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Original Article

Effect of growth stages, culture media, and processing methods on the component variations of *Bletilla formosana* and comparison of its component contents to commercial Rhizoma Bletillae crude drugs

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ABSTRACT

Rhizoma Bletillae, a traditional Chinese medicine (TCM), and *Bletilla formosana* (Hayata) Schltr. (endemic to Taiwan) is widely distributed throughout the island. This study used militarine, cinnamic acid, 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol (BHMD), and 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene (DHMD) as marker compounds along with high pressure liquid chromatography (HPLC) for quantitative analysis of *B. formosana*. Through mass reproduction by tissue culture, *B. formosana* was analyzed according to different growth stages, culture media, drying methods, and processing treatments, and then compared with different commercial Rhizoma Bletillae crude drugs. The results showed that the levels of almost all component contents in the vegetative phase were higher than those in the flowering phase. The militarine content in the mature tuber was higher than that in other components and plant sections. The results of this study indicated that the ideal harvest time for *B. formosana* is from September to October. In the different culture media, Medium 2 (peat soil: snake wood: nacrite: vermiculite = 1:2:1:1) and Medium 3 (sandy loam: snake wood: nacrite: vermiculite = 5:2:2:1) offered higher productivities and provided the best growth conditions. However, Medium 4 (snake wood: nacrite: vermiculite = 3:1:1) gave the highest content of the four compounds in its tuber. The processing treatment applied to fresh tubers was steam heating for a short time (10 minutes or 45 minutes) and then drying at 50 °C. This study also revealed that *B. formosana* had higher component contents than the commercial crude Rhizoma Bletillae drugs.

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1. Introduction

Rhizoma Bletillae (Orchidaceae, *Bletilla*), known as Bai ji, is a common traditional Chinese medical (TCM) material originally documented in the Shen Non Ben Tsao Ging and other medical books. The dried tuber of *Bletilla striata* (Thunb.) Reichb. f. is a major source of the medical material. The species endemic in Taiwan is heterogeneous to *B. formosana* (Hayata) Schltr., *B. striata* (Thunb.) Reichb. f. Its tuber can be used as Rhizoma Bletillae medical material and can also be regarded as an ornamental plant [1].

Most recent relevant studies have focused on isolating and purifying the contents of Rhizoma Bletillae, and more than 80 components have been isolated to date [2–15]. In *B. formosana* (Hayata) Schltr., the components isolated and identified from its pseudobulbs include militarine, excelsioside, gymnoside IX, benzyl alcohol, *trans*-coumaric acid methyl ester, coelonin, batatasin III, cinnamic acid, 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol (BHMD), and 4,7-dihydroxy-1- *p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene (DHMD) [16,17]. Numerous bioactivities including antimitotic and relative immunity activities have been studied in *B. striata* [18–20]; but few researchers have analyzed the components in *B. formosana*, and even fewer have studied the growth stages, culture media, and processing methods of the *Bletilla* plant.

Wild Bletilla plants are rare and currently only a few plants are being artificially cultured. Prior to planting, the soil must be plowed to a depth of 30 cm, raked, and furrowed 15–20 cm high to facilitate drainage [20]. The *Bletilla* plant can be separately propagated using the tuber. Newly budded tuber harvested between September and October was chosen for planting in Sichuan Province, China. The tubers were cut from the connection site and were then planted in a line spaced 33 cm apart in holes 10–13 cm deep. Three tuber pieces were arranged into a triangle in each hole so the new buds would face outward. Each hectare was planted with 15,000 tuber pieces, which were then covered with 1200–1500 kg ash or mature compost per hectare. Then soil was added to the top of the furrow [21].

Following harvesting, the leaf, stem, and fibrous roots were removed. These must be processed immediately after cleaning or they will turn black. The tubers were washed, boiled, sun dried, and de-horned to produce white Rhizoma Bletillae [22]. Previous reports have suggested many processing methods such as boiling for 10 minutes, steaming for 3–5 minutes and then removing the peels, oven or sun drying, and cutting fresh Bai ji into pieces for direct drying [23–25].

These reports revealed that differences in culturing management and processing treatments after harvesting might have a great impact on Bai ji productivity and quality. Our previous study [17] analyzed the components including militarine, cinnamic acid, BHMD, and DHMD, and the antioxidant activity of the *Bletilla* plants [*B. formosana* (Hayata) Schltr. f. and *B. formosana* (Hayata) Schltr. f. *kotoensis* (Hayata) T.P. Lin] collected from different regions in Taiwan. Militarine was reported beneficial for treating dementia [26]. DHMD, BHMD, and related components showed cytotoxicity to tumor cell lines including Hep G2, HL-60, Skov-3, and A431 [27]. Cinnamic acid has been reported to have antibacterial, antiviral, and antifungal properties [28]. The objective of this study was to use militarine, cinnamic acid, BHMD, and DHMD as marker compounds to conduct quantitative analysis by high pressure liquid chromatography (HPLC). *B. formosana* was analyzed according to different growth stages, culture media, drying methods, and processing treatments, and the results were compared with the components of different commercial crude Rhizoma Bletillae drugs.

2. Methods

2.1. Materials

Commercial crude Rhizoma Bletillae drugs were purchased from local Chinese herbal medicine markets in Taipei City, Miaoli County, Taichung City, Chiayi County, Tainan City, and Pingtung County. *B. formosana* plants used in this study were collected from Wutai Township, Pingtung County, Taiwan. These samples were then cultivated in a greenhouse at the National Pingtung University of Science and Technology (NPUST), and were verified by Professor Hsieh, Ching-Hsiang, Department of Plant Industry, NPUST. Voucher specimens were maintained in the corresponding author's laboratory at NPUST.

2.2. Chemicals and reagents

Militarine, cinnamic acid, DHMD, and BHMD were purified and identified from Rhizoma Bletillae in our laboratory [25]. Quercetin (98%; Sigma Chemical Co., St. Louis, MO, USA) was used as an internal standard. Sodium carbonate was purchased from Showa (Japan). Saccharose was from Nihon Shiyaku Industries (Japan). Activated carbon was from Nippon Shiyaku Kogyo (Japan). Hyponex No.1, with a nutrition elements ratio of N:P:K = 7:6:19, was purchased from Hyponex Chemical Co. (New York, USA).

The 95% ethanol was provided by the Taiwan Tobacco and Wine Board (Taipei, Taiwan, ROC). Acetonitrile and methanol (HPLC grade) were from Merck (Darmstadt, Germany). *n*-Hexane, ethyl acetate, acetone, methanol, *n*-butanol, and chloroform were from Riedel-de Haën (Seelze, Germany). Phosphoric acid was from Kanto Chemical (Tokyo, Japan). Ultra-pure distilled water with a resistivity greater than 18.2 $M\Omega/cm^2$ was obtained from a Millipore mini-Q system (Millipore, Bedford, MA, USA). HPLC samples were filtered through 0.45-μm Millipore membrane filters (Millipore). All other reagents were of analytical grade.

2.3. Propagation of *B. formosana*

The ripened fruits of *B. formosana* were sterilized with sodium hypochlorite, and seeds from the fruits were cultured in the media supplemented with 50 g/L mashed potato, 50 g/L mashed banana, 2 g/L Hyponex No.1, 2 g/L tryptone, 2% sucrose, 2 g/L activated carbon, and 0.75% agar. The seeds were cultured at 25 ± 2°C under a constant illumination of 2000 lux for 16 hours/day. After 2.5 months, the seeds germinated. When the seedlings were 3–5 cm in length, they were

transferred to a new medium for subculturing. After 2.5 months, the seedlings were 10–15 cm in length and were transferred to 4-inch pots (with a medium supplement peat soilage: nacrite: vermiculite: sand ratio of 2:1:1:2, mixed with 10% cow manure fertilizer).

2.4. Plant culture and investigation

Regenerate plantlets were transplanted to 4-inch (diameter of 12 cm) pots (with a medium supplement peat soilage: nacrite: vermiculite: sand ratio of 2:1:1:2, mixed with 10% cow manure fertilizer) in January 2006. Three months later, the seedlings were 20-cm each in length and had 4–6 leaves. We randomly sampled 45 plants from April 2006 to March 2007, and conducted monthly measurements of plant height, number of leaves, leaf length, leaf width, mature tuber length, mature tuber width, mature tuber fresh weight, mature tuber dry weight, number of lateral buds, bud length, lateral bud leaf count, lateral bud leaf length, lateral bud leaf width, young tuber fresh weight, and young tuber dry weight. The measurements were followed by HPLC analyses.

2.5. Different culturing media of cultivated B. formosana

In April, when the seedlings were 10–15 cm in length, they were transferred to 4-inch pots. Peat soil, snake wood (*Cyathea lepifera*), sandy loam, nacrite, vermiculite, carbonized rice husk, and coconut fiber are common culturing media for Orchidaceae plants because these media have excellent air permeability and stability characteristics. In this study, six different combinations of culture media were designed for testing and named Media 1–6 as listed below. Each medium was mixed with 10% cow manure fertilizer.

Medium 1 – peat soil: sandy loam: nacrite: vermiculite = 2:1:1:1.

Medium 2 – peat soil: snake wood: nacrite: vermiculite = 1:2:1:1.

Medium 3 – sandy loam: snake wood: nacrite: vermiculite = 5:2:2:1.

Medium 4 – snake wood: nacrite: vermiculite = 3:1:1.

Medium 5 – carbonized rice husk: coconut fiber: sand = 1:1:1.

Medium 6 – peat soil: nacrite: vermiculite: sand = 2:1:1:2.

The plant tubers and leaves were harvested and cleaned in February 2007. All fresh tubers were then dried in a 50°C oven for 48 hours. The dried leaves and tubers were pulverized and stored at −20°C for future HPLC analyses.

2.6. Different drying methods for each part of the cultivated B. formosana

In April, the tissue cultured seedlings reached a length of 10–15 cm and were transplanted to 4-inch pots (medium supplemented with peat: nacrite: vermiculite: sand = 2:1:1:2, and mixed with 10% cow manure fertilizer). The plants were harvested in February 2007 at which time the plants were cleaned and flowers, leaves, stems, tubers, and roots were immediately separated. All samples were freeze-dried, 50°C oven-dried, and 90°C oven-dried separately and each treatment was processed for 48 hours. The dried flowers, leaves, stems, tubers, and roots were pulverized and stored at −20°C for future HPLC analyses.

2.7. Different methods for processing cultivated B. formosana tubers

The tubers were processed by steaming or boiled in water for different treatment periods of 10, 45, and 150 minutes. Then, each of the processed tubers was dried in a 50°C oven for 48 hours separately. The dried tubers were pulverized and stored at −20°C for future HPLC analyses.

2.8. Preparation of standard and internal standard solution

All the standards were weighed and dissolved in 70% methanol to give sequential concentrations of militarine 4000.0 μg/mL, cinnamic acid 130.0 μg/mL, BHMD 200.0 μg/mL, and DHMD 120.0 μg/mL. These were prepared as standard stock solutions.

Quercetin (500.0 μg/mL) was prepared as an internal standard stock solution.

2.9. Preparation of sample solutions

A 1.0-g sample was reflux extracted with 100 mL methanol at 80°C for 3 hours. Each solution was then filtered, evaporated, and adjusted to 5 mL by adding 75% methanol. Meanwhile, quercetin, the internal standard, was simultaneously added to each solution to a concentration of 250 μg/mL. The final solutions were subjected to subsequent HPLC analysis after filtration through a 0.45-μm membrane filter.

2.10. HPLC instruments and conditions [24]

HPLC was performed on a Hitachi system equipped with a DG-2410 degasser, L-7100 pump, L-7420 UV/Vis detector, and an L-7200 auto-sampler (Tokyo, Japan). Peak areas were calculated with D-7000 HSM software (Tokyo, Japan). A Waters ODS-2 column (4.6 mm I.D. × 250 mm, Milford, USA) was used. The mobile phase was a mixture of 5% acetonitrile (solvent A) and 60% acetonitrile (solvent B) for linear gradient elution programmed as follows: 0–15 minutes, 20–32% B; 15–25 minutes, 32–35% B; 25–70 minutes, 35% B; 70–80 minutes, 35–50% B; 80–120 minutes, 50–60% B; 120–125 minutes, 60–68% B; 125–130 minutes, 68–75% B; 130–140 minutes, 75–20% B. The flow rate was 1.0 mL/min, with a detection wavelength of UV 220 nm. The column temperature was 30°C. Twenty microliters of each sample solution prepared as above was injected into the HPLC for analysis. The results were quantified by interpolation into a linear regression plot made from the standard solutions.

2.11. Statistical analysis

One-way ANOVA and Duncan's multiple range tests were used to analyze and compare data. SAS (Statistical Analysis System, USA) software was used and the critical value for the limit of significance was set at $p < 0.05$.

3. Results

3.1. Plant properties in cultivated *B. formosana*

Table 1 shows the monthly variation of plant properties in *B. formosana* recorded from April 2006 to March 2007. Plant height ranged from 24 cm to 39 cm between April and September and sharply increased from April to June, before losing the aerial parts at the start of the droop period in October. The color of the sword-shaped leaves mostly ranged from yellowish to dark green. Leaf length varied from 7.73 cm to 31.68 cm, and width varied from 0.59 cm to 2.13 cm. From April to September, the cultivated *B. formosana* had raceme-inflorescence with 5–10 white or pinkish flowers. The full-flowering phase lasted from May to July, with the fruit phase lasting from May to September.

B. formosana has underground tubers and roots. The mature tubers had an irregular rhombus shape, with a length ranging from 18.95 mm to 25.61 mm, width ranging from 11.53 mm to 19.50 mm, an average fresh weight of 1.89 g/tuber, and an average dry weight of 0.3 g/tuber. Each plant developed 1–3 new lateral buds at the start of the summer season beginning in June. From the end of the flowering phase in October 2006, the number of lateral buds kept constant at 1.96–1.80 to March 2007.

3.2. Component contents in each part of cultivated *B. formosana*

3.2.1. Tuber component contents

The tubers of cultivated *B. formosana* were either covered or not by soil and this produced a difference in color. The covered tubers were white and the uncovered tubers were green. The contents of the marker components for the two colors of tubers are shown in Table 2. The white tubers had significantly higher militarine content (70.04 mg/g), while the dark green tubers had higher contents of the other three marker components.

The militarine, cinnamic acid, BHMD, and DHMD contents of the mature *B. formosana* tubers were examined and the results are presented in Table 3. Militarine was the major component and was found in much greater amounts than the other three components. The levels of militarine in the mature tubers varied from 27.53 mg/g to 40.51 mg/g, cinnamic acid from 203.27 μg/g to 673.20 μg/g, BHMD from 279.02 μg/g to 882.31 μg/g, and DHMD from 117.56 μg/g to 623.74 μg/g. The contents of all components were at lower levels during the full-flowering phase, from May to July, than in other months. This may possibly be due to the transportation of nutrition from underground components to aerial components. Then, in the droop period beginning in November 2006, the contents of all aerial components gradually increased to a high value until March 2007. Taken together, these data indicate that the contents of nearly all components were higher in the vegetative phase than those in the flowering phase.

The component contents of the newly developed young tubers were investigated from October 2006 to March 2007 and the results are presented in Table 3. The levels of militarine varied from 33.13 mg/g to 36.29 mg/g, cinnamic acid from 225.84 μg/g to 603.44 μg/g, BHMD from 530.73 μg/g to 737.48 μg/g,

Table 1 – Monthly variation of plant properties in cultivated *Bletilla formosana*.

	Plant height (cm)	Number of leaves	Leaf length (cm)	Leaf width (cm)	Mature tuber length (mm)	Mature tuber width (mm)	Mature tuber F.W. (g)	Mature tuber D.W. (g)	Number of lateral buds	Lateral bud length (cm)	Leaf number of lateral bud	Leaf length of lateral bud (cm)	Leaf width of lateral bud (cm)	Young tuber F.W. (g)	Young tuber D.W. (g)
2006 Apr.	31.54	3.84	27.05	1.84	18.95	11.53	0.84	0.15							
2006 May	34.95	4.08	28.82	2.01	20.22	14.80	1.51	0.38							
2006 Jun.	38.42	4.12	31.68	2.13	23.68	16.76	2.21	0.57	1.68	0.93					
2006 Jul.	36.72	3.12	30.53	1.95	23.60	15.77	2.01	0.45	1.84	2.16					
2006 Aug.	30.65	1.76	25.14	1.52	25.29	17.39	2.01	0.39	2.12	12.49	3.16				
2006 Sep.	24.12	1.68	20.26	1.33	23.06	17.55	1.99	0.29	1.74	17.65	3.39				
2006 Oct.	9.98	1.12	7.73	0.59	25.61	16.33	1.92	0.29	2.92	34.78	4.72	27.45	2.67		
2006 Nov.					25.34	17.13	1.96	0.25	1.96	36.60	5.12	30.88	2.73	0.77	0.14
2006 Dec.					20.08	16.54	1.62	0.20	1.80	36.88	4.68	30.56	2.45	0.89	0.17
2007 Jan.					20.40	18.22	2.00	0.20	1.80	38.84	5.00	31.84	2.56	0.99	0.19
2007 Feb.					22.19	16.41	2.66	0.19	1.80	37.40	4.54	32.03	2.49	1.42	0.29
2007 Mar.					20.48	19.50	1.97	0.24	1.80	37.50	4.72	30.57	2.53	1.62	0.34

D.W. = dry weight; F.W. = fresh weight.

Table 2 – Effects of different colors of tubers on component contents in *Bletilla formosana*.

Tuber color	Contents			
	Militarine (mg/g D.W.)	Cinnamic acid (μg/g D.W.)	BHMD (μg/g D.W.)	DHMD (μg/g D.W.)
Dark green	55.57 ± 3.23^{b}	963.30 ± 24.96^{a}	981.01 ± 20.80^{a}	531.02 ± 23.00^{a}
Light green	41.82 ± 0.70^{c}	586.49 ± 20.69^{c}	736.52 ± 21.03^{b}	293.65 ± 8.62^{b}
White	70.04 ± 2.07^{a}	668.99 ± 21.65^{b}	521.05 ± 24.59^{c}	280.31 ± 11.50^{b}

The covered tubers were white and the uncovered tubers were green.
Values represent mean ± standard deviation of three samples. Means with different letters (a, b, c) in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test. BHMD = 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol; DHMD = 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene; D.W. = dry weight.

Table 3 – Monthly variation of each component content from mature tuber, young tuber, leaf, root, and lateral bud of cultivated *Bletilla formosana* from 2006 to 2007.

	Date	Contents			
		Militarine (mg/g D.W.)	Cinnamic acid (μg/g D.W.)	BHMD (μg/g D.W.)	DHMD (μg/g D.W.)
Mature tuber	Apr. 2006	27.88 ± 0.41^{f}	673.20 ± 5.10^{a}	320.50 ± 11.49^{h}	117.56 ± 3.36^{i}
	May 2006	29.45 ± 1.02^{e}	203.27 ± 8.35^{g}	326.97 ± 13.15^{h}	162.61 ± 5.37^{g}
	Jun. 2006	27.53 ± 1.35^{f}	215.33 ± 1.19^{g}	279.02 ± 7.27^{i}	143.29 ± 2.65^{h}
	Jul. 2006	30.06 ± 0.29^{e}	240.89 ± 4.13^{f}	360.72 ± 1.73^{g}	167.46 ± 4.54^{g}
	Aug. 2006	34.26 ± 0.43^{d}	312.80 ± 2.98^{d}	470.83 ± 13.14^{f}	208.38 ± 5.34^{f}
	Sep. 2006	35.50 ± 0.20^{c,d}	345.77 ± 7.40^{c}	555.30 ± 22.23^{e}	472.85 ± 18.40^{d}
	Oct. 2006	35.54 ± 0.82^{c,d}	315.34 ± 1.95^{d}	562.00 ± 11.90^{e}	434.83 ± 14.81^{e}
	Nov. 2006	36.61 ± 0.40^{c}	255.85 ± 6.67^{e}	596.69 ± 23.91^{d}	463.71 ± 16.05^{d}
	Dec. 2006	38.19 ± 0.55^{b}	321.21 ± 8.78^{d}	795.60 ± 7.71^{a,b}	590.38 ± 18.17^{b}
	Jan. 2007	39.73 ± 1.27^{a}	401.28 ± 14.09^{b}	822.31 ± 33.65^{a}	462.87 ± 11.82^{d}
	Feb. 2007	39.79 ± 0.45^{a}	392.94 ± 4.41^{b}	709.78 ± 30.36^{c}	623.74 ± 11.86^{a}
	Mar. 2007	40.51 ± 1.43^{a}	322.39 ± 10.03^{d}	779.86 ± 5.01^{b}	539.13 ± 11.01^{c}
Young tuber	Oct. 2006	36.29 ± 0.26^{a}	225.84 ± 5.20^{c}	737.48 ± 31.88^{a}	287.16 ± 3.33^{c}
	Nov. 2006	34.99 ± 0.20^{b}	598.51 ± 18.10^{a}	530.73 ± 11.33^{d}	220.62 ± 6.30^{d}
	Dec. 2006	34.38 ± 0.29^{bc}	501.06 ± 10.00^{b}	583.80 ± 15.18^{c}	213.88 ± 7.77^{d}
	Jan. 2007	33.42 ± 0.44^{c,d}	520.14 ± 8.02^{b}	643.93 ± 13.06^{b}	329.73 ± 7.01^{b}
	Feb. 2007	33.71 ± 0.15^{c,d}	577.26 ± 15.52^{a}	586.80 ± 18.84^{c}	342.70 ± 3.07^{b}
	Mar. 2007	33.13 ± 1.18^{d}	603.44 ± 14.43^{a}	657.04 ± 21.96	373.15 ± 11.93^{a}
Leaf	Apr. 2006	10.40 ± 0.12^{a}	306.10 ± 12.08^{d}	496.23 ± 20.02^{d}	16.39 ± 0.26^{c}
	May 2006	3.91 ± 0.08^{d}	417.91 ± 4.71^{b}	720.96 ± 7.75^{b,c}	16.57 ± 0.08^{c}
	Jun. 2006	4.92 ± 0.04^{c}	441.80 ± 12.98^{a}	744.78 ± 14.89^{b}	Trace
	Jul. 2006	5.58 ± 0.07^{b}	355.27 ± 4.48^{c}	1008.08 ± 18.54^{a}	20.21 ± 0.14^{a}
	Aug. 2006	3.66 ± 0.12^{e}	188.92 ± 8.91^{f}	411.92 ± 13.53^{e}	18.34 ± 0.12^{b}
	Sep. 2006	3.27 ± 0.10^{f}	221.67 ± 1.30^{e}	408.29 ± 13.55^{e}	16.63 ± 0.01^{c}
	Oct. 2006	2.88 ± 0.01^{g}	359.71 ± 15.82^{c}	704.14 ± 30.86^{c}	18.64 ± 0.86^{b}
Root	Apr. 2006	38.53 ± 0.90^{a}	191.24 ± 8.07^{f}	104.45 ± 4.09^{c,d}	105.97 ± 3.29^{c,d}
	May 2006	37.74 ± 0.77^{a,b}	312.59 ± 5.47^{a}	169.57 ± 4.30^{b}	103.18 ± 2.71^{d}
	Jun. 2006	36.49 ± 1.40^{a,b,c}	271.51 ± 9.08^{b,c}	122.01 ± 3.49^{c}	93.27 ± 3.59f^{g}
	Jul. 2006	36.84 ± 1.76^{a,b,c}	262.76 ± 7.69^{c}	126.77 ± 1.00^{c}	92.11 ± 2.29^{g}
	Aug. 2006	31.77 ± 1.22^{e}	200.30 ± 4.39^{f}	118.50 ± 3.06^{c}	96.84 ± 4.02^{e,f}
	Sep. 2006	31.87 ± 0.82^{e}	217.61 ± 0.86^{e}	123.72 ± 2.11^{c}	99.41 ± 1.39^{e}
	Oct. 2006	31.12 ± 0.91^{e}	224.40 ± 5.52^{e}	206.86 ± 10.16^{a}	93.65 ± 3.32^{f,g}
	Nov. 2006	32.96 ± 0.51^{d,e}	250.80 ± 5.93^{d}	204.27 ± 4.79^{a}	104.86 ± 0.61^{c,d}
	Dec. 2006	34.56 ± 1.45^{c,d}	197.14 ± 5.35^{f}	63.84 ± 2.29^{e}	104.12 ± 1.36^{c,d}
	Jan. 2007	38.65 ± 1.46^{a}	223.79 ± 1.14^{e}	205.77 ± 8.28^{a}	134.23 ± 3.15^{a}
	Feb. 2007	35.54 ± 1.59^{b,c}	281.30 ± 8.33^{b}	107.83 ± 2.36^{c,d}	108.02 ± 3.45^{c}
	Mar. 2007	34.85 ± 0.21^{c,d}	270.33 ± 9.45^{b,c}	85.19 ± 0.80^{d,e}	126.12 ± 2.95^{b}
Lateral bud	Jul. 2006	19.16 ± 0.78^{a}	1079.25 ± 1.02^{a}	500.16 ± 3.35^{c}	24.03 ± 0.39^{c}
	Aug. 2006	18.92 ± 0.52^{a}	756.42 ± 2.71^{b}	749.28 ± 26.91^{b}	39.07 ± 1.17^{b}
	Sep. 2006	15.45 ± 0.64^{b}	581.34 ± 1.53^{c}	865.04 ± 22.93^{a}	54.83 ± 1.28^{a}

Values represent mean ± standard deviation of three samples. Means with different letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
BHMD = 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol; DHMD = 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene; D.W. = dry weight.

and DHMD from 213.88 μg/g to 373.15 μg/g. Militarine content reached its highest level in October and then gradually decreased from November 2006 to March 2007. Meanwhile, the contents of cinnamic acid, BHMD, and DHMD gradually increased from November 2006 to March 2007.

Comparing the content of each component in mature tubers and young tubers, militarine and DHMD had higher levels in the mature tubers from October 2006 to March 2007. However, the contents of cinnamic acid and BHMD had higher levels in the young tubers.

3.2.2. *Leaf component contents*

Militarine and DHMD levels in the leaves of *B. formosana* were the lowest for all organs (Table 3). Militarine content varied from 2.88 mg/g to 10.40 mg/g, while cinnamic acid varied from 188.92 μg/g to 441.80 μg/g, BHMD from 408.29 μg/g to 1008.08 μg/g, and DHMD from 16.39 μg/g to 20.21 μg/g. During the full-flowering phase (May–July 2006), all components had higher contents than in other months, except for DHMD contents in June 2006 (Table 3). This may be due to the transportation of nutrition from aerial components to underground components. The results indicate that nearly all component contents in leaves were higher during the flowering phase than those in the vegetative phase.

3.2.3. *Root component contents*

Cinnamic acid and BHMD levels in the roots of *B. formosana* were the lowest for all organs (Table 3). Militarine content varied from mg/g 31.12 to 38.65 mg/g, and had its lowest levels from August to October 2006. Cinnamic acid varied from 191.24 μg/g to 312.59 μg/g. BHMD varied from 63.84 μg/g to 206.68 μg/g. DHMD varied from 92.11 μg/g to 134.23 μg/g, and showed no obvious difference from April 2006 to March 2007 (Table 3).

3.2.4. *Bud component contents*

Militarine levels varied from 15.45 mg/g to 19.16 mg/g. Cinnamic acid varied from 581.34 μg/g to 1079.25 μg/g, gradually decreasing from July to September 2006. BHMD varied from 500.16 μg/g to 865.04 μg/g. DHMD varied from 24.03 μg/g to 54.83 μg/g, gradually increasing from July to September 2006 (Table 3).

3.3. *Component contents in B. formosana tubers grown in different culture media*

Table 4 presents the results of the four marker components in *B. formosana* tubers cultured in six different media. *B. formosana* cultured in Medium 2 and Medium 3 had higher biomass (0.28 g and 0.30 g, respectively), while plants cultured in Medium 5 had the lowest biomass (0.13 g). Militarine content varied from 31.05 mg/g to 36.65 mg/g, and those cultivated in Media 1, 3, and 5 had highest content, followed by Medium 4. Cinnamic acid, BHMD, and DHMD contents were highest in Medium 4 and Medium 5, respectively. Of the six culturing media, Medium 4 resulted in *B. formosana* of the highest overall content of the four marker components.

3.4. *Component contents in each part of cultivated B. formosana for different drying methods*

Table 5 showed the marker components in the seven parts of *B. formosana*, including tubers, leaves, roots, and flowers analyzed by HPLC after hot-wind drying or lyophilizing. The results showed that the different drying methods for the *B. formosana* tubers resulted in significant differences.

Higher militarine content (41.83 mg/g) was obtained when the tubers were hot-wind dried at 90°C. Cinnamic acid content was higher (441.21 μg/g) in tubers that were hot-wind dried at 50°C. BHMD content was higher (865.20 μg/g and 833.64 μg/g) in tubers that were hot-wind dried at 50°C and 90°C, respectively. DHMD content was higher (415.83 μg/g and 414.68 μg/g) in tubers that were hot-wind dried at 90°C and lyophilized, respectively. Leaves had higher contents of the four marker components when they were hot-wind dried at 50°C.

Table 4 – Effect of different culture media on four component contents in tubers of *Bletilla formosana*.

Medium	Tuber D.W. (g)	Contents			
		Militarine (mg/g D.W.)	Cinnamic acid (μg/g D.W.)	BHMD (μg/g D.W.)	DHMD (μg/g D.W.)
C1	0.19	36.23 ± 0.41^{a}	362.94 ± 10.49^{c}	$754.18 \pm 16.26^{b,c}$	501.42 ± 11.78^{a}
C2	0.28	32.64 ± 0.59^{b}	466.65 ± 11.49^{b}	746.91 ± 17.25^{c}	462.75 ± 16.28^{b}
C3	0.30	36.65 ± 0.25^{a}	483.17 ± 14.19^{b}	783.00 ± 7.25^{b}	408.36 ± 12.41^{c}
C4	0.18	35.87 ± 0.55^{a}	533.62 ± 18.27^{a}	919.63 ± 28.53^{a}	487.71 ± 4.47^{a}
C5	0.13	36.52 ± 0.75^{a}	465.77 ± 13.64^{b}	521.41 ± 14.41^{d}	356.17 ± 8.44^{d}
C6	0.25	31.05 ± 0.96^{c}	468.20 ± 14.48^{b}	344.87 ± 16.05^{e}	217.93 ± 6.18^{e}

Values represent mean ± standard deviation of three samples. Means with different letters (a, b, c, d, e) in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
C1 – Peat soilage: sandy loam: nacrite: vermiculite = 2:1:1:1.
C2 – Peat soilage: snake wood: nacrite: vermiculite = 1:2:1:1.
C3 – Sandy loam: snake wood: nacrite: vermiculite = 5:2:2:1.
C4 – Snake wood: nacrite: vermiculite = 3:1:1.
C5 – Carbonized rice husk: coconut fiber: sand = 1:1:1.
C6 – Peat soilage: nacrite: vermiculite: sand = 2:1:1:2.
BHMD = 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol; DHMD = 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene; D.W. = dry weight.

Table 5 – Effect of different drying methods and parts on four component contents in cultivated *Bletilla formosana*.

Parts	Drying method	Contents			
		Militarine (mg/g D.W.)	Cinnamic acid (μg/g D.W.)	BHMD (μg/g D.W.)	DHMD (μg/g D.W.)
Flower	Freeze dry	26.06 ± 0.55[a]	1,139.69 ± 15.20[a]	535.55 ± 22.31[a]	28.68 ± 1.20[a]
	50°C	19.06 ± 0.56[b]	329.81 ± 8.33[b]	458.31 ± 16.28[b]	28.60 ± 1.50[a]
	90°C	—	—	—	—
Leaf	Freeze dry	3.67 ± 0.13[a]	442.55 ± 10.86[a]	406.69 ± 14.70[b]	44.46 ± 1.22[b]
	50°C	3.85 ± 0.75[b]	490.21 ± 17.02[b]	493.89 ± 27.10[a]	53.12 ± 1.56[a]
	90°C	—	—	—	—
Stem	Freeze dry	22.62 ± 0.91[b]	847.22 ± 31.02[b]	150.28 ± 1.19[c]	54.62 ± 1.20[a]
	50°C	32.39 ± 0.88[a]	1,138.04 ± 33.11[a]	538.20 ± 23.92[a]	16.38 ± 0.04[c]
	90°C	25.03 ± 1.31[b]	668.57 ± 24.34[c]	212.92 ± 4.18[b]	19.39 ± 0.36[b] b
Tuber	Freeze dry	37.70 ± 0.78[b]	328.04 ± 4.04[c]	728.47 ± 23.24[b]	414.68 ± 13.10[a]
	50°C	39.50 ± 1.25[a]	441.21 ± 6.61[a]	865.20 ± 39.73[a]	355.74 ± 4.55[b]
	90°C	41.83 ± 1.49[a]	340.15 ± 11.17[b]	833.64 ± 24.97[a]	415.83 ± 13.92[a]
Root	Freeze dry	29.90 ± 0.41[a]	135.61 ± 3.12[b]	114.98 ± 2.34[b]	134.01 ± 3.86[c]
	50°C	31.48 ± 1.46[a]	171.30 ± 4.28[a]	185.09 ± 8.48[a]	200.65 ± 6.82[a]
	90°C	32.51 ± 1.45[a]	50.46 ± 0.61[c]	126.83 ± 5.48[b]	154.96 ± 4.27[b]

Values represent mean ± standard deviation of three samples. Means with different letters (a, b, c) in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
BHMD = 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol; DHMD: 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene; D.W. = dry weight; — = no test.

Meanwhile, flowers had higher contents of the four marker components when they were lyophilized.

Aside from militarine, roots had higher contents for all marker components when they were hot-wind dried at 50°C. Aside from DHMD, stems had higher contents for all marker components when they were hot-wind dried at 50°C, and stems had higher DHMD content (54.62 μg/g) when they were lyophilized.

3.5. *Component contents for different processing methods of each part in cultivated B. formosana*

This study also investigated the variations of the four marker components in the tubers of *B. formosana* when the tubers were boiled or steamed, and then hot-wind dried at 50°C. The results are shown in Table 6. Processed *B. formosana* had higher militarine content than the unprocessed one, except in tubers that were boiled for 150 minutes. Cinnamic acid in processed *B. formosana* was lower than in the unprocessed one, except in tubers that were boiled for 10 minutes and 45 minutes. BHMD in processed *B. formosana* was lower than in the unprocessed one, except in tubers that were steamed for 10 minutes and 150 minutes. DHMD in processed *B. formosana* was higher than in the unprocessed one, except in tubers that were steamed for 10 minutes. Overall, the processed *B. formosana* tubers could increase the marker components, and the most effective processing method was steam processing for 150 minutes.

3.6. *Component contents in commercial crude Rhizoma Bletillae drugs*

The contents of the four marker components varied significantly in 10 kinds of commercial crude *Rhizoma Bletillae* drugs from different sources, as shown in Table 7. The commercial crude drugs were quite different from each other and the

Table 6 – Effect of different processing methods on four component contents in tuber of cultivated *Bletilla formosana*.

Processing methods	Contents			
	Militarine (mg/g D.W.)	Cinnamic acid (μg/g D.W.)	BHMD (μg/g D.W.)	DHMD (μg/g D.W.)
0 min–50°C	39.50 ± 1.25[c]	441.21 ± 6.61[c]	865.20 ± 39.73[bc]	355.74 ± 4.55[d]
S-10 min–50°C	41.98 ± 1.68[b]	362.60 ± 6.81[d]	900.77 ± 31.90[ab]	283.70 ± 10.09[e]
B-10 min–50°C	40.69 ± 1.52[bc]	476.66 ± 25.09[b]	636.29 ± 17.77[d]	386.22 ± 9.65[bc]
S-45 min–50°C	43.43 ± 1.55[a]	336.45 ± 9.54[e]	846.13 ± 29.89[c]	527.43 ± 8.20[a]
B-45 min–50°C	40.96 ± 1.48[b]	510.14 ± 9.91[a]	667.69 ± 24.43[d]	371.34 ± 2.85[c,d]
S-150 min–50°C	43.70 ± 1.77[a]	362.82 ± 6.69[d]	925.15 ± 15.83[a]	394.22 ± 16.62[b]
B-150 min–50°C	34.65 ± 0.94[d]	335.14 ± 8.51[e]	466.49 ± 23.74[e]	374.03 ± 8.46[c,d]

Values represent mean ± standard deviation of three samples. Means with different letters (a, b, c, d, e) in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
B = boiling treatment; BHMD = 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol; DHMD = 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene; D.W. = dry weight; S = steam treatment.

Table 7 – Contents of each marker component from various sources of commercial Rhizoma Bletillae in Taiwan.

Commercial crude drugs	Contents			
	Militarine (mg/g D.W.)	Cinnamic acid (μg/g D.W.)	BHMD (μg/g D.W.)	DHMD (μg/g D.W.)
A	15.58 ± 0.68^{b}	1492.96 ± 33.50^{a}	110.82 ± 3.34^{c}	39.91 ± 2.10^{c}
B	4.27 ± 0.09^{c}	198.54 ± 6.85^{d}	274.67 ± 9.87^{a}	169.12 ± 4.86^{b}
C	15.63 ± 0.40^{b}	282.32 ± 5.42^{c}	256.44 ± 4.04^{b}	222.52 ± 2.16^{a}
D	19.04 ± 0.63^{a}	491.02 ± 13.65^{b}	121.10 ± 1.48^{c}	37.47 ± 0.73^{c}
E	22.76 ± 0.71^{b}	343.22 ± 11.74^{c,d}	1065.48 ± 18.20^{a}	497.92 ± 8.72^{b}
F	25.18 ± 0.67^{a}	295.19 ± 8.57^{c,d}	647.92 ± 3.09^{d}	618.36 ± 13.35^{a}
G	21.36 ± 0.23^{c}	241.44 ± 0.79^{d}	717.94 ± 18.90^{c}	484.07 ± 3.19^{b,c}
H	16.51 ± 0.69^{f}	392.00 ± 22.64^{b,c,d}	546.53 ± 26.13^{e}	463.30 ± 33.39^{b,c}
I	25.94 ± 0.29^{a}	574.46 ± 17.34^{a}	824.17 ± 1.08^{b}	449.34 ± 22.89^{c}
J	17.83 ± 0.57^{e}	833.56 ± 51.16^{a}	663.26 ± 41.67^{d}	355.62 ± 21.72^{d}

Values represent mean ± standard deviation of three samples. Means with different letters (a, b, c, d, e, f) in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
Commercial crude drugs A–J were purchased from different local Chinese herbal medicine markets.
BHMD = 1,8-bi(4-hydroxybenzyl)-4-methoxyphenanthrene-2,7-diol; DHMD = 4,7-dihydroxy-1-*p*-hydroxybenzyl-2-methoxy-9,10-dihydrophenanthrene; D.W. = dry weight.

cultivated *B. formosana*. Among the 10 commercial crude drugs, militarine was the major *B. formosana* component, ranging from 4.27 mg/g to 25.94 mg/g (6.07 fold). The content of cinnamic acid ranged from 198.54 μg/g to 1492.96 μg/g (7.52 fold). The content of BHMD ranged from 110.82 μg/g to 1065.48 μg/g (9.61 fold). The content of DHMD ranged from 37.47 μg/g to 618.36 μg/g (16.50 fold). The results might be due to the difference in the botanical origin of the source, planting region, fertilizer management, cultivation method, harvest season, storage environment, and inconsistencies in the refining process of each commercial crude Rhizoma Bletillae drug. The results showed that the quality as well as component contents of *Rhizoma Bletillae* materials commercially available in Taiwan varied significantly.

4. Discussion

Plant breeding, agricultural operations, and gene control are all methods for optimizing plant nutrition and the bioactivity of chemical compositions in plants [29–31]. In agricultural strategy, collocating treatments after harvesting, processing, and storing can prevent the loss of compounds [32]. The components of Chinese herbal medicines are complicated and require processing to reduce toxicity and prevent side effects. Masaki et al (1998) revealed that isoflavones and astragaloside in *Astragali membranaceus* could be affected by different hot-wind drying methods [33].

The results of monthly component changes in *B. formosana* (Hayata) Schltr. indicated that militarine was the most significant of the four components elicited by HPLC from different plant organs with tubers and roots containing the highest militarine content; the levels in the leaves were significantly lower.

Roots had significantly lower cinnamic acid and BHMD contents. The results also showed that leaves had a barely detectable level of DHMD. Monthly variations were also noted in leaves and lateral buds, and variations within months were also found across different components and plant organs.

With regard to variations at the different growing stages, component levels in tubers in the flowering to fruiting stages (April to September) were found significantly lower than those during the nutrition growth stages (October to the following March). Component levels varied in leaves, roots, and lateral buds during the reproductive and nutrition growth stages, but these variations were not significant. When comparing the levels of each component in young and mature tubers harvested in the same month, it was found that militarine and DHMD contents were significantly higher in mature tubers that those in young tubers, indicating variances of the component levels in *B. formosana* with the plant's growth stages.

Processing for Rhizoma Bletillae materials usually involve impurities removal followed by hot-wind direct drying, boiling, or steaming for different time periods [20,23]. Previous reports indicate that Rhizoma Bletillae contains significant amounts of volatile compounds and mucus. Thus, hot processing of Rhizoma Bletillae is generally avoided to prevent damage to essential oils and mucus that would compromise its clinical effects [34]. According to this study, most of the compounds in *B. formosana* were not significantly decreased when they were hot processed, except for 150 minutes of steam processing. Except for the leaves, no significant increase or decrease was found in the component contents of *B. formosana* when every part was lyophilized. Generally, short-duration steam processing of *B. formosana* could slightly raise the contents of the four marker components.

The culturing media for Orchidaceae must be porous, aired, and sterile and feature good quality drainage and water retention. The common media may be made by mixing sphagnum, coconut fiber, peat, carbonized rice husk, humus, and river sand according to a certain proportion for culturing. Seedlings of *Anoectochilus formosanus* Hayata, an Orchidaceae plant like *B. formosana*, can grow in suitable media including peat soil: coconut fibers: vermiculite = 2:1:2, vermiculite: humus = 1:1, vermiculite: tree fern: humus: carbonized rice husk = 5:1:3:1 [35,36]. In this study, Medium 2 (peat soil: fine tree fern: vermiculite: pearl stone = 1:2:1:1) and Medium 3

(sandy loam: fine tree fern: vermiculite: pearl stone = 5:2:1:1) produced the most tubers. Medium 4 (fine tree fern: vermiculite: pearl stone = 3:1:1) produced the least tubers. This indicates that the culturing medium did affect the productivity and bioactivity of *B. formosana*. The productivity, quality, and quantity of each component must be considered.

The four marker components all varied significantly from each of the commercial crude Rhizoma Bletillae drugs. The results revealed that the quality of the drug materials was inconsistent and, due to the lack of a quality standard for commercial crude Rhizoma Bletillae drugs, the species used in drugs could not be identified. The properties of the drug materials were influenced by many factors including place of origin, culturing conditions, processing procedures, and storage conditions. The appearance of the Rhizoma Bletillae materials was inconsistent. Previous reports have compared many alternatives of Rhizoma Bletillae and while construction in tissues was largely consistent, slight differences in appearance and size make it difficult to identify the species of Rhizoma Bletillae materials based on appearance and size alone [37].

Comparing the components in the vegetative stage (October to March the following year) between *B. formosana* and Rhizoma Bletillae materials showed that, given the same extract concentrations, every component in the commercial crude Rhizoma Bletillae drugs was lower than that in the cultured *B. formosana*. Comparing the inner structure of *B. formosana*, Orchid Island *B. formosana* and commercial crude Rhizoma Bletillae drugs showed that wild *B. formosana* had roughly the same structure as the commercial crude drugs, except for some small differences in the epidermal cells, layers of fiber cells, starch grains, and needle crystals [1,38]. Acute toxicity tests showed the same LD_{50} for methanol extracts of *B. formosana* tubers and commercial crude Rhizoma Bletillae drugs, although *B. formosana* tubers had the effect of shortening bleeding and thromboplastic time, making endemic species *B. formosana* tubers a highly feasible alternative to Rhizoma Bletillae [1,38]. However, several problems including the plant's thinness, small size, and lack of cultivation origin must first be overcome. In addition, variations in quality, quantity, and characteristics in Rhizoma Bletillae planted under identical conditions must be further evaluated to select species that contain the best component genotypes for propagation.

5. Conclusion

In summary, culturing methods, harvest time, and the cultivation process used for the crop had important effects on production and quality. The best culture conditions for *B. formosana* were combinations of peat soil: snake wood: nacrite: vermiculite = 1:2:1:1) or sandy loam: snake wood: nacrite: vermiculite = 5:2:2:1. The monthly variations in components showed that tubers had the highest contents of militarine. In the vegetative phase, almost all component contents were higher than those in the flowering phase. September to October is proposed for harvesting time. The rhizomes, leaves, and flowers also had high component contents. All of them are valuable for development and use in a diverse range of applications.

The recommended processing treatment for fresh Taiwan *B. formosana* tubers is steam heating for a short time (10 minutes or 45 minutes) and then drying at 50°C. The results of the four-component analysis showed a higher value than imported commercial crude drugs, indicative of the developmental potential of Taiwan *B. formosana*.

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