

Anti-Inflammatory and PPAR Transactivational Effects of Oleanane-Type Triterpenoid Saponins from the Roots of *Pulsatilla koreana*

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Abstract

In this study, 23 oleanane-type triterpenoid saponins were isolated from a methanol extract of the roots of *Pulsatilla koreana*. The NF- κ B inhibitory activity of the isolated compounds was measured in TNF α -treated HepG2 cells using a luciferase reporter system. Compounds 19-23 inhibited TNF α -stimulated NF- κ B activation in a dose-dependent manner, with IC₅₀ values ranging from 0.75-8.30 μ M. Compounds 19 and 20 also inhibited the TNF α -induced expression of iNOS and ICAM-1 mRNA. Moreover, effect of the isolated compounds on PPARs transcriptional activity was assessed. Compounds 7-11 and 19-23 activated PPARs the transcriptional activity significantly in a dose-dependent manner, with EC₅₀ values ranging from 0.9-10.8 μ M. These results suggest the presence of potent anti-inflammatory components in *P. koreana*, and will facilitate the development of novel anti-inflammatory agents.

Key Words: *Pulsatilla koreana*, NF- κ B inhibitory activity, Tumor necrosis factor- α , PPAR

INTRODUCTION

The *Pulsatilla* genus, *Pulsatilla koreana* Nakai (Ranunculaceae) from Korea is an important herb in traditional medicine used to treat amoebic dysentery and malaria (Kim *et al.*, 2004). Phytochemical studies on *P. koreana* roots have demonstrated the presence of protoanemonin, deoxypodophyllotoxin, oleanane, and lupane-type triterpenoid saponins (Martín *et al.*, 1990; Kim *et al.*, 2002; Kim *et al.*, 2004; Bang *et al.*, 2005; Lee *et al.*, 2010; Suh *et al.*, 2010). Root extracts of *P. koreana* possess antitumor, antibiotic, and anti-inflammatory activities (Martín *et al.*, 1990; Cuong *et al.*, 2009; Yang *et al.*, 2010). Although, *P. koreana* contains a wide variety of metabolites and bioactivities, the active components that inhibit nuclear factor kappa B (NF- κ B) and activate peroxisome proliferator-activated receptor (PPAR) have not been identified.

NF- κ B is a family of Rel domain-containing proteins that includes RelA, RelB, c-Rel, NF- κ B1, and NF- κ B2. NF- κ B activation has been linked to multiple pathophysiological conditions such as cancer, arthritis, asthma, inflammatory bowel disease, and other inflammatory conditions (Baldwin, 2001). NF- κ B activation by various stimuli, including the inflammatory cyto-

kines tumor necrosis factor alpha (TNF- α) and interleukin-1 (IL-1), T-cell activation signals, growth factors, and stress inducers cause transcription at κ B sites, which are involved in a number of diseases, including inflammatory disorders and cancer (Baldwin, 2001; Pande and Ramos, 2005). In the present study, the effects of compounds 1-23 (oleanane-type triterpenoid saponins that were isolated from a methanol extract of the roots of *Pulsatilla koreana*) on TNF α -induced NF- κ B transcriptional activity in human hepatocarcinoma (HepG2) cells were evaluated using an NF- κ B-luciferase assay.

PPAR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. It is predominantly expressed in adipose tissue, adrenal glands, and the spleen (Moraes *et al.*, 2006; Sharma and Staels, 2007). Three isoforms have been identified: PPAR α , PPAR γ , and PPAR β (δ). PPARs regulate the expression of genes involved in the regulation of glucose, lipid, and cholesterol metabolism by binding to specific peroxisome proliferator response elements (PPREs) in the enhancer sites of target genes (Berger and Moller, 2002; Balint and Nagy, 2006; Barish *et al.*, 2006; Haluzik and Haluzik, 2006). Accordingly, compounds that modulate PPARs function are attractive for the treatment of type

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2 diabetes, obesity, metabolic syndrome, inflammation, and cardiovascular disease (Kuroda *et al.*, 2010). In this study, 23 oleanane-type triterpenoid saponins were isolated from a methanol extract of the roots of *P. koreana*. Their inhibition of NF- κ B and activation of PPAR were measured in HepG2 cells using luciferase reporter systems.

MATERIALS AND METHODS

Chemical and sample preparation

Compounds 1-23 were isolated from the roots of *P. koreana* and identified in our previous reports. Sulfasalazine and bezafibrate (positive control) were the products of Sigma-Aldrich. All other chemicals and reagents were of analytical grade. The tested compounds and positive control were dissolved in DMSO.

Plant material

Dried roots of *P. koreana* were purchased from herbal market, Kumsan, Chungnam, Korea in March 2009 and identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 09106) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and isolation

Dried roots of *P. koreana* (2.0 kg) were extracted with MeOH under reflux for 10 h (7 L \times 3 times) to yield 500.0 g of extract. This extract was suspended in water and partitioned with ethyl acetate to yield 37.0 g ethyl acetate extract and 463.0 g water extract. The water extract was partitioned with *n*-BuOH to yield 130.0 g BuOH extract. The ethyl acetate extract was subjected to silica gel column chromatography with a gradient of CHCl₃-MeOH (50:1, 20:1, 10:1 and 1:1; 2 L for each step) to give 6 fractions (Fr. E1-E6). The fraction E4 was separated using an YMC column with a MeOH-actone-H₂O (0.25:0.3:1-1.3:1.3:1, 1.2 L for each step) elution solvent to give compound 23 (75.0 mg). The fraction E5 was separated using an YMC column with a MeOH-H₂O (3.2:1 1.4 L) elution solvent to give compound 11 (19.0 mg).

The *n*-BuOH extract was subjected to silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (5:1:0.1, 2:1:0.1 and 0:1:0; 3 L for each step) to give 6 fractions (Fr. B1-B6). The fraction B3 was separated using a silica gel column with CHCl₃-MeOH-H₂O (5:1:0.1, 4:1:0.1 and 3:1:0.1, 1 L for each step) to give 4 sub-fractions (Fr. B3.1-B3.4). Fraction B3.1 was separated using an YMC column with a MeOH-H₂O (4.5:1, 1.1 L) elution solvent to give compound 20 (120.0 mg). Fraction B3.2 was separated using an YMC column with a MeOH-acetone-H₂O (2.5:0.7:1, 2.5 L) elution solvent to give compounds 8 (78.0 mg) and 19 (30.0 mg). Fraction B3.3 was separated using an YMC column with a MeOH-acetone-H₂O (1.5:0.7:1, 750 mL) elution solvent to give compound 22 (180.0 mg). Fraction B3.4 was separated using an YMC column with a MeOH-acetone-H₂O (2:0.5:1, 2.5 L) elution solvent to give compound 9 (2.1 g). The fraction B4 was separated using a silica gel column with CHCl₃-MeOH-H₂O (3.5:1:0.1, 2:1:0.1 and 1:1:0.2, 1 L for each step) to give 4 sub-fractions (Fr. B4.1-B4.4). Fraction B4.3 was separated using an YMC column with a MeOH-acetone-H₂O (10.5:1:1-0.85:2:1, each 550 mL) elution solvent to give compound 10 (25.0 mg).

Water extract was chromatographed on a column of high-

ly porous polymer (Diaion HP-20) and eluted with H₂O and MeOH, successively, to give 4 fractions (Fr. W1-W4). Fraction W3 was subjected to silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (6:1:0.1, 4:1:0.1, 2:1:0.1 and 0:1:0; 4 L for each step) to give 6 fractions (Fr. W3.1-W3.6). Fraction W3.3 using an YMC column with a MeOH-acetone-H₂O (1:0.3:1-1:0.4:1.4, 650 mL for each step) elution solvent to give compounds 2 (50.0 mg), 3 (44.0 mg), 13 (17.0 mg), 14 (77.0 mg), and 16 (38.0 mg). Fraction W3.4 was separated using an YMC column with a MeOH-H₂O (1.3:1-2.5:1, 750 mL for each step) elution solvent to give compounds 1 (44.0 mg) and 12 (110.0 mg). Fraction W3.5 was separated using an YMC column with an acetone-MeOH-H₂O (0.25:1:1-0.32:1:1, 600 mL for each step) elution solvent to give compounds 4 (78.0 mg) and 18 (460.0 mg). Fraction W3.6 was separated using a silica gel column with CHCl₃-MeOH-H₂O (1.2:1:0.15, 1.5 L) to give compound 15 (130.0 mg). Fraction W4 was subjected to silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (2.5:1:0.1, 1.5:1:0.15 and 0:1:0; 3 L for each step) to give 3 fractions (Fr. W4.1-W4.3). Fraction W4.1 was further chromatographed on RP chromatography column with acetone-MeOH-H₂O (0.7:1.5:1-1:2:1, 1 L for each step) to yield compounds 7 (12.0 mg) and 21 (196.0 mg). Fraction W4.2 was further chromatographed on RP chromatography column with acetone-MeOH-H₂O (0.6:1:1-1:1.7:1, 750 mL for each step) to yield compounds 5 (300.0 mg) and 17 (12.0 mg). Compound 6 (36.0 mg) was isolated from W4.3 using RP chromatography column with acetone-MeOH-H₂O (0.3:1.7:1).

Their structures were elucidated as cernuoside A (1) (Zhang *et al.*, 2000), hederacholchiside E (2) (Yang *et al.*, 2010), beesioside Q (3) (Tommasi *et al.*, 2000), 3-O- β -D-glucopyranosyl (1 \rightarrow 4)- β -D-glucopyranosyl (1 \rightarrow 3)- α -L-rhamnopyranosyl (1 \rightarrow 2) [β -D-glucopyranosyl(1 \rightarrow 4)]- α -L-arabinopyranosyl oleanolic acid 28-O- α -L-rhamnopyranosyl (1 \rightarrow 4)- β -D-glucopyranosyl (1 \rightarrow 6)- β -D-glucopyranoside (4) (Liu *et al.*, 2012), hederacoside B (5) (Majester-Savornin *et al.*, 1991), raddeanoside R17 (6) (Sun *et al.*, 2008), 3-O- β -D-glucopyranosyl (1 \rightarrow 3)- α -L-rhamnopyranosyl (1 \rightarrow 2) [β -D-glucopyranosyl (1 \rightarrow 4)]- α -L-arabinopyranosyl oleanolic acid (7) (Schenkel *et al.*, 1991), 3-O- β -D-glucopyranosyl (1 \rightarrow 3)- α -L-rhamnopyranosyl (1 \rightarrow 2)- α -L-arabinopyranosyl oleanolic acid (8) (Bang *et al.*, 2005), raddeanoside R13 (9) (Bang *et al.*, 2005), 3-O- β -D-glucopyranosyl (1 \rightarrow 4)- β -D-glucopyranosyl (1 \rightarrow 3)- α -L-rhamnopyranosyl (1 \rightarrow 2) [β -D-glucopyranosyl(1 \rightarrow 4)]- α -L-arabinopyranosyl oleanolic acid (10) (Mimaki *et al.*, 1999), 3-O- β -D-glucopyranosyl (1 \rightarrow 4)- β -D-glucopyranosyl (1 \rightarrow 3)- α -L-rhamnopyranosyl (1 \rightarrow 2) [β -D-glucopyranosyl(1 \rightarrow 4)]- α -L-arabinopyranosyl oleanolic acid (11) (Mimaki *et al.*, 1999), hederacholchiside F (12) (Yang *et al.*, 2010), fatsiaside G (13) (Li *et al.*, 1990), *Pulsatilla* saponin F (14) (Tran *et al.*, 2011), pulsatilloside F (15) (Li *et al.*, 2013a), patrinia saponin H3 (16) (Kang *et al.*, 1997), hederasaponin D (17) (Tran *et al.*, 2011), cernuoside B (18) (Zhang *et al.*, 2000), scabioside C (19) (Bang *et al.*, 2005), hederoside C (20) (Li *et al.*, 1990), *Pulsatilla* saponin D (21) (Shimizu *et al.*, 1978), kalopanaxsaponin H (22) (Ye *et al.*, 1996), and scabioside A (23) (Baykal *et al.*, 1997) (Fig. 1). Their structures were elucidated on the basis of spectroscopic data and comparison of 1D- and 2D-NMR and mass spectral data with reported values.

Cell lines and culture

Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad,

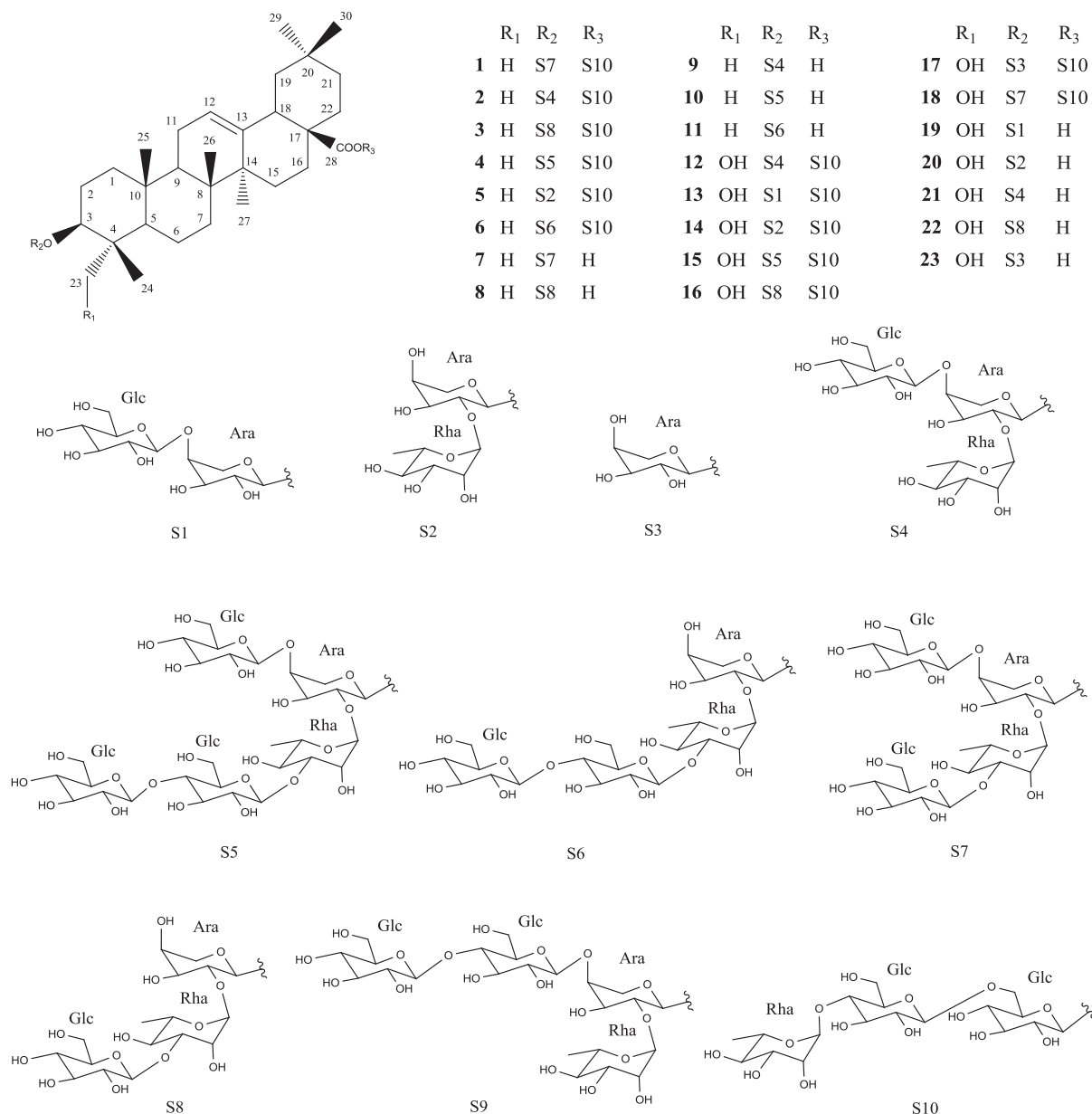


Fig. 1. Structures of compounds 1–23 from the roots of *P. koreana*.

CA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 10 µg/mL streptomycin, at 37°C and 5% CO₂. Human TNF-α was purchased from ATgen (Seoul, Korea).

Cell toxicity assay

Cell-Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) was used to analyze the effect of compounds on cell toxicity according to the manufacturer’s instructions. Cells were cultured overnight in 96-well plate (~1×10⁴ cells/well). Cell toxicity was assessed after the addition of compounds on dose-dependent manner. After 24 h of treatment, 10 µl of the CCK-8 solution was added to triplicate wells, and incubated for 1 h. Absorbance was measured at 450 nm to determine viable cell

numbers in wells.

NF-κB-Luciferase assay

HepG2 cells were maintained in Dulbecco’s modified Eagles’ medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 µg/mL streptomycin at 37°C and 5% CO₂. The luciferase vector was first transfected into HepG2 cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg²⁺ and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10⁵ cells per well in a 12-well plate and

grown. After 24 h, cells were transfected with inducible NF- κ B firefly luciferase reporter and constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM+0.5% FBS+0.1 mM NEAA+1 mM sodium pyruvate+100 units/ml penicillin+10 μ g/ml streptomycin) and cells were pretreated for 1 h with either vehicle (1% DMSO in water) and compounds, followed by 1 h of treatment with 10 ng/mL TNF α for 23 hr. Unstimulated HepG2 cells were used as a negative control (-), PDTC was used as a positive control. Dual Luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization (Kim et al., 2010).

RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted using Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Approximately 2 μ g total RNA was subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega, Madison, WI, USA) for 1 h at 42°C. PCR for synthetic cDNA was performed using a Taq polymerase pre-mixture (TaKaRa, Japan). The PCR products were separated by electrophoresis on 1% agarose gels and stained with EtBr. PCR was conducted with the following primer pairs: iNOS sense 5'-TCATCCGCTATGCTGGCTAC-3', iNOS antisense 5'-CTCAGGGTCACGGCCATTG-3', ICAM-1 sense 5'-CTGCAGACAGTGACCATC-3', ICAM-1 antisense 5'-GTC-CAGTTTCCCGGACAA-3', β -actin sense 5'-TCACCCACACT-

GTGCCCATCTACG-3', and β -actin antisense 5'-CAGCG-GAACCGCTCATTGCCAATG-3'. The specificity of products generated by each set of primers was examined using gel electrophoresis and further confirmed by a melting curve analysis. HepG2 cells were pretreated in the absence and presence of compounds for 1 h, then exposed to 10 ng/mL TNF α for 6 h. Total mRNA was prepared from the cell pellets using Easy-blue. The levels of mRNA were assessed by RT-PCR.

PPRE-Luciferase assay

HepG2 cells were seeded at 1.5×10^5 cells per well in 12-well plates and grown for 24 h before transfection. An optimized amount of DNA plasmid (0.5 μ g of PPRE-Luc and 0.2 μ g of PPAR-inpCMV) was diluted in 100 μ L of DMEM. All cells were transfected with the plasmid mixture using WelFect M Gold (WelGENE Inc.) as described by the manufacturer. After 30 min of incubation at room temperature, the DNA plasmid solution (100 μ L) was introduced and mixed gently with cells. After 24 h of transfection, the medium was changed to TOM (Transfection Optimized Medium, Invitrogen) containing 0.1 mM NEAA, 0.5% charcoal-stripped FBS, and the individual compounds (test group), dimethyl sulfoxide (vehicle group), or benzafibrate (positive control group). The cells were then cultured for 20 h. Next, the cells were washed with PBS and harvested with $1 \times$ passive lysis buffer (200 μ L). The intensity of emitted luminescence was determined using an LB 953 Autolumat (EG&G Berthold, Bad Wildbad, Germany).

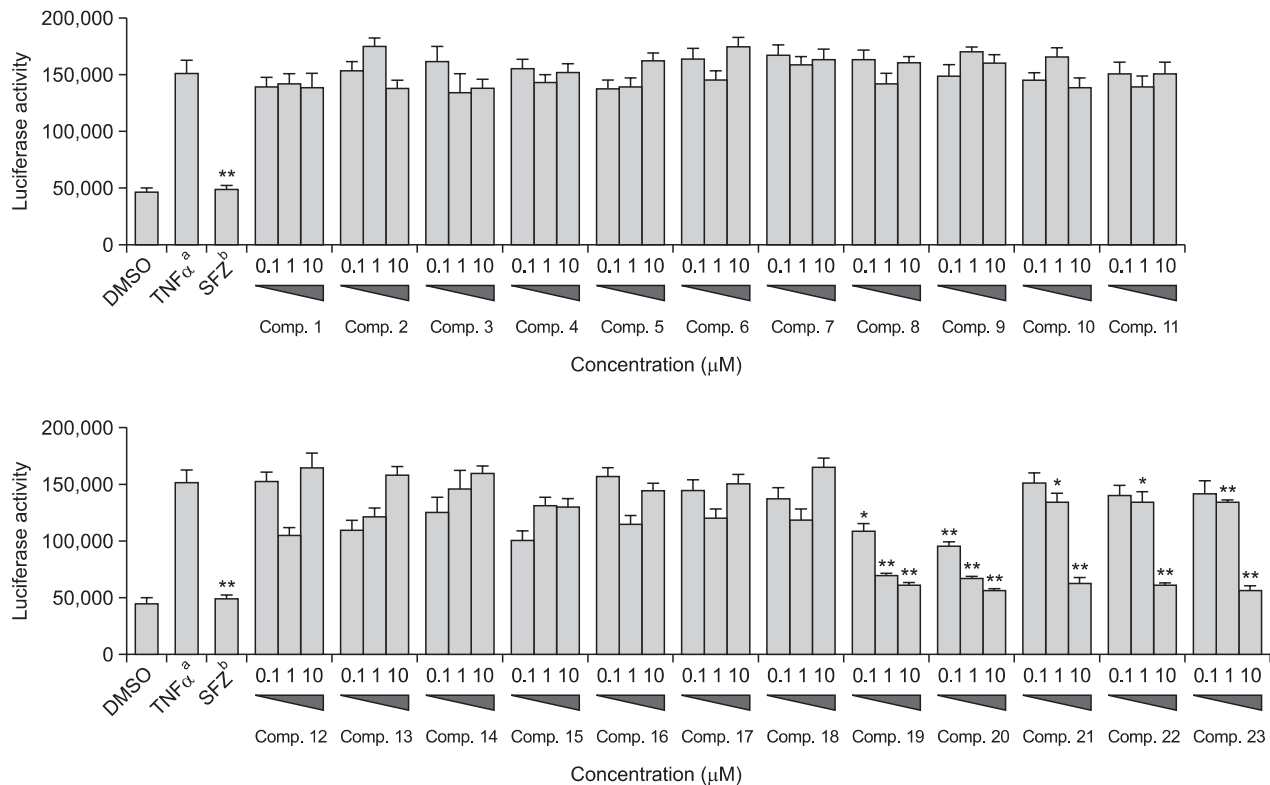


Fig. 2. Effects of compounds 1–23 on the TNF α -induced NF- κ B luciferase activity in HepG2 cells. ^aStimulated with TNF α . ^bStimulated with TNF α in the presence of 1–23 (0.1, 1, and 10 μ M) and sulfasalazine. SFZ: sulfasalazine, positive control (10 μ M). Statistical significance is indicated as * ($p < 0.05$) and ** ($p < 0.01$) as determined by Dunnett’s multiple comparison test.

Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. All results are expressed as the mean ± S.E.M. Data was analyzed by Dunnett's multiple comparison test. Upon observation of a statistically significant effect, the Newman-Keuls test were performed to determine the difference between the groups. A *p* value *(<0.05) and **(<0.01) were considered to be significant.

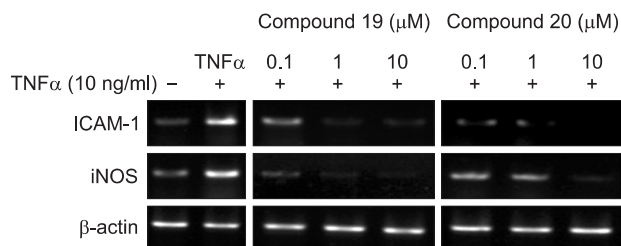


Fig. 3. Effects of compounds 19 and 20 on iNOS and ICAM-1 mRNA expression in HepG2 cells. -: cells were treated without 10 μg/mL TNFα and compounds; +: cells were treated with 10 μg/mL TNFα only; + 0.1, 1, 10: cells were treated with 10 μg/mL TNFα and compounds.

RESULTS

Compounds 1-23 inhibit NF-κB activity in HepG2 cells

The NF-κB inhibitory activity of compounds 1-23 was evaluated using TNFα-induced NF-κB luciferase reporter assay. Cell viability was measured using Cell-Counting Kit (CCK)-8. The results showed that compounds 1-23 were not cytotoxic in HepG2 cells at the tested concentrations (data not shown). HepG2 cells were treated with 10 ng/mL TNFα, which increased NF-κB transcriptional activity compared with untreated control cells. Transfected HepG2 cells were pre-treated with various concentrations (0.1, 1, and 10 μM) of compounds 1-23, and then stimulated with TNFα. Sulfasalazine (SFZ) was used as a positive control (Fig. 2). Data revealed that compounds 19-23 significantly inhibited TNFα-induced NF-κB transcriptional activity in a dose-dependent manner, with IC₅₀ values of 1.12 ± 0.36, 0.75 ± 0.15, 8.30 ± 3.61, 8.10 ± 2.55, and 7.50 ± 1.88 μM, respectively. Compound 20 was the most effective, and was more potent than the positive control, SFZ (IC₅₀=0.9 μM). However, the other compounds (1-18) were inactive at the tested concentrations (IC₅₀>10 μM, data not shown).

Effect of compounds 19 and 20 on the expression of NF-κB target genes

NF-κB target genes include iNOS and ICAM-1, which play important roles in the inflammatory response (Wong and Menendez, 1999; Ley *et al.*, 2007). The ability of compounds 19 and 20 to inhibit the transcription of iNOS and ICAM-1 was

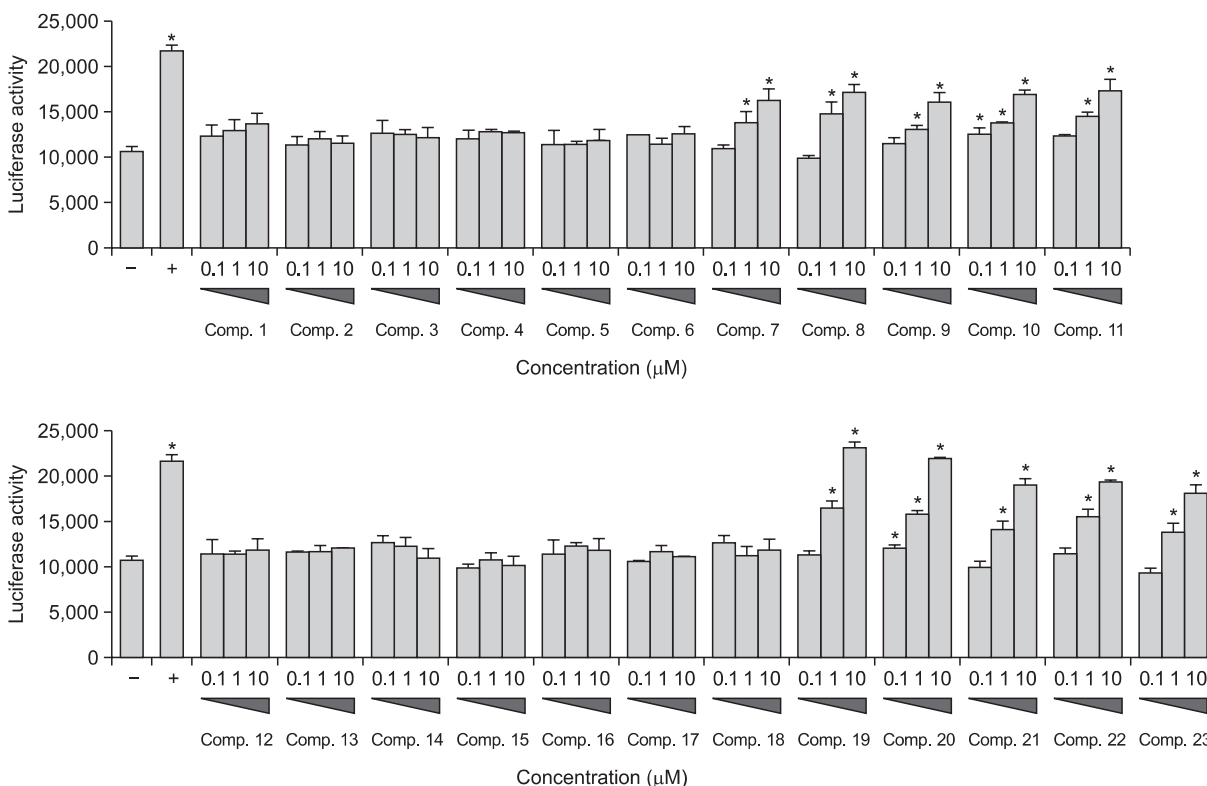


Fig. 4. PPARs transactivational activity of compounds 1–23 in HepG2 cells. (-) Vehicle group; (+) positive control (1 μM): benzafibrate. Statistical significance is indicated as * (*p* < 0.05) as determined by Dunnett's multiple comparison test.

assessed (Fig. 3). Both compounds inhibited the expression of iNOS and ICAM-1 mRNA significantly in a concentration-dependent manner, suggesting that they inhibited the transcription of these genes. The housekeeping, gene β -actin was unchanged by the same concentrations of compounds 19 and 20.

PPAR transactivational activity of compounds 1-23

We evaluated the effects of compounds 1-23 on PPAR activity using a nuclear transcription PPRE cell-reporter system. The PPAR-responsive luciferase reporter construct, used carries a copy of the firefly luciferase gene under the control of a minimal CMV promoter, with tandem repeats of the PPRE sequence. Activated PPAR binds to the PPRE and activates transcription of the luciferase reporter gene. Benzafibrate was used as the positive control. HepG2 cells were co-transfected with the PPRE luciferase reporter and PPAR expression plasmids (Fig. 4). Compounds 7-11 and 19-23 activated the transcriptional activity of PPARs significantly in a dose-dependent manner, with EC_{50} values of 9.8 ± 1.7 , 8.4 ± 2.0 , 10.8 ± 4.2 , 9.1 ± 1.3 , 8.2 ± 1.8 , 0.9 ± 0.2 , 1.7 ± 0.5 , 4.2 ± 1.2 , 1.9 ± 0.3 , and 6.9 ± 1.0 μ M, respectively. Compound 19 was the most effective and was equivalent to the positive control, benzafibrate ($IC_{50}=0.9$ μ M). The remaining compounds were inactive at the tested concentrations ($EC_{50}>20$ μ M).

DISCUSSION

The aim of this study was to identify novel inhibitors of NF- κ B among 23 compounds isolated from the roots of *P. koreana*. In previously study, triterpenoid saponins from *P. koreana* showed anticancer, enhanced immunity, and inflammatory activities (Li *et al.*, 2013a). However, this is the first report describing NF- κ B inhibitory and PPAR activating effects of these compounds. The NF- κ B inhibitory activities and structural properties of compounds 1-23 allowed us to infer information regarding the structure-function relationship. Compounds 19-23 had strong activity because the C-3 of the aglycone was linked to a sugar chain and C-28 was linked to a carboxyl group. Compounds (1-18), which have two sugar chains linked to C-3 and C-28, were active. Therefore, a sugar chain at C-3 and a carboxyl group at C-28 are likely to be key functional elements. The presence of a methyl group at C-23 of the aglycone (compounds 7-11), also resulted in no activity. This suggests that the hydroxyl group at C-23 plays an important role in the anti-inflammatory activity. These observations are consistent with previous reports (Mimaki *et al.*, 2004; Zhang *et al.*, 2011; Li *et al.*, 2013b). Interestingly, compounds 19 and 20 exhibited stronger activity; they contained a disaccharide (Ara-Glc and Ara-Rha, respectively) linked to C-3. These data might be useful to evaluate the structure-function relationship of other triterpenoid saponins.

Compounds 7-11 and 19-23 significantly activated the transcriptional activity of PPARs. These results were similar to the structure-function relationships of cytotoxic activity. These results suggest that compounds 7-11 and 19-23 are promising PPAR agonists. PPAR α/γ , PPAR $\gamma/\beta(\delta)$ dual, and PPAR $\alpha/\gamma/\beta(\delta)$ agonist combinations can achieve a broad spectrum of metabolic effects and reduce undesired weight gain and mortality rates by improving insulin sensitivity and decreasing obesity, dyslipidemia, and hypertension. They also

exert beneficial effects on inflammatory markers (Shearer and Billin, 2007). Therefore, additional studies of individual PPAR subtypes are need to determine how the compounds influence the response to inflammatory stimuli.

In this study, 23 oleanane-type triterpenoid saponins were isolated from a methanol extract of *P. koreana* roots. To our knowledge, this is the first report describing NF- κ B inhibitory and PPAR activating effects of oleanane-type triterpenoid saponins from *P. koreana*. Importantly, these results suggest that oleanane-type triterpenoid saponins are the major bioactive components from this plant that affect inflammation. These results might be useful to evaluate the structure-function relationships of other triterpenoid saponins. Our findings also suggest the presence of anti-inflammatory components in *P. koreana*, and will facilitate the development of novel anti-inflammatory agents.

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