

RESEARCH ARTICLE

The chemical constituents of *Piper kadsura* and their cytotoxic and anti-neuroinflammatory activities

Ki Hyun Kim¹, Jung Wook Choi¹, Sang Un Choi², Sang Keun Ha³, Sun Yeou Kim^{3,4}, Hee-Juhn Park⁵, and Kang Ro Lee¹

¹Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, Suwon, Korea, ²Korea Research Institute of Chemical Technology, Teajeon, Korea, ³Graduate School of East-West Medical Science, Kyung Hee University, Global Campus, Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do, Korea, ⁴East-West Integrated Medical Science Research Centre, Kyung Hee University, Global Campus, Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do, Korea, and ⁵Department of Pharmaceutical Engineering, Sangji University, Wonju, Korea

Abstract

The *n*-hexane and CHCl₃ soluble fractions of the MeOH extract of the aerial parts of *Piper kadsura* were found to potentially inhibit nitric oxide (NO) production in LPS-activated BV-2 cells, a microglial cell line. From the active fractions, a new stereoisomer of guaiane sesquiterpene, 1 α ,5 β -guai-4(15)-ene-6 β ,10 β -diol, kadsuguain A (**1**) and a new cyclohexadienone, kadsuketanone A (**2**), together with twelve known compounds (**3–14**) were isolated. The structures of these compounds were elucidated by extensive NMR spectral studies. The absolute configuration of **2** was determined by circular dichroism (CD) spectra. Compounds **2**, **6**, and **11–14** significantly inhibited both nitric oxide (NO) and prostaglandin E₂ (PGE₂) production in the LPS-activated microglia cells. In addition, compounds **4**, **6**, and **11–14** exhibited cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT15 human tumour cells.

Keywords: Piper kadsura, Piperaceae, anti-neuroinflammatory activity, cytotoxicity

Introduction

Piper kadsura Ohwi (Piperaceae), is a vine-like plant with medicinal uses that naturally inhabits the forests throughout Korea, Japan, and Taiwan at low to medium altitudes and gives off a strong spicy incense [1]. Its stem, known as a haifengteng, has been used as an indigenous medicine for the treatment of asthma and arthritic conditions [2,3]. Moreover, its fruit has been used for cooking in Japan, as it is similar to pepper and is used for improving digestive function [4]. Several lignans and neolignans have been isolated from the genus *Piper*, and various constituents including amides, lignans, terpenes, and cyclohexanes have been isolated from *P. kadsura* [2,5–8]. The extract and components of this plant have anti-human hepatitis B virus [9], anti-platelet activating factor (PAF) [5,10], anti-insect feeding [11] and anti-inflammatory activities [2]. We have found that several spicy plants can prevent or delay the onset and progression of neurodegenerative

disorders [12]. The *n*-hexane and CHCl₃ soluble fractions of the MeOH extract of this spicy plant showed a potent inhibitory effect on nitric oxide (NO) production in LPS-activated BV-2 cells, a microglia cell line. Thus, in the course of our continuing search for biologically active compounds from natural Korean medicinal sources, we have reported the isolation of neolignans as active principles of *P. kadsura* for anti-neuroinflammatory activity [12]. In our continuing study on this source, we further isolated a new stereoisomer of guaiane sesquiterpene, 1 α ,5 β -guai-4(15)-ene-6 β ,10 β -diol, kadsuguain A (**1**) and a new cyclohexadienone, kadsuketanone A (**2**), together with twelve known compounds (**3–14**), from its *n*-hexane and CHCl₃ soluble fractions. The structures of these compounds were elucidated by extensive NMR spectral studies, including 2D-NMR experiments. The ability of the isolated compounds (**1–14**) to inhibit NO production was evaluated in LPS-activated BV-2 cells,

Address for Correspondence: Kang Ro Lee, Natural Products Laboratory, School of Pharmacy, Sungkyunkwan University, 300 Chonchon-dong, Jangan-ku, Suwon, Korea. Tel: 82-31-290-7710; Fax: 82-31-290-7730; E-mail: krlee@skku.ac.kr

(Received 11 February 2010; revised 20 May 2010; accepted 20 May 2010)

a microglial cell line. Furthermore, compounds (**1–14**) were evaluated for their cytotoxicities against the A549, SK-OV-3, SK-MEL-2, and HCT15 cell lines.

Materials and methods

General experimental procedures

All melting points were determined on a Gallenkamp melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). CD spectra were measured on a Jasco J-715 spectropolarimeter. UV spectra were recorded with a Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu, Tokyo, Japan). FAB and HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Peabody, MA, USA). NMR spectra, including ^1H - ^1H COSY, HMQC, HMBC, and NOESY experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^1H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative HPLC used a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, NY, USA). Silica gel 60 (Merck, Darmstadt, Germany, 230–400 mesh) and RP-C₁₈ silica gel (Merck, 230–400 mesh) were used for column chromatography. Merck precoated Silica gel F₂₅₄ plates and RP-18 F_{254s} plates were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with 10% H₂SO₄ in C₂H₅OH (v/v). The packing material for the molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Plant material

The aerial parts of *P. kadsura* were collected in Jeju island, Korea, in October, 2006, and were identified by one of the authors (K.R.L.). A voucher specimen (SKKU 2006-10) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation

The dried aerial parts of *P. kadsura* (3 kg) were extracted with 80% MeOH two times at room temperature and filtered. The filtrate was evaporated in vacuum to obtain a MeOH extract (300 g), which was suspended in distilled H₂O (7.2 L) and then successively partitioned with *n*-hexane, CHCl₃, and *n*-BuOH, yielding 49, 12, and 26 g, respectively. The *n*-hexane soluble fraction (49 g) was separated on a silica gel (230–400 mesh, 550 g) column eluted with *n*-hexane-EtOAc (10:1 → 4:1) to yield five fractions (A–E). Fraction A (4.3 g) was separated further on a silica gel (230–400 mesh, 150 g) column eluted with *n*-hexane-EtOAc (10:1) and purified by preparative normal-phase HPLC, using a solvent system of *n*-hexane-EtOAc (30:1) over 30 min at a flow rate of 2 mL/min (Apollo Silica 5 μ column; 250 × 10 mm;

Shodex refractive index detector) to obtain compounds **5** (15 mg, t_R 15.5 min) and **10** (5 mg, t_R 17.5 min). Fraction C (6.1 g) was separated on a silica gel (230–400 mesh, 150 g) column eluted with *n*-hexane-EtOAc (5:1) to afford 4 fractions (Fr. C1 to Fr. C4). Fr. C2 (1.2 g) was separated further on a silica gel (230–400 mesh, 100 g) column eluted with *n*-hexane-EtOAc (4:1) and purified by preparative normal-phase HPLC, using a solvent system of *n*-hexane-EtOAc (10:1) to yield compounds **3** (32 mg, t_R 13.5 min) and **4** (34 mg, t_R 15 min). Fr. C4 (500 mg) was purified by preparative reversed-phase HPLC, using a solvent system of 80% MeCN over 30 min at a flow rate of 2 mL/min (Econosil RP-18 10 μm column; 250 × 10 mm; Shodex refractive index detector), to afford compounds **1** (6 mg, t_R 12.5 min), **6** (7 mg, t_R 14 min), and **11** (8 mg, t_R 18.5 min). The CHCl₃ soluble fraction (12 g) was separated on a silica gel (230–400 mesh, 350 g) column eluted with *n*-hexane-CHCl₃-MeOH (3:4:0.5) to yield six fractions (F–K). Fraction I (1.5 g) was separated on a RP-C₁₈ silica gel (230–400 mesh, 150 g) column eluted with 50% MeOH and Sephadex LH-20 column (CH₂Cl₂-MeOH, 1:1) and purified by preparative normal-phase HPLC, using a solvent system of CHCl₃-EtOAc-MeOH (5:3:0.5) to yield compounds **2** (32 mg, t_R 10.5 min), **7** (10 mg, t_R 12 min), **8** (6 mg, t_R 12.5 min), and **9** (8 mg, t_R 14 min). Fraction J (750 mg) was separated over a RP-C₁₈ silica gel (230–400 mesh, 70 g) column eluted with 50% MeOH and purified by preparative reversed-phase HPLC, using a solvent system of 45% MeCN to yield compounds **12** (5 mg, t_R 15.5 min) and **13** (16 mg, t_R 17.5 min). Fraction K (650 mg) was separated on a RP-C₁₈ silica gel (230–400 mesh, 60 g) column eluted with 45% MeOH and Sephadex LH-20 column (100% MeOH) and purified by preparative normal-phase HPLC, using a solvent system of CHCl₃-MeOH (20:1) to yield compound **14** (7 mg, t_R 12.5 min).

Kadsuguain A (1)

Colourless gum; 6 mg. $[\alpha]_D^{25} +9.8$ (c 0.2, MeOH); IR (KBr) ν_{max} : 3388, 2946, 1658, 1457, 1211, 1028, and 676 cm⁻¹; FAB-MS (positive mode): m/z 239 [M + H]⁺; HR-FAB-MS (positive mode): m/z 239.2015 [M + H]⁺, (calcd. for C₁₅H₂₇O₂: 239.2011); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1.

Kadsuketanone A (2)

White powder; 32 mg. Mp 130–132°C; $[\alpha]_D^{25} -5.6$ (c 1.2, CHCl₃); UV (MeOH) λ_{max} (log ϵ): 284 (6.3) nm; CD (MeOH, c 1.6 × 10⁻⁴ M) $\Delta\epsilon$ (nm): -12.2 (270), +7.3 (315), and +6.4 (335); IR (KBr) ν_{max} : 3390, 2947, 1662, 1606, 1455, 1215, 1181, 1023, and 673 cm⁻¹; FAB-MS (positive mode): m/z 211 [M + H]⁺; HR-FAB-MS (positive mode): m/z 211.0965 [M + H]⁺, (calcd. for C₁₁H₁₅O₄: 211.097); ^1H NMR (500 MHz, CDCl₃) δ : 5.6–5.53 (1H, m, H-8), 5.5 (1H, s, H-3), 5.48 (1H, s, H-6), 5.07 (1H, dd, J =1.5, 5.5 Hz, H-9a), 5.05 (1H, dd, J =1.5, 12 Hz, H-9b), 3.78 (3H, s, OCH₃-2), 3.66 (3H, s, OCH₃-5), 2.61 (2H, dd, J =1.5, 6.5 Hz, H-7). ^{13}C NMR (125 MHz, CDCl₃) δ : 182.3 (C-4), 174.9 (C-2), 150.5 (C-5), 131.3 (C-8), 119.9

(C-9), 112.5 (C-6), 101.1 (C-3), 72.4 (C-1), 56.5 (OCH_3 -2), 55.4 (OCH_3 -5), 45.4 (C-7).

Piperolactam B (14)

Yellowish gum; 7 mg. UV (MeOH) λ_{max} (log ϵ): 225 (4.5), 267 (4.6), 295 (4.5), 335 (4), and 372 (4) nm; IR (KBr) ν_{max} : 3358, 2943, 1681, 1545, 1453, 1306, 1021, and 676 cm^{-1} ; FAB-MS (positive mode): m/z 296 [M + H]⁺; ¹H NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 12.00 (1H, br s, NH), 9.98 (1H, d, $J=8$ Hz, H-5), 7.99 (1H, d, $J=8$ Hz, H-8), 7.63 (1H, td, $J=8, 2$ Hz, H-6), 7.54 (1H, dd, $J=8, 2$ Hz, H-7), 7.45 (1H, s, H-9), 4.67 (3H, s, OCH_3 -2), 3.84 (3H, s, OCH_3 -3). ¹³C NMR (125 MHz, $\text{C}_5\text{D}_5\text{N}$) δ : 168.6 (C=O), 156.6 (C-4), 154.2 (C-2), 140.8 (C-3), 135.2 (C-10), 134.5 (C-8a), 129.2 (C-8), 128.4 (C-5), 128.1 (C-4b), 126.6 (C-7), 125.7 (C-6), 124.6 (C-10a), 113.5 (C-4a), 106.6 (C-1), 105.8 (C-9), 63.2 (OCH_3 -2), 61.6 (OCH_3 -3).

Measurement of NO production and cell viability in LPS-activated BV-2 cells

The BV-2 microglia cells were stimulated with 100 ng/mL LPS in the presence or absence of samples for 24 h. The nitrite present in the culture media, a soluble oxidation product of NO, was measured by a Griess reaction. The supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader (Emax, Molecular Device, Sunnyvale, CA, USA). Sodium nitrite was used as a standard to calculate the nitrite concentration [13]. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [14]. *N*^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, USA), a well-known NOS inhibitor, was tested as a positive control.

Measurement of PGE₂ production in LPS-activated BV-2 cells

The BV-2 microglia cells were stimulated with 100 ng/mL LPS in the presence or absence of samples for 24 h, and the media was collected. The supernatant from the culture medium was harvested and used for measuring the level of prostaglandin E₂ (PGE₂). PGE₂ was measured by a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol.

Cytotoxicity assay

A sulphorhodamine B bioassay (SRB) was used to determine the cytotoxicity of each compound against four cultured human cancer cell lines, A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT-15 (colon cancer cells) [15]. Doxorubicin (Sigma, St. Louis, MO, USA, $\geq 98\%$) was used as a positive control.

Results and discussion

The MeOH extract of *P. kadsura* was fractionated by solvent (*n*-hexane, CHCl_3 , *n*-BuOH), and then each fraction was evaluated by assessing NO production in LPS-activated BV-2 cells, a microglia cell line. The *n*-hexane and CHCl_3 soluble fractions showed a potent inhibitory effect on NO production. The active fractions were subjected to a series of chromatographic methods, followed by semi-preparative HPLC to afford a new stereoisomer of guaiane sesquiterpene (**1**) and a new cyclohexadienone (**2**), along with twelve known compounds (**3–14**) (Figure 1).

Kadsuguain A (**1**) was obtained as a colourless gum, whose molecular formula was determined to be $\text{C}_{15}\text{H}_{26}\text{O}_2$

Table 1. The ¹H and ¹³C NMR data for compound **1** (δ in ppm, 500 MHz for ¹H and 125 MHz for ¹³C, in CDCl_3)^a.

Position	δ_{H}	δ_{C}	HMBC (H→C)
1	2.22 (m)	53.4	C-3, C-5, C-6, C-10, C-14
2	1.82 (m)	26.3	C-4, C-5, C-10
3 α	2.18 (m)	30.1	C-1, C-5, C-15
3 β	2.38 (m)		
4		154.2	
5	2.62 (t, 10.5)	55.6	C-2, C-4, C-6, C-7, C-15
6	3.5 (t, 10.5)	69.3	C-4, C-5, C-7, C-8
7	1.58 (m)	48.7	C-5, C-6, C-9, C-12, C-13
8 α	1.31 (m)	19.7	C-6, C-10, C-11
8 β	1.77 (m)		
9 α	1.65 (m)	37.2	C-7, C-14
9 β	1.8 (m)		
10		73.3	
11	2.23 (m)	27.9	C-8, C-12, C-13
12	0.93 (d, 7)	21.5	C-7, C-13
13	0.86 (d, 7)	16.5	C-7, C-12
14	1.18 (s)	29.7	C-1, C-9, C-10
15	5.01 (s)	108.7	C-3, C-5
	5.05 (s)		

^aJ values are in parentheses and reported in Hz; the assignments were based on ¹H-¹H COSY, HMQC, HMBC and NOESY experiments.

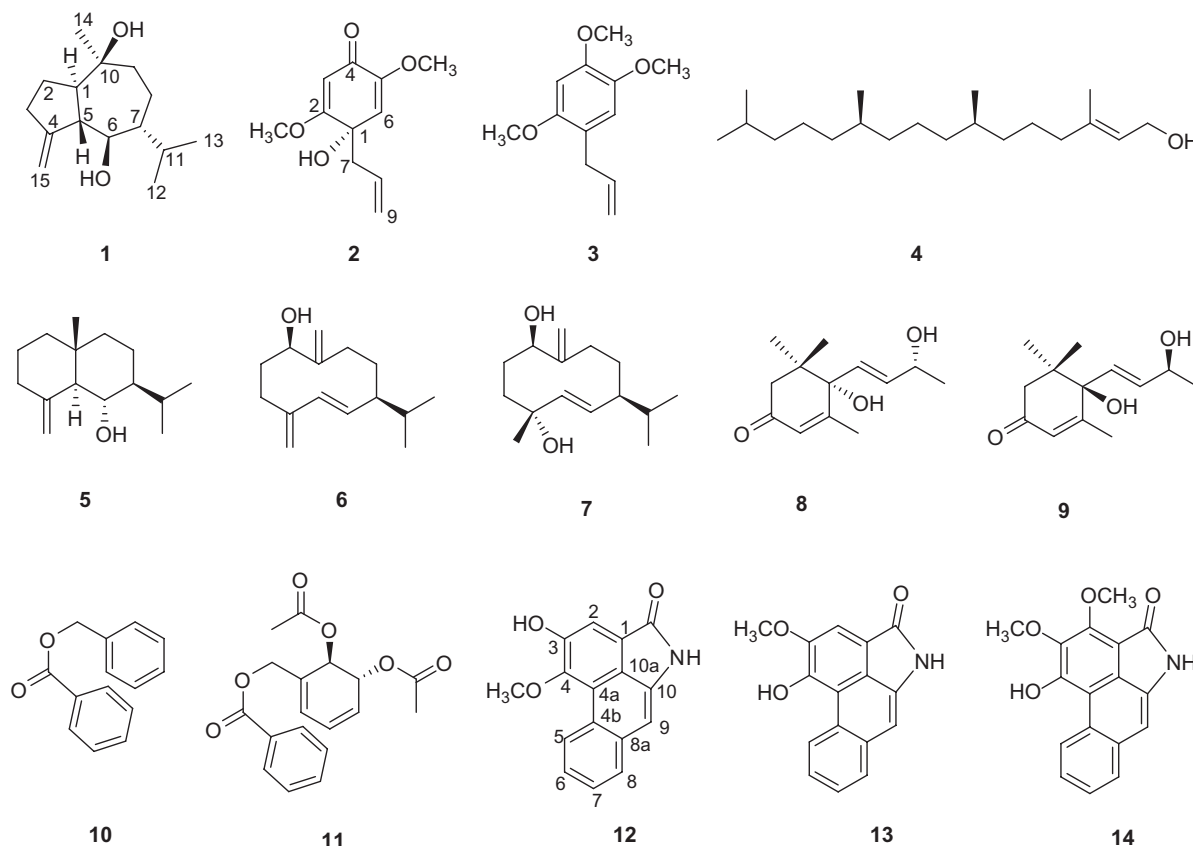


Figure 1. Chemical structures of 1-14.

by combined analysis of its positive-ion HR-FAB-MS showing the fragment ion $[M + H]^+$ peak at m/z 239.2015 (calcd. for $C_{15}H_{27}O_2$: 239.2011) and ^{13}C NMR spectral data. Its IR spectrum showed a hydroxyl (3388cm^{-1}) and an olefinic group (1658cm^{-1}). The 1H NMR spectrum of **1** showed signals for two secondary methyl groups at δ 0.93 (3H, d, $J=7$ Hz) and 0.86 (3H, d, $J=7$ Hz), a tertiary methyl group at δ 1.18 (3H, s), and an oxygenated CH group at δ 3.5 (1H, t, $J=10.5$ Hz). Two broad singlet signals at δ 5.05 (1H, s) and 5.01 (1H, s) corresponding to an exocyclic olefinic CH_2 group were also observed. The ^{13}C NMR and Distortionless Enhancement by Polarization Transfer (DEPT) spectra of **1** indicated the presence of three methyl groups at δ 29.7 (C-14), 21.5 (C-12), 16.5 (C-13), five CH_2 groups including an olefinic one at δ 108.7 (C-15), five CH groups, including an oxygenated one at δ 69.3 (C-6), and two quaternary C-atoms, one of which was an oxygenated signal at δ 73.3 (C-10) and the other was an olefinic signal at δ 154.2 (C-4). The 1H and ^{13}C NMR signals of **1** were assigned unambiguously by further detailed analysis of the 1H - 1H COSY, HMQC, and HMBC spectra. The COSY spectrum showed a correlation system starting at 3- H_2 , continuing via 2- H_2 , 1-H, 5-H, 6-H, 7-H, 8- H_2 and ending at 9- H_2 . At 7-H there was branching that ended at 12- H_3 and 13- H_3 via 11-H. HMBC correlations observed from H-1, H-2, and H-8 to C-10 (δ 73.3), from H-14 to C-10 (δ 73.3), and from H-1 and H-9 to C-14 (δ 29.7) indicated the connectivity of C-10 to C-1 and C-9, forming a seven-member ring

with a methyl group and an OH group attached at C-10. HMBC correlations were also observed between H-15 and C-3 (δ 30.1), as well as C-5 (δ 55.6), showing the connection of C-4 to C-3 and C-5, forming a five-member ring with an exomethylene group attached at C-4. Thus, the planar structure of **1** was derived as guai-4 (15)-ene-6,10-diol. The large coupling constant ($J_{1,5} = 10.5$ Hz) indicated that the junction of the guaianes rings was *trans* [16]. The mutual NOESY correlations H-1/ H_6 , H-6/ H_8 , H-1/ H_8 , H-1/ H_{12} , H-1/ H_{13} , H-6/ H_{12} , H-6/ H_{13} , and H-5/ H_7 and the absence of the correlations H-1/ H_5 , H-5/ H_{12} , and H-5/ H_{13} as well as the large coupling constant between H-5 and H-6, and between H-6 and H-7 (each 10.5 Hz) indicated that H-5 and H-7 were positioned at the same orientation (β -form) and H-1 and H-6 were then on the opposite side (α -form) [17]. The configuration of C-10 is then determined by the NOESY correlations H-1/ H_{14} and H-6/ H_{14} , and the lack of the correlations H-5/ H_{14} and H-7/ H_{14} , suggested that methyl group (δ 29.7) at C-10 was α -oriented in equatorial position [16-18]. The downfield resonance (δ 2.62) of the H-5 proton, due to a *cis* spatial relationship with the OH-10, also supported this point [16]. To establish the absolute configuration of **1**, the modified Mosher's method was performed [19]. However, compound **1** failed to be esterified by (S)- or (R)-MTPA chloride, presumably due to the hindrance of the vicinal isopropyl group at C-7. Thus, the structure of **1** was determined as 1 α ,5 β -guai-4(15)-ene-6 β ,10 β -diol and

named kadsuguain A. An 1-epimer of **1** has been reported previously [18].

Kadsuketanone A (**2**) was obtained as an optically active white powder (mp 130–132°C; $[\alpha]_D^{25}$ –5.6), whose molecular formula was determined to be $C_{11}H_{14}O_4$ from the $[M + H]^+$ peak at m/z 211.0965 (calcd. for $C_{11}H_{15}O_4$: 211.0970) in the positive-ion HR-FAB-MS. The IR spectrum demonstrated the presence of a hydroxyl group (3390 cm^{-1}) and α,β -unsaturated ketone function (1662 cm^{-1}). The UV spectrum of **2** showed dienone absorption at λ_{max} 284 nm. The ^1H NMR spectral data of **2** showed two olefinic signals at δ 5.5 (1H, s, H-3), and 5.48 (1H, s, H-6). A set of ABX signals at δ 5.6–5.53 (1H, m, H-8), 5.07 (1H, dd, $J=1.5, 5.5$ Hz, H-9a), and 5.05 (1H, dd, $J=1.5, 12$ Hz, H-9b) and one methylene at δ 2.61 (2H, dd, $J=1.5, 6.5$ Hz, H-7) were assigned to the allyl group. The ^{13}C NMR spectra showed 11 signals, including one carbonyl carbon at δ 182.3 (C-4), six carbons for olefinic carbon at δ 174.9 (C-2), 150.5 (C-5), 131.3 (C-8), 119.9 (C-9), 112.5 (C-6), and 101.1 (C-3), one quaternary carbon at δ 72.4 (C-1), one methylene carbon at δ 45.4 (C-7), and two methoxy carbons at δ 56.5 (OCH_3 -2), and 55.4 (OCH_3 -5). The ^1H and ^{13}C NMR signals of **2** were assigned unambiguously by further detailed analysis of the HMQC and HMBC experiments. The HMBC correlations of H-6/C-2, C-4 and H-3/C-1, C-5 indicated that compound **2** was a cyclohexadienone derivative. The HMBC spectrum showed that H-7 was correlated to C-1, C-2, and C-6, suggesting that the allyl group was located at C-1. Two methoxy protons at δ 3.78 (3H, s, OCH_3 -2) and 3.66 (3H, s, OCH_3 -5) were assigned at C-2 and C-5, respectively, according to the HMBC correlations with the carbon signals at δ 174.9 and 150.5, respectively. The structure of **2** was determined based on the above considerations, this was found to be similar to the partial structure of burchellin isolated from this plant [2,20]. The CD spectrum of **2** exhibited a first negative Cotton effect at 270 nm, a second positive Cotton effect at 315 nm and a third positive Cotton effect at 335 nm, the Cotton effects were considered to be due to the enone chromophore,

indicating the *S*-configuration at C-1 [21]. Therefore, compound **2** is a new cyclohexadienone derivative, named kadsuketanone A, a rare analogue to occur in natural sources.

The structures of the known compounds were identified as isoasarone (**3**) [22], *trans*-phytol (**4**) [23], junenol (**5**) [24], *ent*-germacra-4(15),5,10(14)-trien-1 β -ol (**6**) [25], germacra-5,10(14)-dien-1 β ,4 β -diol (**7**) [26], blumenol A (**8**) [27], blumenol B (**9**) [28], benzyl benzoate (**10**) [29], *trans*-2,3-diacetoxy-1-[(benzoyloxy)methyl]-cyclohexa-4,6-diene (**11**) [30], aristolactam A II (**12**) [31], and piperolactam A (**13**) [32] by comparison of their spectroscopic data with reported values. Piperolactam B (**14**) was also isolated from this source, and the ^1H NMR data of piperolactam B isolated from *Piper longum* was reported previously [33]. However, the assignments of the NMR data required correction. The chemical shift (δ 4.67) for one of the methoxy groups in **14** indicated that the methoxy group was located at C-2 in **14**, which was mostly shown at δ 4.4–4.6 due to the influence of the *peri*-carbonyl group of the lactam ring [34,35]. The resonances of piperolactam B (**14**) were reassigned unambiguously by 2D NMR (^1H - ^1H COSY, HMQC, HMBC and NOESY).

Neuroinflammation can cause neuronal damage in neurodegenerative diseases [36]. Brain inflammation results from activation of microglia, the resident immune cells. Activated microglia cells produce excessive pro-inflammatory substances such as NO, cytokines, and prostaglandins [37]. The NO and PGE_2 produced by activated microglia is a major factor involved in neuroinflammation [38]. Here, the anti-neuroinflammatory effects of **1–14** were evaluated by using LPS-activated BV-2 microglia cells. Compounds **2**, **6**, and **11–14** significantly inhibited the NO production in LPS-stimulated BV-2 cells. They were more potent than L-NMMA, an inducible NO synthase (iNOS) inhibitor, in inhibiting NO production. Compound **2** was the strongest inhibitor (Table 2), but the other compounds were not active (up to 20 μM). Moreover, compounds **2**, **6**, and **11–14** significantly reduced PGE_2 production in the LPS-stimulated microglia (Figure 2).

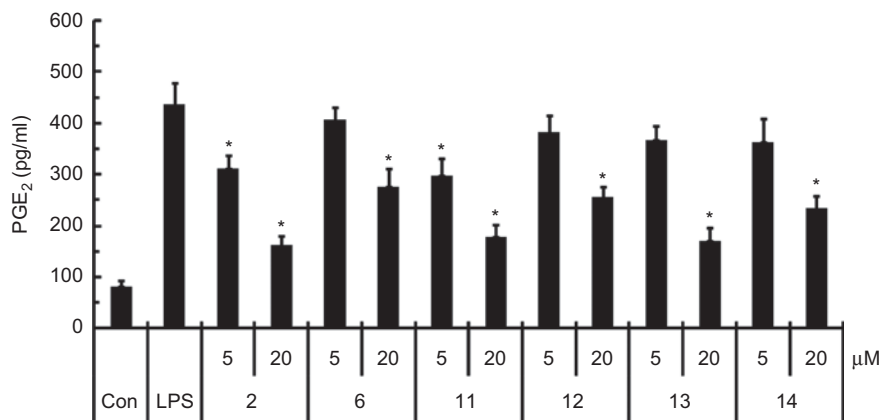


Figure 2. The effects of compounds **2**, **6**, and **11–14** on PGE_2 production in LPS-stimulated BV-2 microglia cells. PGE_2 was assessed by using a competitive enzyme immunoassay kit after treatment with LPS (100 ng/mL) for 24 h in the presence or absence of compounds **2**, **6**, and **11–14** (5 and 20 μM). All data are presented as the mean \pm SEM of three independent experiments. * $p < 0.05$ indicates statistically significant differences compared to treatment with LPS alone.

Table 2. The effects of compounds **1–14** and L-NMMA on LPS-induced NO production in BV-2 microglia cells.

Compound	Inhibition (%) ^a		IC ₅₀ (μM)
	5 μM	20 μM	
1	na ^b	na	-
2	47.5	83.8	5.62
3	na	na	-
4	na	na	-
5	na	na	-
6	24.2	54.7	17.4
7	na	na	-
8	na	na	-
9	na	na	-
10	na	na	-
11	46	71.4	6.71
12	21.9	75	9.14
13	46.3	82.5	6.32
14	27.5	57.3	16.5
L-NMMA	10	54.2	17.7

^aValues are the inhibition of NO production relative to the LPS control (n=3). ^bna, not active.

Table 3. Cytotoxicity of compounds **1–14** against four cultured human tumour cell lines using the SRB assay *in vitro*.

Compound	IC ₅₀ (μM) ^a			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	83.3	>100	80	95.4
2	>100	>100	58.2	>100
3	96.3	>100	85.5	>100
4	32.9	20.8	28.1	21.8
5	>100	>100	>100	>100
6	17.8	23.1	28.2	24
7	83.1	>100	97.6	>100
8	>100	>100	>100	>100
9	98.2	>100	>100	>100
10	96.6	>100	>100	>100
11	>100	52	37.5	64.2
12	20.5	54.5	58.2	>100
13	10.1	18.3	8.3	27.8
14	21.7	14.4	11.6	21.3
Doxorubicin ^b	0.06	0.08	0.04	0.22

^aIC₅₀ values against cell lines, defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*. ^bDoxorubicin as positive control.

The isolated compounds **1–14** were also evaluated for their cytotoxic activities against A549, SK-OV-3, SK-MEL-2, and HCT15 human tumour cell lines using the SRB assay. Compounds **4**, **6**, and **11–14** exhibited cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT15 cells, but the other compounds were found to be inactive (Table 3).

In summary, compounds **2**, **6**, and **11–14** isolated from *P. kadsura* exhibited anti-neuroinflammatory activity by suppressing the release of NO and PGE₂ in LPS-stimulated microglia cells. These results suggest that the active compounds might be good lead compounds to modulate neurological diseases associated with inflammatory processes.

Acknowledgements

The authors would like to thank Do Kyun Kim, Eun Jung Bang, and Jung Ju Seo at the Korea Basic Science Institute for the NMR and MS spectral measurements.

Declaration of interest

This study was supported by a grant from the Seoul R&BD Program (10524) funded by the Seoul Metropolitan Government, Republic of Korea.

References

- Lin TT, Lu SY. Piperaceae. In Flora of Taiwan. Taipei: Editorial Committee of the Flora of Taiwan, 1996;Vol.II:624–631.
- Lin LC, Shen CC, Shen YC, Tsai TH. Anti-inflammatory neolignans from *Piper kadsura*. J Nat Prod 2006;69:842–844.
- Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jain A, Tyagi OD, Prasad AK, Wengel J, Olsen CE, Boll PM. Phytochemistry of the genus Piper. Phytochemistry 1997;46:597–673.
- Shogakukan eds. Dictionary of Chinese Materia Medica. Tokyo: Shanghai Scientific Technological Publishers Shougakukan, 1985:230.
- Han GQ, Dai P, Xu L, Ma J, Li CL, Zheng QT. PAF inhibitors: Neolignans from *Piper kadsura*. Planta Med 1990;56:583–584.
- Chang MN, Han GQ, Arison BH, Springer JP, Hwang SB, Shen TY. Neolignans from *Piper futokadsura*. Phytochemistry 1985;24:2079–2082.
- Konishi T, Konoshima T, Daikonya A, Kitanaka S. Neolignans from *Piper futokadsura* and their inhibition of nitric oxide production. Chem Pharm Bull 2005;53:121–124.
- Matsui K, Munakata K. Four new neolignans from *Piper futokadzura*. Tetrahedron Lett 1976;48:4371–4374.
- Huang RL, Chen CF, Feng HY, Lin LC, Chou CJ. Anti-hepatitis B virus of seven compounds isolated from *Piper kadsura* (CHOISY) OHWI. J Chin Med 2001;12:179–190.
- Shen TY, Hussaini IM. Kadsurenone and other related lignans as antagonists of platelet-activating factor receptor. Methods Enzymol 1990;187:446–454.
- Matsui K, Wada K, Munakata K. Insect antifeeding substances in *Parabenzoin praecox* and *Piper futokadzura*. Agric Biol Chem 1976;40:1045–1046.
- Kim KH, Choi JW, Ha SK, Kim SY, Lee KR. Neolignans from *Piper kadsura* and their anti-neuroinflammatory activity. Bioorg Med Chem Lett 2010;20:409–412.
- Ha SK, Lee P, Park JA, Oh HR, Lee SY, Park JH, Lee EH, Ryu JH, Lee KR, Kim SY. Apigenin inhibits the production of NO and PGE₂ in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model. Neurochem Int 2008;52:878–886.
- Sargent JM, Taylor CG. Appraisal of the MTT assay as a rapid test of chemosensitivity in acute myeloid leukaemia. Br J Cancer 1989;60:206–210.
- Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–1112.
- Bruno M, Torre MC, Rodriguez B, Omar AA. Guaiane sesquiterpenes from *Teucrium leucocladum*. Phytochemistry 1993;34:245–247.
- Wei HH, Xu HH, Xie HH, Xu LX, Wei XY. Sesquiterpenes and lignans from *Tephrosia vogelii*. Helv Chim Acta 2009;92:370–374.
- Anderson M, Bergendorff O, Shan R, Zygmunt P, Sterner O. Minor components with smooth muscle relaxing properties from scented myrrh (*Commiphora guidotti*). Planta Med 1997;63:250–254.
- Su BN, Park EJ, Mbwanbo ZH, Santarsiero BD, Mesecar AD, Fong HHS, Pezzuto JM, Kinghorn AD. New chemical constituents of

- Euphorbia quinquecostata* and absolute configuration assignment by a convenient Mosher ester procedure carried out in NMR tubes. *J Nat Prod* 2002;65:1278-1282.
20. Horne DA, Yakushijin K, Buchi G. Biomimetic synthesis of the neolignans kadsurenone, denudatin B, *O*-methyl-liliflodione, and liliflol B. *Tetrahedron Lett* 1999;40:5443-5447.
 21. Sato S, Obara H, Kumazawa T, Onodera J, Furuhashi K. Synthesis of (+),(-)-model compounds and absolute configuration of carthamin; A red pigment in the flower petals of safflower. *Chem Lett* 1996;25:833-834.
 22. De Santos BV, Da-Cunha EVL, De Chaves MC, Gray AI. Phenylalkanooids from *Piper marginatum*. *Phytochemistry* 1998;49:1381-1384.
 23. Kim KH, Lee KH, Choi SU, Kim YH, Lee KR. Terpene and phenolic constituents of *Lactuca indica* L. *Arch Pharm Res* 2008;31:983-988.
 24. Cardona L, Garcia B, Gimenez JE, Pedro JR. A shorter route to the synthesis of (+)-junenol, isojunenol, and their coumarate esters from (-)-santonin. *Tetrahedron* 1992;48:851-860.
 25. Choi SZ, Choi SU, Lee KR. Phytochemical constituents of the aerial parts from *Solidago virga-aurea* var. *gigantea*. *Arch Pharm Res* 2004;27:164-168.
 26. San Feliciano A, Medarde M, Gordaliza M, Lucas MJ. Structure elucidation of germacrane alcohols from *Juniperus communis* subsp. *Hemisphaerica*. *J Nat Prod* 1995;58:1059-1064.
 27. Cutillo F, Dellagreca M, Previtera L, Zarrelli A. C13 norisoprenoids from *Brassica fruticulosa*. *Nat Prod Res* 2005;19:99-103.
 28. Abe F, Yamauchi T. Megastigmanes and flavonoids from the leaves of *Scorodocarpus borneensis*. *Phytochemistry* 1993;33:1499-1501.
 29. Faler CA, Joullie MM. Aminolysis of allyl esters with bislithium aryl amides. *Tetrahedron Lett* 2006;47:7229-7231.
 30. Kodpinid M, Sadavongvivad C, Thebtaranonth C, Thebtaranonth Y. Structures of β -senepoxide, tingtanoxide, and their diene precursors. Constituents of *Uvaria ferruginea*. *Tetrahedron Lett* 1983;24:2019-2022.
 31. Priestap HA. ^{13}C NMR spectroscopy of aristolochic acids and aristololactams. *Magn Reson Chem* 1989;27:460-469.
 32. Kim JK, Kim YH, Nam HT, Kim BT, Heo JN. Total synthesis of aristolactams via a one-pot Suzuki-Miyaura coupling/aldol condensation cascade reaction. *Org Lett* 2008;10:3543-3546.
 33. Desai SJ, Prabhu BR, Mulchandani NB. Aristolactams and 4,5-dioxoaporphines from *Piper longum*. *Phytochemistry* 1988;27:1511-1515.
 34. Olsen CE, Tyagi OD, Boll PM, Hussaini FA, Parmar VS, Sharma NK, Taneja P, Jain SC. An aristolactam from *Piper acutisleginum* and revision of the structures of piperolactam B and D. *Phytochemistry* 1993;33:518-520.
 35. Chen YC, Chen JJ, Chang YL, Teng CM, Lin WY, Wu CC, Chen IS. A new aristolactam alkaloid and anti-platelet aggregation constituents from *Piper taiwanense*. *Planta Med* 2004;70:174-177.
 36. Liu B, Hong JS. Role of microglia in inflammation-mediated neurodegenerative diseases: Mechanisms and strategies for therapeutic intervention. *J Pharmacol Exp Ther* 2003;304:1-7.
 37. McGeer PL, McGeer EG. The inflammatory response system of brain: Implications for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res Rev* 1995;21:195-218.
 38. Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci* 1997;20:132-139.