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Flavonoid glycosides from *Microtea debilis* and their cytotoxic and anti-inflammatory effects

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ABSTRACT

Two new 5-*O*-glucosylflavones, 5-*O*-β-D-glucopyranosyl cirsimaritin (**1**) and 5, 4′-*O*-β-D-diglucopyranosyl cirsimaritin (**2**), four known flavonoids, cirsimarin (**3**), cirsimaritin (**4**), salvigenin (**5**), 4′, 5-dihydroxy-7-methoxyflavone (**6**), and a norisoprenoid, vomifoliol (**7**), have been isolated from the aerial parts of *Microtea debilis*. All isolates were tested for cytotoxicity in human cancer cell lines (Hep G2, COLO 205, and HL-60) and anti-inflammatory activities in LPS-treated RAW264.7 macrophages. Compound **6** was found to be a potent inhibitor to nitrite production in macrophages. Compounds **2**, **4**, **6**, and **7** showed moderate anti-proliferative activity against COLO-205 cells with IC_{50} values of 7.1, 13.1, 6.1, and 6.8 μM, respectively.

1. Introduction

Microtea debilis Sw. (Phytolaccaceae), commonly known as weak jumby pepper, is a small creeping annual weed originating from South America. It is used in folk medicine to treat cough, stomach ache [1], acute renal failure, and proteinuria [2,3]. It has been reported that the anti-proteinuric effect of the plant is probably related to the adenosine-A_1 antagonistic action of cirsimarin, one of the major flavonoids found in *M. debilis* [2,3]. Cirsimarin was also found to exert a strong lipolytic activity with 20 times more potency than caffeine to trigger lipid mobilization [4]. In vitro, cirsimarin induced a dose-dependent inhibition of lipid peroxidation, indicating moderate antioxidant activity, and exerted a direct action on hydrogen peroxide production by adipose tissue cells [5].

As a part of our continued effort to find phytochemicals with antitumor and anti-inflammatory activities from herbs, a 95% ethanol extract of the aerial parts of *M. debilis* was subjected to column chromatography over SP 70, MCI GEL CHP-20P, and Sephadex LH-20 resins to afford a total of seven pure compounds. Two of the isolates were unusual flavone glycosides possessing an *O*-β-D-glucopyranosyl functionality at the C-5 position, 5-*O*-β-D-glucopyranosyl cirsimaritin (**1**) and 5, 4′-*O*-β-D-diglucopyranosyl cirsimaritin (**2**). Five known compounds were identified as cirsimarin (**3**) [3], cirsimaritin (**4**) [3], salvigenin (**5**) [6,7], 4′, 5-dihydroxy-7-methoxyflavone (**6**) [8], and vomifoliol (**7**) [9,10] by comparison of the observed spectroscopic data with those of the literature. Compounds **5**–**7** were isolated from this plant for the first time.

2. Experimental

2.1. General

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. FT-IR was performed on a Perkin-Elmer BX system (Perkin-Elmer Instruments, Norwalk, CT) with a

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MIRacle ATR accessory (Pike Technologies, Madison, WI). UV spectra were acquired on a Shimadzu, UV-1700 UV–Visible Spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on an Inova-400 (^{1}H at 400 MHz) instrument (Varian Inc., Palo Alto, CA) with DMSO-d_6 as the solvent (Aldrich Chemical Co., Allentown, PA). The 2D correlation spectra were obtained using standard gradient pulse sequences of Varian VNMR software and performed on 4-nuclei PFG AutoSwitchable or PFG Indirect Detection probes. HRESIMS was run on a Waters Micromass LCT (Waters Corporation, Milford, MA) or a Thermo Scientific LTQ Orbitrap mass spectrometers (Thermo-Finnigan, San Jose, CA). GC–MS analysis was carried out on an Agilent HP 6890 Series Gas Chromatograph system and Agilent HP 5973 Mass Spectrometer (Santa Clara, CA) with Rxi-1ms capillary GC column (60 m × 0.25 mm ID × 1.0 μm). HPLC analysis was performed on an Agilent 1100 LC series and the column used was 250 × 4.6 mm i.d., 5 μm, Luna C-8 (Phenomenx Inc.) with a flow rate of 1.0 mL/min. The solvent system consisted of a linear gradient that started with 5% (v/v) MeCN in 0.1% TFA/H_2O, increased to 95% MeCN over 40 min, and increased to 100% MeCN within 5 min. At the end of the run, 100% MeCN was allowed to flush the column for 5 min and an additional 10 min of post run time was set to allow for equilibration of the column. The UV detector was set at 280 nm wavelength and column temperature was ambient.

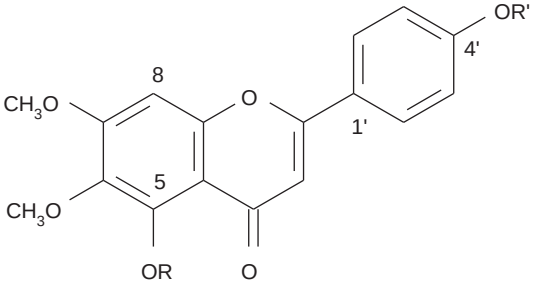
2.2. Plant material

The aerial parts of *M. debilis* were collected in Surinam. The plant was identified by macroscopic examination, TLC, and HPLC methods compared with authentic sample. A voucher specimen (MT105003) was deposited in the Herbarium of Naturex, Inc. (South Hackensack, NJ).

2.3. Extraction and isolation

The air-dried and powdered aerial parts (420 g) were extracted with 95% EtOH three times at 50 °C. The combined EtOH extracts were concentrated (61 g) and was chromatographed on SP 70 resin (Sigma Chemical Co., St. Louis, MO) (1.0 L, 5.5 cm × 75 cm), eluted with gradient H_2O–EtOH system (1:0, 10:1, 5:1, 3:1, 2:1, 1:1, 1:2, and 0:1). In each gradient step, 2.5 L of eluent was used and 0.5 L was collected as one fraction. A total of 40 fractions were collected in which fractions with similar HPLC chromatograms were combined and concentrated. Fractions 10–11 were chromatographed over MCI GEL CHP-20P (Mitsubishi Kasei Co.) (100 mL, 2.5 cm × 40 cm) and eluted with gradient H_2O–MeOH (9:1 → 0:1). The fractions collected from CHP-20P were further purified on Sephadex LH-20 (Sigma Chemical Co.) (100 mL, 2.5 cm × 40 cm) resulted in the isolation of **1** (31 mg, t_R = 10.0 min). In the same manner, repeated chromatography of different fractions using MCI GEL CHP-20P and Sephadex LH-20 yielded **2** (5 mg, t_R = 8.1 min, fractions 7–8), **3** (120 mg, t_R = 12.7 min, fractions 12–15), **4** (65 mg, t_R = 27.4 min, fractions 21–36), **5** (3 mg, t_R = 27.8 min, fraction 37), **6** (4 mg, t_R = 29.2 min, fractions 39–40), and **7** (4 mg, t_R = 9.9 min, fractions 7–8).

Compound (**1**, Fig. 1), 5-*O*-β-D-glucopyranosyl cirsimaritin, yellow amorphous powder, $[\alpha]_D^{25}$ −69.9 (c 0.76, DMSO); UV (MeOH) 217, 235, 266, and 330 nm; IR ν_{max} 3310, 3194, 2164, 2016, 1638, 1605, 1514, 1453, 1355, 1253, 1049, 1010, and 832 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS: *m/z* 477.1415 $[M+H]^+$ (calcd for $C_{23}H_{25}O_{11}$, 477.1397).



1 R = β-D-glucopyranosyl, R' = H
2 R = R' = β-D-glucopyranosyl
3 R = H, R' = β-D-glucopyranosyl
4 R = R' = H

Fig. 1. Structures of **1–4** from *Microtea debilis*. **1** R = β-D-glucopyranosyl, R′ = H. **2** R = R′ = β-D-glucopyranosyl. **3** R = H, R′ = β-D-glucopyranosyl. **4** R = R′ = H.

Compound (**2**, Fig. 1), 5, 4′-*O*-β-D-diglucopyranosyl cirsimaritin, yellow amorphous powder, $[\alpha]_D^{25}$ −1.2 (c 0.25, DMSO); UV (MeOH) 218, 268, and 325 nm; IR ν_{max} 3358, 2363, 2020, 1637, 1605, 1514, 1455, 1355, 1253, 1116, 1010, and 833 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS: *m/z* 639.1929 $[M+H]^+$ (calcd for $C_{29}H_{35}O_{16}$, 639.1925).

2.4. Aglycone analysis

A total of 10 mg of **1** was dissolved in 1 mL of H_2O along with 0.1 mL of conc. HCl. The solution was heated at 65 °C for 2 h and dried under vacuum. The residue was washed with H_2O three times to remove sugar and the aglycone (**4**) was obtained for NMR analysis. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.83 (1H, s, H-3), 6.91 (1H, s, H-8), 7.95 (2H, d, *J* = 6.0 Hz, H-2′, 6′), 6.92 (2H, d, *J* = 6.0 Hz, H-3′, 5′), 3.72 (3H, s, 6-OCH_3), 3.90 (3H, s, 7-OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.1 (C-2), 102.7 (C-3), 182.3 (C-4), 152.1 (C-5), 131.9 (C-6), 158.7 (C-7), 91.6 (C-8), 152.7 (C-9), 105.1 (C-10), 121.1 (C-1′), 128.6 (C-2′ and 6′), 116.0 (C-3′ and 5′), 161.4 (C-4′), 56.5 (7-OCH_3), 60.1 (6-OCH_3). ESIMS: *m/z* 315 $[M+H]^+$, $C_{17}H_{15}O_6$.

2.5. Sugar analysis

A solution of **1** or **2** (2.0 mg each) in 1 N HCl (1 mL) was stirred at 85 °C for 3 h. The solution was evaporated under a stream of N_2. The residue was dissolved in 0.1 mL of Tri–Sil Z (*N*-trimethylsilylimidazole: pyridine: 1:4, Pierce Biotechnology, Rockford, IL) and the mixture was allowed to react at 60 °C for 15 min. After drying under a stream of N_2, the residue was dissolved in 1 mL of water and partitioned with 1 mL of CH_2Cl_2. The CH_2Cl_2 layer was analyzed by GC–MS (Rxi-1ms GC column, temperatures for inlet injection 200 °C; temperature gradient system was used for the oven, starting at 120 °C for 1 min and then raised to 280 °C at rate of 40 °C/min). D-Glucose was identified for **1** and **2** by comparison with retention time of authentic D-glucose (t_R = 9.89 min) after treatment in the same manner with Tri–Sil Z.

Table 1
NMR data of **1** and **2** (DMSO-d_6).

pos.	1			2		
	δ_H (J in Hz)	δ_C [a] mult.	HMBC [b]	δ_H (J in Hz)	δ_C mult.	HMBC
2		161.6, qC			160.8 qC	
3	6.74 s	105.5CH	2,4,10,1′ NOE: 2′,6′	6.85 s	106.3 CH	2,4,10,1′ NOE: 2′,6′
4		177.5 qC			177.4 qC	
5		148.2 qC			148.0 qC	
6		139.9 qC			139.8 qC	
7		158.2 qC			158.2 qC	
8	7.26 s	97.8 CH	4,6,7,9,10	7.33 s	97.8 CH	4,6,7,9,10
9		154.0 qC			153.9 qC	
10		110.8 qC			110.8 qC	
6-OCH_3	3.79 s	61.0 CH_3	6	3.79 s	60.8 CH_3	6
7-OCH_3	3.95 s	56.7 CH_3	7; NOE: 8	3.97 s	56.6 CH_3	7, NOE: 8
1′		121.1 qC			123.7 qC	
2′	7.94 dd (6.8,1.6)	128.3 CH	2,4′,6′	8.06 d (8.8)	127.9 CH	2,4′,6′
3′	6.92 dd (6.8,1.6)	116.1 CH	1′,4′,5′	7.19 d (8.8)	116.5 CH	1′,4′,5′
4′		161.1 qC			160.1 qC	
5′	6.92 dd (6.8,1.6)	116.1 CH	1′,3′,4′	7.19 d (8.8)	116.5 CH	1′,3′,4′
6′	7.94 dd (6.8,1.6)	128.3 CH	2,2′,4′	8.06 d (8.8)	127.9 CH	2,2′,4′
1″	4.77 d (7.6)	105.4 CH	5	4.79 d (8.0)	105.2 CH	5
2″	3.34 m	74.1 CH	1″, 3″	3.38 m	74.0 CH	
3″	3.14 m	77.8 CH		3.13 m	77.7 CH	
4″	3.23 m	69.9 CH		3.25 m	69.8 CH	
5″	3.22 m	76.6 CH		3.45 m	76.6 CH	
6″	3.69 d (11.6)	60.8 CH_2		3.71 m	60.6 CH_2	
	3.34 m			3.65 m		
1‴				5.03 d (7.6)	99.8 CH	4′, NOE: 3′,5′
2‴				3.33 m	73.2 CH	
3‴				3.17 m	77.2 CH	
4‴				3.20 m	69.6 CH	
5‴				3.48 m	76.5 CH	
6‴				3.67 m	60.6 CH_2	

[a] Carbon multiplicities were determined by DEPT experiments.
[b] Two and three bonds H–C correlations.

2.6. Cytotoxic analysis

The COLO-205 cell line was isolated from human colon adenocarcinoma (ATCC CCL-222); human promyelocytic leukemia (HL-60) cells were obtained from American Type Culture Collection (Rockville, MD). The human Hep G2 hepatocellular carcinoma cells (BCRC 60025) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). COLO-205 and HL-60 cell lines were grown at 37 °C in 5% CO_2 atmosphere in RPMI. Hep G2 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY), 100 units/mL of penicillin and 100 μg/mL of streptomycin, and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air. Selected compounds were dissolved in dimethyl sulfoxide (DMSO). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [11]. Briefly, human cancer cells were plated at a density of 1×10^5 cells/mL into 24 well plates. After overnight growth, cells were pretreated with series of concentration of test compounds for 24 h. The final concentration of DMSO in the culture medium was <0.05%. At the end of treatment, 30 μL of MTT was added, and the cells were incubated for a further 4 h. Cell viability was determined by scanning with an enzyme-linked immunosorbent assay reader with a 570 nm filter.

2.7. Nitrite assay

The RAW264.7 cells were treated with isolated compounds and LPS (*Escherichia coli* O127:E8, molecular weight, 60 kDa, Sigma Chemical Co.) together or LPS alone. The supernatants are harvested and the amount of nitrite, an indicator of NO synthesis, is measured by use of the Griess reaction. Briefly, supernatants (100 μL) are mixed with the same volume of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) in duplicate on 96-well plates. After incubation at room temperature for 10 min, absorbance at 570 nm is measured with an ELISA reader (Thermo Labsystems Multiskan Ascent, Finland).

3. Results and discussion

Compound **1** was obtained as a light yellow amorphous powder. The HRESIMS displayed a protonated molecular ion at m/z 477.1415 $[M+H]^+$, suggesting a molecular formula of $C_{23}H_{24}O_{11}$. The IR spectrum of **1** showed absorptions indicative of a hydroxyl group at 3310 cm^{-1} and an aromatic ring at 1638 and 1605 cm^{-1}. Its UV spectrum showed

Table 2
Effect of **1**–**7** on LPS-induced nitrite production in RAW 264.7 macrophages.

	1	**2**	**3**	**4**
Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
LPS	37.6 ± 1.5	37.6 ± 1.5	37.6 ± 1.5	37.6 ± 1.5
20 μg/mL	30.1 ± 1.1	24.9 ± 1.9	28.8 ± 1.3	20.1 ± 1.5
40 μg/mL	31.0 ± 2.4	25.2 ± 1.5	27.2 ± 1.5	12.2 ± 2.1
	5	**6**	**7**	
Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	
LPS	37.6 ± 1.5	37.6 ± 1.5	37.6 ± 1.5	
20 μg/mL	28.0 ± 0.8	2.1 ± 0.9	25.5 ± 1.4	
40 μg/mL	24.0 ± 0.9	0.0 ± 0.2	18.4 ± 2.9	

maximum absorptions at 217, 235, 266, and 330 nm, typical of a flavone. The ^{13}C NMR spectral signals revealed the presence of a flavonoid with two methoxy groups (δ 56.7, 61.0) and one glucopyranosyl unit (δ 105.4, 74.1, 77.8, 69.9, 76.6, and 60.8). The ^{1}H NMR spectrum of **1** contained signals of a 4′-hydroxy substituted B-ring with an AB spin system at δ 7.94 (2H, dd, J = 6.8, 1.6 Hz, H-2′, 6′) and 6.92 (2H, dd, J = 6.8, 1.6 Hz, H-3′, 5′). The presence of a singlet at δ 7.26 was attributed to the proton at H-8 in ring A. The observed long-range correlation of two *O*-methyl protons at δ 3.79 (3H, s, OCH_3) to C-6 (δ 139.9) and δ 3.95 (3H, s, OCH_3) to C-7 (δ 158.2) in the HMBC indicated the location of the two methoxy groups at C-6 and C-7, respectively. The aglycone obtained from the acid hydrolysis of **1** was identified as cirsimaritin (**4**) by comparison of NMR data with those in literature [3], indicative of **1** a cirsimaritin glucoside. The glucosyl moiety was determined to be attached at the C-5 hydroxyl group due to an upfield shift of the C-5 signal from δ 152.1 in **4** [3] to δ 148.2 in **1**, and was confirmed by the observed correlation between H-1″ and C-5 in the HMBC spectrum. The coupling constant of H-1″ (δ 4.77, d, J = 7.6 Hz) suggested a β-anomeric configuration for the glucosyl and its D-configuration was determined via GC analysis of chemical derivatives of the glucose of **1** obtained by acid hydrolysis and the same derivative of standard D-glucose. Thus, **1** was identified as 5-*O*-β-D-glucopyranosyl cirsimaritin based on the above spectroscopic data and chemical analysis.

The HRESIMS of **2** displayed protonated molecular ion at m/z 639.1929 $[M+H]^+$, suggesting a molecular formula of $C_{29}H_{34}O_{16}$. The IR and UV spectra of **2** were merely identical to those of **1**. The ^{13}C NMR spectrum of **2** displayed very similar signals to those of **1**, specifically a flavone with two methoxy groups (δ 56.6 and 60.8), but with two glucopy ranosyl units (Table 1). The two methoxy groups and one glucosyl were determined at C-6, C-7, and C-5 positions, respectively by the observed correlations of the two *O*-methyl protons and anomeric H-1″ to C-6, C-7, and C-5, respectively in HMBC (Table 1). It was thus suggested that **2** had the same skeleton as **1**, and the site of the remaining glucopyranosyl moiety was considered at the C-4′ hydroxyl group by the comparison of the ^{13}C NMR data of the B-ring between **2** and cirsimarin (4′-*O*-cirsimaritin glucopyranoside, **3**), where similar chemical shifts of C-1′ to C-6′ were found between **1** and **3** [3]. The direct evidence came from the observed long-range H–C correlation of H-1‴ (δ 5.03, d, J = 7.6 Hz) to C-4′ (δ 160.1) in the HMBC spectrum and NOE correlation of H-1‴ to H-3′, H-5′ (δ 7.19, d, J = 8.8 Hz) in the ROESY spectrum. The β-anomeric configurations for the two glucosyl moieties were assigned based on the coupling constants of H-1″ (δ 4.79, d, J = 8.0 Hz) and H-1‴ (J = 7.6 Hz). The D-configuration glucose in **2** was determined in the same manner as that described for **1**. Therefore, the new isolate **2** was identified as 5, 4′-*O*-β-D-diglucopyranosyl cirsimaritin.

Table 3
Effect of **1**–**7** on the growth of various human cancer cells.

Compound	HL-60	Cell line	COLO 205
IC_{50} (μM)		Hep G2	
1	>100	>100	>100
2	>100	50.4 ± 2.9	7.1 ± 1.0
3	>100	>100	>100
4	61.0 ± 2.2	>100	13.1 ± 0.6
5	>100	>100	>100
6	55.9 ± 3.1	50.0 ± 4.8	6.1 ± 0.3
7	55.6 ± 0.5	45.5 ± 2.0	6.8 ± 0.5
Doxorubicin [a]	5.0 ± 0.6	10.0 ± 4.3	11.9 ± 4.9

Each experiment was independently performed three times and expressed as mean ± SE.
[a] Positive control.

To investigate the anti-inflammatory effect, the isolated compounds **1**–**7** were incubated at 20 and 40 μg/mL with lipopolysaccharide (LPS)-activated RAW 264.7 cells for 24 h. Compound **6** displayed potent activity against nitrite production in macrophages induced by LPS (100 ng/mL), while **4** and **7** were moderately active, **2**, **3**, and **5** weakly active, and **1** inactive (Table 2). In addition, these compounds were tested for cytotoxicity against three human cancer cell lines, Hep G2, COLO-205, and HL-60. The results showed that **2**, **4**, **6**, and **7** exhibited moderate anti-proliferative activity against COLO-205 cells with IC_{50} values of 7.1, 13.1, 6.1, and 6.8 μM, respectively. Compounds **1**–**7** were inactive against the other two cell lines (Table 3).

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