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ISOLATION AND CYTOTOXIC EVALUATION OF COMPOUNDS FROM THE TWIGS OF *MILLETTIA LEPTOBOTRYA* DUNN

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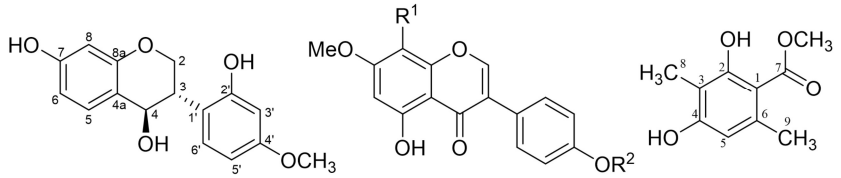
Summary

Chemical investigation of the ethyl acetate extract from the twigs of *Millettia leptobotrya* Dunn led to the isolation of five isoflavonoids (**1**–**5**) and one phenolic derivative (**6**). Their structures were established through comprehensive NMR spectroscopic analyses and comparison with published literature and identified as (+)-*trans*-4,7,2′-trihydroxy-4′-methoxyisoflavanol (**1**), prunetin (**2**), 5-hydroxy-4′,7-dimethoxyisoflavone (**3**), 5,4′-dihydroxy-7,8-dimethoxyisoflavone (**4**), isotectorigenin, 7, 4′-dimethyl ether (**5**), and atraric acid (**6**). This study represents the first report of compounds **1**, **5**, and **6** from the genus *Millettia*, further contributing to the phytochemical characterization of this species. The cytotoxic activities of compounds **1**, **4**, and **5** were evaluated against KB, HepG2, A549, and MCF-7 human cancer cell lines using the MTT assay. Only compound **1** showed weak cytotoxicity against KB, HepG2, A549, and MCF-7 cancer cells, with IC_{50} values of 44.92, 48.70, 64.68, and 49.23 µg/mL, respectively.

Keywords: *Millettia leptobotrya*; Fabaceae; *Isoflavonoid; Cytotoxic.*

1. Introduction

The genus *Millettia* (Fabaceae) comprises approximately 200 species distributed worldwide [1], of which about 22 species are recorded in Vietnam [2]. Phytochemical investigations of *Millettia* species have revealed a diverse array of secondary metabolites, including flavonoids, isoflavonoids, alkaloids, coumarins, phenolic compounds, and terpenoids. Many of these constituents have demonstrated noteworthy biological activities, such as anti-inflammatory, antibacterial, antioxidant, and cytotoxic effects [3],[4],[5],[6],[7]. *Millettia leptobotrya* Dunn is distributed mainly in southern Yunnan, China, and the mountainous regions of northern Vietnam. In traditional medicine, its roots and leaves have been used to treat bone fractures, traumatic injuries, and rheumatoid arthritis [8]. Despite its ethnomedicinal significance, phytochemical investigations of *M. leptobotrya* remain highly limited. To date, only one study has described the chemical constituents of its twigs, with particular emphasis on flavonoid compounds [8]. However, the chemical diversity and biological potential of this species have not yet been comprehensively investigated, and its biological activities remain poorly reported. As part of our ongoing search for bioactive secondary metabolites from Vietnamese medicinal plants, the present study was undertaken to further investigate the phytochemical constituents of the twigs of *M. leptobotrya* collected in Vietnam and to evaluate their cytotoxic activities, considering the reported cytotoxic potential of metabolites from the genus *Millettia* [3],[4],[5],[6],[7]. This study reports the isolation and structural elucidation of six compounds from the ethyl acetate (EtOAc) extract of the twigs of *M. leptobotrya*, together with an assessment of their cytotoxic activities against several human cancer cell lines (Fig. 1).



1 **2:** R^1 = H, R^2 =H **6**

3: R^1 = H, R^2 = Me

4: R^1 = OMe, R^2 = H

5: R^1 = OMe, R^2 = Me

Fig. 1. Structures of isolated compounds **1-6** from *M. leptobotrya* twigs.

2. Materials and Methods

2.1. Plant material

The twigs of *Millettia leptobotrya* Dunn (Fabaceae) were collected in Ngoc Lac, Thanh Hoa Province, Vietnam, in April 2025. The plant material was taxonomically identified by Dr. Nguyen The Cuong (Institute of Biology, Vietnam Academy of Science and Technology (VAST)). A voucher specimen (No. VN-1636B) has been deposited at the Institute of Chemistry, VAST.

2.2. General experimental procedures

Optical rotations were recorded on an Atago Polax-2L polarimeter (Tokyo, Japan). NMR spectra were recorded on a Bruker AM600 FT-NMR spectrometer (Rheinstetten, Germany) operating at 600 MHz and 150 MHz for ^{1}H NMR and ^{13}C NMR spectra, respectively. Column chromatography (CC) was performed on *silica gel* (Kieselgel 60, 230-400 mesh, Merck, Darmstadt, Germany) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). For thin layer chromatography (TLC), pre-coated *silica gel* 60 F_{254} (0.25 mm thickness, Merck) was used. Compounds were visualized under UV radiation (254 and 365 nm) and by spraying with a vanillin reagent consisting of vanillin (1%, w/v), acetic acid (10%, v/v), and H_2SO_4 (5%, v/v) in EtOH, followed by heating with a heat gun.

2.3. Cytotoxicity Assay (MTT method)

The cytotoxic activities of isolated compounds were evaluated *in vitro* against KB, HepG2, A549, and MCF-7 cell lines. The cells were cultured at 37 ^{o}C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a 5% CO_2 incubator. Cells between 5 and 20 passages were used for the assays. The cytotoxic activity was measured by using a modified MTT assay [9]. Viable cells were seeded in 96-well plates at a density of 3 × 10^4 cells/mL. Cells were treated with various concentrations of test compounds (2, 8, 32, and 128 µg/mL) and then incubated at 37 ^{o}C for 72 h in fresh DMEM. Cells were subsequently incubated at 37 ^{o}C with MTT (0.5 mg/mL) for 4 h. After removal of the supernatant, formazan crystals were dissolved in dimethyl sulfoxide, and the optical density was measured at 540 nm. Ellipticine was used as a positive control. All cytotoxicity assays were performed in triplicate in three independent experiments. The percentage of growth inhibition was calculated from the optical density (OD) values according to the following equation: Cell growth inhibition (%) = ($OD_{control\ (+)}$ – OD_{sample})/($OD_{control\ (+)}$– OD_{blank}) × 100%. The IC_{50} values, defined as the concentrations of the test compounds required to inhibit 50% of cell growth, were determined from the concentration–response curves using nonlinear regression analysis. The results are presented as the mean ± standard deviation (SD) of three independent experiments.

2.4. Extraction and Isolation

Dried twigs of *M. leptobotrya* (1.1 kg) were powdered and extracted with MeOH (4 times × 4 L) under ultrasonic conditions at room temperature for 2 h each. The combined extracts were concentrated under reduced pressure to afford a crude MeOH extract (68.0 g). This extract was suspended in H_2O (0.5 L) and successively partitioned with *n*-hexane (4 times × 0.5 L) and EtOAc (4 times × 0.5 L) to afford the *n*-hexane extract (8.9 g) and EtOAc extract (11.2 g), respectively. The EtOAc extract (11.2 g) was subjected to *silica gel* CC and eluted with a CH_2Cl_2 – MeOH (0 - 30% MeOH) gradient system to yield eight fractions (F1 – F8). Combined fractions F2 and F3 (2.5 g) were chromatographed over *silica gel* using an *n*-hexane – acetone (0-20% acetone) gradient to yield five subfractions (F2.1 – F2.5). Subfraction F2.2 (170 mg) was purified by Sephadex LH-20 CC (100% MeOH), followed by *silica gel* CC eluted with an *n*-hexane – EtOAc gradient to afford compound **3** (2.5 mg). Subfraction F2.3 (210 mg) was separated by *silica gel* CC using an *n*-hexane – EtOAc (0-20% EtOAc) gradient to afford compounds **5** (1.5 mg) and **6** (2.0 mg), respectively. Fraction F4 (210 mg) was chromatographed over Sephadex LH-20 (100% MeOH) to give four subfractions (F4.1 – F4.4). Subfraction F4.4 (50 mg) was further purified by *silica gel* CC using *n*-hexane – acetone (10-30% acetone) gradient to afford compound **1** (3.0 mg). Fraction F5 (420 mg) was separated by Sephadex LH-20 CC (100% MeOH) to provide three subfractions (F5.1 – F5.3). Subfraction F5.3 (170 mg) was subjected to *silica gel* CC (*n*-hexane – acetone gradient, 0-100% acetone) to

yield five subfractions (F5.3.1 – F5.3.5). Further purification of subfraction F5.3.2 (18 mg) by *silica gel* CC (CH_2Cl_2−acetone gradient, 0-100% acetone) afforded compound **4** (1.4 mg). Subfraction F5.3.5 (25 mg) was further purified by Sephadex LH-20 CC (100% MeOH) to yield compound **2** (2.5 mg).

(+)-Trans-4,7,2'-trihydroxy-4'-methoxyisoflavanol (1): Pale-yellow oil, $[\alpha]_D^{25}$ +34.2 (*c* 0.03, MeOH), ^{1}H-NMR (600 MHz, $CDCl_3$), ^{13}C-NMR (150 MHz, $CDCl_3$): Table 1.

Prunetin (2): Yellow powder, ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$): Table 1.

5-Hydroxy-4',7-dimethoxyisoflavone (3): White crystals, ^{1}H-NMR (600 MHz, $CDCl_3$), ^{13}C-NMR (150 MHz, $CDCl_3$): Table 1

5,4'-Dihydroxy-7,8-dimethoxyisoflavone (4): White solid, ^{1}H-NMR (600 MHz, $CDCl_3$), ^{13}C-NMR (150 MHz, $CDCl_3$): Table 1.

Isotectorigenin 7, 4′-dimethyl ether (5): Yellow amorphous powder, ^{1}H-NMR (600 MHz, $CDCl_3$), ^{13}C-NMR (150 MHz, $CDCl_3$): Table 1.

Atraric acid (6): White amorphous powder, ^{1}H-NMR (600 MHz, $CDCl_3$): δ_H (ppm) 12.00 (1H, s, OH), 6.21 (1H, s, H-5), 3.92 (3H, s, CH_3O-7), 2.46 (3H, s, H-9), 2.10 (3H, s, H-8). ^{13}C-NMR (150 MHz, $CDCl_3$): δ_C (ppm) 172.6 (C=O), 163.2 (C-4), 158.1 (C-2), 140.1 (C-6), 110.6 (C-5), 108.5 (C-3), 105.3 (C-1), 51.8 (OCH_3), 24.1 (C-9), 7.7 (C-8).

3. Results and Discussion

Compound **1** was obtained as a pale-yellow oil and was optically active $[\alpha]_D^{25}$+34.2 (*c* 0.03, MeOH) (*ref.* $[\alpha]_D^{25}$ +21.6 (*c* 0.019, MeOH) [11]). The ^{1}H-NMR spectrum of **1** indicated the presence of two ABX aromatic systems corresponding to two trisubstituted benzene rings at δ_H 7.38 (1H, d, *J* = 8.4 Hz, H-5), 6.54 (1H, dd, *J* = 2.4, 8.4 Hz, H-6), 6.41 (1H, d, *J* = 2.4 Hz, H-8), and 7.12 (1H, d, *J* = 9.0 Hz, H-6′), 6.45 (1H, dd, *J* = 2.4, 9.0 Hz, H-5′), 6.44 (1H, d, *J* = 2.4 Hz, H-3′). In addition, a methoxy singlet at δ_H 3.77 (3H, s, OCH_3), together with the aliphatic proton signals at δ_H 5.49 (1H, d, *J* = 6.6 Hz, H-4), 4.23 (1H, dd, *J* = 4.8, 10.8 Hz, H_a-2), 3.62 (1H, t, *J* = 10.8 Hz, H_b-2), 3.52 (1H, ddd, *J* = 4.8, 6.6, 10.8 Hz, H-3) were also observed. Analysis of the ^{13}C-NMR and HSQC spectra of compound **1** confirmed the presence of 16 carbons, comprising one methoxy group at δ_C 55.5 (4′-OCH_3), one methylene group at δ_C 66.6 (C-2), eight methine groups at δ_C 132.2 (C-5), 124.7 (C-6′), 109.8 (C-6), 106.4 (C-5′), 103.7 (C-8), 96.9 (C-3′), 78.6 (C-4), and 39.5 (C-3), and six non-protonated carbons at δ_C 112.7 (C-4a), 119.1 (C-1′), 157.1 (C-7), 156.7 (C-8a), 160.7 (C-2′), and 161.2 (C-4′). The downfield chemical shifts of C-2 (δ_C 66.6), C-4 (δ_C 78.6), C-7 (δ_C 157.1), C-4′ (δ_C 161.2), and C-2′ (δ_C 160.7) suggested their linkage to oxygen atoms. Comprehensive analyses of the 1D and 2D NMR spectra established compound **1** as an isoflavan-4-ol derivative. In the HMBC spectrum, the correlation from the methoxy protons at δ_H 3.77 to C-4′ (δ_C 161.2) confirmed the location of the methoxy group at C-4′. Furthermore, additional key HMBC correlations from H-5 (δ_H 7.38) to C-4 /C-7/C-8a, from H-4 (δ_H 5.49) to C-2/C-8a, from H-2 (δ_H 4.23, 3.62) to C-1′/C-4/C-8a, from H-3 (δ_H 3.52) to C-2′/C-2, and from H-6′ (δ_H 7.12) to C-3/C-2′/C-4′ were consistent with the proposed structure of 4,7,2′-trihydroxy-4′-methoxyisoflavanol. The relative configurations at C-3 and C-4 were assigned on the basis of the $^3J_{H3–H4}$ value, taking into consideration the half-chair conformation of the pyran ring and the equatorial orientation of the C-3 aryl group in isoflavan-4-ols. The observed value of 6.6 Hz for **1** was thus consistent with a *trans* configuration of H-3 and H-4, whereas a value of ca. 3.0 Hz has been reported for the corresponding *cis* relationship [10]. This assignment was further supported by the close agreement of the ^{13}C NMR chemical shifts of C-3 (δ_C 39.5) and C-4 (δ_C 78.6) with those reported for the corresponding *trans* isoflavan-4-ol (δ_C 39.9 and 79.1, respectively) [11]. Based on the above spectroscopic evidence, the positive optical rotation value, and comparison with literature data, compound **1** was identified as (+)-*trans*-4,7,2′-trihydroxy-4′-methoxyisoflavanol [12],[13],[14]. This compound has previously been reported to inhibit nitric oxide (NO) production, with an IC_{50} value of 35.41 μM [15].

Compound **2** was obtained as a yellow powder. The ^{13}C-NMR and HSQC spectra of compound **2** revealed the presence of sixteen carbon signals, comprising one carbonyl group (δ_C 180.9), one methoxy group (δ_C 55.8), seven methine groups, and seven non-protonated carbons. The

characteristic NMR signals, including an aromatic proton singlet at δ_H 7.86 assigned to H-2, a downfield carbon resonance at δ_C 152.7 (C-2), and a conjugated carbonyl carbon at δ_C 180.9 (C-4), clearly indicated the presence of an isoflavone skeleton. The ^{1}H-NMR spectrum also displayed a methoxy singlet at δ_H 3.88 (3H, s), two *meta*-coupled aromatic protons at 6.39 (1H, d, J = 2.4 Hz, H-6), 6.41 (1H, d, J = 2.4 Hz, H-8), an A_2B_2 spin system at δ_H 7.41 (2H, J = 9.0 Hz, H-2′, H-6′), 6.91 (2H, J = 9.0 Hz, H-3′, H-5′), together with a chelated hydroxyl proton at δ_H 12.83 (1H, s), indicating a 5-hydroxy-7-methoxy-substituted isoflavone bearing a *para*-disubstituted B ring. The distinct HMBC correlation from the methoxy protons (δ_H 3.88) to C-7 (δ_C 165.6) located the methoxy group at C-7. Accordingly, compound **2** was identified as prunetin by comparison of its spectroscopic data with those reported in the literature [16],[17]. This compound was previously isolated from *Millettia nitida* and has been reported to inhibit NO production with an IC_{50} value greater than 30 μg/mL [18].

Compound **3** was isolated as white crystals. The ^{1}H- and ^{13}C-NMR spectroscopic data of compound **3** closely resembled those of compound **2**, indicating that both compounds shared the same isoflavone skeleton. The ^{1}H-NMR spectrum exhibited the characteristic signals of an isoflavone, including a singlet aromatic proton at δ_H 7.86 (1H, s, H-2), two *meta*-coupled aromatic protons at δ_H 6.38 (1H, d, J = 2.4 Hz, H-6) and 6.40 (1H, d, J = 2.4 Hz, H-8), an A_2B_2 spin system at δ_H 7.46 (2H, d, J = 9.0 Hz, H-2′/H-6′) and 6.98 (2H, d, J = 9.0 Hz, H-3′/H-5′), together with a chelated hydroxyl proton at δ_H 12.85 (1H, s). In addition to the methoxy signal observed for compound **2** [δ_H 3.88 (3H, s), δ_C 55.8], compound **3** displayed an additional methoxy group at δ_H 3.85 (3H, s) and δ_C 55.4, indicating the presence of two methoxy substituents. The ^{13}C-NMR spectrum further supported this assignment by showing seventeen carbons, including two methoxy carbons and one conjugated carbonyl carbon at δ_C 180.9. Comparison of the NMR spectroscopic data with those reported in the literature led to the identification of compound **3** as 5-hydroxy-4′,7-dimethoxyisoflavone [19].

Compound **4** was obtained as a yellow powder. The ^{1}H-NMR spectrum of compound **4** revealed the presence of the proton signals for two methoxy groups at δ_H 3.90 (3H, s) and 3.96 (3H, s). In the downfield region, the characteristic signals of an A_2B_2 aromatic ring system were observed at δ_H 7.40 (2H, J = 9.0 Hz, H-2′, H-6′) and 6.90 (2H, J = 9.0 Hz, H-3′, H-5′). Additionally, two aromatic proton singlets at δ_H 7.93 and 6.45, together with a chelated hydroxyl proton at δ_H 12.66 (1H, s), were also noted. The ^{13}C-NMR and HSQC spectra of compound **4** revealed seventeen carbon signals, including two methoxy groups, six methines, and nine non-protonated carbons (Table 1). The chemical shifts of C-2, C-5, C-7, and C-4′ suggested their linkage to oxygen atoms. The characteristic signals in the NMR data of compound **4** clearly indicated the presence of an isoflavone skeleton. The HMBC correlations from the methoxy protons at δ_H 3.96 to C-7 (δ_C 158.6) and from the methoxy protons at δ_H 3.90 to C-8 (δ_C 128.7) established the locations of the methoxy groups at C-7 and C-8, respectively. Based on these spectroscopic data and comparison of its NMR data with those reported in the literature, compound **4** was identified as 5,4′-dihydroxy-7,8-dimethoxyisoflavone [19].

Compound **5** was isolated as a yellow powder. The NMR data of compound **5** were very similar to those of compound **4**, except for the presence of an additional methoxy group at δ_H 3.84 (3H, s). The ^{1}H-NMR spectrum also showed an A_2B_2 aromatic spin system at δ_H 7.46 (2H, d, J = 8.4 Hz, H-2′, H-6′) and 6.99 (2H, d, J = 9.0 Hz, H-3′, H-5′), together with two aromatic singlets at δ_H 7.93 (H-2) and 6.45 (H-6). Comparison of the ^{1}H- and ^{13}C-NMR data of compounds **4** and **5** revealed a downfield shift of C-4′ from δ_C 156.2 in compound **4** to δ_C 159.9 in compound **5**, indicating methylation of the 4′-hydroxy group. Analysis of the HMBC spectrum further confirmed the structure, with a correlation from the methoxy protons at δ_H 3.84 to C-4′ (δ_C 159.9), establishing the methoxy group at C-4′. The NMR data of compound **5** was consistent with those of isotectorigenin 7, 4′-dimethyl ether [20]. Based on these spectroscopic data and comparison with the reported literature values, compound **5** was identified as isotectorigenin 7, 4′-dimethyl ether [20].

Table 1. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz) spectroscopic data of compounds **1 – 5** in $CDCl_3$.

Pos.	Compound 1		Compound 2		Compound 3		Compound 4		Compound 5	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
2	66.6	3.62 (t, 10.8) 4.23 (dd, 4.8, 10.8)	152.7	7.86 (s)	152.7	7.86 (s)	152.9	7.93 (s)	152.8	7.93 (s)
3	39.5	3.52 (ddd, 4.8, 6.6, 10.8)	123.7	-	123.0	-	123.5	-	123.4	-
4	78.6	5.49 (d, 6.6)	180.9	-	180.9	-	181.2	-	181.1	-
4a	112.7	-	106.3	-	106.3	-	105.5	-	105.3	-
5	132.2	7.38 (d, 8.4)	162.8	-	162.8	-	158.2	-	158.2	-
6	109.8	6.54 (dd, 2.4, 8.4)	98.3	6.39 (d, 2.4)	98.2	6.38 (d, 2.4)	95.9	6.45 (s)	95.9	6.45 (s)
7	157.1	-	165.6	-	165.6	-	158.6	-	158.6	-
8	103.7	6.41 (d, 2.4)	92.5	6.41 (d, 2.4)	92.5	6.40 (d, 2.4)	128.7	-	128.7	-
8a	156.7	-	157.9	-	158.0	-	149.8	-	149.6	-
1′	119.1	-	123.1	-	123.7	-	122.7	-	122.8	-
2′	160.7	-	130.4	7.41 (d, 9,0)	130.1	7.46 (d, 9.0)	130.4	7.40 (d, 9.0)	130.2	7.46 (d, 8.4)
3′	96.9	6.44 (d, 2.4)	115.6	6.91 (d, 9.0)	114.2	6.98 (d, 9.0)	115.7	6.90 (d, 9.0)	114.2	6.99 (d, 9.0)
4′	161.2	-	156.0	-	159.9	-	156.2	-	159.9	-
5′	106.4	6.45 (dd, 2.4, 9.0)	115.6	6.91 (d, 9.0)	114.2	6.98 (d, 9.0)	115.7	6.90 (d, 9.0)	114.2	6.99 (d, 9.0)
6′	124.7	7.12 (d, 9.0)	130.4	7.41 (d, 9.0)	130.1	7.46 (d, 9.0)	130.4	7.40 (d, 9.0)	130.2	7.46 (d, 8.4)
7-OCH_3	-	-	55.8	3.88 (s)	55.8	3.88 (s)	56.4	3.96 (s)	56.4	3.95 (s)
8-OCH_3	-	-	-	-	-	-	61.8	3.90 (s)	61.8	3.89 (s)
4′-OCH_3	55.5	3.77 (s)	-	-	55.4	3.85 (s)	-	-	55.4	3.84 (s)
5-OH	-	-	-	12.83 (s)	-	12.85 (s)	-	12.66 (s)	-	12.69 (s)

Compound **6** was isolated as a white amorphous powder. The ^{1}H-NMR spectrum of **6** displayed the signals corresponding to two methyl singlets at δ_H 2.46 (3H, s) and 2.10 (3H, s), one methoxy singlet at δ_H 3.92 (3H, s), and one aromatic singlet at δ_H 6.21 (1H, s). Analysis of the ^{13}C-NMR spectrum, together with the HSQC spectrum, revealed ten signals, including one carbonyl group at δ_C 172.6 (C=O); two methyl groups at δ_C 24.1 (C-9) and 7.7 (C-8); one methine group at δ_C 110.6 (C-5); five sp^2 aromatic carbons at δ_C 163.2 (C-4), 158.1 (C-2), 140.1 (C-6), 108.5 (C-3) and 105.3 (C-1); and one methoxy group at δ_C 51.8 (CH_3O-7). The downfield chemical shifts of C-4 (δ_C 163.2) and C-2 (δ_C 158.1) suggested their linkage to oxygen atoms. In the HMBC spectrum, the correlations from H_3-8 (δ_H 2.10) to C-2/C-3/C-4, and from H_3-9 (δ_H 2.46) to C-1/C-5/C-6 revealed that these two methyl groups were located at C-3 and C-6, respectively. Furthermore, the HMBC correlation from the methoxy protons (δ_H 3.92) to the carbonyl carbon C-7 (δ_C 172.6) confirmed the presence of a methyl ester group. The substitution pattern of the aromatic ring and the location of the ester functionality were established by analysis of the HMBC data and further supported by comparison with literature values. Accordingly, compound **6** was identified as atraric acid [21],[22].

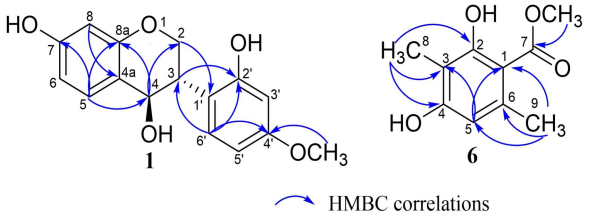


Fig. 2. Main HMBC correlations of compounds **1** and **6**

In the present study, six compounds (**1**–**6**) were isolated from the twigs of *M. leptobotrya*. Among them, compound **2** has previously been reported from this species [8], whereas compounds **3** and **4** are reported herein for the first time from *M. leptobotrya*. At the genus level, compound **3** was previously isolated from the root bark of *M. mannii* [23], while compound **4** was identified from the heartwood of *M. leucantha* [24]. The occurrence of these isoflavones in several *Millettia* species further supports the chemotaxonomic significance of isoflavonoids in *Millettia*. Notably, compounds **1** and **5** are reported from the genus *Millettia* for the first time. Although compound **5** has previously been detected in members of the Fabaceae family, such as *Clitoria ternatea*, based on HR-LC-MS analysis [25], this study provides the first isolation and full spectroscopic characterization of this compound from a Fabaceae species. Similarly, compound **1** has been reported from other Fabaceae species, including *Bolusanthus speciosus* [12] and *Gueldenstaedtia delavayi* [26], supporting its occurrence as a shared metabolite among different members of the Fabaceae family. To the best of our knowledge, compound **6** has not previously been reported from any members of the Fabaceae family. The discovery of this phenolic metabolite in *M. leptobotrya* further broadens the known phytochemical diversity of both the genus *Millettia* and the Fabaceae family.

Compounds **2**, **3**, and **6** have previously been reported to exhibit cytotoxic activities against various human cancer cell lines [21],[27],[28]. However, no information regarding the cytotoxic activities of compounds **1**, **4**, and **5** is available in the literature. Therefore, compounds **1**, **4**, and **5** were evaluated for their *in vitro* cytotoxic activities against four human cancer cell lines (KB, HepG2, A549, and MCF-7) using the MTT assay. As shown in Table 2, compound **1** exhibited weak cytotoxic activity against all four cell lines, with IC_{50} values ranging from 44.92 to 64.68 µg/mL, whereas compounds **4** and **5** displayed no significant activity under the tested conditions.

Table 2. Cytotoxic activities of compounds **1**, **4**, and **5** against human cancer cell lines

Compound	IC_{50} (µg/mL)			
	KB	**HepG2**	**A549**	**MCF-7**
1	44.92±2.85	48.70±2.95	64.68±2.89	49.23±3.48
4	110.0±4.24	115.03±3.67	98.91±4.11	109.24±4.51
5	> 128	> 128	> 128	> 128
Ellipticine	0.49±0.01	0.59±0.01	0.63±0.04	0.57±0.03

4. Conclusion

In summary, six compounds, including (+)-*trans*-4,7,2′-trihydroxy-4′-methoxyisoflavanol (**1**), prunetin (**2**), 5-hydroxy-4′,7-dimethoxyisoflavone (**3**), 5,4′-dihydroxy-7,8-dimethoxyisoflavone (**4**), isotectorigenin 7, 4′-dimethyl ether (**5**), and atraric acid (**6**) were isolated from the twigs of *M. leptobotrya* Dunn and structurally elucidated by analyses of 1D and 2D NMR spectra and comparison with the literature data. Among them, compounds **1** and **5** are reported from the genus *Millettia* for the first time, while compound **6** represents the first report of this compound from the family Fabaceae. Evaluation of the cytotoxic activities of compounds **1**, **4**, and **5** against KB, HepG2, A549, and MCF-7 human cancer cell lines revealed that compound **1** exhibited weak cytotoxic activity, and compounds **4** and **5** were inactive under the tested conditions. These findings expand the phytochemical knowledge of *M. leptobotrya* and provide the first report on the cytotoxic activities of compounds **1**, **4**, and **5**.

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