

• 技术与方法 •

Screening of Effective Ingredients of Traditional Chinese Herb *Epimedium koreanum* Nakai by Combination of Co-cultured ECV304/C6 Cells as an *in vitro* BBB Model with HPLC-MS Analysis

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Abstract The blood-brain barrier (BBB) serves as a physiological protective barrier between the central nervous system (CNS) and the systemic circulation. For CNS drugs, ready permeating across the BBB is necessary for efficacy. To select BBB permeable drug candidates, an *in vitro* BBB model using co-cultured ECV304/C6 cells was adopted in this study. The transendothelial electrical resistance showed that the cultured ECV304 cell monolayer was of integrity and vitality. The active components of *Epimedium koreanum* Nakai, a widely used traditional Chinese herb, were screened with this *in vitro* BBB model, and then analyzed by HPLC-MS. We found that 13 compounds at least from the extract of *Epimedium* could permeate across BBB model, in which icariin and baohuoside-I were identified. These results indicated that the co-cultured ECV304/C6 model combined with HPLC-MS analysis was a potential tool for screening of BBB permeable drug candidates from plant-derived materials.

Key words blood-brain barrier; *in vitro* model; transendothelial electrical resistance; *Epimedium koreanum* Nakai; HPLC-MS

利用体外血脑屏障模型结合高压液相-质谱技术 筛选传统中药——朝鲜淫羊藿有效成分

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摘要 血脑屏障(blood-brain barrier, BBB)是位于中枢神经系统(central nervous system, CNS)和中枢系统环境间的一层生理保护屏障。凡是作用于CNS的药物,必须先通过BBB。为了寻找能够进入CNS的药物,通过细胞培养时间优化和跨膜电阻测定等,建立了ECV304/C6共培养通过BBB药物筛选模型。并将该模型应用于从传统中药淫羊藿的提取物中,筛选可能作用于CNS的活性成分,结合高压液相色谱-质谱联用技术(HPLC-MS),对筛选出的化合物进行鉴定分析。研究结果表明,淫羊藿提取物中至少有13种成分能够穿越BBB模型,其中2种成分被确认为淫羊藿苷和宝藿苷I,为CNS药物开发的早期快速筛选提供了实验依据。

关键词 血脑屏障; 体外模型; 跨膜电子; 朝鲜淫羊藿; 高压液相色谱-质谱联用技术
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Epimedium koreanum Nakai (*Epimedium*) is a well-known traditional Chinese herb widely used as a tonic , aphrodisiac and antirheumatic supplement for over a millennium in eastern countries , especially in China , Japan and Korea. The active constituents of *Epimedium* are complicated and the total flavone extract of *Epimedium* has been reported to reverse the attenuation of monoamine neurotransmitters by stimulating the neuroendocrine-immunological network in the old rat hypothalamus ^[1] , and improve the cerebral function after stroke ^[2]. Icariin (ICA) and baohuoside-I are the two main flavones in the extract of *Epimedium* , and the former was demonstrated to pass through the blood brain barrier (BBB) to exert strong neuropharmacological effects ^[3]. However , there is no report about whether baohuoside-I could pass through the BBB to protect the nervous system. Therefore , screening the permeability of the flavone extract across BBB is the key problem to find new potential central nervous system drug candidates from plant-derived materials.

The cell of *in vitro* BBB model is usually from the bovine brain endothelium ^[4~5]. Recently , different cell lines , such as Madin-Darby canine kidney (MDCK) cell ^[6] , Caco-2 cell ^[7] , and ECV304 cell , are adopted to establish *in vitro* BBB model for the “immortalization” character ^[8]. In the present study , the permeability of flavone extract from *Epimedium koreanum* Nakai across the co-cultured ECV304/C6 *in vitro* BBB model was screened. The separation and analysis of the drug candidates were processed by HPLC coupled with mass spectrometry (LC-MS) analytical technique.

1 Materials and Methods

1.1 Chemicals

RPMI 1640 medium , calf serum (CS) and Hanks' Balanced Salt Solution (HBSS) were obtained from Gibco-BRL (Gaithersburg , MD , USA) . Acetonitrile and formic acid (HPLC-grade) , fluorescein sodium , dimethyl sulfoxide (DMSO) , penicillin and streptomycin were purchased from Sigma-Aldrich Inc. (Saint Louis , MO , USA) .

1.2 Preparation of flavone extract from *Epimedium*

Epimedium koreanum Nakai (*Epimedium*) was collected from Changbai Mountain (Jilin , China) and identified by Professor Xianhua Tian , College of life sciences , Shaanxi Normal University. After drying , all parts of *Epimedium* were pulverized into powder. The powder was filtrated by a 0.45 mm sieve and weighed to get 60.0 g , which then was extracted with 75% ethanol at 85°C (3 L) . After the liquid phase was filtered , mixed , and extracted with mineral ether (3 × 200 mL) , the ethanol layer was separated by

polyamide column chromatography and 75% ethanol eluent was collected. The eluent was lyophilized (ALPHA1-2 Freeze Drying , Germany) to obtain the flavone extract of *Epimedium*.

1.3 Co-culture of ECV304/C6

When rat C6 glioma cells (Wuhan cell library of China) were cultured to confluence , they were trypsinized (2×10^5 cells/mL) and planted in a 24-well plate to attach (2 ~ 3 hours) . ECV304 cells (2×10^5 cells/mL , Wuhan cell library of China) were cultured in medium consisting of RPMI 1640 supplemented with 10% (V/V) heat-inactivated calf serum , 100 IU/mL of penicillin and 100 mg/mL of streptomycin before planting in the cell culture inserts (Millicell-PCF , 0.4- μ m pore size , 12-mm , surface 0.71 cm² , Millipore Co. , USA) . ECV304-containing inserts with a polycarbonate membrane were placed in the 24-well plate with C6 cells. The ECV304-containing inserts sat above the base of the well with four plastic supports and there was no physical contact between the C6 cells and the ECV304 cells. The culture medium was replaced daily from day 4 and the co-cultured cells were incubated at 37°C , 90% relative humidity , and 5% CO₂ atmosphere.

1.4 Measurement of transendothelial electrical resistance

Transendothelial electrical resistance (TEER) was measured with a Millicell-ERS Voltohmmeter (Millipore Co. , USA) . After the Ag/AgCl electrodes of Millicell-ERS were sterilized with 70% ethanol for 15 min , one electrode was positioned inside the insert and the other in the outside liquid , and the resistance was read. To get non-drifting readings , the electrodes had to be clamped using a stand. TEER was monitored daily in co-cultured ECV304 cells for 16 days. Once obtaining the resistance value , blank values (blank insert without cells) were first subtracted , and then the resultant value was multiplied by the membrane area (0.71 cm²) .

1.5 Evaluation of the permeability of flavone extract from *Epimedium*

Culture medium was aspirated from the inside insert and outside liquid , and sodium fluorescein (Mr 375 , Sigma , Germany) transport studies were used to assess co-cultured ECV304/C6 cells system integrity. Co-cultured cells had grown 12 days and TEER was higher over 150 Ω cm². After 1 hour of pre-incubation in HBSS (pH 7.4) instead of culture medium , HBSS containing 0.02% sodium fluorescein was introduced to the inside of the inserts. Sample aliquots (100 μ L) were taken from the outside of the insert at fixed intervals (0 , 30 , 60 , 90 , 120 , 150 , 180 min) . The amount of sodium fluorescein appearing in the outside of the insert was measured by spectrophotometry (POLARstar OPTIMA , Germany) at 490 nm.

1.6 Screening procedure

The flavone extract (4.0 mg) was dissolved in 2 mL sterile DMSO, and the solution was filtrated through 0.22 μm filtrater. Before the experimentation, co-cultured cells had grown 12 days and TEER was higher than 150 Ωcm^2 . All experiments were performed in a 5% CO_2 atmosphere at 37°C. Co-cultured cells were pre-incubated in HBSS (pH 7.4) for 30 minutes. Solution in the insert was replaced by HBSS containing 0.04 mg/mL flavone extract for 2 hours. Screening samples were collected from the outside of the insert, lyophilized, dissolved in methanol before filtered through a 0.45 μm filter, and then underwent LC-MS analysis.

1.7 LC-MS Parameters and Characteristics

The separation of the flavone was carried out on a 5 μm Phenomenex Luna C18 column (250 \times 4.6 mm) by using Agilent 1100 Series HPLC with a diode-array detector at 30°C. The eluents were (A) acetonitrile and (B) 50 mmol/L formic acid. The gradient elution of mobile phase was 25% (A) for 0 ~ 20 min, 25% ~ 35% (A) for 20 ~ 60 min, and 35% ~ 60% (A) for 60 ~ 70 min at the flow rate of 1.0 mL/min. HPLC separation of the flavone sample was detected at 270 ~ 340 nm. As for LC-MS analysis, mass spectrometry (Bruker, Leipzig, Germany) via ESI interface was used. Ultrapure helium (He , 99.999%) was used as the collision gas, and high-purity nitrogen (N_2 , 99.999%) as the nebulizing gas. The ionization mode was positive. MS parameters were as follows: scan range m/z = 200 ~ 900; nebulizer flow 35.0 psi; dry gas flow rate 8.0 L/min; dry gas temperature 325°C; capillary 4000 V; skimmer 40 V.

1.8 Statistical analysis

Statistical analysis was carried out with the SPSS 10.0 for Windows software package (Statistica). Data are expressed as the mean \pm S. E. M. of 3 independent experiments. Student's t -test was used and P value < 0.05 was considered to be statistically significant.

2 Results and Discussion

2.1 ECV304/C6 model for BBB

ECV304 cell line shows a robust endothelial phenotype, develops a raised TEER when co-cultured with rat C6 glioma cells^[9-10]. This model mimics the development of BBB features when peripheral endothelial cells are cultured with astrocytes^[11]. In addition, co-cultured ECV304/C6 cells developed other characteristics of brain endothelium, including expression of transferrin receptors and P-glycoprotein^[12].

2.2 Measurements of transendothelial electrical resistance

Resistance is inversely proportional to

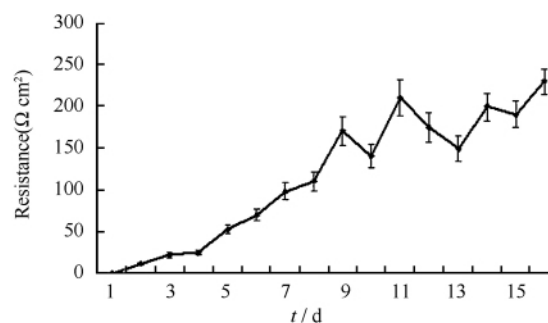


Fig. 1 Changes in transendothelial electrical resistance (TEER) with days in the co-cultured ECV304/C6 cells

Each symbol and bar indicated the mean \pm S. E. M. of three experiments

permeability, and in this instance reflects permeability to small ions that carry electrical current. To measure TEER, two electrodes were placed on the opposite sides of the insert, and transendothelial resistance caused by the developing cell layer was measured with Millicell ERS Voltohmmeter. TEER was obtained after subtraction of the cell-free inserts electrical resistance. ECV304 cells were inducible with glial cells. Fig. 1 showed that TEER increased from day 4. From day 4 to 11, the TEER varied from $25.46 \pm 14.52 \Omega\text{cm}^2$ (mean \pm S. E. M., $n = 3$) to $210.84 \pm 15.63 \Omega\text{cm}^2$ ($n = 3$). TEER reached a plateau from day 11 to 16. For this reason the major findings from the drug screening experiments in co-cultured ECV304/C6 cells system were performed at cultured day 12.

2.3 ECV304 cells permeability study

The cell layers integrity and cell vitality in the experimental condition were assayed by evaluating sodium fluorescein transport across the monolayer. The permeability across the cell layers was measured at given intervals to determine the extent to which the cell tight junctions opened. Fluorescein sodium, a fluorescent marker for the paracellular pathway, was used as an internal control in every test to verify tight junction integrity of the cell layers during the assay. It was considered to be a "tight layer" when the amount of sodium fluorescein outside the insert was less than 1% of the initial amount inside the insert^[13-14]. About 0.75% (less than 1%) of sodium fluorescein was detected within 3 hours outside the insert, demonstrating confluence and cell vitality for all the experimental time.

2.4 Identification of detected components

High-throughput mass spectral analyses are becoming more important in pharmaceutical study. For the identification of biologically active components in complex samples, LC-MS is a powerful approach to solve chemical structure of drug candidates because MS provides abundant structural information and facilitates the structural identification of unknown compounds.

The absorption spectra of the flavone extract from

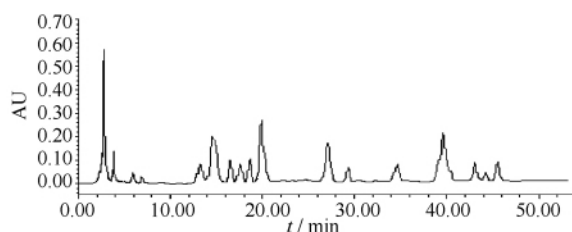


Fig. 2 HPLC fingerprint chromatogram of flavone extract from *Epimedium koreanum* Nakai

The separation of the flavone was carried out on a 5 μ m Phenomenex Luna C18 column (250 mm \times 4.6 mm) by using Agilent 1100 Series HPLC with a diode-array detector at 30°C. The eluents were (A) acetonitrile and (B) 50 mmol/L formic acid. The gradient elution of mobile phase was 25% (A) for 0 ~ 20 min, 25% ~ 35% (A) for 20 ~ 60 min, and 35% ~ 60% (A) for 60 ~ 70 min at the flow rate of 1.0 mL/min. HPLC separation of the flavone sample was detected at 270 ~ 340 nm

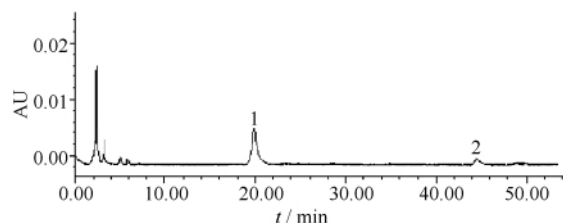


Fig. 3 HPLC fingerprint chromatogram of drug candidates transferring across BBB

For peak 1 at the retention time of 20.03 min, the pseudomolecular ion $[M + H]^+$ was at m/z 677.2, as shown in Fig. 4A. Referring to Yuan *et al.* [15], the corresponding compound was identified as icariin. For peak 2 at the retention time of 44.20 min, the pseudomolecular ion $[M + H]^+$ was at m/z 515.1, as shown in Fig. 4B. Referring to Yuan *et al.* [15], the corresponding compound was identified as baohuoside-I (Icariside II).

Epimedium were recorded from 270 to 340 nm. The results showed more peaks and more sensitive signals at 292 nm. Therefore, HPLC analysis was monitored at 292 nm for the flavone extract and the bioactive compounds obtained from outside the insert liquid. Fig. 2 is a typical HPLC fingerprint chromatogram of the flavone components extracted from Epimedium. Fig. 3 is the HPLC fingerprint chromatogram of the transported across the cell layers compounds. Two peaks were detected in the fingerprint, which are less than those in the extract fingerprint. The possible structures of two peaks in the fingerprint chromatogram were deduced in Fig. 4.

Icariin, a major flavonoid isolated from Epimedium, was shown to promote vascular function as well as inhibit endothelial cell dysfunction by elevating endothelial nitric oxide synthase (eNOS) derived nitric oxide (NO) [16 ~ 18]. In addition, Icariin possessed potent antidepressant-like activities which were at least in part mediated by reducing the dysfunction in the HPA axis [19]. Baohuoside-I was demonstrated to inhibit hypoxia-inducible factor-1 α in human osteosarcoma cells [20], and melanogenesis in melanocytes [21].

Continued exploration of the endogenous transport system, apart from the passive diffusion processes over the lipid membrane (transcellularly) or tight junctions between of the cells (paracellularly), drug candidates can be transferred through the lipid layer of biological membranes using specialized protein carriers, such as active transport and facilitated diffusion. However, lipophilic drug candidates could rapidly distribute to the cell membranes of the endothelium, and both active and passive transport occur simultaneously

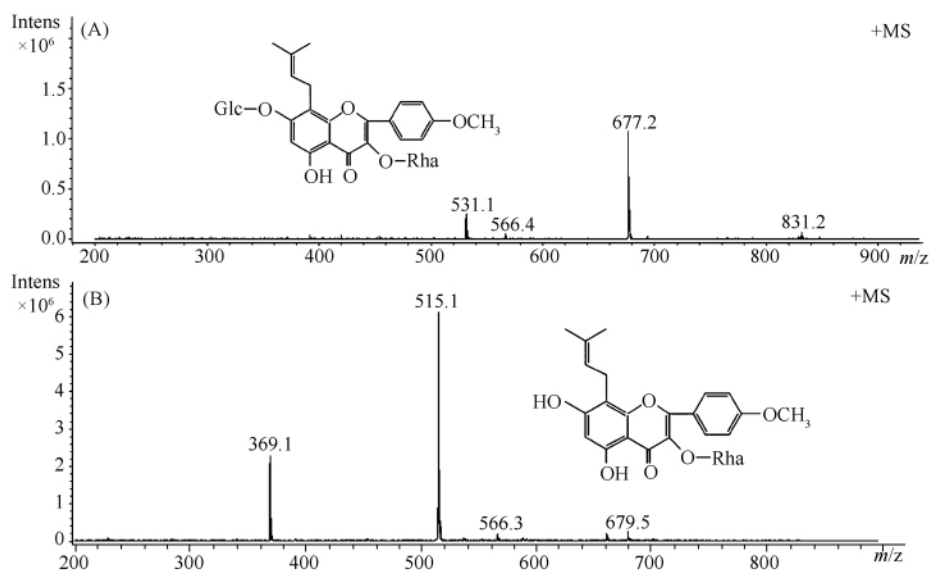


Fig. 4 Product ion mass spectra of $[M + H]^+$ of compound 1 (A) and compound 2 (B) The ionization mode was positive. MS parameters were as follows: scan range m/z = 200 ~ 900; nebulizer flow 35.0 psi; dry gas flow rate 8.0 L/min; dry gas temperature 325 °C; capillary 4 000 V; skimmer 40 V

depending on lipophilic drugs' physiochemical properties. The paracellular route is usually the main route of absorption for hydrophilic drug candidates. Icariin has already been reported to transfer across BBB to CNS^[22], and here, we first found that baohuoside-I also possesses the ability of transferring across BBB.

3 Conclusions

In this study, we adopted a co-cultured ECV304/C6 model *in vitro* to screen that icariin and baohuoside-I from *Epimedium koreanum* Nakai. The two drug candidates might be able to transfer across the BBB. These results suggested that co-cultured ECV304/C6 model coupled with LC/MS assay may help to facilitate the BBB permeable drug discovery from many plant materials at very early stages.

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