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# Isolation and Tandem Mass Fragmentations of an Anti-inflammatory Compound from *Aralia elata*

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One-step isolation of a saponin from *Aralia elata* was undertaken using high-speed counter-current chromatography coupled with evaporative light scattering detection. A triterpenoid saponin, elatoside F, was purified with 96.8% purity using a two-phase-system comprising chloroform-methanol-water-isopropanol. The yield was 35.0 mg from 348.2 mg of the enriched saponin fraction. *In vitro* anti-inflammatory study demonstrated that elatoside F inhibited lipopolysaccharide-induced nitric oxide production, as well as nuclear factor κB activation, in a dose-dependent manner. Two types of mass ionization technique were compared on elatoside F to investigate characteristic fragmentation patterns. MALDI-TOF tandem mass spectrometric fragmentation patterns of sodiated ions provided structural information on glycosidic cleavages and on extensive cross-ring cleavages. Electrospray ionization multiple-stage tandem mass fragmentation of both sodiated and lithiated ions could provide information on glycosidic cleavages. All observed tandem mass fragmentation spectra provided valuable elatoside F structural information when unknown samples from crude extracts are under screening by mass spectrometry.

**Key words:** *Aralia elata, In vitro* anti-inflammatory activity, High-speed counter-current chromatography, Tandem mass spectrometry, Triterpenoid saponin, Elatoside F

## INTRODUCTION

High-speed counter-current chromatography (HSCCC), first introduced in 1980, is a type of liquid-liquid partition chromatography without solid supports. HSCCC provides outstanding benefits, such as high recovery of pure compounds, relatively low solvent consumption, ease of scale-up, and direct introduction of crude samples to the column. HSCCC eliminates irreversible adsorption of samples on solid supports which is a drawback in conventional column chromatography (Marston and Hostettmann, 1994; Ito, 2005). The introduction of evaporative light scattering detection (ELSD) to HSCCC, which measures the scattered light generated by non-volatile analyte particles produced by the droplet nebulization of the effluent, has been used successfully in analysis of many bioactive compounds from natural products. HSCCC-ELSD is a good technique to separate and purify saponins, because conventional column chromatography has many drawbacks related to saponin's inherent features, including high polarity, thermal liability, weak UV absorption, and low abundance in natural products. Therefore, HSCCC has been of interest in the separation of various saponins (Oleszek and Bialy, 2006; Ha et al., 2007; Ganzera et al., 2001; Fuzzati, 2004; Kim et al., 2007).

The stem and root bark of *Aralia elata* (Araliaceae) have been used as a folk medicine in east Asian countries for the treatment of diabetes, gastric ulcer, hepatitis, and inflammatory diseases such as rheumatoid arthritis (Ma et al., 2005). Many triterpenoid saponins

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in *A. elata* have been reported to have hypoglycemic activity and cytoprotective effects on carbon tetrachloride-induced hepatic injury (Kim et al., 2005). The 3-*O*-monodesmoside structure of oleanolic acid, where C-3 is linked to sugars, has been reported as essential to inhibitory effects on ethanol absorption in rats as well as producing potent hypoglycemic effects in an oral glucose tolerance test in rats (Yoshikawa et al., 1993, 1994, 1995, 1996). In addition, the 28-*O*-bisdesmoside structure, where both C-3 and C-28 are linked to sugars, possessing α-L-arabinofuranosyl moiety at the 4-position of the glucuronic acid has exhibited hypoglycemic activity (Yoshikawa et al., 1996). The bisdesmoside structures have also been found in triterpenoidal saponins having cytoprotective effects against carbon tetrachloride-induced hepatic injury (Saito et al., 1993).

Recently, electrospray ionization mass spectrometry (ESI-MS) has gained interest in the analysis of natural products. An advantage of ESI-MS when analyzing natural product constituents is its high sensitivity, which is much higher than nuclear magnetic resonance (NMR) instruments. In addition, tandem MS ($MS^n$) analyses of molecules cationized with metal ions can provide interesting structure information. For example, ESI-$MS^n$ has been applied to isomeric differentiation of saponins on $[M+Li]^+$ ions (Song et al., 2004). Furthermore, characteristic fragmentation behavior of some glucuronide-type triterpenoid saponins using ESI-MS has been reported (Li et al., 2005). It was found that the carboxyl and hydroxyl groups at the C-3' position of the glucuronyl residue were important sites for fragmentation behavior. In another study, ginsenosides containing different numbers of glycosyl groups were differentiated based on the formation of characteristic ginsenoside-acetate adduct anions and deprotonated ginsenosides generated by ESI of methanolic solutions (Wan et al., 2006). Liquid chromatography (LC)-ESI-$MS^n$ has been successfully employed for structural analysis of platycosides in *Platycodon grandiflorum* (Na et al., 2008). LC-ESI-MS also has a role in metabolism and pharmacokinetic studies of saponins, as *in vivo* studies of ginsenoside Rh2 and Rb1 on rats (Qian et al., 2005, 2006), and the determination of protodioscin in rat plasma (Wang et al., 2007) have been reported. However, little studies have reported fragmentation patterns of saponins by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry. Therefore, it is worth investigating the tandem mass fragmentations of the purified saponin, elatoside F, of which structure was fully characterized by spectroscopic techniques.

The present work describes a one-step isolation of a triterpenoid saponin, elatoside F, from the enriched saponin fraction of *A. elata* root bark by HSCCC-ELSD. The structure was characterized by $^{1}$H-NMR-, $^{13}$C-NMR-spectroscopy, MALDI-TOF-, and ESI mass spectrometry. To our knowledge, no previous studies have reported on the anti-inflammatory activities of elatoside F, which leads us to evaluate such activity *in vitro*. In addition, positive ion modes of MALDI-TOF $MS^2$ and ESI-$MS^n$ fragmentation patterns of elatoside F were demonstrated.

## MATERIALS AND METHODS

### Materials

Roots of *A. elata* were collected from plants cultivated at the Natural Products Research Institute, Seoul National University, Korea, in October 2000 and a voucher specimen (No. 001005) was maintained. The plant was authenticated by Prof. Ki Hwan Bae in College of Pharmacy, Chungnam National University. All organic solvents used for HSCCC were of analytical grade and were purchased from Fisher Scientific (Pittsburg, PA, USA). Distilled and deionized water (NANO pure Diamond, Barnstead, Dubuque, IO, USA) was used in all solutions and dilutions. Chromatographic grade acetonitrile was used for HPLC analysis. Reversed-phase $C_{18}$ resin (Diaion HP-20) was purchased from Merck (Darmstadt, Germany). LPS (*Escherichia coli* serotype 0127:B8), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and *N*-*p*-tosyl-*l*-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma Chemical Co. (St. Louis, MO. USA).

### Preparation of crude extract

Dried root bark (1.0 kg) was sliced and refluxed 3 times in a 95% methanol solution for 5 h in a water bath. The solution was filtered through gauze, evaporated to remove methanol and water *in vacuo*, and dried to yield a powdered extract (97.9 g). The extract was dissolved in a 10% methanol solution and successively partitioned with *n*-hexane, chloroform, ethyl-acetate, and butanol to produce hexane-soluble (5.8 g, 6.0%), chloroform-soluble (4.9 g, 5.0%), ethyl acetate-soluble (4.9 g, 5.0%) and butanol-soluble (60.7 g, 62.0%) fractions. The remaining layer was evaporated to yield a water fraction (21.5 g, 22.0%). The butanol-soluble fraction (6.3 g) was loaded onto a reversed-phase $C_{18}$ open column (Diaion HP-20; Merck) (60 cm ×6 cm, column volume 1700 mL). The column was sequentially eluted with water, then (30%, 60%, 80%, 95%) methanol. The 80% methanol fraction was evaporated, lypo-

philized, and stored at 4°C prior to HSCCC separation.

### HSCCC instrument, experimental condition, and procedures

Preparative HSCCC was performed using a TBE-300A HSCCC (Shanghai Tauto Biotech Co. Ltd, China) with a 1.6 mm inner diameter and a 260 mL capacity. At distances 3.8-5.7 cm from the coil to the holder shaft ($r$) and 5 cm from the column axis to the central axis of the centrifuge ($R$), the $\beta$ value ($\beta$ = r/R) varied from 0.50 (internal terminal) to 0.8 (external terminal). The revolution speed of the apparatus was regulated with an electronic controller (0-1000 rpm). Solvent was pumped into the column by HPLC pump (Hitachi L-6200), and the eluent was continuously monitored by connecting the outlet of the coiled column with an ELSD system (Sedex 55; Sedere, Vitry-sur-Seine, France) through a split valve. The multilayer coiled column was first filled with the upper stationary phase. The apparatus was then rotated at 800 rpm and the lower mobile phase was pumped into the head end of the column at a flow rate of 1.3 mL/min. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the outlet, the sample solution was injected through the sample port. The sample was prepared by dissolving 348.2 mg of enriched saponin fraction (the prepared 80% methanol fraction) in a 20 mL mixture of the lower and upper phases (1:1, v/v) of the HSCCC solvent system. The eluent was monitored at the coiled column outlet through a split valve. The ELSD system probe temperature was 70°C, system gain was 2.0, and the nebulizer gas nitrogen adjusted to 1.5 bar. Each peak was collected based on the elution profile and injected into RP-HPLC. After separation was complete, stationary phase retention was measured in the column contents forced out of the column with pressurized $N_2$.

### RP-HPLC analysis

Analytical RP-HPLC of the enriched saponin fraction and of each peak collected from HSCCC was performed on a Hitachi L-6200 instrument equipped with a Sedex 55 ELSD (Sedere) and a SIL-9A auto injector (Shimadzu, Kyoto, Japan), and a Zorbax SB-Aq $C_{18}$ column (150 mm×4.6 mm, 5 μm particle size) from Agilent Technologies (Palo Alto, CA, USA). The elution gradient solvent system was as follows: eluent A, water; eluent B, acetonitrile; gradient, 0-6 min (18-23% B), 6-48.5 min (23-40% B), 48.5-68.5 min (40-100% B), 68.5-77 min (100% B), and then equilibrated with 18% B for 10 min at a flow of 1 mL/min. The ELSD system probe temperature was 70°C, gain was 5, and the $N_2$ nebulizer was 2.5 bar.

### NMR and mass spectrometry

The $^{13}$C-NMR spectrum was recorded on a 300 MHz spectrometer (JNM-LA300; Jeol, Tokyo, Japan), and the $^{1}$H-NMR spectrum was recorded on a Bruker 500 MHz spectrometer (Avance 500, Germany). The compound was dissolved in pyridine-$d_5$ and TMS was used as an internal standard. The positive ion mode of MALDI-TOF mass spectroscopy was performed on a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) equipped with a Nd:YAG laser (wave length 355 nm) using DHB as a matrix. The samples were analyzed at 25 kV source acceleration with two-stage reflectron in the MS mode. In tandem MS/MS mode, collision energy, i.e. the potential difference between the source acceleration voltage (8 kV) and the floating collision cell (7 kV), was set to 1 kV. All positive ion ESI-MS$^n$ spectra were acquired using a Finnigan LCQ DECA XP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The source was operated at 45 kV of ion spray voltage, 13 V of capillary voltage, 250°C of capillary temperature, and with $N_2$ sheath gas set at 80 arbitrary units. A sample was introduced by direct injection through a 3.0 μL direct loop. The elution solvent was a mixture of water containing 0.1% acetic acid/methanol (1:10, v/v). For $[M+Li]^+$ adducts, the sample was dissolved in methanol containing 1 μM LiCl (Lin et al., 2007). In ESI-MS$^n$, $[M+Na]^+$ or $[M+Li]^+$ was selected as a precursor ion, and the cone voltage varied from 30-35 V to optimize intensity of the parent ion. Analyses were conducted at ambient temperature.

### Cell culture

RAW 264.7 murine macrophages were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and resuspended in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL of streptomycin. The cells were plated in a 75 cm$^2$ tissue culture flask, and stored in a 95% air, 5% $CO_2$ humidified atmosphere incubator (Vision Scientific, Bucheon, Korea) at 37°C.

### Cell viability assay

The effect of elatoside F on RAW 264.7 cell viability was investigated using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Briefly, RAW 264.7 cells were seeded onto a 96-well plate at a density of 1×10$^4$ cells per well and incubated at 37°C for 24 h. The cells were treated with different concentrations of elatoside F. After 24 h, 10 μL of CCK-8 solution was added to the wells, and incubation continued for another 3 h. The resulting culture color was assayed at 450 nm

using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA).

### Reporter gene assay

RAW 264.7 cells harboring the pNF-κB-SEAP-NPT reporter construct (from ATCC) were plated at a density of $1\times10^5$ cells per well in a 24-well cell culture plate with 500 μL of culture medium and incubated for 24 h. The cells were then pretreated with elatoside F for 2 h before stimulation with LPS (1 μg/mL) for 18 h. Aliquots of cell-free culture media were heated at 65°C for 6 min, and reacted with SEAP assay buffer [2 M diethanolamine, 1 mM $MgCl_2$, 500 μM 4-metylumbelliferyl phosphate (MUP)] in darkness at 37°C for 1 h. Fluorescence of the SEAP/MUP reaction product was measured in relative fluorescence units (RFU) using a 96-well plate fluorometer (Gemini XS; Molecular Devices) with excitation at 360 nm and emission at 449 nm. TPCK, an inhibitor of NF-κB, were used as a positive control (Moon et al., 2001; Ahn et al., 2003).

### Nitrite assay

RAW 264.7 cells were seeded onto a 24-well culture plate at a density of $1\times10^5$ cells per well with 500 μL of culture medium and incubated for 24 h. The medium was replaced with fresh medium containing different concentration of elatoside F. Then, LPS (1 μg/mL) was added to each well 2 h later. After 22 h, NO production was determined by measuring the accumulation of nitrite. Culture supernatants (100 μL) were mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphtylethylenediamine dihydrodhloride, and 5% phosphoric acid in distilled water) at room temperature for 15 min. Mixture absorbance was determined at 540 nm with a Gemini XS multiwell plate reader (Molecular Devices).

### Statistical analysis

Values are expressed as means ± S.D of the results of three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's t-test were applied to assess the statistical significance of differences among study groups. A value of $p < 0.05$ was chosen as the criterion of statistical significance.

## RESULTS AND DISCUSSION

### Selection of two-phase solvent system on HSCCC

HPLC chromatograms of *n*-butanol and the 80%

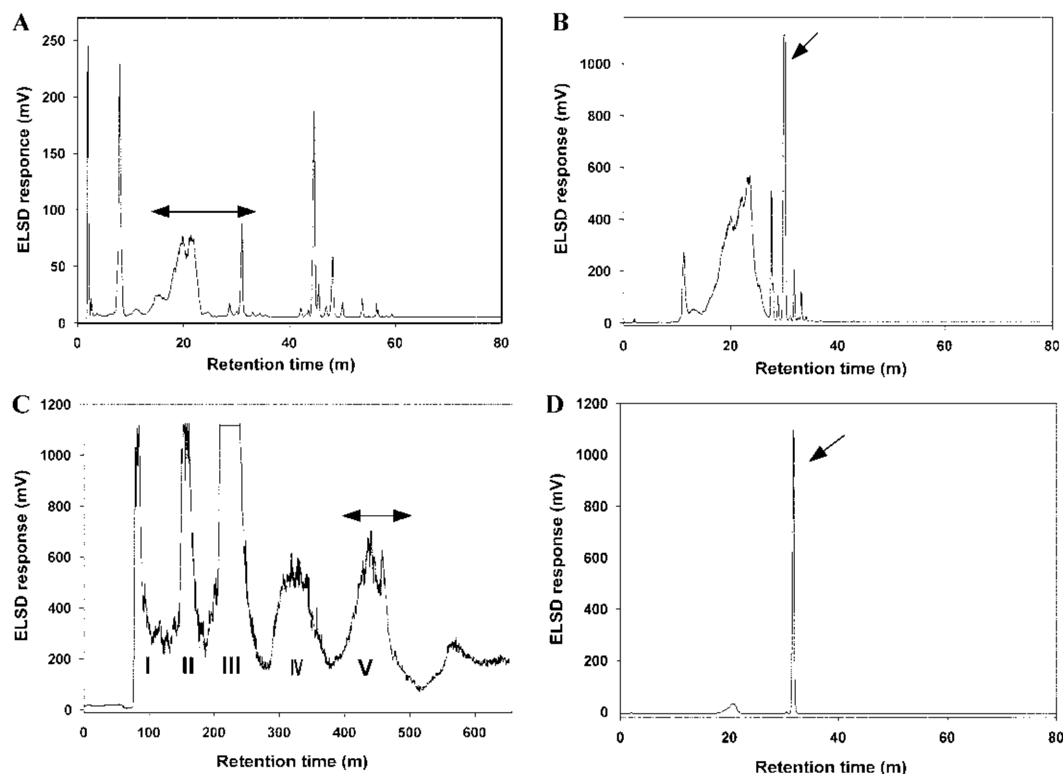


**Fig. 1.** HSCCC-ELSD and RP-HPLC-ELSD chromatograms. The arrow indicates the target compound (compound **1**) throughout the purification procedures. **A** RP-HPLC chromatogram of *n*-butanol extract, **B** RP-HPLC chromatogram of 80% methanol fraction isolated by Dianion HP-20 column, **C** HSCCC chromatogram of 80% methanol fraction. Fraction V was pooled and analyzed by RP-HPLC, and **D** RP-HPLC chromatograms of compound **1** purified by HSCCC.

methanol fraction (enriched saponin fraction) derived from the crude root extract were shown in Fig. 1(a) and (b), respectively. The enriched saponin fraction was subjected to HSCCC to purify the target compound (compound **1**, see Fig. 2 for the structure). Selection of a suitable solvent system for the target compound is important in HSCCC. The selected solvent system should provide an ideal partition coefficient range ($0.2 \le K \le 5$) to the analytes and a short settling time (< 20 sec) (Lu et al., 2007). In order to separate the target compound efficiently, stationary phase retention should be over 50%, and the ratio of separation factor ($\alpha = K_1/K_2$) should be > 1.5 (Ito 2005; Lu et al., 2007; Baldermann et al., 2007; Shi et al., 2007). The chloroform-methanol-water solvent system is widely used and is appropriate for separation of glycoside compounds. Table I summarizes two-phase solvent systems tested, as well as *K* values and settling times. The solvent system of chloroform-methanol-water-isopropanol (4:3:2:1) separated compound **1**; however, the purity was below 80% (data not shown).

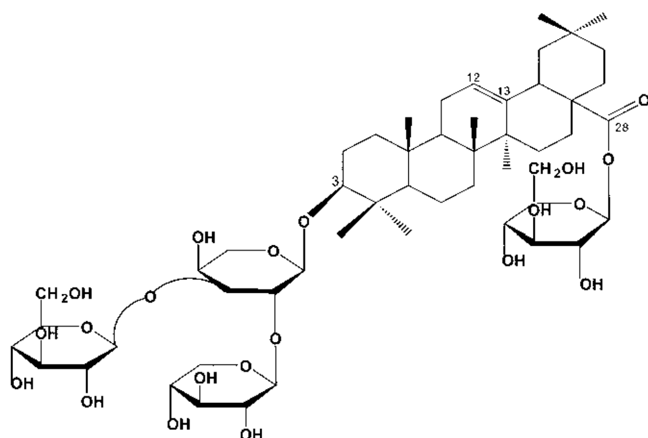


**Fig. 2.** Structure of compound **1**, elatoside F, 28-*O*-*β*-D-glucopyranosyl oleanolinic acid 3-*O*-[*β*-D-xylopyranosyl (1→2)] [*β*-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside.

When the ratio of water (4:3:3:1, v/v) was changed to more polar, the K value increased and the settling time was down to 20 sec. The resulting compound **1** production was 96.8% pure with a single peak in analytical RP-HPLC-ELSD (Fig. 1(d)). Retention of the stationary phase was about 64%. Alteration of other conditions, such as flow rate and revolution speed, were also investigated. The results showed that a flow rate 1.3 mL/min was optimal in both peak resolution and reservation of the stationary phase. Rotation speed > 800 rpm caused excessive sample band broadening, while speed < 800 rpm showed poor peak resolution. Therefore, a flow rate of 1.3 mL/min with a revolution speed of 800 rpm were used throughout the remainder of the study.

## Optimization of HPLC conditions

RP-HPLC for analysis (*n*-butanol and enriched saponin fractions) and each peak purified from HSCCC was optimized. When water-acetonitrile was used as a mobile phase in a gradient elution, major peaks showed baseline separation with a flow rate of 1 mL/min. ELSD was selected for detection system since the majority of saponins in *A. elata* are known to contain oleanolic acid saponins having very weak UV absorbance (Shi et al., 2007).

## Structure characterization

$^1$H-NMR and $^{13}$C-NMR spectra were acquired to elucidate the structure of compound **1** that was purified by HSCCC. All carbon chemical shifts in 75 MHz $^{13}$C-NMR were well correlated with the previous reported compound (Yoshikawa et al., 1996). In addition, chemical shifts in 500 MHz $^1$H-NMR were well correlated with reference data (Satoh et al., 1994): aglycone H-3 (1H, 3.19, dd-like), H-12 (1H, 5.39, brs), H-18 (1H, 3.19, dd-like), H-23 (3H, 1.25, s), H-24 (3H, 1.07, s), H-25 (3H, 0.82, s), H-26 (3H, 1.07, s), H-27 (3H, 1.28, s), H-29 (3H, 0.88, s), H-30 (3H, 0.85, s),

**Table I.** The partition coefficients (*K*) of compound **1** in different solvent systems

| solvent systems (v/v) | *K* value[a] | settling time (s) |
|---|---|---|
| metylene chloride/methanol/water/isopropanol (4:3:2:1) | 2.88 | 22 |
| metylene chloride/methanol/water/isopropanol (4:3:3:1) | 3.44 | 20 |
| metylene chloride/methnol/water/isopropanol (5:6:4) | 12.2 | 21 |
| chloroform/methanol/water (5:6:4) | 13.3 | 18 |
| chloroform/methanol/water/isopropanol (5:6:4:1) | 2.12 | 22 |
| chloroform/methanol/water/isopropanol (4:3:2:1) | 0.818 | 19 |
| chloroform/methanol/water/isopropanol (4:3:3:1) | 4.19 | 20 |
| chloroform/methanol/water/butanol (4:4:2:1) | 1.13 | 21 |
| chloroform/methanol/water/propanol / ethyl acetate (4:3:2:1:1) | 9.66 | 30 |

[a]*K* vaule was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

anomeric protons 3-$O$-Ara-1' (1H, 4.73, d, $J$ = 7.2 Hz), 2'-$O$-Xyl-1'' (1H, 5.40, d, $J$ = 7.9 Hz), 3'-$O$-Glc-1''' (1H, 5.32, d, $J$ = 7.8 Hz), 28-$O$-Glc-1'''' (1H, 6.33, d, $J$ = 8.1 Hz). Four anomeric proton signals indicated the presence of four sugar units, one bonded as a glycosyl ester (δ 6.33) and the others as glycosides (δ 4.73, 5.32, 5.40). From the coupling constant information of anomeric protons ($J$ = 7.2-8.1 Hz), the configuration of all anomeric protons was determined to be axial to the H-2 proton. MALDI-TOF and ESI-MS showed a molecular ion peak $[M+Na]^+$ at $m/z$ 1067.4 and 1067.9, respectively indicating a molecular mass of compound **1** of 1044. Based on the analytical data, the structure of compound **1** was confirmed as elatoside F (Fig. 2).

### Effect of elatoside F on cell viability

The cytotoxic effect of elatoside F *in vitro* was measured on mouse RAW 264.7 cells shown in Fig. 3(a). The results indicated that elatoside F at concentrations of 0.96-96 μM did not affect cell viability. Therefore, this concentration range was used throughout the procedures.

### Effect of elatoside F on NO production in LPS-induced RAW 264.7 cells

Cells were treated with LPS (1 μg/mL) for 18 h after treatment with or without elatoside F (0.96, 9.6, 48, and 96 μM) for 2 h. During 22 h of incubation, RAW 264.7 macrophages in the resting state produced 5.98 ± 0.12 μM nitrite. When LPS (1 μg/mL) was added,

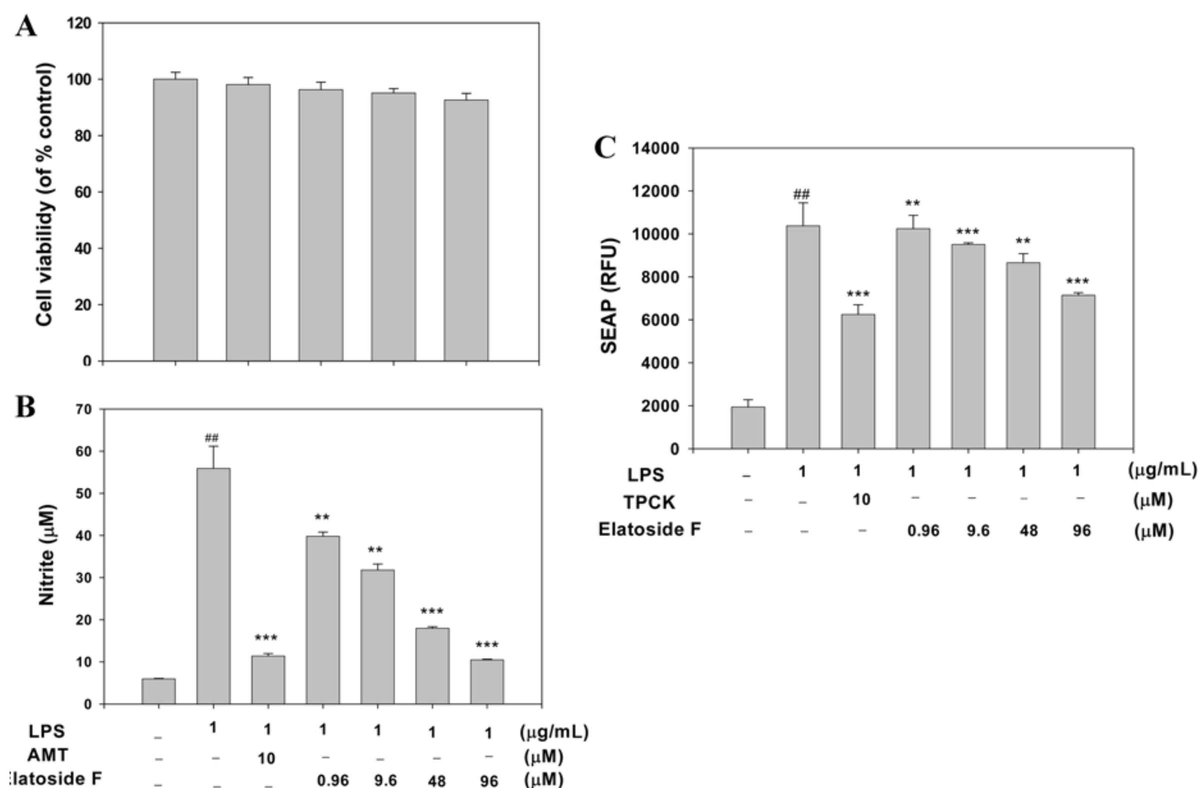


**Fig. 3.** Measurement of anti-inflammatory activity. **A**: Effect of elatoside F on cell viability in RAW 264.7 macrophages. RAW 264.7 cells were cultured with the indicated concentrations of elatoside F at 37°C in a 96-well plate for 24 h. Cell viability was evaluated as described in experimental section, and is expressed as a percentage of the control without the addition of elatoside F. The values are expressed as the means ± S.D. of three individual experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences from the control. **B**: Effect of elatoside F on LPS-induced nitrite production in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with the indicated concentration of elatoside F for 2 hr and treated with LPS (1 μg/mL). After 22 hr incubation, the amount of nitrite in the culture supernatants was measured by the Griess reaction assay, as described in experimental section. The values are expressed as the means ± S.D. of three individual experiments. $^{\#\#}p < 0.01$ indicates significant differences from the unstimulated control group. **$p < 0.01$ and ***$p < 0.001$ indicate significant differences from the LPS-treated group. **C**: Effects of elatoside F on LPS-mediated NF-κB transcriptional activity. RAW 264.7 cells harboring NF-κB-SEAP-NPT reporter construct were pretreated with the indicated concentrations of elatoside F for 2 hr and treated with LPS (1 μg/mL). After 18 hr incubation the SEAP activity was measured in RFU, using a microplate flourometer. The values are expressed as the means ± S.D. of three individual experiments. $^{\#\#\#}p < 0.001$ indicates significant differences from the unstimulated control group. **$p < 0.01$ and ***$p < 0.001$ indicate significant differences from the LPS-treated group.

nitric oxide (NO) production was increased to 55.91 ± 5.26 μM. The accumulation of nitrite, a stable metabolite of NO, was determined by Griess reaction in culture medium. As shown in Fig. 3(b), the addition of elatoside F inhibited LPS-induced NO production in a dose-dependent manner resulting in 28.8 ± 1.8%, 43.2 ± 2.6%, 67.9 ± 0.66%, and 81.23 ± 0.29% inhibition at 0.96, 9.6, 48, and 96 μM of elatoside F, respectively.

### Effect of elatoside F on inhibition of LPS-induced NF-κB activation in transfectant RAW 264.7 cells

Mouse RAW 264.7 macrophages harboring a pNF-κB-SEAP-NPT construct, encoding four copies of the κB sequence and with a SEAP gene as a reporter, were pretreated with elatoside F (Moon et al., 2001). As shown in Fig. 3(c), such pretreatment inhibited LPS-stimulated NF-κB activation in a dose-dependent manner resulting in 8.96 ± 4.89%, 20.38 ± 19.71%, and 33.68 ± 2.55% inhibition at 9.6 μM, 48 μM, and 96 μM elatoside F concentrations, respectively. Tosyl phenylalanyl chloromethyl ketone (TPCK) as a positive control also showed inhibitory activity.

### Tandem mass fragmentations

MALDI-TOF $MS^2$ and ESI-$MS^n$ were performed to compare fragmentation patterns of two ionization techniques. In general, two types of fragmentation are common on sugar residues: glycosidic cleavages and cross-ring cleavages. Glycosidic cleavage of a bond linking two sugar rings provides information on sequence and branching while cross-ring cleavage of two bonds on one sugar residue provides information on linkages. Our fragmentation mass spectra are annotated using the Domon and Costello nomenclature system (Domon and Costello, 1988). Ions retaining the charge on the reducing terminus are named X (cross-ring), Y, and Z, whereas ions retaining the charge on the non-reducing terminus are named A (cross-ring), B, and C.

MALDI-TOF MS showed $[M+Na]^+$ at *m/z* 1067.4 along with $[M+K]^+$ at *m/z* 1083.4 on the positive ion mode (data not shown). As shown in Fig. 4(a), elatoside F was analyzed by MALDI-TOF $MS^2$ on a sodiated parent ion at *m/z* 1067.4. 2,5-Dihydroxy benzoic acid (DHB) was used as a matrix which is reported to be suitable for the analysis of neutral sugars. DHB gave abundant $[M+Na]^+$ signals suggesting $Na^+$ cationization of elatoside F under MALDI-TOF conditions. Most of the peaks obtained were from the fragmentations of sugar residues. As shown in Fig. 4(b), glycosidic bond cleavage B-, C-, and Y- were major ions providing sequence information of glycans. Cross-ring cleavages

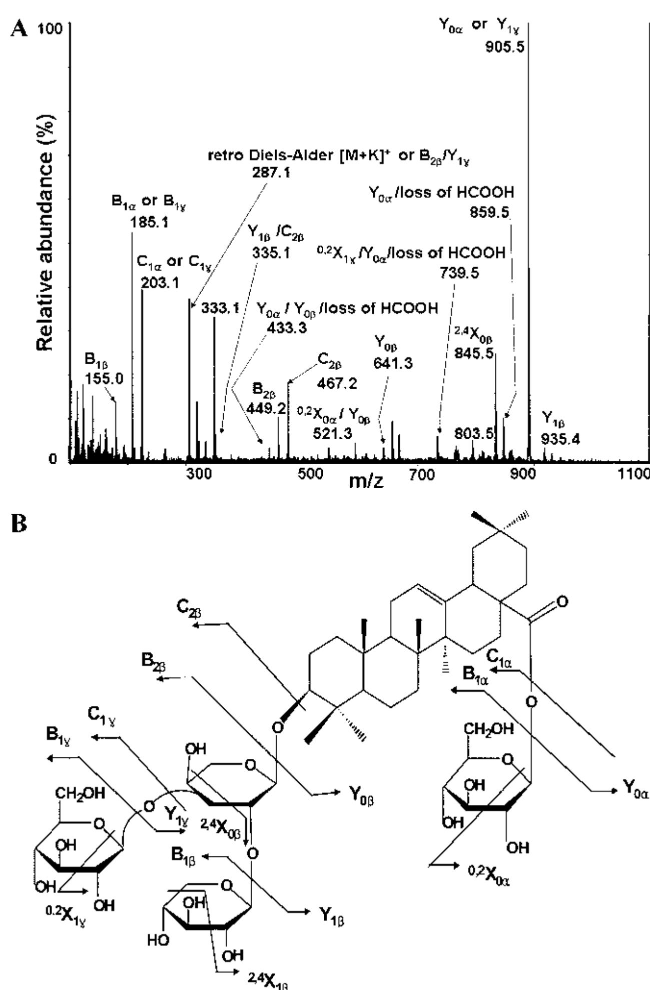


**Fig. 4.** The positive ion mode of MALDI-TOF/TOF spectrum (**A**), and the fragmentation patterns of elatoside F (**B**).

($^{0,2}$X- and $^{2,4}$X-) were also observed. As shown in Fig. 4(a) and (b), a loss of hexose ($Y_{0\alpha}$ or $Y_{1\gamma}$) was a major fragmentation that generated a peak at *m/z* 905.5. The sequential loss of 60 Da (*m/z* 845.5) suggests a pentose cross-ring cleavage ($^{2,4}X_{0\beta}$). This cross-ring cleavage suggests that a hexose was connected to a pentose *via* a 1→3 linkage at C-3. The sequential loss of 46 Da from *m/z* 905.5 was observed at *m/z* 859.5, which indicates the loss of HCOOH (46 Da) of C-28 ester. The loss of 46 Da from the precursor ion (*m/z* 905.5) could be the result of a McLafferty + 1 rearrangement (Zheng et al., 2007), in which the γ-H atom was transferred to C-28 followed by a cleavage between α-C and C-28 bond, thereby generating HCOOH. The peak at *m/z* 739.5 is a cross-ring cleavage of a hexose ($^{0,2}X_{1\gamma}$) at C-3 together with a $Y_{0\alpha}$/loss of HCOOH. This observation confirmed that a hexose is present at non-reducing end of C-3. Peaks at *m/z* 935.4, 803.5, and 641.3 were the sequential loss of two pentoses and a hexose from the parent ion suggesting the existence of

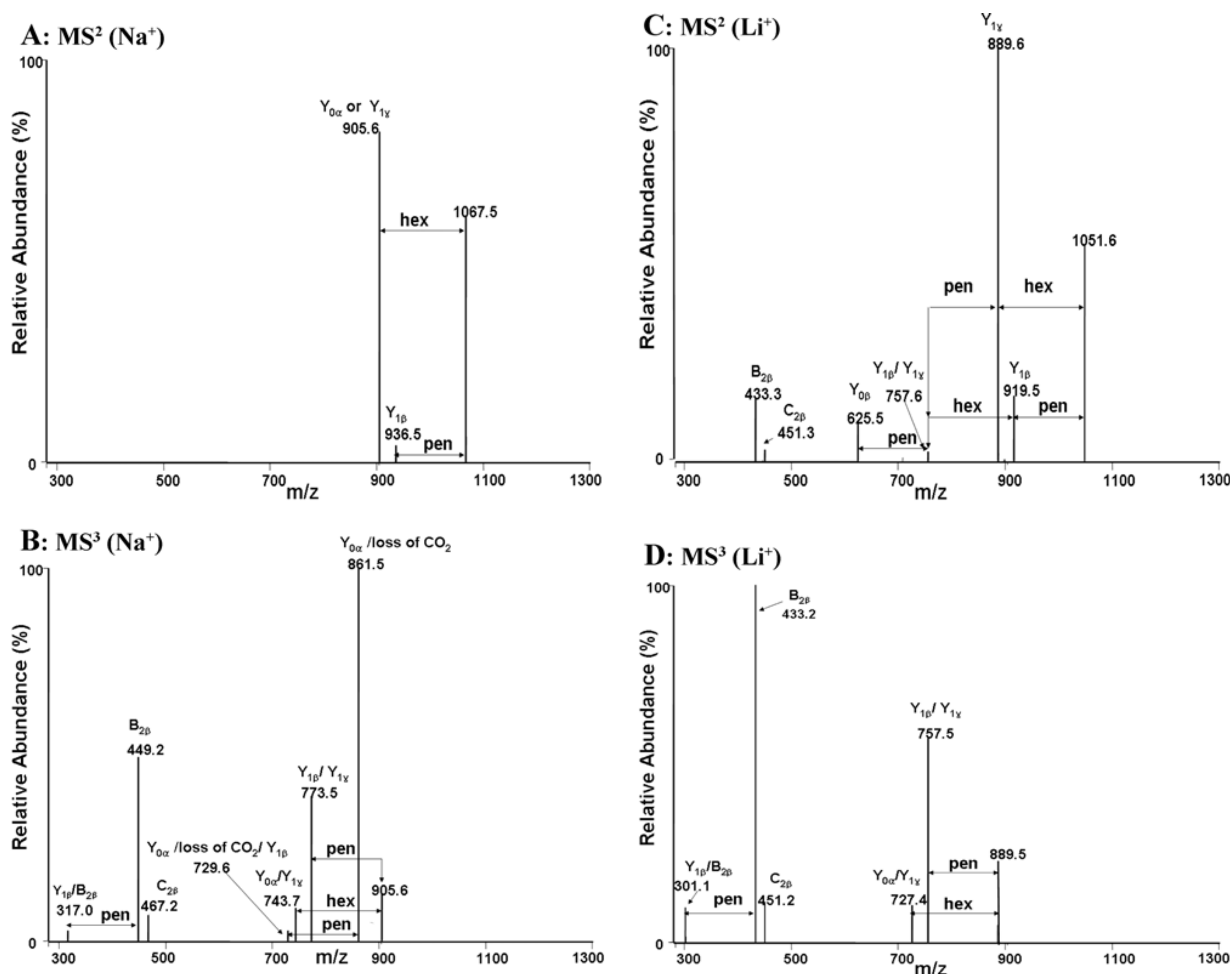


**Fig. 5.** The positive ion mode of ESI-MS$^2$ and MS$^3$ spectra of elatoside F on both Na$^+$ (**A** and **B**) and Li$^+$ (**C** and **D**) adducts. **A**: MS$^2$ spectrum of ion at *m/z* 1067.5, **B**: MS$^3$ spectrum of ion at *m/z* 905.6, **C**: MS$^2$ spectrum of ion at *m/z* 1051.6, and **D**: MS$^3$ spectrum of ion at *m/z* 889.5.

a trisaccharide residue attached to aglycone. Interestingly, an 'internal pentose loss' phenomenon [M+Na-pentose-pentose]$^+$ (*m/z* 803.5) of precursor ion *m/z* 1067.4 was observed, which is not consistent with previous reports. In general, 'internal monosaccharide residue loss' on proton adducts, not on sodium adducts, have been reported on fucosylated *N*-glycans, sulfatides, glycosphingolipids, and *O*-diglycosyl flavonoids (Wuhrer et al., 2006; Hsu and Turk, 2004; Tadano-Aritomi et al., 2003; Ma et al., 2000). In *O*-diglycosyl flavonoids, 'internal glucose loss' was observed to be dependent on aglycone types (Ma et al., 2000). In our results, both a hexose and HCOOH (46 Da) were lost from *m/z* 641.3 generating a peak at *m/z* 433.3 and confirming a glucose with an ester bond at C-28. The cross-ring cleavage ($^{0,2}X_{0\alpha}$) of a glucose attached to C-28 was observed at *m/z* 521.3. The peak at *m/z* 287.1 has two possibilities: a sodium adduct of glycosidic cleavages ($B_{2\beta}/Y_{1\gamma}$) or a potassium adduct of *retro* Diels-Alder fragmentation of the D/E ring. *Retro* Diels-Alder fragmentation confirmed that the aglycone had a double bond (C-12 and C-13) on the C ring. Further fragmentation studies on *m/z* 287.1 are necessary to verify *retro* Diels-Alder fragmentation.

As shown in Fig. 5(a-d), ESI-MS$^n$ of sodiated and lithiated ions showed mainly glycosidic cleavages providing sequence information on the sugars. As shown in Fig. 5(a), ESI-MS$^2$ of [M+Na]$^+$ molecular ion peak (*m/z* 1067.5) showed Y-cleavages at *m/z* 905.6 ($Y_{0\alpha}$ or $Y_{1\gamma}$) and 936.5 ($Y_{1\beta}$). As shown in Fig. 5(b), ESI-MS$^3$ of *m/z* 905.6 fragmented into three pathways generating ions at *m/z* 861.5 ($Y_{0\alpha}$/loss of $CO_2$), 773.5 ($Y_{1\beta}/Y_{1\gamma}$), and 743.7 ($Y_{0\alpha}/Y_{1\gamma}$). It is interesting to note that the cleavage of the glycosidic bond ($Y_{0\alpha}$) with $CO_2$

loss preferentially occurred in the $MS^3$ spectra. A pentose loss from $m/z$ 861.5 confirmed a pentose attached to C-3 at non-reducing end. The ion at $m/z$ 449.2 ($B_{2\beta}$) corresponded to [hexose+pentose+pentose+ $Na]^+$ suggesting three sugars attached to the aglycone. The ion at $m/z$ 317.0 corresponding to [hexose+ pentose+$Na]^+$ was from a pentose loss of $m/z$ 449.2 ($B_{2\beta}$).

ESI-$MS^2$ and -$MS^3$ spectra of lithiated ions are shown in Fig. 5(c-d). The molecular ion at $m/z$ 1051.6 indicated that elatoside F was successfully cationized with $Li^+$. The $MS^2$ fragmentation ions of elatoside F were mainly composed of ions from glycosidic cleavages at C-3. As shown in Fig. 5(c), the two fragmentation pathways of $m/z$ 1051.6 ($m/z$ 1051.6 $\rightarrow$ 889.6 $\rightarrow$ 757.6 and $m/z$ 1051.6 $\rightarrow$ 919.5 $\rightarrow$ 757.6) demonstrated that three sugars were attached to aglycone in a branched structure. However, the loss of $CO_2$ was not observed in the $[M+Li]^+$ fragmentation ions under the current ESI-$MS^n$ conditions.

In conclusion, a one-step isolation method for HSCCC of elatoside F, a triterpenoid saponin from *A. elata* root bark, was developed. The developed HSCCC method is deemed useful for the purification of a triterpenoid saponin, elatoside F. In addition, this method provided simple purification of mg quantities of this bioactive compound. Elatoside F showed *in vitro* anti-inflammatory activity on LPS-induced responses in mouse RAW 264.7 cells by inhibition of both NO production and NF-κB activity in a dose-dependent manner. DHB matrix was successfully introduced for MALDI-TOF of elatoside F providing abundant $[M+Na]^+$ signals, which are useful for structural determination of sugars. Elatoside F was ionized through metal cationization ($Li^+$ and $Na^+$) under ESI-MS conditions, and the $MS^n$ spectra showed peaks corresponding to the cleavage of glycosidic bonds.

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