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Crude extract and solvent fractions of *Calystegia soldanella* induce G1 and S phase arrest of the cell cycle in HepG2 cells

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Abstract. The representative halophyte *Calystegia soldanella* (L) Roem. et Schult is a perennial vine herb that grows in coastal dunes throughout South Korea as well as in other regions around the world. This plant has long been used as an edible and medicinal herb to cure rheumatic arthritis, sore throat, dropsy, and scurvy. Some studies have also shown that this plant species exhibits various biological activities. However, there are few studies on cytotoxicity induced by *C. soldanella* treatment in HepG2 human hepatocellular carcinoma cells. In this study, we investigated the viability of HepG2 cells following treatment with crude extracts and four solvent-partitioned fractions of *C. soldanella*. Of the crude extract and four solvent fractions tested, treatment with the 85% aqueous methanol (aq. MeOH) fraction resulted in the greatest inhibition of HepG2 cell proliferation. Flow cytometry showed that the 85% aq. MeOH fraction induced a G0/G1 and S phase arrest of the cell cycle progression. The 85% aq. MeOH fraction arrested HepG2 cells at the G0/G1 phase in a concentration-dependent manner, and resulted in decreased expression of cyclin D1, cyclin E, cyclin-dependent kinase (CDK)2, CDK4, CDK6, p21, and p27. Additionally, the 85% aq. MeOH fraction treatment also arrested HepG2 cells in the S phase, with decreased expression of cyclin A, CDK2, and CDC25A. Also, treatment with this fraction reduced the expression of retinoblastoma (RB) protein and the transcription factor E2F. These results suggest that the 85% aq. MeOH fraction exhibits potential anticancer activity in HepG2 cells by inducing G0/G1 and S phase arrest of the cell cycle.

Introduction

Cancer is an obstinate disease with high morbidity and mortality, and the incidence rate is predicted to increase over the coming years (1). Although various therapies have been developed for the treatment of cancer, the mortality rate remains high (2,3). Hepatocellular carcinoma is one of the most common gastrointestinal malignancies. It is the sixth-leading cause of cancer-related death in the United States (4). The prognosis of hepatocellular carcinoma is poor due to high malignancy. Although biochemical and clinical studies have led to significant advances, the 7-year (2004-2010) survival rate remains <18% (4). Most of the poor prognoses were associated with recurrence and metastasis following treatment, including curative resection (5). Common treatments including surgery, chemotherapy, radiotherapy, interventional treatment and liver transplantation could only provide limited clinical results (6). Traditional chemotherapy and radiotherapy cause intrinsic and potential cytotoxicity in normal cells, and extended use of these therapies can lead to drug resistance and side effects, such as hair loss, vomiting, nausea, and the occurrence of secondary cancers (1). Due to the limitations of conventional therapies, it is important to find safer, more targeted anticancer agents. New outstanding strategy for cancer chemoprevention and chemotherapeutic are also required to including the cell cycle arrest and apoptosis of cancer cells that grow abnormally by deregulating the cell cycle control. Recently, there has been growing interest in searching for novel and effective anticancer agents from natural sources, especially from marine organisms (7-19).

Halophytes are plants that tolerate high salt concentrations, and can grow in salt marshes, mangrove swamps, seashores, coastal sand dune regions, and estuarine environments (20). Environmental stress and ecological factors, such as drought, salt spray, floods, high temperature, low capillary water holding activity of sandy soil, low nutrients, and water availability affect the plant's metabolism and survival (21-23). In these environments, halophytes need to conform to develop stress adaptation responses for survival. As a result, these salt marsh plants are predicted to be an important source in the search for novel and unique bioactive secondary metabolites (24-30).

Calystegia soldanella (L) Roem. et Schult (Convolvulaceae), a representative halophyte and endemic plant, is found on

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coastal sand dunes and foredunes where the environmental stresses are significant. These plants are perennial vine herbs with ubiquitous distribution in the coastal dune areas of South Korea, East Asia, Europe, and the Pacific (31). This plant has long been used as an edible and medicinal herb to cure rheumatic arthritis, sore throat, dropsy, and scurvy (32). Some studies have shown that this plant species exhibits various biological activities. Another species, *C. japonica*, which has been used as a traditional medicine to treat urination problems, fever, or diarrhea in Chinese and oriental herb medicine (33,34). Moreover, *C. soldanella* has been shown to exhibit a number of biological activities, including anti-inflammatory, antiviral, antifungal, anticancer, and analgesic properties, and more specifically, inhibition of protein tyrosine phosphate 1B (PTP1B) (35-42). Methanol extracts of *C. soldanella* decreased NO production, iNOS protein, and mRNA expression in LPS-activated Raw 264.7 cells (35). Water extracts of *C. soldanella* induced anti-inflammatory and analgesic effects in mice (36). Alkyl *p*-coumarates of an *n*-hexane fraction from a *C. soldanella* extract inhibited PTP1B activity *in vitro* (37). Resin glycosides from *C. soldanella*, calysolins V-IX, X-XIII, and XIV-XVII, induced antiviral activity against the herpes simplex virus type 1 (HSV-1) (39-41,43-46). An active fraction of *Ipomoea carnea* subsp. fistulosa (Convulvulaceae) induced antifungal activity in *Colletotrichum gloeosporioides* and *Cladosporium cucumerinum* (42).

Active components from *C. soldanella* are nortropane alkaloids, anthocyanin, coumaric acids, and flavonoids (47-50). Moreover, chloroform extracts showed both cytotoxic activities [ED_{50} 2 μg/ml in UISO (squamous cell cervix carcinoma); ED_{50} 7 μg/ml in KB (nasopharyngeal carcinoma)] and antibacterial (MIC 14.7 μg/ml in *Bacillus subtilis*) (43,44). Methanol extract also exhibited potential cytotoxicity against A549 lung (IC_{50} 8.0 μg/ml) and Col2 colon (IC_{50} 27.4 μg/ml) cancer cells (38). However, studies of the anticancer effect of *C. soldanella* have not been extensive focused on cytotoxicity. To find active components with anticancer activity, this study investigated the cytotoxic activity of crude extract and four solvent-partitioned fractions of *C. soldanella* in HepG2 human hepatocellular carcinoma cells. Furthermore, the 85% aqueous methanol (aq. MeOH) fraction, which exhibited the greatest cytotoxic effect, was evaluated for cell cycle distribution and the expression of several cell cycle checkpoint proteins.

Materials and methods

Plant material. The C. *soldanella* whole plant was collected from Gijang, Busan, Korea in July, 2013 by Professor Y. Seo. A voucher specimen was deposited at the Herbarium of the Division of Marine Environment and Bioscience, Korea Maritime and Ocean University, Korea. The collected sample was briefly air-dried under shade, chopped into small pieces, ground into a powder, and stored at -25˚C.

Extraction and fractions. Samples (800 g) were extracted for 2 days with methylene chloride (CH_2Cl_2; 10 L x 2) and methanol (MeOH; 10 L x 2). The combined crude extracts (106.51 g) were evaporated under reduced pressure and partitioned between CH_2Cl_2 and water. The organic layer was further partitioned into *n*-hexane (19.19 g) and 85% aq. MeOH (22.47 g). The aqueous layer was also fractionated with *n*-butanol (BuOH; 10.48 g) and water (57.66 g), successively.

Cell culture. The HepG2 human hepatocellular carcinoma cells (ATCC HB-8065) were obtained from the American Type Culture Collection (ATCC; MD, USA). Cells were cultured in modified essential medium (MEM) supplemented with 10% fetal bovine serum containing 50 μg/ml penicillin, 25 μg/ml amphotericin B, and 50 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C. The medium was changed 2 or 3 times every week.

Cell viability assay. Cell viability was evaluated using the CytoX cell viability assay kit (LPS solution, Daejeon, Korea). The cells were seeded at a density of $1x10^5$ cells/well in a 96-well plate. After 24 h, the cells were washed with serum-free medium (SFM) for 4 h and the media were replaced with fresh SFM containing different concentrations of samples. After 24 h of incubation, 20 μl of CytoX solution was added to each well and incubated for 4 h. The amount of formazan crystals was determined by measuring the absorbance at 450 nm using a FilterMax F5 microplate reader (Molecular Devices LLC, CA, USA). Cell viability was estimated by comparison with the relative absorbance value of the untreated sample.

Cell cycle analysis. Cells were seeded at a density of $1x10^4$ cells/well and treated with different concentrations of sample for 24 h. Control and treated cells were harvested, washed in cold phosphate-buffered saline (PBS), fixed in 70% ethanol, and stored at 4˚C. The resulting cells were stained with 200 μl of Muse cell cycle reagent at room temperature for 30 min in the dark prior to analysis. DNA content was assessed with the Muse cell analyzer (EMD Millipore Co., CA, USA).

Western blot analysis. Following treatment with different concentrations of samples, cells were washed twice with PBS and lysed in RIPA buffer [1% Nonidet™ P-40, 1 mM EDTA, 50 mM Tris (pH 7.4), 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF]. The cell lysates were centrifuged at 12,000 rpm for 15 min at 4˚C and the supernatants were collected. The protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Inc., IL, USA). The proteins were treated with SDS sample buffer and heated at 95˚C for 10 min. The protein samples were separated by 12% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore Corp., MA, USA). The membranes were blocked by incubation with 1% bovine serum albumin (BSA) in Tris-buffered saline-Tween-20 [TBS-T; 10 mM Tris-HCl, 150 mM NaCl (pH 7.5) containing 0.1% Tween-20] at room temperature for 1 h and incubated for 3 h with primary antibodies against GAPDH, cyclin D, cyclin E, cyclin A, cyclin-dependent kinase (CDK)2, CDK4, CDK6, CDC25A, p21, p27, retinoblastoma (RB), and E2F (Santa Cruz Biotechnology, Inc., TX, USA). The membranes were washed three times with TBS-T and incubated for 2 h with the appropriate HRP-conjugated goat anti-rabbit, goat anti-mouse, or rabbit anti-goat secondary antibodies (Santa Cruz Biotechnology, Inc.) diluted to 1:10,000 in

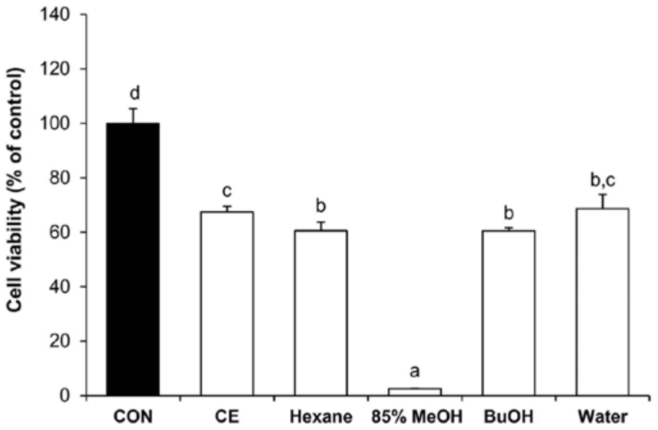

Figure 1. Cell viability of HepG2 cells following treatment with 50 µg/ml crude extract or solvent fractions of *Calystegia soldanella*. The effect of treatment of crude extract and solvent fractions of *C. soldanella* on cell viability was measured in HepG2 cells by CytoX assay. Cells were treated with a concentration of 50 µg/ml crude extract or solvent fractions of *C. soldanella*. Data are presented as the mean ± standard deviation (SD) from three independent experiments. The different letters represent significant differences ($p<0.05$) as determined by Duncan's multiple range test.

TBS-T with 1% BSA. The respective proteins were detected using a chemiluminescent substrate (Advansta, CA, USA) and visualized on a GeneSys imaging system (SynGene Synoptics, Ltd., London, UK).

Statistical analysis. The data are presented as mean ± standard deviation (SD). Differences between the means of the individual groups were analyzed using an analysis of variance (ANOVA) with Duncan's multiple range tests performed in SPSS software (SPSS Inc., IL, USA). A p-value <0.05 was considered to indicate statistical significance.

Results

Crude extracts and solvent fractions of C. soldanella decrease the viability of HepG2 cells. Effects of the crude extract and the four solvent fractions of C. *soldanella* on the proliferation of HepG2 cells were examined using the CytoX cell viability assay kit. As shown in Fig. 1, the growth of HepG2 cells was inhibited at a concentration of 50 µg/ml. The crude extract inhibited cell proliferation by 37%. The crude extract was fractioned into *n*-hexane, 85% aq. MeOH, *n*-BuOH, and water soluble fractions, treatment which inhibited proliferation by 39, 97, 40 and 31%, respectively. Of the four solvent fractions tested, the 85% aq. MeOH fraction caused the greatest inhibition of HepG2 cell proliferation.

The 85% aq. MeOH fraction from C. soldanella decreases the viability of HepG2 cells. To determine the effect of the 85% aq. MeOH fraction from *C. soldanella* on the viability of HepG2 cells, the cells were treated with 3, 6, 12, 25, or 50 µg/ml of the 85% aq. MeOH fraction for 24 h. As shown in Fig. 2, treatment with 85% aq. MeOH reduced the viability of HepG2 cells in a concentration-dependent manner compared with the control group (3 µg/ml, 95.9%; 6 µg/ml, 90.6%; 12 µg/ml, 71.1%; 25 µg/ml, 20.8%; and 50 µg/ml, 22.7%). In subsequent experiments, cells were treated with 3, 6, or 12 µg/ml of the 85% aq. MeOH fraction from *C. soldanella* for 24 h.

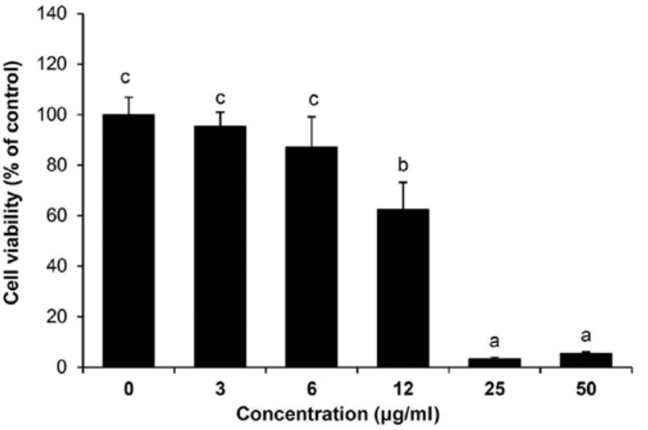

Figure 2. Cell viability of HepG2 cells following treatment with the *C. soldanella* 85% aqueous methanol (aq. MeOH) fraction. The effects of treatment with the 85% aq. MeOH fraction from *C. soldanella* on cell viability were determined in HepG2 cells by CytoX assay. Cells were treated with the indicated concentrations of the 85% aq. MeOH fraction of *C. soldanella*. Data are presented as the mean ± SD from three independent experiments. The different letters at all concentrations represent significant differences ($p<0.05$) as determined by Duncan's multiple range test.

Table I. Induction of G0/G1 and S arrest in HepG2 cells following treatment with the 85% aq. MeOH fraction of *C. soldanella*.

	% of cells		
Concentration (µg/ml)	G0/G1	S	G2/M
0	60.47±0.85^a	12.87±0.21^a	25.20±0.87^c
3	63.80±1.42^b	14.57±0.71^b	19.60±1.37^b
6	64.97±0.90^b	16.10±2.16^c	15.43±0.31^a
12	69.60±3.44^c	16.77±1.59^c	16.40±0.70^a

The cells were treated with the indicated concentrations of the 85% aq. MeOH fraction from *C. soldanella* for 24 h. The cells were collected, fixed, and stained with propidium iodide for flow cytometric analysis. The different letters at all concentrations represent significant differences ($p<0.05$) as determined by Duncan's multiple range test.

The 85% aq. MeOH fraction from C. soldanella induces a G0/G1 and S arrest in HepG2 cells. Flow cytometric analysis of the cell cycle of HepG2 cells showed that the number of cells in G0/G1 phase significantly increased from 60.47±0.85% in the control group to 63.80±1.42, 64.97±0.90 and 69.60±3.44% in the groups treated with the various concentrations of the C. *soldanella* 85% aq. MeOH fraction (Table I). In addition, the number of cells in S phase significantly increased from 12.87±0.21% in the control group to 14.57±0.70, 16.10±2.16 and 16.77±1.59% in the groups treated with the *C. soldanella* 85% aq. MeOH fraction. The population of HepG2 cells in G2/M was significantly reduced following treatment with the

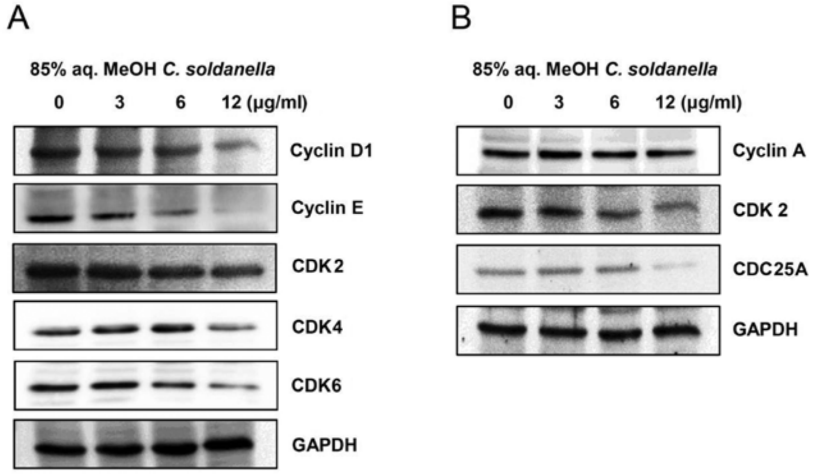

Figure 3. Downregulation of G0/G1 and S phase-associated cyclins and CDKs in HepG2 cells following treatment with the 85% aq. MeOH fraction of *C. soldanella*. (A) HepG2 cells were treated with the indicated concentrations of the 85% aq. MeOH fraction from *C. soldanella* for 24 h. The cell lysates were separated, and equal amounts of total cell lysate were subjected to SDS-PAGE analysis. G0/G1-associated protein levels of cyclin D1, cyclin E, CDK2, CDK4, and CDK6 were examined by western blotting. The bands were normalized to an internal control, GAPDH. (B) S phase-associated protein levels of cyclin A, CDK2, and CDC25A were examined by western blotting. Data are presented as the mean ± SD (p<0.05) from three independent experiments.

85% aq. MeOH fraction from *C. soldanella*. These results suggest that treatment with the *C. soldanella* 85% aq. MeOH fraction arrests HepG2 cells in the G0/G1 and S phases of the cell cycle, and that the reduced viability of HepG2 cells following treatment with the 85% aq. MeOH fraction is likely the result of these cell cycle blocks.

The 85% aq. MeOH fraction from C. soldanella regulates cell cycle checkpoint proteins in HepG2 cells. To investigate the cell cycle arrest induced by the 85% aq. MeOH fraction from *C. soldanella* in HepG2 cells, the expression of G0/G1 phase cell cycle checkpoint proteins, including cyclin D1, cyclin E, CDK2, CDK4, and CDK6, was examined. As shown in Fig. 3A, the 85% aq. MeOH fraction of *C. soldanella* significantly decreased the protein levels of cyclin D1, cyclin E, CDK2, CDK4 and CDK6.

Treatment with 3, 6, or 12 μg/ml of the C. *soldanella* 85% aq. MeOH fraction significantly reduced cyclin D1 (81.9, 64.2 and 23.5%) and cyclin E (62.5, 50.4 and 24.0%) expression in a concentration-dependent manner. Also, treatment with 3, 6, or 12 μg/ml of the 85% aq. MeOH fraction reduced CDK4 expression in HepG2 cells compared with the control group by 114.1, 109.7 and 78.5%, respectively. Moreover, treatment with 3, 6, or 12 μg/ml of the 85% aq. MeOH fraction reduced CDK6 expression in HepG2 cells compared with the control group by 96.3, 68.7 and 46.5%, respectively.

To investigate the cell cycle arrest of HepG2 cells induced by treatment with the 85% aq. MeOH fraction from *C. soldanella*, the expression of S phase cell cycle checkpoint proteins, including cyclin A, CDK2, and CDC25A, was examined. As shown in Fig. 3B, the 85% aq. MeOH fraction from *C. soldanella* significantly decreased the protein levels of cyclin A, CDK2, and CDC25A. In particular, treatment with 3, 6, or 12 μg/ml of the *C. soldanella* 85% aq. MeOH fraction resulted in significantly reduced CDK2 expression in a concentration-dependent manner with values of 78.5, 56.8 and 47.5%, respectively.

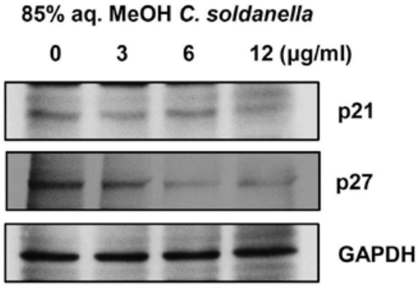

Figure 4. Effects of the 85% aq. MeOH fraction of *C. soldanella* on the level of CDK inhibitors in HepG2 cells. The cells were treated with the indicated concentrations of the 85% aq. MeOH fraction from *C. soldanella* for 24 h. The cell lysates were separated, and equal amounts of total cell lysate were subjected to SDS-PAGE analysis. Protein levels of p21 and p27 were examined by western blotting. Data are presented as the mean ± SD (p<0.05) from three independent experiments.

Treatment with the C. soldanella 85% aq. MeOH fraction decreases the expression of CDK inhibitors in HepG2 cells. Cyclin D/CDK4/6 and cyclin E/CDK2 complexes are important for the cell cycle transition from G1 into S phase, and these complexes are negatively regulated by CDK inhibitors, such as p21 and p27. As shown in Fig. 4, treatment with the 85% aq. MeOH fraction of *C. soldanella* significantly decreased the expression of p21 and p27.

Treatment with 3, 6, or 12 μg/ml of the 85% aq. MeOH fraction from *C. soldanella* significantly reduced p21 expression in a concentration-dependent manner, with values of 85.6, 86.1 and 70.4%, respectively. Also, treatment with 3, 6, or 12 μg/ml of the 85% aq. MeOH fraction from *C. soldanella* reduced p27 expression in HepG2 cells compared with the control group by 89.4, 74.3 and 50.9%, respectively.

Treatment with the 85% aq. MeOH fraction of C. soldanella downregulates RB phosphorylation and E2F expression in HepG2 cells. As cyclin D and cyclin E-induced CDK activity converges in hyperphosphorylation of the RB protein, the

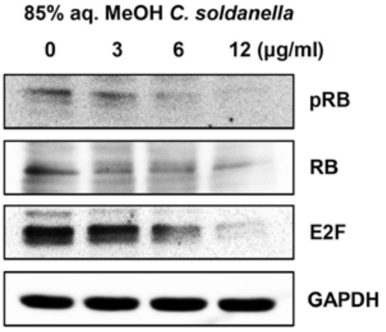

Figure 5. Effects of the 85% aq. MeOH fraction of *C. soldanella* on the level of pRB, RB, and E2F in HepG2 cells. The cells were treated with the indicated concentrations of the 85% aq. MeOH fraction from *C. soldanella* for 24 h. The cell lysates were separated, and equal amounts of total cell lysate were subjected to SDS-PAGE analysis. Protein levels of pRB, RB, and E2F were examined by western blotting. Data are presented as the mean ± SD ($p<0.05$) from three independent experiments.

effect of treatment with the 85% aq. MeOH fraction from *C. soldanella* on the phosphorylation status of RB was examined using western blotting. As shown in Fig. 5, treatment with the 85% aq. MeOH fraction significantly decreased the expression of phosphorylated RB (pRB) and RB.

E2F is an important transcription factor for cell cycle progression from G1 to S phase and DNA synthesis. The effect of the 85% aq. MeOH fraction from *C. soldanella* on the level of E2F was examined. Treatment with the *C. soldanella* 85% aq. MeOH fraction significantly reduced the expression of E2F. In particular, treatment with 3, 6, or 12 μg/ml of the 85% aq. MeOH fraction resulted in significantly reduced E2F expression in a concentration-dependent manner, with values of 84.6, 65.1 and 42.1%, respectively.

Discussion

We screened cytotoxicity of crude extract from *C. soldanella* against various human cancer cell including HepG2 hepatocellular, AGS gastric, HT-29 colon, and MCF-7 breast cancer cell in 50 μg/ml concentration. As a result, the cytotoxicity against HepG2 (37%) and HT-29 (36%) cancer cells was the greater compared to AGS (27%) and MCF-7 (14%) cancer cells (data not shown). This study reports the anticancer effect in HepG2 human cancer cells for the first time.

The purpose of this study was to investigate the viability of crude extracts and four solvent-partitioned fractions from *C. soldanella* in HepG2 human hepatocellular carcinoma cells. *C. soldanella* was extracted with methylene chloride and methanol, and the combined extract was partitioned into the *n*-hexane, 85% aq. MeOH, *n*-BuOH, and water fractions. The crude extract and four solvent fractions were examined using a cell viability assay, in which the 85% aq. MeOH fraction showed the greatest inhibition of proliferation in HepG2 cells at a concentration of 50 μg/ml (97% compared with the control group; Fig. 1). The effect of the 85% aq. MeOH fraction from *C. soldanella* on cell viability was examined in HepG2 cells. Treatment with the *C. soldanella* 85% aq. MeOH fraction reduced viability concentration-dependently (Fig. 2).

Apoptosis (regulated cell death), occurs during normal homeostasis, disease, and development, and is characterized by morphological changes, including cell shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation, and an increase in the population of sub-G1 cells (51,52). The 85% aq. MeOH fraction of *C. soldanella* induced apoptotic nuclear morphological changes in HepG2 cells. Thus, the 85% aq. MeOH fraction increased the rate of apoptosis compared with the control (12 μg/ml, 44.27%; and control, 20.85%; data not shown).

Because treatment with the 85% aq. MeOH fraction resulted in the greatest inhibition of cell growth, we evaluated the cell cycle distribution and expression of cell cycle checkpoint proteins. As shown in Table I, treatment with the *C. soldanella* 85% aq. MeOH fraction induced G0/G1 arrest (12 μg/ml, 69.60%; and control, 60.47%) and S phase arrest (12 μg/ml, 16.77%; and control, 12.87%) in HepG2 cells. Therefore, our results suggest that treatment with the 85% aq. MeOH fraction reduces cell growth of HepG2 cells through cell cycle arrest in G0/G1 and S phase and induces apoptosis.

Cancer cells exhibit deregulation of the cell cycle, increased apoptosis, and activation of signaling pathways that result in abnormal growth. Cyclins and CDKs are critical for appropriate regulation of the cell cycle, and altered formation of cyclin/CDK complexes has been shown to increase or decrease cell growth and affect proliferation and/or differentiation by apoptosis (53,54). Cyclin D/CDK4/6 complexes and cyclin E/CDK2 complexes are critical factors for progression through the G0/G1 phase of the cell cycle. These factors are negatively regulated by CDK inhibitors, such as p21 and p27 (55,56). To investigate the cell cycle arrest induced by treatment with the 85% aq. MeOH fraction from *C. soldanella* in HepG2 cells, expression of the G0/G1 phase cell cycle proteins, including cyclin D1, cyclin E, CDK2, CDK4, CDK6, p21, and p27, was examined. As shown in Figs. 3A and 4, the 85% aq. MeOH fraction of *C. soldanella* significantly decreased the protein levels of cyclin D1, cyclin E, CDK2, CDK4, CDK6, p21, and p27.

Cyclin A, CDK2, and CDC25A are important factors for the S phase of the cell cycle. CDC25A is activated by cyclin A/CDK2 complexes. These complexes allow for progression of the cell cycle, and increased expression of CDC25A promotes cell growth (57,58). We have demonstrated that treatment with the *C. soldanella* 85% aq. MeOH fraction significantly decreased the protein levels of cyclin A, CDK2, and CDC25A (Fig. 3B).

The cell cycle proteins E2F and pRB are known to play important roles in cell cycle progression from G1 to S phase. Dephosphorylation of RB inhibits cell cycle progression by interacting with transcription factors of the E2F family, but phosphorylation of RB induces cell cycle progression by reducing pRB/E2F complexes (55,56). We showed that treatment with the 85% aq. MeOH fraction decreased expression of E2F and pRB, thus inhibiting the G1-S phase transition in HepG2 cells (Fig. 5). Overall, the *C. soldanella* 85% aq. MeOH fraction exhibited anticancer activity in HepG2 cells by blocking the G0/G1 and S phases of the cell cycle and by decreasing the expression of important cell cycle check point proteins.

Previous studies have investigated the potential cytotoxic effects of MeOH and chloroform extracts from *C. soldanella* against human cancer cells, including A549 lung cancer cells and Col2 colon cancer cells (38). This report reveals for the first time the anticancer effect in HepG2 human hepatocellular carcinoma cells. The 85% aq. MeOH fraction from *C. soldanella* should be considered for its therapeutic potential in hepatocellular cancer treatment. It will be necessary to identify the components of the 85% aq. MeOH fraction with high performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), and mass spectroscopy (MS). Determining the composition of the *C. soldanella* 85% aq. MeOH fraction is important.

Acknowledgements

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