Anticancer Activity of *Asparagus cochinchinensis* Extract and Fractions in HepG2 Cells

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Received October 18, 2010; Accepted January 4, 2010

***Asparagus cochinchinensis* Merrill (Liliaceae) has been traditionally used for the treatment of cancer in Korea and China, but its anticancer activity and underlying mechanism remain to be defined. Anticancer activities were investigated on fractions obtained from *A. cochinchinensis* 70% ethanol extract (ACE-EtOH) in human hepatocellular carcinoma HepG2 cells. Ethylacetate fraction from *A. cochinchinensis* extract (ACE-EA), more effective than other fractions, induced apoptosis of HepG2 (IC_{50}=72.33±0.34 μg/mL), as revealed by apoptotic feature observation, increased capase-3 activity and Poly ADP ribose polymerase cleavage, and decreased expression of X-linked inhibitor of apoptosis protein in a dose-dependent manner. Protein levels of autophagy-related molecules, microtubule-associated protein 1 light chain 3 α and beclin 1, appeared to be induced by ACE-EA, suggesting ACE-EA exhibits anti-cancer activity with induction via both apoptosis and autophagy signaling pathways in HepG2 cells.**

Key words: anticancer activity, apoptosis, *Asparagus cochinchinensis*, autophagy, HepG2

The pathophysiology of hepatocellular carcinoma (HCC) is not entirely understood; there are a variety of risk factors, such as exposure to hepatitis viruses, heavy alcohol intake, nonalcoholic fatty liver disease, diabetes, obesity, and foodstuffs contaminated with aflatoxin and hemochromatosis [Aravalli *et al*., 2008]. Moreover, hepatocarcinogenesis is a complicated process associated with the accumulation of aberrant genetic and epigenetic changes. Therefore, several studies have focused on the elucidation of the physiological, cellular, and molecular mechanisms of hepatocarcinogenesis, as well as molecular profiling for biomarker identification with the goal of developing new treatment strategies [Llovet and Bruix, 2008; Hui, 2009].

Historically, natural sources have yielded a variety of therapeutic agents, including anticancer agents. Several herb-based constituents and extracts have been reported to reduce tumor growth and inhibit metastasis in human HCC HepG2 cell-based *in vitro* and *in vivo* models [Chung *et al*., 2004; Cheung *et al*., 2007].

Because the root of *Asparagus cochinchinensis* was historically believed to possess numerous therapeutic properties, including anticancer and anti-inflammatory activities, it has been traditionally used in the therapeutic regimen for more than 2,000 years, but its functional role has been evaluated in only a few studies [Huang, 1993; Lee *et al*., 2009].

Therefore, in the present study cytotoxicity of *A. cochinchinensis* extract (ACE) and its fractions in HepG2 cells were examined. The ethylacetate fraction of *A. cochinchinensis* extract (ACE-EA) was found to inhibit the viability of HepG2 cells. The fraction-dependent

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doi:10.3839/jksabc.2011.031

cytotoxicity of ACE in HepG2 cells was then monitored by caspase-3 activity assay and western blot analysis to collect further evidence of cell death via apoptosis and autophagy.

Material and Methods

Plant material and sample preparation. *A. cochinchinensis* was collected from the Namhae, Korea in June 2007. Botanical identification was confirmed by morphological characteristics and by analysis of Internal Transcribed Spacer (ITS) sequences, which showed 100% identification with the *A. cochinchinensis* ITS region (accession no. AB195579) in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny. A voucher specimen (no. KIOM200701000240) was deposited at the herbarium of the Center of Herbal Resources Research (Korea Institute of Oriental Medicine, Daejeon, Korea). The dried and pulverized roots of *A. cochinchinensis* were extracted three times with 70% ethanol (with 2 h reflux), and the extract was concentrated under reduced pressure and lyophilized. The extract powder was dissolved in distilled water and sequentially fractionated by the polarities of solvents using *n*-hexane (Hx), methylene chloride (MC), ethyl acetate (EA), *n*-butanol (BuOH), and water. Subsequently, the five fractions obtained were lyophilized. The yield of dried extracts from starting plant materials were 15.4% (70% ethanol, ACE-EtOH), 0.01% (*n*-hexane fraction, ACE-Hx), 0.03% (methylene chloride fraction, ACE-MC), 0.03% (ethyl acetate fraction, ACE-EA), 0.25% (butanol fraction, ACE-BuOH), and 5.96% (H_2O fraction, ACE- H_2O). The lyophilized powder was dissolved in 10% dimethyl sulfoxide (DMSO) and then filtered through a 0.2-μm syringe filter to create stock solution.

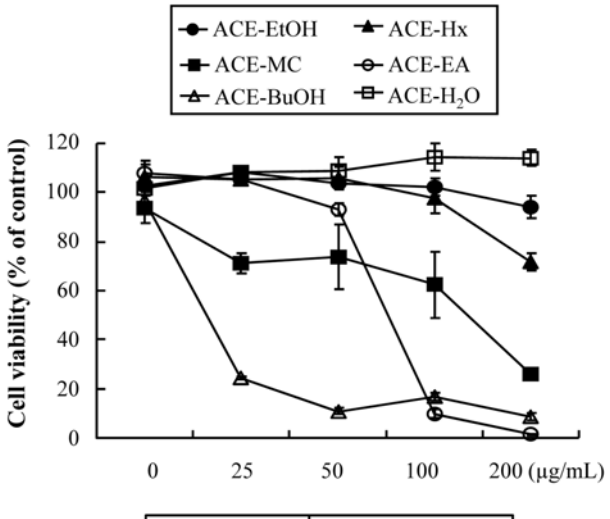
Cell culture. All materials for cell culture were purchased from HyClone (Logan, UT). HepG2 cells were cultured in Minimum Essential Medium with Earle's Balanced Salts with L-glutamine (MEM/EBSS) supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin with a change of medium every 3 days in humidified atmosphere of 5% CO_2 at 37°C.

Cell viability assay. Cells were plated in a 96-well plate at 1×10^4 cells/well. After 24 h, cells were treated with serially diluted extract or fractions and incubated for an additional 48 h. Cell viability was then measured by the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer's protocol. The experiment was performed in triplicate, and cell viability was presented as percent of the control.

Caspase-3 activity assay. Cells were plated in a 96-well plate at 1×10^4 cells/well. After 24 h incubation at (5% CO_2, 37°C), cells were treated with serially diluted extracts or fractions and incubated for additional 24 h. Then, caspase-3 activity was measured in triplicate by using Caspase-Glo 3/7 Reagent (Promega, Madison, WI) according to the manufacturer's protocol.

Apoptotic nuclear condensation. Cells were plated in a 96-well plate at 1×10^4 cells/well. After 24 h incubation at (5% CO_2, 37°C), cells were treated with serially diluted extracts or fractions and incubated for additional 48 h. After the medium was aspirated, cells were washed twice with PBS and subjected to 4'-6-diamidino-2-phenylindole (DAPI) staining, the apoptotic features of the cells were examined by fluorescence microscope with a DP Controller (Olympus Optical, Tokyo, Japan).

Western blot analysis. Cells were treated with ACE-EA for 1 day, homogenized in buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM PMSF, and 1 protease inhibitor cocktail tablet (Roche, Mannheim, Germany) at 4°C, and centrifuged at 10,000×g for 15 min. The supernatant protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL). Samples (20 μg) were mixed with a sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 15 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad, Hercules, CA). Proteins separated on the gels were transferred onto nitrocellulose membranes (Scheicher & Schnell BioScience, Keene, NH), and membranes were stained with Ponceau S to confirm efficiency of transfer and equal protein loading. The stained membranes were washed and incubated in a blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk). The membranes were incubated for 2 h at room temperature with 1:1000 diluted primary antibodies (all antibodies were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing with blocking buffer three times for 15 min, membranes were probed with secondary antibody (1:2000 dilutions) for 1 h. They were washed three times for 15 min and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) using the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).



	IC_{50} (μg/mL)
ACE-MC	127.55±14.58
ACE-EA	75.92±0.08
ACE-BuOH	16.09±0.24

Fig. 1. Effect of ACE-EtOH and its fractions on cytotoxicity in HepG2 cells. Cell viability was measured as described in 'Materials and Methods'. In brief, cells were incubated with each fraction for 48 h, and then cell viability was measured.

Results

Effect of ACE-EtOH and it's fractions on cytotoxicity in HepG2 cells. The cytotoxic activities of HepG2 cells in ACE-EtOH and five fractions (ACE-Hx, ACE-MC, ACE-EA, ACE-BuOH, and ACE-H_2O) were evaluated. When cells were treated with ACE-EtOH and fractions for 48 h, ACE-BuOH, ACE-EA, and ACE-MC (in order of decreasing strength) exhibited strong cytotoxicity in HepG2 cells (Fig. 1). ACE-Hx exhibited mild cytotoxicity at high concentrations, but neither ACE-EtOH nor ACE-H_2O showed any cytotoxicity (Fig. 1).

Effect of cytotoxic ACE fractions on induction of apoptosis. Cancer cell death is distinguished by several mechanisms, including necrosis, apoptosis, and autophagy. Apoptosis in most cells is characterized by caspase-3 activation. Therefore, the effects of the cytotoxic ACE fractions (ACE-MC, ACE-EA and ACE-BuOH) on caspase-3 activation were evaluated. Only ACE-EA triggered the induction of caspase-3 activity, at high concentration of 200 μg/mL (Fig. 2A). To confirm the induction of apoptosis, the chromatin condensation by ACE-EA was observed by DAPI staining, and the chromatin condensation was observed in cells treated with ACE-EA (Fig. 2B).

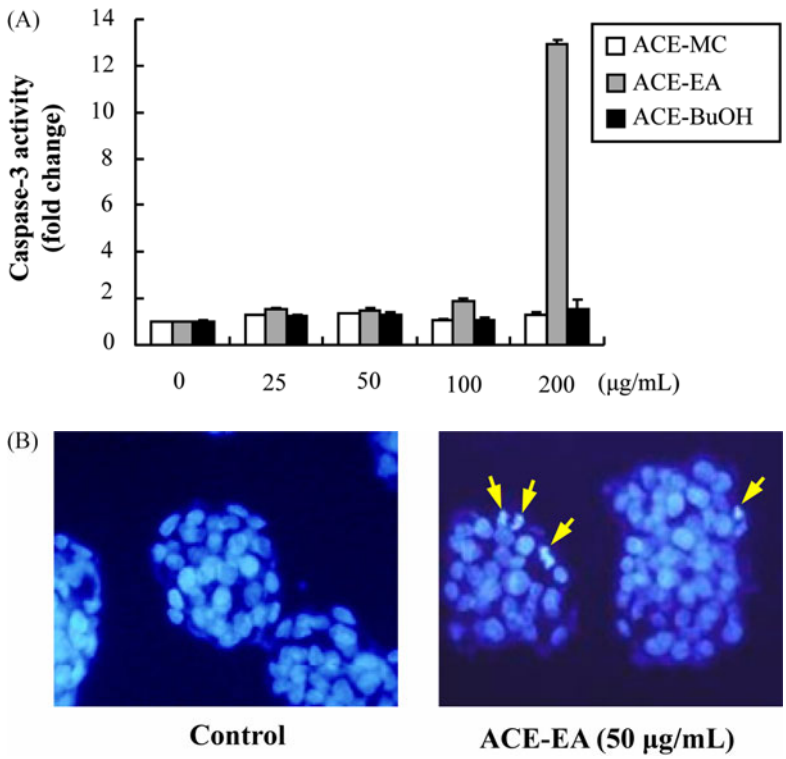


Fig. 2. (A) Effect of cytotoxic ACE-fractions on caspase-3 activation. (B) Effect of ACE-EA fraction on apoptotic chromatin condensation in HepG2 cells. Cells (1×10^4 cells/well) in a 96-well plate were incubated with each fraction for 24 h and then caspase-3 activity was measured as described in 'Materials and Methods'. Chromatin condensation was observed by DAPI staining.

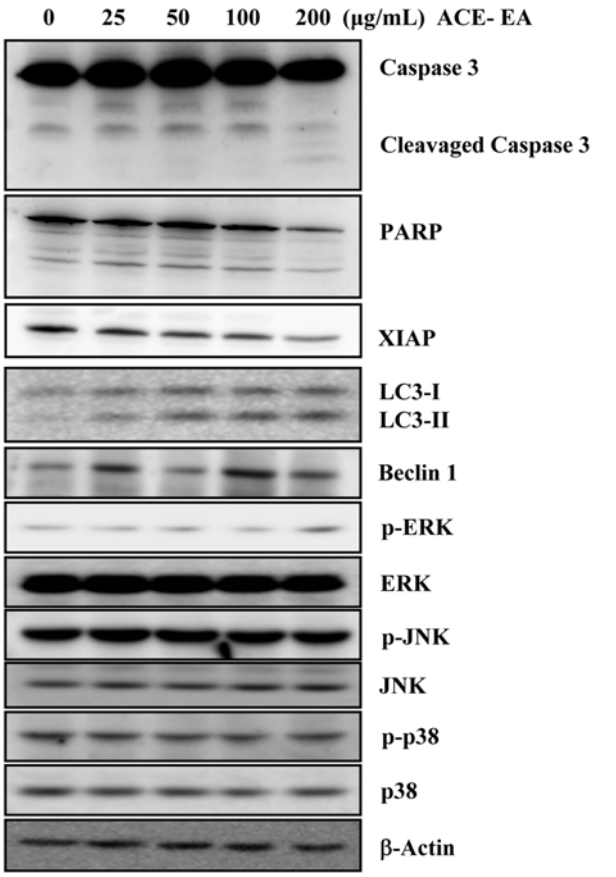


Fig. 3. Effect of ACE-EA on protein levels of apoptosis and autophagy-related molecules, and activation of MAP kinases. Effects of ACE-EA on the protein expression levels of apoptosis and autophagy-related molecules, and the activation of MAP kinases were evaluated by western blot analysis. Actin was used as a positive control.

Effects of ACE-EA on protein levels of apoptosis and autophagy-related protein expression. Apoptosis induced by ACE-EA was monitored by measuring the expression levels of apoptosis-related proteins in HepG2 cells. ACE-EA dose-dependently induced the cleavage of PARP and was associated with decreased levels of the anti-apoptotic protein XIAP (Fig. 3). Because the induction of apoptosis and autophagy by an herbal extract was recently reported in HepG2 cells (Lin *et al*., 2007), the effect of ACE-EA on the protein levels of autophagy-related protein expression was further evaluated (Fig. 3). Levels of microtubule-associated protein 1 light chain 3α (MAP1LC3A, also known as LC3), a specific marker of autophagy, were dose-dependently increased by ACE-EA. In particular, ACE-EA was associated with increased levels of LC3-II protein, which correlates with increased formation of autophagosomes. ACE-EA was also associated with the increased levels of beclin 1 (a mammalian ortholog of Atg6), a positive regulator of autophagic vacuole formation.

Effect of ACE-EA on activation of mitogen-activated protein (MAP) kinases. Effects of ACE-EA on the activation of MAP kinases involved in apoptotic and/or autophagic cell death were evaluated. ACE-EA induced phosphorylation of ERK and was associated with increased levels of JNK protein. The protein levels of both total p38 and phosphorylated p38 were slightly reduced in the presence of ACE-EA.

Discussion

A. cochinchinensis was historically believed to possess numerous therapeutic properties, including anticancer and anti-inflammatory activities, but its functional role has been examined experimentally in only a few studies [Huang, 1993; Lee *et al*., 2009]. In the present study, the anti-cancer activities of ACE extract and fractions in human HCC HepG2 cells were evaluated. Of the ACE-EtOH and the five fractions, ACE-BuOH, ACE-EA, and ACE-MC exhibited strong cytotoxicity in HepG2 cells, but only ACE-EA triggered the induction of caspase-3 activity, which is used to characterize apoptosis. The induction of apoptosis by ACE-EA was also confirmed by observing the chromatin condensation. Furthermore, western blot analysis revealed that ACE-EA dose-dependently induced the cleavage of PARP and led to decreased levels of the anti-apoptotic XIAP proteins. The down-regulation of XIAP in HepG2 cells has been shown to inhibit cell viability and increase chemosensitivity [Chen *et al*., 2006].

Recently, the induction of apoptosis and autophagy by an herbal extract was reported in HepG2 cells [Lin *et al*., 2007]. In the present study, ACE-EA increased the protein levels of the autophagy-related molecules LC3 and beclin 1, which are involved in the autophagic vacuole formation. Autophagy is a dynamic process in which cellular components are degraded by the cell recycling system [Klionsky and Emr, 2000]. The importance of autophagy in the regulation of cancer development and progression was recently recognized; observations in cellular and animal models and clinical samples have suggested a variety of physiological and pathological roles for autophagy, but its relevance with regard to the destiny of cancer cells is still controversial [Tsuchihara *et al*., 2009]. Although autophagy can be used as a protective mechanism leading to prolonged survival in cancer cells subjected to starvation, autophagy-related cell death has also been regarded as a potential mechanism for suppressing cancer cell growth [Sandra and Amato, 2010]; however, considering the results of the present study showing that ACE-EA induced the apoptosis with the increase of autophagy-related molecules, our results

suggest that ACE-EA could trigger autophagy, as well as activate the apoptotic signaling. Kiyono *et al*. [2009] reported on the relationship between autophagy and apoptosis in HepG2 cells, showing that transforming growth factor (TGF)-β, which regulates cell growth, differentiation, and apoptosis of various types of cells, activates autophagy earlier than the execution of apoptosis.

MAP kinases have been shown to be involved in the process of apoptotic and/or autophagic cell death. In the present study, ACE-EA at a high concentration was found to slightly induce the phosphorylation of ERK in HepG2 cells. ERK activation by anticancer drugs has been reported in HepG2 cells; the induction of apoptosis by norcantharidin or doxorubicin is accompanied by ERK activation [Chen *et al*., 2003; Choi *et al*., 2008]. The protein levels of both total p38 and phosphorylated p38 were slightly reduced in the presence of ACE-EA. The chemopreventive effect of resveratrol in liver cancer cells has been shown to be involved in the downregulation of the p38 signaling pathway [Parekh *et al*., 2010]. Additionally, the inhibition of JNK attenuated the TGF-β-induced authophagy, suggesting that JNK activation is involved in the induction of autophagy [Kiyono *et al*., 2009]. In the present study, ACE-EA was found to be associated with increased levels of total JNK protein, but not the phosphorylated JNK. However, considering the results showing that siRNA silencing of JNK reduces HepG2 cell susceptibility to apoptosis, JNK expression or activation could exert a crucial role in the process of ACE-EA–induced apoptotic and/or autophagic cell death [Lauricella *et al*., 2006]. Further study is required to elucidate the precise mechanism of action by which ACE-EA triggers the death of HepG2 cells. Our results suggest that ACE-EA may possess bioactive components that trigger apoptosis in HepG2 cells. Several components isolated from *A. cochinchinensis,* including furostanol oligoglycosides (aspacochiosides A, B, and C), 3-*O*-[(alpha-L-rhamnopyranosyl-(1→4))(beta-D-glucopyranosyl)]-26-*O*[beta-D-glucopyranosyl]-(25*S*)-5beta-spirostane-3beta-ol, C-27 spirosteroids (asparaconsins A and B), and phenolic compounds (3'-hydroxy-4'-methoxy-4'-dehydroxynyasol, asparenydiol, nyasol, 3'-methoxynyasol, 1,3-bis-di-*p*-hydroxyphenyl-4-penten-1-one, and *trans*-coniferyl alcohol) have been reported [Shi *et al*., 2004; Zhang *et al*., 2004]; however, only few functional studies on the constituents of *A. cochinchinensis* have been performed. Among the compounds that have been investigated, nyasol (*cis*-hinokiresinol) was reported to be an estrogen receptor β-selective agonist [Paruthiyil *et al*., 2009] that exhibits antiprotozoal [Oketch-Rabah *et al*., 1997], antifungal [Iida *et al*., 2000], anti-oxidant [Song *et al*., 2007], anti-inflammatory, and *in vitro* cytotoxic activities against human ovarian carcinoma and hepatoma, with IC_{50} values of 30.6 and 29.4 μM, respectively [Yang *et al*., 2004].

In conclusion, ACE-EA exhibited the anti-cancer activity with the induction of apoptosis and autophagy in HepG2 cells. Further studies to evaluate the *in vivo* anticancer efficacy of ACE-EA and identify the bioactive components in ACE-EA to exhibit its anti-cancer activity will be required for developing ACE-EA and/or its bioactive components as one of naturally derived anti-cancer agents.

Acknowledgments. This work was supported by the project, 'Construction of the Basis for Practical Application of Herbal Resources' funded by the Ministry of Education, Science and Technology (MEST) of Korea to the Korea Institute of Oriental Medicine (KIOM). We thank the 'Classification and Identification Committee' of the Korea Institute of Oriental Medicine for the critical authentication of plant and for the helpful discussions.

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