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Full Length Research Paper

Preparative separation of hyperoside of seeds extract of *Saposhnikovia divaricata* by high performance counter-current chromatography

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Hyperoside was isolated and purified from the seeds of *Saposhnikovia divaricata* for the first time by high performance counter-current chromatography (HPCCC) using a solvent system of ethyl acetate-*n*-butanol-ethanol -water (1:1:0.1:2, *v/v/v/v*). By injecting ca. 500 mg of *n*-butanol extract of *S. divaricata* seeds for five times, a total of 232.7 mg of hyperoside was purified from 2.5 g of the *n*-butanol extract of *S. divaricata* seeds, at 96.3% purity as determined by high performance liquid chromatography (HPLC). The identification of the purified compound was achieved by congruent retention time, ultraviolet (UV) spectra and the data of high-performance liquid chromatography- electrospray ion source mass spectroscopy (LC-ESI-MS^n) in the positive mode with that of the authentic standard and literature reports.

Key words: *S. divaricata* seeds, hyperoside, HPCCC.

INTRODUCTION

Saposhnikovia divaricata (Turcz.) Schischk is an important medicine in China, and the dried root has been used to treat headache, generalized aching, inflammatory symptoms and cancer (Okuyama et al., 2001; Xue et al., 2000). The chromones are the major bioactive constituents in the root of *S. divaricata* (Dai et al., 2008; Jiang et al., 2006; 2007; Sasakiet al., 1982; Gui et al., 2011).

However, compared to the many reported methods on the dried root of *S. divaricata* (Okuyama et al., 2001; Jiang et al., 2007; Sasaki et al., 1982; Gui et al., 2011), effective methods for the isolation, purification and structural characterization of constituents in seeds of *S. divaricata* are scarce. HPCCC methods have become an effective alternative to the conventional chromatographic techniques for the separation of hyperoside from some plant extracts (Sheng et al., 2009; Xie et al., 2010). The present paper describes the successful preparative separation and purification of hyperoside from the seeds of *S. divaricata* for the first time using preparative HPCCC.

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EXPERIMENTAL

Chemicals and reagents

The seeds of *S. divaricata* were purchased from HeiLongJiang NongKe; hyperoside was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing China). All organic solvents used for HPCCC separation were of analytical grade and purchased from Beijing Chemicals (Beijing, China); Acetonitrile and acetic acid were HPLC grade and purchased from Fisher Scientific Company. Water was purified on a Milli-Q water purification system (Millipore, France).

Sample preparation

The seeds of *S. divaricat*a (500 g) was milled to powder and extracted with 2000 ml of 80% aqueous ethanol for ten times at room temperature, each for 12 h. Each time, the extraction mixture was filtered and the combined filtrates were concentrated to dryness in *vacuo* at 40°C. The extract was re-dissolved in 500 ml of water and was then defatted five times each with 500 ml hexane. The water layer was then extracted successively five times each with 500 ml of *n*-butanol. The combined *n*-butanol layer was concentrated to dryness in *vacuo* at 40°C, which yielded 11.02 g. The *n*-butanol extract was stored at -20°C before HPCCC separation.

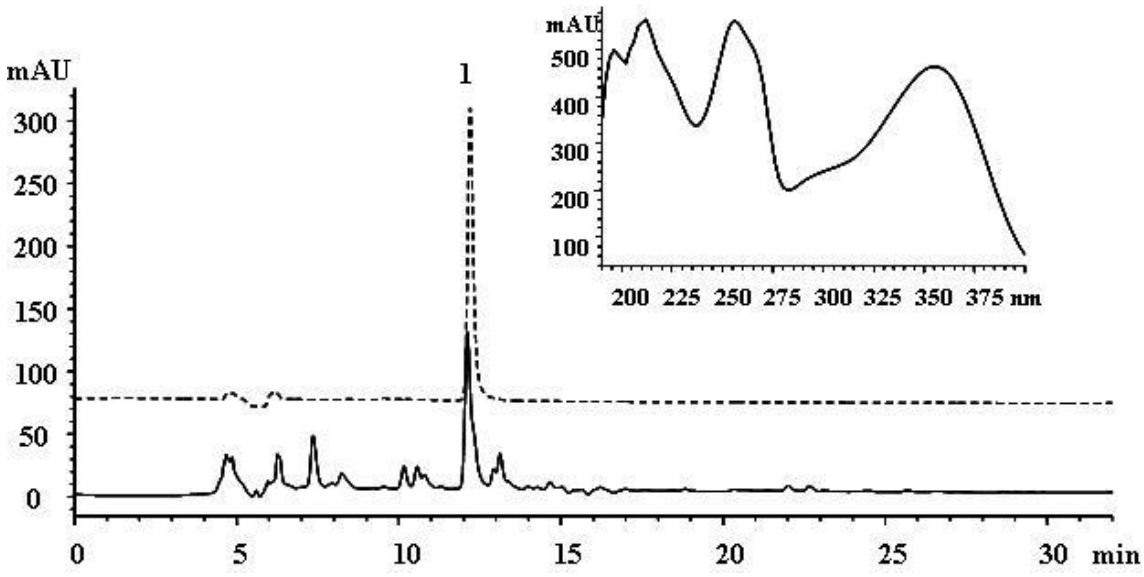

Figure 1. HPLC profiles of the *n*-butanol extract of the seeds of *S. divaricata* (A), purified compound 1 (B) and UV spectra of purified compound. The related compound was identified as hyperoside.

HPLC analysis

An agilent technology 1100 series HPLC system equipped with a quaternary pump, a degasser, thermostatic auto-sampler and a photodiode array detector (DAD) was used for the analysis of hyperoside in the *n*-butanol extract of *S. divaricata* seeds, the partition coefficient (*K*) and to monitor the fractions collected from the HPCCC separation. The HPLC method following the same procedures published earlier (Gui et al., 2011). Briefly, an Agilent Zorbax Extend C_{18} column (250 × 4.6 mm, 5 µm) was used. The binary mobile phase consisted of acetonitrile (solvent A) and water containing 0.5% acetic acid (solvent B). The flow-rate was kept constant at 0.5 ml/min for a total run time of 25 min. The system was run with a gradient program: 0 to18 min: 80% B to 40% B; 18 to 20 min, 40% B to 80% B; and 20 to 25 min, 80% B to 80% B. Peaks of interest were monitored at 254 nm by a DAD detector.

HPCCC separation

Preparative HPCCC was carried out in a Spectrum HPCCC (DE, England). The apparatus has one coil of 132 ml for preparation. The HPCCC system was equipped with two check pumps (Smartline Pump 100, KNAUER, Germany), a 2500 sensitive UV detector (KNAUER, Germany). The rotation speed can be regulated with a speed controller in the range of 0 to 1600 rpm. Sample injection was accomplished through an injection valve with a 10-ml sample loop. A solvent system of ethyl acetate-*n*-butanol-ethanol-water (1:1:0.1:2, *v/v/v/v*) was selected for separating compound 1 in Figure 1. This system provided a *K* value of 1.16 for compound 1. The 132 ml coil was used and the apparatus was set in the reversed phase mode. The entire coiled column was first filled with the upper phase, which serves as the stationary phase.

The temperature was held at 30°C. The rotation rate of the apparatus was set at 1600 rpm and the lower phase (mobile phase) was pumped into the column at a flow rate of 3 ml/min. When the hydrodynamic equilibrium was established, a sample (ca. 500 mg) dissolved in 6 ml of the mixture of ethyl acetate-*n*-butanol-ethanol-water (1:1:0.1:2, *v/v/v/v*) was loaded into the injection valve after the system reached hydrodynamic equilibrium. Then time was recorded after the injection. The effluent from the outlet of the column was continuously monitored by a UV detector at 254 nm and collected into test tubes with a fraction collector set at 2 min for each tube. Fractions from the HPCCC that had the same single peak as determined by HPLC were combined and freeze-dried. The purity of the peak as determined by HPLC was 96.3% for compound 1. The purified compound was stored at -20°C before LC-ESI-MS^n analyses.

LC-ESI-MS for Identification

A Thermo Scientific LCQ Fleet mass spectrometer was connected to Thermo Scientific Surveyor Liquid chromatography (LC) Plus system via electrospray ionization (ESI) interface (ThermoFisher, USA). The LC-ESI-MS method following the same procedures published earlier (Gui et al., 2011). Briefly, the operating parameters in the positive ion mode were as follows: the sheath gas and auxiliary flow rates were set at 30 and 5 (arbitrary unit), respectively. The capillary voltage was set at 34.98 V and its temperature was controlled at 350□ C. The entrance lens voltage was fixed at -35 V and the multipole retention factor (RF) amplitude was set at 406 V. The ESI needle voltage was controlled at 4.5 kV. The multipole lens 1 offset was -15 V. The electron multiplier voltage was set at - 980 V for ion detection.

RESULTS AND DISCUSSION

The *n*-butanol extract of *S. divaricata* seeds and the fractions corresponding to compound 1 isolated by HPCCC were analyzed by HPLC and the results are given in Figure 1, a good separation was achieved within 25 min. Peak 1 was separated and detected with retention times at 12.11 min. Tentative identification of this peak was achieved by congruent retention time, UV

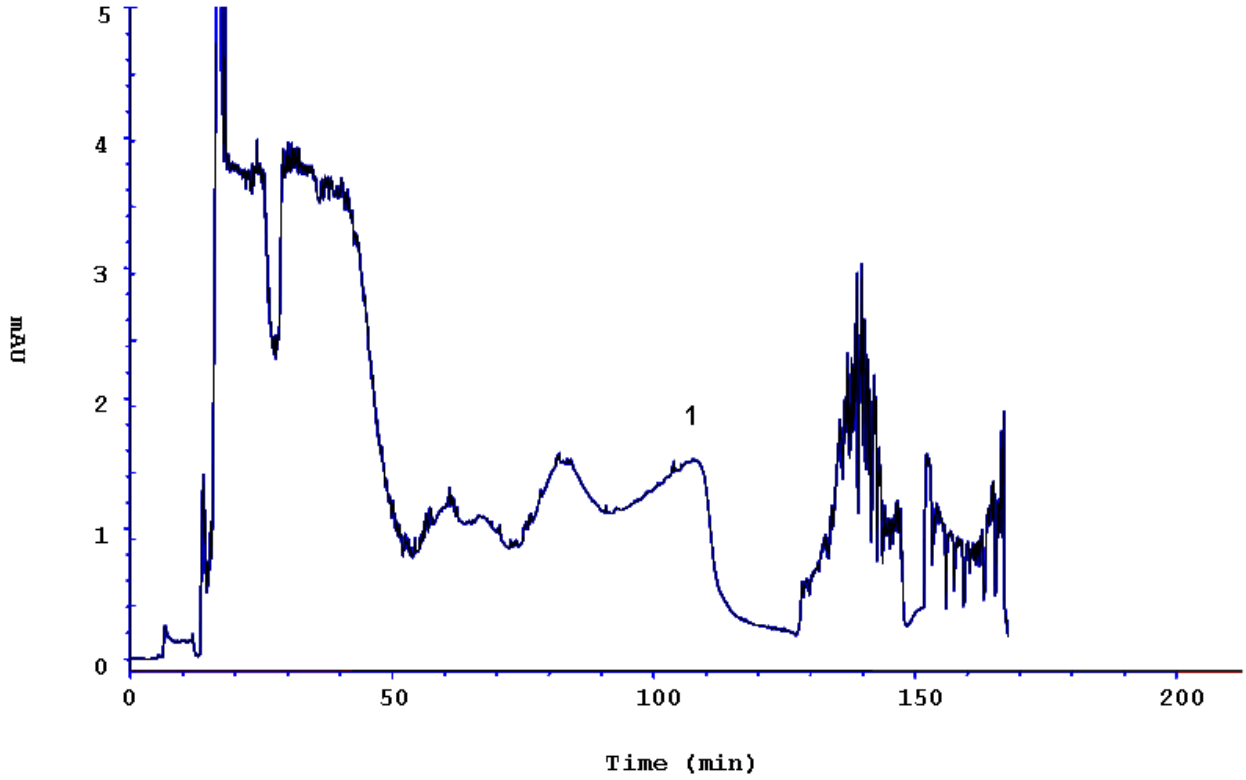

Figure 2. The HPCCC chromatogram of the *n*-butanol extract of *S. divaricata* seeds. The coil volume was 132 ml; The temperature was held at 30°C; The rotation rate of the apparatus was set at 1600 rpm; The lower phase (mobile phase) was 3 ml/min; Sample (ca. 500 mg) dissolved in 6 ml of the mixture of ethyl acetate-*n*-butanol-ethanol-water (1:1:0.1:2, *v/v/v/v*); UV detector at 254 nm.

spectra with that of the authentic standard and the peak 1 was identified as hyperoside. In our experiment, a solvent system containing ethyl acetate-*n*-butanol-ethanol- water (1:1:0.1:2, *v/v/v/v*) was selected to separate compound 1, which has been used in HPCCC to separate four chromones from *S. divaricata* root (Gui et al.. 2011). We found that this system was suitable for the separation of compound 1 from the *n*-butanol extract of *S. divaricata* seeds. The *K* value for compound 1 in this system was at 1.16. When this solvent system was applied to the HPCCC separation with a sample load of ca.500 mg and flow rate at 3 ml/min, the compound of interest was separated within 130 min (Figure 2).

A total of 232.7 mg of hyperoside was purified from 2.5 g of the *n*-butanol extract of *S. divaricata* seeds, at 96.3% purity as determined by HPLC. To further investigate the structure of this peak, LC-ESI-MS^n experiment was attempted. Under LC-ESI-MS conditions, compound related to the peak 1 in Figure 1 exhibited intense molecular ions $[M+H]^+$ at *m/z* 465 in the positive mode and low intensity dimer $[M+Na]^+$ ion at *m/z* 487, from which the molecular weight of peak 1 was confirmed to be 464, the same as that for hyperoside (Li et al., 2005; Chen et al., 2002). In the LC-ESI-MS^2 experiment, the ion at *m/z* 465 formed one major fragment ion at 303. The ion at *m/z* 303 was produced directly from the parent ion of *m/z* 465 due to the loss of a galactosyl moiety. By comparing the molecular weight information and LC-ESI-MS^n data with that of the authentic standard and literature report (Li et al., 2005; Chen et al., 2002), the identities of compound 1 were confirmed as hyperoside.

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