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Quantitative Analysis of the Flavonoid Content in the Leaves of *Boehmeria nivea* and Related Commercial Products

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Abstract – Content analysis of flavonoids (epicatechin, epicatechin gallate, and rutin) present in the leaves of *Boehmeria nivea* (originating from Geumsan-myeon, Biin-myeon, Hansan-myeon, and Baeksu-eup) and their commercial products (ramie tteok, ramie songpyeon, ramie bory-tteok, and ramie tea) was conducted by HPLC. The content of epicatechin, epicatechin gallate, and rutin was highest in the leaves of *B. nivea* from Geumsan-myeon (0.138 mg/g), Baeksu-eup (1.654 mg/g) and Geumsan-myeon (12.205 mg/g), respectively. With respect to commercial products, the content of epicatechin and epicatechin gallate was highest in ramie tea, with concentrations of 1.879 and 1.090 mg/g, respectively. Given these flavonoid concentrations, *B. nivea* leaf extracts have the potential to be used as additives in natural medicinal products, health supplements, and beverages.
Keywords – *Boehmeria nivea*, Epicatechin, Epicatechin gallate, Rutin, HPLC

Introduction

Boehmeria nivea, which belongs to the family Urticaceae, is distributed throughout Asia including the Philippines, India, China, Korea, and Thailand. It is a herbaceous perennial plant with broad (6 - 12 cm) and long (7 - 15 cm) heart-shaped leaves that appear silvery due to the dense small hairs on the underside.[1] *B. nivea* is commonly referred to as China grass, white ramie, green ramie, and rhea, and has been used as far back as 5,000 BC in Egyptian mummy cloths. Furthermore, the leaves of this plant have been used as sources of tea, cloths, and tteok (traditional Korean rice cakes).[2,3]

B. nivea leaves are also commonly used in folk remedies as a diuretic and anti-pyretic, and has are thought to possess hepatoprotective, anti-oxidant, and anti-inflammatory properties.[4] Previous studies have indicated that these leaves contain kiwiionoside, rutin, uracil, 3-hydroxy-4-methoxy-benzoic acid, cholesterol, α-amyrin, nonacosanol, emodin, emodin-8-*O*-β-glucoside, physcion, polydatin, catechin, potassium nitrate, β-sitosterol, epicatechin, and epicatechin gallate.[5-7] Furthermore, the leaves of *B. nivea* contain a large amount of phenolic compounds, which were able to inhibit angiotensin I-converting enzyme.[6] In the roots of a related species, *B. tricuspis* contains several epicatechin dimers such as (–)-epiafzelechin-(–)-epicatechin-4,8(or 6)-dimer, and (–)-epicatechin-(–)-epicatechin-4,8-(or 6)-dimer.[8] Among them, epicatechin, epicatechin gallate, and rutin are polyphenolic compounds known as flavonoids, which are known for their anti-oxidant, anti-inflammatory, anti-tumorigenic, anti-bacterial, anti-viral, and anti-allergenic properties.[9-16]

This study utilized HPLC to analyze the concentrations of epicatechin, epicatechin gallate, and rutin in the leaves of *B. nivea* originating from four different locations (Geumsan-myeon, Biin-myeon, Hansan-myeon, and Baeksu-eup), and in commercial products (ramie tteok, ramie songpyeon, ramie bory-tteok, and ramie tea).

Experimental

Plant material – The leaves of *B. nivea* were cultivated and collected by Yeonggwang Agricultural Technology & Extension Center, Korea. The collection areas for *B. nivea* were Geumsan-myeon, Biin-myeon, Hansan-myeon, and Baeksu-eup, Korea.

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Commercial products – Commercial products (tteok, songpyeon, bory-tteok, and ramie tea) containing *B. nivea* leaves were purchased from Yeonggwang-gun, Korea. Among them, tteok, songpyeon, and bory-tteok were made up of *B. nivea* leaves and wheat flour. Ramie tea was made up of dried *B. nivea* leaves.

Instruments and reagents – Methanol (MeOH), *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) were purchased from SamChun Pure Chemical Co. (Pyeongtaek, Korea). Epicatechin (**1**), epicatechin gallate (**2**), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Electron ionization-mass spectrometry (EI-MS) was conducted with a Jeol JMS-600W mass spectrometer (Tokyo, Japan). NMR spectra were recorded with a Bruker AVANCE 500 NMR spectrometer (Bremen, Germany) using TMS as the internal standard. Chemical shifts are reported in parts per million (δ), and coupling constants (*J*) are expressed in Hertz. An Eyela rotary evaporator system (Tokyo, Japan) under reflux *in vacuo* was used for evaporation. Thin-layer chromatography was conducted with Kiesel-gel 60 F_{254} plates (silica gel, 0.25 mm layer thickness; Art. 5715, Merck Co., Darmstadt, Germany), and compounds were visualized by spraying with 10% H_2SO_4 in MeOH, followed by heating to 100 ºC. Sephadex LH-20 (20 - 100 μm) was purchase from Sigma-Aldrich. HPLC chromatograms of flavonoids were recorded with a Waters Breeze system (Massachusetts, USA) equipped with a Waters 1525 binary HPLC pump and a 2489 system UV/VIS detector. Water and MeOH used in this study were of HPLC grade, and all other reagents were of analytical grade.

Isolation and identification of rutin (3) – *B. nivea* leaves (2 kg) were dried finely powdered and immersed in MeOH for 3 h (4 L × 8) under reflux at 65 ºC-75 ºC. The solvent was evaporated *in vacuo* to produce the MeOH extract (294.6 g). This extract was then suspended in distilled water and partitioned with *n*-hexane (106.9 g), CH_2Cl_2 (4.1 g), EtOAc (2.4 g), and *n*-BuOH (23.8 g), successively. The EtOAc fraction (2.4 g) was separated on an LH-20 column (φ 2.0 × 50 cm) using MeOH/water (gradient: 1 : 3 → 1 : 0, v/v). Nine fractions were obtained by combining those with similar R_f on TLC behavior (1 → 9). Among them, compound **3** was isolated from fraction 7 by recrystallization with MeOH (Fig. 1).

Compound **3**: FAB-MS *m/z*: 611 $[M + H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 1.00 (3 H, d, *J* = 6.0 Hz, Rha CH_3), 3.07-3.69 (12 H, m, *J* = 2 Hz, sugar H), 4.38 (1 H, s, Rha H-1), 5.34 (1H, d, *J* = 7.5 Hz, Glc H-1), 6.19 (1 H, d, *J* = 1.2 Hz, H-6), 6.38 (1 H, d, *J* = 1.2 Hz, H-8), 6.84 (1 H, d, *J* = 8.5 Hz, H-5'), 7.54 (1 H, d, *J* = 2.5 Hz, H-2'), 7.55 (1 H, dd, *J* = 2.5, 8.5 Hz, H-6'), 12.58 (1 H, s, 5-OH); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 156.5 (C-2), 133.2 (C-3), 177.3 (C-4), 161.1 (C-5), 98.6 (C-6), 164.1 (C-7), 93.5 (C-8), 156.5 (C-9), 103.9 (C-10), 121.5 (C-1'), 115.2 (C-2'), 133.2 (C-3'), 148.4 (C-4'), 116.2 (C-5'), 121.1 (C-6'), 101.1 (Glc C-1), 74.0 (Glc C-2), 76.4 (Glc C-3), 70.5 (Glc C-4), 75.9 (Glc C-5), 67.0 (Glc-C6), 100.7 (Rha C-1), 70.3 (Rha C-2), 70.0 (Rha C-3), 72.0 (Rha C-4), 68.2 (Rha C-5), 17.7 (Rha C-6).

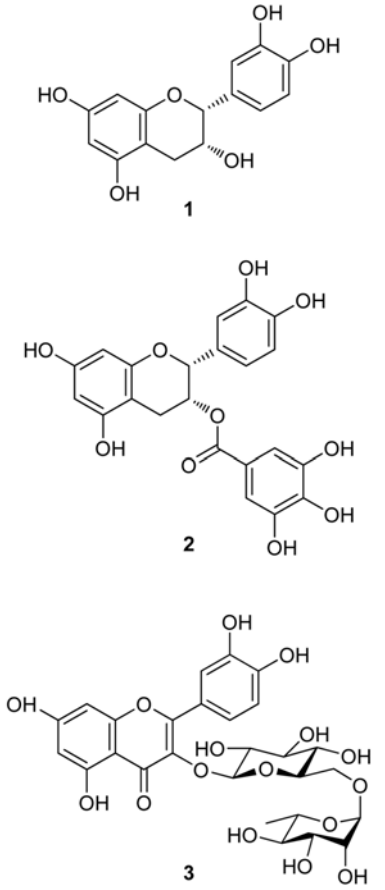


Fig. 1. Structures of compounds **1** - **3**.

Sample preparation – To determine the content of **1**, **2**, and **3** in *B. nivea* leaves and in the commercial products (mentioned above), 50 g of either leaves or commercial products were dried finely powdered and immersed in 50% MeOH (3 × 100 mL) by reflux and evaporation *in vacuo*. The residue was dissolved in 1 mL of MeOH and filtered through a 0.45 μm filter. The resulting solution was used for HPLC analysis.

HPLC conditions- HPLC separation of **1**, **2**, and **3** for qualitative and quantitative analysis was performed using a reverse phase system. A Waters Spherisorb® ODS2 (4.6

× 250 mm, 5 μm) column was used with a mobile phase consisting of water (0.2% acetic acid) and MeOH. The elution program was a gradient solvent system of water and MeOH (80 : 20 to 50 : 50 for 30 min). UV detection was conducted at 280 nm. The injection volume was 10 μL and the flow rate was 0.5 mL/min. All injections were performed in triplicate.

Calibration curve- Stock solutions (1 mg/mL) of **1**, **2**, and **3** were prepared in MeOH, successively reducing the solution content to 50% to create different concentrations. The amount of **1**, **2**, and **3** from *B. nivea* leaves and commercial products were determined from the corresponding calibration curve. The calibration functions of **1**, **2**, and **3** were calculated using the peak area (Y), concentration (X, μg/10 μL), and mean values (n = 5) ± standard deviation.

Results and Discussion

In this study, *B. nivea* leaves were extracted with MeOH, and partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH. The *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH fractions were applied sequentially to open column chromatography over silica gel to yield compound **3**. Compound **3** was obtained as a white powder from the EtOAc fraction. The typical flavonoid signals of **3** were observed in the ^{1}H- and ^{13}C-NMR spectra. The ^{1}H-NMR spectra revealed that it had an ABX system (H-2', -5', and -6'), as demonstrated by the coupling constant signal at δ 7.54 (d, H-2'), 7.55 (dd, H-6'), and 6.84 (d, H-5') in the B-ring structure. Compound **3** is a flavonoid glycoside. The ^{13}C-NMR of **3** can be correlated with the flavonoid moiety of quercetin with rutinose, with the latter comprising glucose and rhamnose units. The FAB-MS spectrum of **3** showed a quasimolecular ion peak at *m/z* 611, corresponding to a molecular formula of $C_{27}H_{30}O_{16}$. Accordingly, the structure of **3** was elucidated as rutin (5,7,3',4'-tetrahydroxyflavone-3-rutinose).[14]

Simultaneous determination of **1**, **2**, and **3** in the leaves of *B. nivea* was conducted by HPLC. The HPLC separation of analytes was conducted using a reverse phase system with a mobile phase consisting of water and MeOH (80 : 20 to 50 : 50 for 30 min) and all identified flavonoids were detected at 280 nm (Table 1). Using an optimized analytical method, compounds **1**, **2**, and **3** in the leaves of *B. nivea* were determined simultaneously (Fig. 2). The concentrations of **1**, **2**, and **3** were detected in *B. nivea* leaves from Geumsan-myeon (0.138, 0.233, and 12.205 mg/g, respectively), Biin-myeon (0.025, 1.227, and 5.510 mg/g, respectively), Hansan-myeon (0.022, 1.230, and 4.969 mg/g, respectively), and Baeksu-eup (0.025, 1.654, and 11.638 mg/g, respectively) (Table 2). The content of **3** was consistently higher than **1** and **2** in *B. nivea* leaves originating from the four areas investigated. Additionally, the concentrations of **1** and **2** were detected in the commercial products ramie tteok (0.0100 and 0.0423 mg/g, respectively), ramie songpyeon (0.055 and 0.039 mg/g, respectively), ramie bory-tteok (0.655 and 0.842 mg/g, respectively), and ramie tea (1.879 and 1.090 mg/g, respectively) (Table 3).

From our analyses, the concentrations of **1** and **3** were highest in the leaves of *B. nivea* from Geumsan-myeon

Table 1. Calibration curves for compounds **1** - **3**.

Compound	t_R	Calibration equation[a]	Correlation factor, r^{2b}
1	23.14	Y = 8.7028X + 25.229	0.9992
2	27.29	Y = 21.129X – 89.66	0.9993
3	24.716	Y = 18239X + 85450	0.9996

[a]Y = peak area, X = concentration of standard (μg/ml).
[b]r^2 = correlation coefficient for three data points in the calibration curve (n = 5).

Table 2. Concentrations of compounds **1** - **3** in the MeOH extracts of the leaves of *B. nivea*.

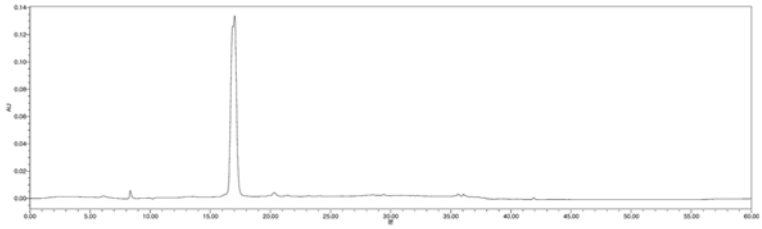
Collection Area	Content (mg/g)			
	1	**2**	**3**	Total
Geumsan-myeon	0.138 ± 0.047	0.233 ± 0.011	12.205 ± 0.355	12.438 ± 0.413
Biin-myeon	0.025 ± 0.024	1.227 ± 0.010	5.510 ± 0.321	6.762 ± 0.355
Hansan-myeon	0.022 ± 0.020	1.230 ± 0.082	4.969 ± 0.350	6.221 ± 0.452
Baeksu-eup	0.025 ± 0.018	1.654 ± 0.181	11.638 ± 0.376	13.317 ± 0.575

Data are represented as the mean ± S.D. (n = 4) in mg/g of the dried samples.

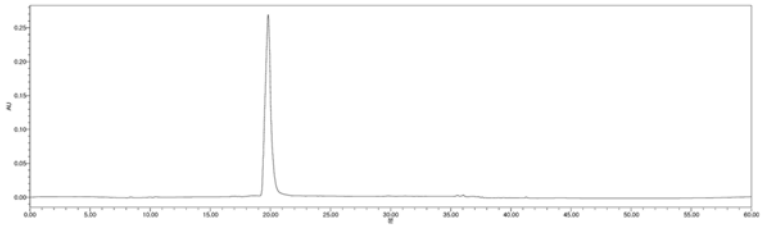
Table 3. Concentrations of **1** and **2** in the MeOH extracts of commercial products from the leaves of *B. nivea*.

Commercial Product	Content (mg/g)		
	1	**2**	Total
Ramie Tteok	0.010 ± 0.001	0.042 ± 0.002	0.052 ± 0.001
Ramie Songpyeon	0.055 ± 0.001	0.039 ± 0.001	0.094 ± 0.001
Ramie Bory-tteok	0.655 ± 0.082	0.842 ± 0.004	1.497 ± 0.078
Ramie Tea	1.879 ± 0.254	1.090 ± 0.017	2.969 ± 0.271

Data are represented as the mean ± S.D. (n = 3) in mg/g of the dried samples.
Tteok, Songpyeon: half-moon-shaped rice cake, and Bory-tteok: traditional Korean rice cakes
Tea: dried leaf of tea



(A)



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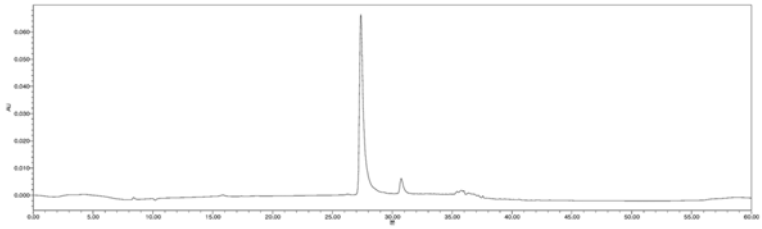

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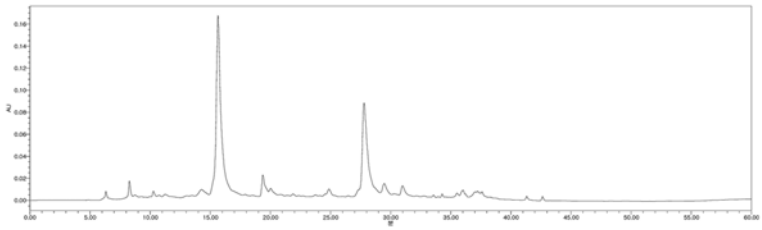

(D)

Fig. 2. HPLC chromatograms of **1** (A), **2** (B), and **3** (C) and the MeOH extract of *B. nivea* from Hansan-myeon (D)

(0.138 and 12.205 mg/g, respectively), and the concentration of **2** was highest in the leaves from Baeksu-eup (1.654 mg/g). The concentration of **1** in *B. nivea* leaves was similar when taken from either Biin-myeon, Hansan-myeon, or Baeksu-eup. The concentration of **2** in *B. nivea* leaves originating from Biin-myeon was similar to that seen in leaves from Hansan-myeon, however the leaves from Geumsan-myeon contained a significantly smaller amount of **2** than that found in the other locations. Lastly, the concentration of **3** in the leaves of *B. nivea* from Geumsan-myeon and Baeksu-eup was similar, and likewise, the concentration of **3** in leaves from Biin-myeon and Hansan-myeon was similar. In the total content of *B. nivea* from Geumsan-myeon, Biin-myeon, Hansan-myeon, and Baeksu-eup, the content of Baeksu-eup was higher than that of Geumsan-myeon, Biin-myeon, and Hansan-myeon because of the different geological features. Baeksu-eup is similar in climate and precipitation. Also, in the total content of commercial products, ramie tteok, ramie songpyeon, ramie bory-tteok, and ramie tea in *B. nivea*, the content of ramie tea was higher than that of ramie tteok, ramie songpyeon, and ramie bory-tteok because of production process. Therefore, ingesting ramie tea is likely one of the best ways to obtain large amounts of **1** and **2**. This may be important medicinally as increased consumption of green tea is associated with lower risks of obesity and obesity-related disorders, including inflammation, cardiovascular disease, and nonalcoholic fatty liver disease.[17]

These results demonstrate that *B. nivea* contains large amounts of **1**, **2**, and **3**, and has the potential to be a new additive for natural medicinal products, health supplements, and beverages.

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