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...
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 successfully 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$_1$, F$_{254a}$ (Merck) plates. Spots were visualized by spraying 10% H$_2$SO$_4$ 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$_2$O (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$_2$O-soluble fraction. The H$_2$O fraction was loaded onto a Diaion HP-20 column and eluted with MeOH–H$_2$O (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$_2$Cl$_2$–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$_2$O (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 plate (1×10$^4$ 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$_{490}$ 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 (IN-tRON 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'-TCATCGCTTATGCTGGCTAC-3', iNOS reverse 5'-CTCAGGTTCACGGGCCATTG-3', COX-2 forward 5'-GCCAGCACTTCACGCATCAG-3', COX-2 reverse 5'-GACCAGGCACCGACCAAAAGACC-3', GAPDH forward 5'-TGTTGCCATCAT-AATGACCCCTT-3', and GAPDH reverse 5'-CTCCACCGAGTACTCAGCGG-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 oleane-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 with...
Liang et al. Oleanane-triterpenoids from P. stipuleanatus inhibit NF-κB

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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-
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 onco-genes [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-arthritis 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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