Flavonol Glycosides from *Muehlenbeckia platyclada* and Their Anti-inflammatory Activity

Chiao-Ting YEN,[a] Pei-Wen HSIEH,[b] Tsong-Long HWANG,[b] Yu-Hsuan LAN,[c] Fang-Rong CHANG,[a,d] and Yang-Chang WU*[,a,d,e]

[a] *Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University; Kaohsiung 807, Taiwan:* [b] *Graduate Institute of Natural Products, Chang Gung University; Tao-Yuan 333, Taiwan:* [c] *College of Pharmacy, China Medical University; Taichung 404, Taiwan:* [d] *Center of Excellence for Environmental Medicine, Kaohsiung Medical University; Kaohsiung 804, Taiwan: and* [e] *National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center; Kaohsiung 804, Taiwan.*

Received September 25, 2008; accepted November 18, 2008; published online December 3, 2008

A new flavonol, morin-3-*O*-α-rhamnopyranoside (1), along with four known flavonols, kaempferol 3-*O*-α-rhamnopyranoside (2), kaempferol 3-*O*-β-glucopyranoside (3), quercetin 3-*O*-α-rhamnopyranoside (4) and (+)-catechin (5), were isolated from the methanolic extract of *Muehlenbeckia platyclada*. The structures of these compounds were determined on the basis of chemical and spectroscopic evidence, as well as acid hydrolysis of the original glycoside. Isolates were evaluated for inhibition of generation of superoxide anion, and inhibition of release of neutrophil elastase. Compound 2 showed moderate inhibition of superoxide anion generation with an IC_{50} value of 6.11±0.86 μg/ml; 1, 3 and 5 inhibited neutrophil elastase release with IC_{50} values of 3.82±0.80, 8.61±1.38 and 4.37±0.72 μg/ml, respectively, and were 15-fold more potent than phenylmethylsulfonyl fluoride (PMSF), the positive control, in this anti-inflammatory assay.

Key words *Muehlenbeckia platyclada*; Polygonaceae; flavonoid; superoxide anion generation; neutrophil elastase

Four species of *Muehlenbeckia* belong to the plant family Polygonaceae. Only one species, *Muehlenbeckia platyclada* (F.V. MUELL) Meisn, is found in Taiwan.[1] In previous studies, the genus *Muehlenbeckia* exhibited oxytoxic, analgesic and wound-healing activities.[2,3] Several chemical constituents have been identified from this genus, including flavonoids, acetophenones, lignans, and anthraquinone.[2,4—6]

M. platyclada, commonly known as "ribbon-bush" in Taiwan and China, is an herbal medicine used for treatment of poisonous snake bites and fracture injuries, as well as for alleviating fever and detoxification.[7] However, a phytochemical investigation has never been done. In a continuing search for biologically active constituents from natural sources, the methanolic extract of this species was found to have inhibitory effects on the generation of human neutrophil superoxide anion and the release of neutrophil elastase induced by formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Table 1).

Neutrophils play an important role in the defense against invasion by microorganisms and in the pathogenesis of chronic obstructive pulmonary disease (COPD), rheumatoid arthritis and asthma.[8,9] Activated neutrophils can secrete a series of cytotoxins, including superoxide ($O_2^{\cdot-}$), granule proteases, and bioactive lipids.[9—12] Regulating neutrophil activation by using chemical agents has been proposed as a way to ameliorate inflammatory diseases.[9]

Bioassay-directed fractionation led to isolation of a new flavonoid glycoside, along with four known flavonoids [kaempferol 3-*O*-α-rhamnopyranoside (**2**), kaempferol 3-*O*-β-glucopyranoside (**3**), quercetin 3-*O*-α-rhamnopyranoside (**4**) and (+)-catechin (**5**)] from the extract of *M. platyclada* (Figs. 1, 2). Known compounds were identified by comparison of their physical and spectroscopic data with literature values.[13—16] The structural elucidation of the new compound and the anti-inflammatory effects of **1**—**5** are reported herein.

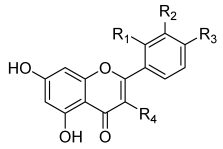
Compound **1** was isolated as a brown powder with the molecular formula, $C_{22}H_{24}O_{10}$, deduced from the HR-ESI-MS (*m*/*z* 471.1270 $[M+Na]^+$, Calcd 471.1267). Its IR spectrum revealed the carbonyl group of a 4-pyrone (1652 cm^{-1}) and a hydroxyl group (3352 cm^{-1}). UV data, as well as a violet coloration with ferric chloride and pink color in the Shinoda test,[17] indicated that **1** was a flavonoid. On acid hydrolysis of **1**, morin and rhamnose were liberated and identified by HPLC. The ESI-MS (negative mode) spectrum of compound **1** showed a deprotonated ion peak at *m*/*z* 447.2 and product ions were observed at *m*/*z* 301.1 ($[M-H-146]^-$), suggesting a rhamnosyl residue in the molecule. This assignment was confirmed by the ^{1}H-NMR spectrum, which showed the typical flavonol α-L-rhamnopyranoside proton pattern at δ_H 0.94 (3H, d, H-6″)/δ_C 17.7, 3.57—3.60 (2H, m, H-4″, 5″)/δ_C 73.3 and 72.0, 3.78 (1H, dd, H-3″)/δ_C 72.2, 4.22 (1H, dd, H-2″)/δ_C 71.9, and 5.35 (1H, d, H-1″)/δ_C 103.5.[15] In addition, the ^{1}H-NMR spectrum showed three aromatic proton signals at δ 7.32 (H-3′), δ 7.29 (H-5′) and δ 6.91 (H-6′), indicative of the presence of ring B of a 2′,4′-substituted flavonoid (Table

Table 1. Inhibitory Effects of Crude Extracts on Superoxide Anion Generation and Elastase Release by Human Neutrophils in Response to fMLP/CB

Crude extracts	Superoxide anion	Elastase
	IC_{50} (μg/ml)[a] or (Inh %)	IC_{50} (μg/ml)[a] or (Inh %)
$CHCl_3$ extract	(25.66±4.98)**	(23.04±4.46)***
BuOH extract	7.04±1.49**	16.11±0.37***
H_2O extract	(23.94±3.56)***	(18.29±5.94)*
DPI[b]	0.7±0.4	
PMSF[b]		130.9±29.1

Percentage of inhibition (Inh %) at 10 μg/ml. Results are presented as mean±S.E.M. (n=3). * p<0.05, ** p<0.01, *** p<0.001 compared with the control value. *a*) Concentration necessary for 50% inhibition (IC_{50}). *b*) Diphenyleneiodonium (DPI) and phenylmethylsulfonyl fluoride (PMSF) were used as positive control in anti-inflammatory.

* To whom correspondence should be addressed. e-mail: yachwu@kmu.edu.tw



	R_1	R_2	R_3	R_4
1	OH	H	OH	rhamnosyl
2	H	H	OH	rhamnosyl
3	H	H	OH	glucosyl
4	H	OH	OH	rhamnosyl

Fig. 1. Flavonol Glycosides Isolated from *M. platyclada*

1, morin-3-*O*-α-rhamnopyranoside; **2**, kaempferol 3-*O*-α-rhamnopyranoside; **3**, kaempferol 3-*O*-β-glucopyranoside; **4**, quercetin 3-*O*-α-rhamnopyranoside.

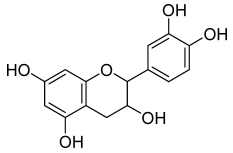


Fig. 2. The Structure of (+)-Catechin

3), and two *meta*-coupled protons at δ 6.21 (J=2.0 Hz) and 6.38 (J=2.0 Hz), characteristic of flavonoids with 5,7-oxygenated substitutions. An anomeric methine at δ_H 5.35 (1H, d, 1.4 Hz)/δ_C 103.5 confirmed a monosaccharide moiety on the flavonol. Moreover, cross peaks [H-6′ (δ 6.91) to C-2 (δ 159.3)/C-1′ (δ 122.8)/C-4′ (δ 149.8)/C-5′ (δ 116.4) and H-1″ (δ 5.35) to C-3 (δ 136.2)/C-2″ (δ 71.9)] in the HMBC spectrum confirmed the presence of a morin structure and 3-*O*-linkage of the rhamnosyl moiety, respectively. Therefore, the structure was established as shown in Fig. 1 and named morin-3-*O*-α-rhamnopyranoside.

The morin structure occurs commonly in plants of *Psidium guajava* (Myrtaceae), and bioactivities associated with this structure include antioxidant and antimicrobial effects.[18,19] While flavonoids are widespread in *Muehlenbeckia* plants, the morin structure (2′,4′,3,5,7-oxygenated flavonoid) has been found for the first time in this genus, specifically in *M. platyclada*.

Furthermore, the isolates were subjected to two anti-inflammatory assays, superoxide anion generation and elastase release of human neutrophils induced by fMLP (Table 2). Compounds **1**, **3** and **5** showed inhibitory effects on the release of neutrophil elastase, with IC_{50} values of 3.82, 8.61 and 4.37 μg/ml, respectively, and were 15-fold more potent than PMSF as a positive control in this anti-inflammatory assay (p<0.001). Compound **2** exhibited a selective inhibitory effect on generation of neutrophil superoxide anion, with an IC_{50} value of 6.11 μg/ml. According to previous studies,[20,21] flavonoids are direct inhibitors of human neutrophil elastase. In the present study, comparison of the data of compounds **1**, **2** and **4** showed that when hydroxyl group was changed to hydrogen group at R_1 position, the activity of inhibition of elastase release reduced, but at the same time, while hydroxyl group was substituted by hydrogen group at R_2 position, the activity increased (Fig. 1, Table 2). Additionally, compound **3** (kaempferol 3-*O*-β-glucopyranoside) was more potent than **2** (kaempferol 3-*O*-α-rhamnopyranoside), suggesting that 3-*O*-glucosyl substitution was effective against human neutrophil elastase release. On the basis of these findings, the preliminary structure–activity relationships for anti-inflammatory effects were proposed. Therefore, the inhibition of neutrophil elastase release by the methanolic extract of *M. platyclada* was mainly due to the presence of morin 3-rhamnopyranoside, kaempferol 3-glucopyranoside, and (+)-catechin.

Table 2. Inhibitory Effects of Compounds on Superoxide Anion Generation and Elastase Release by Human Neutrophils in Response to fMLP/CB

Compound	Superoxide anion	Elastase
	IC_{50} (μg/ml)[a] or (Inh %)	IC_{50} (μg/ml)[a] or (Inh %)
1	(33.13±4.37)***	3.82±0.8***
2	6.11±0.86***	(16.47±5.27)*
3	(25.65±2.35)***	8.61±1.38***
4	(34.35±5.85)**	(16.58±4.08)*
5	N[b]	4.37±0.72***
DPI[c]	0.7±0.4	
PMSF[c]		130.9±29.1

Percentage of inhibition (Inh %) at 10 μg/ml. Results are presented as mean±S.E.M. (n=3—4). *p<0.05, **p<0.01, ***p<0.001 compared with the control value. *a*) Concentration necessary for 50% inhibition (IC_{50}). *b*) **5** reacts with cytochrome C. *c*) Diphenyleneiodonium (DPI) and phenylmethylsulfonyl fluoride (PMSF) were used as positive control in anti-inflammatory.

Table 3. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Morin-3-*O*-α-rhamnopyranoside Isolated from *M. platyclada* (CD_3OD)

Position	δ_H (multiplicity)	J_{H-H} (Hz)	δ_C	HMBC correlation
2			159.3	
3			136.2	
4			179.6	
5			163.2	
6	6.21 (1H, d)	2.0	99.9	C-5, C-7, C-8, C-10
7			165.9	
8	6.38 (1H, d)	2.0	94.7	C-7, C-9, C-6, C-10
9			158.5	
10			105.9	
1′			122.8	
2′			146.4[a]	
3′	7.32 (1H, d)	2.2	117.0	C-2′, C-4′, C-1′, C-5′
4′			149.8[a]	
5′	7.29 (1H, dd)	2.2, 7.6	116.4	C-4′, C-6′, C-1′, C-3′
6′	6.91 (1H, d)	7.6	123.0	C-1′, C-5′, C-4′, C-2
1″	5.35 (1H, d)	1.4	103.5	C-3, C-2″
2″	4.22 (1H, dd)	1.4, 3.3	71.9	
3″	3.78 (1H, dd)	3.2	72.2	
4″	3.57—3.60 (1H, m)		73.3	
5″	3.57—3.60 (1H, m)		72.0	
6″	0.94 (3H, d)	5.8	17.7	C-4″, C-5″

a) Interchangeable.

Experimental

General Experimental Procedures Compound purity was checked by preparative TLC on precoated silica gel plates (Merck, Kieselgel 60 F_{254}, layer thickness 0.25 mm). Melting points were determined on a Melt-Temp II apparatus, optical rotations were measured with a JASCO P-1020 digital polarimeter, and UV spectra were obtained on a Hitachi 200-20 spectrophotometer in CD_3OD. NMR spectra, both 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, TOCSY, HSQC, HMBC and NOESY), were performed at 200 and 50 MHz for ^{1}H and ^{13}C, respectively, and obtained on a Varian NMR spectrometer. ^{1}H-NMR: CD_3OD as solvent, δ=3.31 ppm and ^{13}C-NMR: CD_3OD as solvent, δ=49.0 ppm. Low resolution ESI-MS spectra were obtained on an API 3000^{TM} (Applied Biosystems) in positive or negative mode (solvent: CH_3OH), and high resolution ESI-MS spectra on a Bruker Daltonics APEX II 30e spectrometer in positive or negative mode (solvent: CH_3OH). Shimadzu LC-6AD pumps, a SPD-M10A UV–Vis detector, and Develosil,

ODS 5 μm (250×4.6 mm i.d.) and preparative ODS 5 μm (250×21.2 mm i.d.) columns were employed for HPLC.

Plant Material *M. platyclada* was collected from Yongkang, Tainan, Taiwan, in September 2006. Samples were authenticated and deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan.

Extraction and Isolation The air-dried plant (1.2 kg) of *M. platyclada* was extracted four times at room temperature with CH_3OH. Combined CH_3OH extracts were evaporated under vacuum and distilled to yield a dried green residue (62.84 g). The latter was dissolved in $CHCl_3$ and extracted with H_2O. The H_2O solution was partitioned with BuOH to give a H_2O layer (37.74 g) and a BuOH layer (15.5 g). The BuOH extract was further separated on RP-18 column (VersaPak^{TM}, 40×150 mm, Supelco) and MPLC with a gradient of 80% H_2O/MeOH, 60% H_2O/CH_3OH, 40% H_2O/CH_3OH, 20% H_2O/CH_3OH and CH_3OH (each 1000 ml) to yield five fractions (A—E). Fraction C (740 mg) was separated on Sephadex LH-20 with CH_3OH to give five subfractions (C1—C5). Subfraction C4 was subjected to column chromatography on silica gel. Elution started with $CHCl_3$/CH_3OH 20/1 to yield **1** (6.08 mg, $CHCl_3$/CH_3OH 5/1), kaempferol 3-*O*-α-L-rhamnopyranoside (**2**) (14.1 mg, $CHCl_3$/CH_3OH 10/1) and kaempferol 3-*O*-β-glucopyranoside (**3**) (3.84 mg). Fraction D (1.12 g) was separated on Sephadex LH-20 with CH_3OH to give five subfractions (D1—D5). Subfraction D4 was further purified by RP-18 column (LiChroprep, 40—63 mm, Merck) and preparative HPLC (CH_3OH/H_2O 65/35, flow rate 3 ml/min, detection at 280 nm) to yield quercitrin (**4**) (8.7 mg) and (+)-catechin (**5**) (14.5 mg).

Morin-3-*O*-α-rhamnopyranoside (**1**): Brown amorphous powder. UV (CH_3OH) λ_{max} nm (log ε): 260 (4.41), 351 (4.26); mp: 270—280 °C; $[\alpha]_D$ −20.8° (c=0.003, CH_3OH); IR, ν (KBr) cm^{-1} 3352, 1652, 1606, 1510, 1355, 1301, 1109, 1088; ^{1}H-NMR (CD_3OD, 200 Hz): see Table 3; ^{13}C-NMR (CD_3OD, 50 Hz): see Table 3; HR-ESI-MS m/z: 471.1270 $[M+Na]^+$, Calcd 471.1267 for $C_{22}H_{24}O_{10}Na$.

Acid Hydrolysis The mixture of compound **1** and 1% aqueous HCl (1 ml) was heated in a capped tube on water bath at 85 °C for 2 h. Then, methanol was added to the resulting solution and sonicated for 10 min. The solution was re-filtered prior to HPLC injection. The water eluate was collected and submitted to HPLC analysis of the sugar. A further eluate with MeOH was collected and submitted to HPLC analysis of the flavonoid aglycone.

Assays of Superoxide Anion Generation and Elastase Release of Human Neutrophils Induced by fMLP All isolates from the entirety of *M. platyclada* were purified before bioassay (purity >99%). The assays were carried out according to established protocols.[14)]

Acknowledgement We gratefully acknowledge financial support for this project from the National Science Council, National Science Technology Program/Biotechnology and Pharmaceuticals, National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center, and Center of Excellence for Environmental Medicine (KMU-EM-97-2.1a). We also thank Mr. Yun-Zhao Chen for providing plant materials.

References

1) Boufford D. E., Hsieh C. F., Huang T. C., Chashi H., Yang Y. P., Lu S. Y., "Flora of Taiwan," 2nd ed., Vol. II, ed. by Editorial Committee of the Flora of Taiwan, Taipei, 1996, p. 294.
2) Erazo S., Muñoz O., García R., Lemus I., Backhouse N., Negrete R., San Feliciano A., Delporte C., *Z. Naturforsch. C*, **57**, 801—804 (2002).
3) Villegas L. F., Fernández I. D., Maldonado H., Torres R., Zavaleta A., Vaisberg A. J., Hammond G. B., *J. Ethnopharmacol.*, **55**, 193—200 (1997).
4) Autore G., De Tommasi N., Aquina R., Borrelli F., Tartaglia D., Mascolo N., *Phytother. Res.*, **10**, 202—206 (1996).
5) Pressland A. J., Keenan F. J., *Plant Protect. Quart.*, **1**, 62—66 (1985).
6) Pablo M., Lilia G. G., *Cienciay Nat.*, **14**, 2—10 (1973).
7) Je-Chian F., Hsiang-Pei Y., Jen-Chen Wu., "Quanzhou Herbal Medicine," ed. by Quanzhou Committee on Science and Technology Quanzhor City Bureau Quanzhou Institute of Medicine Science, ROC, 1961.
8) Nathan C., *Nat. Rev. Immunol.*, **6**, 173—182 (2006).
9) Hwang T. L., Hung H. W., Kao S. H., Teng C. M., Wu C. C., Cheng S. J., *Mol. Pharmacol.*, **64**, 1419—1427 (2003).
10) Ross D., Van Bruggen R., Meischl C., *Microb. Infect.*, **5**, 1307—1315 (2003).
11) Witko-Sarsat V., Rieu P., Descamps-Latscha B., Lesavre P., Halbwachs-Mecarelli L., *Lab. Invest.*, **80**, 617—653 (2000).
12) Borregaard N., *Curr. Opin. Hematol.*, **3**, 8—11 (1996).
13) Nørbæk R., Nielsen J. K., Kondo T., *Phytochemistry*, **51**, 1133—1138 (1999).
14) Kazuma K., Noda N., Suzuki M., *Phytochemistry*, **62**, 229—237 (2003).
15) Zhong X. N., Otsuka H., Ide T., Hirata E., Takushi A., Takeda Y., *Phytochemistry*, **46**, 943—946 (1997).
16) Bilia A. R., Morelli I., Hamburger M., Hostettmann K., *Phytochemistry*, **43**, 887—892 (1996).
17) Shinoda J., *J. Pharm. Soc. Jpn.*, **48**, 214—220 (1928).
18) Bourama N., Annelise L., Byung H. U., Robert A., Mamadou E. K. Kone, *Biochem. Syst. Ecol.*, **33**, 309—312 (2005).
19) Arima H., Danno G.-i., *Biosci. Biotechnol. Biochem.*, **66**, 1727—1730 (2002).
20) Siedle B., Hrenn A., Merfort I., *Planta Med.*, **73**, 401—420 (2007).
21) Melzig M. F., Loser B., Ciesielski S., *Pharmazie*, **56**, 967—970 (2001).