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Effect of Apoptosis Induction of *Ailanthus altissima* on Human Lung Carcinoma Cells

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ABSTRACT

We investigated the inhibitory effects of solvent extracts from *Ailanthus altissima* in A549 human lung cancer cell. *A. altissima* has been recognized as a traditional healthy food due to its various biological activities against hypertension, strokes, fever, pain, neuralgia, inflammation, and cancer effects. Recently, it has been reported that the extracts of various wild vegetables show strong anti-cancer properties by induction of apoptosis. However, the mechanisms of their cytotoxicity in human lung cancer cells have been poorly understood. The present study was investigated the effects of solvent extracts from *A. altissima* on cell growth and apoptosis on A549 human lung cancer cells. A treatment of *A. altissima* inhibited the growth of A549 cells in a dose-dependent manner by inducing apoptosis. Especially, the chloroform fraction showed the highest anti-cancer effect among five kinds of fractions. And also, induction of apoptosis by chloroform fraction were associated with down-regulation of Bcl-2, and up-regulation of pro-apoptotic Bax expression. From these results, *A. altissima* may have a therapeutic potential in human lung cancer cells and as a functional food.

Key words - *Ailanthus altissima*, Lung cancer, Apoptosis, Bax, Bcl-2

I. INTRODUCTION

Apoptosis, an important process in cell development and maintenance of tissue homeostasis, plays an essential role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells (Kaufmann & Hengartner, 2001; Jin & El-Deiry, 2005). The relationship between apoptosis and cancer has been a recent focus. Apoptosis provides a number of useful clues upon developing effective therapies, and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells (Jemal *et al*., 2011). Therefore, induction of apoptosis has become a principal mechanism by which anticancer therapy is effective. Recent scientific efforts have focused on the potential roles of extracts of traditional herbs as alternative and complementary medications for cancer treatment.

Ailanthus altissima (Simaroubaceae) well-known 'tree-of-heaven', is used in Chinese traditional medicine. It has been used to treat cold and gastric diseases and anti-proliferation (Kim *et al*., 1994), anti-inflammatory activity (Jin *et al*., 2006), central nervous system depressants (Crespi-Perellino *et al*., 1988), plant growth regulation and insecticidal effects (Pascual-villalobos & Robledo, 1998).

In several studies *on A. altissima* extracts, phenolic compounds, flavonoid compounds, merosin, tannin phlobaphen, ailanthone, amarolide, acetylamarolide were identified (Kazuya *et al*., 1994; Barakat, 1998). The phenolic compounds are primarily composed of 3,4,5-trimethoxyphenol, p-coumaric acid, vanillin, vanillic acid. The flavonoid compounds are primarily composed

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of 5,7-dihydroxychromone- 7-neohesperidoside, naringin. And these components have been reported their biological activities such as antimicrobial effect (Lee *et al.*, 2002), cell cycle regulation (Hwang *et al.*, 2002), improvement of liver function (Kim *et al.*, 1994) and acute lymphocytic leukemia (Kim & Lee, 1997). Although many biological activities of *A. altissima* were reported, apoptosis induction of *A. altissima* on human cancer cell has not been reported. Thus, this study investigated how *A. altissima* fractions affects cell growth and apoptosis of A549 human lung cancer cell. The anticancer mechanism of *A. altissima* fractions was also elucidated by analyzing expressions of apoptosis-related molecules, including Bax and Bcl-2 gene.

II. Materials and Methods

2.1 Reagents

The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), RPMI1640 medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen Life Technologies Inc. (Carlsbad, CA, USA). RNeasy plus mini kit and SYBR Green mix were purchased from Qiagen (Valencia, CA, USA). Other reagents were used of analytical grade.

2.2 Sample Preparation and Fractionation

The Korean wild edible vegetable, *A. altissima* was purchased from the Plant Extract Bank (Dae-jeon, Korea). The dried *A. altissima* was milled into powder of 40 mesh particle size and extracted with 70% ethanol by stirring for 24 hr at room temperature. The extract was filtered, and the residue was extracted in duplicate, under the same conditions. Subsequently, the filtrates were combined and evaporated under vacuum (EYELA N-1000, Tokyo Riakikai Co., Ltd. Japan) and then lyophilised with a Bondiro Lyophpride freeze dryer (Ilshine Lab Co. Ltd., Korea) at -70℃ under reduced pressure (< 20 Pa). The dried ethanol extracts were then suspended in water (500 ml) and further fractionated, by additional extraction with n-hexane, chloroform, and n-butanol in a stepwise manner (Fig. 1.). Each fraction powder was dissolved in DMSO and diluted with PBS (pH 7.4) to the desired final concentration. And filtered through a 0.45 μm syringe filter (Advanced MFS. Inc., Dublin, CA, USA) before use.

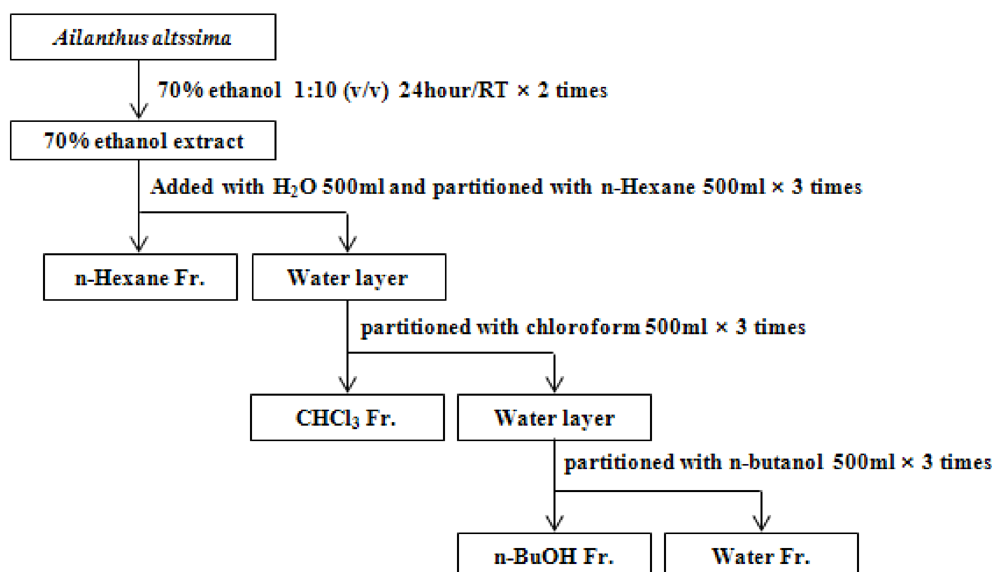

Fig. 1. Procedure of extract and fraction layers of *Ailanthus altissima.*

2.3 Cells and Culture

Raw 264.7, mouse macrophage cell line, (KCLB no.40071) and A549 human lung carcinoma cell line (KCLB no.10185) were purchased from the Korean cell line bank (Seoul, Korea). The cell lines were grown in RPMI1640 medium or DMEM with 10% FBS and 1% penicillin-streptomycin and incubated at 37℃ in 5% CO_2.

2.4 Cell Cytotoxicity Assay

Exponentially growing A549 or Raw 264.7 cells were seeded in 96-well culture plates. After 4 hr, cells were treated with various concentrations (1, 10, or 100 µg/ml) of each fraction for 48 hr. 10 µl of MTT solution was added and the cells were incubated for another 4 hr. After removing the media, DMSO was added to each well to dissolve MTT-formazan product. The resulting absorbance was measured at 540 nm (Hansen *et al.*, 1989). Cell cytotoxicity was expressed as percentage of viable cells of treated samples to control samples. All tests were performed in triplicate.

2.5 Real-time RT-PCR Analysis

To determine the expression levels of Bax and Bcl-2, real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using a real-time thermal cycler Qiagen rotorgene Q, in accordance with the manufacturer's instructions. The cell was treated with each fraction of *A. altissima* and cultured for 2 days in DMEM with 10% FBS. Thereafter, cDNA was synthesised from the total RNA isolated from cells. The PCR reaction was performed using 2X SYBR Green mix. All results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following primer sequences were used for the real-time RT-PCR: GAPDH; 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' (forward), 5'- AGC CTT CTC CAT GGT GGT GAA GAC-3' (reverse), Bax; 5'-ATG GAC GGG TCC GGG GAG-3' (forward), 5'-TCA GCC CAT CTT CTT CCA-3' (reverse), Bcl-2; 5'-CAG CTG CAC CTG ACG-3' (forward), 5'-ATG CAC CTA CCC AGC-3' (reverse). All reactions were performed in triplicate.

2.6 Statistical Analysis

Statistical analysis was performed with SPSS statistical software (version 17.0). Descriptive statistics were used to calculate the mean and standard error of the mean (SEM). One-way analysis of variance was performed and, when significance (p<0.05) was found, the differences of the mean values were identified with Duncan's multiple range tests.

III. Results and Discussions

3.1 Effect of the *Ailanthus altissima* fractions on cell cytotoxicity

Four organic solvents (n-hexane, chloroform, n-butanol and aqueous residue) were used in a stepwise manner to extract the anticancer components from a ethanol extract of *A. altissima*. The effect of the *A. altissima* fractions on the growth of A549 and Raw 264.7 cells were examined by MTT assay. In order to screen the cytotoxic effects, we performed the cytotoxicity of the fractions in Raw 264.7 cells exposed to various concentrations (1, 10, or 100 µg/ml). The $CHCl_3$ fraction inhibited cell proliferation at 100 µg/ml (Fig. 2.) On the basis of this result, we determined the appropriate concentration to be 10 and 1 µg/ml for cell treatment. As shown in Fig. 3, among the all fractions tested, the $CHCl_3$ fraction appeared to be most potent growth inhibition of A549 cells. The percent cytotoxicity of A549 cells exposed to the $CHCl_3$ fraction at 1 and 10 µg/ml was 9.55 ± 1.4, and 30.18 ± 2.6%, respectively, compared with the controls (p<0.05). As results obtained in this research, De-Feo *et al.* (2005) reported that the $CHCl_3$ fractions of *A. altissima* root had high anti-cancer effect in HeLa cell and these growth inhibition by inducing apoptosis. So, we also expect that *A. altissima* will be induce apoptosis in A549 cell.

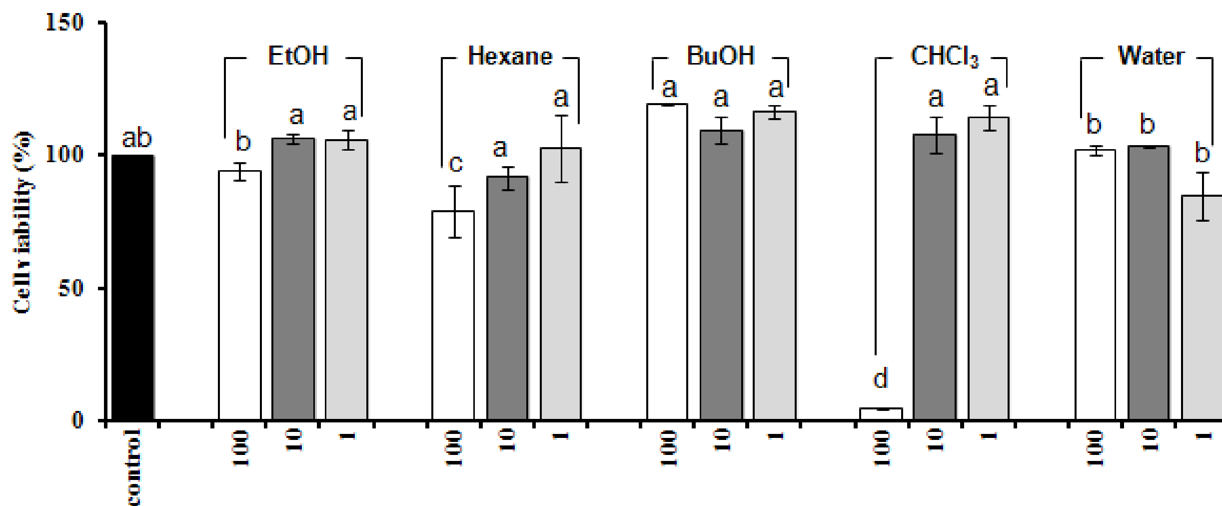

Fig. 2. Cell viability of *Ailanthus altissima* fractions in Raw 264.7 cells. Cell viability in the control group was taken as 100%, Values are the mean of three determinations ± SEM. Values expressed different letters in the same concentration among fractions are significantly different at $p<0.05$.

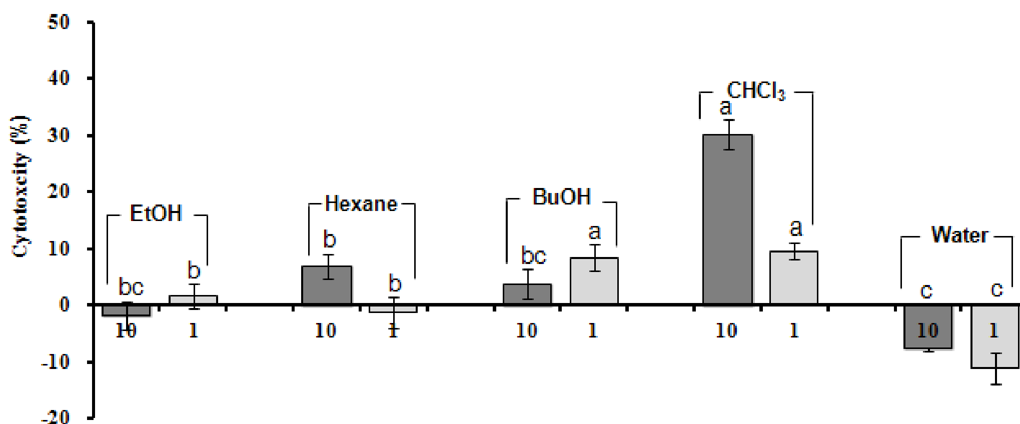

Fig. 3. Cell cytotoxicity of *Ailanthus altissima* fractions in A549 human lung cancer cells. Cellular cytotoxicity was determined as the percentage of control. Values are the mean of three determinations ± SEM. Values expressed different letters in the same concentration among fractions are significantly different at $p<0.05$.

3.2 Altered Bax and Bcl-2 gene expression by *Ailanthus altissima* in A549 cells

To investigate the mechanism of apoptosis, the expression of pro- and anti- apoptotic genes was studied by real time RT-PCR. A number of studies, it has been reported that the progress of apoptosis is regulated by the expression of several transcriptional genes. One of these genes is member of the Bcl-2 family (Vaux & Korsmeyer, 1999). The Bcl-2 family can be classified into two functionally distinct groups: anti-apoptotic genes and pro-apoptotic genes. Bcl-2, an anti-apoptotic gene, is known for regulating the apoptotic pathways and protecting cell death, while Bax, a pro-apoptotic gene of the family, is expressed abundantly and selectively during apoptosis, promoting cell death (Oltvai *et al.*, 1993). And also, some groups have shown in various cancer cell lines that wild plants can lead to cell death by inducing apoptosis through

regulation of Bax/Bcl-2 expression (Chiu *et al.*, 2006; Hwang *et al.*, 2011; Puangpronpitag *et al.*, 2011). Our data showed that *A. altissima* regulated the expressions of apoptosis-regulating transcriptional genes in A549 cells (Fig. 4.). Furthermore, the expression of Bax was significantly increased compared to the control in all cells treated with fractions. Especially, $CHCl_3$ fraction-treated cells were increased the mRNA level approximately 2-fold higher than control group and the expression of Bcl-2 was decreased significantly compared to the control (p<0.05). Further study is needed to demonstrate active compounds from *A. altissima* $CHCl_3$ fraction that are responsible for regulating the apoptotic signaling pathway or the cellular differentiation signaling pathway.

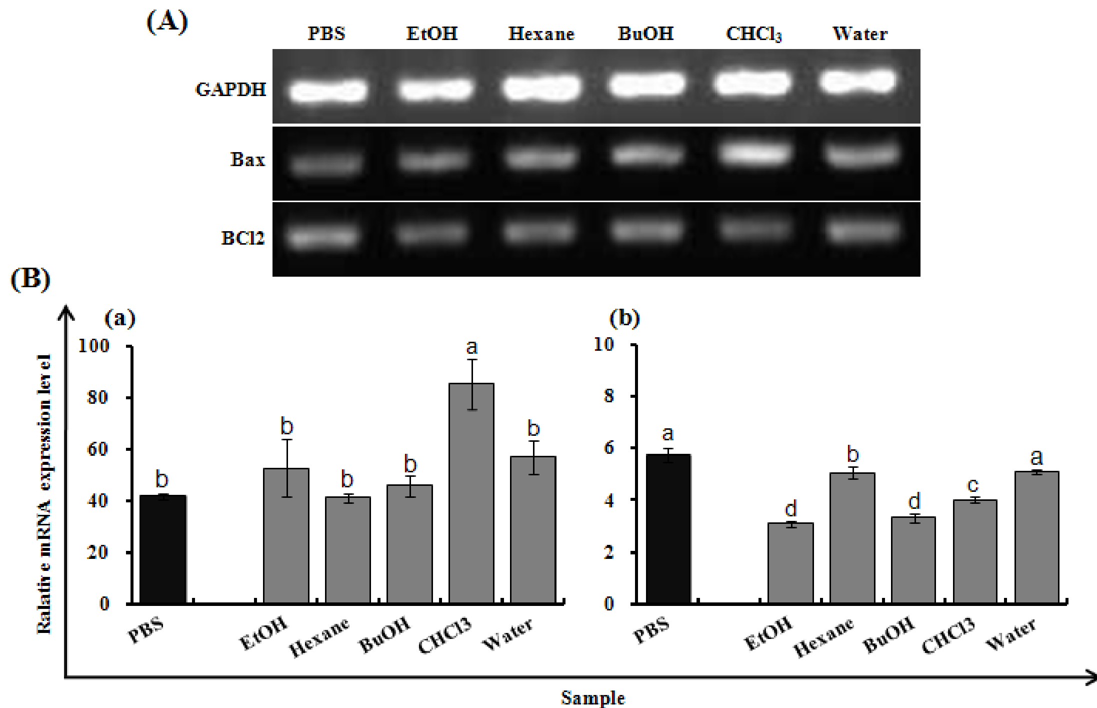

Fig. 4. mRNA expression of Bax and Bcl-2 in A549 cells treated with *Ailanthus altissima* fractions (A) Electrophoresis of the PCR products in an agarose gel (1.5%). (B) The ratio of Bax (a), and Bcl-2 (b) in A549 to GAPDH mRNA levels. Values are the mean of three determinations ± SEM. Values expressed different letters in the same concentration among fractions are significantly different at p<0.05.

Ⅳ. Acknowledgement

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Abbreviation

DMEM : Dulbecco’s modified eagle’s medium
DMSO : Dimethyl sulfoxide
FBS : Fetal bovine serum
GAPDH : Glyceraldehyde 3-phosphate dehydrogenase
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
KCLB : Korean cell line bank
PBS : Phosphate-buffered saline
SEM : Standard error of the mean