Preparative separation of 3-O-methylkaempferol from *Caragana leucophloea* by high-speed counter-current chromatography and its antimicrobial activity

Haifeng Gao¹, Chao Luo¹, Lan Wang², Jihua Wang¹, Bingbing Zheng¹, Youliang Peng¹ and Ligang Zhou¹*

¹College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China.
²College of Plant Science, Tarim University, Alar 843300, Xinjiang, China.

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3-O-methylkaempferol was successfully separated from the ethyl acetate fraction of the crude ethanol extract from *Caragana leucophloea* by high-speed counter-current chromatography (HSCCC) with n-hexane-chloroform-methanol-water-acetic acid (0.5:3.5:3.0:2.0:0.05, v/v) as the two-phase solvent system. 3-O-Methylkaempferol was examined to have strong antibacterial activity with the minimum inhibitory concentration (MIC) values ranging from 12.5 to 50 μg/ml, and the median inhibitory concentration (IC₅₀) values from 7.42 to 25.83 μg/ml. The IC₅₀ value of 3-O-methylkaempferol on the spore germination of *Magnaporthe oryzae* was 56.56 μg/ml. This is the first time to isolate 3-O-methylkaempferol from *C. leucophloea*. Preparative separation of 3-O-methylkaempferol from *C. leucophloea* by HSCCC should be an effective and rapid method.

**Key words:** Leguminosae, *Caragana leucophloea*, preparative separation, 3-O-methylkaempferol, high-speed counter-current chromatography, antimicrobial activity.

INTRODUCTION

High-speed counter-current chromatography (HSCCC), a support free liquid-liquid partition chromatographic technique, eliminates the irreversible adsorptive loss of sample onto the solid support. Furthermore, the HSCCC method permits direct introduction of crude extracts or fractions into the coil tube without additional treatments, hence it has been successfully applied to isolate and purify a number of natural products (Ito, 1986; Wei et al., 2009; Huang et al., 2010; Wang et al., 2010).

*Caragana leucophloea* Pojark. belongs to leguminosae and is mainly distributed in the provinces of Xinjiang, Gansu and Inner Mongolia of Northwest China. It is also distributed in the surrounding countries such as Tajikistan, Kyrgyzstan, Kazakhstan and Mongolia (Niu, 1999). The roots of *C. leucophloea* have been used to cure irregular menstruation, leucorrhea, numbness and pain caused by arthritis, edema due to the deficiency of spleen, lactation insufficiency, and traumatic injury in traditional Chinese medicine (Meng et al., 2009). To the best of our knowledge, there are no reports on the metabolites of this plant species in the literature though some species in genus *Caragana* were reported to be rich with flavonoids, stilbenoids, terpenoids, and lectins (Meng et al., 2009). The aim of this investigation was to develop an efficient method to isolate and purify 3-O-methylkaempferol, a flavonol from *C. leudophloea* by HSCCC. The antimicrobial activity of 3-O-methylkaempferol was also evaluated.

MATERIALS AND METHODS

**Plant materials**

The aerial parts of *C. leudophloea* Pojark. were collected in June 2008 at Kelamayi (80°44' to 86°1'E, 44°7' to 46°8'N) of Xinjiang Province of China, and was authenticated by Professor Ping Yan of Shihze University of Xinjiang. A voucher specimen of this collection (BSMPMI-200806001) was deposited at the Herbarium of the Institute of Chinese Medicinal Materials, China Agricultural University. The plant materials were left to dry in the shade at room temperature to a constant weight.
Preparation of the crude extract and solvent fractions

The air-dried and powdered aerial parts (1.0 kg) of C. leucophloea were soaked in 95% ethanol (5 L) at room temperature for three times at an interval of 7 days (3 × 5 L). After the filtrate was combined and concentrated under vacuum at 50°C, the brown residue (95.5 g, 9.55% w/w) was suspended in water and extracted with petroleum ether, then with ethyl acetate, and last with n-butanol, to obtain petroleum ether fraction (32.0 g, 3.20% w/w), ethyl acetate fraction (13.1 g, 1.31% w/w), n-butanol fraction (27.2 g, 2.72% w/w), and aqueous fraction (20.5 g, 2.05% w/w), successively.

High performance liquid chromatography analysis

The analytical high performance liquid chromatography (HPLC) system consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector, and a CBB-20A lite system controller. Luna reversed-phase C18 column (4.6 mm × 250 mm, 5 μm, Phenomenex, Torrance, USA) was used for separation by using a mobile phase of methanol-H2O (55: 45, v/v) at a flow rate of 1.0 ml/min. The injection volume for each time was 10 μl; the temperature was maintained at 40°C, and UV detection at 254 nm. Methanol used for HPLC analysis was of chromatographic grade and purchased from Xilong Chemical Company of China.

Selection of the two-phase solvent system

The composition of the two-phase solvent system (Table 1) was selected according to the partition coefficient (K value) of 3-O-methylkaempferol. The K-value was determined by HPLC as follows: 1.0 mg of 3-O-methylkaempferol, which had been purified and identified in our laboratory, was added to a test tube to which 2.0 ml of the lower phase of the pre-equilibrated two-phase solvent system was added. After the compound was thoroughly dissolved, equal volume of the upper phase of the pre-equilibrated two-phase solvent system was added and shaken violently for several times. Finally, the upper and lower phases were analyzed by HPLC. The K-value was calculated according to the ratio of the peak areas. K = A2/A1, where A2 was the peak area of the upper phase, and A1, the peak area of the lower phase.

Preparation of the two-phase solvent systems and sample solution

The selected solvent system, n-hexane-chloroform-methanol-water-acetic acid (0.5: 3.5:3:0.2:0.05, v/v/v), was prepared by adding all the solvents to a separation funnel at room temperature according to the volume ratios and thoroughly equilibrated by shaking repeatedly, and two phases were separated. Both two phases were obtained and degassed by sonication for 30 min prior to use. The sample solution was prepared by dissolving the ethyl acetate fraction (about 500 mg for each time) in a 20-ml of the mixture of equal volume of lower and upper phases of the solvent system used for HSCCC separation, and sonicated for several minutes before loaded into the column.

HSCCC separation procedure

Preparative HSCCC was carried out with a model TBE-300B (Tauto Biotech, China) instrument. The apparatus was equipped with a polytetrafluoroethylene tube (diameter of tube was 2.6 mm, and total volume was 280 ml) composing of three preparative coils and a 20-ml sample loop. The HSCCC system was equipped with a TBP-5002 pump and TBD-2000 UV detector operating at 254 nm (Tauto Biotech, China), and a WH500-USB workstation (Wuhao, China). The experimental temperature was 25°C adjusted by a constant temperature circulating implement (Boyikang, China). All organic solvents used for HSCCC were of analytical grade and bought from Beijing Chemical Company. The coil column was first entirely filled with the stationary phase (the upper phase) of the solvent system. Then the apparatus was rotated at 850 rpm, and the mobile phase (the lower phase) was pumped into the column at a flow rate of 3 ml/min. After the mobile phase front emerged, and hydrodynamic equilibrium was established in the column, the sample solution was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile. The temperature of the apparatus was set at 25°C. After the separation was completed, the solvent in the coil column was ejected with nitrogen gas. Volumetric measurement showed that the retention ratio of the stationary phase solvent was 81.40%.

Analysis and identification of 3-O-methylkaempferol

The ethyl acetate solvent fraction, HSCCC peak 2 fraction, upper and lower phases of the two-phase solvent system, and 3-O-methylkaempferol were analyzed by HPLC. The linear equation of 3-O-methylkaempferol by HPLC analysis was Y = 2.7587X + 40.809, where Y was the peak area, and X was the sample concentration (μg/ml). Melting point of 3-O-methylkaempferol was determined on an XT4-100B microscopic melting-point apparatus (Tianjin Tianguang Optical Instruments Company, China) and uncorrected. NMR spectra were recorded on a Bruker Avance DRX-500 (1H at 500 MHz and 13C at 125 MHz) spectrometer. ESI-MS spectrum was recorded on a Bruker Esquire 6000 LC/MS spectrometer. HPLC-TOF-MS analysis was carried out with an Agilent 1260 HPLC-6520 Q-TOF-MS System. The structure of 3-O-methylkaempferol was identified according to the physicochemical and spectrometric data, and confirmed by comparison with literature data.

Antimicrobial activity assay

Thin layer chromatography (TLC) bioautography examination of the

Table 1. The partition coefficient (K value) of 3-O-methylkaempferol with two-phase solvent system by HPLC analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>Two-phase solvent system and its volume ratio (v/v)</th>
<th>K value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloroform-methanol-water-acetic acid (4.0:2:0:2.0:0.05)</td>
<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform-methanol-water-acetic acid (4.0:3:0:2.0:0.05)</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform-methanol-water-acetic acid (4.0:4:0:2.0:0.05)</td>
<td>0.51</td>
</tr>
<tr>
<td>4</td>
<td>n-Hexane-chloroform-methanol-water-acetic acid (0.5:3.5:3.0:2.0:0.05)</td>
<td>0.89</td>
</tr>
<tr>
<td>5</td>
<td>n-Hexane-chloroform-methanol-water-acetic acid (1.0:3.0:3.0:2.0:0.05)</td>
<td>1.39</td>
</tr>
</tbody>
</table>
Antimicrobial activity of 3-O-methylkaempferol was evaluated by both antibacterial and antifungal activity assays. The microplate spectrophotometer (PowerWave HT, BioTek Instruments, USA) was employed to measure light absorption values of the samples. A modified broth dilution-colorimetric assay by using the chromogenic reagent 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA) was used to detect antibacterial activity of the samples according to our previous report (Liu et al., 2010). Five gram-negative (Agrobacterium tumefaciens ATCC 11158, Escherichia coli ATCC 29425, Pseudomonas lachrymans ATCC 11921,Ralstonia solanacearum ATCC 11636 and Xanthomonas vesicatoria ATCC 11633) and one gram-positive (Bacillus subtilis ATCC 11852) bacteria were selected for antibacterial activity assay. The spore germination assay by using rice blast fungus Magnaporthe oryzae (strain 131) was employed to detect the antifungal activity of the samples (Liu et al., 2009). The minimum inhibitory concentration (MIC) value to bacteria was defined as the lowest sample concentration that inhibited visible growth, as indicated by the MTT staining. The median inhibitory concentration (IC_{50}) value was calculated using the linear relation between the inhibitory probability and concentration logarithm according to the method of Sakuma (1998).

RESULTS AND DISCUSSION

HPLC analysis of the ethyl acetate fraction

After TLC-bioautography examination of the samples, one antibacterial spot was screened in the ethyl acetate fraction of the crude ethanol extract from C. leucophloea. The ethyl acetate fraction was further analyzed by HPLC (Figure 2A) to show a main peak with its retention time as 13.577 min, and UV \( \lambda_{\text{max}} \) (methanol) as 266 and 352 nm, respectively (Figure 2Aa). The best separation conditions were gained using methanol-H\(_2\)O (55:45, v/v) as the solvent system eluted at a flow rate of 1.0 ml/min at the detection wavelength of 254 nm. After HPLC-TOF-MS analysis on the main peak, an HR-ESI-MS \( m/z \) 299.0561 [M-H] was obtained and the molecular formula was determined as C\(_{16}\)H\(_{19}\)O\(_6\) (Figure 2Ab). The result was in accordance with that of the structural identification of 3-O-methylkaempferol.

Selection of suitable two-phase solvent system for HSCCC

Successful separation by HSCCC largely depends upon the selection of a suitable two-phase solvent system, which needs an ideal range of the partition coefficient (K) for the target compound (Ito, 2005). In our test, different solvent systems containing chloroform-methanol-water-acetic acid or n-hexane-chloroform-methanol-water-acetic acid were examined to optimize the K values (Table 1) of 3-O-methylkaempferol by HPLC analysis. The most appropriate K value as 0.89 was obtained with a two-phase solvent system composed of n-hexane-chloroform-methanol-water-acetic acid at its volume ratio of 0.5 : 3.5 : 3.0 : 2.0 : 0.05 (v/v), which was selected to further isolate and purify 3-O-methylkaempferol by HSCCC in the present study.

Separation of 3-O-methylkaempferol and chemical structural identification

As the ethyl acetate fraction (13.1 g) had a main compound with its higher content and stronger antimicrobial activity than any other solvent fraction, it was further fractionated with HSCCC. Under the optimal...
Figure 2. HPLC analysis and UV spectr

A

B

mAU

mAU
conditions, six peak fractions were obtained in one-step elution in about 5 h. The HSCCC chromatogram is shown in Figure 1. The total yield was calculated as 4.18 g for peak 1 fraction, 2.20 g for peak 2 fraction, 0.52 g for peak 3 fraction, 0.24 g for peak 4 fraction, 0.16 g for peak 5 fraction, and 0.31 for peak 6 fraction. All the six peak fractions were examined by both TLC and HPLC analysis. Only peak 2 fraction was examined as a pure compound (with 96.78% purity). HPLC chromatogram is shown in Figure 2B. Its physicochemical and spectrometric data were given as follows: Yellow amorphous powder (MeOH); m.p. 252 to 255°C; ESI-MS m/z 301 [M+H]+; 1H-NMR (DMSO-d6, 500 MHz) δ (ppm), 3.77 (3H, s, OCH3-3), 12.67 (1H, s, OH-5), 6.19 (1H, d, J = 2.0 Hz, H-6), 10.81 (1H, s, OH-7), 6.42 (1H, d, J = 2.0, H-8), 7.92 (H, d, J = 7.0 Hz, H-2'), 6.93 (1H, d, J = 7.0 Hz, H-3'), 10.23 (1H, s, OH-4'), 6.93 (1H, d, J = 7.0 Hz, H-5'), 7.92 (H, d, J = 7.0 Hz, H-6'); 13C-NMR (DMSO-d6, 125 MHz) δ (ppm), 155.6 (C-2), 137.6 (C-3), 59.7 (OCH3-3), 177.9 (C-4), 161.2 (C-5), 98.5 (C-6), 164.1 (C-7), 93.7 (C-8), 156.3 (C-9), 104.2 (C-10), 120.5 (C-1'), 130.1 (C-2'), 115.6 (C-3'), 160.1 (C-4'), 115.6(C-5'), 130.1 (C-6'). After comparing the data with those reported in the literature (Wang et al., 1989; Nakatani et al., 1991; Lee et al., 2003), the compound was identified as 3-O-methylkaempferol; the structure is shown in Figure 3.

Antimicrobial activity of 3-O-methylkaempferol

3-O-Methylkaempferol was tested for antimicrobial activity; MIC and IC50 values are summarized in Table 2. The MIC values on the test bacteria ranged from 12.5 to 50 μg/ml, and IC50 values from 7.42 to 25.83 μg/ml. Among the test bacteria, E. coli and R. solanacearum were the most sensitive to 3-O-methylkaempferol. The IC50 value of 3-O-methylkaempferol on the spore germination of M. oryzae was 56.56 μg/ml. There are no reports on the antimicrobial activity of 3-O-methylkaempferol except for its antipoliovirus activity (Robin et al., 2001).

In conclusion, 3-O-methylkaempferol as a main antimicrobial compound was successfully separated from C. leucophloea by HSCCC with n-hexane-chloroform-methanol-water-acetic acid (0.5: 3.5: 3.0: 2.0: 0.05, v/v) as the two-phase solvent system. The content of 3-O-
methylkaemperol was 16.79% in the ethyl acetate fraction, 2.30% in the crude ethanol extract, and 0.22% in the dried aerial parts of C. leucophloea, respectively. The present study will lay a foundation for the large scale preparation of 3-O-methylkaemperol from the aerial parts of C. leucophloea by using HSCCC. It also provides additional data for supporting the utilization of C. leucophloea as medicine. Greater research efforts should be devoted to confirming antimicrobial activity of 3-O-methylkaemperol which may be applied in food, agriculture and medicinal industry as a source of antimicrobial agent. Separation and purification of other compounds from C. leucophloea are in progress.

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