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Quantitative Analysis of Bioactive Compounds in the Fruits of *Crataegus pinnatifida* by High-Performance Liquid Chromatography

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Abstract – In order to facilitate the quality control of the fruits of *Crataegus pinnatifida*, a simple, accurate and reliable HPLC method was developed for the simultaneous determination of the three bioactive compounds: chlorogenic acid (**1**), rutin (**2**), and hyperin (**3**), which were selected as the chemical markers of *C. pinnatifida*. Separation was achieved on an Agilent Eclipse XDB-C18 column with a gradient solvent system of 0.1% trifluoroacetic acid aqueous-acetonitrile at a flow-rate of 1.0 mL/min and detected at 254nm. All three calibration curves showed good linearity ($R^2 > 0.998$). The recoveries of three marker compounds were in the range of 94.87~111.52 %. The contents of chlorogenic acid (**1**), rutin (**2**), and hyperin (**3**) of the fruits of *C. pinnatifida* collected from 23 district markets in Korea, Japan, and China were 0.16~0.65 mg/g, 0.07~1.24 mg/g, and 0.03~0.62 mg/g, respectively. The results demonstrated that this method is simple and reliable for the quality control of the fruits of *C. pinnatifida*.
Keywords – Fruits of *Crataegus pinnatifida*, HPLC, Chlorogenic acid, Rutin, Hyperin

Introduction

Crataegus pinnatifida Bunge (Rosaceae) is a perennial tree which grows widely in Korea. The fruits of *C. pinnatifida* have been used for gastric malignancy and diarrhea (Perry, 1980). On biological studies of principles from this fruit, the polyphenols have been elucidated as a cancer chemopreventive effect (Kao *et al*., 2007). Isolation of many classes of biological active phenolics and triterpenoids such as hyperin, quercetin, rutin, chlorogenic acid, corosolic acid, uvaol, ursolic acid, and 3-oxo-ursolic acid were reported to have a wide range of activities, including anti-inflammatory, anti-cancer, anti-HIV-1 protease, anti-hyperlipidemic, and anti-chitin synthase II activities (Kim *et al*., 2011; Ye *et al*., 2010; Ahn *et al*., 1998; Jeong *et al*., 1999; Min *et al*., 1999; Min *et al*., 2000). Furthermore, the leaves of this plant have been recognized to possess type 2 anti-diabetics and anti-hyperlipidemics activities (Wang *et al*., 2011). Several triterpenes and flavonoids were also reported from the leaves of *C. pinnatiida* (Zhang *et al*., 2001; Zhang & Xu, 2003; Liu & Yu, 2006). In addition, a variety of biological studies, the *Crataegus* species has emerged as a potent candidate of natural occurring therapeutic agents for obesity due to their compounds, such as flavonoids, triterpenoids, and polyphenols (Huang *et al*., 2010; Luo *et al*., 2009; Kuo *et al*., 2009).

Generally, flavonoids and triterpenoids were believed to be the beneficial components and were chosen as marker compounds for the quality evaluation, standardization of *C. pinnatifida*, and their preparation (Ying *et al*., 2009; Cheng *et al*., 2007; Cui *et al*., 2006). However, due to multiple compounds that might be associated with the therapeutic functions, a single or a few marker compounds could not be responsible for the overall pharmacological activities of this species. Therefore, it is urgently needed to establish a comprehensive quality evaluation method based on analysis of a variety of structural active compounds in order to accurately reflect the quality of this herbal drug. Our present study aims to develop a simple and validated HPLC method for the simultaneous determination of biologically active compounds from the fruits of *C. pinnatifida*, namely chlorogenic acid (**1**), rutin (**2**), and hyperin (**3**) (Fig. 1)

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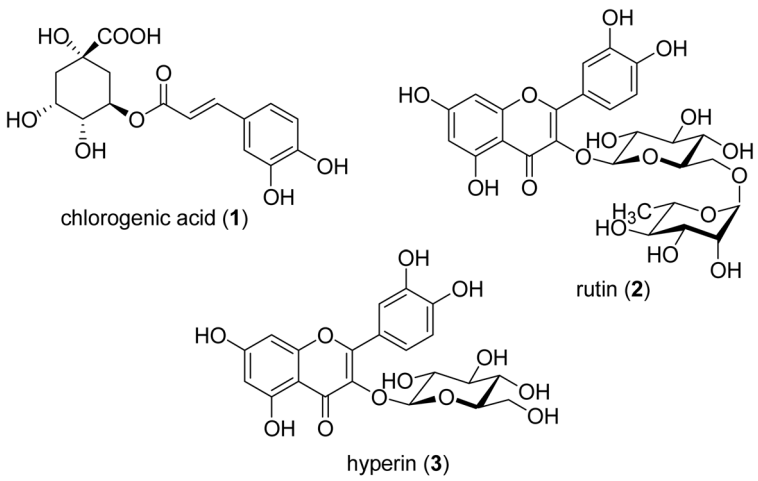

Fig. 1. Structure of chlorogenic acid (**1**), rutin (**2**), and hyperin (**3**) isolated from *C. pinnatifida*.

Experimental

General – HPLC grade MeOH and acetonitrile were purchased from Merck K GaA (Darmstadt, Germany). Distilled and deionized water were obtained from the central instrument center (Catholic University of Daegu, Daegu, Korea) and used throughout the study. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Missouri, USA). Others solvents and reagents were of analytical grade. The reference compounds **1 - 3** were supplied from Prof. Jae-Sue Choi, Pukyung National University, Korea. The purities of compounds were determined to be greater than 95% by normalization of the peak areas detected by HPLC analyses. The internal standard of methyl paraben was purchased from the Sigma-Aldrich (Missouri, USA). The 23 batches of the fruits of *C. pinnatifida* were collected from Korea, Japan, and China markets: 11D1001 (purchased from Jecheon, cultivated in Korea), 11D1002 (purchased from Jecheon, cultivated in Korea), 11D1003 (purchased from Sancheong, cultivated in Korea), 11D1004 (purchased from Gyeongju, cultivated in Korea), 11D1005 (purchased from Gyeongju, cultivated in Korea), 11D1006 (purchased from Gyeongju, cultivated in Korea), 11D1007 (purchased from Jecheon, cultivated in Korea), 11D1008 (purchased from Gyeongju, cultivated in Korea), 11D1009 (purchased from Gyeongju, cultivated in Korea), 11D1010 (purchased from Gyeongju, cultivated in Korea), 11D1011 (purchased from Ulsan, cultivated in Korea), 11D1012 (purchased from Gyeongju, cultivated in Korea), 11D1013 (purchased from Gyeongju, cultivated in Korea), 11D1014 (purchased from Gyeongju, cultivated in Korea), 11D1015 (purchased from Gyeongju, cultivated in Korea), 11D1016 (purchased from Ulsan, cultivated in Korea), 11D1017 (purchased from Tokyo, cultivated in China), 11D1018 (purchased from Sandong, cultivated in China), 11D1019 (purchased from Seomseo, cultivated in China), 11D1020 (purchased from Sandong, cultivated in China), 11D1021 (purchased from Habuk, cultivated in China), 11D1022 (purchased from Habuk, cultivated in China), and 11D1023 (purchased from Oklim, cultivated in China). The origin of sample was identified by Prof. Je Hyun Lee, Dongguk University, Korea and voucher specimens were deposited in Catholic University of Daegu, Korea.

HPLC apparatus and chromatographic conditions – The chromatographic system for quantitative analysis consisted of a 306 pump (Gilson, USA), 811C dynamic mixer (Gilson, USA), UV/VIS-156 detector (Gilson, USA), 231 XL sample injector (Gilson, USA), and GILSON UniPoint data processor (Gilson, USA). The chromatographic separation of analyses was performed carried out on an Agilent Eclipse XD8-C18 (Agilent Technologies, USA; 5 μm, 4.6 × 150 mm) performed at ambient temperature using a MetaTherm (Varian, USA). The auto-sampler was also set at ambient temperature. Data was collected and analyzed using Gilson Millennium software. The mobile phase consisting of 0.1% TFA in water (A) and acetonitrile (B) was run with gradient elution at a flow rate of 1.0 mL/min. The linear gradient elution was set as follows: 0~40 min; 10% B → 60% B. The injection volume was 10 μL (Fig. 2). UV absorption was monitored at 254 nm. The column temperature was maintained at 30 °C. Quantification was conducted using an internal standard method based on the peak area ratio of the analyte/IS *versus* the amount of each analyte.

Preparation of standard solutions – Based on the

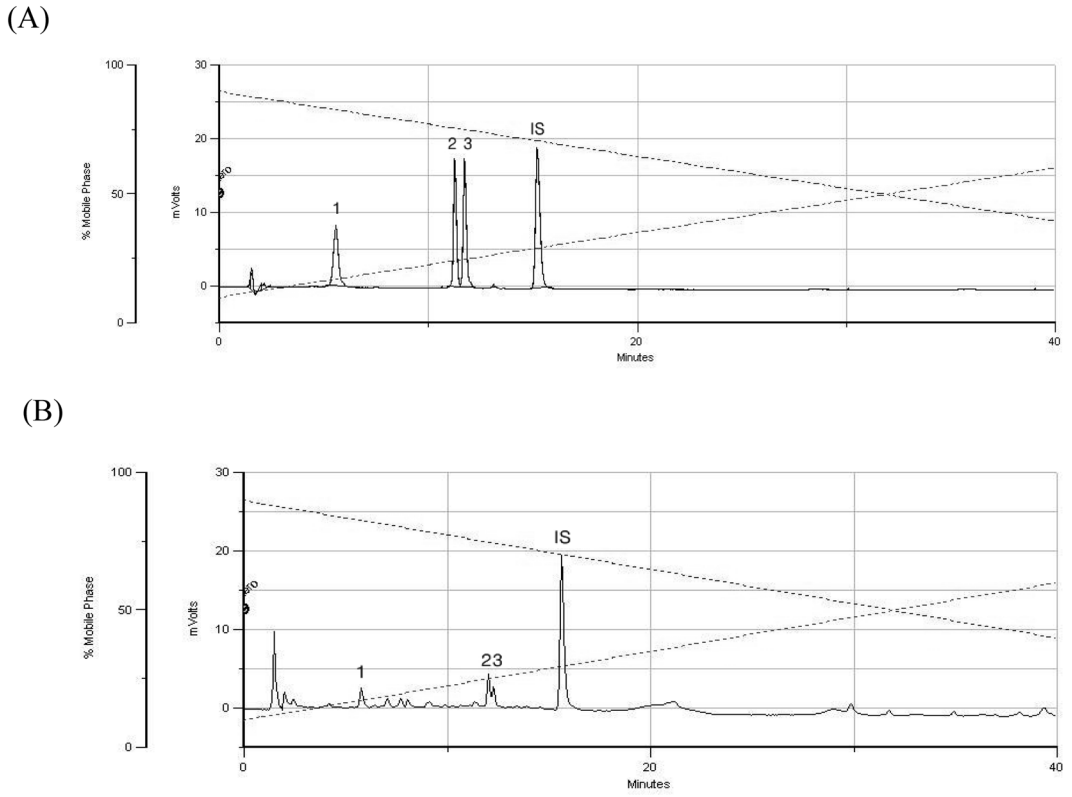

Fig. 2. HPLC chromatogram of chlorogenic acid (**1**), rutin (**2**), and hyperin (**3**) isolated from the fruits of *C. pinnatifida* and crude drug (B).

solubility of each component in DMSO, a stock standard solution was prepared by dissolving 1.00 mg of each compound **1 - 3** in 5 mL DMSO. Four additional calibration levels were prepared by diluting this stock solution with 70% EtOH. These solutions were stored away from light at 5 ºC.

Linearity, calibration range, limit of detection, and quantification – DMSO stock solution, which contained three compounds, was prepared and diluted to an appropriate concentration for the construction of calibration curves. Four concentration levels of the mixed standard solution were injected in triplicate. The calibration curves were constructed by plotting the peak area ratio (compound/IS methyl paraben) *versus* the amount of each compound. The good linearity (correlation coefficient values $R^2 > 0.998$) was achieved in relatively wide concentration ranging from 1.25 to 20 μg/mL for all the compounds (Fig. 3). The lowest concentration of working solution was diluted with 70% methanol to yield a series of appropriate concentrations, and the limit of detection (LOD) and quantification (LOQ) under the chromatographic conditions were separately determination at signal-to-noise ratio (S/N) of about 3 and 10, respectively. The data are summarized in Table 1.

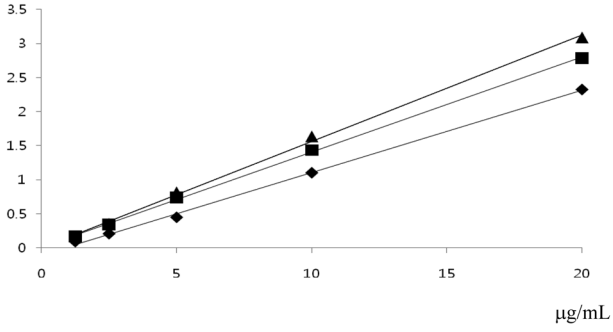

Fig. 3. Calibration curve of (◆)-chlorogenic acid (**1**), (■)-rutin (**2**), and (▲)-hyperin (**3**).

Accuracy – Recovery test was used to evaluate the accuracy of the assay. Accurate amounts of the three standards were added into a sample of *C. pinnatifida,* which was quantified previously. The mixture was extracted and compounds using the above-established method. Each sample was analyzed in triplicate. For comparison, a blank sample (not spiked with standard compounds) was prepared and analyzed. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. As shown in Table 2, the recovery rates were in the range 94.87 ~

Table 1. Calibration data for compounds **1-3** (n = 3)

Compound[a]	Regression equation ($y = ax + b$)[b]	R^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	$y = 0.0143x - 0.029$	0.9986	1.25 ~ 20.0	0.1	0.25
2	$y = 0.023x + 0.012$	0.9994	1.25 ~ 20.0	0.1	0.25
3	$y = 0.0184x + 0.02$	0.9982	1.25 ~ 20.0	0.1	0.25

[a]**1** (chlrogenic acid); **2** (rutin); **3** (hyperin), [b]y is the peak area ratio, x is the corresponding injection concentration (μg/mL), a is the slope and b is the intercept of the regression line.

Table 2. Analytical results of recoveries

Compound	Original (μg/mL)	Added (μg/mL)	Determined (μg/mL)	Recovery (%)	RSD (%)
1	0.40	2.0	2.53	106.71	0.43
		5.0	5.18	95.66	0.05
		10.0	10.91	105.12	0.64
2	0.18	2.0	2.08	94.87	0.51
		5.0	5.46	105.60	0.16
		10.0	10.83	106.51	0.15
3	0.47	2.0	2.69	111.24	0.41
		5.0	5.68	104.23	0.39
		10.0	11.62	111.52	0.30

Table 3. Effect of extraction solvent on the yields (mg/g) of compounds **1 - 3**

Compound	Content (mg/g)			
	MeOH[a]	70% MeOH	EtOH	70% EtOH
1	0.31 ± 0.02	0.4 ± 0.02	0.23 ± 0.05	0.36 ± 0.1
2	0.15 ± 0.04	0.18 ± 0.04	0.11 ± 0.02	0.13 ± 0.03
3	0.41 ± 0.8	0.47 ± 0.7	0.31 ± 0.4	0.42 ± 0.6

[a] extract solvent

111.52%, and their RSD values were less than 0.7%.

Sample preparation – Samples (0.10 g) were weight accurately and extracted with 10 mL 70% methanol by sonication for 60 min. After filtration using filter membrane 0.45 μm (Whatman, Maidstone, UK), 10 μL of the aqueous sample solution containing the internal standard (methyl paraben) was injected into the HPLC system in triplicate. The content of each compound was determined from the corresponding calibration.

Results and Discussion

In order to achieve a complete extraction of the studied components from the fruits of *C. pinnatifida* four solvent systems, including methanol, 70% methanol, ethanol, and 70% ethanol, were tested. The extraction efficiencies of all of the components from each of the solvent extraction systems were obtained and compared. The results indicated that, for chlorogenic acid (**1**), rutin (**2**), and hyperin (**3**), the 70% methanol and 70% ethanol solvent systems were demonstrated to be more efficient than the methanol and ethanol solvent systems (Table 3). From compounds, aqueous solvent system was exhibited to be more efficient than organic solvent system. In addition, the effect of the extraction time and methods on extraction efficiency was investigated by using three different methods, i.e. shake, reflux and sonication for 30, 60 and 120 min. The results demonstrated that sonication for 60 min by using 70% methanol was the preferred procedure.

An HPLC method was developed in order to separate and quantify the major compounds in the fruits of *C. pinnatifida.* To obtain chromatograms with a good separation, initial screening experiments showed that the mobile phase needed to be acidic. As a result, acetonitrile and 0.1 % TFA aqueous were chosen as the eluting solvent system to give the desired separation and acceptable tailing factor within the running time of 20 min. The best separations, with respect to resolution and peak symmetry, were observed with an Agilent Eclipse XDB-C18 80 Å column.

According to the UV spectra of the compounds **1 - 3** in

Table 4. Contents of three compounds in samples of Crataegi Fructus (n = 3)

Sample	Content (μg/g)		
	1	**2**	**3**
11D1001	0.39 ± 0.02	0.34 ± 0.03	0.10 ± 0.01
11D1002	0.38 ± 0.03	0.58 ± 0.02	0.24 ± 0.01
11D1003	0.24 ± 0.01	0.33 ± 0.01	0.15 ± 0.01
11D1004	0.37 ± 0.01	0.18 ± 0.01	0.05 ± 0.01
11D1005	0.43 ± 0.21	0.48 ± 0.01	0.21 ± 0.02
11D1006	0.52 ± 0.01	0.52 ± 0.01	0.29 ± 0.01
11D1007	0.47 ± 0.04	0.54 ± 0.01	0.26 ± 0.01
11D1008	0.44 ± 0.01	0.54 ± 0.01	0.26 ± 0.02
11D1009	0.65 ± 0.03	1.24 ± 0.02	0.62 ± 0.01
11D1010	0.38 ± 0.01	0.39 ± 0.01	0.19 ± 0.01
11D1011	0.22 ± 0.01	0.16 ± 0.01	0.08 ± 0.01
11D1012	0.44 ± 0.01	0.07 ± 0.01	0.03 ± 0.00
11D1013	0.33 ± 0.01	0.36 ± 0.01	0.15 ± 0.01
11D1014	0.48 ± 0.01	0.29 ± 0.01	0.13 ± 0.01
11D1015	0.50 ± 0.01	0.23 ± 0.02	0.10 ± 0.01
11D1016	0.21 ± 0.01	0.20 ± 0.01	0.11 ± 0.01
11D1017	0.27 ± 0.02	0.42 ± 0.01	0.23 ± 0.01
11D1018	0.31 ± 0.01	0.20 ± 0.01	0.12 ± 0.00
11D1019	0.30 ± 0.01	0.45 ± 0.01	0.27 ± 0.00
11D1020	0.45 ± 0.01	0.43 ± 0.02	0.30 ± 0.00
11D1021	0.43 ± 0.02	0.16 ± 0.01	0.12 ± 0.01
11D1022	0.16 ± 0.01	0.16 ± 0.01	0.11 ± 0.00
11D1023	0.58 ± 0.01	0.24 ± 0.01	0.15 ± 0.00

the range from 200 to 600 nm, 254 nm was set for monitoring three phenolic compounds. The peaks of the three compounds were assigned by spiking the samples with reference standards and comparison of their UV, mass spectra and retention times. Representative chromatograms of standards mixture and *C. pinnatifida* sample monitored at 254 nm were showed in Fig. 2.

The established analytical method was then applied to quantitatively analyze three compounds **1** - **3** in various samples of *C. pinnatifida*, using the regression equation as described above. Their contents were summarized in Table 4. The contents of three compounds varied significantly in the remaining samples. The assay of standard compounds showed chlorogenic acid (0.16~0.65 mg/g), rutin (0.07~1.24 mg/g), and hyperin (0.03~0.62 mg/g), respectively. For example, the content of chlorogenic acid (**1**) and rutin (**2**) were found to be the main components in all tested samples. The content of three compounds were higher in the sample of cultured Korea (chlorogenic acid, 0.40 mg/g; rutin, 0.4 mg/g; hyperin, 0.19 mg/g: average value of 11D1001~11D1016) than cultured China (chlorogenic acid, 0.36 mg/g; rutin, 0.29 mg/g; hyperin, 0.18 mg/g: average value of 11D1007~ 11D1023). These large variations might be explainable by seasonal or geographic variations, used part, processing method, harvest time, and storage in the compound contents.

Three bioactive compounds were selected as chemical markers of the *C. pinnatifida*. In this study, a simple, accurate and reliable analytical method for simultaneous quantification of the three active components in the fruits of *C. pinnatifida* were developed using high-performance liquid chromatography. Separation was achieved on an Agilent Eclipse XDB-C18 column (5 μm, 150 × 4.6 mm i.d.) with a gradient solvent system of 0.1% trifluoroacetic acid aqueous-acetonitrile, at a flow rate of 1.0 mL/min, and detected at 254 nm. The developed assay has been applied successfully to quantify the three compounds in 23 batches of the *Crataegus* species collected from different markets. The variation in contents of active compounds greatly influences the quality, stability and therapeutic effects of this medicinal herb. Therefore, the simultaneous determination of bioactive multi-components can play an important role in the quality evaluation, used part, and on guidance for good agriculture practice of *C. pinnatifida*.

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