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A rapid method for simultaneous determination of 15 flavonoids in *Epimedium* using pressurized liquid extraction and ultra-performance liquid chromatography

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Abstract

Herba Epimedii (family Berberidaceae), *Yinyanghuo* in Chinese, is one of commonly used Chinese medicines. Flavonoids are considered as its active components. In this study, a rapid ultra-performance liquid chromatography (UPLC) method was developed for simultaneous determination of 15 flavonoids, including hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I in different species of *Epimedium*. The analysis was performed on Waters Acquity UPLC system with an Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 μm) and gradient elution of 50 mM acetic acid aqueous solution and acetonitrile within 12 min. All calibration curves showed good linearity ($R^2 > 0.9997$) within test ranges. The LOD and LOQ were lower than 0.13 and 0.52 ng on column, respectively. The R.S.D.s for intra- and inter-day of 15 analytes were less than 5.0% at three levels, and the recoveries were 95.0–103.7%. The validated method was successfully applied to quantitatively analyze 15 flavonoids in different species of *Epimedium*. The results showed there were great variations among the contents of investigated flavonoids. Hierarchical clustering analysis based on characteristics of 15 investigated compounds peaks in UPLC profiles showed that 37 samples were divided into 3 main clusters, which were in accordance with their flavonoids contents. The simulative mean chromatogram of the high content cluster was generated to compare the samples from different species and/or locations of *Epimedium*. Four flavonoids including epimedin A, B, C and icariin were selected as markers for quality control of the species of *Epimedium* used as *Yinyanghuo*.

Keywords: Flavonoids; *Epimedium*; Ultra-performance liquid chromatography (UPLC); Pressurized liquid extraction (PLE); Hierarchical clustering analysis; Similarity evaluation

1. Introduction

The genus *Epimedium* is widespread in Asia, Europe and the Middle and Far East. It comprises about 50 species found throughout the world [1]. According to the Chinese Pharmacopoeia, the dried aerial parts of *Epimedium brevicornu* Maxim., *Epimedium sagittatum* (Sieb. et Zucc.) Maxim., *Epimedium pubescens* Maxim., *Epimedium wushanense* T.S. Ying and *Epimedium koreanum* Nakai were used as *Yinyanghuo*, a well-known Chinese herbal medicine [2]. The flavonoids have been reported possessing multiple biological activities, such as anti-osteoporosis, immunological function modulation and anti-tumor actions [3–7]. In addition, among more than 130 compounds identified in different species of *Epimedium*, most are the flavonoids [8,9]. Thus, determination of flavonoids is necessary for quality control of *Epimedium*. Up to date, a series of methods, including UV–vis spectrophotometry [10–12], thin layer chromatography (TLC) [13–16], high performance liquid chromatography (HPLC) [15,17–20], micellar electrokinetic chromatography (MEKC) [21–24] and capillary zone electrophoresis (CZE) [25–27], have been reported to quantify the level of flavonoids in *Epimedium*. However, these methods suf-

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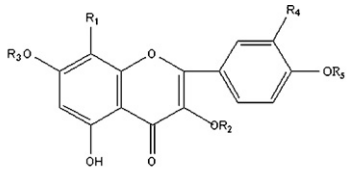
fered from long analysis time, low resolution and sensitivity and/or few analytes.

Ultra-performance liquid chromatography (UPLC) makes it possible to perform very high-resolution separations in short periods of time with little solvent consumption [28–30], which utilizes solid phase particles of 1.7 μm diameter to achieve superior theoretical plates and resolution. And it has attracted wide attention of pharmaceutical and biochemical analysts [31–35]. In addition, it is well known that interaction of multiple chemical compounds contributes to the therapeutic effect of Chinese medicine. Therefore, the analysis of multiple components is necessary and helpful to control the quality of Chinese medicine.

This study developed a rapid and reliable pressurized liquid extraction (PLE) and UPLC method to analyze 15 flavonoids, including hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I, in *Epimedium*. The validated method was applied for assay of 37 samples from 17 species of *Epimedium*. The contents of flavonoids in different species of *Epimedium* were also compared.



Compounds	R_1	R_2	R_3	R_4	R_5	M.W
hexandraside E	$-CH_2CH=C(CH_3)_2$	-glc	-glc	-H	-H	678
kaempferol-3-O-rhamnoside	-H	-rha	-H	-H	-H	432
hexandraside F	$-CH_2CH=C(CH_3)_2$	-rha(3-1)glc	-glc	-H	$-CH_3$	838
epimedin A	$-CH_2CH=C(CH_3)_2$	-rha(2-1)glc	-glc	-H	$-CH_3$	838
epimedin B	$-CH_2CH=C(CH_3)_2$	-rha(2-1)xyl	-glc	-H	$-CH_3$	808
epimedin C	$-CH_2CH=C(CH_3)_2$	-rha(2-1)rha	-glc	-H	$-CH_3$	822
icariin	$-CH_2CH=C(CH_3)_2$	-rha	-glc	-H	$-CH_3$	676
epimedoside C	$-CH_2CH=C(CH_3)_2$	-H	-glc	-H	-H	516
baohuoside II	$-CH_2CH=C(CH_3)_2$	-rha	-H	-H	-H	500
caohuoside C	$-CH_2CH=C(CH_3)_2$	-rha	-H	-OH	$-CH_3$	530
baohuoside VII	$-CH_2CH=C(CH_3)_2$	-rha(4-1)glc	-H	-H	$-CH_3$	676
sagittatoside A	$-CH_2CH=C(CH_3)_2$	-rha(2-1)glc	-H	-H	$-CH_3$	676
sagittatoside B	$-CH_2CH=C(CH_3)_2$	-rha(2-1)xyl	-H	-H	$-CH_3$	646
2''-O-rhamnosyl icariside II	$-CH_2CH=C(CH_3)_2$	-rha(2-1)rha	-H	-H	$-CH_3$	660
baohuoside I	$-CH_2CH=C(CH_3)_2$	-rha	-H	-H	$-CH_3$	514

Fig. 1. Chemical structures of 15 investigated compounds.

2. Experimental

2.1. Chemicals, reagents and materials

Acetonitrile and acetic acid for liquid chromatography were purchased from Merck (Darmstadt, Germany). Absolute ethanol used for extraction was purchased from Riedel-de Haën (Seeize, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

Hexandraside E, kaempferol-3-*O*-rhamnoside, hexandraside F, epimedin A, epimedin B, epimedin C, icariin, epimedoside C, baohuoside II, caohuoside C, baohuoside VII, sagittatoside A, sagittatoside B, 2″-*O*-rhamnosyl icariside II and baohuoside I (Fig. 1) were separated and purified in our lab. The purity of all compounds is more than 95% (determined by HPLC). The structures are confirmed by their UV, MS, ^{1}H NMR and ^{13}C NMR data compared with the literatures [36–46].

The materials of *Epimedium* were collected and identified by Professor Baolin Guo, one of the authors (Table 1). The voucher specimens of these samples were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao SAR, China.

2.2. Sample preparation

Sample preparation was performed by using pressurized liquid extraction on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA) under optimized conditions. In brief, dried powder of *Epimedium* (0.25 g) was mixed with diatomaceous earth in a proportion (1:1) and placed into an 11 ml stainless steel extraction cell, respectively. The extraction cell was extracted under the optimized conditions: solvent, 70% ethanol; particle size, 60–80 mesh; temperature, 120 °C; static extraction time, 10 min; pressure, 1500 psi; static cycle, 1 and the number of extraction times, 1. Then the extract was transferred into a 25 ml volumetric flask which was made up to its volume with extraction solvent and filtered through a 0.2 μm Nylon membrane filter (Whatman, UK) prior to injection into the UPLC system.

2.3. UPLC analysis

All analyses were performed on a Waters Acquity UPLC system (Waters, MA, USA) including binary solvent manager, sampler manager, column compartment and PDA detector, connected to a Waters Empower 2 software. An Acquity UPLC BEH C18 column (50 mm × 2.1 mm I.D., 1.7 μm) also from Waters was used. The column temperature was maintained at 25 °C. The standards and samples were separated using a gradient mobile phase consisting of water with 50 mM acetic acid (A) and acetonitrile (B). The gradient condition is: 0–2 min, 20–24% B; 2–4 min, 24–26% B; 4–5 min, 26–32% B; 5–12 min, 32–35% B; 12–15 min, 35–100% B; and finally, reconditioning the column with 20% B isocratic for 3 min after washing column with 100% B for 2 min. The flow rate was 0.25 ml/min and the injection volume was 1 μl. The peaks were detected at 270 nm.

2.4. Data analysis

Hierarchical clustering analysis was performed by SPSS 14.0 for windows (SPSS Inc., Chicago, IL, USA), which comprise a number of "procedures" – graphical, statistical, reporting, processing and tabulating procedures – that enable simple and rapid data evaluation. Ward's method, a very efficient method for the