**Full Length Research Paper**

**Liquid chromatographic (LC) determination of four bioactive compounds in the *Portulaca oleracea* L.**

Lan Cheng¹, Zhongzhe Cheng¹, Haixing Liu², Hui Zhang¹#, Wenjie Zhang¹, Yang Du¹, Yunjiao Wang¹, Haibo Li², Xixiang Ying¹ and Tingguo Kang¹*

¹School of Pharmacy, Liaoning University of Traditional Chinese Medicine, 77 Shengming 1 Road, DD Port, Dalian, 116600, China.

²School of Basic Medical Sciences, Liaoning University of Traditional Chinese Medicine, Shenyang, 79 Chongshang East Road, Shenyang 110032, China.

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The present article described a simple high-performance liquid chromatographic (HPLC) method for the simultaneous determination of hesperidin, caffeic acid, ferulic acid and *p*-coumaric acid in *P. oleracea* L., using a Phenomsil C₁₈ analytical column (5 µm, 250 × 4.6 mm, Feinami Technologies, Beijing, China) and mobile phase consisting of methanol-acetonitrile-tetrahydrofuran-0.1% glacial acetic acid (8:2:18:72, v/v/v/v). The calibration curves of the four components were linear (r²>0.9995) over the concentration range of 0.75 to 20.0 μg/ml. The mean recoveries were 96.9 to 98.5%. The results indicate that the HPLC method developed can easily be applied to the determination of four bioactive compounds in the *P. oleracea* L.

**Key words:** High-performance liquid chromatographic (HPLC), *Portulaca oleracea* L., simultaneous determination.

**INTRODUCTION**

*Portulaca oleracea* L., a well-known traditional Chinese medicine (TCM) recorded in the Chinese Pharmacopoeia, is used for the treatment of febrifuge, antiseptic, hemostasia and antidiarrhea (PRC, 2010). Some bioactive components in *P. oleracea* L. have been reported to be hesperidin (HP), caffeic acid (CA) (Yang et al., 2007), ferulic acid (FA) and *p*-coumaric acid (*p*-CA) (Xiang et al., 2005) (Figure 1). As a cosmopolitan plant, many attentions were paid on its pharmacological studies, such as antioxidant (Lim and Quah, 2007), neuropharmacological (Radhakrishnan et al., 2001) and anti-inflammatory (Chan et al., 2000) and so on. Also, these bioactive components contribute to many pharmacological actions such as HP for decreasing the cholesterol level in rat (Monforte et al., 1995), CA for inhibiting the tumor promotion and possessing antioxidant activity as well as FA (Huang et al., 1988; Maurya and Devasagayam, 2010) and *p*-CA for antibacterial and antifungal effects (Aziz et al., 1998), and so on. In previous literature, a spectrophotometric analysis was carried out to determine the appropriate content of total flavonoids (Zhu et al., 2010), and a capillary electrophoresis method with electrochemical detection was employed for quantitative determination of five flavonoids in *P. oleracea* L. (Xu et al., 2006). However, to our knowledge, there has been no published report of measuring four compounds (HP, CA, FA and *p*-CA) simultaneously with HPLC method. The aim of this study is to develop an assay to estimate the contents of four compounds with isocratic reverse phase HPLC method in *P. oleracea* L.

**EXPERIMENTAL**

**Plant material**

*P. oleracea* L. was collected from Shenyang (Liaoning, China) in September 2009, and identified by Professor Yanjun Zhai. Voucher

*Corresponding author. E-mail: kangtingguo@hotmail.com. Tel: +86-0411-87586005. Fax: +86-0411-87586078.

#This author contributed equally.
specimens (No. 2009090-2009100) were deposited at School of Pharmacy, Liaoning University of traditional Chinese medicine.

Reagents
HP, CA and FA were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and p-CA was provided by Nanchang Beta Biotech Co., Ltd. (Nangchang, China). Water was purified with Milli-Q® Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). Methanol, acetonitrile and tetrahydrofuran were all of HPLC grade were provided by Damao Chemical Reagent Plant (Tianjin, China). All other reagents were of analytical grade purchased from Jinfeng Chemical Factory (Tianjin, China).

Chromatographic system and conditions
HPLC analysis was carried out on an Agilent 1100 series HPLC (Palo Alto, CA, USA) incorporating a UV detector. The separation was obtained using a Phenomsil C18 column (5 µm, 250 × 4.6 mm, Feinami Technologies, Beijing, China) at ambient temperature. The mobile phase consisted of a mixture of methanol-acetonitrile-tetrahydrofuran-0.1% glacial acetic acid (8:2:18:72, v/v/v/v), which was passed under vacuum through a 0.45 µm membrane filter before use. The analysis was performed under isocratic conditions at a flow rate of 1 ml/min, injection volume of 20 µl and the total run time was 20 min. Chromatograms was recorded at 283 nm for HP, 322 nm for CA, FA and p-CA.

Sample preparation
The dried whole plant of *P. oleracea* L. (35 g) was extracted with 60% ethanol (350 ml) twice by refluxing 2 h, then collected and combined the filtrates, and ethanol was evaporated under reduced pressure. After that, the residue was passed through an AB-8 macro porous resin column (5 × 120 cm, Shanghai, China). To eliminate the impurity, the column was eluted with 200 ml water, and eluted with 400 ml of 60% ethanol. After removing the ethanol of 60% ethanol fraction in vacuo, the supernatant was dried on a water bath. Subsequently, the dried power (0.5 g) was extracted with 30 ml of a mixture of methanol-water (40:60, v/v) in an ultrasonic bath for 45 min. The supernatants were filtered with 0.45 µm membrane filter to obtain the filtered solution, and an aliquot (20 µl) of filtrate was injected into the high-performance liquid chromatographic (HPLC) system.

Method validation
Stock standard solutions of the HP, CA, and p-CA were prepared with methanol and FA with 70% methanol. The concentrations of HP, CA, FA and p-CA were 0.204, 0.208, 0.203, and 0.17 mg/ml, respectively. Seven calibrators of the four analytes were prepared by dilution of stock solutions. The calibration curves of four analytes were obtained by plotting the HPLC-generated peak areas versus the series of concentrations of dilute stock solutions. The data achieved from this analysis was subjected to regression analysis. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined from signal to noise ratio (S/N) of 3:1 and (S/N) of > 10, respectively. Assay precision was estimated by calculating the RSD (%) of six replicate injections of four standards and six different samples of *P. oleracea* L.

The recovery was determined by adding known amount of the standard substances, approximately 1 times the levels detected in unspiked samples prior to extraction, and these spiked samples were prepared by using the proposed procedure. The found amount of analyte subtracted that of unspiked sample was divided by the added known amount of the standard substance, and the extraction recovery was calculated as percentage.

For determination of stabilities, the same extracted samples solution were determine at 0, 6, 12, 18, and 24 h and the RSD (%) values were calculated.

RESULTS AND DISCUSSION

HPLC analysis
An isocratic liquid chromatographic (LC) method was obtained after investigating several mobile phases
HPLC chromatogram of four standard substances (1: HP, 2: CA, 3: FA, 4: p-CA) (A) and the *Portulaca oleracea* L. (B). Mobile phase was methanol-acetonitrile-tetrahydrofuran-0.1% glacial acetic acid (8:2:18:72, v/v/v/v); analytical column: Phenomsil C<sub>18</sub> column (5 µm, 250 × 4.6 m); flow rate: 1 ml/min; injection volume: 20 µl; detection at 283 nm for hesperidin, 322 nm for caffeic acid, ferulic acid and p-coumaric acid, respectively.

Figure 2. HPLC chromatogram of four standard substances (1: HP, 2: CA, 3: FA, 4: p-CA) (A) and the *Portulaca oleracea* L. (B). Mobile phase was methanol-acetonitrile-tetrahydrofuran-0.1% glacial acetic acid (8:2:18:72, v/v/v/v); analytical column: Phenomsil C<sub>18</sub> column (5 µm, 250 × 4.6 m); flow rate: 1 ml/min; injection volume: 20 µl; detection at 283 nm for hesperidin, 322 nm for caffeic acid, ferulic acid and p-coumaric acid, respectively.

consisting of mixtures of methanol, acetonitrile, tetrahydrofuran and water at different percents. In addition, to improve the peak shape and resolution, the addition of different ratios of glacial acetic acid in the solvent was tried. Eventually, a mixture of methanol-acetonitrile-tetrahydrofuran-0.1% glacial acetic acid (8:2:18:72, v/v/v/v) was used to obtain good elution for analytes. Our attempts using a single wavelength over the range of 280 to 330 nm to determine four analytes simultaneously were unsuccessful. However, stronger signals were observed when the wavelength was set at 283 nm for HP and 322 nm for CA, FA and p-CA, respectively. Figure 2 shows typical chromatograms of the standard substances (A), and the sample of *P. oleracea* L. (B), indicating the absence of interference from the adjacent peaks. The retention times for HP, CA, FA and p-CA were 8.0, 10.2, 11.8 and 14.7 min, respectively. The total run time was 20 min.

To optimize the extraction procedure, methanol, water and their combination with different ratios were tried, eventually, a mixture of methanol-water (40:60, v/v) gave the best recovery for the analytes.

Method validation

**Linearity, limit of quantification (LLOQ) and limit of detection (LOD)**

Seven-point calibration curve over the concentration range of 2.5 to 17.5, 0.75 to 10.0, 1.25 to 15.0 and 1.0 to 20.0 µg/ml for HP, CA, FA and p-CA, respectively, were used to evaluate the linearity. The regression equations and coefficients were showed in Table 1.

The limit of detection (LOD) of the analytes were 0.0310, 0.0374, 0.0226 and 0.0025 µg/ml, and the lower limit of quantification (LLOQ) of the four component, defined as the lowest concentration on the calibration curve, were 2.50, 0.75, 1.25, and 1.00 µg/ml, respectively.
Table 1. Linearities of the four investigated compounds.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Regression equation</th>
<th>r</th>
<th>Linear range (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP</td>
<td>$Y = 10.919X - 0.0756$</td>
<td>0.9995</td>
<td>2.50-17.5</td>
</tr>
<tr>
<td>CA</td>
<td>$Y = 41.489X - 0.0608$</td>
<td>0.9997</td>
<td>0.75-10.0</td>
</tr>
<tr>
<td>FA</td>
<td>$Y = 40.722X + 0.0351$</td>
<td>0.9998</td>
<td>1.25-15.0</td>
</tr>
<tr>
<td>p-CA</td>
<td>$Y = 25.427X - 0.0264$</td>
<td>0.9995</td>
<td>1.00-20.0</td>
</tr>
</tbody>
</table>

Table 2. Results of recovery experiments.

<table>
<thead>
<tr>
<th>S/N</th>
<th>HP (%)</th>
<th>CA (%)</th>
<th>FA (%)</th>
<th>p-CA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95.6</td>
<td>98.2</td>
<td>98.8</td>
<td>97.2</td>
</tr>
<tr>
<td>2</td>
<td>97.1</td>
<td>97.8</td>
<td>97.4</td>
<td>96.7</td>
</tr>
<tr>
<td>3</td>
<td>98.4</td>
<td>97.6</td>
<td>98.1</td>
<td>98.1</td>
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<tr>
<td>4</td>
<td>98.0</td>
<td>99.3</td>
<td>96.3</td>
<td>97.1</td>
</tr>
<tr>
<td>5</td>
<td>95.3</td>
<td>99.1</td>
<td>98.7</td>
<td>96.9</td>
</tr>
<tr>
<td>6</td>
<td>97.5</td>
<td>98.7</td>
<td>99.6</td>
<td>99.0</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>96.9±1.27</td>
<td>98.5±0.69</td>
<td>98.15±1.16</td>
<td>97.5±0.87</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.3</td>
<td>0.71</td>
<td>1.2</td>
<td>0.90</td>
</tr>
</tbody>
</table>

**Precision**

Six replicate injections of four standards were used to evaluate the intra and inter-day precision. Both the intra and inter-day precision for HP, CA, FA and p-CA were less than 0.8, 2.9, 1.9 and 1.4%, respectively. The precision was also estimated by analyzing six different accurately weighed and prepared samples of *P. oleracea* L. and the RSDs of the four analytes were no more than 0.6, 2.2, 2.9 and 1.8%, respectively, indicating the procedure of sample preparation has a good reproducibility.

**Recovery**

To validate the accuracy of the developed method, the recoveries of the four analytes were determined by six samples spiked with known amounts of each standard. The results showed that the mean recoveries ranged from 96.9 to 98.5% with RSD (%) values less than 1.3% (Table 2).

**Stability**

The stability test was carried out by determining the standard solution and sample solution respectively. The stock solutions of the four compounds were found to be stable at ambient temperature over the period of 0 to 24 h. The stabilities of the four analytes in the solution of a mixture methanol-water (40:60, v/v) were investigated during the storing period of 0 to 24 h and the results were not more than 3%, suggesting that the sample prepared solution was stable under the experimental conditions of the regularly analytical procedure.

**Simultaneously determine four components**

HPLC incorporating UV detector was employed to simultaneously determine the four bioactive components in the *P. oleracea* L. (*n* = 10). The contents of HP, CA, FA, and p-CA were 0.144±0.031, 0.055±0.012, 0.090±0.003 and 0.122±0.036 μg/g in *P. oleracea* L., respectively.

**Conclusions**

The isocratic HPLC method was developed for the simultaneous determination the four bioactive components in the *P. oleracea* L. The linearity, recovery and stability of the developed method were validated, respectively. The developed method can be helpful for quality control of *P. oleracea* L.. Moreover, further study is needed to clarify its potency and safety in future.

**ACKNOWLEDGEMENTS**

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REFERENCES


