Screening for Potential Antibreast Cancer Components From *Prunellae Spica* Using MCF-7 Cell Extraction Coupled with HPLC-ESI-MS/MS

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**Abstract**

*Prunellae Spica* (PS), the dry spikes of *Prunella vulgaris* L., is a medicinal herb widely distributed in Asia and Europe. As a traditional Chinese medicine, PS has been used for the treatment of mastitis, infectious hepatitis, and hypertension. The oral solution and some compounds (polysaccharide, ursolic acid, and betulinic acid) of PS have been reported to show activities against breast cancer. In this study, Michigan Cancer Foundation-7 (MCF-7) cell extraction coupled with high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) analysis was applied to screen for potential antibreast cancer ingredients from PS. Protocatechuic acid (1), protocatechualdehyde (2), caffeic acid (3), and rosmarinic acid (4) were identified as high-affinity components. The cytotoxic activities of these compounds were evaluated in MCF-7 cells using Cell Counting Kit-8 assay. All the compounds displayed cytotoxicity to MCF-7 cells, but protocatechualdehyde and caffeic acid exhibited significant cytotoxicity with half-maximal inhibitory concentration values of 10.9 μM and 26.8 μM, respectively. This study provides the first report of the successful usage of cell extraction coupled with LC-MS/MS to screen active ingredients from PS. This method can be used as a screening tool for bioactive constituents in natural products.

**Keywords**

*Prunellae Spica*, cell extraction, HPLC-MS/MS, MCF-7, bioactive components screening

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**Introduction**

*Prunellae Spica* (PS), called “Xia-ku-cao” in traditional Chinese medicine (TCM), is the dry spikes of *Prunella vulgaris* L. PS has long been used as a TCM for the treatment of mastitis, infectious hepatitis, and hypertension. Phytochemical studies have shown that PS mainly contains flavonoids, triterpenes, phenolic acids, and steroids. These compounds exhibit diverse and significant properties such as anticancer, neuroprotective, antiallergic, and anti-inflammatory activities. PS also exhibited significant cytotoxicity against lung, breast, colon, and gastric cancers. Among these, breast cancer is the most common. It has been reported that an extract of Xia-ku-cao could prevent the progression of breast cancer. PS polysaccharide inhibits the viability of human breast associated fibroblasts (CAFs) by inhibiting the expression of basic fibroblast growth factor. Ursolic acid and betulinic acid from PS could be potential therapeutic agents against estrogen-dependent tumors by inhibition of estrogen signaling. However, except for the polysaccharide, ursolic acid, and betulinic acid, there has been no report about antibreast cancer ingredients from PS.

The classic procedures for finding bioactive components in TCM are time-consuming and laborious, which include extraction and purification of constituents, followed by pharmacological evaluation using either in vivo or in vitro methods. Modern pharmacological studies have demonstrated that most drugs should interact with either receptors or channels on cells or membranes to elicit activity. Several new techniques, such as cell membrane chromatography, biomembrane affinity
chromatography, and cell extraction coupled with high-performance liquid chromatography–mass spectrometry (HPLC–MSn) have been successfully used as a basis for the hypothesis of bioactive components in Chinese medicines. 13–17

In this current study, cell extraction coupled with HPLC–MS/MS was applied to screen for active ingredients from PS.

Results and Discussion

HPLC revealed 4 peaks in the extract of PS (EPS). By comparing their chromatographic characteristics and major MS and MS/MS fragments with standards (Table 1), the compounds were identified as protocatechuic acid (1), protocatechualdehyde (2), caffeic acid (3), and rosmarinic acid (4). The same 4 peaks were also detected in the Michigan Cancer Foundation-7 (MCF-7) cell extracts, but these were not detected in the last wash elution. Thus, there was no doubt that these 4 compounds from EPS could bind to some receptors or channels of MCF-7 cells.

As shown in Table 2, all 4 compounds showed cytotoxicity to MCF-7 cells, but compounds 2 and 3 showed greater inhibitory effects, with half-maximal inhibitory concentration (IC50) values of 10.9 µM and 26.8 µM, respectively. The effects of compounds 2 and 3 were different from the others at the concentration of 100 µM (Figure 1).

In this study, potential antibreast cancer components were extracted from Xia-ku-cao using MCF-7 cell extraction and analyzed by HPLC-electrospray ionization (ESI)-MS/MS; 4 bioactive compounds were determined. Previous studies have reported that polysaccharides from PS inhibited the viability of breast CAFs with an IC50 value of 8.39 µg/mL. 12 Moreover, ursolic acid and betulinic acid isolated from PS could induce cell death in estrogen receptor-positive MCF-7 cells with IC50 values of approximately 80 µM and 60 µM, respectively. 6 In the present study, protocatechualdehyde and caffeic acid showed a significant inhibitory effect on MCF-7 cells, with IC50 values of 10.9 µM and 26.8 µM, respectively. This study indicated that cell extraction coupled with LC-MS/MS was a rapid and useful approach for screening potential bioactive components in TCM. However, in this study, only 4 compounds were identified. Some active compounds with low content could not be detected and the possible reason was that they might be removed in the washing steps. Although this method may have some limitations, compared with the conventional procedures, the application of cell extraction coupled with HPLC-ESI-MS/MS for screening bioactive components of TCMs is rapid, convenient, systemic, and reliable.

Materials and Methods

Materials and Reagents

PS was bought from Guangdong Tiantai Pharmaceutical Co., Ltd. and authenticated by Professor Quan Zhu. A voucher specimen (No. 20171108) was deposited at the School of Stomatology and Medicine, Foshan University. Protocatechuic acid (lot: 5809), protocatechualdehyde (lot: 5351), caffeic acid (lot: 5617), and rosmarinic acid (lot: 5235) were obtained from Shanghai Nature-standard Technology Service Co., Ltd. (Shanghai, China); their purity was >98% by HPLC.

Table 1. Characterization of Potential Bioactive Compounds From EPS by HPLC-ESI-MS/MS.

| Compound | Rt (min) | MS (m/z) | MS/MS (m/z) |
|----------|----------|----------|-------------|
| 1        | 5.79     | 153      | 108, 91, 65, 63 |
| 2        | 7.06     | 137      | 91, 65, 63    |
| 3        | 8.46     | 179      | 135, 108, 89, 65 |
| 4        | 12.08    | 359      | 161, 135, 133, 123, 73 |

MS, mass spectrometry; HPLC, high-performance liquid chromatography; EPS, extract of Prunellae Spica; ESI, electrospray ionization.

Table 2. Cytotoxicity of Compounds 1-4 in MCF-7 Cells.

| Concentration (µM) | Inhibition rate (%) | IC50 (µM) | Inhibition rate (%) | IC50 (µM) | Inhibition rate (%) | IC50 (µM) | Inhibition rate (%) | IC50 (µM) |
|--------------------|---------------------|-----------|---------------------|-----------|---------------------|-----------|---------------------|-----------|
| 5                  | 41.5 ± 4.2          | >100      | 43.8 ± 5.0          | 10.9      | 36.0 ± 4.6          | 26.8      | 24.8 ± 3.5          | >100      |
| 10                 | 36.3 ± 4.1          |          | 48.3 ± 1.6          |           | 33.9 ± 1.2          | 24.6 ± 2.1|                     |           |
| 50                 | 38.9 ± 1.5          |          | 64.0 ± 2.2          |           | 61.0 ± 3.7          | 30.0 ± 2.8|                     |           |
| 100                | 24.6 ± 2.7          |          | 66.8 ± 1.56         |           | 63.2 ± 4.2          | 36.3 ± 7.3|                     |           |
| 150                | 33.8 ± 2.4          |          | 75.2 ± 0.9          |           | 66.5 ± 2.6          | 50.7 ± 2.8|                     |           |

IC50, half-maximal inhibitory concentration; MCF-7, Michigan Cancer Foundation-7.

Data are presented as means ± standard deviation (N = 3).
Phosphate-buffered saline (PBS, pH 7.4, pH 4.0) buffers were prepared in our laboratory. HPLC-grade acetonitrile was purchased from Merck Company (Darmstadt, Germany) and HPLC-grade formic acid from Tedia Company Inc. (Fairfield, USA). Deionized water was prepared using a Millipore MilliQ-Plus system (Millipore, Bedford, MA, USA). Human breast cancer cells, MCF-7, were obtained from First People’s Hospital of Foshan City. Roswell Park Memorial Institute (RPMI) Medium 1640 basic (lot: 8117030), trypsin–ethylenediaminetetraacetic acid (EDTA) (0.05%, lot: 1888583), penicillin–streptomycin liquid (lot: 1741838), and fetal bovine serum (lot: 42F0266K) were purchased from Thermo Fisher Scientific Co., Ltd. (Shanghai, China), and Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories (Shanghai, China). All other chemicals used in this study were of analytical grade. All solvents and samples were filtered through 0.22 µm nylon membranes before use.

**Preparation of EPS**

PS was dried and ground to powder, then 5 g was immersed in 40 mL of water for 0.5 hours and refluxed for 1 hour, twice. The combined extracts were concentrated to dryness at 60°C under vacuum, dissolved in 10 mL PBS to make a 10 mg/mL solution, and filtered through a 0.22 µm membrane. The filtrate was used as a sample for HPLC-ESI-MS/MS analysis and MCF-7 biospecific extraction.

**Preparation of Mixed Standard Solution**

Appropriate amounts of protocatechuic acid, protocatechueraldehyde, caffeic acid, and rosmarinic acid were accurately weighed and dissolved in the initial mobile phase to prepare a 54 µg/mL protocatechuic acid, 54 µg/mL protocatechueraldehyde, 54 µg/mL caffeic acid, and 35 µg/mL rosmarinic acid solution.

**MCF-7 Cell Extraction and Sample Preparation**

The cell extraction and sample preparation methods were designed based on our previous report. Human breast cancer cell line MCF-7 was grown in RPMI 1640 complete medium (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.05% (v/v) trypsin–EDTA, penicillin (100 mg/L), and streptomycin (100 mg/L). Cells were passaged at a split ratio of 1:3 every 3 days. MCF-7 cells were calculated and inoculated into a transwell with 3 × 10⁴ cells/L and incubated under 5% carbon dioxide (CO₂) at 37°C for 30 minutes. Then, 1 mL of EPS was added and incubated for 60 minutes. The drug solution was removed, and the cells were washed with 1.5 mL PBS, 5 times. Finally, the MCF-7 cells were washed with 1.5 mL of PBS (pH 4.0) as the biospecific extraction solution. Last eluate of PBS (1.5 mL), the extract of MCF-7 cells and EPS, and the standard sample solution were extracted with 1.5 mL of acetic ether, 3 times, respectively. The extracts were combined and concentrated to dryness at 40°C under vacuum. The residue was dissolved in 250 µL initial mobile phase and centrifuged at 1600 g for 5 minutes. The supernatant was transferred to an autosampler vial and applied to the HPLC-ESI-MS/MS system for analysis.

**Chromatographic and Mass Spectrometric Conditions**

Separation was performed with an Agilent SB-C18 reserved-phase column (100 mm × 2.1 mm i.d., 1.8 µm) with a mobile phase consisting of acetonitrile (A) and water–0.1% formic acid (B). The gradient elution conditions were: 0-1 minute, 1% A; 1-5 minutes, 1%-12% A; 5-20 minutes, 12%-55%; and 20-25 minutes, 55%-95% A. The flow rate was 0.3 mL/min. The column temperature was set at 30°C, the detection wavelength was 290 nm, and the injection volume was 5 µL.

An Agilent 1290 LC (Agilent company, USA) and Sciex TripleTOF 4600 Triple quadrupole mass spectrometer (AB Sciex company, USA) were employed for the sample analysis. Ionization was achieved using electrospray in the negative mode. Ultra-high purity helium was used as the collision gas and high purity nitrogen as the nebulizing gas. The mass detector was optimized to obtain maximum yields of (M − H)⁻ ions of the compounds. The mass parameters in the negative ion mode were optimized as follows—ion spray voltage: −4.5 kV; ion source gas 1 pressure: 50 arbitrary units; curtain gas

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**Figure 1.** Michigan Cancer Foundation-7 cells were treated with 100 µM of compounds 1-4 for 24 hours. Cells were stained with live/dead assay reagent for 15 minutes and then analyzed under a fluorescence microscope. Scale bar, 200 µm.
were rinsed 3 times using PBS. The cells (1 × 10^6 cells/mL) were seeded in 96-well plates at a concentration of 3 × 10^4 cells per well (0.1 mL medium) and incubated for 24 hours.

Cytotoxicity Tests in MCF-7 Cells

The IC₅₀ of compounds 1-4 in MCF-7 cells was measured using CCK-8 assay, as previously described. First, cells were seeded in 96-well plates at a concentration of 3 × 10⁴ cells per well (0.1 mL medium) and incubated for 24 hours (37°C with 5% CO₂ and 100% humidity). After that, cells were incubated for another 24 hours with different concentrations of compounds 1-4 (5, 10, 50, 100, 150 µM). Cells incubated with the fresh medium were used as a control. After that, 20 µL CCK-8 was added to each well and the plate was further incubated for 4 hours. Finally, the absorbances were detected by a microtiter plate reader (Spectramax 190, Molecular Devices, USA) at 450 nm. Each well was evaluated in triplicate in 3 independent experiments. The inhibition rate (%) is expressed as the percentage of 1 − (ODtest − ODblank)/(ODcontrol − ODblank), where ODtest is the optical density of the cells exposed to the compounds 1-4, ODcontrol is the optical density of the control sample, and ODblank is the optical density of the wells without MCF-7 cells. The IC₅₀ was calculated by SPSS software.

Live/Dead Staining

The cytotoxicity of the compounds was evaluated by live/dead cell dyeing. Firstly, cells were seeded in 6-well plates at a concentration of 3 × 10⁴ cells per well and incubated for 24 hours. After that, the cells were incubated for another 24 hours with compounds 1-4 (100 µM). After trypsin digestion, the cells were rinsed 3 times using PBS. The cells (1 × 10⁶ cells/mL) were stained using a live/dead staining kit (Invitrogen, USA) for 15 minutes and then observed under a fluorescence microscope (Zeiss, Germany).

Declaration of Conflicting Interests

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