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Original Article

Rapid identification and quantitative analysis of chemical constituents of *Gentiana veitchiorum* by UHPLC-PDA-QTOF-MS

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ABSTRACT

Gentiana veitchiorum Hemsl., Gentianaceae, a traditional Tibetan medicine, was used for the treatment of liver jaundice with damp-heat pathogen, as well as for headache and chronic pharyngitis. A rapid ultra-performance liquid chromatography, photodiode array detector, quadrupole time-of-flight mass spectrometry method was developed for the fast and accurate identification and quantification of the chemical constituents of *G. veitchiorum*. In fact, eighteen compounds were detected and identified on the basis of their mass spectra, fragment characteristics and comparison with published data. Especially, the MS fragmentation pathways of iridoid glycosides and flavone *C*-glycosides were illustrated. Five compounds among them were quantified by UHPLC-PDA, including swertiamarin, gentiopicroside, sweroside, isoorientin, and isovitexin. The proposed method was then validated based on the analyses of linearity, accuracy, precision, and recovery. The overall recoveries for the five analytes ranged from 96.54% to 100.81%, with RSD from 1.05% to 1.82%. In addition, ten batches of *G. veitchiorum* from different areas were also analyzed. The developed method was rapid and reliable for both identification and quantification of the chemical constituents of *G. veitchiorum*, especially for simultaneous qualitative and quantitative analysis of iridoid glycosides and flavone *C*-glycosides.

Introduction

Gentiana veitchiorum Hemsl., belonging to the family of Gentianaceae, was known as "bangjianwenbao" in traditional Tibetan medicine. It is distributed at altitudes of 2500–4800 m in Qinghai, Sichuan and Tibet provinces of China and used for the treatment of liver jaundice with damp-heat pathogen, as well as for headache and chronic pharyngitis. Most Chinese people drink it as a herbal infusion (Liu et al., 2006a,b).

Modern pharmacological studies showed that *G. veitchiorum* can inhibit the LPS induced pulmonary alveolar macrophages TNF-alpha expression (Hou et al., 2011), the formation of liver fibrosis (Li et al., 2008), and showed significant antibacterial activity on methicillin resistant *Staphylococcus aureus* (Liu et al., 2011). Meanwhile, the extract of *G. veitchiorum* demonstrated significantly anti-RSV effect *in vitro* and *in vivo* (Wei et al., 2011) and improved chronic bronchitis (Geng et al., 2010). Moreover, a formula containing the flower of *G. veitchiorum* showed certain therapeutic effects against pulmonary injuries induced by bleomycin in rats (Liang et al., 2011). Our previous study has also confirmed that *G. veitchiorum* could protect the liver against CCl_4-induced damage in mice, and this hepatoprotective effect was due partly to its ability of scavenging free radicals (Zhang et al., 2014).

Although pharmacological effects of *G. veitchiorum* have been well investigated in previous literatures, the chemical analyses of *G. veitchiorum* are still limited. Previous chemical investigations of *G. veitchiorum* showed that iridoid glycosides and flavone compounds were the main chemical constituents (Zou et al., 2010; Yang et al., 2008). Gentiopicroside could be one of the most important chemical constituents of *G. veitchiorum*. An HPLC method was also developed to quantify gentiopicroside in *G. veitchiorum* (Liu et al., 2006a,b). However, the use of only one compound as a standard reference for the evaluation of the chemical constituents in complex mixture of herbal medicines is not sufficient (Zhou and He, 2014). Therefore, a UHPLC-QTOF-MS method has been developed as an efficient and powerful tool for simultaneous qualitative and quantitative analysis of complicated mixture of compounds in herbal medicines, especially for unknown compounds.

In comparison to previous study, quadrupole time-of-flight mass spectrometry (Q-TOF-MS) is more sensitive and accurate via

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performing exact mass neutral loss scan together with MS and MS/MS spectra. Moreover, some compounds can be confirmed by the presences of diagnostic fragments and/or neutral losses in MS/MS spectra. The candidate compounds can be further identified by detecting exact m/z values for characteristic fragments and/or neutral eliminations, which are diagnostic for each class of natural products (Zhang et al., 2015; Li et al., 2015). Therefore, in the current study, we developed a rapid and sensitive UHPLC-PDA-QTOF-MS method based on exact mass neutral loss scan to simultaneously analyze known and unknown constituents in *G. veitchiorum* in a short time. In total, eighteen compounds were identified or tentatively characterized. Furthermore, quantitative analysis of five bioactive components was performed by UHPLC-PDA. Ten batches of *G. veitchiorum* samples from different collecting spots were investigated by the established method. The present study can provide important information for quality control of *G. veitchiorum*. Meanwhile, it might provide the chemical evidence for explaining the therapeutic effects of *G. veitchiorum*.

Material and methods

Plant material

Samples of *Gentiana veitchiorum* Hemsl., Gentianaceae, were collected in Sichuan province, Qinghai province, Tibet province or purchased in local markets at the Sichuan province. The material was authenticated by Prof. Hao Zhang (Professor of Pharmacognosy, School of Pharmacy, Sichuan University). Voucher specimens were deposited in Herbarium Centre, Southwest University for Nationalities, Sichuan, China.

Chemicals and reagents

Chemical reference standards of swertiamarin (No. 1402121), gentiopicroside (No. 1406017), sweroside (No. 1406009), isoorientin (No. 1406014), and isovitexin (No. 1406011) were purchased from Chengdu Kangbang Biological Technology CO., LTD (Chengdu, China). The purity of each chemical was above 98%, as confirmed by HPLC. Methanol was HPLC grade from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other reagents and chemicals were of analytical grade. All solutions were passed through a 0.22 μm PTFE membrane (Cherish Technology CO., LTD, Beijing, China) before injecting into the UHPLC system.

Preparation of standard solutions

Standards of swertiamarin, gentiopicroside, sweroside, isoorientin, and isovitexin were dissolved in methanol to produce five stock solutions (about 1 mg/ml), which were stored at 4 °C. Working solutions of standards were prepared by diluting the stock solutions with methanol into different concentrations.

Preparation of sample solutions

G. veitchiorum was collected and then dried. The samples were cut into small sections and ground to powder in a mill. The powder (0.5 g) was extracted with methanol (2 × 10 ml) by sonication (30 min, at room temperature), the solutions combined and adjusted to 25 ml volume. The sample solutions were filtered through a 0.22 μm PTFE membrane filter. The filtrate was stored at 4 °C in a refrigerator before being used for UHPLC analysis.

Method validation of UHPLC-PDA

Following the analytical procedure guideline of ICH 2005, the developed UHPLC-PDA method was validated based on the linearity, precision, repeatability, and recovery.

(1) Limits of quantification and detection
The standards stock solutions were diluted to a series of appropriate concentrations with methanol. The diluted solutions were analyzed by UHPLC. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting the diluted solution with known concentrations of the standards. LOD and LOQ were defined as the signal-to-noise ratios (S/N) equal to 3 and 10, respectively.

(2) Linearity
The stock solutions of the five standards were mixed for construction of calibration curves. Six levels of the solution concentration were determined in duplicate, and calibration curves were then established by plotting the peak area versus the concentration of each analyte. The regression equation was calculated as follows: $Y = aX + b$, where Y is the peak area and X is the concentration of the standard solutions.

(3) Precision, repeatability and recovery
The intra- and inter-day precisions were investigated by analyzing a mixed standard solution in five replicates during a single day and by duplicating the experiments on three consecutive days. *G. veitchiorum* was analyzed in five replicates with the proposed method to confirm the repeatability of the developed approach. The relative standard deviation (RSD) was calculated as a variation of precision and repeatability. A recovery experiment was carried out to further evaluate the accuracy of the method. Three different quantities (low, medium, and high) of the standards were spiked into a known amount of *G. veitchiorum* sample (0.25 g). Then, the resultant sample was extracted and analyzed with the established method and triplicate experiments were performed at each level. The recovery percentages for the five compounds were calculated based on the following equation:

$$\text{Recovery}\ (\%) = \frac{\text{detected amount-original amount}}{\text{spiked amount}} \times 100.$$

UHPLC-PDA-QTOF-MS analysis

UHPLC-PDA-QTOF-MS analysis was performed on the Waters Acquity UHPLC I-Class system (Waters, USA). Sample was separated in an Acquity HSS C_{18} column (100 mm × 2.1 mm, 1.8 μm) with a C_{18} pre-column (Waters, USA). The column was set at the temperature of 35 °C. The mobile phase was a mixture of 0.1% formic acid–water (A) and methanol (B), with an optimized linear gradient elution as follows: 0–15 min, 20–50% B. The injection volume was 1.0 μl. The flow rate was 0.20 ml min^{-1}. Detector wavelength was set at 254 nm.

Mass spectrometry was performed on the Waters definition accurate mass quadrupole time-of-flight (Q-TOF) XevoG2-S mass spectrometer with electrospray ionization (ESI) source (Waters MS Technologies, UK). Samples were scanned in both positive and negative ion modes to get the complementary information for structural identification. All the parameters were set as follows: mass range, m/z 100–1500; the flow rate of drying gas (N_2), 800 l/h; drying gas temperature, 450 °C; cone gas, 30 l/h; source temperature, 100 °C; capillary voltage, 2500 V; and cone voltage, 40 V.

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Table 1
Linear regression, precision, repeatability, and recovery data of five analytes from *Gentiana veitchiorum*.

Compounds	Regression equations	r	Linear range	Precision (RSD)		Repeatability	Recovery		LOD	LOQ
			($\mu g\,ml^{-1}$)	Intra-day	Inter-day	(RSD)	Average	(RSD)	($\mu g\,ml^{-1}$)	($\mu g\,ml^{-1}$)
Swertiamarin	$Y = 8.1711 \times 10^{-6}X - 315{,}852.9$	0.9998	0.07–2.30	2.03%	2.58%	2.45%	100.04%	1.82%	0.51	1.26
Gentiopicroside	$Y = 3.7819 \times 10^{-6}X - 11{,}742.0$	0.9997	0.15–2.36	1.34%	2.30%	1.67%	100.81%	1.91%	0.46	1.03
Sweroside	$Y = 7.6114 \times 10^{-6}X - 10{,}172.2$	0.9995	0.06–2.00	1.56%	2.71%	1.38%	98.78%	1.33%	0.53	1.65
Isoorientin	$Y = 9.5069 \times 10^{-6}X - 102{,}206.5$	0.9999	0.09–2.01	1.18%	2.01%	2.13%	98.12%	1.12%	0.33	1.43
Isovitexin	$Y = 6.4399 \times 10^{-6}X - 11{,}628.2$	0.9999	0.11–2.12	1.31%	2.41%	1.28%	99.11%	1.05%	0.56	1.01

Results and discussion

Method validation of UHPLC-PDA

To achieve the best resolution for each detected peaks within shorter time, the organic solvent, column type, mobile phase, flow rate of mobile phase, and column temperature were optimized. The developed method for the quantification of analytes was validated by determining the linearity, limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day precisions, repeatability and recovery. Swertiamarin, gentiopicroside, sweroside, isoorientin and isovitexin showed good linearity with high correlation coefficient ($r > 0.9999$) within the tested range. The LOD (S/N = 3) and LOQ (S/N = 10) for the five standard analytes were in the range of 0.33–0.56 ng/ml and 1.01–1.65 ng/ml, respectively.

Precision and repeatability of the UHPLC-PDA method were also validated for the five analytes. Relative standard deviations (RSD) of intra-day, inter-day precision, and repeatability were 1.18–2.03%, 2.01–2.71%, and 1.28–2.45%, respectively, indicating high precision and good repeatability. The five analytes were stable in prepared sample solution when placed in the autosampler at 4 °C for 24 h, with the RSD of 1.18–2.13%. Five replicate tests were performed. The developed method was also accurate with recoveries of 98.12–100.81%, and with the RSD of 1.05–1.91%. The above data showed that the developed method was sensitive and accurate for quantitative determination of these analytes. The results are summarized in Table 1.

Quantitative analysis

The developed UHPLC-PDA analytical method was subsequently applied to the simultaneous determination of *G. veitchiorum* from different regions in China. The representative chromatogram was shown in Fig. 1(A,C). The five analytes (swertiamarin,

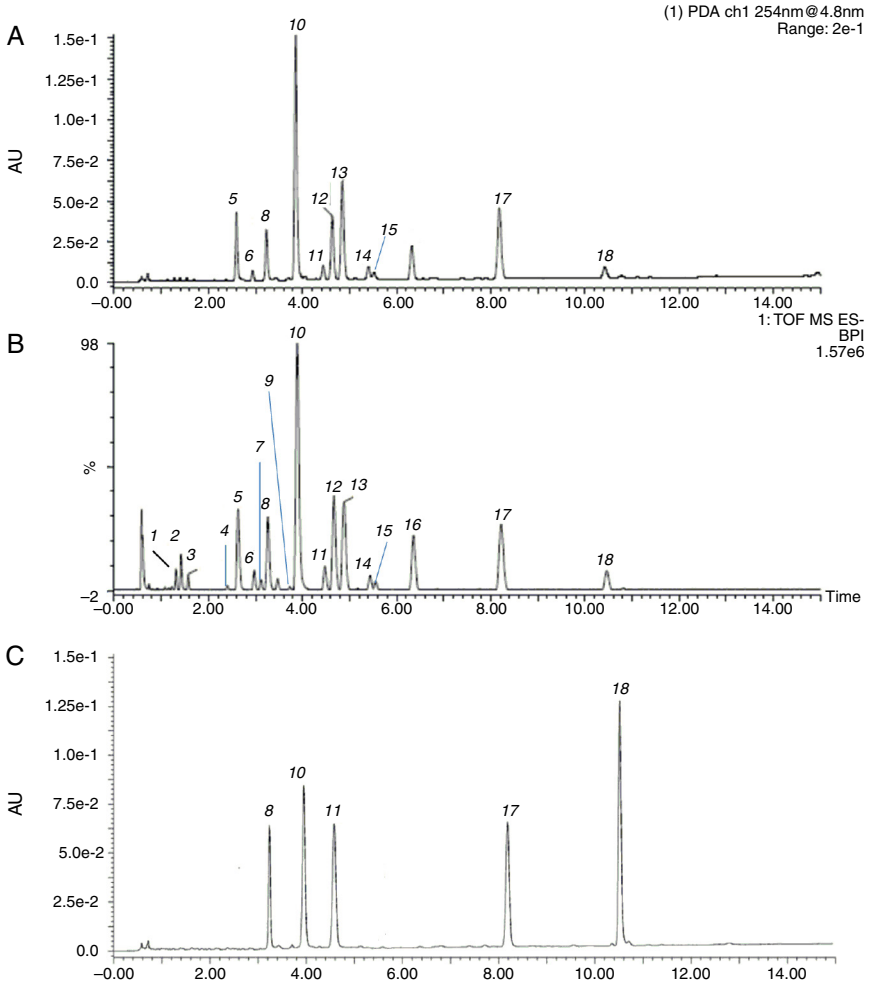

Fig. 1. The representative PDA and BPI chromatogram of *Gentiana veitchiorum* based on UHPLC-PDA-QTOF-MS analysis (A, PDA chromatogram; B, BPI chromatogram; C, standards: 8, swertiamarin; 10, gentiopicroside; 11, sweroside; 17, isoorientin; 18, isovitexin).

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Table 2
Quantification of swertiamarin, gentiopicroside, sweroside, isoorientin, and isovitexin from different spot samples of *Gentiana veitchiorum*.

Samples	Swertiamarin ($mg\,g^{-1}$)	Gentiopicroside ($mg\,g^{-1}$)	Sweroside ($mg\,g^{-1}$)	Isoorientin ($mg\,g^{-1}$)	Isovitexin ($mg\,g^{-1}$)
S2015001 from Hongyuan, Sichuan	5.49 ± 0.02	2.69 ± 0.02	5.82 ± 0.13	6.02 ± 0.09	3.69 ± 0.02
S2015002 from Kangding, Sichuan	5.06 ± 0.05	3.01 ± 0.03	5.56 ± 0.22	5.81 ± 0.14	3.01 ± 0.08
S2015003 from Luhuo, Sichuan	2.62 ± 0.02	2.47 ± 0.02	4.92 ± 0.15	5.92 ± 0.05	2.66 ± 0.05
S2015004 from Ruoergai, Sichuan	4.89 ± 0.04	2.46 ± 0.04	5.52 ± 0.21	4.82 ± 0.12	2.46 ± 0.07
S2015005 from Hainanzhou, Qinghai	2.01 ± 0.10	2.23 ± 0.02	2.44 ± 0.08	3.74 ± 0.08	3.21 ± 0.04
S2015006 from Linzhi, Tibet	3.07 ± 0.12	3.01 ± 0.06	3.51 ± 0.14	2.91 ± 0.14	3.00 ± 0.08
S2015007 from local market of Sichuan	2.13 ± 0.09	2.54 ± 0.06	4.15 ± 0.21	3.15 ± 0.03	2.50 ± 0.11
S2015008 from local market of Sichuan	2.98 ± 0.05	2.21 ± 0.09	3.67 ± 0.16	3.67 ± 0.18	2.44 ± 0.12
S2015009 from local market of Sichuan	2.24 ± 0.03	2.78 ± 0.09	3.24 ± 0.16	2.34 ± 0.16	2.61 ± 0.03
S2015010 from local market of Sichuan	3.65 ± 0.11	2.12 ± 0.02	4.12 ± 0.43	4.21 ± 0.07	2.66 ± 0.05

gentiopicroside, sweroside, isoorientin, and isovitexin) were representative compounds of iridous and flavone glycosides in the family of Gentianaceae and had been reported to exhibit significantly cytoprotective effects (Oztürk et al., 2006). Therefore, the developed UHPLC-PDA method could be used for comprehensive evaluation of the quality of *G. veitchiorum*. The results of determining standards analytes of *G. veitchiorum* from different collecting spots were shown in Table 2. It was found that the five analytes can be detected in all of the samples. The total contents of the five analytes in samples from Hongyuan and Kangding counties of Sichuan province were higher than the values from other places. The contents of the five analytes of samples from Qinghai were the lowest. The differentiation may be due to differences in climate, soil, and ecological environment.

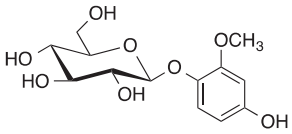
In contrast with a recent study that reported the use of HPLC coupled with UV to establish chromatographic fingerprints and determining the content of one compound in *G. Veitchiorum* (Wang, 2010), the present work identified eighteen compounds within a short period of 15 min, particularly, the simultaneous quantitative analysis of the five main constituents which were difficult to isolate using conventional methods. This result indicated that the developed method was more sensitive and time-saving than the previously reported procedure.

Identification of the detected components of Gentiana veitchiorum

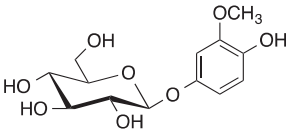
The ethanol extracts of *G. veitchiorum* were analyzed by UHPLC gradient elution with QTOF-MS detection for the first time. To achieve the highest sensitivity for the chemical constituents, the ionization of compounds of *G. veitchiorum* was performed in both negative and positive ion modes. The results showed that negative mode was more sensitive for the detection of these compounds. Typical MS basic peak ion (BPI) chromatogram trace, with numbered peaks of *G. veitchiorum*, was illustrated in Fig. 1(B).

In total, eighteen compounds were well detected in the developed method and tentatively or unambiguously characterized from *G. veitchiorum*. Formic acid was added in the mobile phase in order to improve chromatographic peak resolution and generate adducts $[M-H+HCOOH]^-$, which were helpful for the confirmation of deprotonated $[M-H]^-$, protonated ions $[M+H]^+$ or sodium adducts $[M+Na]^+$.

Compounds **1** and **2** at 1.31 min and 1.42 min displayed a similar deprotonated ion $[M-H]^-$ at *m/z* 301.0914 and 301.0918 with the same molecular formula $C_{13}H_{18}O_8$. The fragment information of **1** at *m/z* 139.0391 $[M-H-162]^-$ and 124.0155 $[M-H-162-15]^-$ could be explained by the loss of one glucosyl together with a methyl groups while compound **2** was observed the loss of the aglycon to form glucosyl ions at *m/z* 179.0555 $[Glu-H]^-$ and 161.0443 $[Glu-H-H_2O]^-$. Compounds **1** and **2** should be assigned as the isomers via their high-resolution mass values. The *Clog P* values of the two compounds were calculated using ChemDraw software. Compound **1** has smaller *Clog P* value while Compound **2** has higher value. They were −0.8251 and −0.7251, respectively. The *Clog P* value of one compound, which was the logarithm of its partition coefficient between *n*-octanol and water log ($C_{octanol}/C_{water}$), was a well-established measure of a compound hydrophilicity. Therefore, compound **1** was firstly eluted. Compounds **1** and **2** were tentatively identified as isotachioside and tachioside (Xiao et al., 2010), respectively. The two compounds were separated and identified for the first time in *G. veitchiorum*.



1



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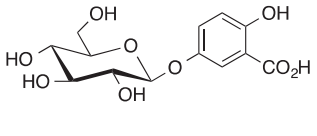
Compound **3** was identified as an organic acid, which showed a deprotonated ion $[M-H]^-$ at *m/z* 315.0707 with the molecular formula $C_{13}H_{16}O_9$. The fragment ion at *m/z* 153.0190 suggesting the loss of a glucose residue $[M-H-162]^-$. The fragment information at *m/z* 109.0284 $[M-H-162-CO_2]^-$ could be explained by the loss of carbon dioxide. Compound **3** was tentatively identified as 5-(β-D-glucopyranosyl)-2-hydroxybenzoic acid (Yeon et al., 2013).

Mass spectrum of compound **4** showed the deprotoned ion $[M-H]^-$ at *m/z* 537.1819 with the molecular formula $C_{23}H_{34}O_{15}$, The fragment information at *m/z* 375.1302 $[M-H-162]^-$ and *m/z* 213.0771 $[M-H-162-162]^-$ could be explained by the consecutive elimination of two hexoses. The structure of this compound was tentatively identified as 6′-*O*-β-D-glucosyl-loganic acid and it has been previously reported in the Gentianaceae family (Suryawanshi et al., 2006).

Compound **5** at 2.63 min showed a deprotonated ion $[M-H]^-$ at *m/z* 375.1283 with the molecular formula $C_{16}H_{24}O_{10}$ in the negative ionization mode. The sodium adduct $[M+Na]^+$ was observed in positive ionization mode. Further CID–MS^2 scan showed that they produced fragment ions at *m/z* 213.0756 $[M-H-Glc]^-$, which could be due to the loss of a hexose. Comparing the data with literature, compound **5** was tentatively characterized as loganic acid and it has been also reported in members of the Gentianaceae family (Suryawanshi et al., 2006).



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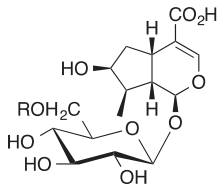
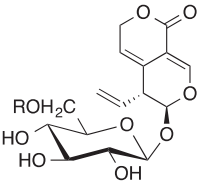


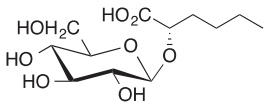
4 =RGlc
5 R=H

Compound **6** at 2.97 min showed an adduct at *m/z* 563.1611 $[M-H+HCOOH]^-$ which confirmed the molecular formula $C_{22}H_{30}O_{14}$ in the negative ionization mode. Sodium adduct at *m/z* 541.1531 $[M+Na]^+$ was observed in positive ionization mode. Further CID–MS^2 scan displayed fragment ions at *m/z* 341.1089 $[2Glu-H]^-$ and *m/z* 179.0557 $[Glu-H]^-$, indicating two glucosyl groups. The further fragments at *m/z* 161.0445, 149.0594, 119.0338, 112.9845 were produced from the glucosyl ion. Therefore, compound **6** was tentatively identified as 6′-*O*-β-D-glucosyl-gentiopicroside (Kakuda et al., 2001).

Compound **7**, the deprotonated ion peak was observed at *m/z* 293.1231 $[M-H]^-$ with the molecular formula $C_{12}H_{22}O_8$ and fragment ions at *m/z* 248.9602 could be explained by the loss of carbon dioxide $[M-H-CO_2]^-$. The structure of this compound was tentatively identified as 2-*O*-glucosyl-hexanoic acid.



6 R=Glc
10 R=H



7

Compounds **9** and **15** at 3.74 min and 5.55 min displayed a similar deprotonated ion peak at *m/z* 755.2043 and 755.2044 with the same molecular formula $C_{33}H_{40}O_{20}$. The fragmentation information at *m/z* 431.0984 $[M-H-2Glc]^-$ could be explained by the loss of two *O*-glucosyl groups, and the strong peaks at *m/z* 341.0653 $[M-H-2Glc-90]^-$ and *m/z* 311.0557 $[M-H-2Glc-120]^-$ indicated another *C*-glucosyl group. Moreover, the ions of **9** and **15** at *m/z* 413.0866 and 413.0872 $[M-H-2Glu-H_2O]^-$ confirmed the existence of the *C*-glucosyl group and located its position at C-6 of the flavone skeleton (Cuyckens and Claeys, 2004). Compounds **9** and **15** may be the isomers with three glucosyl groups. The *Clog P* values of the two compounds were calculated. Compound **9** has the smaller *Clog P* value while compound **15** has the higher *Clog P* value. They were -2.93 and -2.87, respectively. Therefore, compounds **9** and **15** were tentatively identified as isovitexin 4′-*O*-diglucoside and isovitexin 7-*O*-diglucoside (Xu et al., 2009).

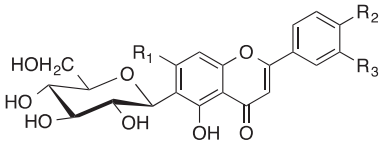
Compound **12** at 4.66 min presented a deprotonated ion peak at *m/z* 593.1504 with a molecular formula $C_{27}H_{30}O_{15}$. The fragmentation information at *m/z* 503.1197 $[M-H-90]^-$, 473.1085 $[M-H-120]^-$, 341.0661 $[M-H-90-162]^-$, and 311.0551 $[M-H-120-162]^-$ indicated one *C*-glucosyl group together with an *O*-glucosyl group (Cuyckens and Claeys, 2004). Fragmental ions at *m/z* 282.0522 and 283.0571 were similar to compound **18**, suggesting the aglycon to be the same as that of isovitexin. Thus, the structure of this compound was tentatively confirmed as isosaponarin (Bergeron et al., 1997).

Compounds **13** and **16** at 4.88 min and 6.36 min displayed their deprotonated ion peaks at *m/z* 609.1457 and 609.1446 with the same molecular formula $C_{27}H_{30}O_{16}$. The fragment information at *m/z* 447.0926 $[M-H-162]^-$ and 285.0393 $[M-H-162-162]^-$ could be explained by the loss of two consecutive hexose units. Two strong peaks at *m/z* 357.0602 $[M-H-162-90]^-$, and 327.0499 $[M-H-162-120]^-$ indicated that one of the hexoses was C-glucosyl, the other is O-glucosyl group. Compounds **13** and **16** must be isomers. Compound **13** has the smaller *Clog P* value while compound **16** has the higher *Clog P* value. They were −1.98 and −1.63, respectively. Therefore, compounds **13** and **16** were tentatively identified as isoorientin 4′-*O*-glycoside (Schaufelberger and Hostettmann, 1987) and lutonarin (Liu et al., 2005), respectively. The two compounds were separated and identified for the first time in *G. veitchiorum*.

Compound **14** displayed a molecular ion at *m/z* 771.1984 $[M-H]^-$ with a molecular formula $C_{33}H_{40}O_{21}$. The deprotonated ion $[M-H]^-$ showed the fragmental ions of *m/z* 447.0927 $[M-H-2Glc]^-$, *m/z* 357.0605$[M-H-2Glc-90]^-$, and *m/z* 327.0499 $[M-H-2Glc-120]^-$. Moreover, the aglycon ion of compound **14** was very similar with that observed for compound **17**. Therefore, compound **14** was tentatively identified as isoorientin 4′-diglucoside (Xu et al., 2009).

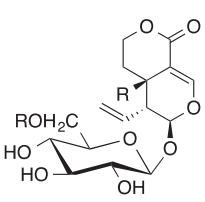
The deprotonated ion peak of compound **17** was observed at *m/z* 447.0925 $[M-H]^-$ with the molecular formula $C_{21}H_{20}O_{11}$ and fragment ions at *m/z* 357.0601 $[M-H-90]^-$, 327.0503 $[M-H-120]^-$ and 285.0393 $[M-H-162]^-$, which could be explained by partially or fully losing of a C-glucosyl group. In addition, RDA cleavage ions of flavonoids were also observed at *m/z* 161.0244 and 117.0335. The structure of this compound was tentatively confirmed as isoorientin, and it has been previously reported in Gentianaceae (Wang et al., 2008; Sasaki et al., 2015).

Compound **18** displayed a deprotonated ion peak at *m/z* 431.0976 $[M-H]^-$ with a molecular formula $C_{21}H_{20}O_{10}$. Furthermore, CID–MS^2 scan showed that they produced fragment ions at *m/z* 311.0554 $[M-H-90]^-$ and 283.0601 $[M-H-120]^-$ showing *C*-glucosyl group, together with RDA cleavage ions of flavonoids at *m/z* 161.0244 and 117.0335, which agreed with previously reported data for isovitexin, which has been reported in the Gentianaceae family (Huang et al., 2015).



9 R_1=OH; R_2=$OGlc^6$-1Glc; R_3=H
12 R_1=OH; R_2=OGlc; R_3=H
13 R_1=OH; R_2=OGlc; R_3=OH
14 R_1=OH; R_2=$OGlc^6$-1Glc; R_3=OH
15 R_1=$OGlc^6$-1Glc; R_2=OH; R_3=H
16 R_1=OGlc; R_2=R_3=OH
17 R_1=R_2=R_3=OH
18 R_1=R_2=OH; R_3=H

Compounds **8**, **10**, and **11** were unambiguously identified as swertiamarin, gentiopicroside, and sweroside by comparing the retention time, UV spectra and MS^2 fragmentation pattern with those of the commercial standards, respectively. The characterization of the remaining fifteen constituents was based on chromatographic retention times, MS fragmentation behaviors, and published data (Table 3). Among these identified compounds, gentiopicroside as the key constituent, played the most important role in the bioactivities described for the herbal remedy, which showed analgesic and anti-inflammatory activities, the protection of liver and pulmonary injuries, and the promotion of bile secretion (Liang et al., 2011).



8 R=OH
11 R=H

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Table 3
Characterization of chemical constituents in *G. veitchiorum* by UHPLC-QTOF-MS.

Peak No.	Retention (min)	Molecular formula	$[M-H]^-$ (error (ppm))	MS/MS fragments ions	$[M+Na]^+$	Identified compounds
1	1.31	$C_{13}H_{18}O_8$	301.0914(−3.0)	139.0391, 124.0155	325.0894	Isotachioside
2	1.42	$C_{13}H_{18}O_8$	301.0918(−1.7)	179.0555, 161.0443, 121.0283, 119.0338	325.0894	Tachioside
3	1.57	$C_{13}H_{16}O_9$	315.0707(−2.9)	153.0190, 109.0284		5-(*β*-D-glucopyranosyl)-2-hydroxybenzoic acid
4	2.40	$C_{23}H_{34}O_{15}$	537.1819(0.0)	375.1302, 213.0771, 169.0865, 129.9758, 113.0236		6′-*O*-*β*-D-glucosyl-loganic acid
5	2.63	$C_{16}H_{24}O_{10}$	375.1283(−2.1)	213.0756, 169.0860, 151.0754, 113.0232	399.1270	Loganic acid
6	2.97	$C_{22}H_{30}O_{14}$	563.1611(−0.7)	517.1556, 341.1089, 221.0656, 179.0557, 161.0445, 149.0594, 119.0338, 112.9845	541.1531	6′-*O*-*β*-D-glucosyl-gentiopicroside
7	3.11	$C_{12}H_{22}O_8$	$[M-H+HCOOH]^-$ 293.1231(−1.7)	248.9602, 229.0387, 131.0701, 113.0231		2-*O*-glucosyl-hexanoic acid
8	3.26	$C_{16}H_{22}O_{10}$	419.1182(−1.9)	409.0893, 179.0551, 161.0446, 141.0180, 119.0336, 112.9843		Swertiamarin
9	3.74	$C_{33}H_{40}O_{20}$	$[M-H+HCOOH]^-$ 755.2043(−1.3)	431.0984, 413.0866, 341.0653, 311.0557, 146.9635, 112.9853		Isovitexin 4′-*O*-diglucoside
10	3.89	$C_{16}H_{20}O_9$	401.1081(−0.7)	391.0801, 355.0004, 179.0558	379.1001	Gentiopicroside
11	4.48	$C_{16}H_{22}O_9$	$[M-H+HCOOH]^-$ 403.1234(−1.5)	393.0944, 357.1202, 195.0662, 125.0232	409.1588	Sweroside
12	4.66	$C_{27}H_{30}O_{15}$	$[M-H+HCOOH]^-$ 593.1504(−0.3)	503.1197, 473.1085, 341.0656, 311.0551, 283.0571, 282.0522	617.1483	Isosaponarin
13	4.88	$C_{27}H_{30}O_{16}$	609.1457(0.3)	489.1037, 447.0926, 357.0605, 327.0499, 298.0467, 285.0393	633.1427	Isoorientin 4′-*O*-glycoside
14	5.43	$C_{33}H_{40}O_{21}$	771.1984(0.9)	447.0927, 429.0822, 357.0605, 327.0499, 285.0394, 112.9850	485.1429	Isoorientin 4′-diglucoside
15	5.55	$C_{33}H_{40}O_{20}$	755.2044(1.2)	575.1420, 431.0979, 413.0872, 341.0658, 311.0553, 282.0522, 161.0239, 112.9847		Isovitexin 7-*O*-diglucoside
16	6.36	$C_{27}H_{30}O_{16}$	609.1446(−1.6)	519.0013, 489.1036, 447.0926, 357.0602, 327.0496, 284.0318	633.1429	Lutonarin
17	8.22	$C_{21}H_{20}O_{11}$	447.0925(−0.4)	357.0601, 327.0494, 298.0469, 285.0393, 163.0030, 133.0282	471.0900	Isoorientin
18	10.47	$C_{21}H_{20}O_{10}$	431.0976(−0.5)	311.0548, 283.0601, 161.0244, 117.0335	455.0945	Isovitexin

Conclusions

The present work is the first report of an accurate, rapid, and reliable analytical method for the simultaneous quantitation and identification of chemical constituents in *G. veitchiorum* by UHPLC-PDA-QTOF-MS. Eighteen compounds were successfully separated by UHPLC and identified or tentatively characterized by performing exact mass neutral loss scan together with MS and MS/MS spectra of QTOF-MS, including six iridoid glycosides, eight flavonoids, three phenolic glycosides, and one glucosylated organic acid. Three iridoid glycosides and two flavonoids were simultaneously determined and quantified in all samples under one run. Therefore, the developed method was a useful and reliable quality control method for a rapid identification and quantification of the chemical constituents of *G. veitchiorum*.

Authors contributions

LS performed the laboratory work and drafted the paper. WCX contributed in MS data analysis; HLL and YZG were responsible for HPLC data analysis; WKS and YMH collected all of the plant samples; ZZF designed the study and supervised the overall project work. Authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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