Structure Characterization and Identification of Triterpenoid Saponins from the Root of *Platycodon grandiflorum* by HPLC/ESI-QTOF-MS/MS

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Triterpenoid saponins, derived from the root of *Platycodon grandiflorum*, have been shown many biological activities, such as anti-inflammatory, antioxidant, antitumor, antiobesity and antihyperlipidemia effects. Nevertheless, the high polarity, non chromophores and low abundance of these compounds in this plant bring difficulty for their isolation and structural determination by traditional chromatographic and spectroscopic techniques. The aim of the present study was to develop a sensitive and rapid high-performance liquid chromatography coupled with electrospray time-of-flight mass spectrometry (HPLC/ESI-QTOF-MS/MS) approach for the structural analysis and identification of triterpenoid saponins. According to the HPLC retention behaviour, the proposed fragmentation pathways provided by high-resolution MS and MS/MS spectra and literature sources, a total of 18 triterpenoid saponins in the crude extract of root of *Platycodon grandiflorum* were identified or tentatively characterized. This method therefore proved to be a very useful technique for the characterisation and structure elucidation of triterpenoid saponins of this plant.

Keywords: Triterpenoid saponins, *Platycodon grandiflorum*, HPLC/ESI-QTOF-MS/MS.

INTRODUCTION

The root of *Platycodon grandiflorum* (RPG), officially documented in the Chinese Pharmacopoeia under the name "Jie Geng", is one of the most commonly used traditional Chinese medicine (TCM) for the treatment of chronic adult diseases and inflammatory diseases. Chemical investigation of root of *Platycodon grandiflorum* revealed that triterpenoid saponins were the major chemical components¹⁻³, which exhibit a variety of pharmacological activities, such as antioxidant⁴, immunological adjuvant⁵, protective effects on the hepatotoxicity induced by chemicals⁶, antiallergic⁷, anticancer⁸, anti-inflammatory⁹, etc. However, because of their high polarity, non chromophores and low content in plants, triterpenoid saponins are difficult to be isolated from root of *Platycodon grandiflorum* by conventional phytochemical methods, which hindered the use of root of *Platycodon grandiflorum* in clinical practice and retarded the process of root of *Platycodon grandiflorum* modernization.

In recent years, the high-performance liquid chromatography coupled with electrospray time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) has been used to separate and characterize the chemical constituents from plants¹⁰⁻¹².

Compared with LC-UV or LC-ELSD, the combination of HPLC and Q-TOF technique not only shortens the analysis time but also provides significant advantages concerning flexibility, selectivity, sensitivity, accuracy and speed for rapid screening target compounds in complex matrices.

The aims of present work are to develop a rapid and reliable HPLC-ESI-QTOF-MS method for analysis of triterpenoid saponins from root of *Platycodon grandiflorum* extract. As a result, a total of eighteen triterpenoid saponins in the crude extract of root of *Platycodon grandiflorum* were identified or tentatively identified.

EXPERIMENTAL

The root of *Platycodon grandiflorum* (RPG) were purchased from Bozhou, Anhui Province, P.R. China, in September 2013. Four saponin standards platycoside G₂, platycoside G₁, platycoside E and polygalacin D were isolated from root of *Platycodon grandiflorum* extract and their chemical structures were confirmed by MS, 1D and 2D NMR. The purities of these standards were no less than 95 % by normalization of the peak areas detected by HPLC/MS.

HPLC grade acetonitrile, methanol and formic acid were purchased from Dikma Company (Dikma, USA). Water was

deionized and double distilled. All other analytical grade reagents were from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Sample preparation: An aliquot of 1 g root of *Platycodon grandiflorum* drug powder was accurately weighed and extracted with 10 mL of methanol:water (70:30, v/v) by ultrasonication for 0.5 h. The solution was centrifuged at 12000 rpm for 10 min and the supernatant was filtered through a 0.22 μ m filter. A volume of 2 μ L was injected into the HPLC-QTOF/MS system for the analysis.

HPLC conditions: The chromatography analytical procedures were performed on an agilent 1260 series (Agilent, Santa Clara, CA, USA) LC system equipped with a binary pump, an online degasser, an auto plate-sampler and a thermostatically controlled column compartment. The columns were maintained at 30 °C. The separation was carried out on an Agilent poroshell 120 EC-C18 column (2.7 $\times$ 100 mm, i.d., 2.7 μ m), preceded by a C18 guard column (4 $\times$ 2 mm; Agilent, CA, USA). The mobile phase consisted of water containing 0.1 % (v/v) formic acid (A) and acetonitrile (B). A gradient program was used as follows: 0-5 min, 5-20 % B; 5-15 min, 20 % B; 15-25 min, 20-30 % B; 25-35 min, 30 % B; 35-40 min, 30-90 % B. The composition was then held at 90 % B for 5 min and returned to initial conditions and maintained 10 min for equilibration. The flow rate was 0.35 mL/min and sample injection volume was 2 μ L.

Mass spectrometry conditions: Mass spectrometry was performed using an agilent 6530 Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) interface and was operated in negative ion mode with parameters set as follows: capillary voltage, 3500 V; fragmentor, 150 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V; pressure of nebulizer, 35 psi; drying gas temperature, 300 °C; sheath gas temperature, 350 °C. Nitrogen was used as sheath and drying gas at a flow rate of 8 and 11 L/min, respectively. The collision energy (CE) was set 45 V and the mass range recorded m/z 100-2000. The accurate-mass capability of the TOF analyzer allowed reliable confirmation of the identity of the detected metabolites, normally with mass errors below 5

ppm in routine analysis, which was sufficient to verify the chemical constituents in root of *Platycodon grandiflorum*. An external calibration solution (Agilent calibration solution A) was continuously sprayed in the ESI source of the QTOF system, employing the ions with m/z 112.9855 (TFA anion) and 1033.9881 [HP-0921 (TFA adduct)] to recalibrate the mass axis, ensuring mass accuracy and reproducibility throughout the chromatographic run. All operations, acquisition and analysis of data were monitored by Agilent HPLC-Q-TOF-MS MassHunter Acquisition Software Version A.01.00 (Agilent Technologies) and operated under MassHunter Acquisition Software Version B.06.00 (Agilent Technologies).

RESULTS AND DISCUSSION

Optimization of Q-TOF-MS/MS conditions: To obtain better detection, four reference compounds platycoside G₂, platycoside G₁, platycoside E and polygalacin D were characterized according to their mass spectra using syringe pump continuous infusion analysis to find their mass spectrometric behaviours and optimize the MS conditions. When the electrospray ionization (ESI) source was operated, good sensitivity and sufficient numbers of fragment ions were obtained. With regard to the four standards, negative ion mode offered better sensitivity than positive ion mode and gave $[M + Cl]^-$ ions as the base peak. Therefore, ESI in the negative ion mode was selected for follow-up analyses.

HPLC-ESI-Q-TOF-MS/MS analysis of root of *Platycodon grandiflorum* extract: Eighteen peaks were identified or tentatively characterized by comparing their retention time, mass spectra fragmentation characteristics and chromatography behaviours with those of authentic standards or the data provided in the literature. The total ion current (TIC) chromatogram for root of *Platycodon grandiflorum* extract was shown in Fig. 1. The retention times, molecular weights, fragment ions and parent ions of the eighteen compounds were summarized in Table-1 and their structures were shown in Fig. 2.

As shown in Fig. 3, peak 1 gave an adducted ion of $[M + Cl]^-$ at m/z 1319.5677 in the first-order mass spectrum. In the MS/MS spectrum, it yielded an important fragment ion

Fig. 1. MS total ion current (TIC) chromatogram of root of *Platycodon grandiflorum* (RPG) extract

TABLE-1
ESI-TOF-MS DATA OF COMPOUNDS IDENTIFIED FROM THE ROOT OF *Platycodon grandiflorum* EXTRACT

Peak	t _R (min)	m.f.	Identification	Ion mode	Mass (m/z)	Fragment ions (m/z)	Ref.
1 ^a	12.889	C ₅₉ H ₉₆ O ₃₀	Platycoside G ₂	[M + Cl] ⁻	1319.5677	1283.5892 [M-H] ⁻ , 1005.4907 [M-H-Rha-Ara] ⁻ , 987.4791 [M-H-Rha-Ara-H ₂ O] ⁻ , 943.4837 [M-H-Rha-Ara-H ₂ O-CO ₂] ⁻ , 843.4635 [M-H-Rha-Ara-Glc] ⁻ , 681.3538 [M-H-Rha-Ara-2Glc] ⁻ , 651.0844 [M-H-Rha-3Glc] ⁻ , 519.3315 [M-H-Rha-Ara-3Glc] ⁻	1
2 ^a	13.467	C ₆₄ H ₁₀₄ O ₃₄	Platycoside G ₁	[M + Cl] ⁻	1451.6100	1415.6333 [M-H] ⁻ , 1325.6057 [M-H-C ₃ H ₆ O ₃] ⁻ , 1253.5928 [M-H-Glc] ⁻ , 1005.4916 [M-H-Xyl-Rha-Ara] ⁻ , 843.4289 [M-H-Xyl-Rha-Ara-Glc] ⁻ , 681.3916 [M-H-Xyl-Rha-Ara-2Glc] ⁻ , 519.3338 [M-H-Xyl-Rha-Ara-3Glc] ⁻	1
3	14.571	C ₆₄ H ₁₀₄ O ₃₃	Platycoside I	[M + Cl] ⁻	1435.6153	1399.6354 [M-H] ⁻ , 989.4923 [M-H-Xyl-Rha-Ara] ⁻	2
4 ^a	14.715	C ₆₉ H ₁₁₂ O ₃₈	Platycoside E	[M + Cl] ⁻	1583.6511	1547.6405 [M-H] ⁻ , 1457.6405 [M-H-C ₃ H ₆ O ₃] ⁻ , 1005.4871 [M-H-Api-Xyl-Rha-Ara] ⁻	1
5	15.887	C ₆₉ H ₁₁₂ O ₃₇	Platycoside D	[M + Cl] ⁻	1567.6564	1531.6714 [M-H] ⁻ , 1441.6811 [M-H-C ₃ H ₆ O ₃] ⁻ , 989.4824 [M-H-Api-Xyl-Rha-Ara] ⁻	3
6	20.600	C ₅₈ H ₉₄ O ₂₉	Deapioplatycodin D ₃	[M + Cl] ⁻	1289.5575	1253.5797 [M-H] ⁻ , 1163.5484 [M-H-C ₃ H ₆ O ₃] ⁻ , 843.4381 [M-H-Xyl-Rha-Ara] ⁻	13
7	21.772	C ₆₃ H ₁₀₂ O ₃₃	Platycodin D ₂	[M + Cl] ⁻	1421.6004	1385.6222 [M-H] ⁻ , 1295.5931 [M-H-C ₃ H ₆ O ₃] ⁻ , 1121.5242 [M-H-Api-Xyl] ⁻ , 843.4371 [M-H-Api-Xyl-Rha-Ara] ⁻ , 681.3805 [M-H-Api-Xyl-Rha-Ara-Glc] ⁻	1
8	22.264	C ₆₅ H ₁₀₄ O ₃₄	2''-O-Acetylplatycodin D ₂	[M + Cl] ⁻	1463.6116	1427.6307 [M-H] ⁻ , 1295.5831 [M-H-Api] ⁻ , 843.4362 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻ , 681.3998 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara-Glc] ⁻	14
9	24.931	C ₅₈ H ₉₄ O ₂₉	Deapioplatycodin D ₂	[M + Cl] ⁻	1289.5571	1253.5789 [M-H] ⁻ , 1163.5493 [M-H-C ₃ H ₆ O ₃] ⁻ , 843.4366 [M-H-Xyl-Rha-Ara] ⁻	15
10	25.491	C ₆₃ H ₁₀₂ O ₃₃	Platycodin D ₃	[M + Cl] ⁻	1421.5994	1385.6216 [M-H] ⁻ , 1253.5776 [M-H-Api] ⁻ , 843.4376 [M-H-Api-Xyl-Rha-Ara] ⁻ , 663.3719 [M-H-Api-Xyl-Rha-Ara-Glc-H ₂ O] ⁻	1
11	25.899	C ₅₉ H ₉₄ O ₂₉	2''-O-Acetylplatycodin D	[M + Cl] ⁻	1301.5600	1265.5805 [M-H] ⁻ , 1223.5675 [M-H-C ₂ H ₂ O] ⁻ , 1133.5397 [M-H-Api] ⁻ , 795.4172 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-H ₂ O] ⁻ , 681.3853 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻	15
12 ^a	26.051	C ₅₇ H ₉₂ O ₂₇	Polygalacin D	[M + Cl] ⁻	1243.5522	1207.5742 [M-H] ⁻ , 1117.5427 [M-H-C ₃ H ₆ O ₃] ⁻ , 665.3906 [M-H-Api-Xyl-Rha-Ara] ⁻	15
13	26.102	C ₆₅ H ₁₀₄ O ₃₃	2''-O-Acetylpolygalacin D ₂	[M + Cl] ⁻	1447.6170	1411.6387 [M-H] ⁻ , 1279.5961 [M-H-Api] ⁻ , 827.4428 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻ , 665.3888 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara-Glc] ⁻	15
14	26.408	C ₅₉ H ₉₄ O ₂₈	2''-O-Acetylpolygalcin D	[M + Cl] ⁻	1285.5632	1249.5831 [M-H] ⁻ , 1207.5731 [M-H-C ₂ H ₂ O] ⁻ , 1117.5462 [M-H-C ₂ H ₂ O-Api] ⁻ , 665.3892 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻	15
15	26.645	C ₅₄ H ₈₆ O ₂₅	Platycoside C	[M + Cl] ⁻	1169.5179	1133.5362 [M-H] ⁻ , 1091.5248 [M-H-C ₂ H ₂ O] ⁻ , 1001.4907 [M-H-Xyl] ⁻ , 855.4426 [M-H-C ₁₁ H ₁₈ O ₈] ⁻ , 723.3965 [M-H-C ₂ H ₂ O-Xyl-Rha-C ₃ H ₆ O ₃] ⁻ , 681.3845 [M-H-C ₂ H ₂ O-Xyl-Rha-Ara] ⁻ , 663.3747 [M-H-C ₂ H ₂ O-Xyl-Rha-Ara-H ₂ O] ⁻ , 619.3812 [M-H-C ₂ H ₂ O-Xyl-Rha-Ara-H ₂ O-CO ₂] ⁻ , 591.3493 [M-H-C ₂ H ₂ O-Xyl-Rha-Ara-C ₃ H ₆ O ₃] ⁻	2
16	27.274	C ₅₉ H ₉₄ O ₂₉	3''-O-Acetylplatycodin D	[M + Cl] ⁻	1301.5611	1265.5790 [M-H] ⁻ , 1223.5679 [M-H-C ₂ H ₂ O] ⁻ , 1133.5379 [M-H-Api] ⁻ , 795.4124 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-H ₂ O] ⁻ , 681.3850 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻	15
17	27.427	C ₆₅ H ₁₀₄ O ₃₃	3''-O-Acetylpolygalacin D ₂	[M + Cl] ⁻	1447.6173	1411.6385 [M-H] ⁻ , 1279.5941 [M-H-Api] ⁻ , 827.4421 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻ , 647.3785 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara-Glc-H ₂ O] ⁻	15
18	27.733	C ₅₉ H ₉₄ O ₂₈	3''-O-Acetylpolygalacin D	[M + Cl] ⁻	1285.5694	1249.5833 [M-H] ⁻ , 1207.5725 [M-H-C ₂ H ₂ O] ⁻ , 665.3897 [M-H-C ₂ H ₂ O-Api-Xyl-Rha-Ara] ⁻	15

Note t_R: Retention time; Ref: literature referred to structural identification; ^aCompared with a reference compound

Fig. 2. Chemical structures of eighteen triterpenoid saponins

at m/z 1283.5892 $[M-H]^-$, which corresponds to $C_{59}H_{96}O_{30}$. In addition, a series of characteristic signals at m/z 1005.4907 $[M-H-Rha-Ara]^-$, 987.4791 $[M-H-Rha-Ara-H_2O]^-$, 943.4837 $[M-H-Rha-Ara-H_2O-CO_2]^-$, 843.4635 $[M-H-Rha-Ara-Glc]^-$, 681.3538 $[M-H-Rha-Ara-2Glc]^-$, 651.0844 $[M-H-Rha-3Glc]^-$ and 519.3315 $[M-H-Rha-Ara-3Glc]^-$ were also observed due to the characteristic cleavage of different sugar chains. By comparison with reference standard, peak **1** was thus identified as platycoside G₂. Peak **2** featured an adducted ion of $[M + Cl]^-$ at m/z 1451.6100 in the first-order mass spectrum. In the MS/MS spectrum, a base peak at m/z 1415.6333 $[M-H]^-$ was observed, which was 132 Da more than that of peak **1**, indicating the existence of Xyl. The ion at m/z 1325.6057 was generated by loss of $C_3H_6O_3$ (90 Da) from the ion at m/z 1415.6333 via the cross-ring cleavage reactions in the Glc residue, as shown in Fig. 4. In addition, other fragment ions at m/z 1253.5928 $[M-H-Glc]^-$, 1005.4916 $[M-H-Xyl-Rha-Ara]^-$, 843.4289 $[M-H-Xyl-Rha-Ara-Glc]^-$, 681.3916 $[M-H-Xyl-Rha-Ara-2Glc]^-$ and 519.3338 $[M-H-Xyl-Rha-Ara-3Glc]^-$ were yielded in the MS/MS spectrum. It showed a similar pathway to peak **1**. Therefore, peak **2** was identified as platycoside G₁ and this identity was confirmed with the authentic standard. Peak **3** gave an adducted ion of $[M + Cl]^-$ at m/z 1435.6153, which was 16 Da (O) less than that of peak **2**, indi-

cating that it may be the deoxidation derivative of platycoside G₁. Subsequent MS/MS investigation revealed major fragments at m/z 989.4923 $[M-H-Xyl-Rha-Ara]^-$ by cleavage of the ester bond at C-28. By comparing with the reported work², peak **3** was tentatively identified as platycoside I. Peak **4** gave an adducted ion of $[M + Cl]^-$ at m/z 1583.6511, which was 132 Da more than that of peak **2**, indicating the presence of an extra sugar moiety (Api). By comparison with the authentic standard, peak **4** was identified as platycoside E. Peaks **5-14** all displayed a series of characteristic fragment ions and neutral losses of triterpenoid saponins. By comparing with the available reference^{1,3,13-15}, these compounds were tentatively identified as platycoside D, deapioplatycodin D₃, platycodin D₂, 2''-O-acetylplatycodin D₂, deapioplatycodin D₂, platycodin D₃, 2''-O-acetylplatycodin D, polygalacin D, 2''-O-acetyl polygalacin D₂ and 2''-O-acetyl polygalacin D, respectively. Peak **15** gave an adducted ion of $[M + Cl]^-$ at m/z 1169.5174 and yielded a characteristic fragment ion by loss of 42 Da, indicating the occurrence of acetyl. The fragment ion at m/z 855.4426 by loss of $^{0,2}B$ ($C_{11}H_{18}O_8$) from $[M-H]^-$ at m/z 1133.5362 was yielded, confirming that acetyl group should be occurred at C3-OH position of Rha, as shown in Fig. 5. By comparison with the previous work², peak **15** was tentatively characterised as platycoside C. Peak **16**

Chemical formula: $C_{59}H_{96}O_{30}$
Molecular weight: 1284

Fig. 3. MS/MS spectra and proposed fragmentation pathways of platycoside G_2

Chemical formula: $C_{64}H_{104}O_{34}$
Molecular weight: 1416

Fig. 4. MS/MS spectra and proposed fragmentation pathways of platycoside G_1

Fig. 5. MS/MS spectra and proposed fragmentation pathways of platycoside C

exhibited an adducted ion of $[M + Cl]^-$ at m/z 1301.5611, 132 Da more than that of peak **15**, indicating the presence of an extra sugar moiety (Api). Owing to unavailability of standard, peak **16** could only be tentatively ascribed as 3''*O*-acetylplatycodin D according to previous publications¹⁵. Similarly, peaks **17** and **18**, with adducted ions of $[M + Cl]^-$ at m/z 1447.6173 and 1285.5694, were, respectively identified as 3''*O*-acetylpolygalacin D₂ and 3''*O*-acetylpolygalacin D, by comparison with the previous work¹⁵.

Conclusion

In this paper, HPLC-ESI-QTOF-MS provides a sensitive, selective and rapid analytical method for successfully characterization triterpenoid saponins from root of *Platycodon grandiflorum* extract, thus avoiding time-consuming isolation of pure constituents. Based on the retention times, MS and MS/MS data with the reference standards and previous reports, eighteen triterpenoid saponins were identified or tentatively characterized from root of *Platycodon grandiflorum* extract. The identification and structural elucidation of the chemical constituents provided essential data for further pharmacological and clinical studies of root of *Platycodon grandiflorum*. The present method will also be helpful for the rapid identification of similar triterpenoid saponins in other herb medicines.

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