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RESEARCH ARTICLE

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

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Abstract
The *n*-hexane and $CHCl_3$ soluble fractions of the MeOH extract of the aerial parts of *Piper kadsura* were found to potently 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 E_2 (PGE_2) 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 $CHCl_3$ 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 $CHCl_3$ 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,

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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 ^{1}H-^{1}H 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 (^{1}H) 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-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. Merck precoated Silica gel F_{254} 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% H_2SO_4 in C_2H_5OH (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 H_2O (7.2 L) and then successively partitioned with *n*-hexane, $CHCl_3$, 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 5μ 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 $CHCl_3$ soluble fraction (12 g) was separated on a silica gel (230–400 mesh, 350 g) column eluted with *n*-hexane-$CHCl_3$-MeOH (3:4:0.5) to yield six fractions (F - K). Fraction I (1.5 g) was separated on a RP-C_{18} silica gel (230–400 mesh, 150 g) column eluted with 50% MeOH and Sephadex LH-20 column (CH_2Cl_2-MeOH, 1:1) and purified by preparative normal-phase HPLC, using a solvent system of $CHCl_3$-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-C_{18} 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-C_{18} 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 $CHCl_3$-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 $C_{15}H_{27}O_2$: 239.2011); ^{1}H (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, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 284 (6.3) nm; CD (MeOH, *c* 1.6×10^{-4} M) Δε (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 $C_{11}H_{15}O_4$: 211.097); ^{1}H NMR (500 MHz, $CDCl_3$) 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, OC$\underline{H}_3$-2), 3.66 (3H, s, OC$\underline{H}_3$-5), 2.61 (2H, dd, *J* = 1.5, 6.5 Hz, H-7). ^{13}C NMR (125 MHz, $CDCl_3$) 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 (O<u>C</u>H$_3$-2), 55.4 (O<u>C</u>H$_3$-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]$^+$; ^{1}H NMR (500 MHz, C_5D_5N) 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, OC<u>H</u>$_3$-2), 3.84 (3H, s, OC<u>H</u>$_3$-3). ^{13}C NMR (125 MHz, C_5D_5N) 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 (O<u>C</u>H$_3$-2), 61.6 (O<u>C</u>H$_3$-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-napthylethylenediamine 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 PGE_2 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 E_2 (PGE_2). PGE_2 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, ≥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 $C_{15}H_{26}O_2$

Table 1. The ^{1}H and ^{13}C NMR data for compound **1** (δ in ppm, 500 MHz for ^{1}H and 125 MHz for ^{13}C, 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
3α	2.18 (m)	30.1	C-1, C-5, C-15
3β	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
8α	1.31 (m)	19.7	C-6, C-10, C-11
8β	1.77 (m)		
9α	1.65 (m)	37.2	C-7, C-14
9β	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)		

[a]*J* values are in parentheses and reported in Hz; the assignments were based on ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments.

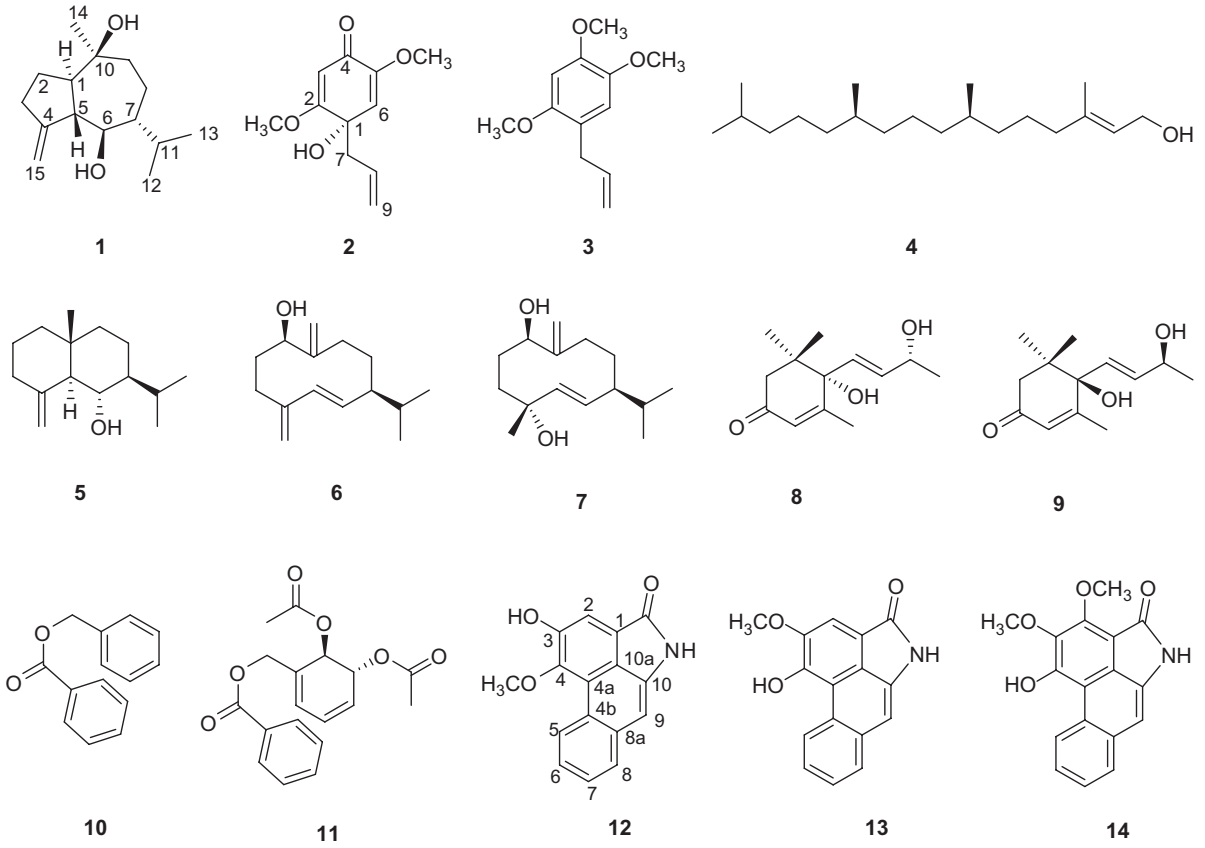

Figure 1. Chemical structures of **1**–**14**.

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 (3388 cm^{-1}) and an olefinic group (1658 cm^{-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 guaiane rings was *trans* [16]. The mutual NOESY correlations H-1/H-6, H-6/H_α-8, H-1/H_α-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 ($OC\underline{H}_3$-2), and 55.4 ($OC\underline{H}_3$-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, $OC\underline{H}_3$-2) and 3.66 (3H, s, $OC\underline{H}_3$-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-1β-ol (**6**) [25], germacra-5,10(14)-dien-1β,4β-diol (**7**) [26], blumenol A (**8**) [27], blumenol B (**9**) [28], benzyl benzoate (**10**) [29], *trans*-2,3-diacetoxy-1-[(benzoy1oxy)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).

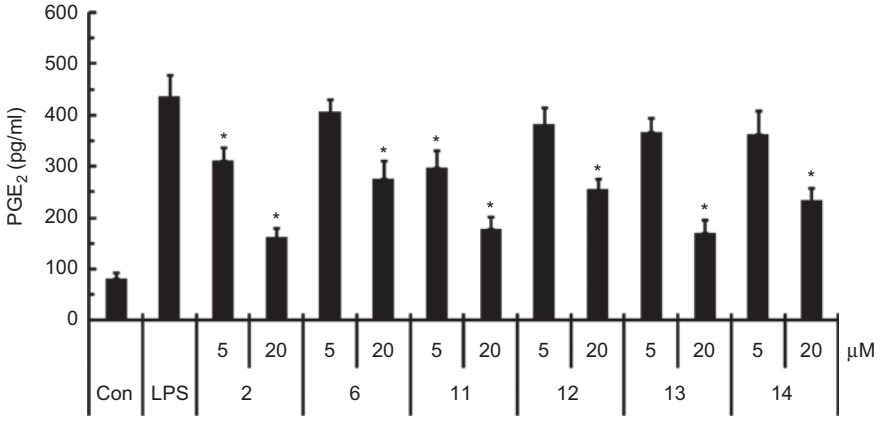

Figure 2. The effects of compounds **2**, **6**, and **11**–**14** on PGE_2 production in LPS-stimulated BV-2 microglia cells. PGE_2 was assessed by using a competitive enzyme immunoassay kit after treatment with LPS (100 ng/mL) for 24 h in the presence or absence of compounds **2**, **6**, and **11**–**14** (5 and 20 μM). All data are presented as the mean ± 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] 5 μM	Inhibition (%) 20 μM	IC_{50} (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

[a]Values are the inhibition of NO production relative to the LPS control (n=3). [b]na, 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

[a]IC_{50} values against cell lines, defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*. [b]Doxorubicin 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 PGE_2 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.

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Declaration of interest

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