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Oleanane-triterpenoids from *Panax stipuleanatus* inhibit NF-κB

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In continuation of our research to find biological components from *Panax stipuleanatus*, four oleanane-type triterpenes (12 to 15) were isolated successively. Fifteen oleanane-type saponins (1 to 15) were evaluated for nuclear factor (NF)-κB activity using a luciferase reporter gene assay in HepG2 cells. Compounds 6 to 11 inhibited NF-κB, with IC_{50} values between 3.1 to 18.9 μM. The effects on inducible nitric oxide synthase and cyclooxygenase-2 by compounds 8, 10, and 11 were also examined using reverse transcription-polymerase chain reaction. Three compounds (8, 10, and 11) inhibited NF-κB activity by reducing the concentration of inflammatory factors in HepG2 cells.

Keywords: *Panax ginseng*, *Panax stipuleanatus*, Oleanane-type triterpenoid, Nuclear factor-κB

INTRODUCTION

Nuclear factor (NF)-κB is a dimeric transcription factor that activates the expression of many genes involved in inflammation, e.g., cytokines interleukin (IL)-1β, IL-2 and tumor necrosis factor (TNF)-α, adhesion molecules, and enzymes including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and 5-lipoxygenase [1,2]. Without stimulation, NF-κB is inactive, but it can be activated by extracellular signals such as TNF-α, IL-1, lipopolysaccharide (LPS), UV light, and phorbol esters [3]. In unstimulated cells, NF-κB is retained in the cytoplasm via interaction with its inhibitor, NF of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB). In response to various pro-inflammatory stimuli, IκB is phosphorylated by the IκB kinase complex. This leads to the ubiquitination and subsequent proteasome-mediated degradation of IκB, allowing NF-κB to enter the nucleus [4]. NF-κB is highly activated at the site of inflammation in diverse diseases such as rheumatoid arthritis, atherosclerosis, asthma, inflammatory bowel disease, and *Helicobacter pylori*-associated gastritis, and it is associated with cancer, cachexia, diabetes, euthyroid sick syndrome, and AIDS. Due to its apparent involvement in a variety of human diseases, NF-κB has been the target of several anti-inflammatory and anti-cancer drugs [5]. In addition, many of the triterpenoids are reported to have anti-inflammatory activity by regulating NF-κB [6].

Panax stipuleanatus Tsai et Feng (Araliaceae) is an herb that grows in southeast Yunnan, China and north Vietnam. In China, this plant has been used traditionally as a tonic and in the treatment of bruises, bleeding, and

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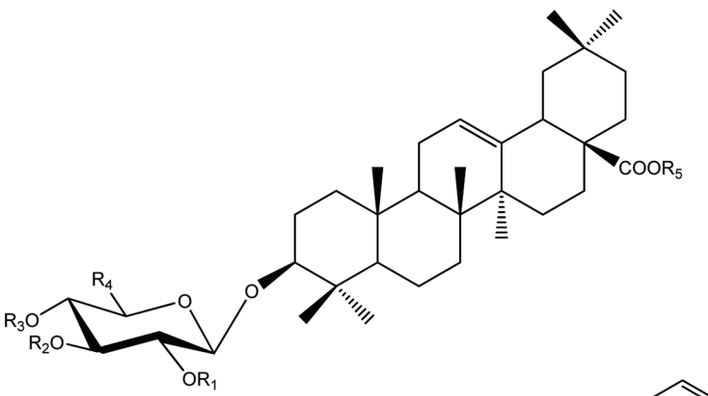
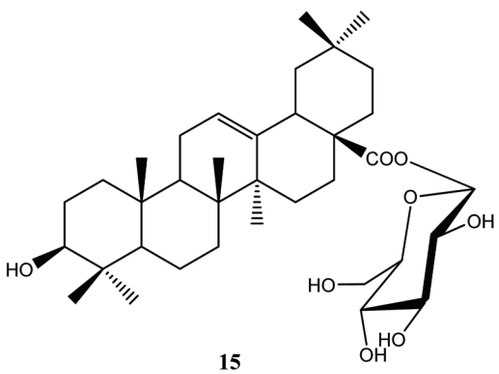


	R1	R2	R3	R4	R5
1	H	Glc I	H	$COOCH_3$	H
2	Xyl	H	H	$COOCH_3$	H
3	H	Glc I	H	COOH	Glc II
4	Xyl	H	H	$COOCH_3$	Glc II
5	Xyl	H	H	COOH	Glc II
6	H	Glc I	Ara(f)	$COOCH_3$	Glc II
7	H	Glc I	Ara(f)	COOH	Glc II
8	H	H	Ara(f)	$COOCH_3$	Glc II
9	H	Glc I	H	$COOCH_3$	Glc II
10	Xyl	H	H	CH_2OH	Glc II
11	H	H	H	COOH	Glc II
12	Xyl	Ara(p)	H	COOH	GlcI
13	Xyl	Gal	H	COOH	H
14	H	Glc I	Ara(f)	$COOCH_3$	H



15

Ara(f or p): α-L-arabino(furanosyl or pyranosyl)
Xyl: β-D-xylopyranosyl
Glc: β-D-glucopyranosyl

Fig. 1. Chemical structures of compounds 1 to 15 isolated from *Panax stipuleanatus*.

muscular pain [7]. To-date, oleanane-type triterpenoid has been reported as the major component of *P. stipuleanatus* [8], and stipuleanosides R1 and R2 have been isolated from the methanol extract of this plant [7]. In a previous chemical investigation of this plant, we isolated eleven oleanane-type triterpenoids (1 to 11), and investigated their anti-cancer activity in HL-60 and HCT-116 human cancer cell lines [9]. In continuation of our research into the biological components of *P. stipuleanatus*, four oleanane-type triterpenes (12 to 15) were isolated successively from the methanol extract (Fig. 1). Their structures were assigned through spectroscopic data analysis using ^{1}H and ^{13}C nuclear magnetic resonance (NMR), correlation spectroscopy, heteronuclear multiple spectroscopy, heteronuclear multiple bond correlation, and mass spectroscopy, and were compared with data in the literature. This paper evaluated the effects of compounds 1 to 15 on TNF-α-induced NF-κB activation in HepG2 cells.

MATERIALS AND METHODS

General experimental procedures

Preparative HPLC was carried out using a Waters HPLC system (600 pump, 600 controller, and a 996 photodiode array detector; Waters, Milford, MA, USA).The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer (Bruker, Karlsruhe, Germany) and tetramethylsilane was used as an internal standard. The electrospray ionization mass spectra were recorded on an Agilent 1100 LC-MSD trap spectrometer (Agilent Technologies, Palo Alto, CA, USA). Column chromatography was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh; Merck, Darmstadt, Germany) and YMC RP-18 resins (30-50 μm; Fuji Silysia Chemical, Aichi, Japan). TLC was performed on DC-Alufolien 60 silica-gel F_{254} (Merck) or DC Platten RP_{18} F_{254s} (Merck) plates. Spots were visualized by spraying 10% H_2SO_4 aqueous and heating for 5 min.

Plant materials

The rhizomes of *P. stipuleanatus* were collected in Vietnam in 2007 and taxonomically identified by Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, Vietnam. The reference specimen (VHKC-0044) has been deposited at the Institute of Natural Products

Chemistry,Vietnam Academy of Science and Technology, Vietnam.

Extraction and isolation

The rhizomes of *P. stipuleanatus* (2 kg) were extracted with MeOH at room temperature for 1 d (10 L×3 times). Evaporation of the combined MeOH extract *in vacuo* gave a residue (200 g), which was then suspended in H_2O (3 L) and extracted with EtOAc (3 L×3 times) to give 4 g of an EtOAc-soluble fraction and 146 g of an H_2O-soluble fraction. The H_2O fraction was loaded onto a Diaion HP-20 column and eluted with MeOH–H_2O (0%, 25%, 50%, 75%, and 100% MeOH) to give five fractions (1A to 1E). Fractions 1D and 1E were combined due to their similar TLC pattern and then separated chromatographically through a silica gel column using a CH_2Cl_2–MeOH (10:1–0:1) gradient to give five fractions (3A to 3E). Fraction 3B was re-chromatographed on a silica gel column with $CHCl_3$–MeOH–H_2O (5:1:0.1) to give six sub-fractions (4A to 4F). Fraction 4D was separated by preparative HPLC eluted with an acetonitrile–water (40:60, 55:45) and gave compound 12 (5 mg), 13 (24 mg), 14 (11 mg) and 15 (112 mg).

Cell culture and chemicals

HepG2 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 10 μg/mL streptomycin at 37°C and 5% CO_2 incubator. Human TNF-α was purchased from ATgen (Seoul, Korea). Pyrrolidine dithiocarbamate, a NF-κB inhibitor, was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cytotoxicity assay

Cell Titer 96 AQUEOUS non-radioactive cell proliferation assay (MTS; Promega, Madison, WI, USA) was used to analyze the effect of compounds on cell viability. Cells were cultured overnight in 96-well plate1×10^4 cells/well). Cell viability was assessed after the addition of compounds at the 10 μM concentrations for 24 h. The number of viable cells was assessed by determination of the A_{490nm} of the dissolved formazan product after addition of MTS for 30 min as described by the manufacturer (Promega).

Reverse transcriptase polymerase chain reaction

Total RNA was extracted with Easy-blue reagent (iNtRON Biotechnology, Seoul, Korea). Approximately 2 μg of total RNA was reverse-transcribed using moloney murine leukemia virus reverse transcriptase and oligo-dT primers (Promega) for 1 h at 42°C. Polymerase chain reaction (PCR) of synthetic cDNA was performed using Taq polymerase pre-mixture (Takara, Shiga, Japan). PCR products were subjected to electrophoresis on 1% agarose gels, and stained with EtBr. PCR was conducted with the following primer pairs: iNOS forward 5'-TCATCCGCTATGCTGGCTAC-3', iNOS reverse 5'-CTCAGGGTCACGGCCATTG-3', COX-2 forward 5'-GCCCAGCACTTCACGCATCAG-3', COX-2 reverse 5'-GACCAGGCACCAGACCAAAGACC-3', GAPDH forward 5′-TGTTGCCATC-AATGACCCCTT-3′, and GAPDH reverse 5′-CTCCACGACGTACTCAGCG-3′. Quantification of PCR products was performed using the Quantity One Program (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All results are expressed as the mean±SEM. Data was analyzed by one-factor analysis of variance. Upon observation of a statistically significant effect, the Newman-Keuls test was performed to determine the difference between the groups. A p-value of <0.05 was considered to be significant.

RESULTS

Identification of isolated compounds from *Panax stipuleanatus*

We previously reported the isolation of eleven oleanane-type triterpenoids (1 to 11) from *P. stipuleanatus* and their anti-cancer activities in HL-60 and HCT-116 cancer cell lines. Four additional compounds were isolated from this plant and identified by comparing their physical and spectroscopic data with those reported in the literature. They were determined as hemslosides Ma2 (12) [10], elatoside A (13) [11], stipuleanoside R_1 methyl ester (14) [12], and oleanolic acid 28-O-β-D-glucopyranosyl ester (15) [10].

Nuclear factor-κB inhibitory effect of isolated compounds in HepG2 cells

To find new NF-κB inhibitors from natural products, we used the nuclear transcription NF-κB cell-reporter system. It is well-known that the pro-inflammatory cytokine, TNF-α activates the NF-κB pathway [13]. After treatment with TNF-α (10 ng/mL), HepG2 cells transfected with a NF-κB luciferase reporter plasmid exhibited an approximately four-fold increase in luciferase signal compared to untreated cells, which represents increased transcriptional activity. Treated HepG2 cells were treated

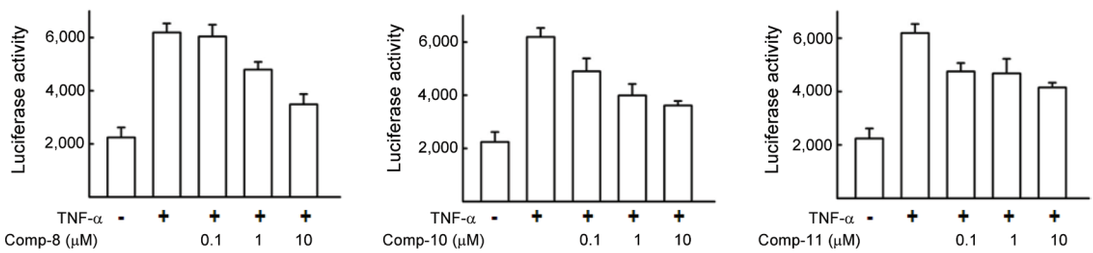


Fig. 2. Effect of compounds on tumor necrosis factor (TNF)-α-induced nuclear factor (NF)-κB luciferase reporter activity in HepG2 cell lines. HepG2 cells transiently transfected with pNF-κB-Luc were pretreated for 1 h with either vehicle dimethyl sulfoxide and compounds, prior to 1 h of treatment with TNF-α (10 ng/mL). Unstimulated HepG2 cells acted as a negative control. Cells were then harvested and luciferase activities were assessed. Results are expressed as relative luciferase activity. Comp, compound.

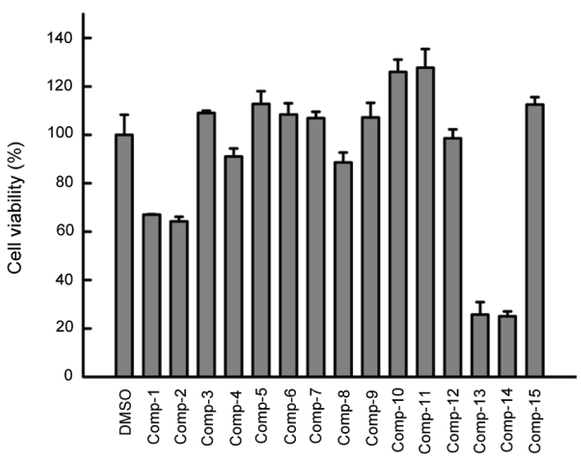


Fig. 3. Cytotoxic effect of compounds on HepG2 cells. HepG2 cells were seeded at a density of 1×10^4 per well and treated with compounds for 24 h. Cells viability was determined using the Cell Titer96 Aqueous One Solution Cell Proliferation Assay. The results are expressed in terms of percentage relative cell viability. All values are means±SEM (*n*=3) vs. control. DMSO, dimethyl sulfoxide; Comp, compound.

Table 1. Inhibitory activity (IC_{50}) for the tumor necrosis factor-α-induced nuclear factor-κB activation of compounds 1 to 15 in HepG2 cells

Compound	IC_{50} (μM)
1	ND
2	ND
3	ND
4	ND
5	ND
6	18.2
7	18.9
8	6.3
9	15.7
10	3.1
11	16.7
12	ND
13	ND
14	ND
15	ND
PDTC[1)]	1.5

ND, not detected; PDTC, pyrrolidine dithiocarbamate.
[1)]Positive control.

with fifteen oleanane-type triterpenes (1 to 15); compounds 6 to 11 showed dose-dependent inhibitory effects on TNF-α-induced NF-κB transcriptional activity and these compounds inhibited NF-κB, with IC_{50} values between 3.1-18.9 μM (Table 1). Among them, compounds, araloside A methyl ester (8), 3-O-β-D-xylopyranosyl (1→2)-β-D-glucopyranosyl-28-O-β-D-glucopyranosyl oleanolic acid (10), and chikusetsusaponin IVa (11) showed significant activity with IC_{50} values 6.3, 3.1, and 16.7 μM, respectively. The compounds 8, 10, and 11 reduced TNF-α-induced NF-κB activation by 36.5 %, 68.5 %, and 38.7 % at 10 μM in a dose-dependent manner (Fig. 2). The cell viability was measured using an MTS assay. The compounds 8, 10, and 11 were no cytotoxic effect at up to 10 μM, indicating that NF-κB inhibition was not due to cell toxicity (Fig. 3). Interestingly, cell viability by treatment of compounds 13 and 14 were markedly reduced, indicating that these compounds may provide a new cytotoxic effect in hepatocarcinoma as a potential anticancer agent (Fig. 3).

Effect of the compounds 8, 10, and 11 on inducible nitric oxide synthase and cyclooxygenase-2 gene expression through the nuclear factor-κB pathway

To confirm the inhibition of NF-κB activity produced by these compounds in the reporter assay, compounds 8, 10, and 11 were selected to examine the effects on the expression of NF-κB target genes by reverse transcription-

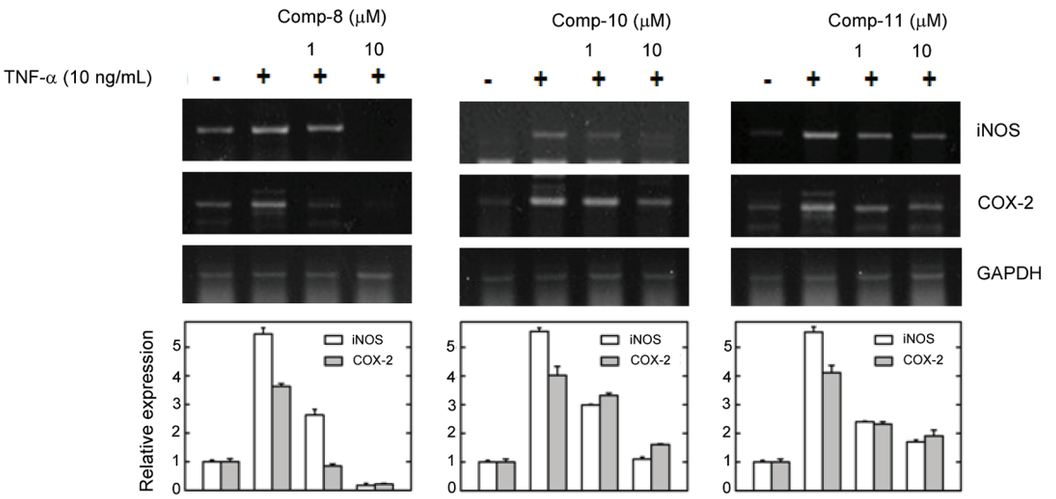


Fig. 4. Effect of compounds 8, 10, and 11 on cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNA expression in HepG2 cell lines. HepG2 cells were pretreated in the absence and presence of compounds 8, 10, and 11 for 1 h before tumor necrosis factor (TNF)-α treatment (10 ng/mL), then exposed to TNF-α for 6 h. Total mRNAs were prepared from the cell pellets using Easy-blue reagent. The relative levels of mRNAs were assessed by reverse transcriptase polymerase chain reaction. Expression levels are displayed as the ratio of iNOS and COX-2 signal strength to a reference gene (GAPDH), compensating for variations in the RNA concentrations. Comp, compound.

polymerase chain reaction. In HepG2 cells, treatment with TNFα (10 ng/mL) significantly upregulated mRNA expression levels of NF-κB target genes, COX-2 and iNOS, by approximately 5.2- and 3.5-fold, respectively. The relative levels of mRNAs were assessed by RT-PCR. Expression levels are displayed as the ratio of iNOS and COX-2 signal strength to a reference gene (GAPDH), compensating for variations in the RNA concentrations [14]. Compounds 8, 10, and 11 significantly inhibited the induction of iNOS mRNA by 30-, 5- and 3-fold, and COX-2 mRNA by 17-, 4-, and 2-fold at 10 μM in dose-dependent manner, indicating that these compounds attenuated synthesis of these transcripts at the transcriptional level (Fig. 4).

DISCUSSION

Oleanane-type triterpenoids are the main constituents of *P. stipuleanatus* and are believed to play a pharmacologically important role, including anti-cancer and anti-inflammatory activity. We isolated total fifteen oleanane-type triterpenoids from *P. stipuleanatus*. Recently, one oleanane saponin oleanolic acid 3-O-β-D-glucopyranosyl(1→3)-α-l-rhamnopyranosyl(1→2)-α-l-arabinopyranoside was reported to inhibit LPS-induced nitric oxide production by down-regulated NF-κB activity in Raw 264.7 cells [15] and elatoside F inhibit LPS-induced nitric oxide production as well as NF-κB activation [16]. Araloside A was reported to be an anti-ulcer compound based on bioassay-guided separation procedure [17].

COX-2 and iNOS are two inducible enzymes that are strongly expressed upon stimulation by diverse factors, such as oxidants, inflammatory factors, LPS, and oncogenes [18]. Recent studies have demonstrated that eukaryotic transcription of NF-κB is involved in the regulation of COX-2 and iNOS expression [19]. Accordingly, many substances developed to-date prevent inflammatory damage by either suppressing the activation of iNOS or COX-2 directly, or by inhibiting NF-κB signaling, which regulates iNOS and COX-2 at the transcriptional level [20].

This study demonstrates that compounds 8, 10, and 11 inhibit TNF-α-induced NF-κB promoter activity in a dose-dependent manner by interfering with gene transcription and the expression of two inducible enzymes, iNOS and COX-2, at the transcriptional level, as demonstrated by reduced mRNA levels in TNF-α-treated HepG2 cells. Our data suggest that compounds 8, 10, and 11 isolated from *P. stipuleanatus* have therapeutic potential as anti-inflammatory, anti-atherosclerotic, and anti-arthritic substances. However, elucidation of the detailed inhibitory mechanisms of the TNF-α-induced NF-κB pathway and subsequent decreases in iNOS and COX-2 gene expression with compounds 8, 10, and 11 requires further investigation.

In summary, this study is the first to demonstrate inhibition of NF-κB activity by compounds isolated from *P. stipuleanatus*. Additionally, all these data support the

pharmacological use of *P. stipuleanatus*, which has been traditionally employed as an herbal medicine for the treatment of inflammatory diseases.

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