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IRIDOID GLUCOSIDES FROM *Barleria lupulina* LINDL.

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Abstract. Four iridoid glycosides, 6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester (**1**), 6-*O*-*p*-methoxy-*trans*-cinnamoyl-8-*O*-acetylshanzhiside methyl ester (**2**), 6-*O*-*cis*-*p*-coumaroylshanzhiside methyl ester (**3**), and ipolamiide (**4**) have been purified and structurally elucidated from *Barleria lupulina* Lindl. (Acanthaceae) collected in Luang Namtha province, Laos. Compound **1**, at a concentration of 128 µg/mL, inhibited the growth of the A549 cancer cell line by 31% and nitric oxide production in RAW 264.7 cells by 30%. This is the first report on the chemical constituents of Lao's *Barleria lupulina*.

Keywords: *Barleria lupulina*, iridoid glycoside, cytotoxicity, anti-inflammatory activity.

1. Introduction

Barleria lupulina Lindl is a small shrub distributed in the Southeast Asia region. In Thailand, the plant is widely used in folk medicine as an anti-inflammatory remedy for insect bites, herpes simplex, and herpes zoster [1]. In Cambodia, the plant is known for treating bruises, bone fractures, wound bleeding, carbuncles, severe sores, and snake bites [2]. The leaves and flowers of this plant are exploited in traditional medicine in Vietnam for various treatments such as reducing swelling, detoxifying, and relieving pain, snake bites, asthma, cough, sore throat, and toothache [3]. In addition, people in central Laos are using this plant for detoxification and abscess treatment [4]. Anti-herpes simplex virus type 2 (HSV-2), antioxidant, antibacterial, anti-inflammatory, anticancer, and antidiabetic activities of the crude extract of many parts of this plant have recently been reported [5]-[10]. Phytochemical studies on different parts of the plant collected in Thailand, Indonesia, and Vietnam have revealed the presence of a number of iridoid

glycosides and some lignans [11]-[18]. However, no reports have yet been made regarding the chemical constituents and biological activity of this plant from Laos. In the course of our investigation on the biologically active compounds from Laotian medicinal plants, *B. lupulina* was collected in Luang Namtha province, Laos, then its chemical constituents and potential activities were studied.

2. Content

2.1. Materials and methods

2.1.1. Plant material

Whole plant specimens of *Barleria lupulina* Lindl. were collected in Luang Namtha province, Laos, from July to December 2021 and identified by Bounnam Xangyaorn, Luangnamtha Teacher Training College, Luangnamtha, Laos. The voucher specimen (BL2021) has been deposited at the Faculty of Chemistry, Hanoi University of Education, Vietnam.

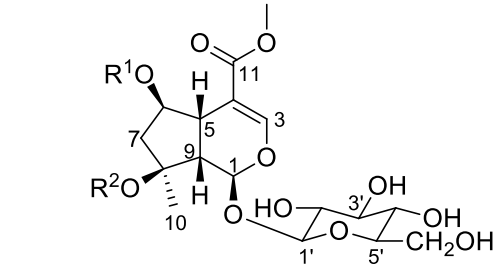
2.1.2. General procedure

Thin layer chromatography (TLC) was carried out on precoated Si gel GF_{254} (Merck). TLC spots were observed at 254, 302, and 366 nm and visualized by spraying with 10% H_2SO_4 in methanol, followed by heating until the spots appeared. Column chromatography was conducted on silica gel 60 (60 - 100 µM, Merck), Sephadex LH-20 (Amersham Pharmacia Biotech), and Diaion HP-20 (Supelco). Preparative medium-pressure liquid chromatography (MPLC) was performed with a Work-21 pump (Lab-Quatec Co., Ltd, Japan) and a Lobar column (Merck), at a flow rate of 1.0 mL/min. Preparative high-performance liquid chromatography (HPLC) was performed on a Jasco PU-2087 instrument with UV-2070 and RI-2031 detectors using a Waters 5C 18-AR-II column (10.0 × 250 mm) at a flow rate of 1.0 mL/min. 1D and 2D NMR (^1H, ^{13}C NMR, HSQC, HMBC) spectra were recorded on a Bruker Avance 600 MHz Instrument.

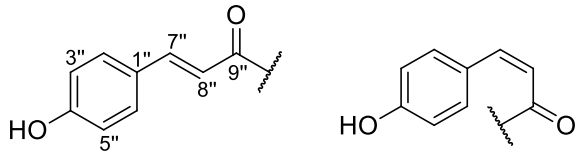
2.1.3. Extraction and Isolation

The whole dried plant of *Barleria lupulina* Lindl. (6.5 kg) was powdered and then extracted with methanol to yield the crude methanol (MeOH) extract (approximately 540 g). This extract was partitioned between *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). The *n*-hexane extract (115 g) was subjected to a Sephadex LH-20 column, using a MeOH/$CHCl_3$ (1/2, v/v) solvent to give 5 subfractions (BLH1-5). Subfraction BLH3 (10.7 g) was chromatographed on a silica gel column, eluting with *n*-hexane/EtOAc gradient (from 5/1 to 1/5, v/v) and EtOAc/MeOH gradient (from 98/2 to 80/20, v/v) to give 19 subfractions (BLH3A-S). Subfraction BLH3O (275.9 mg) was further separated by MPLC with a Lobar column, using $CHCl_3$/MeOH (90/10, v/v) to give 5 subfractions. Then, subfraction BLH3O1 (98.5 mg) and BLH3O2 (93.8 mg) were further purified using the same method by the reversed phase (C-18) silica gel column, eluting with MeOH/H_2O (60/40, v/v) to afford compound **1** (20 mg) and compound **2** (5.9 mg), respectively. The *n*-butanol fraction (68 g) was subjected to a Diaion HP-20 column, eluting with H_2O 100%, H_2O/MeOH gradient (from 95/5 to 30/70, v/v), and MeOH 100% to give 12 fractions (BLB1-12). Subfraction BLB8 (2.13 g) was further separated by using a Sephadex LH-20 column with 100% MeOH to give 3 additional

subfractions. Then, subfraction BLB8A (31.1 mg) was further purified by MPLC, using CH_2Cl_2/MeOH (8/1, v/v), followed by HPLC with reversed-phase (C-18) column, eluting MeOH/H_2O (4/6, v/v) to yield compound **4** (1.7 mg) with the retention time of 28 minutes. Finally, compound **3** (4.8 mg) was collected by sequential chromatography of subfraction BLB11 (9.38 g) on a silica gel column, eluting with CH_2Cl_2/MeOH gradient (6/1 to 1/5, v/v), yielding 13 subfractions. Subfraction BLB11H (2.28 g) was then purified by Sephadex LH-20 column, using MeOH 100%, followed by MPLC with reversed-phase (C-18) column, using MeOH/H_2O (1/1, v/v).

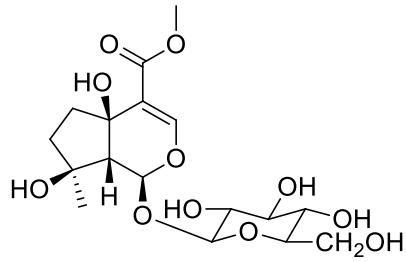


	R^1	R^2
1	*trans-p*-coumaroyl	Ac
2	*p*-methoxy-*trans*-cinnamoyl	Ac
3	*cis-p*-coumaroyl	H



trans-p-coumaroyl

cis-p-coumaroyl



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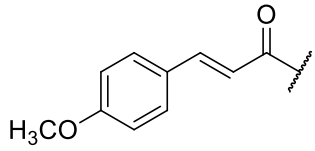

p-methoxy-*trans*-cinnamoyl

Figure 1. Structures of compounds 1-4

2.1.4. Spectral data for compounds 1-4

6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester (**1**): ^{1}H NMR (600 MHz, MeOD): δ_H 7.64 (d, *J* = 15.9 Hz, H-7''), 7.54 (d, *J* = 1.8 Hz, H-3), 7.49 (2H, d, *J* = 8.4 Hz, H-2'', 6''), 6.84 (2H, d, *J* = 8.4 Hz, H-3'', 5''), 6.35 (d, *J* = 15.9 Hz, H-8''), 5.92 (d, *J* = 3.0 Hz, H-1), 5.41 (brs dd, *J* = 2.1, 3.3 Hz, H-6), 4.70 (d, *J* = 8.4 Hz, H-1'), 3.96 (dd, *J* = 2.4, 12.0 Hz, H-6'a), 3.72 (s, 11-OMe), 3.71 (dd, *J* = 6.6, 12.0 Hz, H-6'b), 3.40 (t, *J* = 9.0 Hz, H-3'), 3.37 (m, H-5, H-5'), 3.30 (t, *J* = 9.3 Hz, H-4'), 3.23 (dd, *J* = 8.1, 9.3 Hz, H-2'), 3.07 (dd, *J* = 3.3, 8.7 Hz, H-9), 2.44 (brs d, *J* = 15.6 Hz, H-7a), 2.15 (dd, *J* = 5.4, 15.6 Hz, H-7b), 1.99 (s, 8–OCOMe). 1.60 (s, H-10). ^{13}C NMR (150 MHz, MeOD): δ_C 172.9 (8–OCOMe), 168.5 (C-11), 168.5 (C-9'', CO-coumaroyl), 161.3 (C-4''), 154.5 (C-3), 146.6 (C-7''), 131.2 (C-2'', 6''), 127.1 (C-1''), 116.9 (C-3'', 5''), 115.4 (C-8''), 108.6 (C-4), 100.3 (C-1'), 95.4 (C-1), 89.6 (C-8), 78.8 (C-6), 78.4 (C-5'),

78.0 (C-3'), 74.7 (C-2'), 71.7 (C-4'), 63.0 (C-6'), 51.9 (11-COMe), 50.4 (C-9), 45.2 (C-7), 40.0 (C-5), 22.2 (8–OCOMe), 21.8 (C-10).

6-*O*-*p*-methoxy-*trans*-cinnamoyl-8-*O*-acetylshanzhiside methyl ester (**2**): ^{1}H NMR (600 MHz, MeOD): δ_H 7.67 (d, *J* = 16.2 Hz, H-7''), 7.59 (2H, d, *J* = 8.4 Hz, H-2'', 6''), 7.54 (d, *J* = 1.8 Hz, H-3), 6.99 (2H, d, *J* = 9.0 Hz, H-3'', 5''), 6.41 (d, *J* = 16.2 Hz, H-8''), 5.92 (d, *J* = 3.6 Hz, H-1), 5.42 (dd, *J* = 1.8, 3.6 Hz, H-6), 4.70 (d, *J* = 8.4 Hz, H-1'), 3.94 (dd, *J* = 2.4, 12.0 Hz, H-6'a), 3.86 (s, 4''-OMe), 3.72 (s, 11-OMe), 3.69 (m, H-6'b), 3.38-3.21 (m, H-2'-5' and H-5), 3.07 (dd, *J* = 3.0, 8.4 Hz, H-9), 2.45 (brs d, *J* = 15.0 Hz, H-7a), 2.17 (dd, *J* = 5.4, 15.6 Hz, H-7b), 1.99 (s, 8–OCOMe). 1.60 (s, H-10). ^{13}C NMR (150 MHz, MeOD): δ_C 172.7 (8–OCOMe), 168.5 (C-11), 168.3 (CO-cinnamoyl), 163.3 (C-4''), 154.5 (C-3), 146.3 (C-7''), 131.0 (C-2'', 6''), 128.3 (C-1''), 116.4 (C-8''), 114.5 (C-3'', 5''), 108.6 (C-4), 100.4 (C-1'), 95.5 (C-1), 89.6 (C-8), 78.9 (C-6), 78.4 (C-5'), 78.0 (C-3'), 74.7 (C-2'), 71.7 (C-4'), 63.0 (C-6'), 55.9 (4''-OMe), 51.9 (11-COMe), 50.4 (C-9), 45.2 (C-7), 40.0 (C-5), 22.2 (8–OCOMe), 21.8 (C-10).

6-*O*-*cis*-*p*-coumaroylshanzhiside methyl ester **(3)**: ^{1}H NMR (600 MHz, MeOD): ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 7.69 (2H, d, *J* = 8.7 Hz, H-2'', 6''), 7.47 (d, *J* = 0.6 Hz, H-3), 6.89 (d, *J* = 12.6 Hz, H-7''), 6.77 (2H, d, *J* = 8.7 Hz, H-3'', 5''), 5.84 (d, *J* = 12.6 Hz, H-8''), 5.56 (d, *J* = 4.2 Hz, H-1), 5.17 (brs dd, *J* = 3.0, 4.2 Hz, H-6), 4.70 (d, *J* = 7.8 Hz, H-1'), 3.93 (dd, *J* = 2.1, 11.7 Hz, H-6'a), 3.67 (dd, *J* = 6.6, 11.7 Hz, H-6'b), 3.65 (s, 11-OMe), 3.39 (t, *J* = 9.0 Hz, H-3'), 3.34 (m, overlapped signal, H-5, H-5'), 3.27 (t, *J* = 9.3 Hz, H-4'), 3.22 (dd, *J* = 7.8, 9.0 Hz, H-2'), 2.53 (dd, *J* = 4.2, 9.6 Hz, H-9), 2.28 (dd, *J* = 7.5, 14.1 Hz, H-7a), 1.88 (dd, *J* = 5.1, 14.1 Hz, H-7b), 1.35 (s, H-10). ^{13}C NMR (150 MHz, MeOD): δ_C 168.9 (C-11), 167.7 (CO-coumaroyl), 160.0 (C-4''), 153.3 (C-3), 144.9 (C-7''), 133.7 (C-2'', 6''), 127.7 (C-1''), 117.2 (C-8''), 115.8 (C-3'', 5''), 110.1 (C-4), 99.9 (C-1'), 95.0 (C-1), 79.0 (C-8), 78.9 (C-6), 78.5 (C-5'), 78.0 (C-3'), 74.4 (C-2'), 71.7 (C-4'), 62.9 (C-6'), 51.8 (11-COMe), 51.5 (C-9), 47.7 (C-7), 38.8 (C-5), 25.4 (C-10).

Ipolamiide (**4**): ^{1}H NMR (600 MHz, MeOD): δ_H 7.46 (s, H-3), 5.83 (d, *J* = 1.2 Hz, H-1), 4.60 (d, *J* = 8.4 Hz, H-1'), 3.92 (dd, *J* = 2.4, 12.0 Hz, H-6'a), 3.75 (s, 11-OMe), 3.68 (dd, *J* = 6.6, 12.0 Hz, H-6'b), 3.39 (t, *J* = 9.0 Hz, H-3'), 3.33 (m, overlapped signal, H-5'), 3.29 (t, *J* = 9.3 Hz, H-4'), 3.20 (dd, *J* = 7.8, 9.0 Hz, H-2'), 2.51 (brs, H-9), 2.28 (m, H-6a), 2.11 (m, H-6b), 1.96 (m, H-7a), 1.59 (m, H-7b), 1.17 (s, H-10). ^{13}C NMR (150 MHz, MeOD): δ_C 168.1 (C-11), 152.6 (C-3), 115.3 (C-4), 99.6 (C-1'), 94.3 (C-1), 78.9 (C-8), 78.4 (C-5'), 77.5 (C-3'), 74.4 (C-2'), 71.7 (C-4', 5), 62.9 (C-6'), 61.7 (C-9), 51.6 (11-COMe), 40.4 (C-7), 38.9 (C-6), 23.3 (C-10).

2.1.5. Biological activity assay

**** Cytotoxicity***

Four compounds were tested against the lung carcinoma cell line (A549 CCL-185) following the MTT assay according to the method of Scudiero et al. [19]. The fractions were dissolved in dimethyl sulfoxide and subsequently diluted in water. IC_{50} values were calculated based on the percentage inhibition of cell growth.

**** Nitric oxide (NO) inhibitory activity***

The inhibitory capacity of four compounds on lipopolysaccharides-stimulated NO production of RAW 264.7 cells was evaluated according to the method of Cheenpracha *et al.* [20] with L-NMMA as a reference.

2.2. Results and Discussion

Compound **1** was isolated as an amorphous powder. The analysis of ^{1}H- and ^{13}C-NMR spectra revealed an iridoid structure with the presence of one *β*-glucopyranosyl unit, one acetyl group, and one coumaroyl moiety. The ^{1}H NMR exhibited two pairs of aromatic proton signals (AA'BB' system) at δ_H 7.49 (2H, d, J = 8.4 Hz, H-2'', H-6''), 6.84 (2H, d, J = 8.4 Hz, H-3'', H-5'') and two olefinic protons at δ_H 7.64 and 6.35 (d, J = 15.9 Hz, H-7'', H-8''), indicating a *trans* configuration in the *p*-coumaroyl moiety. An anomeric proton signal was observed at δ_H 4.70 (d, J = 8.4 Hz, H-1'), along with the corresponding anomeric carbon signal at δ_C 100.3 (C-1') in its ^{13}C-NMR spectra. Additionally, the acetyl group was observed at δ_H 1.99 and δ_C 172.9 and 22.2 ppm. The attachment of the acetyl group was assigned to C-8 on the iridoid group, based on the downfield shift of C-8 (δ_C 89.6 ppm) as compared to shanzhiside methyl ester (δ_C 79.0 ppm) [21].

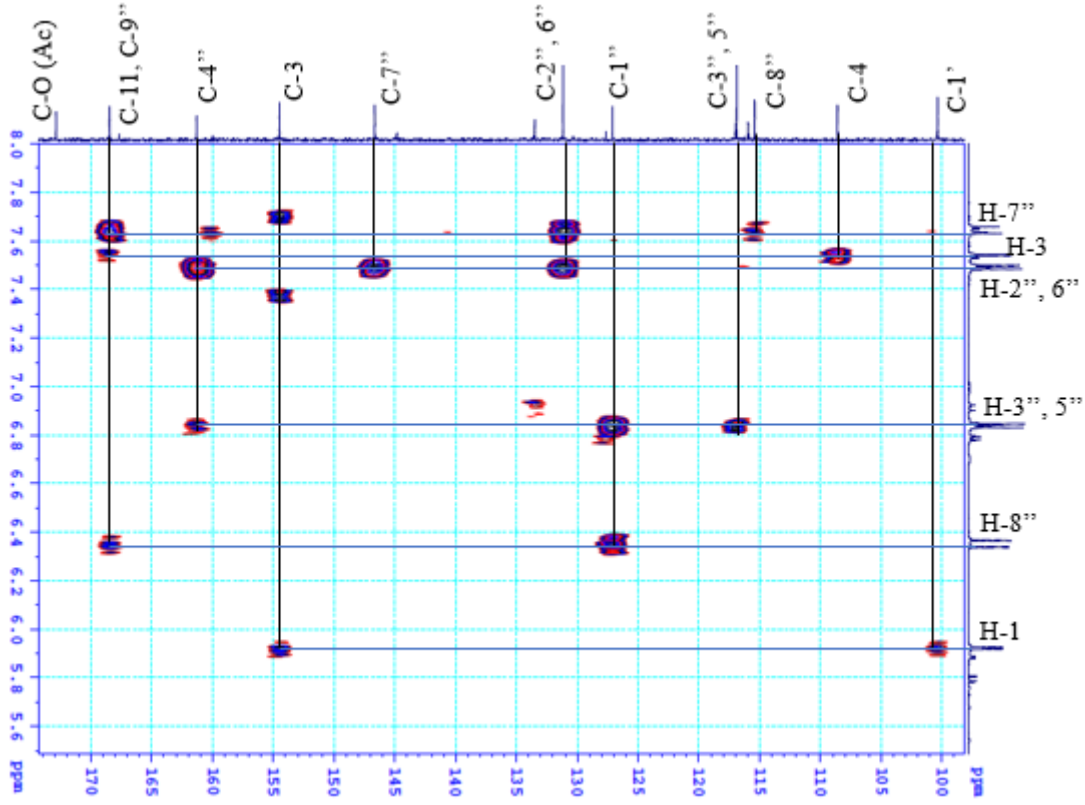

Figure 2. Some important HMBC correlations of compound 1

The glucopyranosyl unit of compound **1** was attached at C-1 due to HMBC correlations between H-1 (δ_H 5.92, d, J = 3.0 Hz) and C-1', and between H-1' and C-1

(δ_C 95.4). Its β-configuration was confirmed by the coupling constants of H-1' (δ_H 4.70, d, J = 8.4 Hz). Furthermore, *p*-coumoroyl moiety was determined to be positioned at the C-6 due to the downfield shift of H-6 (δ_H 5.41, brs dd, J = 2.1, 3.3 Hz), which compared to those of shanzhiside methyl ester (δ_H 4.03, m) [21]. Finally, the structure of compound **1** was determined as shown in Fig. 1 and named 6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester [22]. This compound was previously found in the *n*-BuOH extract of leaves and EtOAc extract of aerial parts of *B. lupulina* collected in Thailand and Vietnam, respectively [14].

Compound **2** was also isolated as an amorphous powder. The ^{1}H and ^{13}C NMR spectra of compound **2** were very similar to those of compound **1**, except for the aromatic methoxy group (δ_H 3.86 and δ_C 55.9 ppm), which was observed in the spectra. There were two pairs of aromatic protons signals (AA'BB' system) at δ_H 7.59 (2H, d, J = 8.4 Hz, H-2'', H-6''), 6.99 (2H, d, J = 9.0 Hz, H-3'', H-5'') in its ^{1}H-NMR, thus, indicating the methoxy group is located at C-4'' (δ_C 163.3 ppm). Therefore, the structure of compound **2** was identified as 6-*O*-*p*-methoxy-*trans*-cinnamoyl-8-*O*-acetylshanzhiside methyl ester [13]. This compound was previously found in the *n*-BuOH extract of leaves and EtOAc extract of aerial parts of this plant, collected in Thailand and Vietnam, respectively [14].

Compound **3** was isolated as an amorphous powder. The ^{1}H and ^{13}C NMR spectra of compound **3** were also very similar to those of compound **1**, except for two features. The major distinction was the absence of an acetyl group signal in the spectra of compound **3**. Another difference was the double bond configuration of the *p*-coumaroyl group. Compound **3** exhibits a *cis* configuration due to its small coupling constant δ_H 6.89 and 5.84 (d, J = 12.6 Hz; H-7'', H-8''). Therefore, the structure of compound **3** was identified as 6-*O*-*cis*-*p*-coumaroylshanzhiside methyl ester [23]. This compound was isolated from *B. lupulina* for the first time.

Compound **4** was also obtained in an amorphous solid. Examination of the NMR spectra data of **4** also revealed the characteristics of an iridoid glucoside; however, unlike the other three compounds, no signals corresponding to the coumaroyl moiety or acetyl group were observed. Analysis of the ^{13}C-NMR spectra indicated that this compound contained 17 carbon signals, similar to those of shanzhiside methyl ester [21]. However, one methine proton (H-5) was absent, and two more non-equivalent methylene proton signals were observed at δ_H 2.28 (m) and 2.11 (m) in the ^{1}H-NMR spectra, which were attributable to H-6a and H-6b. Therefore, the structure of compound **4** was identified as ipolamiide [24]. This compound was previously found in the methanol extract from aerial parts of this plant collected in Thailand [14].

Recently, compound **1** was reported to exhibit a low level of antioxidant activity in the DPPH assay (IC_{50} 486.5 μg/mL) and a 3:1 mixture of compound **1** with its *cis* isomer exhibited antiviral activity against the respiratory syncytial virus (A2 strain) in a cell culture-based cytopathic effect assay (EC_{50} 2.46 μg/mL) [17], [22]. Compound **4** exhibited a potent antiosteoporotic effect (134.6 ± 1.5% at 25 μM) [16]. In this study, the cytotoxicity and anti-inflammatory activity of compound **1** was investigated. The result showed that compound **1** has weak inhibitory activity against the growth of A549 cancer cell lines and nitric oxide production in RAW 264.7 cells.

3. Conclusions

Four secondary metabolites, 6-*O*-*trans*-*p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester (**1**), 6-*O*-*p*-Methoxy-*trans*-cinnamoyl-8-*O*-acetylshanzhiside methyl ester (**2**), 6-*O*-*cis*-*p*-coumaroylshanzhiside methyl ester (**3**), and ipolamiide (**4**) were isolated from Laotian medicinal plant *B. lupulina* for the first time. Their structures were determined by 1D and 2D NMR spectroscopic methods. This research result provides more evidence supporting the traditional use of *B. lupulina* for treating cancer and inflammatory diseases.

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