

## NF- $\kappa$ B Inhibitory Activities of Phenolic and Lignan Components from the Stems of *Acanthopanax divaricatus* var. *albeofructus*

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**Abstract** – *Acanthopanax divaricatus* var. *albeofructus* (ADA) is commonly ingested as a traditional medicine or as a component of a health drink in Korea. In this phytochemical study, nine phenolics (**1** - **9**) and three lignans (**10** - **12**) were isolated from the MeOH extract of the stems of ADA. Chemical structures were elucidated by comparing spectroscopic data with reported values. Nuclear factor kappa B (NF- $\kappa$ B) inhibitory activity of the isolated compounds was evaluated using an NF- $\kappa$ B luciferase assay in HepG2 cells. Among them, compounds **1**, **3** - **8**, and **11** showed significant inhibitory effects on TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activity in a dose-dependent manner, with IC<sub>50</sub> values ranging from 13.25 to 37.36  $\mu$ M. Further studies on potential anti-inflammatory effects and the benefits of phenolic and lignan components from ADA are warranted.

**Keywords** – *Acanthopanax divaricatus* var. *albeofructus* (ADA), Araliaceae, NF-kappa B, phenolic, lignin

### Introduction

*Acanthopanax divaricatus* var. *albeofructus* (ADA) is a deciduous shrub of the Araliaceae family that has been used in traditional oriental medicine due to its tonic and prophylactic functions. In Korea, the leaves, stems and roots of this species have been ingested as a drink and drug. ADA has demonstrated both anticancer and antivirus effects, as well as other functions. In addition, during the course of previous studies, the water extract of ADA fruits has been applied to human peripheral blood mononuclear cells (hPBMC). Moreover, both water and ethanol extracts of ADA leaves have demonstrated antioxidative activity in human dermal fibroblasts.<sup>1-4</sup> As well as Lyu *et al.* further investigated the antioxidant activity of various components of ADA fruits.<sup>5</sup> Phytochemical studies on ADA have determined that its main active constituents are triterpenoids, triterpenoidal saponins, lignans and cyanidins. However, no active NF- $\kappa$ B inhibitory components have not been reported to date. With regards to the *Acanthopanax* genus, recent reports have shown that impressic acid from the leaves and some phenolics from the stems of *A. koreanum* display significant NF- $\kappa$ B

inhibitory activity.<sup>6,7</sup> Therefore, this study is the first to report NF- $\kappa$ B transcriptional inhibitory activities of the phenolic and lignan components of ADA, an important finding for both medicine and food science research.

Nuclear factor kappa B is a protein complex that controls DNA transcription. It is found in nearly all animal cell types and is involved in cellular responses to stimuli, such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens.<sup>8-11</sup> Over the past few years, the transcription of NF- $\kappa$ B, and its regulatory proteins, has emerged as a signaling system of preminent importance in human physiology and in an increasing number of pathologies. NF- $\kappa$ B plays a role in many compartments of the immune system during immune cells differentiation, immune activation, and in the development of lymphoid organs.<sup>12</sup> Furthermore, activation of NF- $\kappa$ B causes transcription at the  $\kappa$ B site, which is involved in several diseases, including inflammatory disorders and cancer. Hence, NF- $\kappa$ B signaling inhibition is an important therapeutic target for the treatment of such diseases.<sup>13</sup> In the present study, twelve compounds were isolated from ADA. The effects of compounds **1** - **12** on TNF- $\alpha$  induced NF- $\kappa$ B transcriptional activity were evaluated in human hepatocarcinoma (HepG2) cells were evaluated using an NF- $\kappa$ B luciferase assay.

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## Experimental

**General experimental procedures** – The NMR spectra were recorded using a JEOL ECA 600 spectrometer ( $^1\text{H}$ , 600 MHz;  $^{13}\text{C}$ , 150 MHz), with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and  $^1\text{H}$ - $^1\text{H}$  correlation spectroscopy (COSY) spectra were recorded using a pulsed field gradient. EI-MS spectra were obtained using a Hewlett Packard HP 5985B spectrometer, ESI-MS using an Agilent 1200 LC-MSD Trap spectrometer. Melting points were determined using an Electro thermal IA-9200 system. Column chromatography was performed using a silica gel (Kieselgel 60, 70 - 230, and 230 - 400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F<sub>254</sub> and RP-18 F<sub>254S</sub> plates (both 0.25 mm, Merck, Darmstadt, Germany), the spots were detected under UV light and using 10% H<sub>2</sub>SO<sub>4</sub>.

**Plant material** – The stems of *Acanthopanax divaricatus* var. *albeofructus* (ADA) were collected from the herbal garden, College of Pharmacy, Chungnam National University in Daejeon, Korea in September 2012 and were identified by Prof. Young Ho Kim, Chungnam National University. A voucher specimen (CNU12104) was deposited at herbarium, College of Pharmacy, Chungnam National University, Korea.

**Extraction and isolation** – Dried stems of ADA (6.0 kg) were extracted three times with MeOH under reflux. The MeOH extract (300.0 g) was suspended in H<sub>2</sub>O (1.6 L) and partitioned with *n*-hexane and EtOAc (each 1.6 L × 3). The EtOAc extract (40.0 g) was subjected to silica gel column chromatography with a gradient of CHCl<sub>3</sub>-acetone-MeOH (1 : 0 : 0 to 1 : 1 : 0.2), to yield seven fractions (Fr. A1-A7). Sub-fraction A2 was further purified by reverse-phase (RP) column chromatography with a MeOH-H<sub>2</sub>O (1 : 2.5 to 1 : 1) elution solvent to yield compounds **1** (15.0 mg) and **8** (5.0 mg). Fr. A3 was further chromatographed in a silica gel column, using a gradient of *n*-hexane-EtOAc-MeOH (6 : 1 : 0.1 to 1 : 1 : 0.2), to yield eight sub-fractions (Fr. A3.1-A3.8). Further purification of Fr. A3.3 was conducted by chromatography in an RP column with an eluent gradient of MeOH-H<sub>2</sub>O (0.8 : 1 to 2.2 : 1) to yield compounds **2** (6.0 mg), **3** (40.0 mg), and **5** (49.0 mg). Fr. A3.5 was further chromatographed in an RP column with MeOH-H<sub>2</sub>O (0.9 : 1) to yield compound **6** (2.0 mg). Fr. A5 was chromatographed in a silica gel column with an eluent gradient of *n*-hexane-EtOAc-MeOH (2.3 : 1 : 0.1 to 1 : 1 :

0.1) to yield four fractions (Fr. 5.1-5.4). Fr. A5.2 was column chromatographed in an RP column, eluting with MeOH-H<sub>2</sub>O (1 : 1) to yield compound **4** (20.0 mg). The H<sub>2</sub>O fraction (100.0 g) was chromatographed on a column of highly porous polymer (Diaion HP-20), and eluted with H<sub>2</sub>O and MeOH (1 : 0-3 : 1-1 : 1-1 : 3-0 : 1) to yield five fractions (Fr. B1-B5). Fr. B2 was subjected to silica gel column chromatography with a gradient of CHCl<sub>3</sub>-MeOH (10 : 1 to 0 : 1) to yield six fractions (Fr. B2.1-B2.6). Fr. B2.5 was separated using an RP column with a MeOH-H<sub>2</sub>O (1 : 1) elution solvent to yield compound **9** (5.0 mg). Fr. B3 was separated by silica gel column chromatography with a gradient of CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (6.5 : 1 : 0.1 to 0 : 1 : 0) to yield four sub-fractions (Fr. B3.1-B3.4). Fraction B3.2 was further chromatographed by silica gel chromatography column with EtOAc-MeOH (10 : 1 to 1 : 1) to yield compounds **7** (7.0 mg), **10** (52.0 mg), and **11** (10.0 mg). Fraction B3.4 was further chromatographed on an RP chromatography column with MeOH-H<sub>2</sub>O (1.5 : 1) to yield compound **12** (10.0 mg).

**Cell culture and reagents** – Human hepatocarcinoma 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% CO<sub>2</sub>. Human TNF-α was purchased from ATgen (Seoul, Korea).

**Cytotoxicity Assay** – A 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. HepG2 cells were cultured overnight in a 96-well plate (~1 × 10<sup>4</sup> cells/well). Cell toxicity was assessed after the addition of compounds in a 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. The absorbance at 450 nm was measured to determine the viable cell numbers.

**NF-κB-luciferase Assay** – The luciferase vector was first transfected into human hepatocarcinoma 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 Mg<sup>2+</sup> 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 × 10<sup>5</sup> cells per well in 12-well plates and grown. After 24 h, cells were transfected with inducible NF-κB 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 µg/mL streptomycin) and cells were pretreated for 1 h with either vehicle (DMSO) and compounds, followed by 1 h of treatment with 10 ng/mL TNF- $\alpha$  for 20 h. Unstimulated cells were used as a negative control (-), apigenin 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.

**Statistical Analysis** – All measurements were performed independently at least triplicate. Data were expressed as the mean  $\pm$  standard deviation (SD). Statistical significance is determined by one-way analysis of variance followed by Dunnett's multiple comparison test,  $P < 0.05$ .

## Results and Discussion

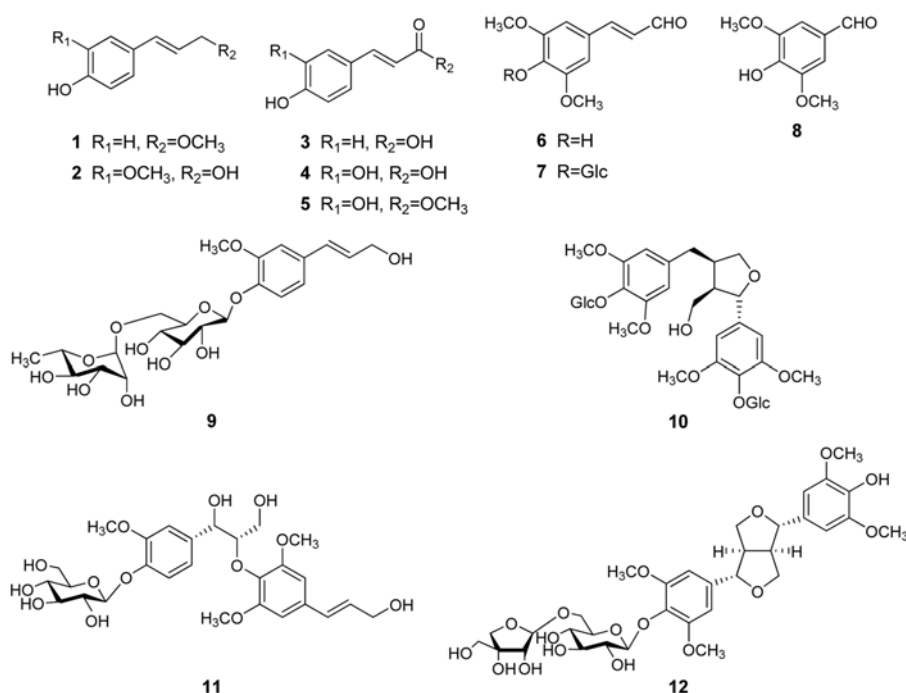
In this study, nine phenolics (**1 - 9**) and three lignans (**10 - 12**) were isolated from the MeOH extract of the stems of ADA. The structures of compounds **1 - 12** were identified based on spectroscopic data, chemical evidence and comparisons with previously reports (Fig. 1). Their structures were elucidated as 4-[(1*E*)-3-methoxy-1-propenyl] phenol (**1**), coniferyl alcohol (**2**), *trans*-4-hydroxycinnamic acids (**3**), *trans*-caffeic acid (**4**), methyl caffeate (**5**), sinapaldehyde (**6**), sinapaldehyde glucoside (**7**), syringaldehyde (**8**), 3-methoxy-1-(3-hydroxy-propen-1-yl)phenyl-

4-*O*- $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  6)- $\beta$ -D-glucopyranoside (**9**), (+)-salvadoraside (**10**), citrusin B (**11**), obtusifoside A (**12**).<sup>6,14-24</sup> Among them, compounds **2 - 10** were the first time reported from ADA, and compounds **1** and **11 - 12** were new found in the Araliaceae family.

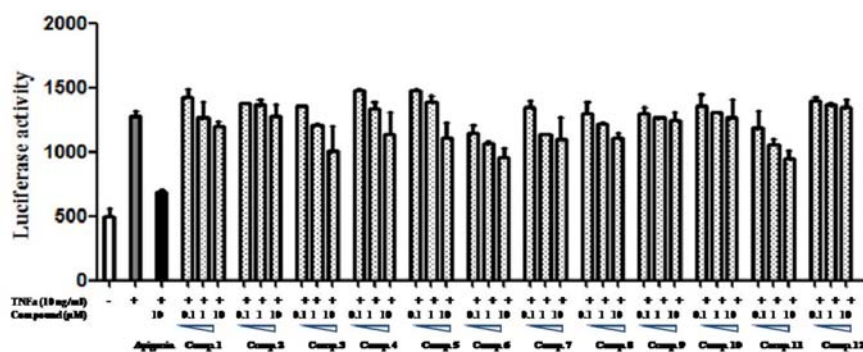
Compounds **1 - 12** were evaluated for cytotoxicity according to the manufacturer's instructions using a Cell-Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) assay, as described in "Materials and Methods". The results indicate that compounds **1 - 12** display no significant cytotoxicity toward HepG2 cells at the tested concentrations (0.1, 1, and 10  $\mu$ M; data not shown).

HepG2 cells were treated with 10 ng/mL TNF- $\alpha$ , which resulted in increased transcriptional activity compared to untreated cells. Transfected HepG2 cells were pretreated with 0.1, 1, and 10  $\mu$ M of the compounds, followed by stimulation with TNF- $\alpha$ . Apigenin was used as a positive control (Fig. 2). The anti-inflammatory activity of compounds **1 - 12** was evaluated by the inhibition of a TNF- $\alpha$ -induced NF- $\kappa$ B luciferase reporter in HepG2 cells. Compounds **1, 3 - 8**, and **11** significantly inhibited TNF- $\alpha$  induced NF- $\kappa$ B transcriptional activity, with IC<sub>50</sub> values of 37.36, 17.71, 20.57, 18.29, 13.25, 23.61, 23.33, and 14.41  $\mu$ M (Table 1), respectively. Whereas compounds **2, 9 - 10** and **12** exhibited weak inhibitory activity, with IC<sub>50</sub> values ranging from 62.22 to 93.67  $\mu$ M.

With regards to the structure-activity relationships of



**Fig. 1.** Structures of isolated compounds **1 - 12** from the stems of *A. divaricatus* var. *albeofructus* (ADA).



The values are means  $\pm$  SDs (n = 3).

Apigenin, positive control (10  $\mu$ M).

Statistical significance is determined by one-way analysis of variance followed by

Dunnnett's multiple comparison test,  $P < 0.05$  versus control.

**Fig. 2** Effects of compounds **1 - 12** on the TNF- $\alpha$  induced NF- $\kappa$ B luciferase reporter activity in HepG2 cells.

**Table 1.** Inhibitory effects of compounds **1 - 12** on the TNF- $\alpha$  induced NF- $\kappa$ B transcriptional activity

Comp.	IC <sub>50</sub> <sup>a</sup> ( $\mu$ M)	Comp.	IC <sub>50</sub> <sup>a</sup> ( $\mu$ M)
<b>1</b>	37.36 $\pm$ 4.03	<b>7</b>	23.61 $\pm$ 5.62
<b>2</b>	93.67 $\pm$ 1.83	<b>8</b>	23.33 $\pm$ 2.02
<b>3</b>	17.71 $\pm$ 5.15	<b>9</b>	62.22 $\pm$ 5.00
<b>4</b>	20.57 $\pm$ 2.79	<b>10</b>	71.12 $\pm$ 3.71
<b>5</b>	18.29 $\pm$ 3.15	<b>11</b>	14.41 $\pm$ 2.03
<b>6</b>	13.25 $\pm$ 3.00	<b>12</b>	64.91 $\pm$ 3.14
Apigenin <sup>b</sup>	4.32 $\pm$ 0.89		

<sup>a</sup> Values are mean  $\pm$  SD (n = 3); <sup>b</sup> Apigenin was used as positive control.

the isolated compounds, compounds **1** and **3 - 8** displayed significantly inhibitory activity, with IC<sub>50</sub> values of 13.25 - 37.36  $\mu$ M. Conversely, compound **2** showed a weak effect (IC<sub>50</sub> = 93.67  $\mu$ M). Comparison of their structures allowed us to discuss the structure-activity relationships of the isolated phenolics. Compared with compounds **1** and **3 - 5**, compound **2** exhibited linkage to the C-3 methoxyl group of 1-(3-hydroxy-propen-1-yl) phenyl, and displayed weak inhibition of NF- $\kappa$ B transcriptional activity, suggested that the benzene ring is superseded by a methoxyl group that may decrease TNF $\alpha$ -induced NF- $\kappa$ B transcriptional activity. However, compounds **6**, **7** and **8** demonstrated significantly increased activity (13.25, 23.61, and 23.33  $\mu$ M). These compounds contain two methoxyl groups at C-3 and C-5 of the skeleton. These results suggest that the two methoxyl groups at C-3 and C-5 of 3,5-dimethoxy-1-(3-hydroxy-propen-1-yl) phenol may play an important role in NF- $\kappa$ B

inhibition. Therefore, these are likely the key functional elements in the phenolic derivatives, indicating that the symmetrical methoxyl group is more important than the single methoxyl group with respect to NF- $\kappa$ B inhibition.

These results led us to conclude that nine phenolic compounds (**1 - 9**) and three lignans (**10 - 12**) from the stems of ADA exhibit significant anti-inflammatory effects by inhibiting TNF- $\alpha$  induced NF- $\kappa$ B activation. Therefore, we suggest that the stems of ADA be used to treat natural inflammatory and metabolic diseases by inclusion in functional foodstuffs and nutraceuticals.

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