



Quantitative analysis of coumarins in *Artemisia keiskeana* and *A. stolonifera* using HPLC/PDA

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Abstract *Artemisia keiskeana* and *A. stolonifera* are plants of the genus *Artemisia*, distributed in various regions, especially China and Korea. They are renowned as medicinal plants with biological and pharmacological activities. Fraxidin, isofraxidin, and daphnoretin are coumarins present in *Artemisia* spp.; however, research on them is limited. Therefore, this study was carried out to quantify the content of these compounds in the aerial parts of *A. keiskeana* and *A. stolonifera* in different regions in Korea. High-performance liquid chromatography was performed with a photodiode array detector and a reverse-phase INNO column. *A. stolonifera* only contained fraxidin with the highest amount found in Yongmun commune. *A. keiskeana* cultivation in Soyang commune gave the highest fraxidin and daphnoretin content. However, isofraxidin was not present in all samples. The findings suggest that the concentrations of the three compounds may differ depending on the growth site and provide a foundation for future studies.

Keywords *A. keiskeana* · *A. stolonifera* · Daphnoretin · Fraxidin · Isofraxidin · Quantitative analysis

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Introduction

The genus *Artemisia* is a large, diverse, and economically crucial genus of the family Asteraceae. Most species in the genus *Artemisia* are herbaceous perennials, with a few annuals or biennials [1]. *Artemisia* spp. are distributed in many places such as the North American region, the Mediterranean region, Asia, Africa, and Australia [2]. *Artemisia* spp. have been used since ancient times as a medicinal herb to treat a number of conditions such as malaria, hepatitis, hypertension, cough, pain, and bacterial or viral infections [3]. Several studies have indicated the antimicrobial and antioxidant activities of *Artemisia* spp. [4]. Furthermore, previous studies have shown that *Artemisia* spp. had noteworthy anti-cancer, anti-inflammatory, and anti-obesity effects *in vitro* [5]. In addition, some important drugs have been isolated from this genus, notably, artemisinin, a herbal medicine from *Artemisia montana*, which is well-known for its antimalarial activity in China [6].

A. keiskeana and *A. stolonifera* both belong to the genus *Artemisia*, which are both East Asian perennial herbs commonly found in Korea and China [7-10]. In China, *A. keiskeana* is used as a traditional medicine to treat gynecological diseases, amenorrhea, bruises, and rheumatic diseases. In addition, it has expectorant activity and antioxidant properties [11]. *A. stolonifera* has been used as a folk medicine for the treatment of eye diseases, fever, and urinary retention [12,13].

Fraxidin and isofraxidin are hydroxycoumarins, which are natural multi-targeted agents isolated from several plants in the genus *Artemisia*. Both have shown the ability to attenuate multiple destructive signaling mediators and therapeutic targets in several diseases [14-19]. In addition, they exhibit biological and pharmacological activities such as antioxidant, cardioprotective, weight loss, anti-osteoarthritis, antimalarial, and neuroprotective effects, with promising anti-cancer and anti-inflammatory effects [20-25]. Daphnoretin is a well-known derivative of biscoumarin [26,27]. Several studies have shown that this compound could exert antifungal and anti-complement activity against Ehrlich

ascites carcinoma in vivo. It is also an activator of protein kinase C, inhibits DNA polymerase β lyase, and suppresses hepatitis B virus in human hepatoma cells [28-31]. Fraxidin, daphnoretin, and isofraxidin are common coumarins found in the Asteraceae family, which can be isolated from the whole plant of *A. keiskeana* [32,33]. However, quantitative information on these compounds in *A. keiskeana* and *A. stolonifera* cultivated in Korea is still limited.

In this study, fraxidin, isofraxidin, and daphnoretin were analyzed quantitatively using the methanol (MeOH) extracts of the aerial parts of *A. keiskeana* and *A. stolonifera*, which were harvested in different regions in Korea, by high-performance liquid chromatography (HPLC) coupled with a photodiode array (PDA) detector.

Materials and Methods

Plant materials

The aerial parts of *A. keiskeana* was collected in three different regions (Soyang commune, Yongmun commune, and Okdo commune, Korea), and the aerial parts of *A. stolonifera* was collected in two different regions (Soyang commune and Yongmun commune, Korea). The plants were identified by Dr. Jae Min Chung, Department of Forest Resource Conservation, Korea National Arboretum, Pocheon, Korea. All samples were deposited at Korea National Arboretum, Pocheon, Korea.

Instruments and reagents

HPLC was performed on a Waters Alliance e2695 Separations Module, USA Quat with pump, autosampler, and Waters 2998 Photodiode Array (PDA) Detector, USA. HPLC-grade solvents such as MeOH, water, trifluoroacetic acid (TFA) and acetonitrile (ACN) were purchased from J. T. Baker (Avantor, PA, USA). Fraxidin, daphnoretin, and isofraxidin (Fig. 1) were provided by the Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong, Korea.

Sample extraction

Dried aerial parts of *A. keiskeana* and *A. stolonifera* of different regions (5 g) were extracted in MeOH (100 mL) under reflux for 3 h, which was repeated three times. The samples were then filtered and evaporated to obtain a concentrated MeOH extract

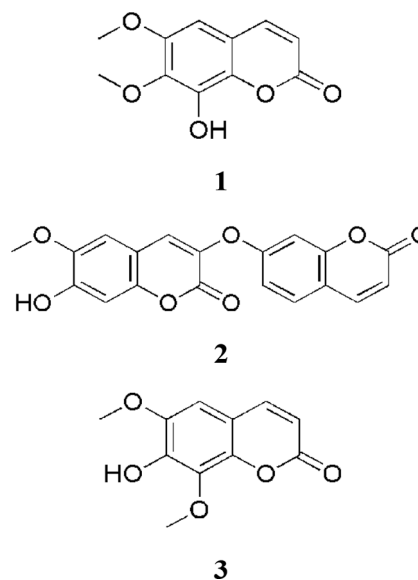


Fig. 1 Chemical structure of fraxidin (1), daphnoretin (2), and isofraxidin (3)

using a vacuum concentrator. The extraction yield was calculated, and the data are presented in Table 1.

Preparation of standard and sample solutions

The extracts of aerial parts of *A. keiskeana* and *A. stolonifera* and standard solutions of fraxidin, daphnoretin, and isofraxidin were dissolved in MeOH to obtain a concentration of 20 mg/mL for the extracts and 1 mg/mL for the three standards. Then, they were sonicated for 30 min and filtered using a 0.45 μ m polyvinylidene fluoride membrane filter.

HPLC conditions

The extracts of aerial parts of *A. keiskeana* and *A. stolonifera* were quantitatively analyzed in a reverse-phase HPLC system using an INNO C18 column (25 cm \times 4.6 mm, 5 μ m) with a gradient elution system using a mobile phase composed of 0.1% TFA in water (A) and ACN (B). The elution conditions were 83% A at 0 min until 10 min, 40% A at 40 min, 0% A at 45 min, 83% A at 50 min, and 83% A at 60 min. The column temperature was retained at 30 $^{\circ}$ C. The injection volume was 10 μ L, the flow rate was 1.0 mL/min, and the wavelength was set at 385 nm.

Table 1 Extraction yield of five samples

Sample	Region	Sample (g)	Extract (g)	Yield (%)
The aerial part of <i>A. keiskeana</i>	Soyang commune	5.0	1.3	26
	Yongmun commune	5.0	1.0	20
	Okdo commune	5.0	0.9	18
The aerial part of <i>A. stolonifera</i>	Yongmun commune	5.0	1.2	24
	Soyang commune	5.0	0.9	18

Table 2 Calibration curve equation for fraxidin (1), daphnoretin (2), and isofraxidin (3)

Compound	t _R	Calibration equation ^a	Correlation factor, r ² ^b
1	19.0	Y = 880.43X + 4812	0.9999
2	31.1	Y = 4402.9X + 9005.6	0.9999
3	16.5	Y = 1966.1X – 6043.8	0.9998

^aY = peak area, X = concentration of the standard (μg/mL)

^br² = correlation coefficient for five calibration data points (n = 3)

Calibration curve

The standard stock solutions of fraxidin, daphnoretin, and isofraxidin were serially diluted to five concentrations, which were used to design the calibration curve. From the calibration curve, linearity was determined based on the correlation coefficient (r²), and the content was quantified on both extracts and dry samples. The calibration function of the three compounds was established with the peak area (Y), concentration (X, μg/mL), and mean value (n = 3) ± standard deviation (Table 2).

Results and Discussion

Coumarins are a group of phenolic compounds widely distributed in plants and exhibit a wide range of biological activities [34,35]. Coumarins have been applied to different therapies such as anti-tumor therapy, anti-cancer therapy, HIV treatment, photochemo-

therapy, and edema treatment. In addition, they are known for their antibacterial, anti-inflammatory, anti-coagulant, and dyeing capabilities [36,37]. Moreover, hydroxycoumarins are powerful antioxidants that can prevent damage caused by free radicals [38]. Fraxidin, daphnoretin, and isofraxidin are coumarins, which potentially have various biological and pharmacological benefits. Daphnoretin could suppress the proliferation of breast cancer cells [39]. However, research on its presence in medicinal herbs is still limited. Although fraxidin and isofraxidin have been reported to be present in *Artemisia* spp., including *A. keiskeana*, *A. campestris*, *A. scotina*, and *A. annua* [4,32,40,41], most studies have only focused on proving the existence of these compounds without specific reports on their content. In addition, information on these compounds in *A. stolonifera* is still scarce.

This study examined the fraxidin, isofraxidin, and daphnoretin content of *A. keiskeana* and *A. stolonifera*, which were cultivated in different areas in Korea, using the HPLC/PDA method. In the

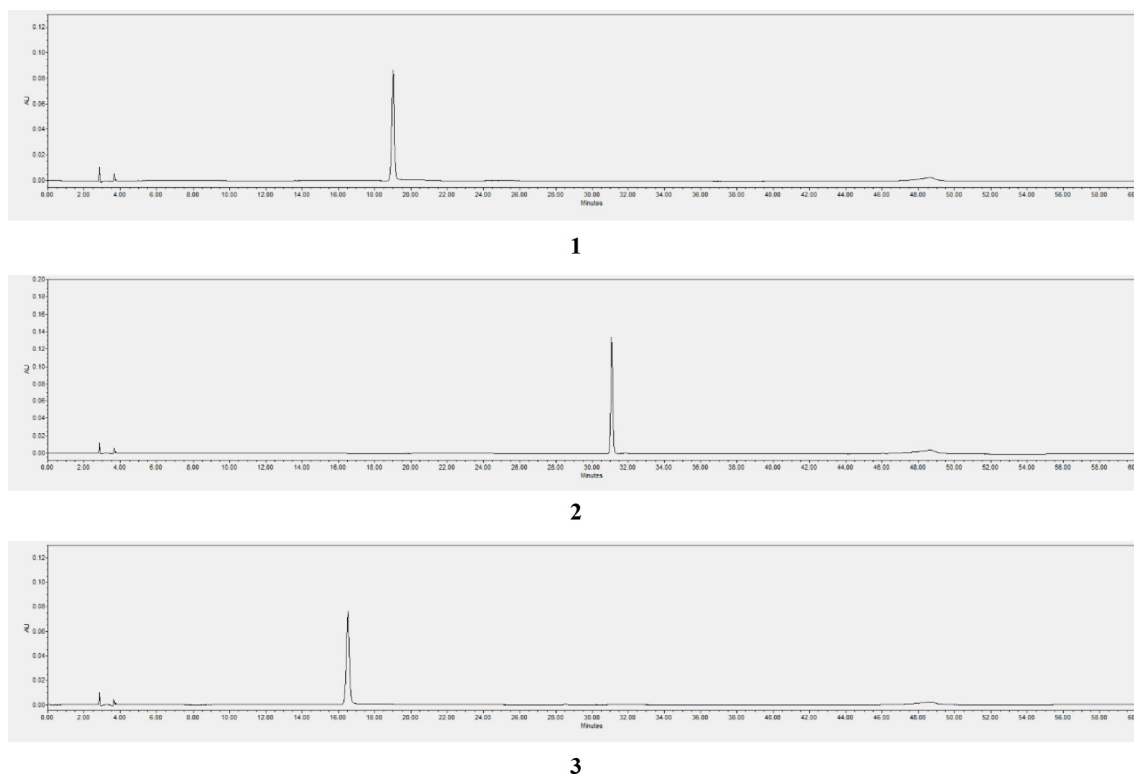


Fig. 2 HPLC chromatograms of fraxidin (1), daphnoretin (2), and isofraxidin (3)

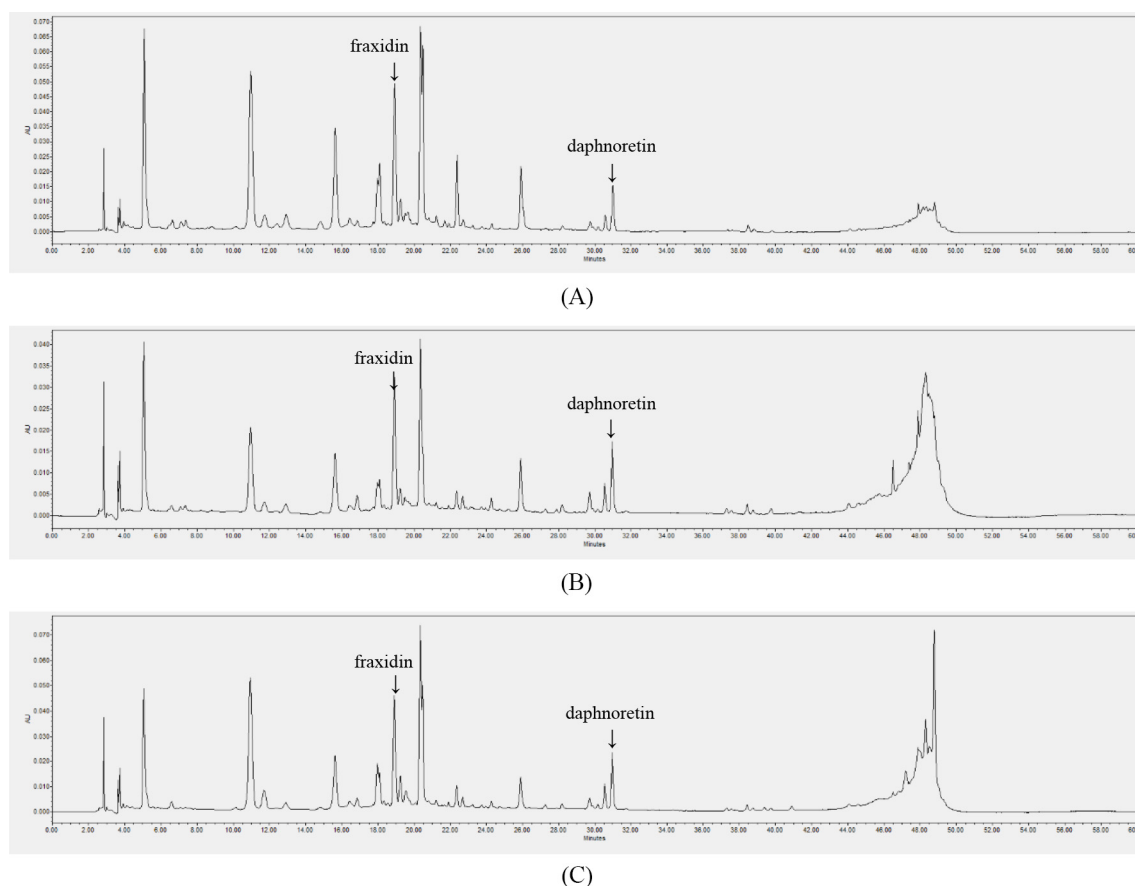


Fig. 3 HPLC chromatograms of the Soyang (A), Yongmun (B), and Okdo (C) samples of *A. keiskeana*

HPLC chromatogram, fraxidin, daphnoretin, and isofraxidin were well separated with retention times of 19.0, 31.1, and 16.5 min, respectively. The HPLC results of the three compounds are shown in Fig. 2. The linear calibration curve equations of fraxidin, daphnoretin, and isofraxidin were $Y = 880.43X + 4812$, $Y = 4402.9X + 9005.6$, and $Y = 1966.1X - 6043.8$, respectively, in which Y represents a given peak area, and X represents the compound concentration. The correlation coefficients (r^2) of all three compounds were higher than 0.9998, demonstrating the good linearity of the method (Table 2). Based on the retention time of three standard compounds and the experiment with the matrix spike samples, the peak of fraxidin, daphnoretin, and isofraxidin in all samples were determined. Through the calibration curve equation, the content of each compound in the samples was determined. The chromatogram of the five samples is shown in Fig. 3, and the quantitative analysis results are summarized in Table 3.

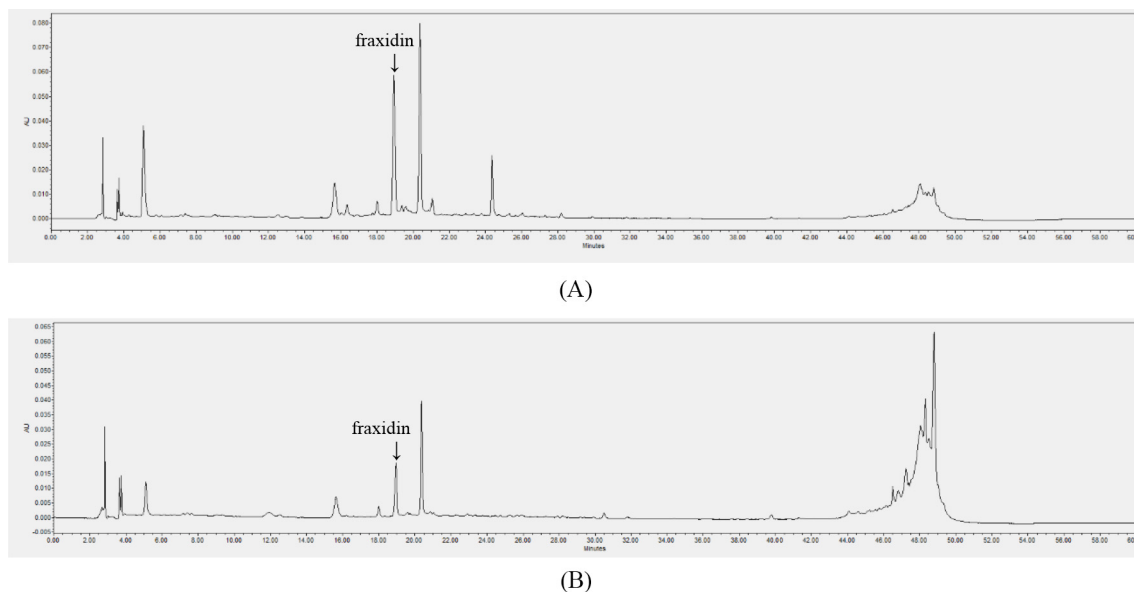
Overall, all samples contained a large amount of fraxidin (ranging from 1.31 to 5.93 mg/g DW), and daphnoretin was found only in *A. keiskeana* at concentrations ranging from 0.26 to 0.36 mg/g DW. However, isofraxidin was not found in all samples. Among samples of *A. keiskeana*, *A. keiskeana* harvested in Soyang commune had the highest content of fraxidin (5.51 mg/g

DW) and daphnoretin (0.36 mg/g DW) compared with the content of samples from the other two regions. On the other hand, the concentrations of these two compounds in *A. keiskeana* grown in Yongmun commune were the lowest (fraxidin: 2.44 mg/g DW; daphnoretin: 0.26 mg/g DW). Among samples of *A. stolonifera*, although grown in the Soyang commune and Yongmun commune and differed only in the commune, the fraxidin content of the two samples was significantly different. In particular, *A. stolonifera* grown in Yongmun commune had a fraxidin content 4.5 times higher than that of *A. stolonifera* grown in Soyang commune (5.93 mg/g DW against 1.31 mg/g DW), and it was the highest among the five surveyed samples (Fig. 4, Table 3).

Some studies have reported the presence of all three compounds in *A. keiskeana*; however, in this study, isofraxidin was not found. The results also showed the presence of only fraxidin in *A. stolonifera*. In addition, the content of fraxidin and daphnoretin in all samples was different. Depending on the growing region, harvest time, light, pH, temperature, and weather conditions, the content of compounds in plants may be different [42]. However, as there has not been a specific study showing that these three compounds are affected by environmental or external factors, it is difficult to compare or provide a specific cause for this difference.

Table 3 Content of fraxidin (1), daphnoretin (2), and isofraxidin (3) in the MeOH extracts of *A. keiskeana* and *A. stolonifera*

Sample		Content (mg/g extract)			Content (mg/g DW)		
		1	2	3	1	2	3
<i>A. keiskeana</i>	Soyang commune	21.19±0.35	1.38±0.03	ND	5.51±0.09	0.36±0.01	ND
	Yongmun commune	12.18±0.06	1.30±0.02	ND	2.44±0.01	0.26±0.00	ND
	Okdo commune	18.11±0.08	1.89±0.01	ND	3.26±0.08	0.34±0.00	ND
<i>A. stolonifera</i>	Yongmun commune	24.70±0.13	ND	ND	5.93±0.03	ND	ND
	Soyang commune	7.26±0.07	ND	ND	1.31±0.01	ND	ND

**Fig. 4** HPLC chromatograms of the Yongmun (A) and Soyang (B) samples of *A. stolonifera*

Therefore, it can only be concluded that, when harvested at different locations, the content of the three compounds will be different.

In conclusion, this study quantitatively analyzed the content of three coumarins (fraxidin, daphnoretin, and isofraxidin) in *A. keiskeana* and *A. stolonifera* harvested in different regions (Soyang commune, Yongmun commune, and Okdo commune). The results showed that depending on the geographical location, the content of the compounds in the plants could be different. Particularly, *A. stolonifera* grown in Yongmun commune and *A. keiskeana* grown in Soyang commune had the highest content of fraxidin and daphnoretin, respectively, whereas isofraxidin was not detected in all samples. To the best of our knowledge, this study was the first report to quantify and compare these three compounds in *A. keiskeana* and *A. stolonifera* from different regions in Korea

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