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Cytotoxic Effects on Human Cancer Cells and Apoptosis of a Sesquiterpene Lactone from *Saussure lappa*

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Abstract – In order to study the cytotoxic properties of sesquiterpenes, dehydrocostus lactone (DL) and costunolide from *Saussurea lappa*, cytotoxicity was measured by SRB method using various human cancer cell lines. Dehydrocostus lactone (DL) and costunolide exhibited significant cytotoxicity against A-549, SK-OV-3, SK-MEL-2, XF-498 and HCT 15 cells. The U937 human leukemia cells treated with DL showed several apoptotic evidences like chromosome condensation and formation of apoptotic bodies. From the results of FACS analysis, early apoptosis was observed by phosphatidylserine externalization detected by annexin V-FITC. Furthermore, these studies determined hypodiploid contents and effects on the cell phase distribution of DL-treated U937 cells. After exposure of U937 cells to 30 μM DL effectively led to G2/M modified cell cycle distribution within 24 hr. These observations suggest that DL can be used efficiently for the cancer treatment.

Keywords ☐ sesquiterpene lactone, *Saussurea lappa*, cytotoxicity, apoptosis, cell cycle

Saussurea lappa has been used as a traditional remedy and have shown to exhibit anti-ulcer, anti-inflammatory, antiviral effects as well as cytotoxic activities against human tumor cells (Bensky and Gamble, 1986; Chen *et al.*, 1995; Jung *et al.*, 1998; Lee *et al.*, 1995). Many chemicals have been identified and characterized in *S lappa,* which include polyphenols, saussureamines and sesquiterpenes such as costunolide and dehydrocostus lactone (Taniguchi *et al.*, 1993; Yoshikawa *et al.*, 1993). Dehydrocostus lactone (DL) has a simple sesquiterpene structure with α-methylen-γ-lactone ring and has been shown to have a variety of pharmacological effects. Several studies reported this compound as an anti-inflammatory agent by inhibiting the expression of inducible nitric oxide synthase (Lee *et al.*, 1999) and suppressing TNF-α and IL-8 production in LPS activated murine macrophages (Lee *et al.*, 1995; Cho *et al.*, 1998). It has also been demonstrated that DL inhibits hepatitis B virus surface antigen gene expression at the mRNA level (Chen *et al.*, 1995). Although the molecular mechanisms of these actions have not been elucidated in detail, it has been believed that the active site of this compound is α-methylen-γ-lactone ring and most of its biological activities were caused from the interaction of this active site with specific enzyme and/or bioactive protein motif (Schmidt, 1997). In the bioactive screening of this compound we studied significant cytotoxicity against human cancer cells and potent inducing activity of apoptotic death in U937, a line of humam leukemia cell.

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MATERIALS AND METHODS

Plant material

Saussureae Radix, the dried root of *Saussurea lappa* (Compositae) (200g) was purchased from the Kyungdong oriental drug market. The methanolic extract (6.5g) of *Saussurea lappa* was successively partitioned with hexane, EtOAc and BuOH. The EtoAc soluble fraction (0.43g) was chromatographed on a silica gel (20g) column eluted with CH_2Cl_2-acetone (100:1, 70:1 and 50:1, 100 ml each) gradients yielding two cytotoxic compounds. Their structures were elucidated by spectral analysis as dehydrocostus lactone (Mathur *et al.*, 1965) and costunolide (Somasekar *et al.*, 1960).

Cell culture

Human cancer cell lines A549 (lung cancer), SK-OV-3 (ovarian cancer), SK-MEL-2 (skin cancer), and XF498 (CNS cancer) and HCT15 (colon cancer) were obtained from

National Cancer Institute (NCI) in the U.S.A., which were currently used in the NCI's *in vitro* anti-cancer drug screening. Stock cell cultures were grown in T-25 flask containg RPMI-1640 medium containing glutamin, sodium bicarbonate and 5% fetal bovine serum (Gibco BRL, Detroit, MI), penicillin (100 U/ml) and streptomycin (10 μg/ml) and U937 cells were obtained from Dr. Kim (IMBG, Seoul National University) and cultured in RPMI 1640 medium containing 10% fetal bovine serum (Gibco BRL, Detroit, MI), penicillin (100 U/ml) and streptomycin (10 μg/ml). The cells were cultured at 37°C 5% CO_2 in fully humidified air. Exponentially growing cells were used throughout this study.

Cytotoxic activity *in vitro*

All experimental procedures followed the NCI's protocol based on the SRB method (Skehan *et al.*, 1990). Experimental cultures were plated in 96-well plates containing 0.15 ml of growth medium per well with a cell density of 5×10^3 (A549 and HCT15), 1×10^4 (SK-MEL-2 and XF498) and 2×10^4 (SK-OV-3) and incubated in 37°C, humidified 5% CO_2 incubator for one day. Then, media were aspirated off and added the test material in triplicate which was dissolved in media at various concentrations. In case of necessity, the test material was dissolved in small amount of dimethylsulfoxide (DMSO), but the final concentration of DMSO in the medium was not exceed 0.5%. After 2 days of continuous drug exposure, the medium was removed, and cell attached to the plastic substrarum were fixed by gentle layering with 0.1 ml of cold 10% trichloroacetic acid (TCA). It was incubated at 4°C for 1 hour followed by wash with tap water five times to remove TCA solution and dried at room temperature overnight. The TCA fixed cells were stained with 0.1 ml of 0.4% sulforhodamine B (SRB) in 1% acetic acid per well for 30 minutes. At the end of the staining period, SRB supernatant was removed out and the remaining cells were rinsed with 1% acetic acid five times. And then the bound dye in cells was extracted with 0.1 ml of 10 mM unbuffered Tris base (pH 10.5) by stirring on a gyratory shaker for 5~10 minutes, followed by measured the optical density at 520 nm by MR700 microplate reader (Dynetech Laboratories). Antitumor activities of the test material at various concentrations were estimated as the net growth % of cells compared with that of control (without test material, net growth=100%). The dose-response curves of test material were constructed and the ED_{50} values were calculated as the concentration of the test material that caused 50% inhibition of cell growth.

Assessment of apoptosis

Morphological examination

U937 cells were harvested by centrifugation and fixed with 4 % paraformaldehyde and then cells were incubated with 1 ml of staining solution containing 8 μg of Hoechst 33258 (Sigma Co. U.S.A.) in dark place for 5 min. After washing with PBS buffer three times 10 μl of stained cells were placed down on a slide and mounted with a cover glass. Microscopic examination was performed with a reflected light fluorescence microscope (Olympus BH-2, Germany).

Flow cytometric analysis

For the cell cycle distribution experiment, 1×10^6 cells per sample were fixed for 1 hr at 4°C with 1 ml of 70% ethanol. Cells were washed twice with PBS and incubated in the dark with 100 μg/ml propidium iodide (PI) solution and 50 μg/ml RNase A at room temperature for 15 minutes. Analysis was performed with a FACS can flow cytometer (Becton Dickinson, San Joes, USA). According to the user's manual of annexin V FITC apoptosis detection kit (Pharmigen Co. U.S.A.), cells were washed with cold PBS and then resuspend cells in 1 × binding buffer. One hundred microliter of cell suspension was transferred to a 5 ml culture tube. After addition of 5 μl annexin V-FITC and 10 μl of PI solution, cells were gently mixed and incubated for 15 min at room temperature in the dark. Analysis was performed by flow cytometry after the addition of 400 μl of binding buffer to each tube (Becton Dickinson, San Joes, U.S.A.).

RESULTS AND DISCUSSION

The effect of the sesquiterpene lactones on the cell viability of A549, SK-OV-3, SK-MEL-2, XF498 and HCT15 cell lines was determined following treatment of samples using SRB method (Skehan *et al.*, 1990). As shown in Table I, dehydro-

Table I. Antitumoral activity of dehydrocostus lactone and costunolide against human cancer cell lines**

Compounds	ED_{50}*				
	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
Dehydrocostus Lactone	2.97	1.83	0.59	1.70	1.55
Costunolide	1.64	1.65	0.55	0.43	1.16

*ED_{50} value against each cancer cell line was defined as a concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

**A 549 (lung cancer), SK-OV-3 (ovarian cancer). SK-MEL-5 (skin cancer), XF498 (CNS cancer), HCT15 (colon cancer)

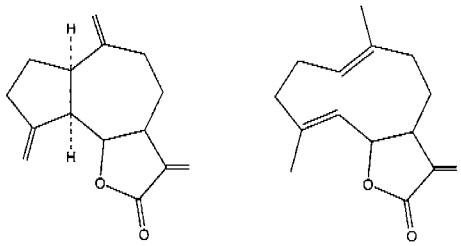

Fig. 1. The structures of dehydrocostus lactone and costunolide from *Saussurea lappa.*

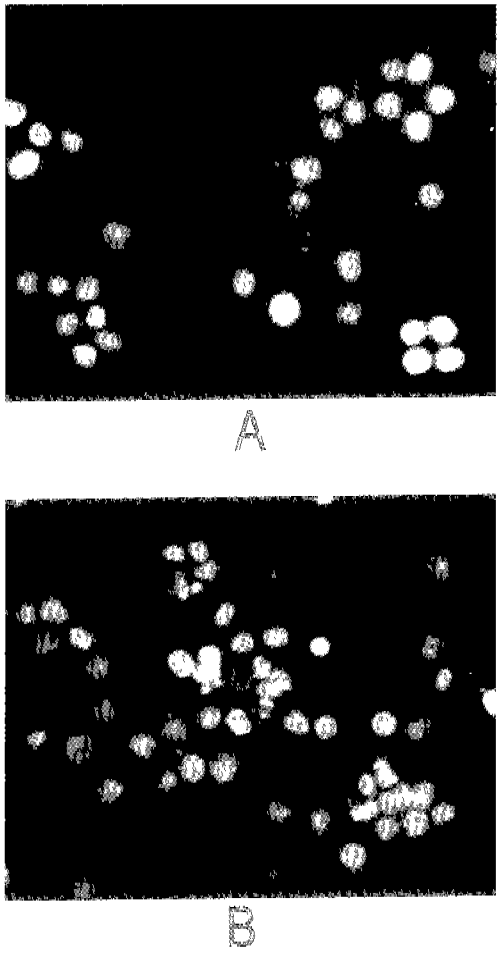

Fig. 2. Morphological features of DL-treated U937 cells. U937 cells were exposed to vehicle (A), or 30 μM DL. After 24 hr, cells were fixed with 4% formaldehyde and stained with Hoechst 33258 followed by fluorescences microscopy (B).

costus lactone and costunolide showed marked activity against each tumor cells with a similar potency. These compounds, especially showed stronger cytotoxicity against SK-MEL-2 cell lines (skin cancer) of their ED_{50} values 0.59 and 0.55 μM, respectively. As a model system for studying of apoptosis by DL, we selected human leukemia cell line U937.

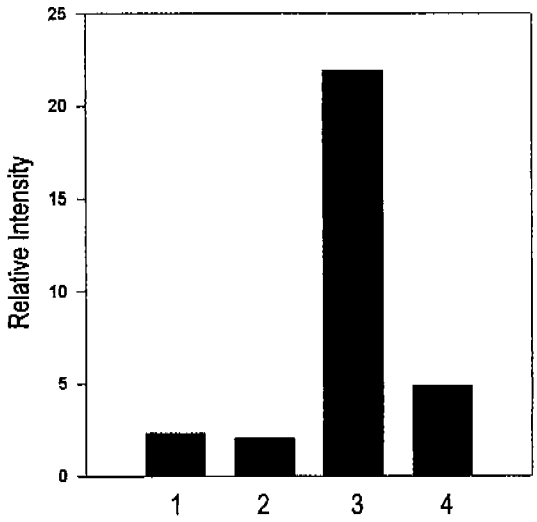

Fig. 3. Induction of early apoptosis by DL in human leukemia cell. annexine V binding to phosphatidylserine were assayed in control cell (1) and DL treated U937 cells, 5 μM for 6 hr (2), 5 μM for 12 hr (3), 30 μM for 24 hr (4).

Because the observation under light microscopy showed that exposure of U937 cells to DL was associated with prominent apoptotic morphology. As shown in Fig. 2, treatment of the cells with 30 μM DL induced cell shirking, condensation of chromatin, and membranous apoptotic bodies within 24 hr. The molecular aspects of apoptosis were assessed by using flowcytometry. In most cell type it has been demonstrated (Martin *et al.*, 1995; Koopman *et al.*, 1994) that phosphatidylserine (PS), a lipid normally confined to the inner leaflet of the plasma membrane, is exported to the outer plasma leaflet in the early stages of apoptosis process. PS externalization is easily detected by staining with a FITC conjugate of annexin V, a protein that has a strong natural affinity for PS, and propidium iodide. Fig. 3 represented the progressive stages of apoptosis induced by treatment with 5 μM DL for 12 hr and at that point, the apoptosis indicies were 21.93%. We also analyzed the action of DL on cell phase distribution for 24 hr after treatments. DL induced a sharp increase in the G2/M phase indicating a blockage in this cell cycle phase (Fig. 4). In the following experiment, flow cytometry analysis of PI labeled cells revealed the appearance of hypodiploid DNA in the DL treated cells. The apoptosis indices were 1.5, 7.7, 22.4 and 14.3% in control, 5 μM for 24 hr, 30 μM for 24 hr and the positive control TNF-α (1000 U/ml) for 24 hr. DL was more potent than TNF-α (1000 U/ml) in inducing apoptosis (Fig. 5). Apoptosis, or programed cell death, is an active process of cellular self destruction (Willie *et al.*, 1980). Although a wide

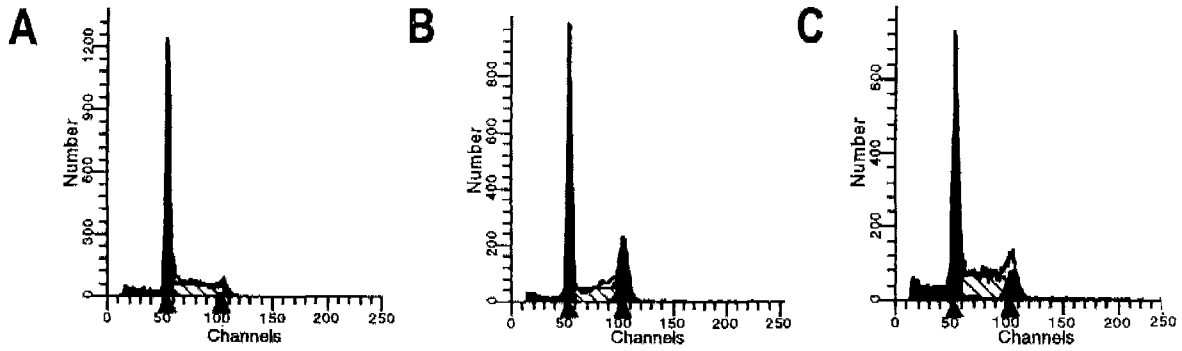

Fig. 4. Percentage of cell phase distribution. Cells treated with DL at 30 μM for 12 hr (B) and 24 hr (C) were measured by flow cytometry after cell staining with PI.

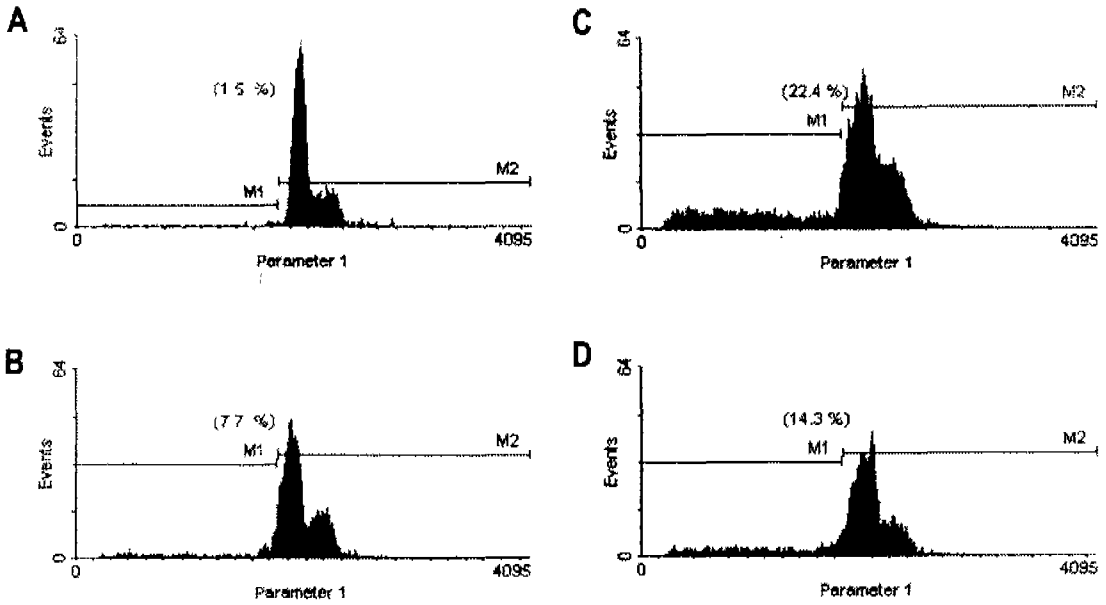

Fig. 5. DL-induced apoptosis of U937 cells as determined by the hypodiploid contents using flow cytometry of PI-stained cells. control (A), 5 μM for 24 hr (B), 30 μM for 24 hr (C), and the positive control TNF-α (1000 U/ml, D) for 24 hr.

variety of stimuli can induce apoptosis, cells undergoing this process exhibit a similar series of changes by stimuli that triggers the process, the cells show a sequences of morphological modification that include nuclei and cytoplasmic condensation with a pronounced decrease in cell volume, chromatic condensation and fragmentation, plasma membrane blebbling and degeneration of the nucleus into membrane bound apoptotic bodies. Chemicals and/or therapy designed to specifically modify the susceptibility of tumor cells to apoptosis provide a new opportunity for the treatment of cancer. Extensive screening of plant extracts has led to the isolation of a large number of sesquiterpene lactones and their derivatives having tumor cytotoxic activities (Jung *et al.*, 1998). Like as several sesquiterpenes isolated from plant sources, these compound showed the possibility as anti-neoclassic agents for human tumor cell. Thapsigargine, a sesquiterpene γ-lactone, which was isolated from the roots of the *Thapsia garganica* showed cytotoxicity against metastatic prostatic cancer (Furuya *et al.*, 1994). Some agents such as helenalin and elephantophin have been reported that they show tumor specific killing as potent alkylating agents (Woerdenbag *et al.*, 1994), and germacranolides from *Carpesium divaricatum* showed significant cytotoxicity against cultured various human tumor cells (Kim *et al.*, 1997). The molecular mechanism of the observed apoptotic effect of DL also remains to be determined. Because induction of cell death by DL was accompanied by G2/M abnormal cell cycle distribution, it is a reasonable suggestion to examine the possibility of topoisomerase inhibition and/or DNA damaging activity. For further understanding of DL-induced apoptosis we now need to

investigate the actions of DL relevant to key molecules which regulate apoptosis.

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