Isolation of Saponins with the Inhibitory Effect on Nitric Oxide, Prostaglandin E_2 and Tumor Necrosis Factor-α Production from *Pleurospermum kamtschaticum*

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Received April 8, 2005; accepted June 14, 2005

As an attempt to search for bioactive natural products exerting antiinflammatory activity, we have isolated two saponins were isolated from the aerial portion of *Pleurospermum kamtschaticum* (Umbelliferae) by nitrite assay activity-directed chromatographic fractionation. They were identified as saikogenin F 3-*O*-{β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-fucopyranoside} (buddlejasaponin IV, 1) and 3β,16β,23,28-tetrahydroxy-11α-methoxyolean-12-ene 3-*O*-{β-D-glucopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-β-D-fucopyranoside} (buddlejasaponin IVa, 2). Compound 1 significantly inhibited nitric oxide (NO) production, and it also significantly decreased prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) release in the lipopolysaccharide (LPS)-activated macrophage Raw 264.7 cells whereas compound 2 was much less active. Saikogenin A (3) and –H (4) were obtained by hydrolyzing 1 and 2. Although these sapogenin showed strong NO inhibition, these effects were caused by the cytotoxic effect on Raw 264.7 cells. These results supported the notion that buddlejasaponin IV is a major inhibitors of NO, PGE_2 and TNF-α production in *P. kamtschaticum*.

Key words *Pleurospermum kamtschaticum*; Umbelliferae; saponin; nitric oxide; prostaglandin E_2; tumor necrosis factor-α

Pleurospermum kamtschaticum HOFFMANN (Umbelliferae) is a perennial herb distributed in Kangwon province Korea.[1] The aerial part of *P. kamtschaticum* are traditionally used to treat colds, arthritis, atherosclerosis and impotence.[2] The constituents of *P. kamtschaticum* have not been reported though Luo *et al.*[3] reported on the isolation of saponins from another *Pleurospermum* spp. Activity-directed fractionation led to the isolation of the two saponins compounds **1** and **2** from *P. kamtschaticum*, which were identified as saikogenin F 3-*O*-{β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-fucopyranoside} (buddlejasaponin IV) and 3β,16β,23,28-tetrahydroxy-11α-methoxyolean-12-ene 3-*O*-{β-D-glucopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-β-D-fucopyranoside} (buddlejasaponin IVa) by physicochemical and spectroscopic data. The structures of the two saponins are shown in Fig. 1. Compounds **1** and **2** have not been previously isolated from *Pleurospermum* spp. These two compounds were tested for their inhibitory effects on nitric oxide (NO) production in an lipopolysaccharide (LPS)-stimulated macrophage cell line (Raw 264.7 cells). NO has diverse physiological roles and also contributes to immune defenses against viruses, bacteria, and other parasites. However, excessive NO production is associated with various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune diseases, chronic inflammation, and atherosclerosis.[4] Previously, we reported on the cytotoxic effects of triterpenes and triterpenoidal saponins against cancer cell lines and on the inhibition of NO production in LPS-activated macrophage cell lines.[5—7]

In general, sapogenins are bioactive principles rather than the saponins themselves. Therefore, saikosapogenin A (**3**) and –H (**4**), which are artifacts rather than genuine aglycones, were obtained from the hydrolysis of the two isolated saponins. However, it was found that these significant NO inhibitory effect of two sapogenins was due to their cytotoxic effect on RAW 264.7 cells. Thus, as a prelude to reveal the underlying mechanisms for the anti-inflammatory effect of compounds **1** and **2**, we evaluated and compared the effects of these compounds isolated from the aerial portion of *P. kamtschaticum* on LPS-induced NO, prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α) release by the macrophage cell line RAW 264.7 in the present study.

MATERIALS AND METHODS

Instruments Melting Points were determined on an Electrothermal digital melting point apparatus. Optical rotations were measured on a JASCO DIP-360 digital polarimeter and IR spectra recorded on a Bomem MB-100 FT-IR spectrometer. FAB-MS were obtained using a JMS HX-110

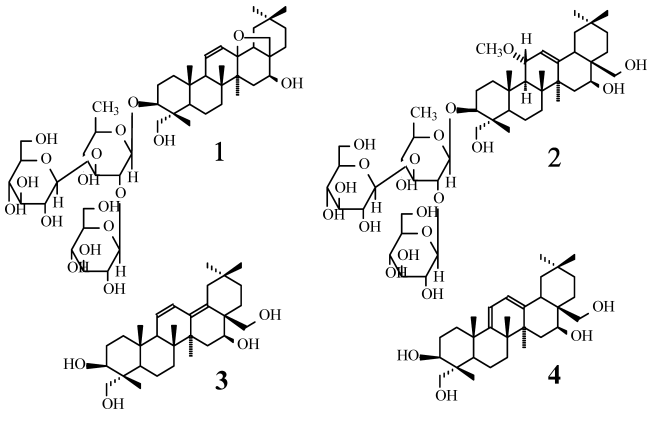

Fig. 1. Structure of Compounds **1**—**4** Obtained from *P. kamtschaticum*

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mass spectrometer. Sample purity was verified by TLC (Si gel, precoated plates, Merck, PF_{254}, 20×20 cm, 0.25 mm).

Plant Material The aerial portion of *P. kamtschaticum* was collected in Taebaek, Gangwon province, Korea and were identified by Dr. Won-Bae Kim (National Alpine Agricultural Experimental Station, RDA, Pyongchang, Korea). A voucher specimen (#natchem-30) has been deposited at Sangji University.

Extraction and Fractionation The aerial portion of *P. kamtschaticum* was dried in the dark and pulverized. This plant material (1.8 kg) was then extracted with MeOH three times for 4 h under reflux. The extract was filtered and evaporated to dryness in a rotatory evaporator under reduced pressure. The concentrated viscous extract obtained was then freeze-dried to give a solid extract (450 g). This solid MeOH extract (430 g) was suspended in 1 l distilled water and partitioned five times with 3 l $CHCl_3$ and concentrated *in vacuo* to give a $CHCl_3$ extract (70 g). The aqueous layer was partitioned five times with 3 l of water-saturated BuOH, and the combined BuOH-soluble fraction was dried *in vacuo* and freeze-dried to give the BuOH extract (180 g).

Isolation of Saponins The BuOH extract (90 g) obtained above was chromatographed on a silica gel column (580 g, 8×70 cm, Merck, Art 7734, Germany) using $CHCl_3$–MeOH–H_2O (7 : 3 : 1, lower phase) as an eluent and collected in 80 ml aliquots. Aliquots corresponding to the eluted volume 4.08—13.6 l were concentrated and rechromatographed on a silica gel column using $CHCl_3$–MeOH–H_2O (65 : 35 : 10, lower phase) and subject to TLC on silica gel plates using the same eluent. Plates were sprayed with 10% H_2SO_4 and showed a violet-colored spot at *Rf* 0.37 ($CHCl_3$–MeOH–H_2O=65 : 35 : 10, lower phase). This corresponding spot was subject to Thin layer chromatography on reverse phase (octadecylsilane, ODS) and developed with MeOH–H_2O (7 : 3). Then, two spots were observed at *Rf* 0.20 and 0.12. So, the corresponding eluted solution from the silica gel column using $CHCl_3$–MeOH–H_2O=65 : 35 : 10 (lower phase) was subjected to octadecylsilane (95 g, 5×70 cm, YMC gel, Japan) column chromatography using MeOH–H_2O (7 : 3) as an eluent. Compounds **1** and **2** were observed at retention volumes of 330—530 ml (compound **2**) and 820—1030 ml (compound **1**). Compound **1** (4 g) and **2** (1.1 g) were also obtained by purifying by repeated ODS column chromatography on the same isolation conditions.

Compound **1** (Buddlejasaponin IV): Amorphous solid, $[\alpha]_D^{25}$ +54.2° (c=0.99, MeOH), FAB-MS (positive): m/z 965.6 $[M+Na]^+$, ^{1}H-NMR (pyridine-d_5, 500 MHz) and ^{13}C-NMR (500 MHz, pyridine-d_5) were identical with those reported in the literature.[8] Compound **2** (Buddlejasaponin IVa): mp 290—294 °C, $[\alpha]_D^{23}$ −6.30° (isopropyl alcohol, c=0.005), IR ν_{max} (cm^{-1}): 3100—3500 (OH), 2919 (CH), 1449, 1388, 1357 1000—1100 (glycosidic C–O), 902, 851; FAB-MS (positive): m/z 997.6 $[M+Na]^+$, FAB-MS (negative) m/z 973.3 $[M-H]^-$ HR-FAB-MS calcd $C_{49}H_{82}O_{19}Na$ 997.5350 observed $C_{49}H_{82}O_{19}Na$ m/z 997.5348. ^{1}H-NMR (pyridine-d_5, 500 MHz) and ^{13}C-NMR (500 MHz, pyridine-d_5) were identical with those reported in the literature.[9]

Acid Hydrolysis of Compounds 1 and 2 Compound **2** (400 mg) was refluxed with 80 ml of 5% HCl in H_2O–MeOH (9 : 1) for 3 h, and extracted with EtOAc, washed with distilled water, and concentrated to yielded 120 mg of crude product. This crude product was chromatographed on a silica gel column using $CHCl_3$–MeOH–H_2O (90 : 7 : 1, lower phase) as eluent to give compounds **3** (45 mg) and **4** (15 mg). Acid hydrolysis of compound **1** was performed under the same conditions as that of compound **2** and showed to produce the same compounds, **3** and **4**, which were observed on silica gel and ODS thin layer chromatography.

Compound **3** (Saikogenin A, 3β,16β,23,28-Terahydroxyolean-11,13(18)-diene): mp 288—290 °C, $[\alpha]_D^{23}$ −37.6° (c=0.85, ethanol), MS m/z (%): 472 (M^+), ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) see Literature data.[10,11]

Compound **4** (Saikogenin H, 3β,16β,23,28-Terahydroxyolean-9(11),12-diene): mp 300 °C, $[\alpha]_D^{23}$ −209.8° (c=0.5, MeOH), ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) see Literature data.[10,11]

Cell Culture and Sample Treatment The RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul). Cells were grown at 37 °C in DMEM containing 10% heat-inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified of 5% CO_2 atmosphere. Cells were incubated with samples at various concentrations and stimulated with LPS at 1 μg/ml.

Nitrite Assay Nitrite accumulation was measured as a measure of NO production using the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine–HCl], incubated at room temperature for 10 min. Absorbance at 550 nm was measured using a microplate reader. Fresh culture medium was used as a blank for all experiments. The amount of nitrite in samples was measured using a standard curve prepared using sodium nitrite solutions.

MTT Assay for Cell Viability Cytotoxicity studies were performed in 96-well plates. RAW 264.7 cells were mechanically scraped and plated at 1×10^5/well in 96-well plates containing 100 μl of DMEM with 10% heat-inactivated FBS and incubated overnight. Samples were dissolved in DMSO, and the DMSO concentrations in all assays did not exceed 0.1%. After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μl of FBS-free medium containing MTT 5 mg/ml. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO 100 μl. The optical density was measured at 540 nm.

PGE_2 and TNF-α Assay PGE_2 and TNF-α level in macrophage culture medium were quantified by EIA kits according to the manufacture's instructions.

RESULTS AND DISCUSSION

P. kamtschaticum is used traditionally to treat cold, atherosclerosis, arthritis and to overcome fatigue. Since it was recently suggested that those diseases are primarily associated with excessive NO formation,[4] we undertook to identify the active principle by focusing on its inhibitory effect on NO formation.

The MeOH extract of *P. kamtschaticum* was fractionated

Table 1. IC_{50} Value of *P. kamtshaticum* Extracts on Nitrite Accumulation in 1 μg/ml LPS-Induced Macrophage 264.7 Cells and on Cytotoxicity

Extract	IC_{50} (μg/ml)	
	NO	Cell survival
MeOH extract	63.1	165.3
$CHCl_3$ fraction	80.3	177.7
BuOH fraction	37.0	80.4
Acid hydrolysate	28.5	107.1

The values are the means of three independent experiments.

and aliquots were subjected to nitrite assay using macrophage RAW 264.7 cells. The acid hydrolysate of the BuOH extract was also tested to examine the effect of a mixed saponin hydrolysate. The inhibitory effect of the MeOH extract and the fractionated extracts are shown in Table 1. These results indicate that the MeOH extract has an inhibitory effect on NO formulation in LPS-induced cells. Since the BuOH fraction showed a higher activity (IC_{50} 37.0 μg/ml) than the $CHCl_3$ fraction (IC_{50} 80.3 μg/ml), we considered that the saponin-containing fraction had the higher bioactivity. Further, it was shown that the IC_{50} value (28.5 μg/ml) of the acid hydrolysate of the saponin fraction (BuOH fraction) was lower than that of the saponin fraction itself (37.0 μg/ml). These results suggest that the hydrolysis of the saponin of this plant may produce a more biologically active mixture.

Silica gel column chromatography and successive ODS column chromatography of the BuOH extract afforded two main saponins, compounds **1** and **2**, which were positive by the Liebermann-Burchard test and the Molish test. Acid hydrolysis of compound **1** produced D-fucose and D-glucose as the sugar moiety and saikogenin A (**3**) and –H (**4**) as the artifact of the sapogenin. Sugar moieties were identified on TLC and the structure of the triterpene produced was established by physicochemical (mp, $[\alpha]_D$) and spectroscopic data (^{1}H-, ^{13}C-NMR, MS). Our physical and spectroscopic data was in accordance with the literature data on buddlejasaponin IV.[8] It has been previously reported that buddlejassaponin IV has an antihepatotoxic effect.[8]

Acid hydrolysis of compound **2** also led to the finding that the sugar moieties were D-fucose and D-glucose, and that the triterpene (artifact) of compound **1** was saikogenin A. Although acid hydrolysis of compounds **1** and **2** produced diverse artifacts of the sapogenin, further chromatographic isolation of the hydrolysates yielded sakogenin A (**3**) and –H (**4**) as artifact sapogenins. Our HMBC and NOESY NMR experiment (data not shown) confirmed the structure of 3β,16β,23,28-tetrahydroxy-11α-methoxyolean-12-ene-3-*O*-{β-D-glucopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-β-D-fucopyrano-side} (buddlejasaponin IVa).[9] And the molecular formula $C_{49}H_{82}O_{19}$ was confirmed by *m/z* 997.6 due to $[M+Na]^+$ in the positive FAB-MS spectrum, *m/z* 973.3 due to $[M-H]^-$ in the negative FAB-MS, and a further *m/z* 997.5348 due to $[M+Na]^+$ in the HR-FAB-MS.

Figure 2A shows the effect of the two isolated saponins on NO formation in LPS-activated macrophage 264.7 cells. The IC_{50} of compound **1** was found to be 5.4 μg/ml and that of **2** was 57.2 μg/ml. L-NIL (10 μM) was used as a positive inhibitor. To examine whether the tested compounds could inhibit the production of pro-inflammatory molecules such as PGE_2 and TNF-α, the cells were pre-incubated with the compounds **1** and **2** for 1 h, and then activated with 1 μg/ml LPS for 24 h. As shown in Figs. 2B and C, compound **1** significantly inhibited the production of PGE_2 and TNF-α in a dose-dependent manner. The cytotoxic effects of compounds **1** and **2** were evaluated in the presence or absence of LPS. Compound **1** and compound **2** did not affect the cell viability of RAW 264.7 cells at the concentrations used (Table 2).

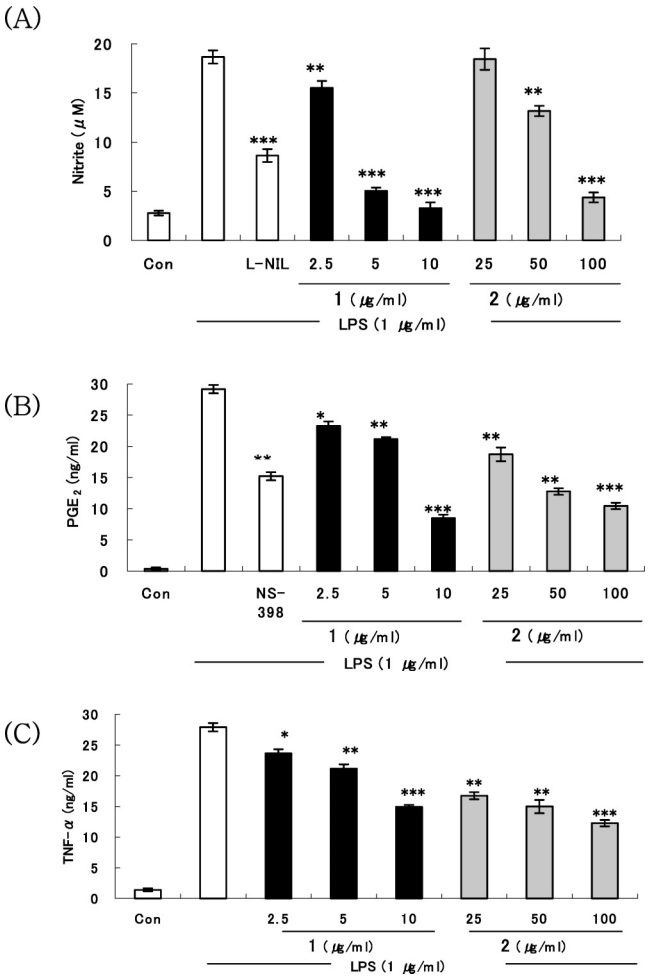

Fig. 2. Effect of Buddlejasaponin IV (**1**) and Buddlejasaponin IVa (**2**) on Nitrite (A), PGE_2 (B), and TNF-α (C) Production by LPS-Induced Raw 264.7 Cells

(A) The cells were pretreated for 1 h with various concentrations of buddlejasaponin IV and buddlejasaponin IVa, and then LPS (1 μg/ml) was added and the cells incubated for 24 h. Control (Con) values were obtained in the absence of LPS or buddlejasaponin IV and buddlejasaponin IVa. 10 μM of L-N^6-(1-iminoethyl)lysine (L-NIL) was present in the assay as a positive control. The values are the means±S.D. from three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$ *vs.* LPS-treated group; the significance of the difference between the treated groups was evaluated using the Student's *t*-test. (B) The conditions of sample treatment were identical to those described for Fig. 2A. 10 μM of NS-398 was used as a positive control in the assay. The values represent the means±S.D. from three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* LPS-treated group; the significance of the difference between the treated groups was evaluated using the Student's *t*-test. (C) The conditions of sample treatment were identical to those described for Fig. 2A. The values represent the means±S.D. from three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* LPS-treated group; the significance of the difference between the treated groups was evaluated using the Student's *t*-test.

These results indicated that the structure of **1** with a 13β,28-epoxy partial structure should have a potent inhibitory effect on NO, PGE_2 and TNF-α formation. They also suggest that buddlejasaponin IV is a major bioactive

Table 2. IC_{50} Value of Compounds **1**—**4** from *P. kamtschaticum* on Nitrite Accumulation in 1 μg/ml LPS-Induced Macrophage 264.7 Cells and on Cytotoxicity

Extract	IC_{50} (μg/ml)	
	NO	Cell survival
1	5.4	30.1
2	57.2	>200
3	18.1	17.2
4	25.8	17.5

The values are the means of three independent experiments.

saponin in *P. kamtschaticum* and thus that its inhibitory effect on NO, PGE_2 and TNF-α formation might be associated with its putative anti-inflammatory effect. Although numerous reports on the biological activities of saikosaponins and saikogenins, *i.e.*, anti-inflammatory,[12] anti-allergic,[13] and anti-angiogenesis[14] and an elevation in intracellular calcium ion concentration,[15] this is the first report on the inhibitory effects of the saponins isolated in the present research on NO, PGE_2 and TNF-α production.

On the other hand, the artifact saikogenin A (**3**) and –H (**4**) were also tested to determine whether or not the artifacts formed during the biodegradation of saponins in the gastrointestinal tract could be responsible for their inhibitory effects on NO formation. The IC_{50} values of compounds **3** and **4** on the NO accumulation were determined to be 18.1 and 25.8 μg/ml respectively. However, it was found that this effect was due to their cytotoxic effect on RAW 264.7 cells (IC_{50}, **3**: 17.2 μg/ml; **4**: 17.5 μg/ml).

Acknowledgements This study (203016-02-1-SB010) was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea. We also thank Mr. Byung-Kee Sohn (Dept. of Chemistry, Pohang Engineering & Technology University, Korea) for performing the NMR measurement.

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