Cytotoxic Constituents from *Podocarpus fasciculus*

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A new diterpene, 16-hydroxy communic acid (1), along with thirty one known compounds including five norditerpenes (2—6), twenty two flavonoids containing four biflavonoids (7—10), nine monoflavonoids (11—19) and nine flavanoid glycosides (20—28), as well as four phenolic constituents (29—32) were isolated from the 95% ethanolic extract of *Podocarpus fasciculus*. The structure of 1 was elucidated using spectral methods. Of these isolates, nagilactone C (2) showed the most significant inhibitory effects against DLD cells (human colon carcinoma) (ED_{50}=2.57 μg/ml) and compounds 7, 8, 10, 11, and 12 had moderate cytotoxic activity against human KB (human oral epithelium carcinoma), Hela (human cervical carcinoma), Hepa (human hepatoma), DLD (colon carcinoma), and A-549 (human lung carcinoma) tumor cell lines. Preliminary structure–activity relationship studies of the isolated diterpenoids and biflavonoids are discussed.

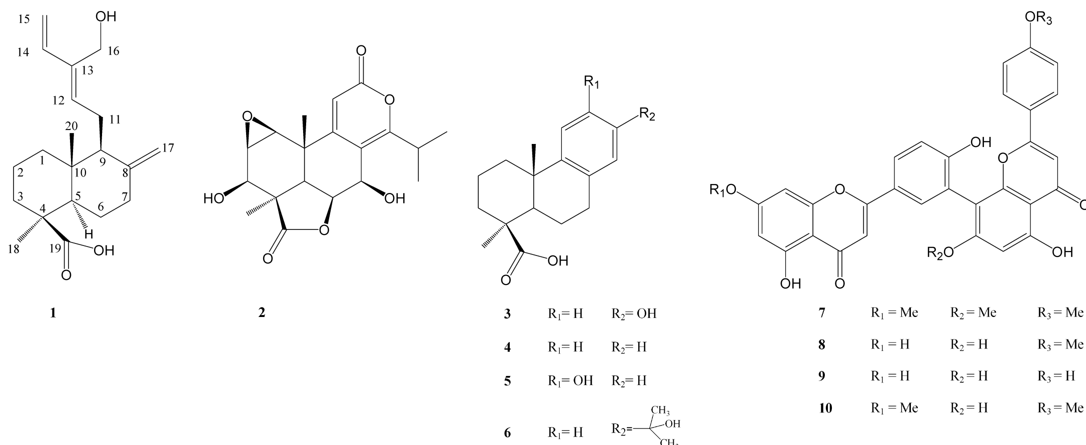
Key words *Podocarpus fasciculus*; Podocarpaceae; 16-hydroxy communic acid; cytotoxic activity

The genus *Podocarpus* (Podocarpaceae) is ancient gymnosperms that grows in scattered parts of east Asia and the southern hemisphere.[1] Phytochemical studies of a number of species in this genus have led to the isolation and elucidation of various terpenoids, and nor- and dinorditerpenoid dilactone groups.[2,3] These isolated constituents have attracted a great deal of interest because of their wide range of biological activities, including antitumor activity,[3,4] plant growth-inhibitory activity,[5] termiticidal activity,[6] insect toxicity,[7] and antifeedant effects.[8]

Recently, we found that the 95% EtOH extract of the stems and leaves of *Podocarpus fasciculus* exhibited cytotoxicity against several human tumor cell lines *in vitro*. Further separation and purification on column chromatograpy and HPLC yielded a new diterpenoid, 16-hydroxy communic acid (**1**), along with 31 known compounds including five norditerpenes (**2**—**6**), four biflavonoids (**7**—**10**), nine monoflavonoids (**11**—**19**), nine flavanoid glycosides (**20**—**28**), and four aromatic derivatives (**29**—**32**). Structural elucidation of **1** was based on spectroscopic analysis, mainly using HR-ESI-MS, IR, ^{1}H-, ^{13}C-, and 2D-NMR experiments. In addition to **1**, most of the other isolates (**2**, **4**—**5**, **7**—**13**, **17**, **29**, **31**, **32**) were evaluated for cytotoxicity in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Compound **1** was obtained as colorless oil. The molecular formula of **1** was determined to be $C_{20}H_{30}O_3$ based on HR-ESI-MS, which exhibited a quasi-molecular ion $[M+Na]^+$ at *m/z* 341.2052 (Calcd 341.2093) and a $[M+K]^+$ at *m/z* 357.1992 (Calcd 357.1832). The IR spectrum of **1** showed carboxyl, vinyl, and terminal methylene ($>C=CH_2$, 1642, 887) groups. In the ^{1}H- and ^{13}C-NMR spectra (Table 1), characteristic signals for two tertiary methyl groups, six methylene groups, two methine groups, one olefinic methine group [δ_H 6.26 (dd, J=17.5, 11.5 Hz)], one terminal olefinic methylene group (δ_C 111.67), an exocyclic methylene group [δ_H 4.38 (s), 4.84 (s), δ_C 107.8 (t), 147.6 (s)], one hydroxymethyl group [δ_H 4.39 (2H, s)], one trisubstituted double bond [δ_H 5.60 (1H, t, J=6.5 Hz), δ_C 137.47, 136.69], and one carboxylic acid [δ_C 183.59] were found.

The ^{1}H–^{1}H COSY spectrum of **1** showed cross peaks of H-1, H-2, and H-3, and cross peaks of H-5, H-6, and H-7, together with quaternary carbons connecting with the respective double- or triple-bonded bonds protons in the HMBC spectrum (C-4/H-2, -3, -5, -18; C-8/H-6, -7, -9, -11, -17; C-



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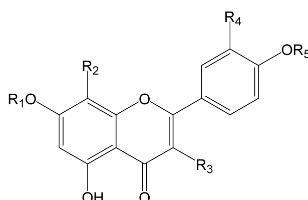
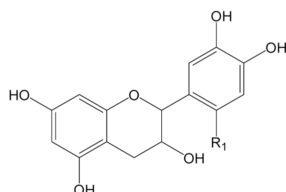
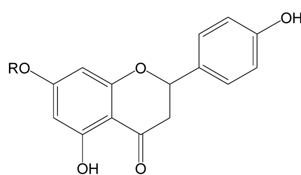


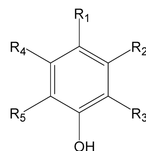
11	R_1= H	R_2= H	R_3= H	R_4= H	R_5= H
12	R_1= H	R_2= H	R_3= OH	R_4= H	R_5= H
13	R_1= H	R_2= H	R_3= OH	R_4= OH	R_5= H
14	R_1= H	R_2= H	R_3= H	R_4= OH	R_5= H
15	R_1= H	R_2= H	R_3= H	R_4= H	R_5= Me
16	R_1= Me	R_2= H	R_3= H	R_4= H	R_5= Me
21	R_1= H	R_2= O-Glc	R_3= H	R_4= OH	R_5= H
22	R_1= Glc	R_2= H	R_3= H	R_4= OH	R_5= H
23	R_1= H	R_2= H	R_3= O-Rha-(1→6)-Glc	R_4= OH	R_5= H
24	R_1= H	R_2= H	R_3= OGlc	R_4= H	R_5= H
25	R_1= H	R_2= H	R_3= OGlc	R_4= OH	R_5= H
26	R_1= Glc	R_2= H	R_3= OH	R_4= OH	R_5= H
27	R_1= H	R_2= H	R_3= O-Rha-(1→6)-Glc	R_4= OMe	R_5= H
28	R_1= Glc	R_2= H	R_3= OH	R_4= H	R_5= H



17 R_1 = H
18 R_1 = OH



19 R = H
20 R = Rha-(1→6)-Glc



29	R_1= H	R_2= H	R_3= OH	R_4= OH	R_5= OH
30	R_1= CH_3	R_2= H	R_3= H	R_4= H	R_5= H
31	R_1= C_2H_5	R_2= H	R_3= H	R_4= H	R_5= H
32	R_1= H	R_2= H	R_3=OCH_3	R_4= OH	R_5= H

10/H-1, -2, -5, -6, -9, -11), suggesting that the partial structure of **1** is comprised of a two-ring fused terpene moiety. Furthermore, based on long-range correlations in the HMBC spectrum, a 3-hydroxymethyl-penta-2,4-diene moiety connected at C-9, an exocyclic methylene group connected at C-8, and two methyl groups connected at C-4 and C-10, respectively, as well as a carboxylic acid connected at C-4, were unambiguously assigned. Accordingly, **1** was designated as a labdane-type diterpene with one carboxylic acid moiety which had a very similar structure to that of communic acid,[9)]

Table 1. ^{13}C- and ^{1}H-NMR Data ($CDCl_3$, 100, 500 MHz) for **1**

Position	C	H (mult, *J* in Hz)	HMBC
1α	39.49 t	1.16 (dt, 5.0, 15.0)	C-2, 5, 10, 20
1β		1.90 (dd, 4.0, 15.0)	C-2, 10
2α	20.12 t	1.55 (m)	C-4, 10
2β		1.83 (m)	
3α	38.14 t	2.17 (m)	C-2, 4, 19
3β		1.07 (m)	C-2, 4, 5
4	44.4 0 s		—
5α	56.44 d	1.36 (dd, 12.0, 2.5)	C-3, 4, 6, 10, 19, 20
6α	26.07 t	2.01(m)	C-5, 7, 8, 10
6β		1.91(m)	
7α	38.70 t	1.98 (m)	C-6, 8, 17
7β		2.42 (m)	C-6, 8, 9, 17
8	147.95 s		—
9α	56.82 d	1.76 (d, 11.5)	C-8, 10, 11, 12, 17, 20
10	40.70 s		—
11A	23.18 t	2.26 (ddd, 15.0, 6.0, 11.5)	C-8, 9, 10, 13
11B		2.52 (ddd, 2.0, 6.0, 15.0)	
12	137.47 d	5.60 (t, 6.5)	C-11, 14, 16
13	136.69 s		—
14	138.91 d	6.26 (dd, 17.5, 11.5)	C-13, 16
15A	111.67 t	5.02 (d, 17.5)	C-13
15B		5.29 (d, 11.5)	
16	57.28 d	4.39 (s)	C-12, 14
17A	108.09 t	4.88 (s)	C-7, 8, 9
17B		4.50 (s)	
18	29.25 q	1.25 (s)	C-3, 4, 5, 19
19	183.59 s		—
20	13.10 q	0.66 (s)	C-1, 5, 9, 10

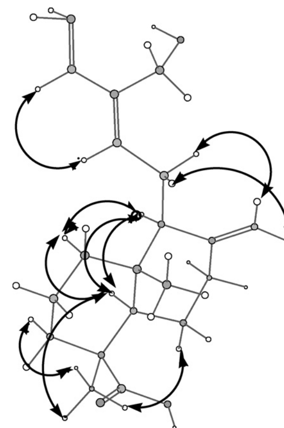


Fig. 1. NOESY (Dashed Arrow) Correlation of **1**

except for the replacement of the methyl by a hydroxymethyl at C-16 in **1**.

On the basis of characteristic NOE correlations in the NOESY spectrum (Fig. 1), the cross peaks of Me-18/H-3α, H-5α, H-6α, H-9α/H-5α, and Me-20/H-1β were observed. Together with the NOE effect between H-12 and H-14 deduced from the 12-*Z* configuration in **1**,[10)] the relative stereochemistry of **1** was determined and the compound was tentatively named 16-hydroxy communic acid. Moreover, five known norditerpene, nagilactone C (**2**),[11)] 7-hydroxy-1,4α-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthrene-1-carboxylic acid (**3**),[12—15)] podocarpa-8,11,13-trin-16-oic acid (**4**),[12—15)] podocarpic acid (**5**),[12—15)] and 15-hydroxydehydroabietic acid (**6**)[16,17)]; 22 flavonoid compounds containing four biflavonoids as heveaflavone (**7**),[18)] podocarpusflavone-A (**8**),[19)] amentoflavone (**9**), and II-4‴,I-7-dimethoxy amentoflavone (**10**)[18)]; the nine monoflavonoids, apigenin

Table 2. Cytotoxic Activity of Compounds (ED_{50}, μg/ml)

Compounds Cell lines[a]	ED_{50} (μg/ml)				
	KB	HeLa	DLD	Hepa	A-549
1	[b]	—[c]	—	[b]	—
2	8.92	[b]	2.57	4.80	[b]
4	—	—	[b]	—	—
5	—	—	[b]	—	—
7	6.15	10.25	12.21	11.56	[b]
8	4.86	14.25	10.56	9.86	[b]
9	—	—	[b]	—	
10	4.56	14.21	10.51	11.23	[b]
11	7.59	4.19	5.48	4.83	6.16
12	8.77	4.85	18.39	4.36	8.24
13	—	—	—	—	[b]
17	—	—	[b]	—	—
29	—	—	[b]	—	—
31	—	—	[b]	—	—
32	—	—	[b]	—	—

a) Key to cell lines used: human oral epithelium carcinoma (KB), human cervical carcinoma (Hela), human colon tumor (DLD), human lung carcinoma (A-549) and human liver carcinma (Hepa) cells. *b*) No test. *c*) —: Inactive (ED_{50}>20 μg/ml).

(**11**),[20—22] kaempferol (**12**),[20—22] quercetin (**13**),[20—22] luteolin (**14**),[23] acacetin (**15**),[20—22] 5-hydroxy-7-methoxy-2-(4-methoxy-phenyl)-chromen-4-one (**16**),[24] catechin (**17**),[25] 6′-hydroxy-catchin (**18**),[25] and naringenin (**19**)[26]; the nine flavanoid glycosides naringin (**20**),[27] orientin (**21**),[28—30] luteolin-7-glucoside (**22**),[28—30] rutin (**23**),[28—30] kaempferol-3-glucoside (**24**),[28—30] quercetin-3-glucoside (**25**),[28—30] quercetin-7-glucoside (**26**), [28—30] and 3′-*O*-methylquercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**27**)[28—30] and kaempferol-7-glucoside (**28**),[28—30] and the four aromatics, benzene-1,2,3,4-tetraol (**29**),[31—34] 4-methyl-phenol (**30**),[31—34] 4-ethyl-phenol (**31**),[31—34] and 5-(3-hydroxy-propyl)-2-methoxy-phenol (**32**)[31—34] were isolated and their structures identified by comparison of the spectral data with those of authentic compounds reported in the cited literature.

Some of the above-mentioned diterpenoids, biflavonoids, aromatics, and several monoflavonoids were evaluated for cytotoxicity against human KB, Hela, Hepa, DLD, and A-549 tumor cell lines. As shown in Table 2, compound **2** showed the most potent cytotoxicity against DLD cells (ED_{50}=2.57 μg/ml). The biflavonoids **7**, **8**, and **10** showed moderate cytotoxicity (ED_{50} *ca.* 4—14 μg/ml) against the human tumor cell lines, whereas **9** (ED_{50}>20 μg/ml) showed no cytotoxicity. In addition, flavonoid **11** (ED_{50}=5.48 μg/ml) had greater inhibitory effects than **12** (ED_{50}=18.39 μg/ml) and **13** (ED_{50}>20 μg/ml) on DLD tumor cells. These preliminary structure–activity relationship studies suggested that the OMe and hydroxy groups in biflavonoids and monoflavonoids, respectively, play a crucial role in mediating cytotoxic activity.

Experimental

General Optical rotations were recorded on a JASCO P-1020 polariete. IR spectra were measured on a Mattson Genesis II spectrophotometer (Thermo Nicolet, Madison, WI, U.S.A.). NMR spectra were recorded on Varian NMR spectrometers (Unity Plus 500 MHz) using $CDCl_3$, CD_3OD, and pyridine-d_5 as solvents for measurement. Low-resolution EI-MS was recorded on a VG Quattro 5022 mass spectrometer. High-resolution ESI-MS were measured on a MAT-95XL high-resolution mass spectrometer. The chemical shifts are given in δ (ppm) and coupling constants in Hz. Sephadex LH-20 and silica gel (Merck 70—230 mesh and 230—400 mesh) (Merck) were used for column chromatography, and precoated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 5% H_2SO_4 and then heating at 110 °C. HPLC separations were performed on a Waters 600 series apparatus with a Waters 996 photodiode array detector, equipped with a 250×5 mm i.d. preparative Cosmosil AR-II column (Nacalai, Tesque).

Plant Material *P. fasciculus* de LAUBENFELS was collected in the midland mountains of Taiwan in July 2003 and identified by Professor Mu-Thiung Kao. A voucher specimen (No. 2003-07-066) has been deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation The dried stems and leaves of *P. fasciculus* (12.4 kg) were chipped, extracted with EtOH (80 l, three times) at 50 °C, and concentrated under reduced pressure. The EtOH extract (1200 g) was partitioned between *n*-hexane and H_2O (1 : 1) to give the *n*-hexane soluble fraction (fr. A, 45 g). Then the H_2O solution was partitioned with $CHCl_3$ and H_2O (3 : 1) to give the $CHCl_3$-soluble fraction (fr. B, 321 g) and the H_2O fraction, respectively.

Fr. A (45 g) was chromatographed on a silica gel column (41×8 cm i.d.) eluted with *n*-hexane–EtOAc (1 : 0→0 : 1) to give 13 fractions (fr. A-1—A-13). Fr. A-6 (15 g, *n*-hexane–EtOAc=5 : 2) was further purified by HPLC (Cosmosil 5C_{18}-AR II, 250×20.0 mm i.d., flow rate: 3 ml/min, 90% MeOH) to obtain **1** (10 mg). Fr. A-7 (5 g, *n*-hexane–EtOAc=2 : 1) was repeatedly chromatographed on a silica gel column (30×2.8 cm i.d.) and then purified by preparative TLC (plate: 20×20 cm, *n*-hexane–$CHCl_3$, 3 : 2) to afford **3** (1.3 mg), **4** (4.5 mg), **5** (3.7 mg), and **6** (2.5 mg). Using preparative TLC (plate: 20×20 cm, *n*-hexane–$CHCl_3$, 3 : 2), **17** (3.1 mg), **18** (1.5 mg), and **27** (1.2 mg) were yielded from the residue of fr. A-7.

Fr. B (321 g) was chromatographed on a silica gel column (46×10 cm i.d.) eluted with $CHCl_3$–MeOH (1 : 0→0 : 1) to afford 20 fractions (fr. B-1—B-20). Fr. B-5 (11 g, $CHCl_3$–MeOH=10 : 1) was repeatedly purified by chromatography on a Sephadex LH-20 column (30×2.8 cm i.d.) with MeOH to give fr. B-5-1—B-5-7, and then fr. B-5-5 (2.5 g) was further purified by preparative TLC (plate: 20×20 cm, $CHCl_3$–MeOH, 10 : 1) to yield **2** (6.5 mg), **7** (8.6 mg), **8** (3.2 mg), **10** (9.2 mg), **11** (2011 mg), and **12** (1204 mg). Fr. B-5-6 (85 mg) was further subjected to preparative TLC (plate: 20×20 cm, *n*-hexane–$CHCl_3$, 1 : 1) to afford **29** (6.2 mg) and **30** (7.2 mg). Fr. B-6 (6 g) was separated by HPLC (Cosmosil 5C_{18}-AR II, 250×20.0 mm i.d., flow rate: 3 ml/min, 60% MeOH) to yield 5 fractions (fr. B-6-1—B-6-5). Further purification with preparative TLC (plate: 20×20 cm, $CHCl_3$–MeOH, 10 : 1) gave **9** (5.8 mg), **13** (22.4 mg), **14** (3.2 mg), and **19** (12.4 mg) from fr. B-6-4 (155 mg), and **22** (7.5 mg), **26** (6.4 mg), **28** (3.3 mg), and **31** (4.2 mg) were yielded from fr. B-6-5 (68 mg), respectively. Fr. B-7 (8 g, $CHCl_3$–MeOH=5 : 1) was chromatographed on a silica gel column (30×2.8 cm i.d.) to give three fractions (fr. B-7-1—B-7-3); then **15** (4.2 mg), **16** (5.2 mg), and **20** (4.8 mg) were yielded by fr. B-7-2 (66 mg) after the same treatment as for fr. B-6-4, and **21** (2.1 mg), **23** (3.4 mg), **24** (5.9 mg), **25** (4.8 mg), and **32** (9.2 mg) were yielded by fr. B-7-3 (87 mg) using preparative TLC (plate: 20×20 cm, $CHCl_3$–MeOH, 5 : 1).

Cytotoxicity Assay The MTT assay against KB, Hela, Hepa, DLD, and A-549 cells was based on methods reported in the literature.[36,37] In brief, the cells were cultured in RPMI-1640 medium. Test samples were prepared at four concentrations. After these cell lines had been seeded in a 96-well microplate for 4 h, 20 μl of sample was placed in each well and incubated at 37 °C for 3 d, and then 20 μl of MTT was added for 5 h. After removing the medium and placing DMSO (200 μl/well) into the microplate with shaking for 10 min, the formazan crystals were redissolved and their absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at the wavelength of 550 nm.

16-Hydroxy Communic Acid (**1**): Colorless oil; $[\alpha]_D^{25}$ +62.61° (c=0.09, MeOH); UV (MeOH) λ_{max} 240 (sh), 213; IR (neat) ν_{max} 3500 (OH), 3100—2800 and 1692 (COOH), 2933, 2831, 1642 and 887 (>C=CH_2), 1468, 1177, 1014. ^{1}H-NMR ($CDCl_3$, 500 MHz): see Table 1; ^{13}C-NMR ($CDCl_3$, 125 MHz): see Table 1; HR-ESI-MS: m/z 341.2052 $[M+Na]^+$ (Calcd for $C_{20}H_{30}O_3Na$: 341.2093), and 357.1992 $[M+K]^+$ (Calcd for $C_{20}H_{30}O_3K$: 357.1832).

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