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Cultivation characteristics and flavonoid contents of wormwood (*Artemisia montana* Pamp.)

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ABSTRACT

The aim of this study was to establish the optimum harvesting time and the content of flavonoids in the leaves, stems, and roots of *Artemisia montana* Pamp. *A. montana* was monitored from June to October in 2012. The yield of *A. montana* at high density (30 × 10 cm) was higher than that of *A. montana* at low density (30 × 20 and 30 cm). Yield in terms of dry weight was increased with an extended growth period and development stage. High yield achieved at 2580 and 2757 kg·10 a^{-1} in September and October, respectively. Among the leaves, stems, and underground plant organs, jaceosidin and eupatilin were mainly detected in the leaves, and the highest levels were observed in June, at values of 66.6 and 158.2 mg·100 g^{-1}, respectively. In contrast, apigenin was the major compound detected in the underground plant organs, with levels ranging from 21.2 to 29.5 mg·100 g^{-1} until September. Therefore, optimal harvest times were between September and October, generating a high yield and adding economic value although a higher level of total flavonoids was observed in crops harvested in June.

Keywords: *Artemisia montana*; Flavonoids; Harvest Time; Plant Density

1. INTRODUCTION

Medicinal plants have long been recognized as natural herbs that have minimal to negligible side effects. As the culture of enhancing human well-being has gained popularity, scientists have engaged in extensive studies of medicinal plants, including studies on natural drugs, herbal cosmetics, and natural pigments [1-3].

In Korea, wormwood has been used as an herb. *Artemisia* spp. belongs to Compositae, and taxonomic estimates have indicated that 200 to 400 species exist worldwide [4]. Approximately 40 species of *Artemisia* are distributed in Korea, of which 26 species are recorded in the *Color Illustrated Book about the Plants in Korea* [5]. In Korean traditional medicine, *Artemisia* spp. is further classified as Chung-ho, Ae-yeop, In-jin, and Am-ryeo. According to the herbal pharmacopoeia, the Ae-yeop pertains to dried medicinal leaves and young stems of *Artemisia argyi* Lev., *A. princeps* var. *orientalis* (Pamp.) Hara., and *A. montana* Pamp. Ae-yeop has been used as a medicinal herb [6]; it imparts warmth to the body and controls blood circulation, body temperature, bleeding, and pregnancy. It has also been used as a remedy for abdominal pain due to complications, diarrhea, chronic diarrhea, hematemesis, epistaxis, melena, and amenorrhea [7]. Ae-yeop contains various compounds such as flavonoids, steroids, phenylpropanoids, terpenoids, peptides, sesquiterpenoids, monoterpenoids, and diterpenoids [8,9]. Among these, flavonoids are known to possess excellent antioxidant activity that effectively eliminates reactive oxygen species, as well as a variety of other biological activities, including anti-cancer and anti-inflammatory activities [10]. The major flavonoids in Ae-yeop include eupatilin, jaceosidin, apigenin, and eupafolin [9,11]. Its pharmacological activities include anti-cancer, anti-inflammatory, anti-diabetic, and anti-allergic activities [12-15]. Eupatilin is known to have strong inhibitory effects on gastric ulcers and has been used as the main raw material for the preparation of natural drugs [16,17]. In addition, the size of *A. montana* commonly used as Ae-yeop is larger than the size of other wormwood species. It has also been proven to have antioxidant and anti-diabetic effects because of components such as caffeic acid, caffeoylquinic acid, catechol,

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hyperoside, and protocatechuic acid making this herb a potential drug resource [18,19]. However, agronomic evaluation of this crop has not been conducted properly because of difficulties in its morphological classification among similar species. To establish "Good Agricultural Practices" (GAP), plant growth characteristics and yields of wormwood (*Artemisia montana* Pamp.) were evaluated on the basis of plant density and harvest times. Consequently, this study examined changes in the flavonoid (apigenin, jaceosidin, and eupatilin) (**Figure 1**) contents on the basis of cultivation characteristics and harvest times of *A. montana*, to develop an economically significant crop.

2. MATERIALS AND METHODS

2.1. Plant Materials

A. montana was collected from Gyeongsangbukdo Ulleung-gun and planted in a test package at the Department of Herbal Crop Research of the Korean National Institute of Horticultural & Herbal Science on May 10, 2012. The plants were stored at KMRH under the Voucher number: MPS0002514 (**Figure 2**). The plants were grown in seedling trays containing 200 holes in a greenhouse at the beginning of March 2012. After planting, the leaves were collected on the 10th day of each month from July to October to investigate the crop characteristics. Samples were harvested five times every month from June to October for analysis of flavonoids. The test package was prepared using 2000 kg·10 a^{-1} base manure and covered with black plastic bags. A randomized complete block design was used in triplicate. For planting density, spacing between furrows and rows was 90 and 30 cm, respectively. The planting intervals were 10, 20, and 30 cm. After planting, 20 specimens were evaluated three times at 30-day intervals. For extraction, block sampling was used. The quantity was converted to the number per 10 a after harvesting in one m^3 test environment.

2.2. Seed Characteristics

To determine the seed characteristics of *A. montana*, 20 seeds were randomly selected in triplicate. The shape, size, and color of the seeds were evaluated. For 1000-seed weight, the average value of 10 measurements was calculated. For germination, seeds with uniform size and color, as well as devoid of pest contamination, were selected using a caliper and microscope. The selected seeds were placed in disposable petri dishes and maintained in constant-temperature incubators set at 15, 20, 25, and 30˚C. The petri dishes were lined with filter paper soaked with distilled water. Germination was defined as the emergence of young leaves and roots of approximately 1 mm in length through the seed coat. The first germination time, bud burst period and germination rate were monitored.

2.3. Equipment and Reagents

Flavonoid standards, jaceosidin, and eupatilin were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chendu, Sichuan, China) and apigenin from Sigma-Aldrich (St. Louis, MO, USA). Seed characteristics were microscopically evaluated (Olympus SZ61; Olympus Co. Tokyo, Japan). Seed germination was monitored in a constant temperature incubator (Multi-Room Incubator, Wisecube, Wonju-si, Korea). Flavonoid analysis based on growth stage was performed using the Agilent 1100 series HPLC system (Agilent Technologies, CA, USA).

2.4. Extraction and Analysis of Flavonoids

Approximately 10 g of powder was extracted from each plant organ of *A. montana* by using methanol (MeOH), thus generating 2.4 g from the leaves, 1.7 g from the stems, and 1.8 g from the roots. Each extract (10 mg) was placed in a 2 mL Eppendorf tube and mixed with 1 mL of MeOH. After 5 min of ultrasonic extraction, the extracts were centrifuged at 3,000 rpm at 4˚C for 5 min. The supernatant was filtered using a 0.45 μm PTFE hydrophilic syringe filter (i.d., 13 mm) and collected in a vial for HPLC.

For the analysis of flavonoids, the Agilent 1100 Series HPLC system (Agilent Technologies, CA, USA) equipped with u-Bondapak TM C18 (10 μm, 3.9 × 300 mm, Waters, MA, USA) was used. Detection was conducted at a wavelength of 354 nm, the flow rate was 1 mL·min^{-1}, and the column oven temperature was set at 30˚C. Approximately 20 μL of the sample was injected using an auto sampler. The mobile phase solvents used were solvent A [Water: H_2PO_4 (99.6: 0.4, v/v)] and solvent B [acetonitrile]. The gradient program used as follows: 0 - 30 min, 30% → 70% solvent B; 30 - 40 min, 70% →

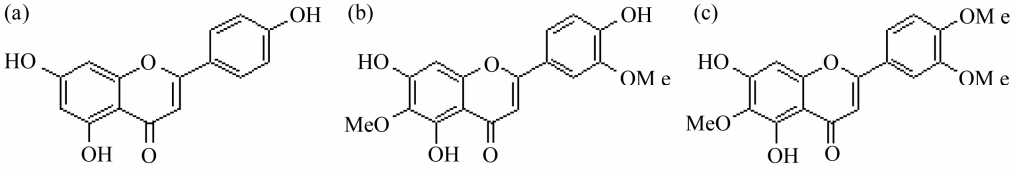

Figure 1. Chemical structure of flavonoids in *A. montana*. (a) apigenin; (b) jaceosidin; (c) eupatilin.

Figure 2. Specimen of *A. montana*. Horticultural traits: *A. montana* is a perennial plant that belongs to Asteraceae and has creeping roots and upright stems. The cauline leaves with hairs are alternately arranged. The size of *A. montana* is larger, compared to the closely related taxa.

100% solvent B; 40 - 50 min, 100% → 30% solvent B; 50 - 55 min, keep 30% solvent B. A stock solution of each flavonoid standards (apigenin, jaceosidin, and eupatilin) was made with 1 mL of MeOH and diluted with MeOH to make 50, 100, 200, 250, and 500 μg·mL^{-1} for standard solutions. After taking 20 μL of each standard solution, HPLC chromatography was conducted to quantify each component. A calibration curve was created using the concentration of the standard solution as the variable. The linear regression equation of the calibration curve of each component was apigenin, y = 7.2412x + 8.8393; jaceosidin, y = 9.6193x + 8.8391; and eupatilin, y = 8.5583x + 6.1677. The coefficient of determination was R^2 = 0.9999. By substituting the HPLC peak area analyzed in each sample for the calibration curve regression equation, the amount of each compound (μg·mL^{-1}) was calculated. By calculating the yield, the extracts were quantified (mg·100 g^{-1} of MeOH extracts).

2.5. Statistical Analysis

Data were analyzed by the application of the Duncan's multiple range test (DMRT, n = 3) at $p \leq 0.05$ using the SAS statistical program (SAS 9.3, SAS Institute Inc., Cary, NC, USA). The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model.

3. RESULTS AND DISCUSSION

3.1. Seed Characteristics

A. montana seeds were oblong in shape, and the hairless achene was wrapped in a white fruit coat. The length, width, and 1000-seed weight were 1.37 mm, 0.52 mm, and 0.110 g, respectively (**Figure 3**). The first germination time was 2 days at 20˚C - 30˚C. However, the first germination time was 3 days at 15˚C. The bud burst period was observed at 20˚C - 30˚C and at 15˚C were 2 days and 5 days, respectively. This trend was similar to that of *A. capillaris*, which is a closely related species [20].

Germination rates at 15˚C, 20˚C, 25˚C, and 30˚C were 84.7%, 90.0%, 92.7%, and 87.3%, respectively, which were slightly higher. The germination rate was the highest at 25˚C (**Figure 3**). The germination rate of *A. montana* increased up to a temperature of 30˚C, and, thereafter, decreased with higher temperature. Thus, 30˚C was considered favorable for the initial germination of *A. montana*. Our results were consistent with the findings of Thompson [21]. Meanwhile, seed germination was closely related to environmental conditions, such as genetic differences, seed maturity, temperature, moisture, oxygen, and sunlight [22]. The temperature has been reported to have the greatest effect on germination rate [23,24]. Thus, when considering the seed characteristics of *A. montana* in this experiment, the optimum germination temperature was 25˚C. This condition can influence the distribution and seeding time of this species.

3.2. Growth Characteristics by Planting Density

The growth characteristics and yields of *A. montana* on the basis of planting density are shown in **Table 1**. Plant height ranged from 168.3 to 176 cm. It appeared that a higher density was often associated with a smaller height. Such findings were contrary to the results of a few studies [3,25,26], suggesting a higher planting density in *Achyranthes japonica*, *Asparagus cochinchinensis*, and *Ligusticum chuanxiong*. Results indicated that the heights of the plants were comparatively higher because of the competition among the species and decrease in light intensity. The results showed a similar tendency to that of Song *et al.* [27], who reported that a higher planting density in *P. sonchifolia* and *W. japonica* led to a lesser height because of competition between species [1]. Leaf dry weight ranged from 32 to 79.3 g. A lower plant density was associated with a higher leaf dry weight. The dry weight of the aerial plant organs per 10 a was the highest in the 30 × 10 cm plots. However, no significant differences were observed between the result and planting distance of 30 × 20 cm. A higher number of aerial plant organs of *A. montana* were associated with a

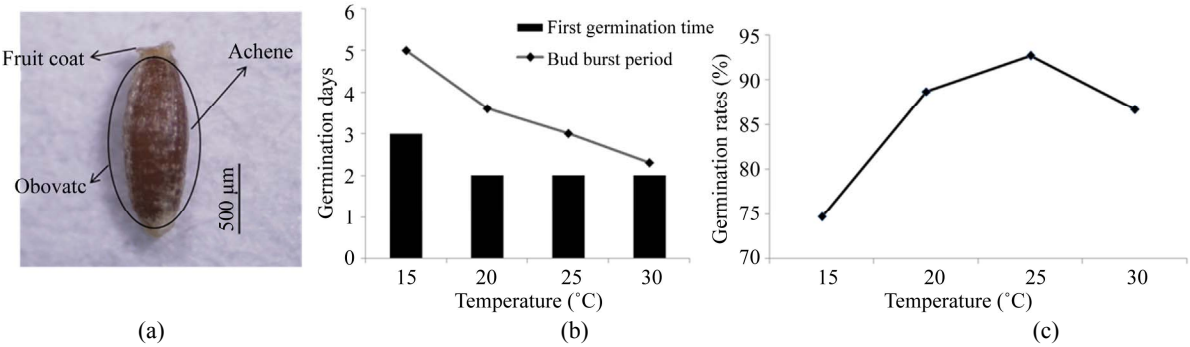

Figure 3. Plant growth characteristics of *A. montana* according to different temperatures. (**a**) seed characteristics; (**b**) first germination time and bud burst period (days); (**c**) germination rates (%).

Table 1. Plant growth characteristics and yields of *A. montana* according to plant density in September.

Plant density (cm)	No. of plant (ea·10 a^{-1})	Plant height (cm)	Stem diameter (mm)	Leaf weight (g, dry weight)	Dry weight ratio (%)	Yield (kg·10 a^{-1}, dry weight)	
						Aerial part organ[a)]	Underground part organ
30 × 10	30,000	168.3 ± 4.10a	8.7 ± 1.40a	32.0 ± 1.50c	55.3a	2580 ± 224.0a	480.0 ± 91.24a
30 × 20	15,000	171.5 ± 6.17a	9.9 ± 1.87a	53.2 ± 8.14b	58.4a	2330 ± 91.65a	532.5 ± 68.74a
30 × 30	9,000	176.0 ± 6.42a	10.2 ± 170a	79.3 ± 4.25a	47.9b	1847 ± 69.18b	559.5 ± 53.31a
F-value		1.41^{NS}	0.70^{NS}	233.53^{***}	12.60^{**}	19.75^{**}	0.93^{NS}

Within each column, values followed by the same letters in a column are not significantly different at $p \leq 0.05$ ($n = 3$). Significance level about F-value is represented at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ^{NS}not significant. [a)]Aerial plant organ indicated above-ground parts including stems and leaves.

greater planting density. These results were similar to that of other medicinal plants such as *A. japonica*, *A. cochinchinensis*, and *L. chuanxiong* [3,25,26].

3.3. Growth Characteristics by Harvest Time

Growth characteristics and yields of *A. montana* on the basis of harvest time were investigated using 30 × 10 cm plots (**Table 2**). The height sharply increased from 122.87 to 169.4 cm during the period of July-August, without showing considerable differences after August. The rainy season in July may have affected the growth of *A. montana* because of the sufficient water and sunlight. After the flowering period in August, growth stopped. The stem diameter was the greatest in August when the growth rate was the highest. Significant differences were observed between August and other times. Leaf dry weight was the highest in October. A longer growth period was associated with a higher yield. Dry weight ratio during the growth period decreased by 44.7% in September and by 71.9% in July. It may be possible that after the rainy season of July to August, the high moisture content caused the higher dry weight ratio; in contrast, in September the hot and dry weather caused the lower levels of moisture content and dry weight ratio. Since September, the dry weight ratio has remained almost constant. Apparently, the reason was that the change in climate after September was not significant. The dry weight of the aerial plant organs by harvest time was 2757 kg·10 a^{-1}, which was the highest in October. No significant differences were observed between the results collected in October, 2757 kg·10 a^{-1}, and the dry weight of the aerial plant organs harvested in September, 2580 kg·10 a^{-1}. Thus, considering leaf dry weights and the yields, the optimal harvesting time for *A. montana* was the period between mid-September and early October.

3.4. Flavonoid Analysis

Jaceosidin and eupatilin were detected only in the leaves, whereas apigenin was detected in the roots (**Table 3**). Contents of jaceosidin and eupatilin with respect to harvest time showed a similar pattern, and the contents in the leaves harvested in June were the highest levels (66.6 and 158.2 mg·100 g^{-1}, respectively). The contents of jaceosidin and eupatilin significantly decreased in July. The contents of jaceosidin and eupatilin in the leaves of *A. princeps* collected in May were the highest (38.6 and 211.4 mg 100 g^{-1}, respectively) [28]. The levels of monoterpene in *A. princeps* were documented the highest level in May 8, and they decreased rapidly after mid-May [29]. Our results were similar to those of previous studies. The level of apigenin in the roots ranged from 21.2 to 29.5 mg 100 g^{-1}. The content increased from June to August and thereafter decreased. These results have also been observed in other medicinal plants such as *A.*

Table 2. Growth characteristics and yields of *A. montana* in different harvest times.

Harvest times	Plant height (cm)	Stem diameter (mm)	Leaf weight (g, dry weight)	Dry weight ratio (%)	Ratio of leaf weight (%)	Aerial part organ[a)] (kg·10 a^{-1}, dry weight)
July	122.8 ± 0.23b	8.9 ± 0.59b	13.5 ± 0.68c	28.1c	43.3a	937.0 ± 15.10c
August	169.4 ± 0.42a	11.7 ± 0.47a	26.0 ± 2.53b	35.1b	31.7b	2,459 ± 62.45b
September	168.3 ± 0.10a	8.7 ± 1.40b	32.0 ± 1.50ab	55.3a	37.2b	2,580 ± 224.0ab
October	165.4 ± 1.27a	9.4 ± 1.04b	39.3 ± 7.66a	51.6a	42.8a	2,757 ± 137.2a
F-value	187.31***	6.15*	21.92***	109.72***	10.46**	115.73**

Within each column, values followed by the same letters in a column are not significantly different at $p \leq 0.05$ ($n = 3$). Significance level about F-value is represented at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ^{NS}not significant. [a)]Aerial plant organ indicated above-ground parts including stems and leaves.

Table 3. Flavonoid contents (mg·100 g^{-1}, $n = 3$) in MeOH extracts of *A. montana* harvested at different development stages from June to October.

Harvest times	Parts	Apigenin	Jaceosidin	Eupatilin
June	Roots	21.2 ± 0.40	ND[a)]	ND
	Stems	tr.[b)]	ND	ND
	Leaves	ND	66.6 ± 2.18	158.2 ± 15.5
July	Roots	24.8 ± 0.97	ND	ND
	Stems	TR	ND	ND
	Leaves	ND	4.5 ± 0.32	14.6 ± 0.26
August	Roots	29.5 ± 0.24	ND	ND
	Stems	TR	ND	ND
	Leaves	ND	17.6 ± 0.32	5.3 ± 0.11
September	Roots	25.7 ± 0.18	ND	ND
	Stems	TR	ND	ND
	Leaves	ND	0.9 ± 0.05	8.8 ± 0.10
October	Roots	11.2 ± 0.12	ND	ND
	Stems	TR	ND	ND
	Leaves	ND	2.1 ± 0.06	11.0 ± 0.10

[a)]ND, not detected. [b)]tr., trace amounts.

capillaris. Capillarisin content in *A. capillaris* increased until flowering time and thereafter decreased [30]. After August, which was the flowering time of *A. montana*, certain ingredients in the underground plant organs decreased.

The levels of active ingredients are influenced by climatic conditions, including precipitation and temperature. For *A. princeps*, whose Ae-yeop was used as herbal drugs, samples collected from April to May showed excellent antioxidant effects. The samples collected from August to September showed high antimicrobial activity [31]. Thus, according to the results of this study, *A. montana* had the potential of serving as natural drugs, and farmers may thus consider wormwood as an economically beneficial crop.

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