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Qualitative and quantitative analysis of phenylpropanoids in cell culture, regenerated plantlets and herbs of *Saussurea involucrata*

Ridao Chen[a], Xiao Liu[a], Jianhua Zou[a], Lin Yang[b], Jungui Dai[a,*]

[a] *State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, PR China*

[b] *College of Life and Environmental Sciences, Minzu University of China, Beijing 100081, PR China*

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ABSTRACT

The major phenylpropanoids in the cell culture, regenerated plantlets and herbs of *Saussurea involucrata* were systematically and comparatively investigated. A total of 17 constituents were identified on the basis of HPLC-DAD/ESI-MS^n and HPLC-ESI-IT-TOF/MS analyses. Among them, 13 constituents were unambiguously identified by comparing the retention time, UV, MS and MS^n spectra of samples with standards/literature, and the other 4 constituents were tentatively assigned on the basis of their UV spectra and MS^n fragmentation patterns. In addition, a quantification method for the simultaneous quantification of 3 major phenylpropanoids syringin, 5-caffeoylquinic acid, and 1,5-dicaffeoylquinic acid was successfully established. The established HPLC quantification method was proved to have excellent linearity, precision, repeatability and accuracy. These studies provide a secondary metabolic profile of the cell cultures of *S. involucrata*, which is valuable for improving the quality control of cell culture and sheds light on the biosynthetic pathway of phenylpropanoids in this species.

1. Introduction

Saussurea involucrata Kar. et Kir. (family Asteraceae) is a rare and endangered traditional Chinese medicinal herb distributed on snowy mountains at altitudes of 4000–5000 m around the *Tianshan* and *A'er Tai* areas in China. Traditionally, it is mainly used for the treatment of rheumatoid arthritis and gynecological disorders [1]. However, due to over exploitation and difficult cultivation, the availability of this herb has become more and more limited. Because plant *in vitro* cultures are able to produce and accumulate many valuable secondary metabolites, cell culture of *S. involucrata* would be a desirable way to alleviate the scarcity of this herb and produce the important active compounds effectively, economically and in an environmentally friendly way [2,3].

During the systematic investigation of the chemical constituents of the cell cultures of *S. involucrata* [4], we found a special cell line possessing a strong ability for producing 1,5-dicaffeoylquinic acid, syringin and 5-caffeoylquinic acid, which are all biosynthesized in the phenylpropanoid pathway. These phenylpropanoids were present in the plants of *S. involucrata* in trace amounts, particularly, 1,5-dicaffeoylquinic acid and syringin [5]. Diverse physiological and pharmacological properties of these natural compounds have been reported in recent studies. For instance, syringin possessed anti-inflammatory and antinociceptive effects [6], hypoglycemic effect [7], antidepressant activity [8] and antitumor activity [9]. On the other hand, 5-caffeoylquinic acid, also named chlorogenic acid, is reported to have anti-inflammatory [10,11] and anti-hepatitis B virus activities [12]. Furthermore, 1,5-dicaffeoylquinic acid displayed potential effects on HIV [13,14] and anti-oxidation activity [15].

Cell culture of *S. involucrata* is an optional approach for producing these valuable phenylpropanoids, especially 1,5-dicaffeoylquinic acid, syringin and 5-caffeoylquinic acid. Accordingly, it is necessary to establish a valid analytical method to characterize the major phenylpropanoids and efficiently control the quality of the cell cultures. In the present study, the major phenylpropanoids in cell culture, regenerated plantlets and herbs of *S. involucrata* were identified by high-performance liquid chromatography coupled with diode array detection and electrospray ion trap tandem mass spectrometry (HPLC-DAD/ESI-MS^n) and liquid chromatography coupled with electrospray ionization hybrid ion trap and time-of-flight mass spectrometry (LC/ESI-IT-TOF-MS) analysis. In addition, an HPLC method for the simultaneous quantification of 1,5-dicaffeoylquinic acid, syringin and 5-caffeoylquinic acid in the cell cultures of *S. involucrata* was developed.

* Corresponding author. Tel.: +86 10 63165195; fax: +86 10 63017757.
E-mail address: jgdai@imm.ac.cn (J. Dai).

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2. Experimental

2.1. Materials and reagents

The herbs of *S. involucrata* were purchased from Xinjiang Uygur Autonomous Region and identified by Prof. Jungui Dai, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College. The cell cultures and regenerated plantlets were maintained in our laboratory [4,16]. Subculture of the cell cultures and regenerated plantlets was performed on solid and liquid MS basal medium [17] supplemented with 0.5 mg/L α-naphthalene acetic acid (NAA), 0.5 mg/L 6-benzyl aminopurine (6-BA), 0.2 mg/L 2,4-dichloro-phenoxyacetic acid (2,4-D), 30 g/L sucrose and 5 g/L agar. The media was adjusted to pH 5.8 with 1 M NaOH before autoclaving at 121 °C for 20 min.

1,5-Dicaffeoylquinic acid, syringin, 5-caffeoylquinic acid, rutin and hispidulin were isolated from the cell cultures or the herbs of *S. involucrata*. Their identities were confirmed by ^{1}H NMR, ^{13}C NMR and MS spectroscopic methods, and their purities were over 99% by HPLC analysis. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); water was purified and deionized by a water purification system from Tautobiotech (Shanghai, China). The formic acid and phosphoric acid used were of HPLC grade from Mreda (Columbia, USA). All the other chemicals used were of analytical grade for laboratory use.

2.2. Instrumentation and analytical conditions

2.2.1. HPLC-DAD/ESI-MS^n analysis

HPLC-DAD/ESI-MS^n analysis was performed on an Agilent 1200 series HPLC system (Agilent Technologies, Boeblingen, Germany) coupled with an LCQ Fleet ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) equipped with an electrospray ionization (ESI) source.

The LC chromatograph consisted of a quaternary pump equipped with an on-line solvent degasser unit, an auto sampler, a column temperature controller and a diode-array detector coupled with an analytical workstation. HPLC analysis was performed on a Shiseido capcell pak C18 MG II column (250 mm × 4.6 mm i.d., 5 μm) at a flow rate of 1 mL/min and the column temperature was maintained at 30 °C. The injection volume was 10 μL. The mobile phase was a gradient elution of solvents A (0.1% formic acid aqueous solution) and B (acetonitrile containing 0.1% formic acid). The gradient program was as follows: 0–30 min, linear change from A–B (92:8, *v/v*) to A–B (84:16, *v/v*); 30–31 min, linear change to A–B (76:24, *v/v*); 31–60 min, linear change to A–B (68:32, *v/v*); 60–70 min, linear change to A–B (40:60, *v/v*); and 70–75 min, linear change to A–B (0:100, *v/v*). The re-equilibration duration was 10 min between individual runs.

For HPLC-DAD/ESI-MS^n analysis, ultra-high purity helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. The optimized ESI source parameters were as follows: sheath gas flow rate, 20 (arbitrary units); auxiliary gas flow rate, 5 (arbitrary units); spray voltage, 5.0 kV; capillary temperature, 350 °C; source collision-induced decomposition (CID), 35 V; tube lens offset voltage, −75 V. The spectra were recorded in the range of *m/z* 100–1000 for full scan MS analysis. The split ratio of effluent from the LC to ion source was 2:1. The data were analyzed using the XCalibur software.

2.2.2. LC-ESI-IT-TOF/MS analysis

To confirm the elemental composition of molecular ions with high accuracy mass, an LC/ESI-IT-TOF-MS experiment was performed on a Shimadzu LC–MS-IT-TOF instrument equipped with a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan). The LC system consisted of a CBM-20A controller, two LC-20AD binary pumps, an SPD-M20A diode array detector, a SIL-20AC autosampler, a CTO-20A column oven and a DGU-20A5 degasser. The LC conditions were the same as those for LC-DAD/ESI-MS^n analysis. The split ratio of effluent from the LC to ion source was 5:1. The optimized MS conditions were as follows: negative ion mode; electrospray voltage, −3.5 kV; detector voltage, 1.7 kV; curved desolvation line (CDL) temperature, 200 °C; heat block temperature, 200 °C; nebulizing gas (N_2), 1.5 L/min; drying gas (N_2) pressure, 100 kPa; scan range, *m/z* 100–1000. Accurate mass determination was corrected by calibration using the sodium trifluoroacetate clusters as reference. The data acquisition and analysis were performed by LCMS Solution Version 3 software (Shimadzu, Kyoto, Japan). The formula predictor function of LCMS Solution was used to predict the chemical formulas.

2.2.3. HPLC–DAD quantitative analysis

For HPLC-DAD quantitative analysis, wavelengths were set at 265 nm for syringin and 327 nm for 1,5-dicaffeoylquinic acid and 5-caffeoylquinic acid. The mobile phase was a gradient elution of solvents A (0.2% phosphoric acid aqueous solution containing 50 mM KH_2PO_4) and B (acetonitrile). The gradient program was as follows: 0–20 min, linear change from A–B (92:8, *v/v*) to A–B (90:10, *v/v*); 20–50 min, linear change to A–B (70:30, *v/v*); and 50–60 min, linear change to A–B (50:50, *v/v*). The other HPLC conditions were the same as those for HPLC-DAD/ESI-MS^n analysis.

2.3. Analytical procedures

2.3.1. Preparation of sample solutions for qualitative and quantitative analyses

The cell cultures were harvested by filtration under reduced pressure after 6, 8, 10, 12, 14 and 16 days of cultivation, then washed with distilled water before being oven-dried at 50 °C to a constant weight. The regenerated plantlets (120 days) and the herbs were also dried under the same conditions. The dried sample was ground with a pestle and mortar to a fine powder (40 mesh) and stored at −20 °C until extraction. One hundred milligrams of powdered sample was weighed, put into a 10 mL-centrifuge tube with 10 mL of 70% aqueous methanol and extracted using ultrasound at room temperature for 15 min. For phenylpropanoid analysis, the solution was filtered through a membrane (0.45 μm pore size) and then injected into the LC.

2.3.2. Preparation of standard solutions for quantitative analysis

A methanol stock solution containing 1,5-dicaffeoylquinic acid (3.5 mg/mL), syringin (5.2 mg/mL) and 5-caffeoylquinic acid (6.1 mg/mL) was prepared and stored at 4 °C in the dark, then diluted to the appropriate concentration range for the establishment of calibration curves. Each calibration curve was analyzed three times with seven different concentrations using the same LC condition as described in Section 2.2.3.

3. Results and discussion

3.1. HPLC/MS analysis of phenylpropanoids

Liquid chromatography coupled with mass spectrometry is a useful tool for the rapid identification of chemicals in herbal extracts. In this work, the major phenylpropanoids in cell cultures, regenerated plantlets and wild plants of *S. involucrata* were investigated and identified by HPLC-DAD/ESI-MS^n and LC/ESI-IT-TOF-MS analysis.

In comparing the retention time, UV, MS and MS^n spectra of compounds with standards/literature, thirteen peaks were unambiguously identified as 3-caffeoylquinic acid (**1**), syringin (**2**), 5-caffeoylquinic acid (**3**), 4-caffeoylquinic acid