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Yield Analysis of Flavonoids in *Acanthopanax divaricatus* and *A. koreanum* Grown using Different Cultivation Methods

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Abstract – High-performance liquid chromatography was performed in order to analyze the changes in the flavonoid content (rutin, hyperin, afzelin, quercetin, and kaempferol) of *Acanthopanax divaricatus* and *A. koreanum*, in response to different cultivation methods (pinching height, planting time, and top dressing). The total flavonoid content of *A. divaricatus* and *A. koreanum* ranged from 0.201 to 0.690 mg/g with different pinching heights, 0.143 to 1.001 mg/g for different planting times, and 0.156 to 1.074 mg/g depending on the rate of fertilizer application. In both *A. divaricatus* and *A. koreanum*, the total flavonoid content in the upper section of the plant was greater than that in the lower section. These results demonstrate which cultivation methods maximize the flavonoid content of *A. divaricatus* and *A. koreanum*, and thus help to optimize flavonoid yields to improve production for nutraceutical, pharmaceutical, and cosmeceutical applications.
Keywords – *Acanthopanax* spp., Culture, Flavonoid, Harvesting, HPLC

Introduction

Acanthopanax species is a perennial herbaceous genus in the family Araliaceae and is widely distributed in East Asia.[1] Most of *Acanthopanax* species grow to 2 - 4 m in height and bear five leaflets. Flowering generally occurs from July to September, and fruits ripen in October.[2] Among the *Acanthopanax* species, *A. divaricatus* has been shown to have anticancer, antiviral, antimutagenic, and immunostimulatory effects.[3,4] *A. koreanum* has been used in the form of tonic as an herbal remedy for dotage, paralysis, arthritis, rheumatism, lameness, and high blood pressure. It has been shown to have anti-inflammatory, immunostimulatory, antioxidant, and PTP1B inhibitory effects.[5-7]

The phytochemicals of *A. divaricatus* and *A. koreanum* are composed of phenylpropanoids, lignans, flavonoids, diterpenoids, triterpenoids, and phytosterols.[8-12] Of these compounds, many of the activities of flavonoids have been identified. Hyperin was found to exhibit strong aldose reductase inhibitory activity;[13] furthermore, hyperin, quercetin, and isoquercitrin all exhibit protective activity against glucose-mediated protein damage.[14] Kaempferol and afzelin were also found to exhibit tyrosinase inhibitory and anti-inflammatory activities.[15]

Acanthopanax species are present both in the wild and under cultivation in many regions of Korea and have attracted commercial interest because of their pharmacological effects. However, it is difficult to produce high-quality *Acanthopanax* specimens consistently, as their pharmacological content varies significantly depending on cultivation area and methods.[16] For example, shading, pinching, fertilizer ratio, and planting time have all been shown to affect the growth and acanthoside D content of both *A. divaricatus* and *A. koreanum*.[17]

Therefore, this study uses high-performance liquid chromatography (HPLC) to investigate the changes in the flavonoid content (rutin, hyperin, afzelin, quercetin, and kaempferol) of *A. divaricatus* and *A. koreanum* in response to differences in cultivation methods.

Experimental

Plant materials – The plants of *A. divaricatus* and *A. koreanum* were treated with different cultivation methods, including pinching at various heights (at 30 and 60 cm on

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March 30, 2007), a range of planting times (March 30, April 15, and April 30, 2007), and different fertilizer top dressing treatments (N-P-K, 10.5-8.5-8.5, 50 kg/10a; 2N-P-K, 21-8.5-8.5, 50 kg/10a; N-2P-K, 10.5-17-8.5, 50 kg/10a; N-P-2K, 10.5-8.5-17, 50 kg/10a; 2N-2P-2K, 21-17-17, 50 kg/10a, on June 10, 2007).

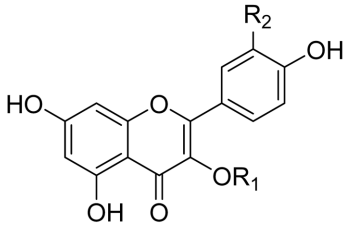
Cultivation conditions and methods – The average temperature and precipitation during cultivation were 13.1 °C and 1,142 mm, respectively. Mature soil was used, and the soil conditions were: pH 7.0, 3.4% soil organic content, 435 ppm available phosphate, and 0.36, 3.0, and 1.2 $cmol^+$/kg of K, Ca, and Mg, respectively.[17] All plants were cultivated under the aforementioned conditions. Tilling was carried out on March 20, during which fully fermented compost (1,500 kg/10a) was added to the soil. *A. divaricatus* and *A. koreanum* seedlings were planted on March 30, 2007. Compound fertilizer (2N-2P-2K, 21-17-17, 50 kg/10a) was applied once on June 10, 2007. All samples were harvested on February 13 of the following year. Upon harvest, stems were divided horizontally, into their upper and lower parts. Experiments were carried out at Yeongcheon Agricultural Technology & Extension Center, Yeongcheon, Korea.

Chemicals and equipment – Acetic acid, acetonitrile, water, and methanol (MeOH) were purchased from J.T. Baker® (USA). Evaporation was conducted using an evaporator system (Eyela rotary system, Tokyo, Japan) under reflux *in vacuo*. HPLC was performed using a Waters Breeze system (Waters Co., Milford, MA, USA) equipped with a Waters 1525 binary HPLC pump and a 2489 system UV/VIS detector. The water and acetonitrile used were both of HPLC grade, while all other reagents were of analytical grade.

Flavonoid preparation – Compounds **1 - 5** were isolated by repeated column chromatography. Compound **1** was isolated from the ethyl acetate fraction of *Fagopyrum tataricum*.[18] Compound **2** was isolated from the ethyl acetate fraction of *A. chiisanensis*.[19] Compounds **3** and **5** were isolated from the ethyl acetate fraction of *Rhododendron mucronulatum* for. *A. albiflorum*.[20] Compound **4** was isolated from the butanol fraction of *Vaccinium koreanum*.[19]

Sample preparation – To analyze the flavonoid content (rutin, hyperin, afzelin, quercetin, and kaempferol) of *A. divaricatus* and *A. koreanum,* 5 g each of dried *A. divaricatus* and *A. koreanum* was extracted by reflux with 50% MeOH (3 × 100 ml) and evaporated *in vacuo*. The residue was dissolved in 1 ml of MeOH and filtered with a 0.45-μm filter. The resulting solution was used for HPLC analysis.

HPLC conditions – The HPLC separation of flavone derivatives for qualitative and quantitative analysis was performed using a reverse phase system. A Discovery® C18 (4.6 × 250 mm, 5 μm) column was used, with a mobile phase that consisted of 0.1% acetic acid and acetonitrile. A gradient solvent system of 0.1% acetic acid and acetonitrile (90:10 to 60:40 for 60 min) was used for the elution program. UV detection was conducted at 350 nm. The injection volume was 10 μl, and the flow rate was 1 ml/min. All injections were performed in triplicate.



Compound	R_1	R_2
1	Rutinose	OH
2	Galactose	OH
3	Rhamnose	H
4	H	OH
5	H	H

Fig. 1. Chemical structures of compounds **1 - 5**.

Calibration curve – A stock solution (1 mg/ml) of each flavonoid was prepared in MeOH and then serially diluted to 50% to obtain solutions of different concentrations. The analyte contents were determined from the corresponding calibration curves. The calibration functions of the flavonoids were calculated using the peak area (Y), concentration (X, μg/10 μl), and mean values (n = 3) ± the standard deviation (SD).

Result and Discussion

Analysis of the differences in the flavonoid content of *A. divaricatus* and *A. koreanum*, among plant sections and in response to different cultivation methods, was performed using HPLC. Compounds **1 - 5** (Fig. 1) were previously isolated from *A. koreanum*, *A. divaricatus*, *A. chiisanensis*, and *A. sciadophylloides*.[12,19,21]

The flavonoids were separated for qualitative and quantitative analysis using HPLC, which was performed using a reverse phase system. The HPLC conditions used in the analysis showed good linearity for the five standard

flavonoids ($r^2 = 0.9999$).[22] The different concentrations of the five standard flavonoids found in *A. divaricatus* and *A. koreanum* were measured according to variations in pinching height, planting time, and fertilizer top dressing, as described in the materials and methods section. The HPLC chromatograms are shown in Fig. 2.

Total flavonoid content of the upper and lower parts of *A. divaricatus* and *A. koreanum* was similar among the different cultivation methods. Pinching (at 30 and 60 cm) yielded a total flavonoid content of 0.349 - 0.395 and 0.245 - 0.315 mg/g in the upper and lower parts of *A. divaricatus,* respectively. In *A. koreanum*, the total flavonoid content was 0.333 - 0.690 and 0.201 - 0.237 mg/g in the upper and lower parts, respectively (Table 1). Different planting times (March 30, April 15, and April 30) yielded plants with a total flavonoid content of 0.358 - 0.397 and 0.143 - 0.266 mg/g in the upper and lower parts of *A. divaricatus*, respectively; similarly, the total content of flavonoids was 0.631 - 1.001 and 0.296 - 0.454 mg/g in the upper and lower parts of *A. koreanum*, respectively (Table 2). Among plants cultivated with different top dressing treatments (N-P-K, 2N-P-K, N-2P-K, N-P-2K, and 2N-2P-2K), the total flavonoid content was 0.278 - 0.342 and 0.156 - 0.252 mg/g in the upper and lower parts of *A. divaricatus*, respectively; and in *A. koreanum*, the total flavonoid content was 0.739 - 1.074 and 0.285 - 0.524 mg/g in the upper and lower parts, respectively (Table 3).

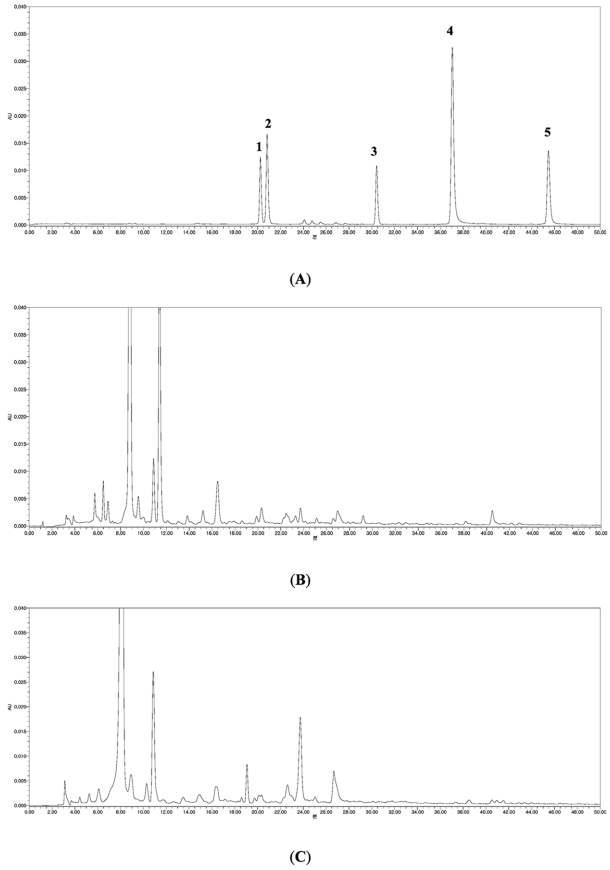


Fig. 2. HPLC chromatograms of compounds **1** - **5** (**A**), the 50% MeOH extracts of the upper part of *A. divaricatus* cultivated with planting time at April 15 (**B**), and *A. koreanum* cultivated with top dressing at N-P-K (**C**).

A previous paper described the effects of shading treatment and harvesting methods on the growth of *A. senticosus*. Specifically, it detailed the effects of using the propagation cutting method as well as the impact of shading treatments, on photosynthetic activity. Additionally, it examined the effects of chilling periods on dehisced seed germination and seedling growth of *A. senticosus*.[23-25] Most previous studies have focused on the physiology of *A. senticosus*. However, there have been few investigations examining the response of *A. senticosus* biochemistry to different cultivation methods. Analysis of *Acanthopanax* species phytochemistry shows that the flavonoid content (rutin, hyperin, afzelin, quercetin, and kaempferol) of *Acanthopanax* species (*A. chiisanensis, A. divaricatus, A. koreanum, A. senticosus*, and *A. sessiliflorus*) fruits was determined to be 9.076, 5.986, 2.175, 1.355, and 4.845 mg/g, respectively.[22] Additionally, the different

Table 1. Contents of compounds **1** - **5** in *A. divaricatus* and *A. koreanum* cultivated by pinching (mg/g)

Sample	Pinching		**1**	**2**	**3**	**4**	**5**	Total
A. divaricatus	30 cm	Upper part	0.008 ± 0.001	0.154 ± 0.008	0.127 ± 0.001	0.071 ± 0.001	0.035 ± 0.001	0.395 ± 0.012
		Lower part	0.006 ± 0.001	0.092 ± 0.001	0.108 ± 0.001	0.071 ± 0.001	0.038 ± 0.001	0.315 ± 0.005
	60 cm	Upper part	0.004 ± 0.001	0.130 ± 0.002	0.110 ± 0.001	0.070 ± 0.001	0.035 ± 0.001	0.349 ± 0.006
		Lower part	0.005 ± 0.001	0.073 ± 0.001	0.086 ± 0.001	0.055 ± 0.001	0.026 ± 0.001	0.245 ± 0.005
A. koreanum	30 cm	Upper part	0.414 ± 0.001	0.090 ± 0.001	0.093 ± 0.001	0.062 ± 0.001	0.031 ± 0.001	0.690 ± 0.005
		Lower part	0.045 ± 0.001	0.040 ± 0.001	0.059 ± 0.001	0.038 ± 0.001	0.019 ± 0.001	0.201 ± 0.005
	60 cm	Upper part	0.182 ± 0.001	0.046 ± 0.001	0.053 ± 0.001	0.034 ± 0.001	0.018 ± 0.001	0.333 ± 0.005
		Lower part	0.078 ± 0.001	0.041 ± 0.001	0.060 ± 0.001	0.038 ± 0.001	0.020 ± 0.001	0.237 ± 0.005

Data are presented as the mean ± SD (n = 3) in mg/g of the dried samples.

Table 2. Contents of compounds **1** - **5** in *A. divaricatus* and *A. koreanum* cultivated with different planting times (mg/g)

Sample	Planting time		**1**	**2**	**3**	**4**	**5**	Total
A. divaricatus	March 30	Upper part	–	0.184 ± 0.005	0.107 ± 0.001	0.071± 0.001	0.035 ± 0.001	0.397 ± 0.008
		Lower part	0.006 ± 0.001	0.057 ± 0.001	0.065 ± 0.001	0.040± 0.001	0.020 ± 0.001	0.188 ± 0.005
	April 15	Upper part	0.002 ± 0.001	0.098 ± 0.002	0.200 ± 0.001	0.063 ± 0.001	0.031 ± 0.001	0.394 ± 0.006
		Lower part	0.001 ± 0.001	0.042 ± 0.001	0.053 ± 0.001	0.031 ± 0.001	0.016 ± 0.001	0.143 ± 0.005
	April 30	Upper part	0.005 ± 0.001	0.101 ± 0.003	0.169 ± 0.004	0.055 ± 0.001	0.028 ± 0.001	0.358 ± 0.010
		Lower part	0.001 ± 0.001	0.078 ± 0.001	0.094 ± 0.001	0.061 ± 0.001	0.032 ± 0.001	0.266 ± 0.005
A. koreanum	March 30	Upper part	0.353 ± 0.001	0.089 ± 0.001	0.096 ± 0.001	0.062 ± 0.001	0.031 ± 0.001	0.631 ± 0.005
		Lower part	0.158 ± 0.001	0.088 ± 0.001	0.105 ± 0.001	0.068 ± 0.001	0.035 ± 0.001	0.454 ± 0.005
	April 15	Upper part	0.673 ± 0.001	0.108 ± 0.001	0.115 ± 0.001	0.070 ± 0.001	0.035 ± 0.001	1.001 ± 0.005
		Lower part	0.097 ± 0.001	0.049 ± 0.001	0.076 ± 0.001	0.046 ± 0.001	0.028 ± 0.001	0.296 ± 0.005
	April 30	Upper part	0.610 ± 0.001	0.134 ± 0.001	0.105 ± 0.001	0.071 ± 0.001	0.035 ± 0.001	0.955 ± 0.005
		Lower part	0.139 ± 0.001	0.079 ± 0.001	0.108 ± 0.001	0.068 ± 0.001	0.039 ± 0.001	0.433 ± 0.005

Data are presented as the mean ± SD (n = 3) in mg/g of the dried samples.

Table 3. Contents of compounds **1** - **5** in *A. divaricatus* and *A. koreanum* cultivated with top dressing (mg/g)

Sample	Top dressing		**1**	**2**	**3**	**4**	**5**	Total
A. divaricatus	N-P-K	Upper part	0.001 ± 0.001	0.088 ± 0.002	0.109 ± 0.001	0.054 ± 0.001	0.026 ± 0.001	0.278 ± 0.006
		Lower part	0.003 ± 0.001	0.070 ± 0.001	0.099 ± 0.001	0.054 ± 0.001	0.026 ± 0.001	0.252 ± 0.005
	2N-P-K	Upper part	0.003 ± 0.001	0.112 ± 0.002	0.095 ± 0.001	0.056 ± 0.001	0.026 ± 0.001	0.292 ± 0.006
		Lower part	0.015 ± 0.001	0.066 ± 0.001	0.074 ± 0.001	0.047 ± 0.001	0.027 ± 0.001	0.229 ± 0.005
	N-2P-K	Upper part	–	0.085 ± 0.001	0.148 ± 0.001	0.070 ± 0.001	0.032 ± 0.001	0.335 ± 0.004
		Lower part	–	0.040 ± 0.001	0.059 ± 0.001	0.039 ± 0.001	0.018 ± 0.001	0.156 ± 0.004
	N-P-2K	Upper part	0.003 ± 0.001	0.135 ± 0.006	0.124 ± 0.001	0.055 ± 0.001	0.025 ± 0.001	0.342 ± 0.010
		Lower part	–	0.063 ± 0.001	0.078 ± 0.001	0.047 ± 0.001	0.022 ± 0.001	0.209 ± 0.004
	2N-2P-2K	Upper part	–	0.082 ± 0.001	0.132 ± 0.001	0.061 ± 0.001	0.028 ± 0.001	0.303 ± 0.004
		Lower part	–	0.049 ± 0.001	0.071 ± 0.001	0.046 ± 0.001	0.023 ± 0.001	0.189 ± 0.004
A. koreanum	N-P-K	Upper part	0.740 ± 0.001	0.116 ± 0.001	0.109 ± 0.001	0.071 ± 0.001	0.038 ± 0.001	1.074 ± 0.005
		Lower part	0.078 ± 0.001	0.053 ± 0.001	0.074 ± 0.001	0.046 ± 0.001	0.042 ± 0.001	0.293 ± 0.005
	2N-P-K	Upper part	0.370 ± 0.001	0.113 ± 0.001	0.126 ± 0.001	0.084 ± 0.001	0.046 ± 0.001	0.739 ± 0.005
		Lower part	0.076 ± 0.001	0.057 ± 0.001	0.089 ± 0.001	0.053 ± 0.001	0.030 ± 0.001	0.305 ± 0.005
	N-2P-K	Upper part	0.569 ± 0.001	0.095 ± 0.001	0.086 ± 0.007	0.054 ± 0.001	0.028 ± 0.001	0.832 ± 0.005
		Lower part	0.093 ± 0.001	0.049 ± 0.001	0.072 ± 0.001	0.046 ± 0.001	0.025 ± 0.001	0.285 ± 0.005
	N-P-2K	Upper part	0.620 ± 0.00[c]	0.120 ± 0.001	0.094 ± 0.001	0.062 ± 0.001	0.032 ± 0.001	0.928 ± 0.005
		Lower part	0.067 ± 0.001	0.059 ± 0.001	0.082 ± 0.001	0.053 ± 0.001	0.034 ± 0.001	0.295 ± 0.005
	2N-2P-2K	Upper part	0.668 ± 0.002	0.117 ± 0.001	0.120 ± 0.001	0.077 ± 0.001	0.037 ± 0.001	1.019 ± 0.006
		Lower part	0.161 ± 0.001	0.089 ± 0.001	0.134 ± 0.001	0.084 ± 0.001	0.056 ± 0.001	0.524 ± 0.005

Data are presented as the mean ± SD (n = 3) in mg/g of the dried samples.

acanthoside D concentrations of both *A. divaricatus* and *A. koreanum* by cultivation methods (shading, pinching, fertilizer ratio, and planting time treatments) were determined to be 0.44 - 3.86, 0.84 - 5.79, 0.61 - 4.30, and 0.77 - 3.51 mg/g, respectively.[17]

Our investigation shows that the requisite conditions for producing the highest flavonoid concentration in *A. divaricatus* were a 30 cm pinching, March 30 planting time, and N-P-2K top dressing. In comparison, for *A. koreanum*, the highest flavonoid concentration was achieved with a 30 cm pinching, April 15 planting time, and N-P-K top dressing. Furthermore, the total flavonoid content in the upper part of the plant was higher than that in the lower part in both *A. divaricatus* and *A. koreanum*.

In conclusion, these results help to advance knowledge of the cultivation methods that would maximize the

flavonoid content of both *A. divaricatus* and *A. koreanum*, thus optimizing flavonoid production procedures for nutraceutical, pharmaceutical, and cosmeceutical applications.

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