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Quantitative Determination of Bioactive Compounds in Some *Artemisia capillaris* by High-Performance Liquid Chromatography

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Abstract – In order to facilitate the quality control of *Artemisia capillaris*, a simple, accurate and reliable HPLC method was developed for the simultaneous determination of the six bioactive compounds: scopolin (**1**), chlorogenic acid (**2**), 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside (**3**), hyperoside (**4**), isorhamnetin 3-O-robinobioside (**5**), and scoparone (**6**), which were selected as the chemical markers of *A. capillaris*. 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 254 nm. All six calibration curves showed good linearity ($R^2 > 0.998$). A simple reversed phase HPLC method was developed for extracting pharmacologically active compounds scopolin, chlorogenic acid, 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside, hyperoside, isorhamnetin 3-O-robinobioside, and scoparone from *A. capillaris* using a binary gradient of acetonitrile : 0.1% trifluoroacetic acid with UV detection at 254 nm. The scopolin (**1**), chlorogenic acid (**2**), 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside (**3**), hyperoside (**4**), isorhamnetin 3-O-robinobioside (**5**), and scoparone (**6**) contents of the herb of *A. capillaris* collected from fifteen district markets in Korea were 0.00~0.90 mg/g, 0.06~7.29 mg/g, 0.06~0.91 mg/g, 0.07~5.05 mg/g, 0.42~13.11 mg/g, and 1.11~29.82 mg/g, respectively. The results demonstrated that this method is simple and reliable for the quality control of *A. capillaris*.
Keywords – *Artemisia capillaris*, HPLC, Isorhamnetin 3-O-robinobioside, Scoparone

Introduction

Artemisia capillaris Thunb. (Compositae) is a medicinal plant, which is distributed in worldwide. The whole part of *A. capillaris* has been used for the food additives and traditional medicines, particularly treatment of hepatitis, inflammation, malaria, cancer, and microbial infections (Cha *et al*., 2009; Aniya *et al*., 2000; Mase *et al*., 2010). On biological studies of principles from this plant, a few coumarins have been elucidated as anticarcinogenic and anti-inflammatory agents (Kim *et al*., 2008; Jang *et al*., 2005). Isolation of many classes of biological active metabolites such as coumarins, essential oils, flavonoids, polyacetylenes, and sterol glycosides were reported to have a wide range of activities, including hepatoprotective, antioxidant, anti-malaria, anti-cancer, anti-viral, anti-fungal, anticomplementary, and interferon inducing (Tan *et al*., 1998). Furthermore, a variety of biological studies, the *Artemisia* genus has emerged as a potent candidates of natural occurring therapeutic agents for diabetes due to their compounds, including coumarins, flavonoids, and cafffeic acids (Cui *et al*., 2009; Logendra *et al*., 2006; Okada *et al*., 1995).

Generally, coumarins were believed to be the beneficial components and were chosen as marker compounds for the quality evaluation, standardization of *Artemisia* genus, and their preparation. 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 the *Artemisia*. 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 these herbal drugs. Our present study aims to develop a simple and validated HPLC method for the simultaneous determination of active coumarins, phenolic compounds, and flavonoids in *A. capillaris*, namely scopolin (**1**), chlorogenic acid (**2**), 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside (**3**), hyperoside (**4**), isorhamnetin 3-O-robinobioside (**5**), and scoparone (**6**) (Fig. 1)

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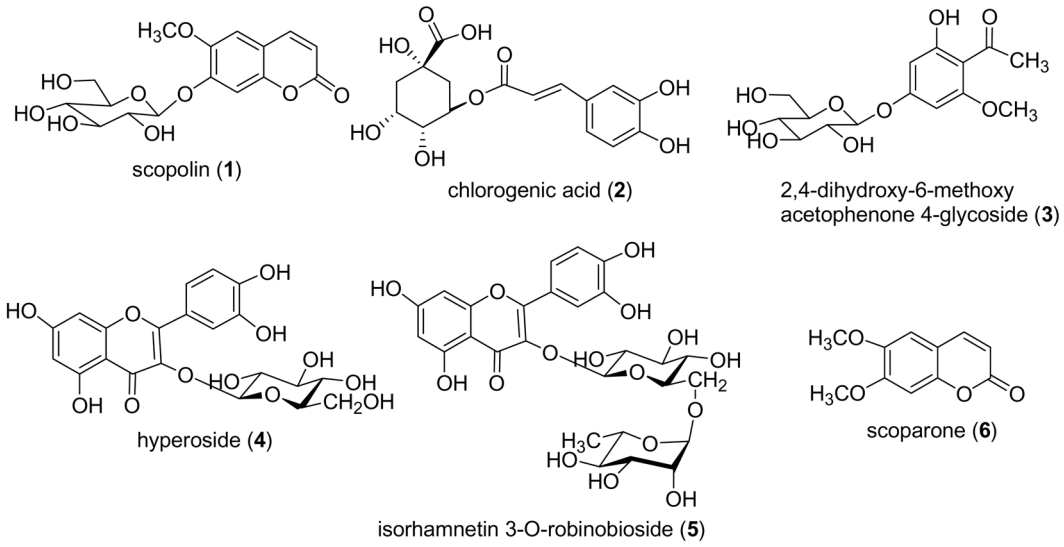

Fig. 1. Structure of scopolin (**1**), chlorogenic acid (**2**), 2,4-dihydroxy-3-methoxy-acetophenone 4-glycoside (**3**), hyperoside (**4**), isorhamnetin 3-O-robinobioside (**5**), and scoparone (**6**) isolated from *A. capillaris*.

Experimental

General – Acetonitrile HPLC grade was purchased from SK Chemicals Company (Ulsan, Korea). Distilled and deionized water were obtained from the instrument center (Catholic University of Daegu, Daegu, Korea) and used throughout the study. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Germany). Others solvents and reagents were of analytical grade. The reference compounds **1 - 6** were supplied from Prof. Jae-Sue Choi, Pukyung National University, Korea. The purities of compounds were determined to be greater than 98% by normalization of the peak areas detected by HPLC analyses. The internal standard of caffeic acid was purchased from the Sigma-Aldrich (German). The 15 batches of the aerial part of *A. capillaris* were collected from Korea and China markets: ACH-1 (collected on Autumn, cultivated in Korea), ACH-2 (collected on Autumn, cultivated in Korea), ACH-3 (collected on Autumn, cultivated in Korea), ACH-4 (collected on Autumn, cultivated in Korea), ACH-5 (collected on Autumn, cultivated in Korea), ACH-6 (collected on Autumn, cultivated in Korea), ACH-7 (collected on Autumn, cultivated in Korea), ACH-8 (collected on Autumn, cultivated in Korea), ACH-9 (collected on Autumn, cultivated in Korea), ACH-10 (collected on Autumn, cultivated in China), ACH-11 (collected on Autumn, cultivated in Korea), ACH-12 (collected on Spring, cultivated in Korea), ACH-13 (collected on Spring, cultivated in China), ACH-14 (collected on Spring, cultivated in China), and ACH-15 (collected on Spring, 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: 0~10 min, 10% B; 10~40 min, 10% → 40% B. The injection volume was 10 μL. UV absorption was monitored at 254 nm. The column temperature was maintained at 30 °C (Fig. 2). 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 solubility of each component in DMSO, a stock standard solution was prepared by dissolving 1.00 mg of each

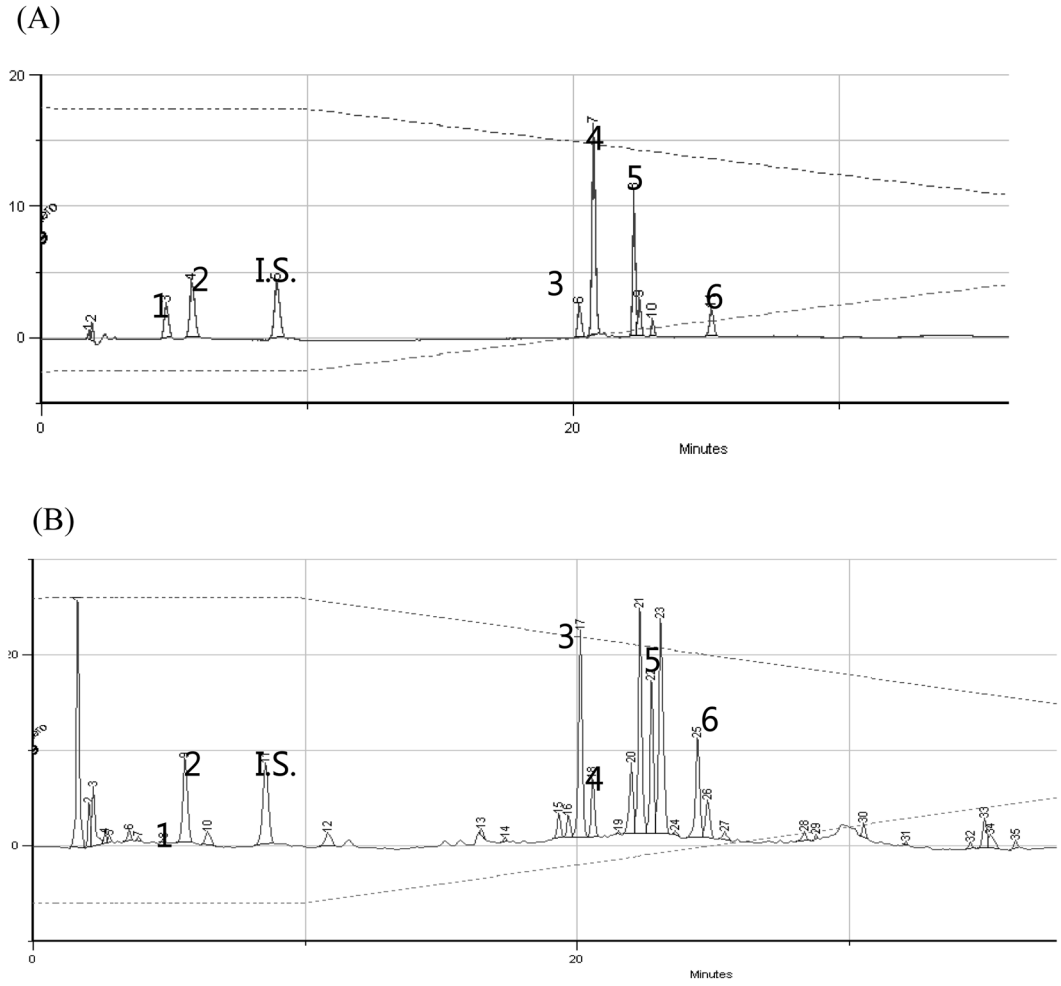

Fig. 2. HPLC chromatogram of scopolin (**1**), chlorogenic acid (**2**), 2,4-dihydroxy-3-methoxyacetophenone 4-glycoside (**3**), hyperoside (**4**), isorhamnetin 3-O-robinobioside (**5**), and scoparone (**6**) isolated from *A. capillaris* and sample (B).

compound **1 - 6** 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 °C.

Linearity, calibration range, limit of detection, and quantification – DMSO stock solution, which contained six 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 caffeic acid) *versus* the amount of each compound. The good linearity (correlation coefficient values $R^2 > 0.998$) was achieved in relatively wide concentration ranging from 1.67 to 167 μg/mL for all the compounds (Fig. 3). The lowest concentration of working solution was diluted with 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.

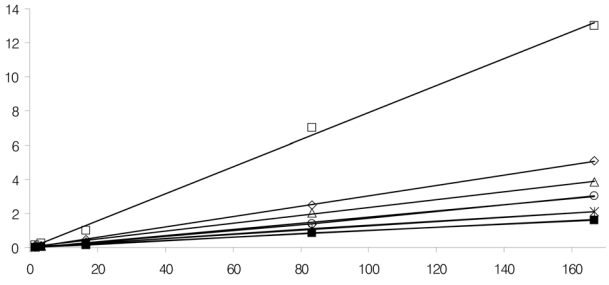

Fig. 3. Calibration curve of (*)-scopolin B (**1**), (△)-chlorogenic acid (**2**), (■)-2,4-dihydroxy-6-methoxyacetophenone (**3**), (□)-hyperoside (**4**), (◇)-isorhamnetin 3-O-robinobioside (**5**), and (○)-scoparone (**6**).

Table 1. Calibration data for 6 compounds **1** - **6** (n = 3)

Compound	Regression equation (y = ax + b)[a]	R^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
1	$y = 0.0126x + 0.013$	0.9996	1.67 ~ 166.7	0.1	0.25
2	$y = 0.023x + 0.03$	0.9982	1.67 ~ 166.7	0.1	0.25
3	$y = 0.0097x + 0.005$	0.9980	1.67 ~ 166.7	0.1	0.25
4	$y = 0.0793x - 0.0248$	0.9975	1.67 ~ 166.7	0.1	0.25
5	$y = 0.0306x - 0.0233$	0.9998	1.67 ~ 166.7	0.1	0.25
6	$y = 0.018x - 0.038$	0.998	1.67 ~ 166.7	0.1	0.25

[a]y is the peak area ratio (peak area/IS area), x is the corresponding injection concentration (mg/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	2.0	1.80	90.21	0.23
		10.0	9.92	99.16	0.32
		16.0	15.90	99.38	0.52
2	3.73	2.0	2.20	110.19	0.46
		10.0	10.19	101.88	1.14
		16.0	16.04	100.22	0.3
3	1.47	2.0	1.86	93.0	2.37
		10.0	10.44	104.4	0.52
		16.0	16.01	100.09	0.22
4	1.90	2.0	1.86	93.38	0.05
		10.0	10.19	101.88	0.11
		16.0	16.24	101.51	0.22
5	4.69	2.0	1.97	98.63	0.87
		10.0	9.53	95.28	4.03
		16.0	16.12	100.73	0.41
6	10.65	2.0	1.89	94.50	1.93
		10.0	9.74	97.4	0.27
		16.0	16.15	100.94	0.80

Accuracy – Recovery test was used to evaluate the accuracy of the assay. Accurate amounts of the six standards were added into a sample of *A. capillaris,* 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 90.21~ 110.19%, and their RSD values were less than 4%.

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 (caffeic acid) 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 aerial part of *A. capillaris* 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 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside (**3**), hyperoside (**4**), isorhamnetin 3-O-robinobioside (**5**), and scoparone (**6**), the 70% methanol and 70% ethanol solvent systems were demonstrated to be more efficient than the methanol and ethanol solvent systems (Table 3). For chlorogenic acid (**2**), the methanol solvent was demonstrated to be more efficient than ethanol. 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 aerial part of *A. capillaris.* 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 40 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** - **6** in the range from 200 to 600 nm, 254 nm was set for monitoring six phenolic compounds. The peaks of the six compounds were assigned by spiking the samples with reference standards and comparison of their UV, mass

Table 3. Effect of extraction solvent on the yields (mg/g) of compounds **1 - 6**

	Content (mg/g)			
	MeOH[a]	70% MeOH	EtOH	70% EtOH
1	–[b]	–	–	–
2	3.15 ± 0.66	3.50 ± 1.58	–	–
3	1.33 ± 0.10	1.98 ± 0.37	0.02 ± 0.01	1.40 ± 0.29
4	1.65 ± 0.14	1.95 ± 0.94	1.52 ± 0.49	1.23 ± 0.38
5	4.14 ± 0.14	4.73 ± 0.30	0.87 ± 0.24	5.42 ± 0.65
6	8.52 ± 0.47	9.77 ± 0.65	5.42 ± 0.21	6.24 ± 0.55

[a]extract solvent; [b]not detected

Table 4. Contents of six compounds in samples of Artemisiae Capillaris Herb (n = 3)

Sample	Content (μg/g)					
	1	**2**	**3**	**4**	**5**	**6**
ACH-1[a]	0.26 ± 0.04	2.05 ± 0.27	0.24 ± 0.01	2.36 ± 0.22	7.46 ± 0.91	13.23 ± 3.03
ACH -2[b]	0.08 ± 0.00	2.24 ± 0.22	0.32 ± 0.02	2.69 ± 0.14	10.32 ± 0.79	21.83 ± 1.05
ACH -3[c]	0.90 ± 0.24	2.49 ± 0.06	0.60 ± 0.01	4.22 ± 0.07	7.98 ± 0.11	29.82 ± 1.18
ACH -4[d]	0.06 ± 0.05	0.89 ± 0.07	0.17 ± 0.06	2.17 ± 0.20	2.26 ± 0.22	8.25 ± 0.61
ACH -5[e]	0.00±0.00	1.62 ± 0.21	0.13 ± 0.04	1.65 ± 0.20	6.31 ± 0.62	12.39 ± 0.51
ACH -6[f]	0.00	0.53 ± 0.06	0.06 ± 0.05	1.05 ± 0.08	2.65 ± 0.02	6.40 ± 0.33
ACH -7[g]	0.22 ± 0.09	2.54 ± 0.27	0.41 ± 0.06	3.88 ± 0.36	10.52 ± 0.93	27.43 ± 5.01
ACH -8[h]	0.26 ± 0.03	3.69 ± 0.07	0.29 ± 0.04	3.51 ± 0.25	4.29 ± 0.20	10.42 ± 0.87
ACH -9[i]	0.00	1.69 ± 0.08	0.27 ± 0.02	1.25 ± 0.06	6.11 ± 0.14	7.68 ± 0.49
ACH -10[j]	0.07± 0.02	0.42 ± 0.10	0.91 ± 0.18	2.185 ± 0.21	1.92 ± 0.51	22.92 ± 3.72
ACH -11[k]	0.00	4.09 ± 0.53	0.48 ± 0.19	5.05 ± 0.92	9.22 ± 0.63	12.83 ± 1.43
ACH -12[l]	0.02 ± 0.01	7.29 ± 0.38	0.83 ± 0.03	2.31 ± 0.16	13.11 ± 1.19	23.44 ± 0.93
ACH -13[m]	0.00	0.98 ± 0.06	0.29 ± 0.04	0.31 ± 0.04	3.09 ± 0.48	5.71 ± 0.39
ACH -14[n]	0.00	2.40 ± 1.76	0.10 ± 0.05	0.07 ± 0.06	1.33 ± 0.40	3.49 ± 0.32
ACH -15[o]	0.00	1.88 ± 0.09	0.64 ± 0.02	0.25 ± 0.00	0.42 ± 0.07	1.11 ± 0.04

[a]ACH-1 (collected on Autumn, cultivated in Korea), [b]ACH-2 (collected on Autumn, cultivated in Korea), [c]ACH-3 (collected on Autumn, cultivated in Korea), [d]ACH-4 (collected on Autumn, cultivated in Korea), [e]ACH-5 (collected on Autumn, cultivated in Korea), [f]ACH-6 (collected on Autumn, cultivated in Korea), [g]ACH-7 (collected on Autumn, cultivated in Korea), [h]ACH-8 (collected on Autumn, cultivated in Korea), [i]ACH-9 (collected on Autumn, cultivated in Korea), [j]ACH-10 (collected on Autumn, cultivated in China), [k]ACH-11 (collected on Autumn, cultivated in Korea), [l]ACH-12 (collected on Spring, cultivated in Korea), [m]ACH-13 (collected on Spring, cultivated in China), [n]ACH-14 (collected on Spring, cultivated in China), and [o]ACH-15 (collected on Spring, cultivated in China)

spectra and retention times. Representative chromatograms of standards mixture and *A. capillaris* sample monitored at 254 nm were showed in Fig. 2.

The established analytical method was then applied to quantitatively analyze six compounds **1 - 6** in various samples of *A. capillaris*, using the regression equation as described above. Their contents were summarized in Table 4. The contents of six compounds varied significantly in the remaining samples. For example, the content of scoparone (**6**) was found to be the most abundant component in all tested samples with less than 0.1%, such as 1, 2, 3, 5, 7, 8, 10, 11, and 12 but it was found to be free or in very small limited amounts in the other samples. The contents of chlorogenic acid (**2**), 2,4-dihydroxy-6-methoxyacetophenone 4-glycoside (**3**), hyperoside (**4**), and isorhamnetin 3-O-robinobioside (**5**) varied with about even more than 70-fold variation. Furthermore, scopolin (**1**) could not be detected in some samples. These large variations might be explainable by seasonal or geographic variations, used part, processing method, harvest time, and storage in the compound contents.

On the basis of antioxidant pharmacological studies which were related to the liver protection mechanism, six bioactive compounds were selected as chemical markers of the *A. capillaris*. In this study, a simple, accurate and reliable analytical method for simultaneous quantification of the six active components in the aerial part of *A. capillaris* 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 six compounds in fifteen batches of the *Artemisia* genus collected from different locations. 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 *A. capillaris*.

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