Preparative isolation of Heteroclitin D from Kadsuriae Caulis using normal-phase flash chromatography

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\textbf{Abstract}
Heteroclitin D (H.D) was successfully isolated from Kadsuriae Caulis by using flash chromatography and recrystallized by methanol, 10.2 mg of H.D was obtained from 4.86 g of crude extract, and the purity determined by HPLC was 99.4%. The structure was identified by UV, IR, MS, and NMR analysis. The fast, simple and efficient method can be applied to the preparation of reference substance of H. D.

\section{1. Introduction}
Kadsuriae Caulis (Dian-Jixueteng in Chinese), the dry stems and rattans of \textit{Kadsuriae interior} A.C. Smith, is a member of Magnoliaceae's family \textsuperscript{[1]}, mainly growing in Yunnan, Guizhou, Guangdong and Guangxi provinces. Heteroclitin D (H.D) is the main active ingredient in Kadsuriae Caulis. Some components, including lignans and triterpenes, also exist in Kadsuriae Caulis. It displays a variety of biological activities such as lipid antioxidant, vascular disease prevention, and anti-tumor effects \textsuperscript{[2–6]}. However, there are few published studies describing the preparative isolation of H.D. Therefore, it is necessary to develop an efficient method for the separation and purification of H. D.

Flash chromatography provides a rapid and inexpensive general method for the preparative separation of mixtures requiring only moderate resolution \textsuperscript{[7]}. It can be applied to normal-phase and reversed-phase separation \textsuperscript{[8]}. Flash chromatography can endure relatively high flow rate with low pressure, offering good separation in a short time under a proper chromatographic condition \textsuperscript{[9]}. The aim of this study was to develop a simple and efficient method for the preparative separation and purification of H.D from Kadsuriae Caulis using normal-phase flash chromatography.
2. Materials and methods

2.1. Materials and reagents

Kadsurae Caulis was purchased from Guangfu Pharmaceutical Co., Ltd. (Yunnan, China). The sample was verified as the dried stems of Kadsurae interior A.C. Smith by Doctor Xin-Jun Xu, Sun Yat-Sen University, China. Methanol was HPLC grade manufactured from SK Chemicals (Korea). Cyclohexane, ethyl acetate and petroleum ether were analytical grade and purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Ultrapure water was obtained from a Milli-QRG purification unit (Millipore, Bedford, MA, USA).

2.2. Apparatus

The separation was performed on a Biotage Isolera Rapid preparation liquid chromatograph with an automatic fraction collector, and a PeakTrak software system (AB company, Sweden). HPLC peak purity analysis was performed on a Waters HPLC (1524 quaternary pump, 717 autosampler, 2996 diode-array detector, USA). GF254 thin layer plate (Taizhou Luqiao Sijia Biochemical Plastic Factory, 5 cm × 10 cm) was used for purity examination. A SB25-12 DTD ultrasonic machine (Scientz Biotechnical Ltd., Ningbo, China), a RE-300 rotational vacuum concentrator (Shanghai Yarong Biochemical Instrument Factory, China), an electronic balance (KERN ABT 220-5DM, 0.1 mg, Germany), a 500 g pendulum pulverizer (Yulang Machinery Equipment Ltd., Guangzhou, China) and a SY-1000E multipurpose thermostat ultrasonic extraction machine (Hongxianglong Biological Technology Ltd., Beijing) were utilized for sample preparation. A UV-2450 UV–VIS (SHIMADZU, Japan), a Finnigan LCQ DECA XP Liquid Chromatography Mass Spectrometer (Thermo, USA), a TENSOR 37 Fourier transformation infra-red spectrometer (Bruker, Germany) and a Bruker Avance III 400 Nuclear Magnetic Resonance Spectrometer (Bruker, Germany) were used for characterization.

2.3. Sample preparation

The dry Kadsurae Caulis (about 270 g) was pulverized and extracted with 2700 mL cyclohexane for 30 min under sonication (duration 6 s, interval 1.5 s, 1000 W, and 40 °C). The procedure was repeated three times. The extracts were combined, then filtered and evaporated to dryness by rotary vaporization at 40 °C under reduced pressure. Finally, about 4.86 g extract was obtained.

2.4. Normal-phase flash chromatography separation

The normal-phase column was 30 cm × 1.5 cm, manually packed with silica gel, the average particle size was 51 μm. The normal-phase column was first moistened by ethyl acetate and petroleum ether (1:4, v/v), and then balanced by 5% ethyl acetate. After that, the extract (dissolved in cyclohexane, about 100 mg/mL) was loaded onto the column and the gradient elution began using ethyl acetate (A) and petroleum ether (B) as the mobile phase. The system was run with a gradient program at 25 mL/min: 0–3 min, 5% A; 3–15 min, 5% → 38% A; and 15–18 min, 38% A. Detection wavelength was set at 330 nm. 250 mg of Kadsurae Caulis extract was dissolved in 2.5 mL of cyclohexane for injection. The effluent from the column was collected into test tubes with a fraction collector set at 8 mL for each tube. Fractions with the same peak and purity were combined, concentrated and dried under reduced pressure. Peak 3 was determined to be H.D by HPLC (Fig. 1).

Fig. 1 Flash chromatogram of Kadsurae Caulis extract (Peak 3 was Heteroclitin D determined by HPLC).

2.5. Purity determination

2.5.1. Preparation of sample solution

An accurately weighed quantity of H.D (after recrystallization) was dissolved in methanol to obtain a solution having a known concentration of about 100 μg/mL for quantification by HPLC-UV. 10 mg/mL of the Kadsurae Caulis extract (S1), 2 mg/mL of H.D (S2) and 0.02 mg/mL of H.D (S3) were used for thin layer chromatography (TLC) determination.

2.5.2. HPLC conditions

The liquid chromatograph was equipped with a 220 nm detector and a Dikma-Diamond C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol–water (70:30, v/v). The flow rate was 1.0 mL/min. The column was maintained at 30 °C and injection volume was 20 μL.

2.5.3. TLC method for examination of impurities in H.D

Silica gel GF254 was used as the stationary phase, petroleum ether and ethyl acetate (4:1, v/v) as the mobile phase were used for TLC. 1 μL of each of the three solutions S1, S2 and S3 were applied to the plate respectively. After developing (about 4 min) and removal of the plate, it was dried in air and visualized under UV245 nm.

3. Results and discussion

3.1. Purification of the isolated compound

H.D was isolated by flash chromatography, but the purity was just 96.5%, which does not meet the standard [10]. Compared with the mobile phase blank control, the impurities appearing in the HPLC chromatogram in 30 min were affirmed to be brought in by the mobile phase, because the impurities in the mobile phase may increase after enriching. Therefore, recrystallization and solvent double distillation have been tried. H.D was dissolved in hot methanol (about 60 °C), and then water was added to the solvent gradually until sediment was separated out. Finally, the purity reached 99.4%, determined by HPLC with a diode array detector. The purity angle was 0.131 and purity requirement (Fig. 2).

2.5.2. HPLC conditions

The liquid chromatograph was equipped with a 220 nm detector and a Dikma-Diamond C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol–water (70:30, v/v). The flow rate was 1.0 mL/min. The column was maintained at 30 °C and injection volume was 20 μL.

3.2. The purity validation of H.D

S1 was the Kadsurae Caulis extract for system suitability testing; S2 and S3 were the H.D sample solution and 1% sample solution respectively for main component self-compare. The result of TLC
showed that not more than 1.0% of any individual impurity was found, which was in accord with that of HPLC.

### 3.3. Characterization of target compound

The chemical structure of the target compound was identified according to the MS, UV, IR, \(^1\)H NMR and \(^{13}\)C NMR data. The structural data are listed as follows, matching the data of H.D [11,12], including ESI-MS: \(m/z: 383.2[M-C_6H_2O_2]^+, 482.9[M+H]^+, 505.2[M+Na]^+, 981.9[2 M+NH_3]^+, 987.0[2 M+Na]^+; \) UV (MeOH) \(\lambda_{max} \text{ nm}: 219, 335; \) IR (KBr) \(\nu_{max} \text{ cm}^{-1}: 2984, 1714, 1650, 1580, 1488, 1452, 1383, 1207, 1060, 1119. \) \(^1\)H NMR and \(^{13}\)C NMR data are summarized in Table 1.

### 3.4. Optimization of flash chromatography conditions

In order to optimize the flash chromatography conditions, the same TLC method as Section 2.5.3 was used. The sample should be dissolved in the solvent system in theory, but the solubility was not good enough. So cyclohexane was used for dissolution, and the higher purity was achieved.

The sample concentration was more suitable to be 100 mg/mL. If the concentration was too high, it would be too sticky for injection. On the contrary, if it was too low, the response was low and would consume more mobile phase. The elution program was compared among 35%, 38% and 40% of ethyl acetate in 15 min. The results showed that the purity was higher with 38% of ethyl acetate (Fig. 3).

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Isolation of Heteroclitin D using flash chromatography

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