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 1H, 13C, 1H-1H 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 (ED50 ca. 4.56-16.24 µg/mL) and induced cell apoptosis in MCF-7 via mainly sub-G1/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 (1H, 13C, 1H-1H COSY, HMQC, and HMBC), as well as confirmed by comparing with the...
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 a 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 CD3OD and pyridine-d5 as solvent. Silica gel (Merck 570-230 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% H2SO4 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 (NRCIM, No. NRCIM200607A1) 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 45°C for three times and the ethanol extracts were combined and concentrated under vacuum. The crude extract was partitioned between *n*-hexane and H2O (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 CH2Cl2/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 CH2Cl2/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 CH2Cl2/MeOH to furnish four fractions (E7A-D), and then Fr. E7B was separated by a RP-HPLC (MeOH/H2O, 55:40) column to yield amentoflavone (AF, 8.2 mg).

**Amentoflavone (AF):** Brown yellow amorphous powder. Mp. 292-296 °C; UV (MeOH) λmax 337, 268, 226 nm; IR (KBr) νmax 3475, 2993, 2923, 1644, 1605, 1567, 1504, 1465, 1251, 1172, 1102, 1040, 842 cm⁻¹; 1H-NMR (pyridine-d5, 400 MHz) see Table 1; Negative ESIMS m/z 537.08 [M-H].

**Podocarpusflavone-A (PF):** Yellow amorphous powder. Mp. 289-292 °C; UV (MeOH) λmax 341, 269, 221 nm; IR (KBr) νmax 3442, 2983, 1652, 1612, 1557, 1510, 1450, 1249, 1178, 1162, 1107, 1030, 823 cm⁻¹; 1H-NMR (pyridine-d5, 400 MHz) and 13C-NMR (pyridine-d5, 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 °C; UV (MeOH/CH2Cl2) λmax 330, 269, 224 nm; IR (KBr) νmax 3399, 2924, 1644, 1606, 1560, 1504, 1445, 1378, 1253, 1182, 1160, 1109, 1036, 831 cm⁻¹; 1H-NMR (pyridine-d5, 400 MHz) and 13C-NMR (pyridine-d5, 100 MHz) see Table 1; Negative ESIMS m/z 565.22 [M-H].

**Heveaflavone (HF):** Yellow amorphous powder. Mp. 262-266 °C; UV (MeOH/CH2Cl2) λmax 328, 270, 225 nm; IR (KBr) νmax 3454, 2925, 1651, 1604, 1503, 1441, 1370, 1259, 1180, 1160, 1110, 1054, 1027, 835 cm⁻¹; 1H-NMR (pyridine-d5, 400 MHz) and 13C-NMR (pyridine-d5, 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, Israel). Other chemicals, such as 3-(4,5-dimethylthiazol-2-yl)-

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

| No. | AF $^1$H NMR ($J$ in Hz) | PF $^1$H NMR ($J$ in Hz) | DAF $^1$H NMR ($J$ in Hz) | HF $^1$H NMR ($J$ in Hz) | DAF $^{13}$C NMR | HF $^{13}$C NMR | DAF $^{13}$C NMR | HF $^{13}$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 \( (J = 1.8 \text{ Hz}) \) | 100.2 d \( (J = 2.0 \text{ Hz}) \) | 99.8 d \( (J = 2.0 \text{ Hz}) \) | 100.0 d \( (J = 2.0 \text{ Hz}) \) | 6.52 d \( (J = 2.0 \text{ Hz}) \) | 98.5 d |
| 7   | 164.5 s                  | 165.6 s                   | 165.7 s                   | 165.7 s                  |                  |                |                |                |
| 8   | 6.77 d \( (J = 2.4 \text{ Hz}) \) | 94.8 d \( (J = 2.4 \text{ Hz}) \) | 94.7 d \( (J = 2.4 \text{ Hz}) \) | 93.0 d \( (J = 2.4 \text{ Hz}) \) | 6.75 d \( (J = 2.0 \text{ Hz}) \) | 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 \( (J = 2.4 \text{ Hz}) \) | 132.6 d \( (J = 2.4 \text{ Hz}) \) | 132.4 d \( (J = 2.4 \text{ Hz}) \) | 132.6 d \( (J = 2.4 \text{ Hz}) \) | 8.39 d \( (J = 2.0 \text{ Hz}) \) | 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 \( (J = 8.4 \text{ Hz}) \) | 117.6 d \( (J = 8.4 \text{ Hz}) \) | 117.0 d \( (J = 8.4 \text{ Hz}) \) | 117.5 d \( (J = 8.0 \text{ Hz}) \) | 7.54 d \( (J = 8.0 \text{ Hz}) \) | 117.1 d |
| 6'  | 7.89 dd \( (J = 8.4, 8.4 \text{ Hz}) \) | 128.2 d \( (J = 8.4, 8.4 \text{ Hz}) \) | 128.1 d \( (J = 8.8, 2.4 \text{ Hz}) \) | 128.3 d \( (J = 8.0, 2.0 \text{ Hz}) \) | 8.06 dd \( (J = 8.0, 2.0 \text{ Hz}) \) | 128.4 d |
| 2'' | 165.8 s                  | 163.9 s                   | 164.3 s                   | 164.3 s                  |                  |                |                |                |
| 3'' | 6.91 s                   | 116.8 s                   | 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 \( (J = 2.4, 8.4 \text{ Hz}) \) | 128.8 d \( (J = 8.8) \) | 128.2 d \( (J = 8.5) \) | 128.4 d \( (J = 8.5) \) | 7.84 d \( (J = 8.5) \) | 128.4 d |
| 3'' | 7.12 dd \( (J = 8.4 \text{ Hz}) \) | 116.8 d \( (J = 8.8) \) | 114.7 d \( (J = 8.5) \) | 114.8 d \( (J = 8.5) \) | 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 \( (J = 8.4 \text{ Hz}) \) | 116.8 d \( (J = 8.8) \) | 114.7 d \( (J = 8.5) \) | 114.8 d \( (J = 8.5) \) | 6.95 d \( (J = 8.5) \) | 114.9 d |
| 6'' | 7.89 dd \( (J = 2.4, 8.4 \text{ Hz}) \) | 128.8 d \( (J = 8.5) \) | 128.2 d \( (J = 8.5) \) | 128.4 d \( (J = 8.5) \) | 7.84 d \( (J = 8.5) \) | 128.4 d |
| 7''-OCH$_3$ | 3.73 s                  | 56.4 q                  | 3.69 s                  | 55.8 q                  | 3.68 s          | 55.8 q |
| 7-OCH$_3$ | 3.59 s                  | 55.3 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\textsubscript{2} at 37 ºC.

**Cytotoxicity Assay**

Cells (3 x 10\textsuperscript{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 x 10\textsuperscript{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 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 ºC, 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\textsubscript{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\textsubscript{2}O, respectively. The EtOAc-soluble portion exhibited cytotoxic activities against four cell lines, KB (ED\textsubscript{50} = 10.05 µg/mL), HEp-2 (ED\textsubscript{50} = 32.35 µg/mL), DLD (ED\textsubscript{50} = 32.46 µg/mL), and MCF-7 (ED\textsubscript{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\textsubscript{30}H\textsubscript{18}O\textsubscript{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\textsuperscript{-1}), conjugated ketone (1644 cm\textsuperscript{-1}), and aromatic rings (1605, 1567, 1504, 1465 cm\textsuperscript{-1}). The UV absorption maxima at 337, 268, and 226 nm suggested that compound AF possessed a flavonoid-core chromophore. The \textsuperscript{13}C NMR data (Table 1) showed the presence of 30 resonances, which corresponded by DEPT analysis to twelve methines, sixteen quaternary carbons, and two conjugated ketones (δ(C) 182.7, 182.6).
The $^1$H NMR spectrum of AF (Table 1) exhibited an A2B2 spin system at $\delta$(H) 7.89 (dd, $J = 8.4, 2.4$, H-2″′), 6.12 (d, $J = 8.4$, H-3″′) and ABX spin system at $\delta$(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′), along with three aromatic singlets at $\delta$(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.

Compounds DAF and HF were obtained as amorphous yellow powders and their molecular formulas were determined to be $C_{31}H_{21}O_{10}$ (m/z 551.13 [M-Na-]) 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 ($\delta$(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
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-G₁ 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 (IC₅₀ = 42.41 and 65.98 µg/mL, respectively). The correlations between the inhibition of Topoisomerase I (IC₅₀ = ca 40 ~ 70 µg/mL) and the inhibition of the growth on MCF-7 cells (IC₅₀ = 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), podocaepusflavone-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-G₁/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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