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Apoptosis of DU145 human prostate cancer cells induced by dehydrocostus lactone isolated from the root of *Saussurea lappa*

Eun Ji Kim [b], Soon Sung Lim [b], So Young Park [a], Hyun-Kyung Shin [a,b], Jong-Sang Kim [c], Jung Han Yoon Park [a,b,*]

[a] *Department of Food Science and Nutrition, Hallym University, Chuncheon 200-702, Republic of Korea*
[b] *Center for Efficacy Assessment and Development of Functional Foods and Drugs, Hallym University, Chuncheon 200-702, Republic of Korea*
[c] *Department of Animal Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea*

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ABSTRACT

Saussurea lappa (*SL*) is a plant regularly utilized in traditional herbal medicine, and *in vitro* cell culture studies have demonstrated that *SL* has anti-ulcer, anti-inflammatory, and anti-tumor properties. In order to explore the possibility that *SL* exerts chemopreventive effects in androgen-independent prostate cancer, we attempted to determine whether the hexane extract of *SL* (HE*SL*) induces apoptosis of DU145 cells, as well as the mechanisms underlying this effect. HE*SL* substantially reduced the number of viable cells and induced apoptosis in DU145 cells in a dose-dependent manner. HE*SL*-induced the cleavage of poly (ADP-ribose) polymerase (PARP) and caspases 8, 9, 7, and 3. HE*SL* increased the protein levels of Bax, Bak, Bok, Bik, truncated Bid (t-Bid), and Bmf with a concomitant increase in the permeability of the mitochondrial membrane and in the release of cytochrome *c* from the mitochondria. The active fraction of HE*SL* was isolated by column chromatography and the structure of the active compound dehydrocostus lactone (DHCL) was identified via ^{1}H NMR and ^{13}C NMR. DHCL promoted apoptosis with increased activation of caspases 8, 9, 7, 3, enhanced PARP cleavage, decreased Bcl-xL expression and increased levels of Bax, Bak, Bok, Bik, Bmf, and t-Bid. We have demonstrated that HE*SL* and its active principle, DHCL, inhibit cell growth and induce apoptosis in DU145 cells.

1. Introduction

Prostate cancer continues to be a major problem in the developing world and remains the most common malignancy in men. In the United States in 2008, an estimated 186,320 men will be diagnosed with prostate cancer, and 28,660 of these cases will prove fatal (Jemal et al., 2008). Mortality from prostate cancer results from metastasis to the bones and lymph nodes and progression from androgen-dependent to androgen-independent prostatic cancer growth (Bruckheimer and Kyprianou, 2000). The androgen-dependent stage of prostate cancer may be treated effectively with androgen deprivation therapy. However, in the majority of patients, the cancer ultimately progresses to the lethal stage of androgen-independence, wherein it no longer responds to androgen deprivation therapy (Aquilina et al., 1997; Kasamon and Dawson, 2004). In such cases, chemoprevention and intervention strategies using anticancer agents are the most promising alternative options currently available.

Saussurea lappa (*SL*) is a well-known plant in Asia, where it is utilized as a medicine, and its roots have been traditionally used for alleviating pain from abdominal distention and tenesmus, indigestion with anorexia, dysentery, nausea, and vomiting (Sun et al., 2003). Previous *in vitro* cell culture studies have shown that *SL* has anti-ulcer (Yoshikawa et al., 1993), anti-inflammatory (Cho et al., 2000), antiviral (Chen et al., 1995), and anti-tumor properties (Ko et al., 2005, 2004). In addition, sesquiterpene lactones isolated from *SL* inhibit the growth of several types of cancer cells (Cho et al., 2004; Oh et al., 2004; Sun et al., 2003). However, the effects of *SL* on prostate cancer and its mechanisms of action have yet to be elucidated.

One of the hallmarks of cancer is the deregulation of apoptosis (Hanahan and Weinberg, 2000), a universal and efficient cellular suicide pathway. Therefore, increasing apoptosis in tumors can be an effective method for chemopreventive and chemotherapeutic intervention in all types of cancer. Apoptosis is controlled by two diverse pathways – the mitochondrial-mediated pathway

Abbreviations: Apaf-1, apoptosis protease-activating factor-1; FasL, fas ligand; HE*SL*, hexane extract of *Saussurea lappa*; HSP 60, heat shock protein 60; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; t-Bid, truncated Bid.

* Corresponding author. Address: Department of Food Science and Nutrition, Hallym University, 39 Hallymdaehak-gil, Chuncheon 200-702, Republic of Korea. Tel.: +82 33 248 2134; fax: +82 33 256 0199.
E-mail address: jyoon@hallym.ac.kr (J.H.Y. Park).

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(Green and Reed, 1998) and the membrane death receptor-mediated pathway (Ashkenazi and Dixit, 1999). The death receptor-mediated pathway is initiated by the interaction of the ligand with its death receptor, which results in the activation of caspase-8 and caspase-3. Caspase-3 then cleaves various substrates, which ultimately leads to apoptosis (Ashkenazi and Dixit, 1999). By way of contrast, the mitochondrial-mediated pathway involves the alteration of mitochondrial membrane permeability, thereby promoting the release of cytochrome *c* from the mitochondria. Cytosolic cytochrome *c* together with apoptosis protease-activating factor-1 (Apaf-1) activates caspase-9, which in turn activates caspase-3, thus resulting in apoptosis (Green and Reed, 1998). Mitochondrial-mediated apoptosis is regulated by the B-cell leukemia/lymphoma 2 (Bcl-2) family proteins, which control mitochondrial membrane permeability (Cory and Adams, 2002).

In order to explore the possibility that *SL* functions as a chemopreventive and/or chemotherapeutic agent in androgen-independent prostate cancer, the objective of the present study was to determine whether a hexane extract of *SL* (HE*SL*) induces apoptosis in androgen-independent DU145 human prostate cancer cells and to characterize the mechanisms underlying this effect. In addition, we isolated an active principle from the HE*SL*, and identified its structure.

2. Materials and methods

2.1. Materials

Reagents were purchased from the following suppliers: DMEM/Ham's F12 nutrient mixture (DMEM/F12) from Gibco BRL (Gaithersburg, MD, USA); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), anti-β-actin antibody, and anti-α-tubulin antibody from Sigma–Aldrich Co. (St. Louis, MO, USA); antibodies against Bcl-xL, Mcl-1, Bak, Bok, Bid, Bik, cleaved caspase-3, cleaved caspase-8, caspase-7, cleaved caspase-9, and cleaved poly (ADP-ribose) polymerase (PARP) from Cell Signaling (Beverly, MA, USA); antibodies against Bcl-2 and Bax from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and cytochrome *c* antibody, phycoerythrin (PE)-conjugated Annexin V (Annexin V-PE) and 7-amino-actinomycin D (7-ADD) from BD Pharmingen (Franklin Lake, NJ, USA).

2.2. Preparation of extract, fractionation, and identification of active compounds

The dried roots of *SL* Clarke were purchased from a local drugstore and identified by Emeritus Professor Hyung Jun Ji (Seoul National University, Seoul, Korea). The dried roots of the *SL* were pulverized to about 30 mesh with a disintegrator. The powder (500 g) was then placed in 2.5 L of *n*-hexane at 30 °C for 12 h and extracted via 30 min of sonication. The sonication procedure was repeated twice. The extract was evaporated under reduced pressure, and the resultant 22 g (4.4%) of thick cream was stored at 4 °C (the hexane extract). The hexane extract (10 g) was subjected to column chromatography in vacuo (VLC) with silica gel (100 g, 70–230 mesh, Merck, Germany) and eluted by a gradient system of *n*-pentane–*n*-hexane–ethyl acetate (10:0:0–0:5:5), yielding 15 fractions (Fr1–Fr15). The ability of each fraction to decrease DU145 cell viability was assessed using the MTT assay as described below, and the resultant active fraction (Fr7) was purified further by repeating the VLC procedure on silica gel (20 g, 200–400 mesh, Merck, Germany) with solvent systems of *n*-hexane–ethyl acetate (10:1–5:5) to yield an active compound (120 mg). The structural identification of the active compound was conducted via ^{1}H NMR and ^{13}C NMR. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX 400 (400 MHz) spectrometer (Bruker, Analytik GmbH, Germany) with tetramethylsilane (TMS) as an internal standard.

2.3. Cell culture

HT-29 and HCT116 human colon cancer cells and androgen-sensitive LNCaP and androgen-insensitive DU145 human prostate cancer cells were acquired from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM/F12 containing 10% fetal bovine serum (FBS) with 100,000 U/L of penicillin and 100 mg/L of streptomycin at 37 °C in 5% CO_2. To determine the effects of the various extracts and of dehydrocostus lactone (DHCL) on the cell viability, we plated cells in multi-well plates with DMEM/F12 containing 10% FBS. After 24 h, the cell monolayers were rinsed and serum-deprived for 24 h with DMEM/F12 containing 1% charcoal-stripped FBS (serum-deprived medium). The medium was then replaced with fresh serum-deprived medium with or without HE*SL* (1–4 mg/L), fractions (4 mg/L), or DHCL (0.25–2 mg/L), and the cells were cultured for 72 h. Viable cell numbers were estimated by the MTT assay as previously described (Kim et al., 2002). We used the serum-deprived medium containing 1% charcoal-stripped FBS to minimize any possible effects of various bioactive compounds present in the FBS. The extracts were dissolved in dimethylsulfoxide (DMSO), and all cells were treated with DMSO at a final concentration of 0.04%.

2.4. Cell cycle analysis

Cells were treated with or without 4 mg/L of the extracts for 24 h as described above. Cells were detached with trypsin–EDTA, fixed in 70% ethanol, and treated with 50 g/L of RNase. The cellular DNA was then stained with propidium iodide, as described previously (Lim et al., 2005). The percentage of cells in sub G1, G1, S, and G2/M phases of the cell cycle were analyzed via flow cytometry utilizing FACScan software (Becton-Dickinson, Franklin Lakes, NJ, USA). The data were then analyzed using Modifit version 1.2 software (Becton-Dickinson).

2.5. Quantitative analysis of apoptotic cells

Cells were treated with the extracts (0–4 mg/L) for 48 h as described above, harvested via trypsinization, and then stained for 15 min with Annexin V-PE and 7-ADD at room temperature in darkness. After staining, we conducted flow cytometry using FACScan software, followed by data analysis using Modifit (Lim do et al., 2007).

2.6. Mitochondrial membrane potential analysis

Alterations in mitochondrial membrane potential were analyzed via flow cytometry using the mitochondrial membrane sensitive cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine (JC-1). JC-1 is a green-fluorescent monomer at low membrane potential, with the membrane potential of energized mitochondria promoting the formation of red-fluorescent J-aggregates. The ratio of red to green-fluorescence of JC-1 depends solely on the membrane potential, with a reduction in this ratio being indicative of membrane depolarization (Reers et al., 1991; Smiley et al., 1991). The DU145 cells were treated with the extracts (0–4 mg/L) for 24 h, harvested via trypsinization, and incubated for 20 min with JC-1 (2 mg/L in PBS) at 37 °C. The stained cells were then washed twice in PBS and analyzed using FACScan (Jung et al., 2006).

2.7. Western blot analysis

We treated cells with the indicated concentrations of the extracts for 36 or 48 h in 100 mm dishes as described above. Total cell lysates were prepared as described previously (Cho et al., 2003), and the cytosolic proteins were separated as previously described by Eguchi et al. (1999). We determined the protein content in the total cell lysates and cytoplasmic fractions using the BCA protein assay kit (Pierce, Rockford, IL, USA). The proteins in the total cell lysates and cytoplasmic fractions were resolved on sodium dodecyl sulfate (SDS) polyacrylamide gels of 4–20% or 10–20%. The separated proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) followed by blocking with 5% skim milk in TBS-T (20 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20). The blots were then incubated for 1 h with a primary antibody and then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody. Signals were detected via an enhanced chemiluminescence method using SuperSignal® West Dura Extended Duration Substrate (Pierce, Rockford, IL). The relative abundance of each band was quantified using the Bio-profile Bio-1D application (Vilber-Lourmat, Marine la Vallee, France), and the expression levels were normalized to β-actin or α-tubulin.

2.8. Statistical analysis

For all studies, three to six independent experiments were conducted with separate batches of cells. The data were expressed as the means ± SEM and analyzed using analysis of variance. Differences between the treatment groups were evaluated by Duncan's multiple range test, using the SAS system for Windows version 8.12 (SAS Institute, Cary. NC, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. HESL inhibits growth and induces apoptosis of DU145 cells

We performed preliminary studies to determine the effects of several HE*SL*s originating from Korea, China, and Japan on the viability of DU145 cells. We noted that these three HE*SL*s induced a marked decrease in the number of viable DU145 cells, and the efficacy of Korean HE*SL* was found to be greater than that of other HE*SL*s. Therefore, the present study utilized the Korean HE*SL* to