Food Chemistry 131 (2012) 1181–1186

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Identification of antioxidant compounds of *Mucuna sempervirens* by high-speed counter-current chromatographic separation–DPPH radical scavenging detection and their oestrogenic activity

Qizhen Du *, Bo Li

Institute of Food and Biological Engineering, Zhejiang Gongshang University, 149 Jiaogong Road, Hangzhou 310035, China

ARTICLE INFO

Article history:
Received 30 March 2011
Received in revised form 16 July 2011
Accepted 22 September 2011
Available online 7 October 2011

Keywords:
Mucuna sempervirens
High-speed counter-current chromatography
Antioxidant activity
Oestrogenic activity
Separation
Identification

ABSTRACT

High-speed counter-current chromatography (HSCCC) was used to separate an ethanolic extract of leaves of *Mucuna sempervirens* into fractions which were then detected their antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The fractions were grouped into seven larger fractions (components) based on their antioxidant activity. The seven components were isolated by preparative HPLC to yield 12 flavonoids and two phenolic acids identified by electrospray ionisation mass spectrometry and nuclear magnetic resonance analyses. The oestrogenic activity of the 12 isolated flavonoids was evaluated by the luciferase assay based on the MVLN cell line. The results indicate that leaves of *M. sempervirens* are a flavonoid-rich resource that may supply antioxidant and oestrogenic compounds to the human body. The HSCCC separation–DPPH radical scavenging detection could be widely applied for rapid screening and isolation of antioxidants from complex plant extracts.

1. Introduction

Antioxidants and oestrogenic flavonoids in foods have received much attention because of their relevance to human health (Breinholt, Hossaini, Svendsen, Brouwer, & Nielsen, 2000; Rice-Evans & Packer, 1998). *Mucuna sempervirens* Hemsl. (Leguminosae) (Tateishi & Ohashi, 1981) is used in Traditional Chinese Medicine, and its activities include activating blood and dissolving stasis, regulating menstruation, and curing rheumatism and physical injuries (Lu & Peng, 2007). There have been several studies on the active constituents of this plant. The leaves contain C-glycosylflavones including 8-C-α-L-arabinosylluteolin, 6,8-di-C-α-L-arabinosylapigenin, and isoorientin (Ishikura & Yoshitama, 1988). The flavonoid content of the leaves is more than 10% on a dry weight basis, and ethanolic extracts from leaves show strong antioxidant activity (Zhang, Liu, Rao, & Feng, 2008). However, the active compounds and their contribution to antioxidant activity need to be further studied.

In previous studies, identification of antioxidant compounds was achieved by isolating target compounds after screening by using the HPLC and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay (Pérez-Bonilla et al., 2011; Zhang, Shi, Wang, & Huang, 2011), by the high-throughput relative DPPH radical scavenging capacity assay (Cheng, Moore, & Yu, 2006), by the HO radical scavenging capacity assay (Moore, Yin, & Yu, 2006), or by the thin layer chromatography (TLC) bioautography assay (Cimpoiu, 2006; Gu, Wu, & Wang, 2009; Jayasinghe, Puvanendran, Hara, & Fujimoto, 2004) and then identifying target compounds by spectral analyses, e.g. mass spectrum (MS) and/or nuclear magnetic resonance (NMR) analyses. The isolation methods used in such studies include TLC, liquid chromatography (LC), high-speed counter-current chromatography (HSCCC) and preparative HPLC. HSCCC is a continuous liquid–liquid partition chromatographic method in which compounds are partitioned between two immiscible liquid phases without solid support or irreversible adsorption (Ito, 2005). The antioxidant activity of all components of a plant extract can be screened, since all the components in the mobile phase and stationary phase can be fractioned (Du, Chen, Jerz, & Winterhalter, 2004; Du, Li, & Ito, 2001; Lu, Pan, & Berthod, 2008). Conventional isolation methods can lead to the loss of activity during the isolation and purification procedures because of the dilution effects and/or decomposition of the active components, especially antioxidants. Due to the advantages of the HSCCC system, we can first separate an extract into fractions, and then detect the antioxidant activity of the fractions for further separation and purification. This procedure is useful for screening minor antioxidant compounds because the amount of each fraction can easily be adjusted for the antioxidant activity assay.

* Corresponding author. Tel./fax: +86 571 88071024 8575.
E-mail address: qizhendu@163.com (Q. Du).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.09.095

In the present study, a crude ethanolic extract of *M. sempervirens* leaves was separated into fractions by HSCCC. The mobile phase used for separation had a stepwise gradient of polarity. The fractions were screened for their antioxidant activity, and grouped into seven larger fractions (components) based on this activity. Specific antioxidant compounds were isolated from these fractions by preparative HPLC. The individual compounds were identified based on their electrospray ionisation mass spectrometry (ESI-MS) and NMR spectra.

Phytoestrogens with oestrogen-like activities derived from various plants could have beneficial roles in treating human oestrogen deficiency (Lim, Ha, Ahn, & Kim, 2011). Phytoestrogens such as flavonoids may exert their effect by directly binding to oestrogen receptors and producing oestrogenic effects (Klotz et al., 1996; Mukherjee, Mukherjee, & Saha, 2005; Zacharewski, 1997). It is hypothesised that flavonoids modulate the endogenous activities of oestrogen receptors to slow or prevent the development of breast and ovarian cancers. Therefore, they may represent a natural alternative for hormone replacement therapy (Limer & Speirs, 2004). MVLN-cells are human breast cancer cells (MCF-7 cells) that have been stably transfected with the pVit-tk-Luc-Neo plasmid. The transfected cells contain a human oestrogen response element (ERE) coupled to the luciferase reporter gene (Demirpence, Duchesne, Badia, Gagne, & Pons, 1993). This cell line has been used successfully to screen phytoestrogens or environmental oestrogen-like chemicals (Bonefeld-Jorgensen, Grünfeld, & Gjermandsen, 2005; Freyberger & Schmuck, 2005; Le Bail, Varnat, Nicolas, & Habrioux, 1998). In this study, the MVLN-cell line was used to evaluate the oestrogenic activity of the flavonoids isolated from the ethanolic extract, which may give support for the traditional use of the plant in regulating menstruation.

2. Materials and methods

2.1. Materials

Fresh leaves of *M. sempervirens* were collected from the Botanical Garden of Hangzhou, China. The leaves (approx. 5 kg) were freeze-dried, yielding 1 kg of sample.

2.2. Extraction

The freeze-dried sample (1 kg) was extracted twice with 5 l 90% ethanol for 2 h at 50 °C. The extracts were combined and evaporated to syrup. The syrup was defatted with ether and lyophilised, yielding 235 g crude extract. A portion of the crude extract (600 mg) was used for HSCCC separation to obtain fractions for the antioxidant assay.

2.3. High-speed counter-current chromatographic separation

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 500-ml column with six-layer coils made from 3.0 mm i.d. polytetrafluoroethylene (PTFE) tubing. A K-1800 Wellchrom preparative 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 25-ml tube racks were utilised to constitute a HSCCC system. The sample solution was prepared by dissolving 600 mg ethanolic extract in 20 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. Then, 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 3.0 ml/min.

Since the crude extract contained components with various polarities, a stepwise elution in the HSCCC separation was carried out. The stationary phase was water saturated with *n*-butanol and ethyl acetate. The stepwise elution was performed with the following solvents: *n*-hexane–ethyl acetate (1:1) for 80 min, *n*-hexane–ethyl acetate (1:2) for 80 min, *n*-hexane–ethyl acetate (1:4) for 80 min, ethyl acetate for 80 min, *n*-butanol–ethyl acetate (1:4) for 80 min, *n*-butanol–ethyl acetate (1:2) for 80 min, *n*-butanol–ethyl acetate (2:2) for 80 min, and *n*-butanol–ethyl acetate (2:1) for 80 min. The effluent was collected in 20-ml fractions by a fraction collector. After the stepwise elution, the solvent was drained from the column and collected in 20-ml fractions. All fractions were assayed for antioxidant activity. The fractions were combined into seven larger fractions (components) on the basis of their antioxidant activity (Fig. 1).

2.4. HPLC analysis and preparation

The antioxidant components from the HSCCC separation were analysed by HPLC, and the main compounds were prepared by preparative HPLC. The analytical HPLC system consisted of an Alliance 2695 separations module, an ODS AQ column (150 × 3.9 mm i.d., 5 μm), a 996 PDA detector, and a Millennium HPLC 2010 processing system (Waters, Milford, USA). The gradient elution was carried out as follows: water-formic acid (99.5:0.5, v/v) 100% to methanol 100% from 0 to 30 min at a flow rate of 0.8 ml/min. For preparative HPLC, a 250 × 20 mm i.d., 15 μm ODS AQ column was employed. The preparative HPLC conditions were determined based on the analytical HPLC results.

2.5. ESI-MS and NMR

All ESI-MS experiments were performed on a Bruker Esquire LC–MS ion trap multiple mass spectrometer (Bremen, Germany) in positive and negative ionisation modes analysing ions up to *m*/*z* 2200. ^{1}H-, ^{13}C-, and DEPT 90/135-NMR spectra were recorded in DMSO-d_6 on a Bruker Avance 500 (Karlsruhe, Germany) with 500 MHz for ^{1}H-, and 125 MHz for ^{13}C-measurements.

2.6. Determination of antioxidant activity

The DPPH radical assay was carried out as described elsewhere (Tapia et al., 2004). The free radical scavenging efficiency of the fractions and isolated compounds was determined by measuring decolouration of the DPPH radical. Briefly, 250 μl of each HSCCC fraction or 250 μl diluted sample (0.4 mg/ml dissolved in methanol) mixed with 40 μl DPPH–methanol solution (0.4 mg/ml) and completed to a final volume of 3 ml with methanol. The DPPH–methanol solution served as the control. The absorbance was measured at 517 nm after the mixture was incubated at 37 °C for

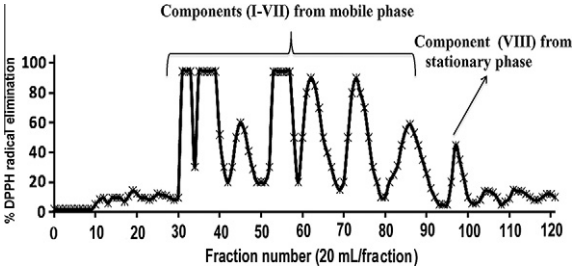

Fig. 1. Antioxidant activity-chromatogram of fractions obtained from HSCCC separation of 600 mg ethanolic *M. sempervirens* leaf extract.