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ORIGINAL ARTICLE

Anticancer activity of *Saussurea lappa* extract by apoptotic pathway in KB human oral cancer cells

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Abstract

Context: Saussurea lappa Dence (Compositae) is used as a traditional herbal medicine to treat abdominal pain and tenesmus in East Asia. Current studies have shown that *S. lappa* has anticancer activity in divergent of cancer cells. However, the effects of *S. lappa* on oral cancer and its mechanisms of action have yet to be elucidated.

Objective: To explore its potential chemotherapeutic effects and mechanism of cell growth inhibition on human oral cancer cells.

Materials and methods: The dried roots of *S. lappa* were used in this study. Cell viability of KB cells was evaluated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay after treatment with 30 μg/ml of methanol extract from the dried roots of *S. lappa*. To understand whether its effect on cell death is related with apoptosis pathway, we performed DNA fragmentation assay, western blot, caspase activity assay and fluorescence-activated cell sorting (FACS) analysis.

Results: Treatment of *S. lappa* extract onto KB cells reduced cell viability significantly with an IC_{50} value of 30 μg/ml. The formation of a DNA ladder was observed starting at the 24 h treatment. In western blotting analysis, the *S. lappa* extract induced the proteolytic processing of caspase-3, -9 and poly (ADP-ribose) polymerase, a significant increase of Bax and marked reduction of Bcl-2. We also confirmed the activation of caspase-3/-7 in living KB cells by fluorescence microscopy.

Conclusion: These results suggested that *S. lappa* extract inhibited cell proliferation through the apoptosis pathway in KB human oral cancer cells.

Keywords

Apoptosis, caspase family, cytotoxic chemotherapy, oral squamous cell carcinoma, traditional herbal medicine

History

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Introduction

Oral squamous cell carcinoma is the most common cancer of the oral and maxillofacial region, with more than 300 000 new cases reported annually worldwide. Based on currently available clinical assessment and treatment methods, patients are often diagnosed at a late stage of the disease, and the 5-year survival rate has remained relatively low (50 ∼ 0%; Schliephake, 2003). Surgical treatment for oral cancer can cause functional and aesthetic impairment, leading to withdrawal and social isolation (Hopper et al., 2004). Complications of radiotherapy can impair wound healing and further complicate surgical salvage after a failed procedure (Bodin et al., 2004). Conventional chemotherapeutic agents have been associated with numerous significant clinical complications, including nausea, hair loss and pancytopenia; thus, alternative and less toxic chemical treatments for oral cancer are required (Yamachika et al., 2004). Therefore, in recognition of nature's potential, several plants screenings have performed since 1960 to replace drugs that have side effects. Today, the taxanes are often the most powerful compounds among all chemotherapeutic drugs, exhibiting a wide range of activity and are of special benefit in fighting metastatic breast, ovarian and lung cancer (Crown et al., 2004; Gligorov & Lotz, 2004).

The dried root of *Saussurea lappa* Dence (Compositae) has been traditionally used for abdominal pain and tenesmus as a traditional medicine in Asia, including Korea and China. *S. lappa* root extract contains resinoids, essential oil, alkaloid, inulin, a fixed oil and other minor constituents such as tannins and sugars (Madhavi et al., 2001). The essential oil of the roots has strong antiseptic, disinfectant and anti-inflammatory properties (Madhavi et al., 2001). Previous study has shown that the ethanol extract of *S. lappa* has anticancer activity (Ko et al., 2004) in AGS gastric cancer cells. Recently, studies reported that the methanol extract of *Gracilaria tenuistipitata* (Yeh et al., 2012) and the hexane extract of *Rheum undulatum* L inhibited oral cancer cell proliferation through apoptosis and

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the down-regulation of specificity protein 1 and surviving (Choi et al., 2011), respectively. However, the effects of *S. lappa* on oral cancer and its mechanisms of action have yet to be elucidated.

One of the hallmarks of cancer is the deregulation of apoptosis (Hanahan & Weinberg, 2000), a universal and efficient cellular suicide pathway. Therefore, increasing apoptosis in cancers can be an effective method for chemopreventive and chemotherapeutic intervention in many types of cancers. Apoptosis, which is a major form of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes (Green & Reed, 1998; Hengartner, 2000; Kaufmann & Hengartner, 2001). Apoptosis can occur *via* a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway and can be induced by various chemotherapeutic agents (Kaufmann & Earnshaw, 2000).

In this study, to explore the possibility that *S. lappa* functions as a chemotherapeutic agent in human oral cancer, we tested the effects of a *S. lappa* extract in inhibiting cell proliferation in KB oral cancer cells and characterized the its underlying mechanisms of action.

Materials and methods

Materials

3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO). Bax, Bcl-2, caspase-3, caspase-9, cleaved caspase-3, cleaved caspse-9 and poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 was purchased from OncoImmunin, Inc. (Gaithersburg, MD).

Plant material and extract preparation

The dried roots of *S. lappa* were purchased from Jeonnam herbal medicine farmer's cooperative (Jeollanam-do, Korea) and identified by Prof. Su-In Cho, School of Oriental Medicine, Pusan National University, Korea. The roots of *S. lappa* (100 g) were ground with a Wiley mill to pass a 1 mm screen and were extracted with 95% methyl alcohol at 40 °C for 5 h. The collected filtrate was dried by evaporation under vacuum at 40 °C using a rotary evaporator (Eyela, Tokyo, Japan). After evaporation, the concentrated extract (3 g) was freeze-dried at −40 °C for 3 days and stored at −20 °C until used. For the treatment, freeze-dried extract was dissolved in 70% dimethyl sulfoxide (DMSO).

Cell culture and reagents

The KB human oral cancer cells were provided by the American Type Culture Collection (Rockville, MD). The cells were incubated in minimum essential medium containing 5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in an atmosphere containing 5% CO_2.

MTT assay

The cell viability test was performed as described previously with minor modifications (Ahn et al., 2008; Park et al., 2008). KB cells were seeded at a density of 5×10^4 cells per well in 12-well plates. After 24 h growth, the cells were treated with methanol extract of *S. lappa* at various concentrations for 24 h and incubation times.

Before testing, the MTT solution (Sigma) was added, and the cells were incubated at 37 °C for 3 h. The culture medium was aspirated, and an acid–isopropanol mixture was added to dissolve the dark blue crystals. The optical density value of the dissolved solute was measured using a microplate autoreader (Winooski, VT) at a wavelength of 570 nm. Three separate experiments were performed for each concentration/exposure time combination.

DNA fragmentation analysis

Approximately 5×10^6 cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K and incubated at 65 °C for 1 h. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and precipitated with ethanol. Genomic DNA was resuspended in Tris-EDTA buffer (pH 8.0) containing 5 μg/ml DNase-free RNase and incubated at 37 °C for 1 h. Genomic DNA was visualized using gel electrophoresis in 1.5% agarose gel (Hayashi et al., 2006).

RNA isolation and reverse transcription polymerase chain reaction analysis

Total RNA was isolated from cell lines using Trizol (Invitrogen) according to the manufacturer's instructions and was reverse-transcribed using the Reverse Transcription System (Promega, Madison, WI). The reverse transcription reaction was performed sequentially for 10 min at 25 °C, for 60 min at 42 °C and for 5 min at 95 °C. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control (Liu et al., 2009). PCR Master Mix reagent (Applied Biosystems, Foster, CA) was used for quantitative polymerase chain reaction (PCR). Primers were designed by Applied Biosystems according to the complementary DNA sequences as follows: Bax (5′-CAG CTG ACA TGT TTT CTG ACG GC-3′, 5′-CTC CCG CCA CAA AGA TGG TCA CG-3′) and Bcl-2 (5′-AGT TCG CCG AGA TGT CCA GGC A-3′, 5′-ACT TGT GGC CCA GAT AGGCAC C-3′). The PCR products were analyzed using gel electrophoresis in 1.2% agarose gel.

Preparation of cell lysates and western blot analysis

The cell pellet was dissolved in lysis buffer (1% Triton-X 100, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and 5 μg/ml leupeptin) and centrifuged for 10 min at 12 000 rpm. Protein concentrations were determined using a BCA Protein Assay Kit (Rockford, IL). The proteins were separated on a 10% SDS-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane followed by western blot analysis. A solution of 5% nonfat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 was used to block nonspecific binding. The membrane was subsequently incubated with antibodies against Bax, Bcl-2, caspase-3, caspase-9, cleaved caspase-3, cleaved

caspse-9 and PARP. After incubation, the blots were extensively washed in TBS containing 0.1% Tween-20. For detection, an ECL kit (Arlington Heights, IL) was used according to the manufacturer's instructions.

Determination of caspase-3/7 activity

The activity of caspase-3/-7 was determined using the cell-permeable fluorogenic substrate PhiPhiLux-G_1D_2 (OncoImmunin), which was used according to the manufacturer's instructions. The cells were incubated for 1 h with PhiPhiLux-G_1D_2. This substrate molecule contains a peptide homo-doubly labeled with a fluorophore. The cleaved substrate has the following excitation and emission peak: excitation = 505 nm and emission = 530 nm. The activity of caspase-3/-7 was visualized by fluorescence microscopy (IX71, Olympus, Tokyo, Japan), as described in the previous study (Kim et al., 2012).

Flow cytometric cell cycle analysis and annexin V-FITC/propidium iodine double staining

The cells were fixed in chilled 75% methanol and stained with a propidium iodine (PI) solution (100 μg/ml RNase and 10 μg/ml PI in phosphate buffered saline) for cell cycle analysis. The cells were stained using a Vybrant® apoptosis assay kit (Molecular Proves, Eugene, OR) followed by labeling with Alexa Fluor® 488 Annexin V and PI for apoptosis analysis. Data acquisition and analysis were carried out using the Cell Lab Quanta™ SC flow cytometer and software (Beckman Coulter Inc., Miami, FL).

Statistical analysis

Data are expressed as the mean ± SEM of at least three individual experiments. Statistical significance was analyzed by using Student's *t*-test for two groups and one way analysis of variance for multi-group comparisons. $p < 0.05$ is considered statistically significant.

Results

The methanol extract of *S. lappa* inhibited the cell proliferation of KB cells

The objective of this study was to investigate potential cytotoxic effect of *S. lappa* extract on KB human oral cancer cells. When treated to KB cells, the methanol extract prepared from the dried root of *S. lappa* showed the growth inhibitory effect in a concentration-dependent manner exhibiting IC_{50} value of 30 μg/ml, approximately (Figure 1A). Time course study at 30 μg/ml concentration revealed that the *S. lappa* extract dramatically decreased cell viability in a time-dependent manner as shown in Figure 1(B).

S. lappa extract induces apoptosis in KB oral cancer cells

To investigate whether *S. lappa* extract causes cell death by apoptosis in KB cells, we evaluated two biochemical events

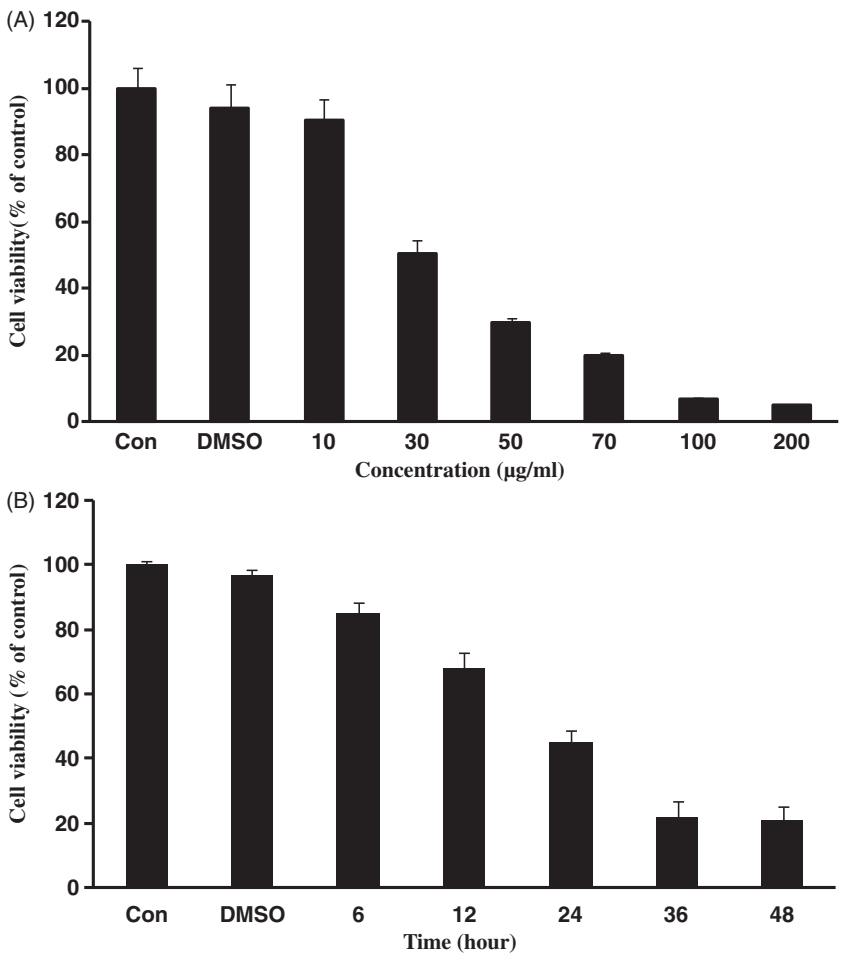

Figure 1. Effect of *Saussurea lappa* extract on cell viability in KB cells. Cells were treated with various concentrations of *Saussurea lappa* extract for 24 h in KB cells (A). 30 μg/ml of *Saussurea lappa* extract treated into cells for different time periods (B). Cell viabilities were determined by the MTT assay. The percentage of cell viability was calculated as a ratio of A570 nm. Results were expressed as percent of the control. Each data point represents the mean ± SEM from three experiments.

specific for apoptosis; genomic DNA laddering, relatively late event in apoptotic pathway and activation of effector molecules. For analyzing the DNA laddering event, KB cells were treated with *S. lappa* extract for 12 and 24 h before preparing genomic DNA. When compared with the control group, 24 h treatment of *S. lappa* extract induced significant fragmentation of genomic DNA (Figure 2A).

In the next series of experiments, we addressed the potential mechanism by which *S. lappa* extract was causing decreased cell viability in KB cells.

Bcl-2 is a member of a large family of cell survival-regulating proteins consisting of both pro- and anti-apoptotic regulators. We therefore determined the level of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) expression in KB cells following treatment with *S. lappa* extract. For both transcriptional (Figure 2B) and translational (Figure 2C) levels, treatment of *S. lappa* extract decreased levels of Bcl-2 in cancer cells, while the level of Bax expression is highly induced after 12-h treatment.

It has been known that the caspase family activation represents one of the earliest known steps in the cell death process. We next explored the mechanism of apoptosis induction by the treatment of *S. lappa* extract in the cancer cells. KB cancer cells exposed to *S. lappa* extract for 12 h exhibited marked activation of caspase-3 and -9 activities (Figure 3A).

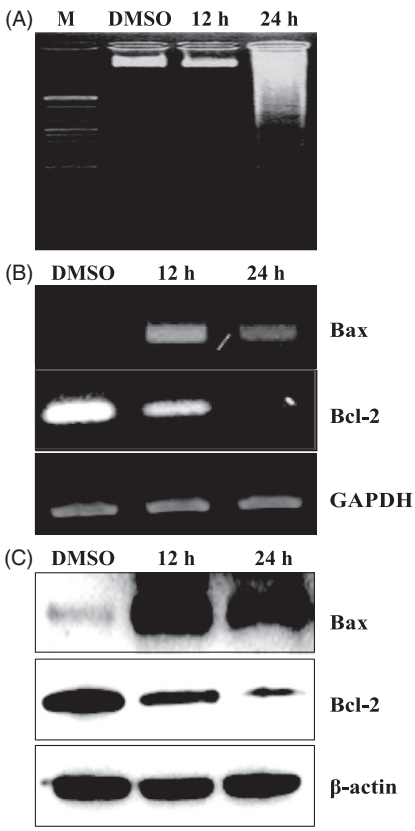

Figure 2. The expression levels of apoptosis-related proteins by treatment with *Saussurea lappa* extract in KB cells. KB cells were seeded at 1×10^6 and were then treated with 30 μg/ml of *Saussurea lappa* extract for the indicated time point (12 h and 24 h). Fragmentation of internucleosomal DNA by *Saussurea lappa* extract treatment in KB cells. Genomic DNA was subjected to 1.5% agarose gel electrophoresis (A). After 12 h, and 24 h *Saussurea lappa* extract treatment, mRNA was determined by RT-PCR (B), and Bax and Bcl-2 protein levels were determined by Western blot analysis (C). Whole cell lysates were separated by 12% SDS-PAGE and probed for Bax, Bcl-2 and β-actin as a loading control.

One of the targets for activated caspase-3 is the DNA repair enzyme PARP. So, we tested the effect of *S. lappa* extract treatment on caspase-3 activity by western blot using a monoclonal antibody against PARP that detects the full length and the cleaved forms of PARP. As expected, the cleavage of PARP protein was evident in *S. lappa* extract-treated cells after 12-h treatment (Figure 3A).

In addition, we also confirmed that the *S. lappa* extract treatment activated the caspase-3/7 in living KB cells by fluorescence microscopy using a cell-permeable fluorogenic caspase-3/7 substrate PhiPhiLux-G_1D_2 (Figure 3B).

Altogether, these findings demonstrate that *S. lappa* extract induces apoptotic cell death in KB oral cancer cells through caspase-3 - and caspase-9-dependent pathways.

Loss of cell membrane asymmetry, detectable by Annexin V staining, represents one of the earliest events in apoptosis. Thus, we performed an Annexin V-PI staining to further demonstrate the *S. lappa* extract-mediated apoptosis. As shown in Figure 4, there was an increase in the number of cell undergoing apoptosis (Annexin V-positive) after treating the cells with 30 μg/ml of *S. lappa* extract. In the DMSO-treated control cells, 8.2% was positive for Annexin V-FITC staining, while *S. lappa* extract treatment resulted in increases of 31.4 and 32.6% for 12 - and 24 h treated cells, respectively.

Discussion

Many plant-derived bioactive constituents, including paclitaxel (from *Taxus brevifolia*), camptothecin (from *Camptotheca acuminata*), podophyllotoxin (from *Podophyllum emodi*) and vinblastine (from *Catharanthus roseus*), have been developed as potential sources of anticancer agents (Hsu et al., 2011). Recent scientific efforts have focused on the potential roles of traditional herbs extracts as alternative and complementary medications for cancer treatment (Kim et al., 2012; Lee et al., 2010; Moon, 2012). Currently, Compelling evidence reported by several investigators suggested different anticancer activities of *S. lappa* extract in both *in vitro* and *in vivo* (Ko et al., 2004, 2005; Sun et al., 2003). However, the effect of the *S. lappa* extract on the growth of KB human oral cancer cell has not been investigated. As shown in Figure 1, *S. lappa* extract inhibited the growth of KB cells in a dose- and time-dependent manner. Therefore, in this study, we intended to elucidate the mechanism of cytotoxic activity of *S. lappa* extract in KB human oral cancer cells.

Apoptosis is characterized by the presence of distinct morphological features and formation of a ladder of genomic DNA fragments (Hu & Kavanagh, 2003). We showed the appearance of a DNA ladder in *S. lappa* extract treated KB cells (Figure 2A). This result suggested that *S. lappa* extract could induce apoptosis in KB cells. Therefore, we performed a series of experiments to identify the mechanism of *S. lappa* extract-induced apoptosis in KB cells. First, we determined the effects of *S. lappa* extract on the level of Bcl-2 family proteins. In a previous study, the cytotoxic effects of *S. lappa* extract on AGS gastric cancer cells were attributed to the regulation of pro-apoptotic factors including Bax, Bad and anti-apoptotic

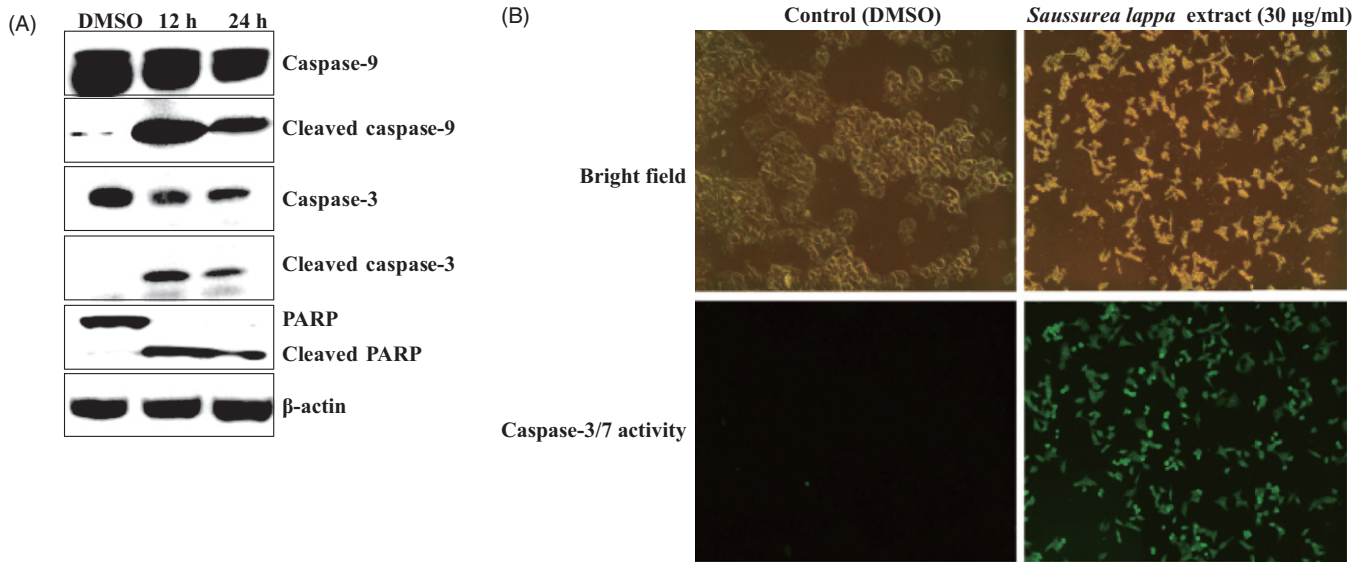

Figure 3. *Saussurea lappa* extract caused caspase-3-dependent apoptosis. Caspase-9, cleaved caspase-9, caspase-3, cleaved caspase-3, PARP and cleaved PARP protein levels were determined by western blot analysis. Whole-cell lysates (50 μg/lane) were subjected to immunoblotting for the indicated proteins. Probing with β-actin was used to show equal protein loading (A). Activation of caspase-3/7 by *Saussurea lappa* extract treatment in living KB cells. The cells were treated with 30 μg/ml of *Saussurea lappa* extract for 24 h and followed by adding specific cell-permeable substrate Phiphilux G_1D_2. Caspase-3/7 activity was visualized by fluorescence microscopy (B).

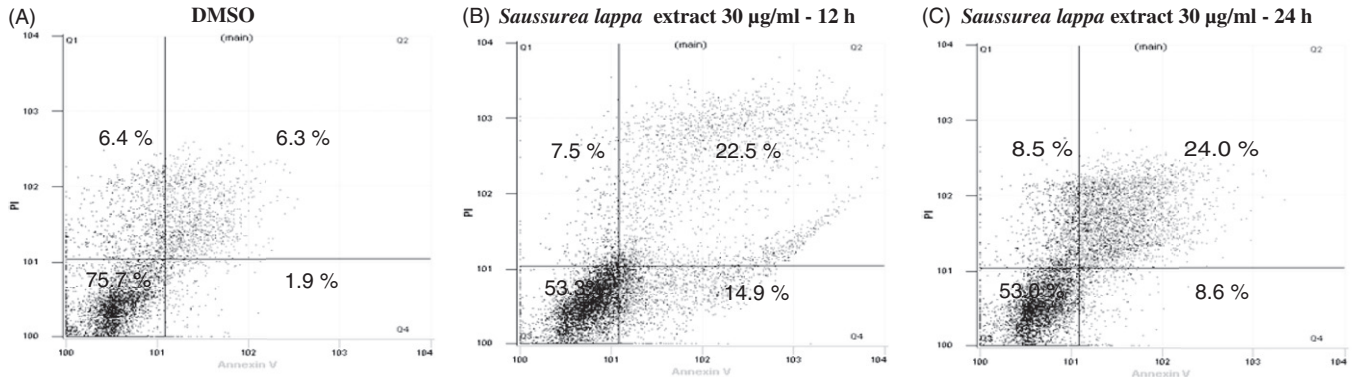

Figure 4. *Saussurea lappa* extract induced apoptosis in KB cells. Cells were treated with 30 μg/ml of *Saussurea lappa* extract for 12 h and 24 h. The cells were stained with Annexin V-FITC and propidium iodine (PI). The apoptotic cells were then analyzed by fluorescence-activated cell sorting (FACS) analysis. This apoptotic data was determined by FACS analysis showing the percentages of lower right quadrant for early and upper right quadrant for late apoptotic cells.

factor including Bcl-2 and Bcl-xl (Ko et al., 2005). In this study, we showed that the treatment of *S. lappa* extract to KB cancer cells induces Bax in both transcriptional (Figure 2B) and translational (Figure 2C) levels. In contrast, Bcl-2 level was gradually decreased in a time-dependent manner. The pro-apototic Bcl-2 family increases mitochondrial membrane permeability thereby induces the activation of initiator caspase. The activation of a family of intracellular cysteine proteases (caspases) is known to play an important role in the initiation and execution of apoptosis induced by various stimuli (Datta et al., 1997; Liu et al., 1997). Among the caspases identified in mammalian cells, caspase-3, -7 and -9 may serve as effectors of apoptotic cells (Cohen, 1997). Thus, we evaluated the effect of *S. lappa* extract on the regulation of caspase activity. The results strongly suggest that its anti-proliferative effect on KB cancer cells is through the induction of initiator capase-9, which in turn activates the executioner caspase-3 (Figure 3A and B). Moreover, we showed that PARP, a cellular substrate of activated caspase-3, is efficiently cleaved, and the Annexin V-FITC positive cells are increased upon treatment with *S. lappa* extract. Altogether, our findings demonstrate that *S. lappa* extract exhibits anti-proliferative effect by inducing caspase-3-dependent apoptosis pathway in KB oral cancer cells

In line with our findings, a previous study showed that costunolide, isolated from the root of *S. lappa*, is a potent inducer of apoptosis, and facilitates its activation *via* reactive oxygen species generation in HL-60 human leukemia cells (Lee et al., 2001). Other investigators also reported that *S. lappa* extract induces apoptotic cell death in AGS human gastric cancer cells (Ko et al., 2004). The powder formulation of *S. lappa* extract is being used for the treatment of gastric cancers either by traditional herbal therapy or combinational therapy (Ko et al., 2005). Therefore, current findings should facilitate further work to examine the beneficial effects of *S. lappa* extract on cancer therapy and to elucidate its mechanism of action.

Conclusions

This study demonstrates that methanol extract of *S. lappa* inhibits cell growth and induces apoptosis in KB human oral cancer cells. The results imply that *S. lappa* extract could be a promising traditional herbal medicine for potential use in oral cancer therapy and that search for new compounds capable of inducing apoptosis is a promising strategy for oral cancer treatment.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. This study was supported by research fund from Chosun University 2010.

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