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Two new phenylpropanoids from *Micromelum integerrimum*

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[ABSTRACT]

AIM: To investigate the chemical and bioactive constituents from the stems and leaves of *Micromelum integerrimum*.

METHOD: The chemical constituents were isolated and purified by silica gel, Sephadex LH-20, and HPLC. Their structures were mainly elucidated on the basis of extensive 1D- and 2D-NMR spectroscopy and mass spectrometry. Their cytotoxicity and antimicrobial activities were tested by the SRB and turbidimetric methods, respectively.

RESULTS: Two new phenylpropanoids and two known coumarins were obtained, and their structures were identified as microintegerrin A (**1**), microintegerrin B (**2**), scopoletin (**3**), and scopolin (**4**). All of the compounds were tested for their cytotoxicity against three cancer cell lines (HeLa, A549, and BGC-823) and for antimicrobial activity against the fungus *Candida albicans* and the bacterium *Staphylococcus aureus*.

CONCLUSION: Two new phenylpropanoids **1** and **2** were isolated and identified from the stems and leaves of *M. intgerrimum*. None of the compounds showed cytotoxic or antimicrobial activity at the tested concentration of 20 μg·mL^{-1}.

[KEY WORDS] *Micromelum integerrimum*; Rutaceae; Phenylpropanoids; Microintegerrin A; Microintegerrin B

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Introduction

Micromelum integerrimum (Buch.-Ham. ex DC.) Wight & Arn. ex M. Roem. (Rutaceae) is a tree distributed widely in Yunnan, Hainan, Guizhou, Guangxi, and Guangdong provinces in China [1]. It has been used for the treatment of colds and trauma, and its roots are used as a folk medicine to relieve stomach pain [1]. Coumarins, alkaloids, and flavonoids have been isolated from the *Micromelum* genus previously [2]. Coumarins and alkaloids have also been characterized from *M. integerrimum* [3-5]. Searching for bioactive compounds from this plant, the chemical investigation of the stems and leaves of *M. integerrimum* was carried out. All of the four isolates were tested for their cytotoxicity on three cancer cell lines (HeLa, A549, and BGC-823) and for antimicrobial activity against *Candida albicans* and *Staphylococcus aureus*.

Results and Discussion

Compound **1** was obtained as yellow oil. The HREI-MS of **1** revealed an ion peak at *m/z* 238.083 8 [M]$^+$, corresponding to the molecular formula $C_{12}H_{14}O_5$ (Calcd. 238.084 1). The IR spectrum of **1** indicated the presence of hydroxyl (3 426 cm^{-1}), aldehyde (2 854 cm^{-1}), carbonyl (1 722, 1 654 cm^{-1}), and phenyl (1 609, 1 509, 1 453, 1 401 cm^{-1}) groups. The UV spectrum showed absorptions indicating the presence of an aromatic ring (233, 277, 324 nm). Compound **1** was identified as a 1, 2, 4, 5-substituted phenylpropanoid by comparison

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of the NMR spectra data (Table 1) with those of methyl 3-(2-hydroxy-4-methoxy-5-propanoylphenyl) propanoate [7]. The ^{1}H NMR spectrum showed two methoxyl singlets at δ 3.63 (s, 3H) and 3.86 (s, 3H), and an aldehyde group at δ 10.08 (s, 1H). The signals of δ 6.44 (1H, s) and 7.53 (1H, s) revealed the presence of a 1, 2, 4, 5-substituted benzene ring. The signals at δ 2.58 (2H, t, J = 7.3 Hz) and 2.83 (2H, t, J = 7.3 Hz) indicated a –CH_2CH_2– fragment. The ^{13}C NMR and DEPT spectra of compound **1** confirmed the existence of two methoxyl groups at δ 52.0 and 56.1, an aldehyde group at δ 189.7, an aromatic ring at δ 99.1, 118.2, 121.7, 131.4, 164.7, and 165.4, two CH_2 at δ 26.2 and 34.7, and a carbonyl at δ 175.4. The 9-OCH_3 was located at C-9 by the cross-peak of δ 3.63 and δ 175.4 in the HMBC spectrum (Fig. 2). Moreover, the location of another methoxyl group was determined by the HMBC correlation between δ 3.86 and δ 165.4 (C-2). The aldehyde group was attached to C-5 on the basis of long-range correlations between δ 10.08 with δ 118.2 (C-5) and 131.4 (C-6). At the same time, long-range correlations from H-8 (δ 2.58) to δ 121.7 (C-1), and from δ 2.83 (H-7) to δ 121.7 (C-1), 165.4 (C-2), 131.4 (C-6) and 34.7 (C-8) showed that C-7 was connected to C-1. Thus, the structure of compound **1** was elucidated and named microintegerrin A (Fig. 1).

Table 1 ^{1}H and ^{13}C NMR spectra data of the compounds 1 and 2

No.	**1**[a]		**2**[a]	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		121.7, C		121.4, C
2		165.4, C		157.1, C
3	6.44 (1H, s)	99.1, CH	6.44 (1H, s)	100.5, CH
4		164.7, C		153.6, C
5		118.2, C		118.3, C
6	7.53 (1H, s)	131.4, CH	6.79 (1H, s)	131.6, CH
7	2.83 (2H, t, 7.3)	26.2, CH_2	2.79 (2H, overlapped)	23.6, CH_2
8	2.58 (2H, t, 7.3)	34.7, CH_2	2.68 (2H, overlapped)	35.4, CH_2
9		175.4, C		176.7, C
10	10.08 (1H, s)	189.7, CH	2.79 (2H, overlapped)	24.4, CH_2
11			2.68 (2H, overlapped)	44.0, CH_2
12				209.1, C
13			2.12 (3H, s)	29.9, CH_3
9-OCH_3	3.63 (3H, s)	52.0, CH_3	3.68 (3H, s)	52.3, CH_3
2-OCH_3	3.86 (3H, s)	56.1, CH_3	3.75 (3H, s)	55.2, CH_3

[a] ^{1}H and ^{13}C NMR spectra of compounds **1** and **2** were recorded on an AV-400 spectrometer in CD_3OD

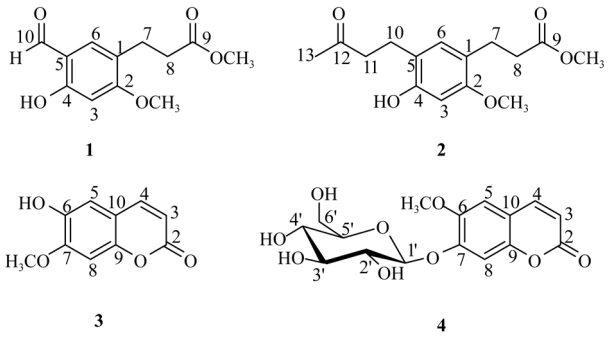

Fig. 1 Structures of compounds 1-4

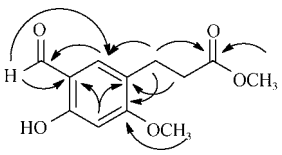

Fig. 2 Key HMBC correlations of compound 1

Compound **2** was obtained as a white powder. The HREI-MS of **2** revealed an ion peak at *m/z* 280.131 3 $[M]^+$, corresponding to the molecular formula $C_{15}H_{20}O_5$ (Calcd. 280.131 1). Comparison of the 1D- and 2D-NMR data of **2** with those of **1** suggested their structures were closely related. The main differences were that the aldehyde attached to C-5 in **1** was absent in **2**, while two methylenes, one carbonyl and one methyl groups were present. In the HMBC spectrum (Fig. 3), long-range correlations from δ 2.68 and 2.79 to δ 118.3 (C-5) suggested that C-10 was connected to C-11. Furthermore, long-range correlations from δ 2.12 (3H, s) to δ 44.0 (C-11) and 209.1 (C-12) showed the existence of –$CH_2CH_2COCH_3$ group. The cross-peak of δ 6.79 (H-6) and δ 24.4 (C-10) revealed that the C-10 was attached to C-5. Thus, compound **2** was determined and named microintegerrin B (Fig. 1).

Compounds **3** and **4** were identified as scopoletin and scopolin by comparison of their spectral data with those in the literature [8-9].

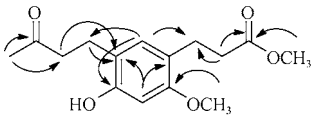

Fig. 3 Key HMBC correlations of compound 2

Compounds **1**−**4** did not show cytotoxicity against HeLa, A549, and BGC-823 cancer cell lines or antimicrobial activities against *C. albicans* and *S. aureus* at the tested concentration of 20 μg·mL^{-1}.

Experimental

General experiment procedures

NMR spectra were recorded on a Bruker AV-400 spectrometer using tetramethylsilane (TMS) as an internal standard. MS spectra were recorded with a VG Autospec-3000 spectrometer. IR spectra were obtained with a Tensor 27 instrument using KBr pellets. UV spectra were recorded on a Shimadzu UV-2410 PC spectrophotometer. Silica gel (100–200 mesh, 200–300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography. Semipreparative HPLC was performed on an Agilent 1100 apparatus equipped with a diode-array detector and an Eclipse XDB-C_{18} (Agilent, 9.4 mm × 250 mm) column. Preparative HPLC was performed on an Agilent 1100 apparatus with a diode-array detector and a Sun Fire ^{TM} Pre C_{18} OBD^{TM} (Waters, 19 mm × 250 mm, 5 μm) column. TLC was carried out on precoated silica gel GF_{254} glass plates (Qingdao Marine Chemical, Inc. China). Spots were first visualized under UV light (254 and 365 nm), followed by spraying with 5% H_2SO_4 in EtOH and then heating.

Plant material

The stems and leaves of *M. integerrimum* were collected in Xishuangbanna, Yunnan Province, China, in September 2011. The plant was identified by Prof. PENG Hua, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (KUN No. 0182256) has been deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

Air-dried stems and leaves of *M. integerrimum* (29 kg) were extracted four times with methanol at 70 °C. After filtration, the solvent was evaporated under reduced pressure to give the MeOH extract (4.5 kg), which was suspended in water, and then partitioned successively with petroleum ether, EtOAc, and *n*-BuOH. The *n*-BuOH extract (400 g) was subjected to a silica gel (100–200 mesh) column, and eluted with a $CHCl_3$−MeOH gradient (1 : 0, 30 : 1, 15 : 1, 9 : 1, 8 : 2, 7 : 3, 1 : 1, 0 : 1) to give 19 fractions (Fr. 1−Fr. 19). Fr. 9 was chromatographed on repeated silica gel (200−300 mesh) columns to yield seven subfractions (B1−B7). B4 was subjected to Sephadex LH-20 with $CHCl_3$-MeOH (1 : 1), and then purified by semipreparative HPLC using H_2O-CH_3CN (7 : 3) to obtain compounds **1** (11.0 mg, purity > 95%) and **2** (22.5 mg, purity > 95%). B1 was purified by preparative HPLC with H_2O-CH_3CN (7 : 3) to afford compounds **3** (45.5 mg, purity > 95%) and **4** (545.3 mg, purity > 95%).

Cytotoxic assay

The cytotoxicity of compounds **1**−**4** against the A549, HeLa, and BGC-823 cancer cell lines was evaluated by the SRB assay with taxol as a positive control [6]. The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2 for 48 h. Aliquots of 90 μL were seeded in 96-well flat-bottomed microtiter plates for 24 h and treated with compounds **1**−**4** at the maximum concentration of 20 μg·mL^{-1}. After incubation for another 48 h, cells were fixed with 25 μL of ice-cold 50% trichloroacetic acid and incubated at 4 °C for 1 h. After washing, air-drying, and staining for 15 min with 100 μL 0.4% SRB in 1% glacial acetic acid, excessive dye was removed by washing with 1% glacial acetic acid. SRB was resuspended in 10 mmol·L^{-1} Tris buffer (100 μL), and the absorbance was measured at 560 nm with a Plate Reader (Molecular Devices, Spectra Max 340). The percentage inhibition of cell growth below 50% is regarded as inactive.

Antimicrobial assay

Compounds **1**–**4** were tested for their antimicrobial activity against *C. albicans* and *S. aureus in vitro* using a turbidimetric method [6]. Miconazole nitrate was used as a positive control. *C. albicans* and *S. aureus* were inoculated in potato dextrose broth (formulated identically to potato dextrose agar (PDA), omitting the agar, prepared in this laboratory) and Mueller Hinton Broth (Oxiod, CM0405, England) to McFarland 0.5 and diluted with the medium to 1 × 10^6 CFU·mL^{-1}. Aliquots of 90 μL were filled in 96-well U-bottomed microplates, and then treated with compounds **1**–**4** at the maximum concentration of 20 μg·mL^{-1}. After culturing at 37 °C for 24 h, the absorbance was measured at 620 nm with the microplate reader. The percentage inhibition of cell growth below 50% is regarded as inactive.

Identification

Microintegerrin A (1) Yellow oil; $C_{12}H_{14}O_5$; ESI-MS *m/z* (%): 261 $[M + Na]^+$ (18); HREI-MS: *m/z* 238.083 8 $[M]^+$ (Calcd. for $C_{12}H_{14}O_5$: 238.084 1); IR (KBr): 3 426, 2 951, 2 928, 2 854, 1 722, 1 654, 1 609, 1 509, 1 453, 1 401, 1 384, 1 285, 1 207, 1 121 and 1 015 cm^{-1}; UV (MeOH) λ_{max} (log ε): 204 (4.0), 233 (3.8), 277 (3.6), and 324 (3.7) nm; ^{1}H and ^{13}C NMR spectral data, see Table 1.

Microintegerrin B (2) White powder; $C_{15}H_{20}O_5$; ESI-MS *m/z* (%): 303 $[M + Na]^+$ (100); HREI-MS *m/z* (%): 280.131 3 $[M]^+$ (Calcd. for $C_{15}H_{20}O_5$: 280.131 1); IR (KBr): 3 441, 3 426, 2 958, 2 926, 1 684, 1 632, 1 573, 1 554, 1 513, 1 453, 1 399, 1 384, 1 307, 1 205, 1 136, 1 107, 1 017, 724, and 612 cm^{-1}; UV (MeOH) λ_{max} (log ε): 203 (4.1), 285 (3.2), and 321 (2.6) nm; ^{1}H and ^{13}C NMR spectral data, see Table 1.

Scopoletin (3) White powder, $C_{10}H_8O_4$, ESI-MS *m/z* (%):

215 $[M + Na]^+$ (100), 407 $[2M + Na]^+$ (6). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 3.95 (3H, s, 7-OCH_3), 6.27 (1H, d, J = 9.2 Hz, H-3), 6.85 (1H, s, H-8), 6.91 (1H, s, H-5), 7.60 (1H, d, J = 9.2 Hz, H-4). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 56.2 (CH_3, 7-OCH_3), 103.2 (CH, C-8), 107.9 (CH, C-5), 111.1 (C, C-10), 112.3 (CH, C-3), 144.0 (CH, C-4), 145.0 (C, C-9), 150.0 (C, C-6), 150.6 (C, C-7), 162.4 (C, C-2).

Scopolin (4) White powder, $C_{16}H_{18}O_9$, ESI-MS *m/z* (%): 377 $[M + Na]^+$ (100), 731 $[2M + Na]^+$ (2). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.10−3.70 (6H, H-2', 3', 4', 5', 6'), 3.80 (3H, s, 6-OCH_3), 5.06 (1H, overlapped, H-1'), 6.31 (1H, d, J = 9.5 Hz, H-3), 7.14 (1H, s, H-5), 7.28 (1H, s, H-8), 7.95 (1H, d, J = 9.5 Hz, H-4). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 56.1 (CH_3, 6-OCH_3), 60.7 (CH_2, C-6'), 69.6 (CH, C-4'), 73.1 (CH, C-2') , 76.8 (CH, C-5') , 77.1 (CH, C-3') , 99.6 (CH, C-1'), 103.0 (CH, C-8), 109.7 (CH, C-5), 112.3 (C, C-10), 113.3 (CH, C-3), 144.3 (CH, C-4), 146.0 (C, C-6), 149.0 (C, C-9), 149.9 (C, C-7), 160.6 (C, C-2).

Conclusion

Phenylpropanoids have been isolated from *M. minutum* and *M. falcatum* [4, 10], but this is the first isolation of phenylpropanoids from *M. integerrimum*. Compounds **1** and **2** might be derived from coumarins. The four isolated compounds **1**−**4** did not show cytotoxicity (HeLa, A549, and BGC-823) or antimicrobial activity (*C. albicans* and *S. aureus*). Several coumarins from the *Micromelum* genus have shown cytotoxicity and antimicrobial activity [2]. In addition, *M. integerrimum*, as a folk medicine for the treatment of cold and trauma, could have antimicrobial activity. So new cytotoxic and antimicrobial coumarins and phenylpropanoids from *M. integerrimum* need further research.

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