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Naturally Occurring Cytotoxic [3′ → 8″]-Biflavonoids from *Podocarpus nakaii*

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Abstract

Bioassay-guided fractionation of the EtOH extract of the dried twigs of *Podocarpus nakaii* Hayata (Podocarpaceae), endemic plant in Taiwan has resulted in isolation of four [3′→8″]-biflavonoid derivatives, amenotoflavone (**AF**), podocarpusflavone-A (**PF**), II-4″,I-7-dimethoxyamentoflavone (**DAF**), and heveaflavone (**HF**). Their structures were determined by physical and extensive spectroscopic analyses such as ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC, and HMBC, as well as comparison with literature values. Compounds **PF** and **DAF** showed significant inhibitions against DLD, KB, MCF-7, HEp-2 tumor cell lines (ED_{50} *ca*. 4.56-16.24 μg/mL) and induced cell apoptosis in MCF-7 via mainly sub-G_1/S phase arrest. Furthermore, these compounds exhibited moderate Topoisomerase I inhibitory activity.

Key words: *Podocarpus nakaii*, [3′→8″]-Biflavonoids, Cytotoxicity, Apoptosis, Topoisomerase I

Introduction

The genus *Podocarpus* (Podocarpaceae) have led to the isolation and elucidation of various diterpenoids and flavonoids, which have shown potentially useful biological activities (Kuo, 2008; Park, 2004). *Podocarpus nakaii* Hayata is an endemic plant commonly grown in the middle mountainous area of Taiwan (Chen, 1993). Naturally occurring biflavonoids consist of two flavonoids linked to each other by either a C-C or a C-O-C bond and display a variety of biological activities, such as 99), neuroprotection (Kang, 2005), antiplasmodia (Dhooghe, 2010), anti-inflammation (Banerjee, 2002), antitumor (Lin, 2000), and osteoblast differentiation stimulation (Lee, 2006). Some papers deal with the bioactivities of the bioflavonoids which are better than that of the corresponding monomer, especially on anti-inflammatory and antitumor activities (Kim, 2008). In this article, we report that the bioassay-guided fractionation of EtOH extract of twigs of *P. nakaii* has resulted in the isolation of four biflavonoids, amenotoflavone (**AF**) (Markham, 1987), podocarpusflavone-A (**PF**) (Markham, 1987), II-4″,I-7-dimethoxyamentoflavone (**DAF**) (Roy, 1987), and heveaflavone (**HF**) (Roy, 1987), and those isolated biflavonoids possess the [3′→8″]-biflavonoid skeleton. Their structures were determined through detailed spectroscopic analyses, involving 1D and 2D NMR experiments (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC, and HMBC), as well as confirmed by comparing with the

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literature data. Biological evaluation for these [3′→8″]-biflavonoids against four human tumor cell lines, including oral epidermoid carcinoma (KB), breast carcinoma (MCF-7), colon adenocarcinoma (DLD) and laryngeal carcinoma (HEp-2), as well as the investigation of apoptosis.

Since DNA topoisomerase I inhibition was served as a target of anticancer drugs, especially camptothecin (CPT) analogues, several cytotoxic mono-flavonoids were demonstrated to possess the DNA topoisomerase I (Topo I) inhibitory effects (Constantinous, 1995; Tselepi, 2011). In this report, among the above isolated biflavonoids, cytotoxic DAF and PF were therefore evaluated for the DNA Topo I inhibition assay.

Materials and Methods

General Experimental Procedure

Optical rotations were measured with JASCO P-2000 polarimeter. Infrared (IR) spectra were measured on a Nicolet AVATAR 320 FT-IR spectrophotometer using a KBr matrix. UV spectra were measured on a Hitachi U-3310 spectrophotometer. ESIMS data were performed on the Waters Quattro Ultima mass spectrometer. 1D and 2D NMR spectra were taken on a Bruker NMR spectrometer (Unity Plus 400 MHz) using CD_3OD and pyridine-d_5 as solvent. Silica gel (Merck 70-230 mesh and 230-400 mesh) were used for column chromatography, and pre-coated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 10% H_2SO_4 and then heating on a hot plate. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with a SPD-10VP UV-VIS detector, equipped with a 250 × 20 mm preparative Cosmosil 5SL-II column.

Plant Material

The twigs of *Podocarpus nakaii* Hayata (Podocarpaceae) was collected in the midland mountains (Nantou) of Taiwan in July 2003 and identified by Professor Mu-Thiung Kao. A voucher specimen (NRICM, No. NRICM200607A1) has been deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation

The dried twigs of *P. nakaii* Hayata (7.2 kg) were extracted with 95% ethanol (40 L) at 45ºC for three times and the ethanol extracts were combined and concentrated under vacuum. The crude extract was partitioned between *n*-hexane and H_2O (1:1) and then the aqueous portion was shaken with EtOAc. The EtOAc-soluble layer (108.5 g) evaporated and subjected to column chromatography using silica gel and a gradient of CH_2Cl_2/MeOH solvent system (from 15:1 to 1:1) to obtain nine fractions (Fr. E1~E9). The Fr. E3 fraction (770 mg) was purified by repeated column chromatography on silica gel eluted with CH_2Cl_2/MeOH (2:1) to afford heveaflavone (**HF**, 5.0 mg) and II-4",I-7-dimethylamenotoflavone (**DAF**, 213 mg). Crystallization of Fr. E5 yielded podocarpusflavone-A (**PF**, 126 mg). Fr. E7 was chromatographed on a silica gel column using a gradient of CH_2Cl_2/MeOH to furnish four fractions (E7A-D), and then Fr. E7B was separated by a RP-HPLC (MeOH/H_2O, 55:40) column to yield amentoflavone (**AF**, 8.2 mg).

Amentoflavone (AF): Brown yellow amorphous powder. Mp. 292-296 ºC; UV (MeOH) λ_{max} 337, 268, 226 nm; IR (KBr) ν_{max} 3475, 2993, 2923, 1644, 1605, 1567, 1504, 1465, 1251, 1172, 1102, 1040, 842 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 400 MHz) and ^{13}C-NMR (pyridine-d_5, 100 MHz) see Table 1; Negative ESIMS *m/z* 537.08 $[M-H]^-$.

Podocarpusflavone-A (PF): Yellow amorphous powder. Mp. 289-292 ºC; UV (MeOH) λ_{max} 341, 269, 221 nm; IR (KBr) ν_{max} 3442, 2983, 1652, 1612, 1557, 1510, 1450, 1249, 1178, 1162, 1107, 1030, 823 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 400 MHz) and ^{13}C-NMR (pyridine-d_5, 100 MHz) see Table 1; Negative ESIMS m/z 551.13 $[M-H]^-$.

II-4",I-7-Dimethoxyamentoflavone (DAF): Yellow amorphous powder. Mp. 235-240 ºC; UV (MeOH) λ_{max} 330, 269, 224 nm; IR (KBr) ν_{max} 3399, 2924, 1644, 1606, 1556, 1504, 1445, 1378, 1253, 1182, 1160, 1109, 1036, 831 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 400 MHz) and ^{13}C-NMR (pyridine-d_5, 100 MHz) see Table 1; Negative ESIMS *m/z* 565.22 $[M-H]^-$.

Heveaflavone (HF): Yellow amorphous powder. Mp. 262-266 ºC; UV (MeOH/CH_2Cl_2)) λ_{max} 328, 270, 225 nm; IR (KBr) ν_{max} 3454, 2925, 1651, 1604, 1503, 1441, 1370, 1259, 1180, 1160, 1110, 1054, 1027, 835 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 400 MHz) and ^{13}C-NMR (pyridine-d_5, 100 MHz) see Table 1; Negative ESIMS *m/z* 579.05 $[M-H]^-$.

Reagents

Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's minimum essential medium (DMEM), phosphate buffered saline (PBS) and trypan blue were purchased from Biological Industries (Kibbutz Beit-Haemek, North District, Isreal). Other chemicals, such as 3-(4,5-dimethylthiazol-2-yl)-

Table 1. ^{1}H- and ^{13}C-NMR spectroscopic data of [3′→8"]-biflavonoids from *P. nakaii* Hayata.

No.	AF		PF		DAF		HF	
	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR (*J* in Hz)	^{13}C NMR	^{1}H NMR (*J* in Hz)	^{13}C NMR
2		164.8 s		164.5 s		165.0 s		164.8 s
3	7.03 s	104.0 d	7.03 s	104.7 d	7.09 s	104.9 d	7.08 s	104.2 d
4		182.7 s		182.6 s		182.8 s		182.7 s
5		162.6 s		162.8 s		162.8 s		162.9 s
6	6.70 d (*J* = 1.8 Hz)	100.2 d	6.72 d (*J* = 2.0)	99.8 d	6.56 d (*J* = 2.0)	100.0 d	6.52 d (*J* = 2.0)	98.5 d
7		164.5 s		165.6 s		165.7 s		165.7 s
8	6.77 d (*J* = 2.4 Hz)	94.8 d	6.80 d (*J* = 2.4)	94.7 d	6.66 d (*J* = 2.4)	93.0 d	6.75 d (*J* = 2.0)	92.8 d
9		158.5 s		158.3 s		158.1 s		158.1 s
10		104.9 s		104.8 s		105.5 s		105.4 s
1'		122.0 s		121.5 s		121.9 s		121.1 s
2'	8.55 d (*J* = 2.4 Hz)	132.6 d	8.47 d (*J* = 2.0)	132.4 d	8.54 d (*J* = 2.4)	132.6 d	8.39 d (*J* = 2.0)	132.3 d
3'		122.5 s		122.2 s		122.9 s		122.3 s
4'		161.9 s		161.1 s		161.7 s		161.2 s
5'	7.38 dd (*J* = 8.4 Hz)	117.6 d	7.50 d (*J* = 8.4)	117.0 d	7.54 d (*J* = 8.8)	117.5 d	7.54 d (*J* = 8.0)	117.1 d
6'	7.89 dd (*J* = 2.4, 8.4 Hz)	128.2 d	7.96 dd (*J* = 8.4, 2.4)	128.1 d	8.03 dd (*J* = 8.8, 2.4)	128.3 d	8.06 dd (*J* = 8.0, 2.0)	128.4 d
2"		165.8 s		163.9 s		164.3 s		164.3 s
3"	6.91 s	116.8 d	6.93 s	104.0 d	6.95 s	104.9 d	6.94 s	104.1 d
4"		183.0 s		182.9 s		183.0 s		183.3 s
5"		162.4 s		162.1 s		162.4 s		162.1 s
6"	6.85 s	100.0 d	6.91 s	99.7 d	6.92 s	98.5 d	6.77 s	96.0 d
7"		163.1 s		163.7 s		163.9 s		163.6 s
8"		105.6 s		103.9 s		104.9 s		105.9 s
9"		155.9 s		155.6 s		155.8 s		155.8 s
10"		104.9 s		105.2 s		122.1 s		105.8 s
1'"		122.1 s		121.5 s		105.8 s		121.1 s
2'"	7.89 dd (*J* = 2.4, 8.4 Hz)	128.8 d	7.84 d (*J* = 8.8)	128.2 d	7.88 d (*J* = 8.5)	128.4 d	7.84 d (*J* = 8.5)	128.4 d
3'"	7.12 dd (*J* = 8.4 Hz)	116.8 d	6.95 d (*J* = 8.8)	114.7 d	6.99 d (*J* = 8.5)	114.8 d	6.95 d (*J* = 8.5)	114.9 d
4'"		162.6 s		162.6 s		162.6 s		162.9 s
5'"	7.12 dd (*J* = 8.4 Hz)	116.8 d	6.95 d (*J* = 8.8)	114.7 d	6.99 d (*J* = 8.5)	114.8 d	6.95 d (*J* = 8.5)	114.9 d
6'"	7.89 dd (*J* = 2.4, 8.4 Hz)	128.8 d	7.84 d (*J* = 8.5)	128.2 d	7.88 d (*J* = 8.5)	128.4 d	7.84 d (*J* = 8.5)	128.4 d
7"-OCH_3							3.73 s	56.4 q
7-OCH_3					3.69 s	55.8 q	3.68 s	55.8 q
4'"-OCH_3			3.59 s	55.2 q	3.59 s	55.3 q	3.58 s	55.4 q

2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and propidium iodide were obtained from Sigma (St. Louis, MO, USA).

Cell Lines and Culture Conditions

Human oral epidermoid carcinoma (KB), human breast carcinoma (MCF-7), human colon adenocarcinoma (DLD) and human laryngeal (HEp-2) cell lines were obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). All of them were maintained in MEM (5% FBS) in a humidified incubator with 5% CO_2 at 37 ºC.

Cytotoxicity Assay

Cells (3 x 10^3 per well) were treated with the isolates at final concentrations of 1, 4, 10, 20 and 40 μM. Incubating for a three-day course, the cell viability was evaluated by a modified MTT assay. In a brief description, formazan conversions through mitochondria can be detected by spectrophotometry in the absorbance at 550 nm and provided a relative estimate of cell viability (Hsu, 2012).

Analysis of Apoptotic Cells by Flow Cytometry

MCF-7 cells (5 × 10^4 per well) were treated with 20, 40 μg/mL of the isolates (dissolved in 2 mL of MEM). After 24 hrs of incubation, the cells were harvested and co-stained with propidium iodide and annexin V-FITC in the dark for 30 minutes. Finally, the cells can be analyzed by flow cytometry (FACSCalibur, Ser. No. E1577, BD) equipped with Cell Quest software (Hsu, 2012).

Topoisomerase I Inhibitory Assays

Topoisomerase I assays were measured by assessing relaxation of supercoiled pBR322 plasmid DNA (Yang Kuo, 2005). Using camptothecin (CPT) as topo I a positive controls (1.61 μg/mL), tested samples were dissolved in 5% (v/v) DMSO and then diluted to appropriate concentrations. In summary, topo I (TopoGen) was mixed with the tested samples and 10 1 volume of assay buffer (100 mM Tris-HCl, 10 mM EDTA, 1.5 M NaCl, 1.0% BSA, 1 M spermidine, and 50% glycerol), and then supercoiled DNA (pBR322) was added. After incubation of topo I mixture for 30 min at 37 °C, 2 λ 10% SDS and 2.5 λ proteinase K were added for 1 h. The reaction mixtures were electrophoresed on a 2% agarose gel (50 V, 20 min; 100 V, 30 min; 110 V, 30 min) and stained with ethidium bromide. Finally, by a densitometer of ImageMaster ® (Fujifilm thermal imaging system, FTI-500), the gels were directly scanned and the area representing supercoiled DNA was calculated. Concentrations for 50% inhibition (IC_{50}) were determined by interpolation from plots of topoisomerase I activity versus inhibitor concentration.

Data Analysis

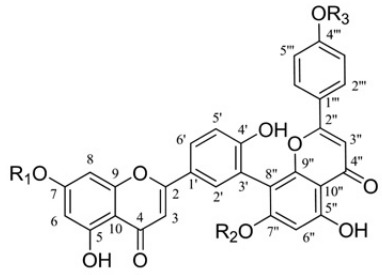
Data were presented as mean ± standard deviation (n = 3). The statistical comparisons were performed by one-way analysis of variance (ANOVA) with Duncan' s test. The significant differences were indicated as $p < 0.01$.

Results and Discussion

Structural elucidation of the isolated [3′→8″]-biflavonoids

The EtOH extract of the dried twigs of *P. nakaii* Hayata was successively extracted and partitioned with *n*-hexane, EtOAc, and H_2O, respectively. The EtOAc-soluble portion exhibited cytotoxic activities against four cell lines, KB (ED_{50} = 10.05 μg/mL), HEp-2 (ED_{50} = 32.35 μg/mL), DLD (ED_{50} = 32.46 μg/mL), and MCF-7 (ED_{50} = 17.25 μg/mL). The EtOAc-soluble layer was further separated and purified by a series of column chromatography and recrystallization to yield four [3′→8"]-biflavonoids, amenotoflavone (**AF**), podocarpusflavone-A (**PF**), II-4″,I-7 -dimethoxyamentoflavone (**DAF**), and heveaflavone (**HF**).

Compound **AF** was isolated as brown yellow amorphous powder, Mp. 292-296 ºC, and its molecular formula was determined to be $C_{30}H_{18}O_{10}$ from the analysis of its negative ESIMS (*m/z* 537.13, $[M-H]^-$) and NMR spectral data (Table 1). The IR spectrum showed the presence of hydroxyl (3475 cm^{-1}), conjugated ketone (1644 cm^{-1}), and aromatic rings (1605, 1567, 1504, 1465 cm^{-1}). The UV absorption maxima at 337, 268, and 226 nm suggested that compound **AF** possessed a flavonoid-core chromophore. The ^{13}C NMR data (Table 1) showed the presence of 30 resonances, which corresponded by DEPT analysis to twelve methines (δ(C) 104.0, C-2; 100.2, C-6; 94.8, C-8; 132.6, C-2′; 117.6, C-5′; 128.2, C-6′; 116.8, C-3″; 100.0, C-6″; 128.8 × 2, C-2‴, C-6‴; 116.8 × 2, C-3‴, C-5‴), sixteen quaternary carbons (δ(C) 164.8, C-1; 162.6, C-5; 164.5, C-7; 158.5, C-9; 104.9, C-10; 122.0, C-1′; 122.5, C-3′; 161.9, C-4′; 165.8, C-2″; 162.4, C-5″; 163.1, C-7″; 105.6, C-8″; 155.9, C-9″; 104.9, C-10″; 122.1, C-1‴; 162.6 C-4‴), and two conjugated ketones (δ(C) 182.7,



Amenotoflavone (**AF**) $R_1 = H, R_2 = H, R_3 = H$
Podocarpusflavone-A (**PF**) $R_1 = H, R_2 = H, R_3 = Me$
II-4‴,I-7-dimethoxyamentoflavone (**DAF**) $R_1 = Me, R_2 = H, R_3 = Me$
Heveaflavone (**HF**) $R_1 = Me, R_2 = Me, R_3 = Me$

Figure 1. Chemcal structures of [3′→8″]-biflavonoids from *P. nakaii* Hayata.

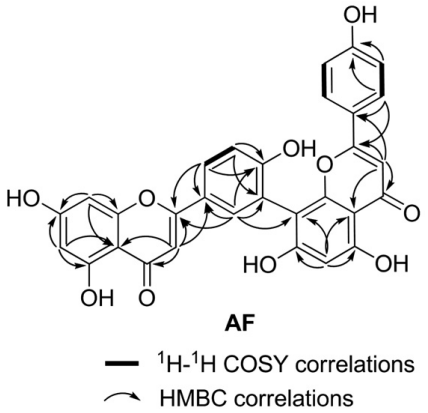


Figure 2. ^{1}H-^{1}H COSY and selected HMB correlations of compound AF.

C-4; 183.0, C-4‴). The ^{1}H NMR spectrum of **AF** (Table 1) exhibited an A_2B_2 spin system at δ(H) 7.89 (dd, *J* = 8.4, 2.4, H-2‴), 6.12 (d, *J* = 8.4, H-3‴), an ABX spin system at δ(H) 7.38 (d, *J* = 8.4, H-5′), 7.89 (dd, *J* = 8.4, 2.4, H-6′) and 8.55 (dd, *J* = 2.4, H-2′), and long-range W coupling spin system at δ(H) 6.78 (d, *J* = 2.0, H-6) and 6.77 (d, *J* = 2.0, H-8), along with three aromatic singlets at δ(H) 7.03, 6.91, and 6.85. The ^{1}H-^{1}H COSY correlations indicated four partial structures, which were also connected by analysis of HMBC correlations, as shown by the arrows in Figure 2. Furthermore, HMBC correlations of H-2′/C-2, C-1′, C-8″ and H-6″/C-5″, C-7″, C-8″ revealed that two flavonol units with a C-C linkage were connected at C-3′ and C-8″ positions in compound **AF**. Based on these findings (MS, UV, and NMR), together with the comparison of reference data (Markham, 1987), the naturally occurring [3′→8″]-biflavonoid, **AF**, was identified.

Compound **PF**, Mp. 289-292 ºC, has a molecular formula of $C_{31}H_{21}O_{10}$ deduced from ESIMS (*m/z* 551.13 $[M-Na]^-$). The presence of a hydroxyl, conjugated ketone, and aromatic ring functionalities in **PF** were evidenced by its IR absorption bands at ν_{max} 3442, 1652, 1612, 1557, 1510 and 1450 cm^{-1}, respectively. The UV spectrum showed absorptions at λ_{max} 341, 269, 221 nm, both showing similarity with those of compound **AF** and suggesting that compound **PF** also possesses a [3′→8″]-biflavonoid system. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) of **AF** were quite similar to those of **PF**, except for the appearance of a methoxyl proton and carbon signals (δ(H) 3.59, s; δ(C) 55.2) in **PF**. Moreover, the HMBC correlations of OMe/C-4‴ clearly determined the methoxyl group at C-4‴ position. Thus, compound **PF** was determined to be podocarpusflavone-A by detailed NMR spectroscopic analysis and comparisons with authentic samples and data reported in the literatures (Markham, 1987).

Compounds **DAF** and **HF** were obtained as amorphous yellow powders and their molecular formulas were determined to be $C_{32}H_{22}O_{10}$ (*m/z* 565.22 $[M-H]^-$) and $C_{33}H_{24}O_{10}$ (*m/z* 580.13 $[M-H]^-$) by ESIMS. The IR, UV, ^{1}H and ^{13}C NMR spectra indicated that both **DAF** and **HF** possess [3′→8″]-biflavonoids as **AF** and **PF**. Comparing the ^{1}H and ^{13}C NMR spectra of **DAF** and **HF** with those of compound **PF**, the additional methoxyl group (δ(C) 55.8) at C-7 in **DAF** and two methoxyl group at C-7 and C-7″ (δ(C) 56.4 and 55.8) in **HF** were inferred. The assignments of compounds **DAF** and **HF** were further established by 1D- and 2D NMR experiments, including ^{1}H-^{1}H COSY, HSQC, and HMBC. On the basis of above observations and comparison with related reports (Roy, 1987), compounds **DAF** and **HF** were determined to be II-4",I-7-dimethoxyamentoflavone and feveaflavone, respectively.

Inhibition of Growth on Cancer Cell Lines by [3′→8″]-biflavonoids

We utilized MTT assay for a three-day course to study the inhibition of cell viability on four cancer cell lines KB, DLD, MCF-7 and HEp-2 by treatment of four naturally occurring [3′→8″]-biflavonoids, compounds **AF**, **HF**, **DAF** and **PF**. At the dosage of 20 μg/mL, compounds **DAF** and **PF** showed moderated anti-proliferative activity against MCF-7 and HEp-2 cell lines, but compounds **AF** and **HF** were invalid. As shown in Table 2, the growth inhibitions of compound **PF** exhibited slightly stronger than **DAF**. Moreover, by a dose-dependent manner in the growth inhibitions of compounds **DAF** and **PF** were more potent on MCF-7 cell line, than that on HEp-2 cells. To make clear the growth inhibitions on MCF-7 cells resulting from growth arrest or apoptosis, we designed some

Table 2. The cytotoxicity of **DAF**, **PF**, and fractions on tumor cell lines.

Samples	ED50 of tumor cell lines (μg/mL)			
	KB	MCF-7	DLD	HEp-2
n-hexane layer	35.70	-	-	-
EtOAc layer	10.05	17.14	32.46	24.56
DAF	4.56	16.24	6.16	13.45
PF	4.67	15.17	4.95	10.87

[a]Human oral epidermoid carcinoma (KB), Human breast carcinoma (MCF-7), Human colon adenocarcinoma (DLD), Human laryerneal carcinoma (HEp-2)

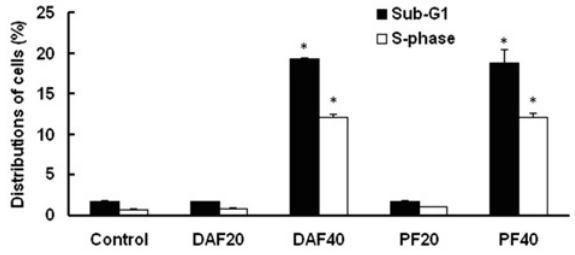


Figure 3. Flow cytometric analysis of the cell distributions of MCF-7 cells. Cells were treated with of compounds DAF and PF (20 or 40 g/mL) for 24 hr. Data were expressed as means ± SD (n = 3). * Significantly different ($p < 0.05$) versus the negative control (without any treatment).

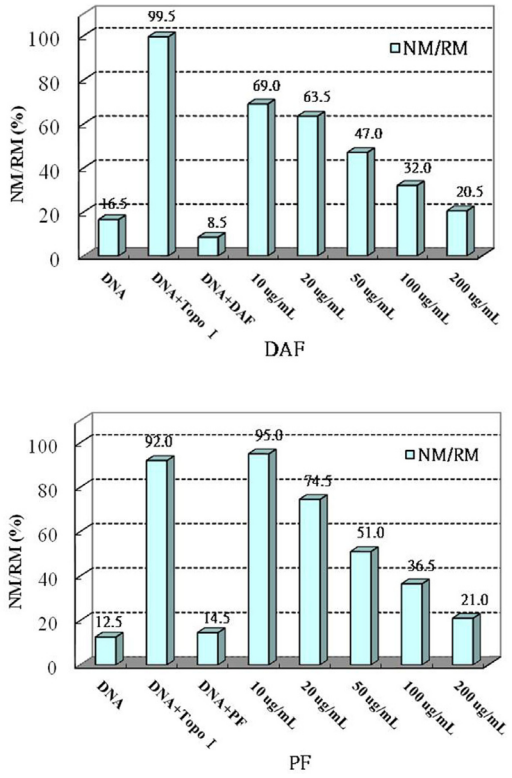


Figure 4. Topoisomerase I inhibitory activities of compounds **DAF** and **PF**.

experiments for the analysis of cell cycle in the next step.

Growth Arrest and Cell Death Induced by DAF and PF on MCF-7 Cells

To study the alterations of cell cycle on MCF-7 cells induced by **DAF** and **PF**, we utilized flow cytometry and the propidium iodide staining method. To analyze the sub-G_1 area and the portion of S-phase, compounds DAF and PF (40 μg/mL, 24 hr) significantly induced about 10 folds of cell deaths and growth arrest in S-phase than the control group (Figure 3). The findings suggested that compounds **DAF** and **PF** were possible to possess two strategies to develop into anti-tumor drugs, which were directly cancer-killing or a good agent for a combined therapy.

Inhibition on Topoisomerase I by DAF and PF

To explore the target of compounds **DAF** and **PF**, we used both of them and CPT for a standard Topoisomerase I inhibitory assay (Figure 4). Compounds **DAF** and **PF** showed a moderated inhibition against Topoisomerase I (IC_{50} = 42.41 and 65.98 μg/mL, respectively). The correlations between the inhibition of Topoisomerase I (IC_{50} = ca 40 ~ 70 μg/mL) and the inhibition of the growth on MCF-7 cells (IC_{50} = ca 20 ~ 40 μg/mL), suggested that Topoisomerase I would be one of the targets for the cytotoxic **DAF** and **PF**. This finding also supported the growth arrest in S-phase induced by compounds **DAF** and **PF**, may possibly result from the inhibition of Topoisomerase I.

Conclusions

In summary, four naturally occurring [3′→8″]-biflavonoids, amenotoflavone (**AF**), podocaepus flavone-A (**PF**), II-4″, I-7-dimethoxyamentoflavone (**DAF**), and heveaflavone (**HF**) have been successfully isolated by bioassay-guided fractionation and identified by spectroscopic data from the EtOH extract of Taiwanese endemic plant, *Podocarpus nakaii* Hayata. Among them, compounds **PF** and **DAF** were proven to have significant cytotoxicity against KB, MCF-7, DLD, and HEp-2 tumor cell lines and may induce MCF-7 cell apoptosis in a dose-dependent manner via mainly sub-G_1/S phase arrest. S phase arrest on apoptosis pathway is a common hallmark induced by DNA topoisomerase I inhibitor; therefore, both **PF** and **DAF** were also demonstrated to possess moderate DNA topoisomerase I inhibitory activities in this report.

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References

Banerjee, T., Valacchi, G., Ziboh, V.A., van der Vliet, A., 2002. Inhibition of TNFa-induced cyclooxygenase-2 expression by amento-flavone through suppression of NF-kB activation in A549 cells. Molecular and Cellular Biochemistry 238, 105-110.

Chen, C.H., 1993. Flora of Taiwan, second edition, Taipei: Editorial Committee of the Flora of Taiwan, Taiwan, 1, pp 564-565.

Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A., Moon, R., 1995. Flavonoids as DNA Topoisomerase Antagonists and Poison: Sturcture-Activity Relationships. Journal of Natural Products 58, 217-225.

Dhooghe, L., Maregesi, S., Mincheva, I., Ferreira, D., Marais, J.P.J., Lemiere, F., Matheeussen, A., Cos, P., Maes, L., Vlietinck, A., Apers, S., Pieters, L., 2010. Antiplasmodial activity of (I-3,II-3)-biflavonoids and other constituents from *Ormocarpum kirkii*. Phytochemistry 71, 785-791.

Hsu, L.C., Hsu, Y.W., Liang, Y.H., Liaw, C.C., Kuo, Y.H., Pan, T.M., 2012. Induction of Apoptosis in Human Breat Adenocarcinoma Cells MCF-7 by Monapurpyridine A, a New Azaphilone Derivative from *Monascus purpureus* NTU 568. Molecular 17, 664-673.

Kang, S.S., Lee, J.Y., Choi, Y.K., Song, S.S., Kim, J.S., Jeon, S.J., Han, Y.N., Son, K.H., Han, B.H., 2005. Neuroprotective effects of naturally occurring biflavonoids. Bioorganic & Medicinal Chemistry Letters 15, 3588-3591.

Kim, H.P., Park, H., Son, K.H., Chang, H.W., Kang, S.S., 2008. Biochemical pharmacology of biflavonoids: Implications for anti-inflammatory action. Archives of Pharmacal Research 31, 265-273.

Kuo, Y.J., Hwang, S.Y., Wu, M.D., Liao, C.C., Liang, Y.H., Kuo, Y.H., Ho, H.O., 2008. Cytotoxic constituents from *Podocarpus* fasciculus. Chemical Pharmaceutical Bulletin 56, 585-588.

Lee, M.K., Lim, S.W., Yang, H., Sung, S.H., Lee, H.S., Park, M.J., Kim, Y.C., 2006. Osteoblast differenatiation stimulating activity of biflavonoids from *Cephalotxus koreana*. Bioorganic & Medicinal Chemistry Letters 16, 2850-2854.

Lin, L.C., Kuo, Y.C., Chou, C.J., 2000. Cytotoxic Biflavonoids from *Selaginella delicatula*. Journal of Natural Products 63, 627-630.

Lin, Y.M., Flavin, M.T., Schure, R., Chang, H.W., Chen, F.C., Sidwell, R., Barnard, D.L., Huffman, J.H., Hern, E.R., 1999. Antiviral Activities of Biflavonoids. Planta Medica 65, 120-125.

Markham, K.R., Sheppard, C., Geiger, H., 1987. ^{13}C NMR studies of some naturally occurring amentoflavone and hinokiflavone biflavonoids. Phytochemistry 26, 3335-3337.

Park, H.S., Yoda, N., Fukaya, H., Aoyagi, Y., Takeya, K., 2004. Rakanmakilactones A-F, new cytotoxic sulfur-containing norditerpene dilactones from leaves of *Podocarpus macrophyllus var. maki*. Tetrahedron 60, 171–177.

Roy, S.K., Qasim, M.A., Kamil, M., Ilyas, M., 1987. Biflavones from the genus *Podocarpus*. Phytochemistry 26, 1985-1988.

Tselpi, M., Papachristou, E., Emmanouilidi, A., Angelis, A., Aligiannis, N., Skaltsounis, A.-L., Kouretas, D., Liadaki, K., 2011. Catalytic Inhibition of Eukaryotic Topoisomerases I and II by Flavonol Glycosides Extracted from *Vicia faba and Lotus edulis* Journal of Natural Products 74, 2362-2370.

Yang Kuo, L.M., Chen, K.Y., Hwang, S.Y., Chen, J.L., Liu, Y.Y., Liaw, C.C., Ye, P.H., Chou, C.J., Shen, C.C., Kuo, Y.H., 2005. DNA Topoisomerase I Inhibitor, Ergosterol Peroxide from *Penicillium oxalicum*. Planta Medica 71, 77–79.