

# Quantitative Determination and Variation Tendencies of Flavonoids in Five *Selaginella* Plant Drugs

Weifang Long<sup>1</sup>, Qi Ding<sup>1</sup>, Yujie Chen<sup>2</sup>, Jiqing Hu<sup>1</sup>, Luyang Li<sup>1</sup>, Fei Zhang<sup>1</sup> and Dingrong Wan<sup>1,3\*</sup>

<sup>1</sup>Department of Pharmacy, College of Pharmacy, South-Central University for Nationalities, Wuhan, China.

<sup>2</sup>Department of Pharmacy, Hainan Provincial Key Laboratory of R & D of Tropical Herbs, School of Pharmacy, Hainan Medical University, Haikou, China.

<sup>3</sup>The Modernization Engineering Technology Research Center of Ethnic Minority Medicine of Hubei Province, China.

## ABSTRACT

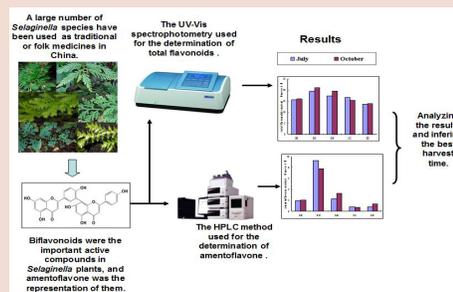
**Introduction:** *Selaginella* species have been used widely for the treatment of inflammatory diseases, diabetes and cancer. In this study, the contents of the total flavonoids and amentoflavone in five *Selaginella* plant drugs [*Selaginella moellendorffii* Hieron. (SM), *Selaginella delicatula* (Desv.) Alston (SD), *Selaginella uncinata* (Desv.) Spring (SU), *Selaginella heterostachys* Baker (SH) and *Selaginella remotifolia* Spring (SR)] from different harvest time were determined by UV-Vis spectrophotometry and HPLC, respectively. The research aimed to preliminarily confirm the appropriate harvest time of *Selaginella* plant drugs. **Methods:** The contents of the total flavonoids and amentoflavone in five *Selaginella* plant drugs from different harvest time will be determined by UV-Vis spectrophotometry and HPLC, respectively. **Results:** The proposed methods were rapid and reliable for the quantitative determination of the total flavonoids and amentoflavone in the five plant drugs. The total flavonoids and amentoflavone contents were significantly different in the five plant drugs, and SM showed the highest content in the both contents. The variation tendencies of the total flavonoids and amentoflavone contents from July to October indicated the diversity and complexity of the accumulation of the active ingredients in plants from the same genus. **Conclusion:** In conclusion, the results provided valuable references for evaluating the quality and preliminarily confirming the appropriate harvest time of *Selaginella* plant drugs.

**Key words:** Amentoflavone content, HPLC, *Selaginella*, Total flavonoids content, UV-Vis spectrophotometry, Variation tendencies.

## SUMMARY

- The study developed methods for quantitative determination of the total flavonoids and amentoflavone contents in five *Selaginella* plant drugs.
- The variation tendencies of the total flavonoids and amentoflavone contents from July to October indicated the diversity and complexity of the accumulation of the active ingredients in plants from the same genus. And the study

showed that confirming the best harvest time of plant drugs had great significance for its quality control.



## PICTORIAL ABSTRACT

**Abbreviations used:** SM: *Selaginella moellendorffii* Hieron, SD: *Selaginella delicatula* (Desv.) Alston, SU: *Selaginella uncinata* (Desv.) Spring, SH: *Selaginella heterostachys* Baker, SR: *Selaginella remotifolia* Spring, UV-Vis spectrophotometry: Ultraviolet-visible spectrophotometry, HPLC: High performance liquid chromatography, RSD: Relative standard deviation.

## Correspondence:

Mr. Dingrong Wan, Department of Pharmacy, College of Pharmacy, South-Central University for Nationalities, Wuhan, Hubei Province-430074, People's Republic of China.

Phone no: 8602767841196, Fax no: 8602767841196

Email: wandr666@163.com

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## INTRODUCTION

The genus *Selaginella* belongs to the family *Selaginellaceae*, mostly distributed in the subtropics and the tropics.<sup>1</sup> There are more than 60 species in China, and a number of them are used as Chinese traditional or folk medicines. The surveys indicated that 15 *Selaginella* species were found in Hubei province, among which 14 species were used as medicines for the treatment of various inflammatory diseases, traumatic hemorrhage and some kinds of cancer.<sup>2,3</sup> Many studies have showed that biflavonoids, such as amentoflavone, were the most important active compounds in *Selaginella* plants, possessing anti-inflammatory, antioxidant, antitumor and antiviral activities.<sup>4-12</sup> In this study, we applied UV-Vis spectrophotometry and HPLC to determine the total flavonoids and amentoflavone contents of the samples collected in different harvest time, trying to evaluate the quality and preliminarily confirm the appropriate harvest time of the five *Selaginella* plant drugs.

## SUBJECTS AND METHOD

### Materials and reagents

The amentoflavone reference substance was purchased from the National Institutes for Food and Drugs Control of China (batch number: 111902-201101). Acetonitrile of HPLC grade was purchased from Tedia Company, Inc., Fairfield, Ohio, USA; the methanol, ethanol, phosphoric acid were of analytical grade. The plant materials were collected from Yichang in Hubei province, China, in July and October, 2012. They were respectively identified as *Selaginella moellendorffii* Hieron., *Selaginella delicatula* (Desv.) Alston, *Selaginella uncinata* (Desv.) Spring, *Selaginella heterostachys* Baker, and *Selaginella remotifolia* Spring by Professor Dingrong Wan (College of Pharmacy, South-Central University for Nationalities, China). All samples were air-dried, pulverized and passed through a 50 mesh sieve.

## Apparatus

The HPLC analysis was performed on a Dionex Ultimate 3000 HPLC System, which was composed of a quaternary pump, an autosampler, a column thermostat, temperature-controlled sample trays, an online degasser and an ultraviolet detector. The total flavonoids content was determined by an UV-Visible spectrophotometer (UV-1800PC, Mapada Co., Shanghai, China).

## Determination of total flavonoids

### Preparation of sample solutions

1.0 g of each sample was accurately weighed, and then 60 mL methanol (90%, v/v) was added and refluxed for 1.5 h. After being cooled to the room temperature, the mixture was weighed again. The loss in weight was replenished with the same solvent, and then, the solution was shaken and filtered. 1.00 mL of the successive filtrate was pipetted into a measuring flask of 50 mL and diluted with 90% methanol for the further use.

### Preparation of standard solution

An amount of 4.51 mg of amentoflavone reference substance was accurately weighed, dissolved in methanol and brought to the volume in a measuring flask of 50 mL. The concentration of the standard solution was  $90.2 \mu\text{g mL}^{-1}$ .

### Selection of the detection wavelength

According to the absorption spectra of the standard solution and the sample solution, both amentoflavone and the sample had strong absorptions at 270 nm. Therefore, 270 nm was chosen as the detection wavelength.

### Optimization of the sample extraction

SD (October 2012) was employed here to explore the optimum extraction process with different extraction solvents, extraction time and material/solvent ratios. The sample solutions prepared under different conditions were determined by measuring the absorbance at 270 nm with an UV-Vis spectrophotometer. The optimum conditions were noted by calculating the extraction yield of total flavonoids on the basis of the calibration curve.

## Determination of amentoflavone

### Preparation of sample solutions

1.0 g of each sample was accurately weighed, and then 60 mL methanol (90%, v/v) was added and refluxed for 1.5 h. After being cooled to the room temperature, the mixture was weighed again. The loss in weight was replenished with the same solvent, and then, the solution was shaken and filtered through a  $0.45 \mu\text{m}$  membrane into an amber glass HPLC vial before analysis.

### Preparation of stock standard solution

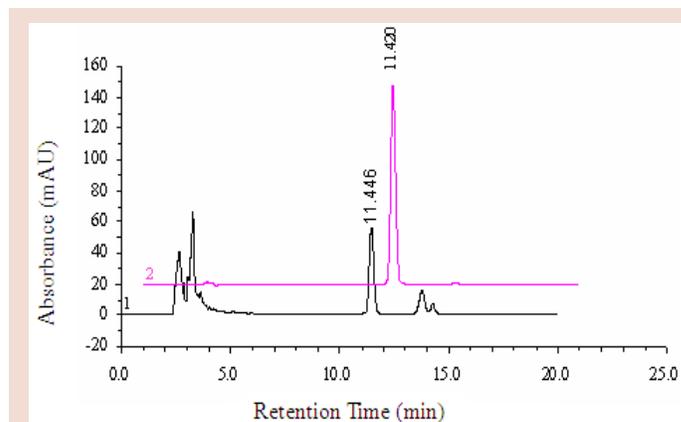
5.05 mg of amentoflavone reference substance was accurately weighed and moved into a volumetric flask, dissolved and made up to 10 mL with methanol. The concentration of the stock standard solution was  $505.0 \mu\text{g mL}^{-1}$ .

### Chromatographic conditions

Based on the results of the full wave scanning of the sample solution and the absorbing wavelength of the standard solution of amentoflavone, 270 nm was chosen as the detection wavelength. Reverse phase separation was performed at  $30^\circ\text{C}$  using a BDS Hypersil C-18 column ( $4.6 \times 250 \text{ mm}$ ) with a particle size of  $5 \mu\text{m}$ . The mobile phase composed of 65% water (containing 0.1%  $\text{H}_3\text{PO}_4$ ) and 35% acetonitrile. The flow rate was kept at  $1 \text{ mL} \cdot \text{min}^{-1}$  and the injection volume was  $10 \mu\text{L}$ .

The chromatographic conditions above were optimized to separate the primary marker peaks of each sample with good resolution ( $R > 1.5$ ) and

theoretical plate numbers (amentoflavone:  $n > 8000$ ). The compounds were identified by comparing the retention times of sample solution with that of the reference standard amentoflavone (Figure 1). The concentration of the amentoflavone was calculated according to the equation of the calibration curve. Three parallel operations were performed for each sample.



**Figure 1:** HPLC chromatograms of reference substance and sample 1. *Selaginella delicatula* (Desv.) Alston; 2. Amentoflavone

## RESULTS

### Determination of total flavonoids by UV-Vis spectrophotometry

#### Calibration curve

Standard solutions of amentoflavone with different volumes (0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 7.00 mL) were spiked into measuring flasks and diluted with methanol to 50 mL, followed by shaking. The absorbances of the solutions were measured at 270 nm using a spectrophotometer against an appropriate blank solution. Using the absorbability as ordinate and the concentration as abscissa, the specification curve was obtained. The calibration curve presented a linear absorbance response within the concentration range of  $0.902\text{--}12.628 \mu\text{g} \cdot \text{mL}^{-1}$ . The regression equation was  $Y = 0.0853X + 0.0011$  with  $r = 0.9999$ , showing a good linearity.

#### Validation

SD collected in October 2012 was used for validation.

To measure precision, the absorbance of the sample solution was measured by an UV-Vis spectrophotometer for six times. The average absorption was 0.390, and the relative standard deviation (RSD) was 0.13%, demonstrating that the instrument used had a high precision.

Repeatability test was carried out with SD powder for six times. The average content of the total flavonoids was  $13.73 \text{ mg} \cdot \text{g}^{-1}$  with RSD of 0.77%, indicating a good repeatability of this method.

As for the stability, the absorbance of the sample solution was measured at 0, 2, 4, 6, 8, 10, 12, 24 h after sample preparation. The results showed that the absorption of the solution was stable (RSD=0.38%) if the measurement was carried out within 24 h.

The accuracy was determined by calculating the recovery. Nine samples solution were prepared according to the previously described method and evenly divided into three groups, then added with 1.00, 2.00, 3.00 mL of amentoflavone (concentration:  $90.2 \mu\text{g} \cdot \text{mL}^{-1}$ ) at each group respectively before analyzing by an UV-Vis spectrophotometer. Recoveries of the total flavonoids obtained are shown in Table 1. The mean recovery was 98.50% and RSD was 1.11%, confirming the accuracy of this method.

**Table 1: Recoveries of total flavonoids in SD (October)**

Flavonoids in the samples (mg)	Spiked amentoflavone amount (mg)	Determined flavonoid amount (mg)	Recovery (%)	Mean recovery(%)	RSD (%)
0.2291	0.0902	0.3171	97.56	-	-
0.2292	0.0902	0.3165	96.78	-	-
0.2292	0.0902	0.3176	98.00	-	-
0.2292	0.1804	0.4050	97.45	-	-
0.2291	0.1804	0.4079	99.11	98.50	1.11
0.2292	0.1804	0.4097	100.06	-	-
0.2291	0.2706	0.4982	99.45	-	-
0.2291	0.2706	0.4982	99.45	-	-
0.2290	0.2706	0.4958	98.60	-	-

**Table 2: Recoveries of amentoflavone in SD (October)**

Amentoflavone in the samples (mg)	Spiked amentoflavone amount (mg)	Determined flavonoid amount (mg)	Recovery (%)	Mean recovery(%)	RSD (%)
1.3195	0.5050	1.8282	98.75	-	-
1.3203	0.5050	1.8396	102.83	-	-
1.3198	0.5050	1.8308	101.19	-	-
1.3199	1.0100	2.3406	101.06	-	-
1.3199	1.0100	2.3345	100.46	100.58	1.19
1.3196	1.0100	2.3254	99.58	-	-
1.3198	1.5150	2.8330	99.88	-	-
1.3198	1.5150	2.8370	100.14	-	-
1.3200	1.5150	2.8561	101.39	-	-

## Determination of amentoflavone by HPLC

### Calibration curve

Different volumes of the stock standard solution (0.10, 0.20, 0.50, 1.00, 2.00, 3.00, 3.50 mL) was accurately pipetted, diluted to a final volume of 10 mL with methanol, and shaken to the uniform to obtain standard solutions with different concentration. Those standard solutions were injected into the HPLC system in duplicate, and the average peak areas were calculated. The regression equation was  $Y=63.415X + 0.3959$  with the correlation coefficient  $r=0.9999$ . The results showed that a good correlation exists between the peak areas (Y) and the amount of amentoflavone (X) within the range of 0.0505-1.7675  $\mu\text{g}$ .

### Validation

In HPLC analysis, the stock solution and powder of SD (collected in October 2012 ) were used for the validation examination.

The precision was evaluated by assaying the sample solution under the selected optimal chromatographic conditions for six times. The RSD of the determination results was 0.09%. The repeatability was estimated by preparing and determining the SD sample simultaneously in six replicates. The RSD of the amentoflavone was 0.45%. These results informed us that the precision and repeatability of the method would suffice for the routine quality control requirements of Chinese herbal medicine.

In addition, the sample solution was assayed at 2, 4, 6, 8, 10, 12, 24 h after sample preparation to assess the stability of amentoflavone. The results manifested that the analyte was stable in 24 h with RSD of 0.35%.

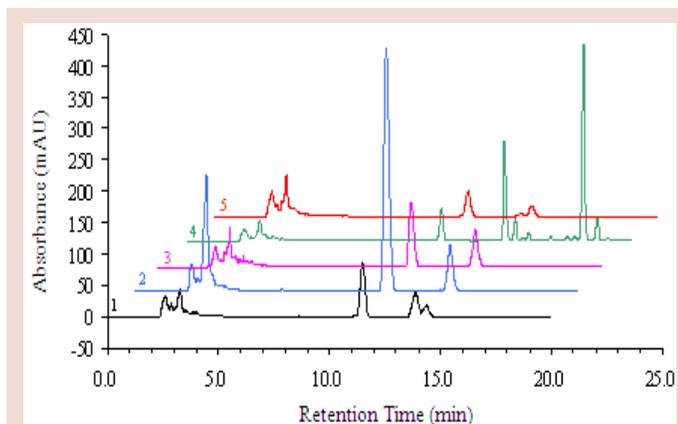
Accuracy was measured by spiking known amount of the analyte at three different concentration levels. The recovery for amentoflavone ranged from 98.75 to 102.83%, while the RSD was 1.19%, indicating a good accuracy of the method (Table 2).

### Sample analysis

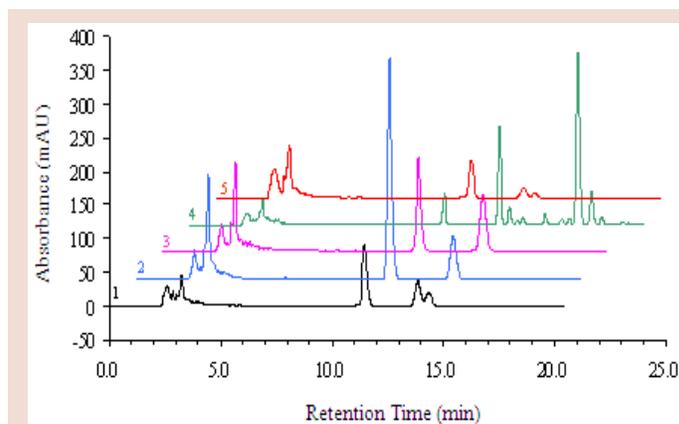
Total flavonoids and amentoflavone (Figure 2 and 3) were determined by the above-mentioned methods. The results are listed in Table 3.

## DISCUSSION

The results showed that the amount of the total flavonoids and amentoflavone in SR and SU samples had no obvious changes between July-collected ones and October-collected ones,. Comparing the amount of amentoflavone and the total flavonoids in SH, it was shown that both of them in October were higher than those in July. The amentoflavone content in SD harvested in autumn was much higher than that in summer, while the total flavonoids content kept changeless. In addition, the varying amount of the total flavonoids in SM was adverse to the content of amentoflavone at different harvest seasons. The results informed us that the contents of the total flavonoids and amentoflavone in the five *Selaginella* plant drugs presented different variation tendencies from summer to autumn, especially in SD and SM. Therefore, we think it maybe not so perfect to evaluate the quality or confirm the harvest time of plant drugs



**Figure 2:** HPLC chromatograms of the samples collected in July 2012  
1. *S. remotifolia* Spring; 2. *S. moellendorffii* Hieron.; 3. *S. heterostachys* Baker; 4. *S. uncinata* (Desv.) Spring; 5. *S. delicatula* (Desv.) Alston



**Figure 3:** HPLC chromatograms of the samples collected in October 2012  
1. *S. remotifolia* Spring; 2. *S. moellendorffii* Hieron.; 3. *S. heterostachys* Baker; 4. *S. uncinata* (Desv.) Spring; 5. *S. delicatula* (Desv.) Alston

**Table 3: Total flavonoids and amentoflavone contents**

Species	Total flavonoids contents (mg·g <sup>-1</sup> )		Amentoflavone contents (mg·g <sup>-1</sup> )	
	July	October	July	October
SR	15.64	15.95	1.96	2.08
SM	19.45	21.11	9.29	7.77
SH	17.41	19.61	2.31	3.30
SU	16.66	15.39	0.83	0.76
SD	13.63	13.75	0.87	1.32

by using the content of a single active compound or a group of active substance as the sole index. With a small proportion of the amentoflavone in the first four plant drugs, it would be more reasonable to evaluate the quality and confirm the best harvest time primarily based on the total flavonoids content with the assistant of amentoflavone content. Therefore, it was feasible to collect SR and SU in summer or in autumn, whereas SH and SD were suggested to be harvested only in autumn. Because the amentoflavone had a high proportion in the total flavonoids (1/3~1/2) in SM, both of them played important roles in evaluating the quality of SM. As a result, it would be appropriate to collect SM in summer or in autumn.

As we all know, Chinese traditional medicines usually contained many complex bioactive chemical constituents. And more often, its bioactivities is not due to the presence of one component but the presence of a mixture of bioactive components. Our studies demonstrated that the amount of the main active components and a single bioactive component had different change tendencies with the growth of medicinal plants. Thus, we think that if a single component has high proportion in a group of active substance, its content will be significant to use for the assessment of the plant drugs. If not, the content of the group of substance should be paid more attention for quality estimation, unless some single component shows particularly strong pharmacological activities.

## CONCLUSION

The methods established in this study had been proved simple, rapid and reliable for quantitative determination of the total flavonoids and amentoflavone contents in the five *Selaginella* plant drugs. And the experimental data provided valuable references for evaluating the quality and preliminarily confirming the appropriate harvest time of *Selaginella* plant drugs.

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## CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

## REFERENCES

1. The Flora of China Editorial Committee. Flora of China, Volume 6. Beijing: Science Press; 2004. p. 86.
2. Wan DR, Chen KL, Zhan YH. Investigations and studies on medicinal plants from *Selaginellaceae* in Hubei Province. *Zhongguo Zhong Yao Za Zhi*. 2005; 30(19): 1507-10.
3. Hsin CH, Wu BC, Chuang CY, Yang SF, Hsieh YH, Ho HY, *et al.* *Selaginella tamariscina* extract suppresses TPA-induced invasion and metastasis through inhibition of MMP-9 in human nasopharyngeal carcinoma HONE-1 cells. *BMC Complement Altern Med*. 2013; 13(1): 234.
4. Su Y, Sun CM, Chuang HH, Chang PT. Studies on the cytotoxic mechanisms of ginkgetin in a human ovarian adenocarcinoma cell line. *Naunyn Schmiede bergs Arch Pharmacol*. 2000; 362(1): 82-90.
5. Zhang JL, Liu ZH, Cao WJ, Chen LY, Xiong XF, Qin SN, *et al.* Amentoflavone inhibits angiogenesis of endothelial cells and stimulates apoptosis in hypertrophic scar fibroblasts. *Burns*. 2014; 40(5): 922-9.
6. Hwang IS, Lee J, Jin HG, Woo ER, Lee DG. Amentoflavone stimulates mitochondrial dysfunction and induces apoptotic cell death in *Candida albicans*. *Mycopathologia* 2012; 173(4): 207-18.
7. Zheng XK, Li KK, Feng WS. Advance in research of chemical constituents in Genus *Selaginella*. *Chinese Journal of New Drugs* 2012; 212(4): 124-33.
8. Setyawan AD. Review: Natural products from Genus *Selaginella* (*Selaginellaceae*). *Bioscience* 2011; 30(1): 44-58.
9. Zou H, Xu KP, Tan GS. Advances in research on chemical constituents and pharmacological activities of Genus *Selaginella*. *Natural Product Research and Development* 2012; 24(3): 1655-70.

10. Lin LC, Kuo YC, Chou CJ. Cytotoxic biflavonoids from *Selaginella delicatula*. J Nat Prod. 2000; 63(5): 627-30.
11. Lin YM, Flavin MT, Schure R, Chen FC, Sidwell R, Barnard DL, *et al.* Antiviral activities of biflavonoids. Planta Med. 1999; 65(2): 120-5.
12. Kim HK, Son KH, Chang HW, Kang SS, Kim HP. Amentoflavone, a plant biflavone: a new potential anti-inflammatory agent. Arch Pharm Res. 1998; 20(4): 406-10.

#### ABOUT AUTHORS



**Miss. Weifang Long:** Presently working as a graduate student in the Department of Pharmacy, College of Pharmacy, South-Central University for Nationalities, Wuhan, China. She has two years of research experience. She has published 3 papers and participated in the writing work of two traditional Chinese folk medicine monographs. Her area of expertise and interest includes identification and quality evaluation of traditional Chinese medicine, research and development of new biologically active substances.



**Mr. Dingrong Wan:** Ph. D., Professor. South-Central University for Nationalities. He has engaged in the study of plant medicine resources and quality evaluation for 33 years. More than 130 papers were published in the study of plant drug resources and quality evaluation.