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Simultaneous Determination of Four Compounds from *Cercidiphyllum japonicum* Using HPLC-UV Analysis

Jeongyeon Kang[1,†], Hyeon Seok Jang[1,†], Ju Yeol Kim[1], Min Sung Lee[2], Young Soo Bae[3], Yongsoo Kwon[1], and Heejung Yang[1,*]

[1]*College of Pharmacy, Kangwon National University, Chuncheon 24341, Korea*
[2]*Wild plants and Seeds Conservation Department, Baekdudaegan National Arboretum, Bonghwa 36209, Korea*
[3]*College of Forest and Environmental Sciences, Kangwon National University, Chuncheon 24341, Korea*

Abstract – *Cercidiphyllum japonicum* is being used for the treatment of obesity and liver fibrosis in Korean local clinics. In the present study, we tried to develop an analytical methodology for the determination of the chemical markers of *Cercidiphyllum japonicum*. Four chemicals, maltol (**1**), chlorogenic acid (**2**), quercetin (**3**), and avicularin (**4**), were selected for method validation, and the analytical conditions were optimized and validated using high-performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV). Additionally, the seasonal variations of four markers were monitored every month for six months. The contents of four chemicals markers were most detected in a sample collected in June.
Keywords – *Cercidiphyllum japonicum*, method validation, maltol, seasonal variation

Introduction

Cercidiphyllum japonicum Sieb. et Zucc (Cercidiphyllaceae) is a deciduous tree growing up to 25 - 30 m in height and is known to be native to East Asian countries, China and Japan. *C. japonicum* is now growing wild in temperate deciduous forests in East Asia (mainly in eastern China and Japan).[1,2] When the leaves fall in the autumn, it has a sweet scent like caramel or ripe apples by a volatile phenolic compound, maltol. In addition, the extract of *C. japonicum* leaves has higher antioxidant activity and was highly likely to be used as a natural fragrance with the antioxidant property.[3,4]

In the study, the validation method of four chemicals from *C. japonicum* has been developed using HPLC-UV analysis. Since the types of four chemicals were quite different each other, it was challengeable to optimize the HPLC analytical conditions for the simultaneous determination. Four compounds, maltol, chlorogenic acid, quercetin, and avicularin, which were selected for the markers of *C. japonicum* have been reported that they have antioxidant, antibacterial, anti-inflammatory and antitumor.[5-10]

Experimental

Chemicals – Maltol was gifted from Dr. Young Soo Bae, an emeritus professor of the College of Forest and Environmental Sciences, Kangwon National University. Three standards, chlorogenic acid, quercetin and avicularin, were purchased from Corescience Inc. (Seoul, Korea). The purities of all of the standards were > 97.0%.

Plant Material – Dried *C. japonicum* leaves were collected from Gandong-myeon, Hwacheon-si, Korea, in 2014 and identified by Dr. Young Soo Bae, an emeritus professor of at the College of Forest and Environmental Sciences, Kangwon National University. The sample was deposited in the Herbarium of the College of Pharmacy, Kangwon National University (KNUPH-CJ-1). HPLC grade solvents were purchased from TEDIA (Fairfield, OH, USA).

Sample preparation – Dried *C. japonicum* leaves were chopped into small pieces of size 2–3 cm. 100 mg of plant material was dissolved in 10 mL of 70% acetone, extracted using an ultrasonic apparatus for 3 h and freeze-dried to a powder before the experiment. The sample and the standard compounds were prepared using 1 mL of 100% methanol. All the samples were filtered through a 0.45-μm PVDF membrane filter before the injection into

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[†]Authors who contributed equally.

*Author for correspondence
Heejung Yang, Ph.D., College of Pharmacy, Kangwon National University, Chuncheon 24341, Korea
Tel: +82-33-250-6919; E-mail: heejyang@kangwon.ac.kr

an HPLC-UV system.

HPLC-UV analysis – The HPLC-UV system consisted of an Agilent 1260 infinity HPLC-UV system connecting of a binary pump, an auto sampler, a thermostated column compartment, and a UV detector (Agilent Technologies Mfg GmbH & Co. KG, Waldbronn, Germany). The analytical column was a Hector-M C18 column (250 mm × 4.6 mm i.d.; 5 μm, RStech, Daejeon, Korea). Flow rate and wavelength were set at 1.0 mL/min and 254 nm, respectively. The mobile phase consisted of H_2O (A) and acetonitrile (B) acidified with 0.1% formic acid, and eluted in gradient mode as follows: 10% B (0–10 min), 10–13% B (10-11 min), 13-17% B (11–20 min), 17-20% B (20–25 min), 20-23% B (25–30 min), 23-26% B (30–35 min), 26-30% B (35–40 min) and 30-35% B (40–45 min). Aliquots of 10 μL were injected using the autosampler for the analyses.

Method Validation – For the development of the validation method for the *C. japonicum* sample, the standard compounds were diluted to the appropriate concentrations based on preliminary studies. A range of five concentrations of each standard was determined under the optimized analytical conditions. The limits of detection (LOD) and quantification (LOQ) were determined under the chromatographic analysis at signal-to-noise (S/N) ratios of 3 and 10, respectively. For the precision study, the intra-day variabilities of the standard compounds were tested by preparing their solutions in three different concentrations and examining them within one day. Meanwhile, the accuracies were determined by the percentages of the recovered amount of each compound after the addition of standards (25, 50, and 100 μg/ml). The relative standard deviation (RSD) was taken to determine of the experiments.

Results and Discussion

The optimization of analytical conditions and development of a validation method have been attempted for the determination of the chemical markers for *C. japonicum* leaves. Since two major peaks at 30.2 and 34.0 min and in the chromatogram were not identified by the LC/MS analysis and a literature inspection, we used four compounds suggested as the chemical markers for the simultaneous determination of *C. japonicum* (Fig. 1). The solvent system using the mixture of H_2O and acetonitrile acidified with 0.1% formic acid was optimized for the clear separation of four standard compounds (Fig. 2). Under the optimized condition, four compounds were well separated from other peaks with high resolution, and were selected for a validation method of *C. japonicum*.

Four standards gave the calibration curves in a wide range of concentrations (25–1000 μg mL^{-1}) and all of the curves showed linear regressions with high correlation coefficient values ($R^2 > 0.997$) (Table 1). Thus, four standards were applied to the next experiments. The LODs and LOQs of the four markers were in the range 1.36–7.18 and 4.11–17.11 ng/mL, respectively, and displayed high sensitivities under the optimized chromatographic condition. The maximum intensities for four compounds were verified at 254 nm.

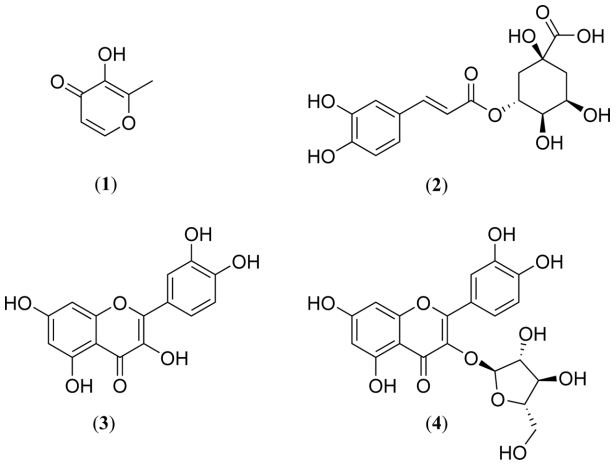

Fig. 1. Four chemical markers of *C. japonicum*.

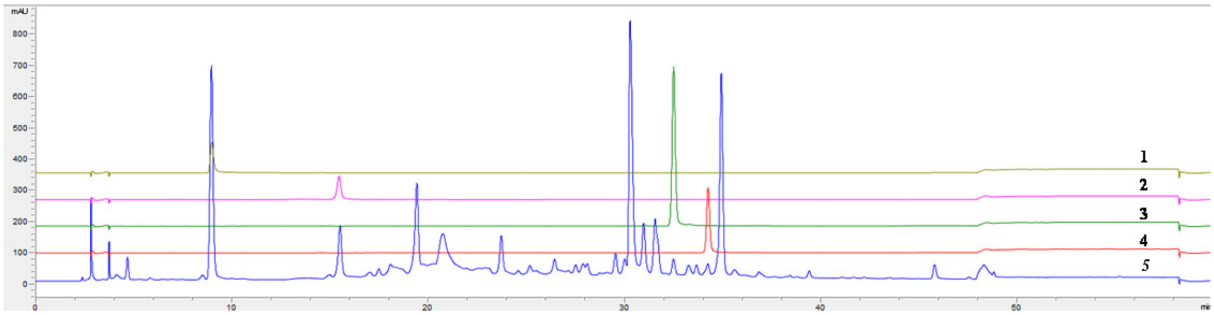

Fig. 2. HPLC chromatogram of *C. japonicum* and four chemical marker obtained at 254 nm; **1** : maltol, **2** : chlorogenic acid, **3** : quercetin, **4** : avicularin, and **5** : the 70% acetone extract of *C. japonicum*.

Table 1. Calibration curves, LOD and LOQ of for four standards from *C. japonicum*

Standard[a]	Linear regression Eq.	R^2	LOD (ng)	LOQ (ng)
1	y = 20.4x – 357.06	0.9998	1.36	4.11
2	y = 18.973x + 116.99	0.9978	5.65	17.11
3	y = 16.451x – 20.326	0.9999	2.04	6.17
4	y = 10.185x + 4.3194	0.9990	2.97	8.99

[a] 1: maltol, 2: chlorogenic acid, 3: quercetin, 4: avicularin

Table 2. Intraday precision test of four standards in *C. japonicum*

Concentration (μg/mL)	RSD (%)			
	1	**2**	**3**	**4**
25	4.39	1.12	3.64	2.50
50	2.10	0.16	6.48	0.85
100	1.09	1.13	1.51	0.26
250	0.67	1.77	5.42	0.54
500	0.99	1.91	1.16	0.36
1000	0.18	0.05	2.51	0.20

[a] 1: maltol, 2: chlorogenic acid, 3: quercetin, 4: avicularin

Table 3. Accuracy test of four standards in *C. japonicum*

Standard[a]	Calculated value (μg/mL)	Measured value (μg/mL)	Accuracy (%)
	189.09	175.62	92.15
1	197.01	185.57	93.63
	213.84	203.12	94.54
	45.74	46.06	100.70
2	84.13	82.72	98.32
	46.06	171.37	105.26
	31.71	30.67	96.73
3	49.90	47.66	95.52
	87.62	83.30	95.08
	107.59	107.07	99.52
4	122.66	117.90	96.11
	149.13	143.76	96.40

[a] 1: maltol, 2: chlorogenic acid, 3: quercetin, 4: avicularin

A precision test was conducted by evaluating the intra-day variances for the four markers. The intra-day test was assayed at three concentrations in a day (Table 2). The intra-day variability for all the chemical markers was less than the RSD of 5%. In addition, four chemical markers showed recoveries with accuracies (in the range of 90 to 110%) and were found to be stable. In this study, the validation method of four chemical markers in *C. japonicum* was successfully established.

Seasonal variations of four standard chemicals were monitored under the optimized conditions (Table 3). The contents of all four standards were most detected in June when the monthly average temperature reached 23.4 °C at the collection area in 2015 (Table 4). The average temperature (25 and 25.6°C) in July and August is higher than June in Korea, but the rainy season in July may disturb the biosynthesis of secondary metabolites.[11] These results were quite different with the seasonal variation of maltol in the previous literature.[12] It could be expected that this difference was due to the different climatic environments with each country.[13] Consequently, the results in the study might be applied to analyze the natural resources containing *C. japonicum* and suggest that we should consider the collection time in order to extract higher content of maltol and antioxidative components.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Table 4. Seasonal changes of four standards in *C. japonicum*

Standard[a]	Content (%)					
	May	Jun	Jul	Aug	Sep	Oct
1	3.02 ± 0.02	4.31 ± 0.16	3.79 ± 0.04	2.98 ± 0.06	2.64 ± 0.01	0.98 ± 0.04
2	1.33 ± 0.02	4.11 ± 0.94	2.62 ± 0.03	0.77 ± 0.04	0.85 ± 0.05	0.97 ± 0.06
3	0.14 ± 0.01	0.46 ± 0.11	0.36 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	0.19 ± 0.02
4	3.60 ± 0.08	8.51 ± 1.88	6.19 ± 0.09	3.45 ± 0.08	3.82 ± 0.10	2.43 ± 0.15

[a] 1: maltol, 2: chlorogenic acid, 3: quercetin, 4: avicularin

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