SOME IMMUNOMODULATORY PRINCIPLES ISOLATED FROM PIPER KADSURA

Yuh-Chi Kuo[1,2], Lie-Chwen Lin[1], Nai-Shian Yang[2], Wei-Jern Tsai[1],

An-Pang Lin[3], Ming-Jen Lee[4] and Cheng-Jen Chou[1]

[1]*National Research Institute of Chinese Medicine,*
[2]*Institute of Biology, Fu-Jen University,*
Taipei, Taiwan
[3]*China Medical College,*
Taichung, Taiwan
[4]*Ming-Jen Lee Chinese Medical United Clinic,*
Taipei, Taiwan

From the ethanol extract of the stem of *Piper kadsura* (Piperaceae), seven compounds-futoquinol; futoenone; (+)-crotepoxide; galgravin; (-)galbelgin; piperlactam **S** and piperolactam **B** were isolated, identified and tested for immunopharmacological activities with human mononuclear cells (HMNC) being used as target cells and cell proliferation determined by ^{3}H-thymidine uptake. The results indicated that compounds futoquinol, galgravin, piperlactam **S** and piperolactam **B** potently inhibited HMNC proliferation and interferon-γ production. Therefore immunosup pressive mechanisms might involve the impairment of cytokines production.

Key words: *Piper kadsura*, Human mononuclear cell, Proliferation, Interferon-γ.

INTRODUCTION

Piper kadsura (Choisy) Ohwi (Piperaceae) is a stems scandent, nodes rooting, and somewhat aromatic plant found in forests at low to medium altitudes throughout Taiwan.[1] The stem of *P. kadsura* (Chinese name: Hai-Feng-Teng) is widely used in Chinese herbal medicine for the treatment of asthma and rheumatic arthritis. A variety of compounds have been found in this plant, including amide, lignans, neolignans, terpenes and oxygenated cyclohexanes.[2] Recently, this plant has received considerable attention because of its reputation for producing

Correspondence to: Cheng-Jen Chou, National Research Institute of Chinese Medicine, No. 155-1, Sec. 2, Li-Nung St., Peitou, Taipei (112), Taiwan, Phone: 886-2-28201999, ext.7101. Fax: 886-2-28264276. E-mail: choucj@cma23.nricm.edu.tw.

neolignans and lignans with platelet activating factor (PAF) antagonist activity and displaying significant tumor-inhibitory activity.[3-7] However there has been a relative scarcity of information pertaining to its immunopharmacological activity. In the course of our search for biologically active substances in nature, we found that the ethanol extract from the stems of *P. kadsura* suppressed human mononuclear cells (HMNC) proliferation[8]. This observation led us to reinvestigate the plant's active principles for the immunomodulatory potentials. In the present studies, HMNCs were used as target cells. The effects of those compounds isolated from the stems of *P. kadsura* on HMNC proliferation and IFN-γ production were determined.

EXPERIMENT

General

Melting points were determined with a Yanagimoto micro melting point apparatus and were uncorrected. IR spectra were taken on a Perkin-Elmer 781 ir spectrometer. Optical rotations were measured on a JASCO DIP-370 polarimeter. FABMS and EIMS spectra were determined respectively on a JEOL JMS-HX 110 spectrometer and a Finnigan GCQ spectrometer. ^{1}H, ^{13}C-, and 2D- NMR spectra were determined on a Bruker ACP-300 spectrometer with deuterated solvents as internal standards. INADEQUATE experiments were carried out on a Varian INOVA 500 MHz NMR spectrometer.

Plant Materials

The stems of *Piper kadsura* (Choisy) Ohwi (syn. *P. futokadsura* Sieb. et Zucc.) were collected at Pettou, Taipei, in August 1997. Plant materials were identified by comparing with (TAI 145842) which had been deposited in the Herbarium of the Department of Botany of National Taiwan University.

Extraction and Isolation

The air-dried stems of *P. kadsura* (16.3 Kg) were extracted with ethanol three times at room temperature. The extract was filtered and concentrated under reduced pressure to give a dark green semi-solid with a very distinctive pepper-like aroma and was partitioned between H_2O and dichloromethane. The organic layers were combined and concentrated under reduced pressure to give 240 g of residue. The dichloromethane extract was subjected to silica gel column chromatography with a gradient of EtOAc in hexane. Thirteen fractions (1-13) were collected. The fractions were collected in 400mL portions and pooled according to their TLC profiles in hexane-EtOAc (85:15 v/v) or hexane/CHCl$_3$/MeOH (3/1.7/0.3 v/v). Fraction 8 (30 g) was further purified by Sephadex LH-20 column (acetone) and silica gel MPLC (EtOAc gradient in hexane) to give futoquinol (**1**, 1.73 g). Fraction 9 (137 g) was dissolved in acetone and gave crystalline futoenone (**2**, 12.03 g). The remaining extract (123 g) was chromatographed on silica

gel CC (2 kg) and eluted with a solvent system (hexane/CHCl$_3$/MeOH, 60/8.5/1.5 v/v). Six fractions (I-VI) were collected. Fraction I (24.4 g) gave compounds **3** (8.45 g), **4** (1.03 g), and **5** (0.47 g) following partitioning on silica gel MPLC (EtOAc gradient in hexane). Chromatography of fraction III (32.3 g) on a Sephadex LH-20 column (acetone) afforded compounds **6** (128 mg), and **7** (21 mg).

Futoquinol (1)

Appeared as colorless fine needles ; mp 96-97 °C (lit.[2] mp 97-98°C).   Spectral data (^{1}H NMR, ^{13}C NMR and EIMS were as reported previously.[10, 11]

Futoenone (2)

Appeared as colorless prisms; mp 197-199 °C (lit.[12] mp 197 °C; lit.[13] mp 192 °C); $[\alpha]_D$ -65.1°(C 1.26, CHCl$_3$) (lit.[13] $[\alpha]_D$ -60.0° (C 0.103, CHCl$_3$)). Spectral data (^{1}H NMR, ^{13}C NMR and EIMS) were as reported previously.[12-14]

(+)-Crotepoxide (Futoxide) (3)

Appeared as colorless needles; mp 148-149 °C (lit.[15] mp 153°C); $[\alpha]_D$ + 67.2° (C 1.31, CHCl$_3$) (lit.[16] $[\alpha]_D$ +71.2° (C 0.34, CHCl$_3$). Spectral data (^{1}H NMR, ^{13}C NMR and EIMS) were as reported previously. [15-17]

Galgravin (4)

Appeared as a colorless powder; mp 116-118 °C (lit.[18] mp 117-119 °C); $[\alpha]_D$ -76.3°(C 1.18, CHCl$_3$) ; ^{13}C NMR(CDCl$_3$, 75.4 MHz) δ 148.9(s), 148.4(s), 134.8(s), 118.5(d), 110.9(d), 109.7(d), 87.2(d), 55.9(q), 55.8(q), 44.3(d), 12.9(q). Spectral data (^{1}H NMR, and EIMS) were as reported previously. [5, 18, 19]

(-)-Galbelgin (5)

Appeared as a colorless powder; mp 141-143 °C (lit.[20] mp 142 °C); $[\alpha]_D$ -98.2°(C 1.10, CHCl$_3$) (lit.[20] $[\alpha]_D$ -85.1° (C 0.047, CHCl$_3$)). Spectral data (^{1}H NMR, ^{13}C NMR and EIMS) were as reported previously. [19, 21]

Piperlactam S (6)

Appeared as yellow needles; mp 242-244 °C (lit.[9] 242-244 °C); IR (KBr) ν max 3460 (OH), 1696 (C=O), 1650, 1620, 1540, 1490, 1320, 990 cm^{-1}; EIMS *m/z* 295[M]$^+$; ^{1}H NMR (DMSO-d$_6$, 500 MHz) and ^{13}C NMR (DMSO-d$_6$, 125 MHz), see Table 1 ; HREIMS *m/z* [M]$^+$ 295.0847 (calcd 295.0845 for C$_{17}$ H$_{13}$NO$_4$).

Piperolactam B (7)

Appeared as a brown yellow powder, mp 228-230 °C (lit.[22] 222-224 °C); spectral data (IR, ^{1}H NMR, ^{13}C NMR and EIMS) were as reported previously. [22]

Biological assays

Lymphoproliferation test

Heparinized human blood (20 mL) was obtained from healthy donors. HMNCs were isolated by the Ficoll-Hypaque method as described previously.[23] The blood was centrifuged at 2500 rpm at 4 °C for 10 min to remove plasma. Blood cells were then diluted with PBS buffer and centrifuged in Ficoll-Hypaque discontinuous gradient at 1500 rpm at room temperature for 30 min. The HMNC layers were collected and washed with cold distilled H_2O and 10X Hank's buffer saline solution to remove red blood cells. The density of the HMNCs was adjusted to 2 $\times 10^6$ cells/mL before use. Cell suspension (100 μL) was introduced into each well of a 96-well flat-bottomed (Nunc 167008, Nunclon, Roskilde, Denmark) plate with or without 5 μg/ml phytohemagglutinin (PHA) (Gibco, Grand Island, NY.). Cyclosporine A (12.5 μM) or various concentrations of the test compounds obtained from *P. kadsura* were cultured with the cells. The plates were then incubated in 5% CO_2-air humidified atmosphere at 37 °C for 3 days. Subsequently, ^{3}H-thymidine (6.70 Ci/mmol, 1 μCi/well, NEN) was added into each well. After 16 hr of incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, MultiMash 2000, Billingshurst, UK). Radioactivity in the filters was measured by a scintillation counter. The inhibitory activity of each compound on HMNC proliferation was calculated by the following equation:

$$\text{Inhibitory activity (\%)} = \frac{\text{Control group (cpm)} - \text{Experimental group (cpm)}}{\text{Control group (cpm)}} \times 100$$

Determination of IFN-γ production

The resting or PHA activated HMNCs (2×10^5 cells/well) were cultured for 3 days with or without cyclosporine A (12.5 μM) or various concentrations of the purified compounds isolated from *P. kadsura*. The cell supernatants were then collected and assayed for IFN-γ concentration by enzyme immunoassay (EIA; Quantikine IFN-γ test kit, R&D systems).

Determination of cell viability

Approximately 2×10^5 of PHA activated HMNC were cultured with the culture medium, 0.1% DMSO, or 100 μM of Compounds **1**, **4**, **6**, or **7** for 4 days. Total, viable, and non-viable cell numbers were counted under the microscope with the help of a hemocytometer following staining by trypan blue. The percentages of viable cells were calculated as follows:

$$\text{Viability (\%)} = \frac{\text{Viable Cell Number}}{\text{Total Cell Number}} \times 100$$

Statistical analysis

Data were presented as mean ± SD. The differences between groups were assessed with Student's *t*-test for statistical significances.

RESULTS AND DISCUSSION

The ethanol extract of the stems of *P. kadsura* was partitioned between water and dichloromethane. Column chromatography of the dichloromethane extract gave seven compounds. They were respectively futoquinol (**1**), futoenone (**2**), (+)-crotepoxide (**3**), galgravin (**4**), (-)-galbelgin (**5**), piperlactam **S** (**6**), and piperolactam **B** (**7**). The structures of those compounds were identified by comparison with existing data from the literatures.[2,5,9-22] Compounds **6** and **7** represent the first report of the occurrence of phenanthrene lactams in this species. Through our integrated ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY, COLOC, and INADEQUATE NMR experiments, the assignment of all carbon signals of compound **6** was carried out (Table 1). However, the signals for C-8a and C-10 had been documented in the literature.[9]

All of these compounds were evaluated for their effects on HMNC proliferation. As shown in Figure 1, the ^{3}H-thymidine uptake in neither the resting nor stimulated states was affected by DMSO (0.1%) treatment. Cyclosporine A was used as a positive control. While it had little effects on ^{3}H-thymidine uptake in resting cells, cyclosporine A

Table 1.   **[1]H- and ^{13}C-Nmr Chemical Shift Assignments [a] of 6 (piperlactam S)**

Position	δ [13]C	δ [1]H	COLOC ([2]J and [3]J)	INADEQUATE
1	117.89 (s)			C=O, C-2
2	114.03 (d)	7.66 (1 H, s)	OH-3	C-1, C-3
3	152.24 (s)		H-2, OH-3	C-2, C-4
4	149.38 (s)		H-2, OCH₃-4, OH-3	C-3, C-4a
4a	120.23 (s)		H-5	C-4, C-5a, C-11
5a	126.31 (s)		H-9	C-4a, C-5, C-8a
5	126.77 (d)	9.12 (1H,dd, J=9.0, 1.5 Hz)		C-5a, C-6
6	125.83 (d)	7.59 (1 H, m)	H-8	C-5, C-7
7	127.37 (d)	7.59 (1 H, m)	H-5	C-6, C-8
8	129.12 (d)	7.99 (1H,dd, J=9.0, 1.5 Hz)	H-6, H-9	C-7, C-8a
8a	134.00 (s)		H-5, H-8, H-9	C-5a, C-8, C-9
9	102.59 (d)	7.36 (1 H, s)	H-8	C-8a, C-10
10	131.88 (s)		H-9	C-9, C-11
11	117.13 (s)		H-2, H-9	C-10, C-4a
C=O	162.10 (s)		H-2	C-1
4-OCH₃	59.47 (q)	4.03 (3 H, s)		
N-OCH₃	64.37 (q)	4.07 (3 H, s)		
3-OH		10.35(s)		

[a] Assignments were based on ^{13}C-DEPT, ^{13}C-^{1}H-COSY, ^{1}H-^{1}H COSY, Coloc and Inadequate spectra.
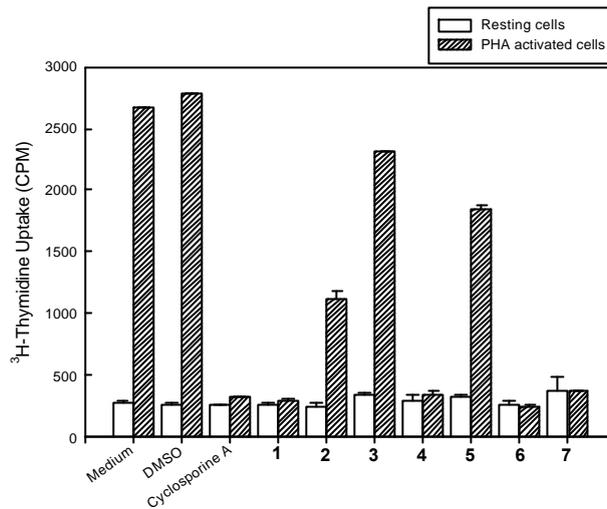
Figure 1. **Effects of compounds 1, 2, 3, 4, 5, 6, and 7 on proliferatoin of HMNC. 2×10^5 HMNC were either in resting state or activated by PHA (5 μg/ml). The cells were treated with or without 12.5 μM of cyclosporine A or 100 μM of all compounds (1-7) for 3 days. Then ^{3}H-thymidine was pulsed for 16 h before harvesting. Radioactivity was counted by scintillation counting. Each bar represents the mean of three independent experiments.**
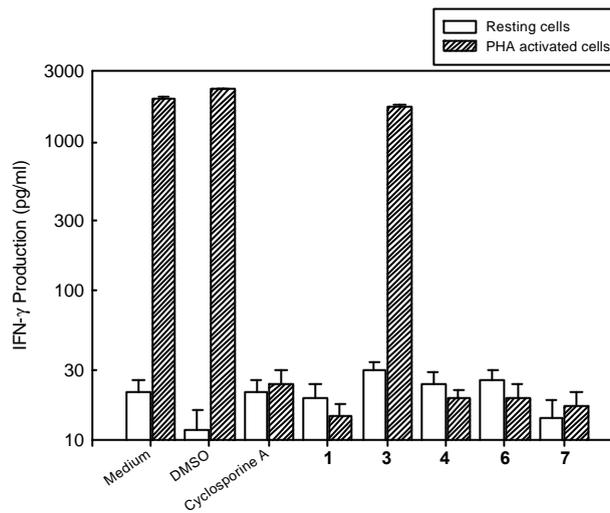
Figure 2. **Effects of compounds 1, 3, 4, 6 and 7 on IFN-γ production by HMNC. Resting or PHA (5 μg/ml) activated HMNCs were cultured with or without 12.5 μM of cyclosporine A or 100 μM of compounds 1, 3, 4, 6 and 7 for 3 days. IFN-γ productions in the cell supernants were assayed by EIA. Each bar represents the mean of three independent experiments.**

inhibited HMNC proliferation activated by PHA. Compounds **3** and **5** had little effect on proliferation in either resting or PHA activated cells. At 100 μM, compound **2** suppressed PHA treated HMNC proliferation to the extent of 60.1 ± 2.5 %. By contrast, the enhanced uptake observe in activated cells were significantly suppressed by compounds **1, 4, 6,** and **7** (P < 0.001). At 100 μM, the inhibitory activities of compounds **1, 4, 6,** and **7** on activated HMNC proliferation were $89.7 \pm 1.3\%$, $87.9 \pm 1.4\%$, $91.1 \pm 2.9\%$, and $86.5 \pm 1.1\%$, respectivelly.
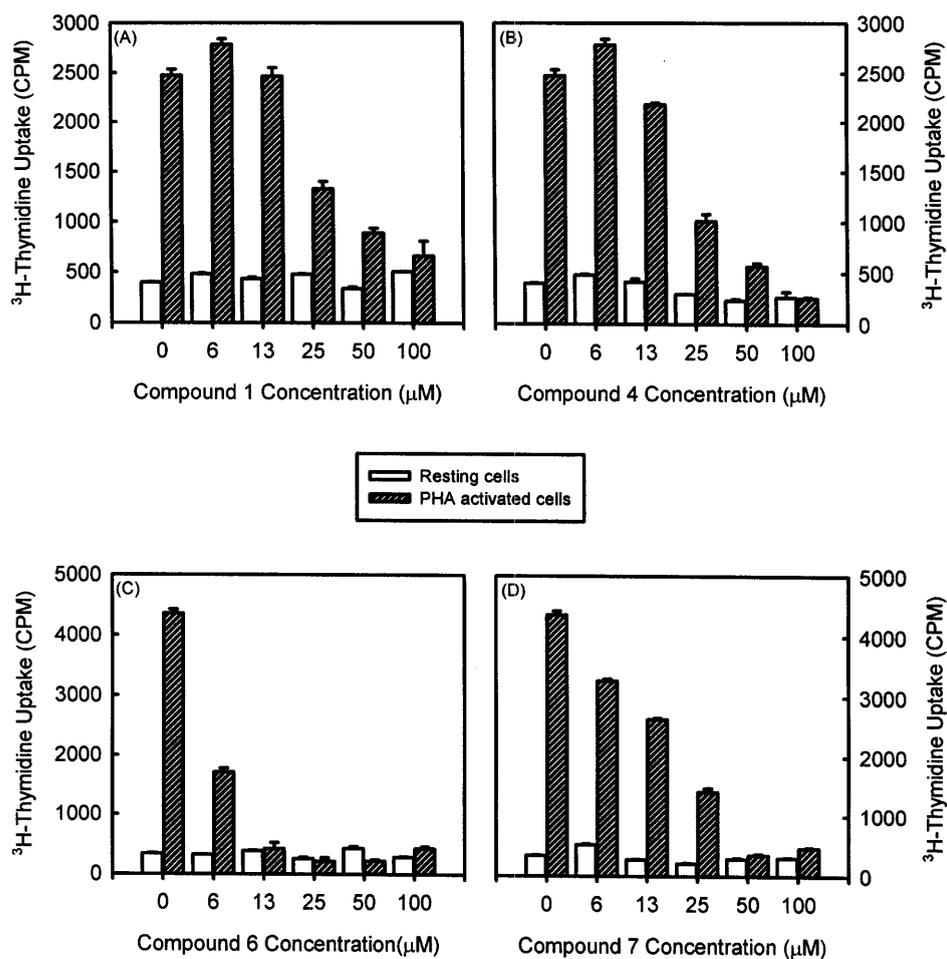
Figure 3. The dosage responses of compounds 1, 4, 6, and 7 on lymphoproliferation of HMNC. 2×10^5 HMNC were either in resting state or activated by PHA (5 µg/ml). The cells were treated with or without various concentrations of compounds 1, 4, 6, and 7 for 3 days. Cell proliferation was determined by the ^{3}H-thymidine uptake. Radioactivity was counted by scintillation counting. Each bar represents the mean of three independent experiments.

To study whether the decrease in HMNC proliferation was related to cytokine production, the cell supernatants were collected, and IFN-γ concentration determined by EIA. The results are shown in Figure 2. The IFN-γ production of HMNC was attenuated by cyclosporine A treatment. Although compound 3 had no effect, IFN-γ production in activated HMNC were significantly suppressed by compounds 1, 4, 6, and 7 (P < 0.001). The results demonstrated that the inhibitory actions of these four compounds on HMNC proliferation and cytokines production were similar to that of cyclosporine A. As shown in Figures 3 and 4, compounds 1, 4, 6, and 7 suppressed lympoproliferation and IFN-γ production of HMNC in a dose dependent manner. The IC_{50} values of compounds 1, 4, 6, and 7 on proliferation of HMNC stimulated with PHA were 34.4 ± 1.3 µM, 28.8 ± 2.1 µM, 5.0 ± 1.5 µM, and 15.5 ± 1.8 µM, respectively. While the IC_{50} values of compounds 1, 4, 6, and 7 on IFN-γ production in HMNC activated with PHA were 14.0 ± 2.2 µM, 3.0 ± 1.3 µM, 12.5 ± 3.0 µM, and 14.2 ± 2.6 µM, respectively. This indicated that compounds 1, 4, 6, and 7 had suppressory activity for HMNC and *P. kadsura* contained immunomodulatory
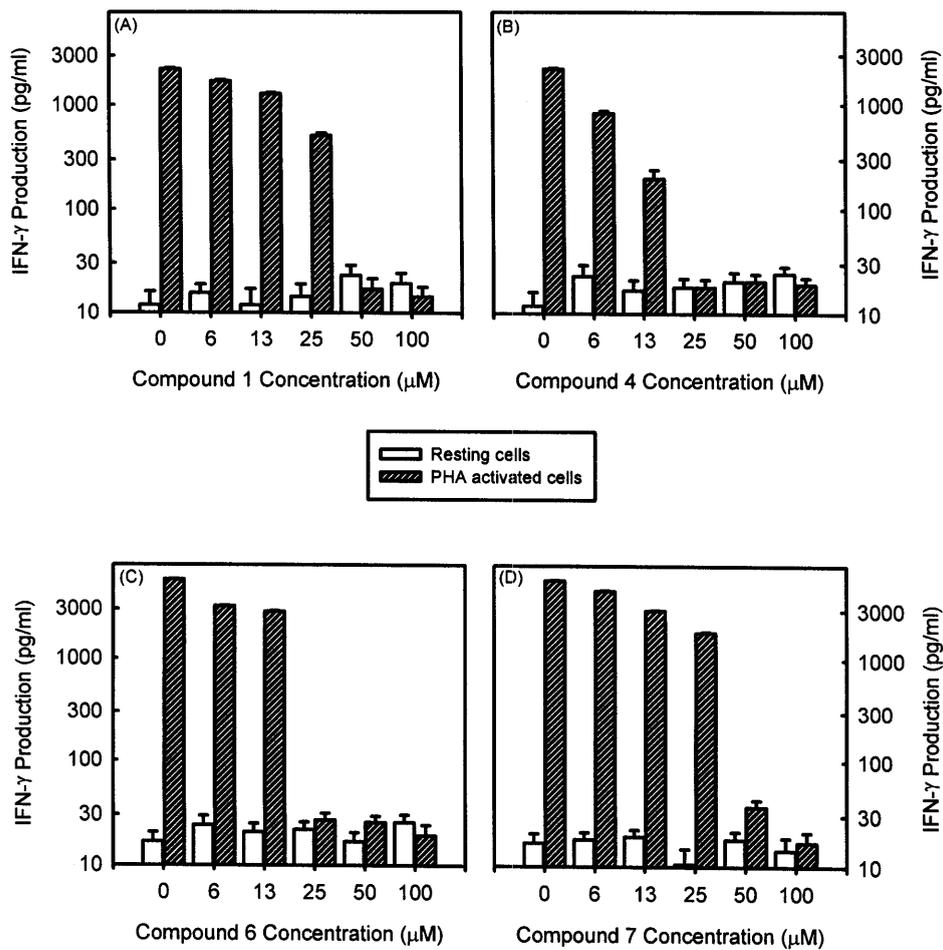
Figure 4. The dosage responses of compounds 1, 4, 6 and 7 for IFN-γ production in HMNC. 2 × 10⁵ HMNC were either in resting state or activated by PHA (5 μg/ml). The cells were treated with or without various concentrations of compounds 1(A), 4(B), 6(C), or 7(D) for 3 days. IFN-γ productions in the cell supernatants were assayed by EIA. Each bar represents the mean of three independent experiments.
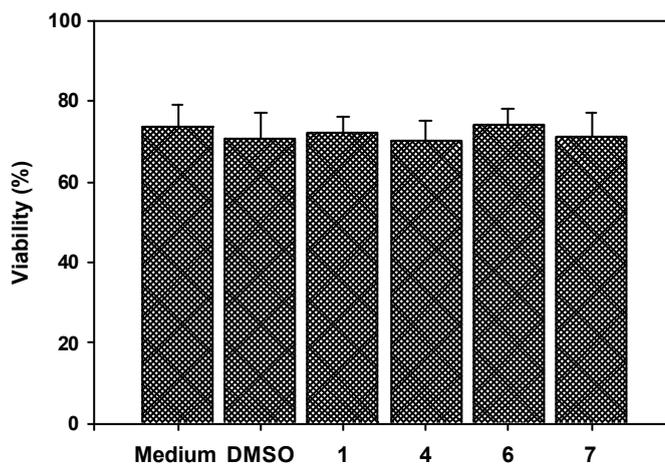
Figure 5. Viability of HMNC cells treated with the compounds 1, 4, 6, or 7. 2 × 10⁵ HMNC cells were stimulated with PHA (5 μg/ml) and treated with medium, 0.1% DMSO, or 100 μM of compounds 1, 4, 6, or 7 for 4 days. Total, viable and non-viable cell numbers were counted after staining by trypan blue on the fourth day. Each bar represents the mean of three independent experiments.

futoquinol (1)

galgravin (4)

piperlactam S (6)
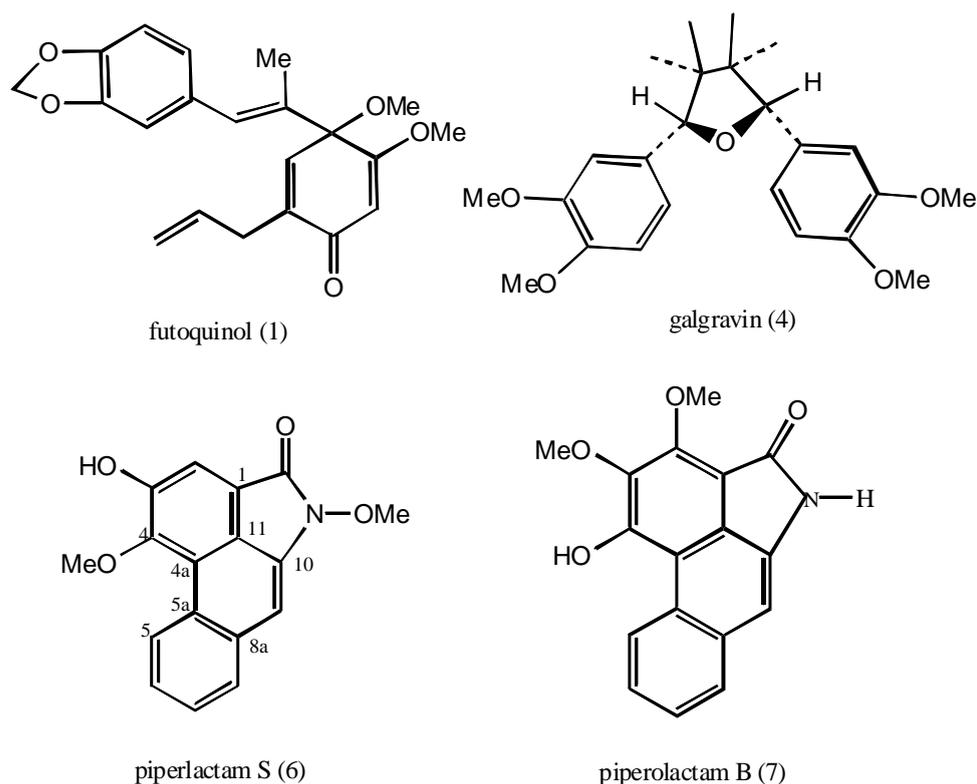
piperolactam B (7)

Figure 6. Structures of the immunomodulatory principles, futoquinol (1), galgravin (4), piperlactam S (6) and piperolactam B(7) from Piper kadsura.

agents. Furthermore, cell viability test indicated that inhibitory effects of these compounds on HMNCs were not through direct cytotoxicity (Fig. 5). The inhibitory mechanisms of compounds **1**, **4**, **6**, and **7** on activated HMNC may involve decreased cytokine production. Plans are underway for the elucidation of their mechanisms of action.

ACKNOWLEDGMENTS

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1,2   1   2   1   3   4   1

1

2

3

4

(2002   3   15   2002   4   24   2002   4   26   )

futoquinol, futoenone, (+)-crotepoxide, galgravin,

(-)-galbelgin, piperlactam **S**   piperolactam **B**

^{3}H-thymidine

futoquinol, galgravin, piperlactam **S**   piperolactam **B**

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