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Phytochemical Identification from *Boehmeria nivea* Leaves and Analysis of (–)-Loliolide by HPLC

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Abstract – Phytochemicals were isolated from leaves of the fiber crop, ramie (*Boehmeria nivea*, Bn), using open column chromatography and medium pressure liquid chromatography. Their structures were identified as β-sitosterol, (–)-loliolide, rutin, and pyrimidinedione by MS, ^{1}H-, and ^{13}C-NMR spectroscopic analysis. Among them, (–)-loliolide was isolated for the first time from *B. nivea*. A content analysis of (–)-loliolide in *B. nivea* collected from different regions and harvest times was conducted by HPLC. The highest content of (–)-loliolide was found in Bn-23 harvested in September. These results will be helpful to use the plant which harvest in September as a high content phytochemical additive in food, health supplements, and medicinal products.
Keywords – *Boehmeria nivea*, Urticaceae, Phytochemical, Identification, Loliolide, NMR

Introduction

Ramie, *Boehmeria nivea* (Bn), is a perennial herbaceous plant belonging to the family Urticaceae. *B. nivea* is often referred to as China grass, white ramie, green ramie, or rhea, and was used in mummy cloths in Egypt during the period 5,000 - 3,300 BC. Moreover, ramie is mainly consisted of cellulose with some impurities called gum such as hemicellulose, pectin, and wax. *B. nivea* is grown as a fiber crop in many countries including China, Japan, Thailand, Philippines, India, and South Korea.[1,2] It has been used as a fabric for centuries because of its excellent fiber.[3] The fibers obtained from the outer part of the stem are one of the strongest and longest fine fabric fibers.[4] Other positive attributes of this fiber are resistance to bacteria, insect attack, and mildew, and its strength increases slightly when wet.[5]

The roots are used in traditional Chinese herbal medication for the treatment of common cold, edema, fever, fetal irritability, urinary tract infections, nephritis, and abortion risk.[6] It has been reported that root extracts exhibited hepatoprotective activities against CCl_4-induced liver injuries and antioxidant effects on $FeCl_2$-ascorbate-induced lipid peroxidation in rat liver homogenate.[7]

B. nivea has been used to prepare tea and in the production of fabric. In Korea, research on the use of *B. nivea* in food, such as various traditional Korean rice cakes, has been carried out.[1,2] In particular, the green leaves of the plant are rich in nutritional factors such as minerals, proteins, vitamins, and various bioactive materials.[8]

Previous studies on the plants of the *Boehmeria* genus led to the isolation of different compounds, such as alkaloids, lignans, flavones, terpenoids, and glycosides.[9-10] *Boehmeria* also comprise behenic acid, ursolic acid, β-sitosterol, cholesterol, kiwiionoside, rutin, uracil, quercetin, α-amyrin, nonacosanol, emodin, emodin-8-*O*-β-glucoside, physcion, polydatin, catechin, epicatechin, and epicatechin gallate.[11,12]

Nevertheless, there has been insufficient research on the bioactive compounds from *B. nivea*. The objective of this study was to determine new bioactive compounds from *B. nivea*. Therefore, our investigation was designed to systematically isolate, identify, and analyze the valuable phytochemicals from *B. nivea*.

Experimental

Plant materials – *B. nivea* from different regions and harvest time (June, July, August, and September) was supplied by the Yeong-Gwang Agricultural Technology

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Table 1. Collection areas for Bn

Sample	Collection area
Bn-02	Seobang variety in Hansan, Seocheon
Bn-07	Taiwan variety in Duwon, Goheung
Bn-10	Ramie in Mangun, Muan
Bn-23	Local variety in Hakgyo, Hampyeong
Bn-38	Local variety in Seocheon-4
Bn-41	White Peel variety in Biin, Seocheon-2
Bn-67	Improved variety in Gwangju, Taiwan variety in Goheung
Bn-76	Natural cross of White Peel variety
Bn-90	Local variety in Baeksu, Yeonggwang

Center, Korea. The Bn collection areas are shown in Table 1.

Apparatus and chemicals – EI-MS was measured with a JEOL JMS-600W (Japan) mass spectrometer and FAB-MS was measured with a JEOL JMS-AX505WA (Japan) mass spectrometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance 300 and 500 NMR spectrometer (Rheinstetten, Germany) using tetramethylsilane as an internal standard. Evaporation was conducted using an Eyela rotary evaporator system (Tokyo, Japan) under reflux *in vacuo*. TLC was performed with precoated silica gel 60 F_{254} (Art. 5715, Merck Co., Germany) plates (20 × 20 cm, 0.25 mm layer thickness). The compounds on the TLC plate were visualized by spraying with 10% sulfuric acid in methanol followed by heating at 100 °C to detect spot color. Medium pressure liquid chromatography (MPLC) separation was carried out on Biotage (Uppsala, Sweden). Open column chromatography was performed on silica gel (200 - 400 Mesh ASTM, Merck Co., Darmstädt, Germany). Solvents such as methanol (MeOH), *n*-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and butanol (*n*-BuOH) (SamChun Pure Chemical Co., Pyeongtaek, Korea) were used as elution solution in open chromatography. HPLC chromatograms were recorded with a Waters Breeze system (Massachusetts, USA) equipped with a Waters 1525 binary HPLC pump and 2489 system UV/VIS detector. Water and MeOH used in this research were of HPLC grade.

Extraction, fractionation, and isolation – Dried and powdered Bn-90 (2 kg) was extracted with MeOH (8 L × 10) under reflux at 65 - 75 °C. The filtrate was concentrated until dry *in vacuo* to afford a dark green material of MeOH extract (294.6 g). The MeOH extract (280.5 g) was suspended in H_2O, and then partitioned successively using *n*-hexane (106.9 g), CH_2Cl_2 (4.1 g), EtOAc (2.4 g), and *n*-BuOH (23.8 g). A portion of the *n*-hexane fraction (95.5 g) was chromatographed on a silica gel column (6 × 80 cm, No.7734) using a stepwise gradient of *n*-hexane-EtOAc (1:0 → 0:1), EtOAc-MeOH (1:0 → 5:5) solvent systems to give 11 sub-fractions. Compound **1** was recrystallized from sub-fraction 4 under $CHCl_3$-MeOH. Sub-fraction 6 was rechromatographed to MPLC eluting with a gradient of *n*-hexane-EtOAc (1:0 → 0:1) solvent systems to afford 10 sub-fractions. Sub-fraction 8 was recrystallized under $CHCl_3$-MeOH to give compound **2**. A portion of the EtOAc fraction (1.4 g) was subjected to MPLC eluting with a gradient of $CHCl_3$-MeOH (1:0 → 5:5) to give 9 sub-fractions. Sub-fraction 7 was recrystallized under $CHCl_3$-MeOH to give compound **3**. A portion of the *n*-BuOH fraction (20 g) was subjected by MPLC on silica gel eluting with a gradient of $CHCl_3$-MeOH (1:0 → 5:5) to give 10 sub-fractions. Sub-fraction 2 was recrystallized under $CHCl_3$-MeOH to give compound **4**.

Compound **1** – White amorphous powder. EI-MS: *m/z* 414 (100) $[M]^+$, 396 (51.1), 369 (11.3), 329 (22.1), 300 (22.5), 271 (21.6), 255 (41.6), 213 (20.1), 145 (20.3), 135 (10.6), 121 (11.2), 105 (14.0), 97 (11.6), 81 (18.4), 69 (13.7). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 0.68 (H-28), 0.81 (H-27), 0.82 (H-26), 0.83 (H-24), 0.94 (H-19), 1.02 (H-29), 3.52 (H-3), 5.38 (H-5). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 12.1 (C-29), 18.9 (C-21), 19.0 (C-26), 19.1 (C-19), 19.6 (C-27), 21.2 (C-11), 23.2 (C-28), 24.5 (C-15), 26.2 (C-23), 28.4 (C-16), 29.1 (C-25), 31.8 (C-2), 32.0 (C-7), 32.1 (C-8), 34.1 (C-22), 36.3 (C-20), 36.6 (C-10), 36.7 (C-10), 37.4 (C-1), 39.8 (C-12), 42.3 (C-4), 42.4 (C-13), 46.0 (C-24), 50.3 (C-9), 56.1 (C-17), 57.0 (C-14), 71.9 (C-3), 121.9 (C-6), 141.0 (C-5).

Compound **2** – Syrup. EI-MS: *m/z* 196 (16.8) $[M]^+$, 178 (55.4), 163 (23.0), 153 (18.4), 140 (41.6), 123 (26.7), 111 (100), 95 (36.0), 85 (32.4), 81 (24.3), 69 (23.6), 57 (40.8). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.31 (H-9), 1.42 (H-10), 1.65 (H-2α), 1.71 (H-4α), 1.84 (H-11), 1.92 (H-2β), 2.45 (H-4β), 4.25 (H-3, m), 5.74 (H-7, s). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 22.6 (C-9), 24.9 (C-11), 30.6 (C-10), 35.9 (C-1), 45.5 (C-4), 47.2 (C-2), 66.6 (C-3), 86.8 (C-5), 111.7 (C-7), 172.0 (C-8), 182.7 (C-6).

Compound **3** – Yellow amorphous powder. FAB-MS: *m/z* 611 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 6.17 (H-6), 6.36 (H-8), 6.83 (H-5'), 7.52 (H-2'), 7.54 (H-6'), 5.33 (glc-1), 3.22 (glc-2), 3.20 (glc-3), 3.36 (glc-4), 3.05 (glc-5), 3.69 (glc-6), 4.38 (rha-1), 3.08 (rha-2), 3.21 (rha-3), 3.06 (rha-4), 3.19 (rha-5), 0.99 (rha-6), 12.5 (5-OH). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 93.6 (C-8), 98.7 (C-6), 103.8 (C-10), 115.1 (C-5'), 116.1 (C-2'), 121.0 (C-1'), 121.5 (C-6'), 133.2 (C-3), 144.7 (C-3'), 148.4 (C-4'), 156.4 (C-2), 156.5 (C-9), 161.1 (C-5), 164.4 (C-7), 177.2 (C-4),

101.2 (glc-1), 75.4 (glc-2), 76.4 (glc-3), 69.9 (glc-4), 74.0 (glc-5), 66.9 (glc-6), 100.7 (rha-1), 70.3 (rha-2), 71.8 (rha-3), 70.5 (rha-4), 68.2 (rha-5), 17.6 (rha-6).

Compound **4** – White amorphous powder. EI-MS: *m/z* 112 (100) $[M]^+$, 83 (2.0), 73 (4.7), 69 (44.0), 57 (4.3). ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 5.43 (H-6), 7.35 (H-5), 10.93 (H-NH), 10.94 (H-NH). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 100.1 (C-5), 142.1 (C-6), 151.4 (C-4), 164.2 (C-2).

Standard and samples preparation – Compound **2** was weighed and dissolved in 50% MeOH to obtain a standard solution (5.0 mg/mL). Aqueous compound solutions were prepared at concentrations of 0.005, 0.05, 0.5, 1, and 5 mg/mL for the construction of a calibration curve. For analysis of compound **2** in *B. nivea*, each 10 g of Bn was extracted with MeOH (3 × 500 ml) by reflux and evaporated *in vacuo*. The residue was dissolved in 1 ml 50% MeOH and filtered with a 0.45-μm filter. The resulting solution was used for the HPLC analysis.

HPLC conditions – HPLC separation of compound **2** for qualitative and quantitative analyses was performed using a reverse phase system. A Discovery® C18 (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 1 mL/min. All injections were performed in triplicate.

Limits of detection and quantification (LOD and LOQ) – Validation of the HPLC method for compound **2** as a standard was performed using the LOD and LOQ. The method linearity was established using triplicate injections in the range of 0.005 - 5 mg/mL. Calibration curves were constructed using linear regression analysis of the peak area ratios (Y) corresponding to compound **2** versus its concentration (X) in mg/mL. The relative standard deviation was used as a measure of repeatability. The percent recoveries were evaluated by calculating the ratio of amount of compound **2** detected versus the amount added. The LOD and LOQ values were separately determined at signal to noise ratios (S/N) of 3 and 10, respectively.

Calibration curve – A stock solution (5 mg/mL) of compound **2** was prepared in 50% MeOH. The contents of the analysis were determined from the corresponding calibration curves. The calibration functions of the compound were calculated using the peak area (Y), concentration (X, μg/10 μL), and mean values (n = 3) ± standard deviation (SD).

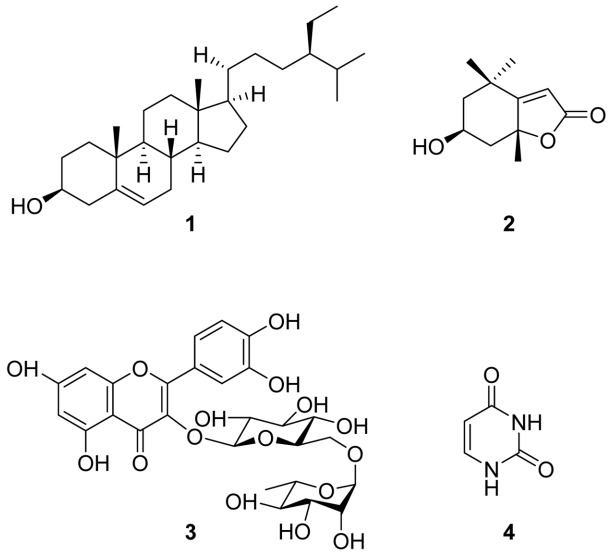

Fig. 1. Structures of compounds **1 - 4** from *B. nivea*.

Result and Discussion

Identification of compounds 1 - 4 from *B. nivea* – Chromatographic separation of *B. nivea* led to the isolation of compounds **1 - 4** (Fig. 1). Their structures were identified through spectral analysis.

Compound **1** was obtained as a white amorphous powder that had a molecular ion peak at *m/z* 414 $[M]^+$ in the EI-MS and a molecular formula of $C_{29}H_{50}O$. ^{1}H-NMR spectra of Compound **1** showed the presence of two methyl singlets at δ 0.68 and 1.02 three methyl doublets that appeared at δ 0.81, 0.82, and 0.94 and a methyl triplet at δ 0.86. Compound **1** also showed protons at δ 5.38, possibly corresponding to that of a trisubstituted olefinic bond. The presence of δ 3.52 (3-OH) was observed. The above spectral data supported the presence of a sterol skeleton having a hydroxyl group at C-3 position with one double bond at C-5/C-6 with six methyl groups. Thus, the structure of **1** was identified as β-sitosterol through comparison with spectral data in the literature.[13] β-Sitosterol is an important nutrient in the diet; it is hydrophobic, and soluble in organic solvents, and considered an excellent biomarker due to its biological activity.[14]

Compound **2** was obtained as a syrup and it showed a molecular ion peak at *m/z* 196 $[M]^+$ in the EI-MS, with a molecular formula of $C_{11}H_{16}O_3$. In the ^{1}H-NMR spectrum, two germinal methyl protons at δ 1.31 (H-9) and 1.42 (H-10) and one olefinic proton at δ 5.74 (H-7) were observed. The ^{13}C-NMR spectrum showed three methyl groups (δ 22.6, 24.9, and 30.6), a trisubstituted olefinic bond (δ

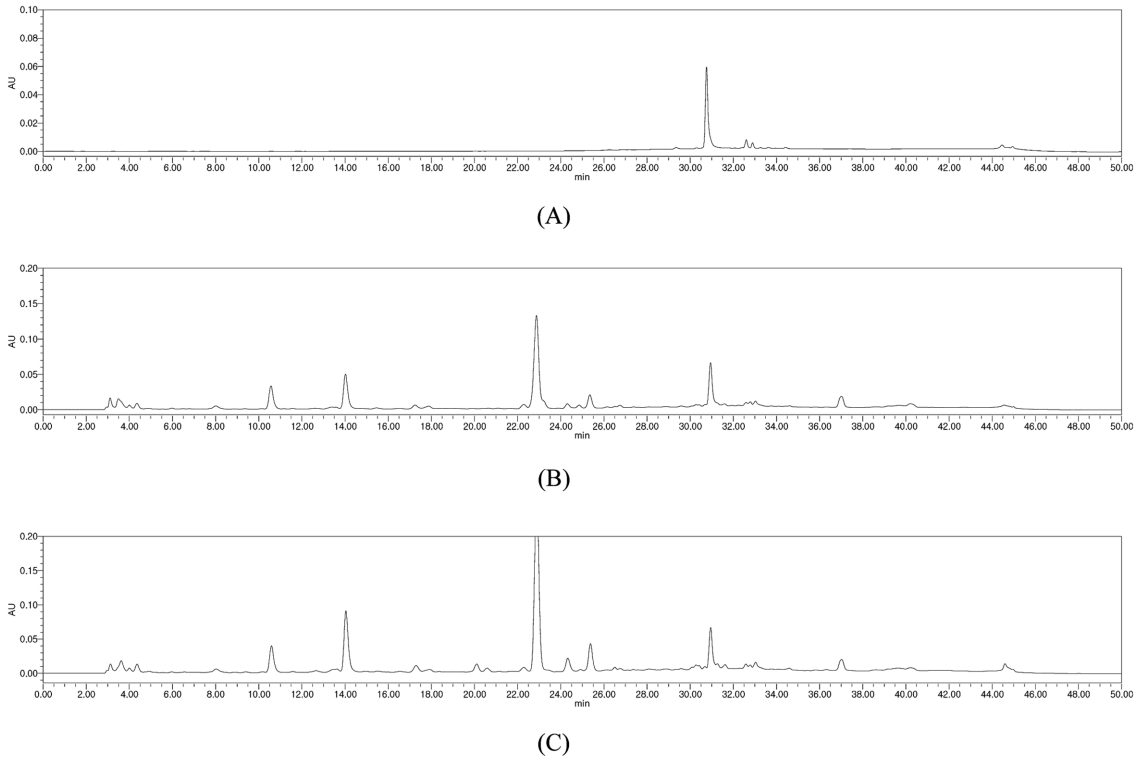

Fig. 2. HPLC chromatograms of (−)-loliolide (A) and the MeOH extract of Bn-23 (B) and -90 (C) collected in September.

111.7 and 170.0), a secondary hydroxyl group (δ 66.6), and a lactone carbonyl (δ 182.7). Accordingly, the structure of **2** was identified as (−)-loliolide through comparison with spectral data in the literature.[15] In previous studies, compound **2** was isolated from marine algae.[16] Biological effects has also been reported for this compound. (−)-Loliolide is a phytotoxic compound and has various effects, such as the inhibition of algal growth, anti-microbial, anti-repellent, and immunosuppressive effects.[17-19]

Compound **3** was obtained as a yellow amorphous powder and it showed a molecular ion peak at *m/z* 611 $[M+H]^+$ in the FAB-MS, with a molecular formula of $C_{27}H_{30}O_{16}$. The ^{1}H-NMR spectrum of **3** exhibited a flavonoid skeleton. The presence of one singlet signal at δ 12.5 (5-OH) was observed. Ring-A proton signals of H-6 and -8 were observed at δ 6.17 and 6.40 (1H, d, J = 1.5 Hz), respectively. In the B-ring structure, compound **3** has an ABX system (H-2', -5' and -6'), each coupling constant signals at δ 6.83 (1H, d, J = 7.5 Hz, H-5'), 7.52 (1H, d, J = 2.0 Hz, H-2'), and 7.54 (1H, dd, J = 7.5, 2.0 Hz, H-6'). The anomeric signal of glucose was observed as a doublet at δ 5.33 (1H, d, J = 7.5 Hz, H-1") and the anomeric signal of rhamnose was observed at δ 4.38. The ^{13}C-NMR spectrum indicated 27 carbon resonances. 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. Accordingly, the structure of **3** was identified as rutin through comparison with spectral data in the literature.[20] Rutin is a flavonoid with anti-diabetic, anti-oxidative, anti-hypertensive, anti-inflammatory, and anti-cancer activities.[21] Anti-oxidant effects of rutin have been shown by other studies where rutin potentiated the anti-lipoperoxidative capacity of α-tocopherol and ascorbic acid, and suppressed oxygen radical overproduction in thalassemic erythrocytes.[22]

Compound **4** was obtained as a white amorphous powder that showed a molecular ion peak at *m/z* 112 $[M]^+$ in the EI-MS, with a molecular formula of $C_4H_4N_2O_2$. ^{1}H-NMR spectra of **4** showed two methine protons at δ 5.43 (H-6) and 7.35 (H-5) and two amide protons at δ 10.93 and 10.94. The ^{13}C-NMR spectrum showed two carbonyl groups at δ 151.4 and 164.2. Thus, the structure of **4** was assigned as pyrimidinedione using spectroscopic analysis and literature survey.[23] Mechanistic studies has conclusively shown that pyrimidinedione acts as a non-nucleoside reverse transcriptase inhibitors (NNRTI) of HIV-1 and as entry inhibitors of both HIV-1 and HIV-2, and thus is the first NNRTI with substantial inhibitory potential against HIV-2.[24]

To the best of our knowledge, from the four known

Table 2. LOD and LOQ of (−)-loliolide

Compound	Calibration equation [a]	r^2 [b]	Linear range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
(−)-loliolide	Y = 5557.1X − 103.46	0.9999	0.005 - 5	0.055	0.142

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

Table 3. The content of (−)-loliolide in MeOH extracts of Bn

Harvest time	Bn-02	Bn-07	Bn-10	Bn-23	Bn-38	Bn-41	Bn-67	Bn-76	Bn-90
June	5.02	6.35	6.93	5.89	4.31	4.24	4.91	5.12	5.46
July	4.32	6.42	7.72	7.97	4.05	4.32	5.65	6.67	5.39
August	3.52	5.17	3.90	4.27	3.26	4.72	3.82	3.30	3.31
September	7.04	7.25	7.43	7.86	6.76	6.38	7.60	6.79	4.77
Total	19.9	25.19	25.98	25.99	18.38	19.66	21.98	21.88	18.93

Data is represented as the mean ± SD (n = 3) in mg/g.

compounds that were isolated, (−)-loliolide (**2**) was isolated for the first time from *B. nivea*.

Quantitative analysis of (−)-loliolide (2) in Bn from different regions and harvest times − Among the isolated compounds, (−)-loliolide (**2**) was isolated for the first time from *B. nivea*. In previous studies, (−)-loliolide (**2**) showed anti-microbial and anti-fungal effects.[19-25] We determined (−)-loliolide (**2**) content in Bn extracts using HPLC. The HPLC chromatograms of (−)-loliolide (**2**) and Bn extract are shown in Fig. 2. (−)-Loliolide (**2**) content of Bn extracts was quantified using linear regression equations. The linear regression data from the extracts showed a strong linear relationship and the resulting equation was valid over the relevant concentration range. The linear calibration equation was Y = 588.44X + 68102, where Y is the peak area and X is the loliolide concentration. The correlation coefficient (r^2) was 0.9989. The LOD and LOQ under our chromatographic conditions were determined at signal to noise ratios (S/N) of 3 and 10, respectively. The LOD and LOQ values for (−)-loliolide (**2**) were determined to be 0.055 and 0.140 mg/mL, respectively (Table 2). The content of (−)-loliolide (**2**) was detected in the leaves of Bn-02, -07, -10, -23, -38, -41, -67, -76, and -90 shown Table 3. The highest content of (−)-loliolide (**2**) was found in Bn-23 and in Bn collected in September. (−)-Loliolide contents in Bn at harvest time continually increased, except in August. The heavy rain in this month could be the reason for this decrease.

In addition, *B. nivea* could be conjugated as potential natural additive in food, cosmetic, and medicinal products, and be useful as a new natural bioactive source. It will be helpful to use the plant which harvest in September as a high content phytochemical additive in food, health supplements, and medicinal products. Future research will focus on the analysis of the other isolated compounds.

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