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Simple and efficient preparation of biochanin A and genistein from *Dalbergia odorifera* T. Chen leaves using macroporous resin followed by flash chromatography

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ABSTRACT

In this study, a simple rand efficient protocol is developed for preparation of biochanin A and genistein from *Dalbergia odorifera* T. Chen leaves using macroporous resin followed by flash chromatography. The adsorption and desorption capacity of 14 macroporous adsorption resins for biochanin A and genistein were evaluated, and AL-2 resin showed better properties for biochanin A and genistein. After treatment with AL-2 resin, the contents of biochanin A and genistein in the enriched product were 6.60-fold and 6.41-fold increased with recovery yields of 87.13% and 84.60%, respectively. Furthermore, the operating parameters of flash chromatography were optimized. The optimal conditions were as follows: stationary phase: silica gel, elution system: *n*-hexane/ethyl acetate, sample/silica gel ratio: 1.3:40 and flow rate: 50 mL/min. After one flash chromatography run, purities of biochanin A and genistein effectively reached over 95%, their recovery yields were 80.13% and 73.11%, respectively. The developed protocol was simple, efficient, scalable and economical, which represented an excellent alternative for the separation and purification of bioactive compounds from plants.

1. Introduction

Flavonoids, a large category of plant polyphenol secondary metabolites, are widely distributed in medicinal herbs, fruits, teas, etc. [1]. They possessed particular interest with regard to human health effects [2]. As a kind of flavonoids, isoflavonoids can be potentially used as clinical therapeutic agents, food additive and health care products [3–8]. *D. odorifera* leaves, a natural renewable resource, usually were discarded as useless material. Our previous studies found that biochanin A and genistein are the main active constituents present in *D. odorifera* leaves [9]. Biochanin A and genistein are naturally occurring plant-derived phytoestrogen, and possess anticancer, antioxidant and antiosteoporosis effect. In view of these beneficial effects, it is necessary to obtain high purity of biochanin A and genistein for further medical studies and applications.

Preparation of isoflavonoids from plant extract is a challenging task because plant extract was a multi-component system. Over the past decade, various physical and chemical techniques for the enrichment of bioactive compounds from plant extract have been investigated, such as liquid–liquid extraction [10,11], membrane filtration [12], ion exchange [13], solid phase microextraction [14] and adsorption–desorption [15]. Among them, adsorption–desorption is considered as an economical and efficient method for preparative enrichment of bioactive components from plant extracts. Macroporous resins adsorption technology has been increasingly viewed as a representative adsorption–desorption for enriching bioactive components [16–18]. Macroporous resins have unique superiorities, such as high mechanical strength, high selectivity, good acid and alkali resistance, low cost, convenience and easy regeneration [19,20]. Open column chromatography is traditionally used for separating bioactive components from plant extracts. However, this method is time-consuming, laborious and requires large volumes of solvents [1]. These limitations warranted to explore a fast and efficient method for preparation of natural products from plant extract. Flash chromatography, also known as medium pressure chromatography, can efficiently overcome these limitations. It is considered as a fast, inexpensive and efficient separation technique for the separation of natural active components from extracts, and is easy to handle. This technique is an excellent alternative for slow and often inefficient gravity-fed chromatography. Compared to traditional gravity-fed

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chromatography, flash chromatography gives a greatly improved reproducibility, higher resolution and recovery yield, and a reduced consumption of organic solvents. Moreover, with a UV detector and an automatic fraction collector, the sample solvent could be collected according to chromatograms profile during elution process.

The aim of this work was to establish a simple and efficient protocol for preparation of biochanin A and genistein from *D. odorifera* leaves using macroporous resin followed by flash chromatography. Up to now, there has never been the report on separation of biochanin A and genistein by flash chromatography. The operating parameters of macroporous resin and flash chromatography were optimized. Hopefully, this work is helpful for the scale-up application for the production of biochanin A and genistein from the *D. odorifera* leaves and other plants.

2. Materials and methods

2.1. Materials, chemicals and adsorbents

The *Dalbergia odorifera* T. Chen leaves were collected from Hainan Province China, and authenticated by Professor Shaoquan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, China. The leaves were dried in shade at room temperature, powdered by a disintegrator and then stored in dark.

Reference compounds biochainin A (4′,5,7-trihydroxyisoflavone-7-glucoside, ⩾98%) and genistein (4′,5,7-trihydroxyisoflavone, ⩾98%) were purchased from Fluka (Buchs, Switzerland). Reagents of HPLC grade including methanol and formic acid were purchased from J&K Chemical LTD. (Beijing, China). Deionized water was produced by a Millipore Direct-Q purification system (Millipore Corp., Bedford, MA, USA). Ethanol of analytical grade was obtained from Tianjin Chemical Reagents Co. (Tianjin, China). The solvents (*n*-hexane, ethyl acetate, chloroform and petroleum) used for preparative flash chromatography were of analytical grade.

Fourteen macroporous resins including AB-8, ADS-5, ADS-11, D-101, FL-1, FL-2, FL-3, HPD500, HPD826, HPD-D, ME-2, AL-2, SA-3 and NKA-9 were purchased from Nankai Hecheng S&T (Tianjin, China) and Bonchem (Hebei, China). Their polarities ranged from non-polar to strong polar. All the resins were pretreated according to the manufacturers' recommendation prior to use in order to remove the monomers and porogenic agents trapped inside the pores during the synthesis process [4,21]. The moisture contents of the tested resins were determined by drying the beads at 100 °C to constant weight in a drying oven for over 24 h. Toyopearl HW-40S gel resin and silica gel (300–400 mesh) were purchased from Tosoh Corporation (Tokyo, Japan) and Qingdao Meigao Chemical Co. Ltd., (Qingdao, China), respectively. TLC (Kieselgel GF254) was purchased from Taizhou Luqiao Sijia Biochemical Plastic Factory (Taizhou, China).

2.2. Preparation of D. odorifera leaves extracts

Pulverized *D. odorifera* leaves (5 kg) were extracted with 30 L of 80% ethanol at room temperature for 3 days, repeated twice. The filtered solutions were gathered and concentrated to dryness by removing the ethanol solvent using a rotary evaporator device (RE52AA, Shanghai Huxi Instrument Co., China) at 40 °C. The dried extracts were obtained, and dissolved in 30% ethanol to get sample solution (5 mg extract/mL) at the concentration of 0.2058 mg/mL for biochanin A and 0.0530 mg/mL for genistein, respectively (Table 1).

2.3. HPLC analysis of biochanin A and genistein

Biochanin A and genistein were analyzed using an Agilent 1200 HPLC system. Chromatographic separation was carried out on a HIQ Sil C_{18}V reversed-phase column (250 mm × 4.6 mm i.d., 5 μm). All samples were filtered through 0.45 μm nylon membranes prior to HPLC analysis. The mobile phase consisted of acetonitrile (A) and water–formic acid (B). The gradient elution program was as follows: 0–5 min, 5–10% A; 5–10 min, 10–28% A; 10–30 min, 28–60% A; 30–35 min, 60% A; 35–37 min, 60–80% A; 37–42 min, 80–100% A; 42–55 min, 100% A. The injection volume was 5 μL. The flow rate and column temperature was 1 mL/min and 30 °C, respectively. Biochanin A and genistein were quantified at wavelength 262 nm. The chromatographic peaks of the analytes were confirmed by comparing their retention times and UV spectrum with those of the reference compounds. Eight experimental points were employed for establishing a calibration curve. The regression lines for biochanin A and genistein were $Y = 84698\ x + 78.7$ ($R^2 = 0.9949$) and $Y = 71148\ x + 1.37$ ($R^2 = 0.9927$), where Y is the peak area of analyte, and x is the concentration of reference compound (mg/mL).

2.4. Enrichment of biochanin A and genistein by macroporous resin

2.4.1. Screening of macroporous resins

Macroporous resins can selectively adsorb and desorb constituents from sample solutions due to their specific physical and chemical properties. The adsorption and desorption capacity of different resins towards biochanin A and genistein were investigated. The adsorption tests were performed as follows: pre-weighed hydrated resins (equal to about 1.0 g dry resin) and 100 mL sample solution were added into 250 mL flasks with stopper. The flasks were shaken in an incubation shaker (120 rpm) for 4 h at 25 °C. After adsorption, the solutions were separated from the resins and analyzed by HPLC. Then, the resins were subsequently desorbed with 100 mL 80% ethanol solution. The flasks were continually shaken (120 rpm) for 6 h at 25 °C. The contents of biochanin A and genistein in desorption solutions were determined by HPLC.

The adsorption properties of resins were evaluated based on the adsorption and desorption capacities and ratio of desorption. The equations were as follows:

Adsorption evaluation:

$$Q_e = \frac{(C_0 - C_e)V_i}{W}$$

where Q_e is the adsorption capacity at adsorption equilibrium (mg/g resin); C_0 and C_e are the initial and equilibrium concentrations of solute in the solutions, respectively (mg/mL); V_i is the volume of sample solution, and W is the weight of the dry resin.

Desorption evaluation:

$$D = \frac{C_d V_d}{(C_0 - C_e)V_i} \times 100\%$$

$$Q_d = \frac{C_d V_d}{W}$$

where Q_d is the desorption capacity after adsorption equilibrium (mg/g resin); D is the desorption ratio (%); C_d is the concentration of solute in the desorption solution (mg/mL); V_d is the volume of the desorption solution (mL); C_0, C_e, W and V_i are the same as described above.

2.4.2. Determination of macroporous resins/sample ratio

A series of adsorptions was carried out with different macroporous resins/sample ratios (2:1, 3:1, 4:1, 4.5:1 and 5:1, w/w) to determine the optimal macroporous resins/sample ratio.