Separation and Purification Technology 104 (2013) 200–207

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

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

Bioassay-guided separation and purification of water-soluble antioxidants from *Carthamus tinctorius* L. by combination of chromatographic techniques

Lingnan Li [a,b], Yi Yang [a], Xueling Hou [a], Dongyu Gu [a], Ba Hang [a], Rahima Abdulla [a], Guirong Wu [b], Xuelei Xin [a], Haji Akber Aisa [a,*]

[a] *Key Laboratory of Xinjiang Indigenous Medicinal Plants Resource Utilization, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Urumqi 830011, China*
[b] *School of Pharmacy, Xinjiang Medical University, Urumqi 830054, China*

ARTICLE INFO

Article history:
Received 24 September 2012
Received in revised form 19 November 2012
Accepted 20 November 2012
Available online 29 November 2012

Keywords:
Carthamus tinctorius L.
Bioassay-guided separation
Combination of chromatographic techniques
High-speed counter-current chromatography
Water-soluble antioxidants

ABSTRACT

A combined chromatographic method using high-speed counter-current chromatography (HSCCC) and Sephadex LH-20 chromatography was established for bioassay-guided separation and purification of water-soluble antioxidants from *Carthamus tinctorius* L. florets. The crude sample II obtained by an initial cleanup step on the AB-8 macroporous resin exhibited a potential ABTS radical cation scavenging activity with the SC_{50} value of 49.28 μg/mL. The HSCCC separation was performed with a two-phase solvent system composed of *n*-butanol-0.1 mol/L HCl (1:1, v/v) at a flow rate of 1.2 mL/min. After a single run, 3 mg hydroxysafflor yellow A (HSYA) with 98% purity was separated as a major component from 20 mg of crude sample II. In order to increase the yield and investigate the minor components, the sample size of HSCCC separation was enlarged to 1 g. Then, HSCCC fractions were subjected to Sephadex LH-20 chromatography and eluted with distilled water at a flow rate of 1.5 mL/min to remove the acid and further separate the components. This separation yielded 184.0 mg of HSYA (**1**), 10.0 mg of 5,4′-dihydroxyflavone-3,6-di-O-*β*-D-glucoside-7-O-*β*-D-glucuronide (**2**), 3.0 mg of 3-O-caffeoylquinic acid (**3**), and 3.2 mg of 4-O-*β*-D-glucosyl-trans-*p*-coumaric acid (**4**) from 1 g crude sample II of *C. tinctorius* L. florets. The purity of the separated compounds were over 95% by HPLC analysis, and their chemical structures were confirmed by MS, ^{1}H NMR and ^{13}C NMR. Compounds 3 and 4 were found from this plant for the first time. Antioxidant activities assayed *in vitro* by ABTS radical cation scavenging showed that the SC_{50} values of the above four compounds were 44.39 ± 1.62 μg/mL, 78.13 ± 1.00 μg/mL, 21.85 ± 1.96 μg/mL, and 31.44 ± 2.06 μg/mL, respectively.

1. Introduction

Carthamus tinctorius L. (safflower), is a highly branched, herbaceous, thistle-like annual plant of Compositae family [1]. There are about 19 species in the genus *Carthamus* which are widely distributed in Middle Asia, Southwestern Asia and Mediterranean areas [2]. Among them, only a few species can be used as food additives and natural pigments. The flowers of Safflower, known as Honghua, have been used as Chinese traditional medicine for the treatment of stroke, coronary heart disease and angina pectoris for over 2500 years [3,4]. And it was also used for anti-tumor [5], antioxidant [6], inhibiting the melanin production [7], treating dysmenorrhea effects [8] and anti-hypertension [9]. The biological activities of safflower have been attributed to several classes of compounds, such as flavonoids [10,11], alkaloids [12,13], steroids, lignans [14] and phenolic compounds [15], which are isolated from different morphological parts of the plant.

In our study, the antioxidant activity found in the extract of safflower prompted us to investigate the chemical constituents and its association with antioxidant. Therefore, *in vitro* assay of the antioxidant activities by ABTS radical cation scavenging activity were carried out for crude samples and purified compounds in order to trace the antioxidant constituents.

It is well known that natural products are constituted by complex mixtures, and contain a massive amount of potential active compounds. Some main ingredients can be chosen as 'marker compounds' for the chemical evaluation or quality control of the plant whereas most of the active compounds are minor ingredients. However, the simultaneous preparation of major compounds and minor active compositions is a big challenge, especially for water-soluble minor compounds. The traditional separation method usually utilizes multiple column chromatography to achieve these goals, such as silica gel, polyamide and Sephadex LH-20 column. However, it is tedious, time consuming, and often leads to

* Corresponding author. Tel.: +86 991 3835679, fax: +86 991 3838957.
E-mail address: haji@ms.xjb.ac.cn (H.A. Aisa).

1383-5866/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.seppur.2012.11.027

loss of activity during the isolation and purification procedures due to dilution effects or decomposition especially for natural antioxidants. Besides, many minor compounds disappear after column chromatographic separation because of irreversibly adsorption onto the solid support during separation [16]. High-speed counter-current chromatography (HSCCC) is a continuous liquid–liquid partition chromatography without solid support matrix [17] and has been widely used for various natural compounds separation [18–21]. Because of high sample recovery, low solvent consuming and no irreversible adsorption [22], it is appropriate for the separation of minor active ingredients from natural products [23–25].

The chromatographic process of HSCCC is based on the partition coefficient (K) value of the target compound between the mobile and stationary phases of an equilibrated two-phase solvent system [26]. However, in our further study, the antioxidant extract of safflower were water-soluble compounds, and many minor ingredients detected by HPLC covers large percent of the crude sample. K values of hydrophilic compounds which were partitioned to the lower aqueous phase are very small in most of the common two-phase solvent systems [27]. Some polar two-phase solvent systems were used for the separation of water-soluble compounds such as carboxylic acids [28], sugars [29], nucleic acids [30], peptides [31–35] and proteins [36,37]. But these solvent systems are not suitable for phenolic compounds in the present study. Since these acidic compounds have two molecular forms, protonated and deprotonated, each with the different K values, and form a broader peak when partly ionized [27]. So the hydrochloric acid was introduced to protonate the phenolic compounds totally to stabilize their K values in the two-solvent system. Because the introduced hydrochloric acid might destroy the natural antioxidants, the separated fractions were eluted with distilled water on a Sephadex LH-20 chromatography column to remove the acid and further separate the minor ingredients. In the present study, bioassay-guided isolation of target compounds using the combined chromatographic method was carried out to identify the compounds responsible for the antioxidant activity.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument employed in this study was a model TBE-300A high-speed counter-current chromatograph (Tauto Biotech, Shanghai, China) with three polytetrafluoroethylene (PTFE) preparative coils (diameter of 2.6 mm, total volume 290 mL). The revolution radius (the distance between the holder axis and the central axis of the centrifuge) was 5 cm, and the β-value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft and R, the revolution radius). The speed of the apparatus could be regulated in a range between 0 and 1000 rpm, where the optimum speed of 850 rpm was used throughout in the present study.

The solvent was pumped into the column with a model TBP-50A constant-flow pump (Tauto Biotech, Shanghai, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV monitor (Binda Yingchuang Technology Co., Ltd., Beijing, China) at 254 nm, and a manual sample injection valve with a 20 mL loop for the preparative HSCCC was used to introduce the sample into the column. Model N2000 workstation (Zhejiang University, Hangzhou, China) was used to draw the chromatogram.

The analytical high-performance liquid chromatography (HPLC) equipment was a DIONEX system including a P680 pump, an ASI-100 automated sample injector, a TCC-100 temperature-controlled column compartment and a UVD170U detector. The analysis was carried out with a Gemini 110A-C_{18} column (5 μm, 4.6 mm × 250 mm) from Phenomenex Inc., Guangzhou, China. Evaluation and quantification of data were made on a Chromeleon WorkStation.

2.2. Chemicals and reagents

Acetonitrile and methanol used for HPLC analysis were of chromatographic grade and purchased from Fisher Scientific Company (Pittsburgh, PA, USA). All organic solvents used for HSCCC were of analytical grade and purchased from Tianjing Chemical Factory (Tianjing, China). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden), AB-8 macroporous resins (The Chemical Plant of NanKai University, Tianjin, China) and ZTC natural clarifying agents II (Tianjing, China) were used in the separation and purification process of the crude sample. ABTS was bought from Sigma–Aidrich (Shanghai Division), and the solutions were freshly prepared in distilled water and kept at dark. Multi-well plates (Greiner) and multi-well plates readers (Molecular Devices, California, USA) were used in the antioxidant activity experiments.

The dried florets of *C. tinctorius* L. were collected from Emin County (Xinjiang, China) and were verified by Professor Zhaomu Wang (Xinjiang Academy of Agricultural Science, Urumqi, China).

2.3. Preparation of crude samples

Dry flowers of *C. tinctorius* L. (800 g) were extracted with the distilled water (10 L) at 80 °C for 20 min. The extraction procedure was repeated two times. The extracts were combined and evaporated to 1600 mL under reduced pressure. Then 8% ZTC II natural clarifying agents were added under constant stirring. After deposited for 24 h, the solution was filtered. About 1200 mL crude sample I was obtained, which was subjected to the AB-8 macroporous resin column (3 cm × 30 cm). The column was firstly eluted by distilled water (4 L) and monitored by the Molisch reaction using sugar control. Molisch reaction is used for Distinguishing carbohydrate and noncarbohydrate [38]. Then, it was eluted by 10%, 30%, 50% ethanol (2 L) in turn, and after evaporation to dryness under reduced pressure at 40 °C, the crude sample II (34 g), III (26 g) and IV (15 g) were obtained, respectively, which were used for evaluation of antioxidant activity.

2.4. Measurement of partition coefficient (K)

The two-phase solvent system was selected according to the partition coefficient (K) of the target components. Different volume ratios of solvent systems were prepared and equilibrated in a separation funnel at room temperature. The K values were determined by HPLC analysis as follows: A suitable amount of crude sample (1 mg) was dissolved in 4.0 mL of the mixture containing equal volume of each phase of the two-phase solvent system in a test tube, and the contents were mixed thoroughly. After the equilibration was completed, the upper phase and the lower phase were analyzed by HPLC separately. The peak area of the upper phase was recorded as A_U and that of the lower phase as A_L. The K value was calculated according to the equation, $K = A_U/A_L$ (Table 1).

2.5. HSCCC separation procedure

The preparative HSCCC was performed with a model TBE-300A HSCCC instrument as follows: The multilayer coil separation column was first entirely filled with the upper phase as the stationary phase, followed by sample injection (the crude sample in 20 mL of a mixture of upper and lower phases), and when the column was