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Ethyl acetate–*n*-butanol gradient solvent system for high-speed countercurrent chromatography to screen bioactive substances in okra

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ABSTRACT

High-speed countercurrent chromatographic separation (HSCCC) possesses the property of zero-loss of sample, which is very useful for the screening of bioactive components. In the present study, the ethyl acetate–*n*-butanol gradient HSCCC solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water was investigated for the screening of bioactive substances. To screen the antiproliferative compounds in okra extract, we used the stationary phase ethyl acetate–*n*-butanol–water (1:1:10) as the stationary phase, and eluted the antiproliferative components by 6-steps of gradient using mobile phases *n*-hexane–ethyl acetate (1:2), *n*-hexane–ethyl acetate (1:4), *n*-hexane–ethyl acetate (0:4), *n*-butanol–ethyl acetate (1:4) *n*-butanol–ethyl acetate (1:2), *n*-butanol–ethyl acetate (2:2), and *n*-butanol–ethyl acetate (2:1). The fractions collected from HSCCC separation with the gradient solvent system were assayed for antiproliferative activity against cancer cells. Bioactive components were identified: a major anti-cancer compound, 4′-hydroxy phenethyl trans-ferulate, with middle activity, and a minor anti-cancer compound, carolignan, with strong activity. The result shows that the gradient solvent system is potential for the screening of bioactive compounds from natural products.

1. Introduction

High-speed counter-current chromatography (HSCCC) is a continuous liquid–liquid partition chromatographic method by partition of components between two immiscible liquid phases without solid support or irreversible adsorption. HSCCC is used for the screening of constituents with bioactivity [1,2]. However, most solvent systems are limited to separating constituents with a narrow range of polarity. It is important to establish a gradient HSCCC solvent system to separate natural products with a wide range of polarity.

Gradient countercurrent chromatographic (CCC) solvent systems mainly involve three types which include pH-gradient, salting-out gradient and solvent gradient. pH-gradient has been used for the separations of the compounds which are sensitive to pH [3–5]. Salting-out gradient method was utilized to the separation of the major chlorogenic acids present in green coffee beans using ethyl acetate-hexane as the stationary phase and an ionic gradient of LiCl (5.0, 2.5 and 0.1 M) as the mobile phase [6]. Solvent gradient has been reported by in a few literatures. A 2-butanol exponential gradient in ethyl acetate-2-butanol-water was used for the separation of anthocyanins from grapes [7]. Two-step methanol gradients made from *n*-hexane–methanol–water (5:5:5, 5:7:3) [8], light petroleum–ethyl acetate–methanol–water (1:1:1.2:0.8, 1:1:1.4:0.6) [9], *n*-hexane–ethyl acetate–methanol–water (5:5:5:5, 5:5:6:4) [10], *n*-hexane–ethyl acetate–methanol–water (0.6:4.0:0.05:1.0, 0.6:4.0:0.7:1.0) [11], *n*-hexane–ethyl acetate–methanol–water (4:6:3:7, 4:6:4:6) [12] has been employed to separate the natural products from plant extracts. A three-step methanol gradient made from light petroleum–ethyl acetate–methanol–water (5:5:5:5, 5:5:6:4, 5:5:6.5:3.5) [13,14] was used in the separation and purification of coumarins, and a three-stepwise gradient *n*-butanol gradient (stationary phase: the upper phase of *n*-hexane–*n*-butanol–0.05 M NaOH (5:1:6); mobile phase: *n*-hexane–*n*-butanol with ratio of 1:1, 1:2 and 1:4) was applied to the separation of ursane triterpenoids [15]. These gradient solvent systems used only one solvent to change the polarity of the mobile phase. However, plant extracts usually contain very complicated constituents with a wide range of polarity. To isolate the complicated constituents,

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it is needed to apply a multi-step elution using a gradient solvent system in which the solvent gradient is made with more than one solvent.

n-Hexane, ethyl acetate and *n*-butanol are three solvents with polarities from low to high. The mobile phases composed of *n*-hexane–ethyl acetate–*n*-butanol could yield multi-step gradient with polarities from low to high through changing the ratio of two solvents ethyl acetate and *n*-butanol, when aqueous phase of *n*-hexane–ethyl acetate–*n*-butanol–water is used as stationary phase. Thus, ethyl acetate-*n*-butanol gradient solvent system based on *n*-hexane–ethyl acetate–*n*-butanol–water is possibly suitable for the separation of complicated constituents. To screen the bioactive compounds from natural products such as ethanolic extracts it is necessary to make a full separation of the constituents with polarities from low to high. Therefore, the present study describes the screening of compounds with anti-cancer activity from okra (*Abelmoschus esculentus* L. or *Hibiscus esculentus* Linn.) [16] by HSCCC separation using ethyl-acetate–*n*-butanol gradient solvent system.

2. Experimental

2.1. Materials

All solvents for extraction and separation were of analytical grade and purchased from Hangzhou Huadong Chemicals Inc., China. Okra (*A. esculentus* L.), were purchased at a local vegetable store in Hangzhou, China. The human ovarian cancer cell line HO8910, the human breast cancer cell line MCF-7, the human myeloid leukemia cell line HL-60 and the human lung adenocarcinoma epithelial cell line A590 were purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. The reagents for the assay of antiproliferation against cancer cells were purchased from Sigma (Shanghai Branch, China).

2.2. Extraction

The freeze-dried sample (1 kg) was extracted twice with 9 l 90% ethanol for 2 h at 50 °C. The extracts were combined and evaporated to syrup. The syrup was defatted with ether and lyophilized, yielding 159 g crude extract. A portion of the crude extract (3 g) was used for HSCCC separation to obtain fractions for an assay to study the antiproliferative activity against HL-60 cells.

2.3. Establishment of gradient solvent system

A stepwise gradient elution was needed in the HSCCC separation because the crude plant extracts contained components with various polarities. In experiments, we found that mobile phases composed of *n*-hexane–ethyl acetate–*n*-butanol with various ratios had a high retention of stationary phase when ethyl acetate–*n*-butanol–water (1:1:10) was used as the stationary phase. The mobile phase *n*-hexane–ethyl acetate provided a low polarity, ethyl acetate with a small part *n*-butanol provided middle polarity, and *n*-butanol with a small part ethyl acetate provided a high polarity.

2.4. Fractionation of the components of antiproliferative activity against HL-60 cells

The high-speed counter-current chromatograph used in the present study was constructed at the Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China. The apparatus was equipped with a 1200-ml column with six-layer coils made from 5.0 mm i.d. polytetrafluoroethylene (PTFE) tubing. A K-1800 Wellchrom preparative high-pressure liquid chromatography (HPLC) pump (Knauer, Germany), a 50-ml sample loop made of 3-mm i.d. PTFE tubing, and a B-684 collector (Büchi, Switzerland) with 50-ml tube racks were used to build the HSCCC system. The separations performed with a "tail to head" mode. The sample solution was prepared by dissolving 4 g ethanolic extract in 45 ml stationary phase solvent (water saturated with *n*-butanol and ethyl acetate). At the beginning of the separation procedure, the column was filled with the stationary phase solvent. The apparatus was rotated at 1000 rpm and the sample solution was injected into the HSCCC system through the sample loop with the mobile phase at a flow rate of 5.0 ml/min. The effluent was collected as the mobile phase started to leave the column. The effluent was collected in 40-ml fractions by a fraction collector. After the stepwise elution, the solvent was drained from the column and collected in 40-ml fractions. All fractions were assayed for the antiproliferative activity against HL-60 cells. The fractions were combined into larger fractions according to their antiproliferative activity against HL-60 cells.

2.5. HPLC analysis of antiproliferative components

HPLC-evaporative light-scattering detector (ELSD) was utilized for the analysis of the antiproliferative components from HSCCC. The analytical HPLC system consisted of an Alliance 2695 separations module, an ODS AQ column (150 × 3.9 mm i.d., 5 μm) and a low-temperature ELSD-LTII (Shimadzu, Japan). The gradient elution was carried out as follows: from 100% water–formic acid (99.5:0.5, v/v) to 100% methanol, from 0 to 40 min, at a flow rate of 0.8 ml/min.

2.6. Preparation of antiproliferative compounds

Preparative HPLC was used for preparation of antiproliferative compounds. A preparative ODS AQ column (250 × 20 mm i.d., 15 μm) was employed. The preparative HPLC conditions were determined based on the analytical HPLC results.

2.7. Identification of the compounds obtained from separation and purification

Electrospray ionization mass spectra (ESI-MS) and nuclear magnetic resonance (NMR) spectra were used for identification of the compounds obtained from separation and purification. All ESI-MS experiments were performed on a Bruker Esquire LC-MS ion trap multiple mass spectrometer (Bremen, Germany) in positive or negative ionization mode analyzing ions up to m/z 2200. ^{1}H, ^{13}C, and DEPT 90/135 NMR spectra were recorded by a Bruker Avance 500 (Karlsruhe, Germany) with 500 MHz for ^{1}H measurements and 125 MHz for ^{13}C measurements.

2.8. Assay of antiproliferative activities against cancer cells

The percentage of growth inhibition was determined by using a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay to measure viable cells [17]. One ml solution of each HSCCC 40-ml fraction was evaporated to dryness under vacuum. The residue was dissolved with 2 ml of dimethyl sulfoxide (DMSO) for assay of antiproliferative activities against cancer cells. A total of 2.5×10^3 cells/well was seeded onto a 96-well plate for 24 h, treated with various concentrations of the samples, and incubated for an additional 3 days at 37 °C. Subsequently, 10 μl of MTT at a concentration of 5 mg/ml was added to each well, and cells were incubated for an additional 5 h. The supernatant was aspirated, and 100 μl of DMSO were added to the wells to dissolve any precipitate. The absorbance was measured at a wavelength of 570 nm using a