Article

Isolation and Simultaneous Quantification of Nine Triterpenoids from *Rosa davurica* Pall.

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Abstract

*Rosa davurica* Pall. has been used as a traditional Oroqen medicine to treat dyspepsia, gastroenterologia, menoxenia and other diseases. In this study, the chemical constituents research on the fruit pulp of *R. davurica* led to the isolation and identification of nine bioactive pentacyclic triterpenoids and five of them were isolated from the plant for the first time. Subsequently a simple and rapid high-performance liquid chromatography (HPLC) method was established for the simultaneous quantification of the nine triterpenoids. The separation was performed on a Merges reverse phase (RP) C\textsubscript{18} (250 × 4.6 mm, 5 μm) column through 40 min gradient delivery of 0.05% phosphoric acid aqueous solution and acetonitrile at a flow rate of 1.2 mL/min at 30 °C and the detection wavelength selected was 210 nm. All of the calibration curves showed good linearity (R\textsuperscript{2} > 0.9990) in the tested ranges. The limit of detection and the limit of quantitation were in the range of 0.21–1.27 μg/mL and 0.63–3.80 μg/mL, respectively. The established method also showed good precision, repeatability and recovery and can be used to the routine quality control of *R. davurica* and other herbs containing pentacyclic triterpenoids.

Introduction

*Rosa davurica* Pall. (Rosaceae) is a deciduous shrub, mainly distributed in the north of China and Japan (1). As a traditional Oroqen medicine, *R. davurica* has long been used to treat various diseases, such as dyspepsia, gastroenterologia, arteriosclerosis, pulmonary tuberculosis, frostbite and menoxenia (2). Modern pharmacological research has revealed that *R. davurica* exhibited the effects of anti-tumor, antiaging, enhance immunity, protecting liver and prevention of cardiovascular diseases (3). In clinical application, it is included in remedies alone or in combination with other herbs to treat thoracalgia induced by cardiovascular diseases and hyperlipidemia, such as Meiguo syrup and Shanmei capsule. Additionally, the fruits of *R. davurica* are often produced to be all kinds of beverages as healthful drinks due to many bioactive and nutritional components in it. *Rosa davurica* contains triterpenoids, flavonoids, tannins and various vitamins (3), in which triterpenoids and flavonoids are considered as the active ingredients in this plant. However, in the pericarps of *R. davurica*, triterpenoids are proved to be the major components (4, 5). Although the chemical research on the roots and leaves of *R. davurica* have been conducted (1, 6), the report of chemical constituents in the pericarps is rare (7, 8). Meanwhile, a simple and precise simultaneous quantification of multiple bioactive triterpenoids in the fruits of *R. davurica* using high-performance liquid chromatography (HPLC) is seldom established so far, due to similar structures and physicochemical properties of triterpenoids leading to difficult separation (9, 10).

In this study, the chemical constituents of the fruit pulp of *R. davurica* were studied and nine pentacyclic triterpenoids (1–9) were isolated and their structures were elucidated on the basis of physicochemical properties and nuclear magnetic resonance (NMR) spectra data. These triterpenoids were euscaphic acid (1), 2-oxo-pomolic acid (2), pomolic acid (3), 3-oxo-19α-hydroxyurs-12-en-28-oic acid (4), ursolic acid lactone (5), betulinic acid (6), ursolic acid (7), betulin (8) and uvaol (9). In these isolated components, compounds 2, 4, 5, 8 and 9 were obtained from *R. davurica* for the first time. A number of literature have frequently reported that these pentacyclic triterpenoids showed...
Various biological activities. Ursolic acid showed a significant effect on antitumor and could effectively decrease blood glucose levels in diabetic mice (11). Betulic acid and betulin had a great potential in anti-HIV and antitumor (12–14). Euscaphic acid could effectively prevent and treat type II diabetes (15). Pomolic acid and 2-oxo-pomolic acid could obviously decrease the melanin content in intracellular of B16-F10 mouse melanoma cells (16). Although these triterpenoids were proved to be the main bioactive components of the R. davurica, especially in the fruit, to date, the simultaneous quantification of these triterpenoids was not reported. Hence, in this study, a simple, rapid and precise HPLC method was established for the simultaneous quantification of the nine triterpenoids in the fruit pulp of R. davurica and the method was proved to be sensitive and reliable according to various validation parameters. This is the first report on simultaneous quantification of the nine triterpenoids in R. davurica by HPLC method and it is feasible for the quality control of the fruit pulp of R. davurica.

**Experimental**

**Instruments, chemicals and reagents**

The NMR spectra including $^1$H and $^{13}$C-NMR were determined on a Bruker AMX-400 spectrometer (Bruker, Germany) with tetramethylsilane (TMS) as an internal standard. Semi-preparative HPLC was performed on a LC-20 (Shimadzu, Japan) instrument equipped with a SPD-M20A detector and a RID-10A detector. Analytical HPLC was performed on a LC-2010A (Shimadzu, Japan) instrument equipped with a low pressure quaternionic pump solvent management system, an online degasser, an auto-sampler and an UV-Vis detector.

Silica gel was obtained from Qingdao Marine (Qingdao, China), Sephadex LH-20 was obtained from GE Healthcare Bio-Sciences AB (Sweden), Acetonitrile (HPLC-grade) was obtained from Kemio Co. (Tianjin, China). Chemicals (analytical-grade) were purchased from Fuyu Chemical Reagent Co. (Tianjin, China).

**Materials**

The fruits of R. davurica were collected in August 2011 from Heihe region of Heilongjiang Province in China. The plant was authenticated by Professor Junxian Wang and a voucher specimen (R. davurica 20110803) was deposited in the laboratory of School of Pharmacy, Xi’an Jiaotong University.

**Isolation of the chemical constituents**

Air dried fruit pulp of R. davurica (3.2 kg) was extracted with methanol (15 L) under reflux for three times (3 h for each time) and then the solution was concentrated in vacuum to give a methanol extract (290.2 g). The partial extract (280.0 g) was suspended in water and successively partitioned with petroleum ether, chloroform, ethyl acetate and $n$-butanol to afford petroleum ether fraction (28.0 g), CHCl$_3$ fraction (35.1 g), EtOAc fraction (38.2 g), $n$-BuOH fraction (78.5 g) and H$_2$O fraction (100.2 g), respectively. With the methods of silica gel column chromatography, Sephadex LH-20, preparative thin-layer chromatography and HPLC, nine triterpenoids were isolated from CHCl$_3$ fraction and EtOAc fraction. The detailed purification process of these compounds is shown in Figure 1.

**Characterization of isolated compounds**

The structural characterization of the nine triterpenoid components were carried out by spectroscopic techniques ($^1$H-NMR, $^{13}$C-NMR). The detailed information of spectra and spectral data are displayed in the Supplementary Information. The $^{13}$C-NMR data of compounds 1–9 are displayed in Supplementary Table I and the $^1$H-NMR and $^{13}$C-NMR spectra of compounds 1–9 are shown in Supplementary Figure 1. The structures of these nine triterpenoids were identified by comparing their spectral data and physicochemical properties with those reported in the literature.

**HPLC analysis**

**Preparation of standard and sample solution**

The stock solutions (0.5 mg/mL) of nine analytes were prepared by precisely weighting and dissolving each in methanol. A mixed standard stock solution was prepared by adding appropriate volume of each stock solution to produce a solution containing 100 μg/mL of each analyte (except for 200 μg/mL of pomolic acid). The mixed stock solution was further diluted to six concentrations. All stock solutions were stored at 4°C and filtered through a 0.45-μm membrane filter before injection (17, 18).

The fruit pulp of R. davurica (2.0 g) was extracted three times with 50 mL methanol under reflux (3 h for each time) and then the solution was concentrated in vacuum to give the methanol extract. The MeOH extract (100.0 mg) of the fruit pulp of R. davurica was accurately weighted and ultrasonically dissolved with 10 mL methanol. The sample solution was stored at 4°C and filtered through a 0.45-μm membrane filter before injection.

**Chromatographic system**

The chromatographic analysis and quantification were carried out with a LC-2010A (Shimadzu, Japan) LC system equipped with a low pressure quaternionic pump solvent management system, an online degasser, an auto-sampler and an UV-Vis detector. A Merges RP-C$_{18}$ (250 × 4.6 mm, 5 μm) column was used for LC separation. The mixture of 0.05% phosphoric acid aqueous solution (A) and acetonitrile (B) was used for gradient elution. The gradient schedule was (i) 0–8 min, 15–75% B; (ii) 8–30 min, 75% B; (iii) 30–32 min, 75–98% B; (iv) 32–40 min, 98–100% B. The column temperature was maintained at 30°C during the analytical procedure. The solvent flow rate was 1.2 mL/min and the detection wavelength was 210 nm. The injection volume was 20 μL.

**Results**

**Phytochemistry**

Through various chromatographic methods, nine pentacyclic triterpenoids were isolated from the fruit pulp of R. davurica. By comparison of their spectral data with those already reported in the literature, these compounds were identified as euscaphic acid (1) (19), 2-oxo-pomolic acid (2) (16), pomolic acid (3) (20), 3-oxo-19α-hydroxyurs-12-en-28-oic acid (4) (21), ursolic acid lactone (5) (22), betulic acid (6) (23), ursolic acid (7) (24), betulin (8) (25) and uvaol (9) (26). In these isolated components, compounds 2, 4, 5, 8 and 9 were isolated from R. davurica for the first time. The structures of the compounds are displayed in Figure 2.
Optimization of chromatographic conditions

According to the UV spectra of the analytes, 210 nm was selected as the detection wavelength. To obtain good separation efficiency within a relative short analysis time, several chromatographic conditions were optimized. Different mobile phases systems (methanol and acetonitrile) and modifiers (formic acid, acetic acid and phosphoric acid); different types of C18 column (Thermo C18, 50 mm × 4.6 mm, 5 µm; TIANHE C18, 150 mm × 4.6 mm, 5 µm; SUNEK C18, 150 mm × 4.6 mm, 5 µm; Megres-C18, 250 mm × 4.6 mm, 5 µm); different column temperatures (25, 30, 35, 40 and 45°C) and different flow rates (0.9, 1.0, 1.1 and 1.2 mL/min) were compared.

Compared with acetonitrile system, the mobile phase containing methanol gave a more serious drift of baseline under gradient elution mode. This can be accounted for by the end absorption of methanol in 210 nm. Therefore, the mixture of acetonitrile and aqueous solution was selected as the mobile phase system. Because of the peak tailing of several peaks, modifier was added to aqueous solution to improve the peak shape. There were no obvious difference in the separation efficiency of using formic acid, acetic acid and phosphoric acid as the modifier, but phosphoric acid was selected considering phosphoric acid can achieve a more stable baseline. So a mixture of acetonitrile and 0.05% phosphoric acid aqueous solution was chosen finally. In the comparison of the four RP-C18 columns, the Megres-C18 column could achieve optimal efficiency in the separation of nine triterpenoids.

In the test of column temperature, the result showed that with the column temperature increasing, the analysis time became shorter; however, when the temperature increased to 45°C, ursolic acid lactone and betulic acid were partly overlapped as shown in Figure 3. Based on the comprehensive consideration of separation
Figure 2. Chemical structures of compounds 1–9. Euscaphic acid (1), 2-oxo-pomolic acid (2), pomolic acid (3), 3-oxo-19α-hydroxyurs-12-en-28-oic acid (4), ursolic acid lactone (5), betulic acid (6), ursolic acid (7), betulin (8) and uvaol (9).

Figure 3. (A) Chromatogram of the mixed standard solution. (B) Chromatogram of the MeOH extract of the fruit pulp of *R. davurica*. euscaphic acid (1), 2-oxo-pomolic acid (2), pomolic acid (3), 3-oxo-19α-hydroxyurs-12-en-28-oic acid (4), ursolic acid lactone (5), betulic acid (6), ursolic acid (7), betulin (8) and uvaol (9).
efficiency and analysis time, 30°C was chosen for the separation. The flow rate was also investigated and it revealed that 1.2 mL/min could afford a relatively short analysis time without changing the separation efficiency, so it was selected as the optimal flow rate.

Method validation

Linearity, limit of detection and limit of quantification

The linearity was studied through the triplicate analysis of mixed standard solution at six proper concentrations. The calibration curves were constructed by plotting the peak areas versus the concentrations of mixed standard solution. The mixed standard solution was further diluted to detect the limit of detection (LOD) and limit of quantification (LOQ) until the S/N ratios for the analytes were 3 and 10, respectively. The detail information of calibration curves, linear range, LOD and LOQ of the nine analytes are displayed in Table I.

Precision, repeatability, stability and recovery

Intraday and interday variance was determined for validating the precision of the method. The intraday precision was performed by measuring a standard solution six times during 1 day while the interday precision was evaluated over 3 consecutive days with two samples per day. Relative standard deviation (RSD), which is used for the evaluation of the precision, ranged from 1.21 to 3.22% (intraday) and 1.19 to 3.99% (interday). The repeatability of this method was evaluated by analyzing six sample solutions prepared in the same way in parallel. The RSD values of the nine analytes ranged from 2.12 to 6.43%. The stability was tested by analyzing the same sample at 0, 2, 4, 8, 12 and 24 h at room temperature and the RSD of the nine analytes ranged from 1.32 to 3.22%. For the further evaluation of the accuracy of the method, recovery test was performed by the method of standard addition. Known amount of stock solutions (0.5 mg/mL) of nine analytes [(1) 0.5 mL, (2) 0.05 mL, (3) 0.1 mL, (4) 0.05 mL, (5) 0.2 mL, (6) 0.2 mL, (7) 2.0 mL, (8) 0.1 mL, (9) 0.1 mL] was added to the accurately weighed R. davurica fruit pulp (1.0 g), extracted, processed, analyzed according to the previous way and six experiments were repeated. The recovery percentage was calculated by the following formula: Recovery (%) = (amount found – amount present)/ amount spiked × 100%. The recovery of the nine analytes ranged from 96.3 to 98.3%. All of this revealed the method of high precision, repeatability, stability and accuracy. The detail information is shown in Table II.

Sample analysis

The developed HPLC method was subsequently applied to simultaneous quantification of the nine triterpenoids in the fruit pulp of R. davurica. The chromatograms of mixed standards and sample solution are shown in Figure 4. The contents of the nine triterpenoids are displayed in Table III.

Discussion

Triterpenoids are the main active ingredients in many traditional Chinese medicine playing important roles in antitumor, antihyperlipidemia and antidiabetes (27, 28). Our previous activity assay demonstrated that the isolated triterpenoids were the active constituents of R. davurica playing the important roles in improving the glucose tolerance in mice and exerting cytotoxic activity in human bladder

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**Table I. Calibration Plots, LOD and LOQ**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Calibration curves</th>
<th>( R^2 )</th>
<th>Linear range (μg/mL)</th>
<th>LOD (μg/mL)</th>
<th>LOQ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( y = 1056x + 215493 )</td>
<td>0.9999</td>
<td>6.25–100</td>
<td>0.21</td>
<td>0.63</td>
</tr>
<tr>
<td>2</td>
<td>( y = 20225x - 3521.2 )</td>
<td>0.9997</td>
<td>2.50–100</td>
<td>0.83</td>
<td>2.50</td>
</tr>
<tr>
<td>3</td>
<td>( y = 8544.1x - 12609 )</td>
<td>0.9995</td>
<td>5.00–100</td>
<td>1.27</td>
<td>3.80</td>
</tr>
<tr>
<td>4</td>
<td>( y = 12493x + 60820 )</td>
<td>0.9999</td>
<td>2.50–100</td>
<td>0.77</td>
<td>2.30</td>
</tr>
<tr>
<td>5</td>
<td>( y = 501.3x - 1500.1 )</td>
<td>0.9992</td>
<td>2.50–100</td>
<td>0.93</td>
<td>2.80</td>
</tr>
<tr>
<td>6</td>
<td>( y = 9114.7x - 14498 )</td>
<td>0.9990</td>
<td>6.25–50</td>
<td>0.83</td>
<td>2.50</td>
</tr>
<tr>
<td>7</td>
<td>( y = 8240x + 36724 )</td>
<td>0.9991</td>
<td>12.50–200</td>
<td>0.77</td>
<td>2.30</td>
</tr>
<tr>
<td>8</td>
<td>( y = 5462.5x + 244932 )</td>
<td>0.9990</td>
<td>2.50–100</td>
<td>0.83</td>
<td>2.50</td>
</tr>
<tr>
<td>9</td>
<td>( y = 20073x + 92301 )</td>
<td>0.9999</td>
<td>6.25–100</td>
<td>0.67</td>
<td>2.00</td>
</tr>
</tbody>
</table>

\( R^2 \), correlation coefficient.

**Table II. Precision, Repeatability, Stability and Recovery of the Nine Analytes with \( n = 6 \)**

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Precision (RSD, %)</th>
<th>Repeatability (RSD, %)</th>
<th>Stability (24 h, RSD, %)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday</td>
<td>Interday</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.38</td>
<td>2.46</td>
<td>6.21</td>
<td>2.39</td>
</tr>
<tr>
<td>2</td>
<td>2.98</td>
<td>1.89</td>
<td>2.11</td>
<td>1.89</td>
</tr>
<tr>
<td>3</td>
<td>2.11</td>
<td>1.66</td>
<td>3.80</td>
<td>2.63</td>
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<td>4</td>
<td>1.73</td>
<td>2.01</td>
<td>5.11</td>
<td>2.52</td>
</tr>
<tr>
<td>5</td>
<td>1.49</td>
<td>1.11</td>
<td>4.21</td>
<td>1.32</td>
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<tr>
<td>6</td>
<td>2.84</td>
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<td>2.12</td>
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<td>8</td>
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<td>1.32</td>
<td>6.43</td>
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<td>9</td>
<td>2.08</td>
<td>1.88</td>
<td>5.21</td>
<td>3.22</td>
</tr>
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</table>
carcinoma T24 cells (data not published). Therefore, to establish a reliable method to simultaneously quantify the nine active triterpenoids components is significant for the quality control of R. davurica. However, these kinds of compounds are difficultly separated from each other due to their similar structures and properties, especially their close polarity and having no good absorbance in UV spectra. Nowadays, HPLC is a routine instrument and commonly used for the isolation and analysis of chemical components in herbs. Thus, to establish a simple and reliable HPLC method to simultaneously determine multiple triterpenoids components is more practical than other methods using the expensive instruments.

In this study, nine pentacyclic triterpenoids were isolated and identified from the fruit pulp of R. davurica by various chromatography methods, five of which were isolated from the plant for the first time. Subsequently, a simple, rapid and reliable HPLC method was established for the simultaneous quantification of the nine triterpenoids. The described HPLC method resulted in a good separation and minimum peak tailing by optimizing various conditions and parameters. The analytical method was fully validated and proved to be accurate as the recovery was >96% (Table II). Both intraday and interday precision were also found to be within 5%. The detector response was found to be linear over the selected concentration range. In addition, results of stability test showed that the time consumed for sample processing and storage of sample up to 24 h did not affect the results.

Table III. The Contents of Triterpenoids 1–9 in the Fruit Pulp of R. davurica with n = 3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Contents (mg/g)</th>
<th>RSD (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>0.260</td>
<td>2.227</td>
</tr>
<tr>
<td>2</td>
<td>0.025</td>
<td>3.332</td>
</tr>
<tr>
<td>3</td>
<td>0.578</td>
<td>1.997</td>
</tr>
<tr>
<td>4</td>
<td>0.024</td>
<td>4.001</td>
</tr>
<tr>
<td>5</td>
<td>0.100</td>
<td>2.543</td>
</tr>
<tr>
<td>6</td>
<td>0.093</td>
<td>3.209</td>
</tr>
<tr>
<td>7</td>
<td>1.095</td>
<td>1.222</td>
</tr>
<tr>
<td>8</td>
<td>0.038</td>
<td>2.319</td>
</tr>
<tr>
<td>9</td>
<td>0.032</td>
<td>3.329</td>
</tr>
</tbody>
</table>

Concluding remarks

In this study, nine active triterpenoids were isolated and elucidated from the fruit pulp of R. davurica and five compounds, namely 2-oxo-pomolic acid, 3-oxo-19α-hydroxyurs-12-en-28-oic acid, ursolic acid lactone, betulin and uvaol were isolated from R. davurica for the first time. Meanwhile, the established HPLC method was simple and reliable for simultaneous quantification of the isolated triterpenoids and the developed method would also be helpful for the quality evaluation of other herbs containing these components.

Supplementary data

Supplementary data are available at Journal of Chromatographic Science online.

Acknowledgements

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