Journal of Chromatography B, 1012 (2016) 42–49

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Preparative separation of polyphenols from water-soluble fraction of Chinese propolis using macroporous absorptive resin coupled with preparative high performance liquid chromatography

Aifeng Li[a,*], Hongzhuan Xuan[b], Ailing Sun[a], Renmin Liu[a], Jichun Cui[a]

[a] College of Chemistry and Chemical Engineering, Liaocheng University, Liaocheng 252000, PR China
[b] College of Life Science, Liaocheng University, Liaocheng 252000, PR China

ARTICLE INFO

Article history:
Received 29 January 2015
Received in revised form 2 December 2015
Accepted 19 December 2015
Available online 11 January 2016

Keywords:
Chinese propolis
Water-soluble fraction
Polyphenols
Macroporous absorptive resin
Preparative high performance liquid chromatography

ABSTRACT

In this study, a preparative separation method was developed for isolation of eleven polyphenols from water-soluble fraction of Chinese propolis using macroporous absorptive resin (MAR) coupled with preparative high performance liquid chromatography (PHPLC). Water-soluble fraction of Chinese propolis was first "prefractioned" using MAR, which yielded four subfractions. The four subfractions were then isolated by PHPLC with an isocratic elution of methanol–water. Finally, eleven polyphenols were purified from Chinese propolis including caffeic acid, ferulic acid, isoferulic acid, 3,4-dimethoxy cinnamic acid, pinobanksin, caffeic acid benzyl ester, caffeic acid phenethyl ester, apigenin, pinocembrin, chrysin and galangin. The purities of the compounds were determined by HPLC and the chemical structures were confirmed by UV and NMR analysis. The method developed was simple, effective, rapid, scalable and economical, and it was a promising basis for large-scale preparation of multiple components from natural products.

1. Introduction

Chinese propolis is a natural sticky substance produced by honeybees, *Apis mellifera* L., from the bud and bark of certain trees and plants [1]. It is thought to have the effect o af improving human health and preventing diseases, so it has been widely used in folk medicine from ancient times. In recent years, Chinese propolis has gained popularity as an additive in food, beverages and cosmetics. And there is a renewed interest in the composition of Chinese propolis and its biological activities. Pharmacological research revealed that it processes various biological activities including antimicrobial, anti-inflammatory, antioxidant, antifungal, antitumor, immunostimulating and wound healing activities [2], so it has been used to treat many ailments such as inflammation, heart disease, diabetes, and even cancers [3]. Its chemical composition is very complex, over 300 constituents have been found in it, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino acids, among which polyphenols have been considered as the primary biologically active compounds [4]. So it was of great interest to develop an effective, simple and rapid method to purify the bioactive components. However, there are few reports with regard to preparative isolation and purification of polyphenols from Chinese propolis mainly due to its compositional complexity.

The traditional separation and purification method of polyphenols from Chinese propolis was silica gel column chromatography [5]. The method was mature, but the steps were very tedious. Furthermore, many kinds of organic solvents were used as the mobile phase in stepwise elution mode, which was hazardous and non-environmentally friendly, the consumption of organic solvents was considerable large, and the mobile phase could not be recycled. Recently, high speed counter-current chromatography [6] was applied to separation and purification of polyphenols from Chinese propolis, but the choice of two phase solvent system was difficult due to lack of theoretical directions. Compared to the above two methods, preparative high performance liquid chromatography (PHPLC) is robust, versatile, effective, and usually rapid [7]. But it is difficult to achieve good resolution in one-step PHPLC separation for crude extract due to the complex composition. Furthermore, the presence of more impurities in crude extract was a great threat to the stationary phase, because it is usually expensive and liable to be contaminated. So, a particular complex mixture often needs prepurification and prefractionation prior to PHPLC separation. Consequently, many techniques, such as solvent/solvent partition,

* Corresponding author.
E-mail address: lxpsdta2001@sina.com (A. Li).

http://dx.doi.org/10.1016/j.jchromb.2015.12.038
1570-0232/

silica gel, macroporous absorptive resin (MAR) and polyamide resin, have been used to simplify the composition and remove most of the impurities.

MAR is a kind of porous high molecular material, which is used to selectively absorb constituents from aqueous solution as well as non-aqueous systems through electrostatic force, hydrogen bonding interaction, complexation, and size sieving action, etc. [8]. It has attracted much attention due to high absorption and desorption capacity, low cost, easy regeneration, and simple techniques. MAR can be used to remove most of the impurities from complex mixtures and enrich some chemicals, so it has been widely used in pharmaceutical industry [9–11].

The aim of the present study was to develope a simple and efficient method for preparation of polyphenols from Chinese propolis. In this work, the water-soluble fraction was first "prefractioned" by MAR to obtain four subfractions. And they were subjected to PHPLC, respectively. Finally, six phenolic acids and five flavonoids were obtained including caffeic acid, ferulic acid, isoferulic acid, 3,4-dimethoxy cinnamic acid, pinobanksin, caffeic acid benzyl ester, caffeic acid phenethyl ester, apigenin, pinocembrin, chrysin and galangin. Their purities were all above 98% as determined by HPLC, and the chemical structures (shown in Fig. 1) were confirmed by UV and NMR analysis.

2. Materials and methods

2.1. Apparatus

Analytical HPLC was performed on Agilent 1100HPLC system (Agilent Inc., America). PHPLC system was produced by Chengdu Gelaipu Technology Co., Ltd. (Chengdu, China). Spherigel ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) used for analytical HPLC and C_{18} SMB 100 column (250 mm × 25.4 mm I. D., 10 μm) used for PHPLC were all produced by Dalian Johnsson Separation Science and Technology Corporation (Dalian, China). ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d6 on a Mercury Plus 400 NMR (Varian Inc., America) at 400 and 100 MHz, respectively. Chemical shift were given in δ (ppm) relative to TMS as internal reference and coupling constants (J) in Hz.

2.2. Reagents

MARs (HPD 100, 400, 500, 600, 722, and 826) were purchased from Cangzhou Bon Absorber Technology Co., Ltd. (Cangzhou, China). Reagents used for HPLC were of chromatographic grade (Yucheng Chemical Factory, Yucheng, China), other reagents were of analytical grade (Jinan Reagents Factory, Jinan, China), and water used was distilled water. All solutions were filtered through a 0.45 μm membrane (Tianjin Keyilong Experimental Equipment Co., Ltd., Tianjin, China) before HPLC analysis.

2.3. Preparation of the crude extract

Chinese propolis was obtained from colonies of honeybees, *A. mellifera* L., in Shandong province of north China. 0.25 kg of Chinese propolis was frozened, milled and extracted with boiling water (4 × 2 L, 2 h). The water extract was filtered, combined and concentrated under reduced pressure with a rotary evaporator until the relative density of the solution was about 1.2. Then 95% ethanol was added to the solution until the concentration of ethanol was about 70%. After 12 h, the supernatant was separated and concentrated under reduced pressure, which yielded 15 g of residue.

2.4. Analytical HPLC conditions

HPLC analysis of all samples including the crude extract, eluents from MAR column chromatography and PHPLC peak fractions was performed on a Spherigel ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm). The mobile phase consisted of methanol (A) and 0.1% phosphoric acid (B) using the following gradient elution program: 0–30 min, 40–100%A. The flow rate was kept at 1.0 mL/min, and the effluent was monitored by a photodiode array detector (DAD) at 280 nm. The column temperature was kept at 30 °C and the injection volume was 20 μL.

2.5. Screening of MARs

Static absorption/desorption tests were performed to select the proper MARs for separation. The resins were pretreated by soaking in ethanol for 24 h. After removal of ethanol, the resins were washed by distilled water and subsequently soaked in 1 M NaOH for 5 h. Then, the resins were washed by distilled water. The washed resins were soaked in 1 M HCl for 5 h. Then, the resins were washed by distilled water thoroughly until the pH was 7. Finally, the pretreated resins were dried by an air dry oven (DHG-9070A, Shanghai Jinghong Laboratory Equipment Co., Ltd., Shanghai, China) at 60 °C to constant weight.

Six aliquots of each pre-treated hydrated resins were accurately weighed, placed into six 250 mL flasks, respectively. Six aliquots of sample solutions were prepared by dissolving suitable amount of the water extract from Chinese propolis into distilled water, respectively. Then the sample solutions were added to the flasks, respectively. Subsequently, the flasks were sealed tightly with a stopper and continually shaken in an incubator shaker (SHA-A, Jintan Chenghui Instrument Co., Ltd., Jintan, China) at 150 rpm for 12 h at 25 °C. The contents of target components in the initial sample solution and the solutions after absorption were analyzed by HPLC.

After absorption equilibrium was reached, the absorbate-laden resins were first filtrated, thoroughly washed by distilled water, and desorbed with 95% ethanol. The flasks were continually shaken at 150 rpm for 12 h at 25 °C. The contents of target components in the desorbed solutions were analysed by HPLC. Each process was repeated for three times.

The following equations were used to calculate the absorption capacity (Q_e), desorption capacity (Q_d), absorption ratio (A) and desorption ratio (D).

$$\text{Absorption capacity: } Q_e = \frac{(C_o - C_e)V_i}{W}$$

$$\text{Desorption capacity: } Q_d = \frac{C_d V_d}{W}$$

$$\text{Absorption ratio: } A(\%) = \frac{C_o - C_e}{C_o} \times 100\%$$

$$\text{Desorption ratio: } D(\%) = \frac{C_d V_d}{(C_o - C_e)V_i} \times 100\%$$

In the equations, Q_e is the absorption capacity at absorption equilibrium resin; Q_d is the desorption capacity after desorption equilibrium; C_o and C_e are the initial and absorption equilibrium concentrations of target components in the solutions, respectively; C_d is the concentration of target components in the desorption solutions; V_i and V_d are the volumes of the initial sample and desorption solutions, respectively. A is the absorption rate (%), and D is the desorption ratio (%). W is the weight of dry resin.