3243). The IR spectrum exhibited absorptions at 3415, 2947, 1711, and 1385  $cm^{-1}$  assignable to hydroxyl, methyl, ketone and methylene functions, respectively. The  $^1H$ -NMR spectrum of **1** showed the following

signals: five tertiary methyl groups at  $\delta_{\text{H}}$  0.78, 0.84, 0.96, 1.14, and 1.63 (each 3H, s); two pairs of germinal oxygenated protons at  $\delta_{\text{H}}$  4.20, 4.21, 4.87, 5.20 (each, d,  $J = 10.8$  Hz); two oxygenated protons at  $\delta_{\text{H}}$  4.39 (d,  $J = 3.6$  Hz) and 4.58 (dt,  $J = 7.2, 3.6$  Hz); and an olefinic proton at  $\delta_{\text{H}}$  5.38 (t,  $J = 3.6$  Hz). The  $^{13}\text{C}$ -NMR spectrum revealed 29 carbon signals, which were further classified by DEPT and HSQC experiments as five methyls, ten methylenes (two oxygenated), six methines (two oxygenated), five quaternary carbons, one trisubstituted double bond ( $\delta_{\text{C}}$  123.3 and 142.9), and one keto carbonyl ( $\delta_{\text{C}}$  214.0) (Table 1). The aforementioned data implied that **1** was a nor-oleanane-type triterpenoid with four hydroxyls. In the HMBC spectrum, two pairs of germinal oxygenated protons at  $\delta_{\text{H}}$  4.20, 4.21, 4.87, 5.20 (H<sub>2</sub>-23, H<sub>2</sub>-24) showed correlations to C-3 ( $\delta_{\text{C}}$  75.2), C-4 ( $\delta_{\text{C}}$  48.1) and C-5 ( $\delta_{\text{C}}$  48.5), indicating that two hydroxyls were linked at C-23 and C-24. The two remaining hydroxyls were placed at C-2 ( $\delta_{\text{C}}$  72.0) and C-3, which were deduced by the HMBC correlations from the proton H-2 to C-1 ( $\delta_{\text{C}}$  44.7), C-3, and C-4, and from H-3 ( $\delta_{\text{H}}$  4.39) to C-1, C-2, C-3, C-4, C-23 ( $\delta_{\text{C}}$  64.1) and C-24 ( $\delta_{\text{C}}$  64.7) (Figure 2). The small coupling constant ( $J_{2,3} = 3.6$  Hz) of H-2 and H-3 indicated the axial position of H-3 and the equatorial position of H-2, which was further confirmed by a NOESY correlation between H-2 and H-3. Moreover, the chemical shifts H<sub>2</sub>-15 at  $\delta_{\text{H}}$  2.55 (d,  $J = 14.4$  Hz), 1.93 (d,  $J = 14.4$  Hz) and C-15 at  $\delta_{\text{C}}$  47.2 were quite different from that reported for oleanane-type triterpenoid [platycoside O, H-15,  $\delta_{\text{H}}$  1.81 (dd,  $J = 15.0, 3.0$  Hz), 2.55 (d,  $J = 12.0$  Hz), C-15 ( $\delta_{\text{C}}$  36.1)] [21]. These results suggested the keto group ( $\delta_{\text{C}}$  214.0) was at C-16, which was confirmed by the HMBC spectrum correlations from H<sub>2</sub>-15, H-17 ( $\delta_{\text{H}}$  1.65), H-18 ( $\delta_{\text{H}}$  2.79) and H-22 ( $\delta_{\text{H}}$  1.34, 2.14) to C-16 ( $\delta_{\text{C}}$  214.0). The  $^1\text{H}$ - $^1\text{H}$  COSY spectrum of **1** indicated the presence of partial structures (Figure 2). Consequently, by comparison with the structure of platycoside O [21], compound **1** was determined to be 2 $\beta$ ,3 $\beta$ ,23,24-tetrahydroxy-28-nor-olean-12-en-16-one, and named platycodonoid A.

Compound **2** was obtained as white amorphous powder. It gave an  $[\text{M}+\text{Na}]^+$  peak at  $m/z$  659.3728 in HRESIMS spectrum corresponding to the molecular formula of  $\text{C}_{35}\text{H}_{56}\text{O}_{10}$  (calcd. for  $\text{C}_{35}\text{H}_{56}\text{O}_{10}\text{Na}$ , 659.3771), which showed a unit of  $\text{C}_6\text{H}_{10}\text{O}_5$  more than that of compound **1**. The 1D-NMR spectra of **2** displayed similarities to those of **1**, except for an additional sugar unit. An anomeric proton signal at  $\delta_{\text{H}}$  5.15 (H-1', d,  $J = 7.8$  Hz), an oxygenated methylene (H<sub>2</sub>-6') at  $\delta_{\text{H}}$  4.35 (t,  $J = 6.0$  Hz) and 4.59 (d,  $J = 10.8$  Hz), four oxymethine protons in the range  $\delta_{\text{H}}$  3.50–4.50 in the  $^1\text{H}$ -NMR spectrum suggested that **2** contained a sugar moiety. In accordance, the  $^{13}\text{C}$ -NMR spectrum displayed six carbon signals at  $\delta_{\text{C}}$  63.0, 72.0, 75.6, 79.0, 79.0, 106.6. The subsequent acid hydrolysis of **2** gave glucose only, which was analyzed by gas chromatography as glucitol acetate [35]. The linkage of this  $\beta$ -D-glucopyranose ( $J = 7.8$  Hz) (Table 1) was confirmed by the HMBC correlation between H-1' and C-3. Therefore, the structure of platycodonoid B (**2**) was elucidated as 2 $\beta$ ,3 $\beta$ ,23,24-tetrahydroxy-28-nor-olean-12-en-16-one 3-*O*- $\beta$ -D-glucopyranoside.

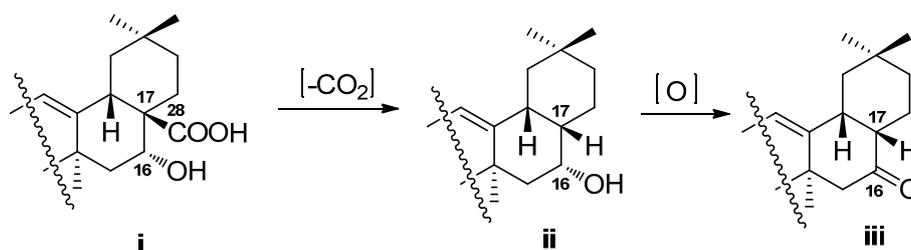
**Figure 2.** Key  $^1\text{H}$ - $^1\text{H}$  COSY (bold lines) and HMBC (H→C) correlations of compound **1**.



**Table 1.**  $^1\text{H}$ -NMR (600 MHz) and  $^{13}\text{C}$ -NMR (150 MHz) data for compounds **1** and **2** (pyridine- $d_5$ ,  $\delta_{\text{H}}$  in ppm,  $J$  in Hz).

Position	1		2	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
1	44.7	2.33 (1H, dd, 13.8, 3.0), 1.30 (1H, m)	45.0	2.08 (1H, m), 1.57 (1H, m)
2	72.0	4.58 (1H, dt, 7.2, 3.6)	70.0	4.76 (1H, m)
3	75.2	4.39 (1H, d, 3.6)	86.4	4.61 (1H, brs)
4	48.1		47.8	
5	48.5	1.82 (1H, brd, 12.0)	48.4	1.85 (1H, d, 12.0)
6	19.2	1.98 (1H, m), 1.83 (1H, brs)	19.4	1.92 (1H, m), 1.61 (1H, m)
7	33.5	1.53 (1H, m), 0.91 (1H, m)	33.4	1.47 (1H, t, 13.8), 1.18 (1H, m)
8	40.1		40.3	
9	47.3	2.49 (1H, t, 5.4)	47.3	2.53 (1H, t, 5.4)
10	37.3		37.7	
11	24.2	2.05 (1H, m), 0.84(1H, m)	24.2	2.04 (1H, m), 0.88 (1H, m)
12	123.3	5.38 (1H, t, 3.6)	123.6	5.42 (1H, brs)
13	142.9		142.9	
14	46.9		47.1	
15	47.2	2.55 (1H, d, 14.4), 1.93 (1H, d, 14.4)	47.1	2.58 (1H, d, 14.4), 1.96 (1H, d, 14.4)
16	214.0		213.8	
17	48.1	1.65 (1H, m)	48.3	1.71 (1H, m)
18	45.1	2.79 (1H, m)	45.2	2.83 (1H, m)
19	47.0	1.40 (1H, t, 13.2), 1.17 (1H, m)	47.3	1.47 (1H, t, 13.2), 1.21(1H, m)
20	31.3		31.4	
21	35.0	1.09 (2H, m)	35.0	1.09 (2H, m)
22	21.5	2.14 (1H, m), 1.34 (1H, m)	21.6	2.17 (1H, m), 1.38 (1H, m)
23	64.1	4.87 (1H, d, 10.8), 4.20 (1H, d, 10.8)	63.8	4.99 (1H, d, 10.8), 4.11 (1H, d, 11.4)
24	64.7	5.20 (1H, d, 10.8), 4.21 (1H, d, 10.8)	63.8	4.80 (1H, d, 10.8), 4.26 (1H, d, 10.8)
25	17.9	0.96 (3H, s)	18.3	0.99 (3H, s)
26	17.6	1.63 (3H, s)	18.0	1.55 (3H, s)
27	27.3	1.14 (3H, s)	27.2	1.17 (3H, s)
29	33.6	0.78 (3H, s)	33.5	0.85 (3H, s)
30	23.7	0.84 (3H, s)	23.8	0.89 (3H, s)
1'			106.6	5.15 (1H, d, 7.8)
2'			75.6	4.05 (1H, t, 8.1)
3'			79.0	4.18 (1H, m)
4'			72.0	4.18 (1H, m)
5'			79.0	3.98 (1H, m)
6'			63.0	4.59 (1H, d, 10.8), 4.35 (1H, t, 6.0)

The origin of compounds **1** and **2** (Scheme 1) was proposed to be the oleanane-type triterpenoids **i** (compound **3–7**). Decarboxylation at C-28 would produce a key intermediate **ii**, which could undergo an oxidation at C-16 to yield **iii** (**1** and **2**).

**Scheme 1.** The plausible biogenetic origin of compounds **1** and **2**.

Compounds **1–7** were evaluated for their anti-proliferative activities against the Hepatic Stellate Cell (HSC)-T6 line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [36]. Colchicine was used as positive control in this study ( $IC_{50}$  value  $< 10 \mu\text{M}$ ). Among the tested compounds, compounds **1**, **3**, **4** and **7** were the most potent, showing  $IC_{50}$  values of 5.27, 1.77, 8.24 and  $1.04 \mu\text{M}$ , respectively. The  $IC_{50}$  values for the remaining compounds, **2**, **5** and **6**, were 69.63, 8150.23, and  $13.36 \mu\text{M}$ , respectively. It was reported that saponins from *Platycodi Radix* prevented the increase in the serum levels of hepatic enzyme markers (alanine aminotransferase and aspartate aminotransferase) and reduced oxidative stress, such as glutathione content and lipid peroxidation, in the liver in a dose-dependent manner [23–30]. The reason why compounds **1**, **3**, **4** and **7** delayed the formation of liver fibrosis need to be further studied. In the structure-activity relationship of these oleanane-type triterpenoids, the presence of a free carboxyl functional group at C-28 seemed not to be related to the hepatoprotective activity (compounds **5** and **6**). When forming C-28 glycosides, the presence of the glycosides affected the activity, and the number of monosaccharides in the sugar moiety increased the activity (compounds **3**, **4** and **7**). In contrast, when came to the 28-nor-oleanane-type triterpenoids **1** and **2**, the aglycone was more active than its corresponding glycoside.

### 3. Experimental

#### 3.1. General

Optical rotations were measured with Perkin-Elmer 341 polarimeter. UV and IR spectra were recorded on Shimadzu UV-2550 and Perkin-Elmer 577 (using KBr disks) spectrophotometers, respectively. NMR spectra were acquired on a Bruker Avance III (600 MHz for  $^1\text{H-NMR}$ , ppm relative to TMS) spectrometer. ESIMS spectra were made on an Agilent 1200 series HPLC and interfaced to an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source, and HRESIMS spectra were made on an Agilent 1290 series HPLC and interfaced to an Agilent 6538 UHD Accurate-Mass Q-TOF LC/MS (Agilent Corporation, Wilmington, DE, USA). GC-MS was conducted on a Thermo Finnigan Trace GC apparatus using an L-Chirasil-Val column ( $25 \text{ m} \times 0.32 \text{ mm}$ , i.d.). Semi-preparation RP-HPLC isolation was achieved with an Agilent 1200 instrument with refractive index detector (RID) using a YMC  $5 \mu\text{m}$  C8 column ( $250 \text{ mm} \times 10 \text{ mm}$ ). Methanol for semi-preparative HPLC was of HPLC-grade (Merck, Darmstadt, Germany). Column chromatography: silica gel (200–300 mesh); macroporous adsorbing resin (D-101, ZTC-1, 0.3–1.2 mm, Tianjin Zhentiancheng Science & Technology Co., Ltd., Tianjin, China); sephadex LH-20 gel (40–70  $\mu\text{m}$ , Amersham Pharmacia Biotech AB, Uppsala, Sweden); silica gel H (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China). All solvents for column chromatography and acid hydrolysis were of analytical grade (Shanghai

Chemical Reagents Company, Ltd., Shanghai, China). Spots of compounds on TLC were developed using 10% H<sub>2</sub>SO<sub>4</sub>-EtOH solution.

### 3.2. Plant Material

Platycodi Radix was collected from Taihe, Anhui Province, China, in September 2010 and was identified by Professor Hanming Zhang of School of Pharmacy, Second Military Medical University. A voucher specimen (No. 20100921) was deposited at the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University.

### 3.3. Extraction and Isolation

Platycodi Radix was air-dried (10 kg) and extracted three times with 70% EtOH (50 L × 3 times) under reflux. The combined extract was concentrated *in vacuo* and suspended in water. The aqueous layer was chromatographed over a macroporous adsorbing resin column eluting with H<sub>2</sub>O, 30% EtOH, 60% EtOH and 95% EtOH. The 95% EtOH-eluted fraction (30 g) was applied to a silica gel column (CHCl<sub>3</sub>-MeOH, 50:1 to 10:1, v/v) and purified by semi-preparative HPLC (MeOH-H<sub>2</sub>O, 4:1) to give compound **1** (10.4 mg). The 60% EtOH-eluted fraction (120 g) was chromatographed on silica gel column eluting with a CHCl<sub>3</sub>-MeOH gradient (30:1 to 2:1, v/v) to afford five subfractions (A-E). Subfraction B (20 g) was chromatographed on a silica gel column (CHCl<sub>3</sub>-MeOH, 10:1 to 5:1, v/v), followed by Sephadex LH-20 column (MeOH-H<sub>2</sub>O, 1:1), and finally separated by semi-preparative HPLC (MeOH-H<sub>2</sub>O, 3:2) to yield compound **2** (9.3 mg). By the same procedure, compounds **3** (20.0 mg), **4** (8.7 mg), **5** (11.2 mg), **6** (9.4 mg) and **7** (7.6 mg) were obtained from subfractions C-E.

### 3.4. Characterization of Compound 1 and Compound 2

Compound **1**: white amorphous powder;  $[\alpha]_D^{22.0} +44.3$  (*c* 0.174, MeOH); IR (KBr)  $\nu_{\max}$  3396, 2943, 2908, 1705, 1639, 1452, 1429, 1381, 1248, 1074, 1043, 898.7 cm<sup>-1</sup>; <sup>1</sup>H-NMR and <sup>13</sup>C-NMR data see Table 1; ESIMS *m/z* 497.4 [M+Na]<sup>+</sup>; HRESIMS *m/z* 497.3243 [M+Na]<sup>+</sup> (calcd. for C<sub>29</sub>H<sub>46</sub>O<sub>5</sub>Na, 497.3243).

Compound **2**: white amorphous powder;  $[\alpha]_D^{22.0} +59.9$  (*c* 0.128, MeOH); IR (KBr)  $\nu_{\max}$  3415, 2947, 1711, 1456, 1433, 1385, 1365, 1134, 1047, 696, 594 cm<sup>-1</sup>; <sup>1</sup>H-NMR and <sup>13</sup>C-NMR data see Table 1; ESIMS *m/z* 659.5 [M+Na]<sup>+</sup>; HRESIMS *m/z* 659.3728 [M+Na]<sup>+</sup> (calcd. for C<sub>35</sub>H<sub>56</sub>O<sub>10</sub>Na, 659.3771).

### 3.5. Acid Hydrolysis of Compound 2

Compound **2** (3.0 mg) was refluxed with 1M HCl (dioxane-H<sub>2</sub>O, 1:1, 2 mL) at 90 °C for 3 h in a water bath. After dioxane was removed, the solution was extracted with EtOAc (2 mL × 3 times). After evaporating to dryness, the monosaccharide portion was analyzed by gas chromatography after conversion of the hydrolysates into corresponding alditol acetates. Only D-glucose was detected. The EtOAc portion was washed with H<sub>2</sub>O and evaporated to yield the aglycone. The aglycone was identified by TLC together with compound **1**.

### 3.6. In Vitro Inhibitory Activity on Cell Proliferation

Tested compounds **1–7** were dissolved in DMSO (final concentration, 0.1%). Inhibitory activity of compounds **1–7** against HSC-T6 cell line was evaluated by the MTT assay [36]. Briefly, cells at the exponential growth phase were harvested and seeded into a flatbottom 96-well plate. A total of 90  $\mu\text{L}$  containing  $5 \times 10^4$  cells was added to each well of the plate and incubated for 24 h in a 5% humidified  $\text{CO}_2$  at 37 °C. HSC-T6 cells were treated with vehicle or compounds at concentration of 0.01, 0.1, 1, 10, 100 and 1000  $\mu\text{g}/\text{mL}$ . After 48 h of incubation at 37 °C, 20  $\mu\text{L}/\text{well}$ , MTT was then added and the plate was again incubated at 37 °C for 4 h. Reduction of MTT to formazan was measured in an ELISA plate reader at 570 nm. Inhibitory activity of compounds **1–7** on cell proliferation (% of control) was calculated as  $100 \times (\text{absorbance of treated compound} - \text{absorbance of background light}) / (\text{absorbance of control} - \text{absorbance of background light})$ . Data were expressed as the mean of the three independent experiment. Colchicin was used as a positive control.

## 4. Conclusions

In conclusion, the phytochemical investigation of the roots extract of *Platycodi Radix* afforded two new triterpenoids, platycodonoids A (**1**) and B (**2**), together with five known triterpenoids: platycodin D (**3**), deapio platycodin D (**4**), 3-*O*- $\beta$ -D-glucopyranosyl polygalacic acid (**5**), 3-*O*- $\beta$ -D-glucopyranosyl platycodigenin (**6**) and polygalacin D (**7**). The structures of the compounds were elucidated on the basis of spectral analysis and chemical evidence and literature comparisons in the case of the known ones. Compounds **1**, **3**, **4** and **7** exhibited significant hepatoprotective activities against HSC-T6 cell lines *in vitro* ( $\text{IC}_{50}$  value < 10  $\mu\text{M}$ ).

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*Sample Availability:* Samples of the compounds 1–7 are available from the authors.