Dichloromethane fractions of *Calystegia soldanella* induce S-phase arrest and apoptosis in HT-29 human colorectal cancer cells

IN-HYE KIM[1*], TAEKIL EOM[1*], JOON-YOUNG PARK[2], HYUNG-JOO KIM[1] and TAEK-JEONG NAM[1]

[1]Future Fisheries Food Research Center, Institute of Fisheries Sciences, Pukyong National University, Busan 46041; [2]Department of Food Science and Nutrition, Pukyong National University, Busan 48513, Republic of Korea

Received September 8, 2021; Accepted November 11, 2021

DOI: 10.3892/mmr.2021.12576

Abstract. *Calystegia soldanella* is a halophyte and a perennial herb that grows on coastal sand dunes worldwide. Extracts from this plant have been previously revealed to have a variety of bioactive properties in humans. However, their effects on colorectal cancer cells remain poorly understood. In the present study, the potential biological activity of *C. soldanella* extracts in the colorectal cancer cell line HT-29 was examined. First, five solvent fractions [n-hexane, dichloromethane (DCM), ethyl acetate, n-butanol and water] were obtained from the crude extracts of *C. soldanella* through an organic solvent extraction method. In particular, the DCM fraction was demonstrated to exert marked dose- and time-dependent inhibitory effects according to results from the cell viability assay. Data obtained from the apoptosis assay suggested that the inhibition of HT-29 cell viability induced by DCM treatment was attributed to increased apoptosis. The apoptotic rate was markedly increased in a dose-dependent manner, which was associated with the protein expression levels of apoptosis-related proteins, including increased Fas, Bad and Bax, and decreased pro-caspase-8, Bcl-2, Bcl-xL, pro-caspase-9, pro-caspase-7 and pro-caspase-3. A mitochondrial membrane potential assay demonstrated that more cells became depolarized and the extent of cytochrome *c* release was markedly increased in a dose-dependent manner in HT-29 cells treated with DCM. In addition, cell cycle analysis confirmed S-phase arrest following DCM fraction treatment, which was associated with decreased protein expression levels of cell cycle-related proteins, such as cyclin A, CDK2, cell division cycle 25 A and cyclin dependent kinase inhibitor 1. Based on these results, the present study suggested that the DCM fraction of the *C. soldanella* extract can inhibit HT-29 cell viability whilst inducing apoptosis through mitochondrial membrane potential regulation and S-phase arrest. These results also suggested that the DCM fraction has potential anticancer activity in HT-29 colorectal cells. Further research on the composition of the DCM fraction is warranted.

Correspondence to: Professor Taek-Jeong Nam, Future Fisheries Food Research Center, Institute of Fisheries Sciences, Pukyong National University, 474 Ilgwang-ro, Busan 46041, Republic of Korea
E-mail: namtj@pknu.ac.kr

*Contributed equally

Key words: apoptosis, *Calystegia soldanella*, dichloromethane fraction, apoptosis, HT-29 cells

Introduction

Colorectal cancer is prevalent and a leading cause of death worldwide (1). The mortality rate of colorectal cancer has been declining over the past number of decades due to early diagnosis using improved screening and treatment strategies. However, the incidence remains high (2). Over the past several decades, in the United States, the incidence and mortality rates of colorectal cancer have been steadily decreasing among those aged >50 years, but the number of those aged between 20 and 49 years is increasing. It is estimated that the incidence and mortality rates of colorectal cancer according to these age groups increase uniformly with economic development due to environmental changes, such as lifestyle, increased obesity and overall lifespan extension, and the consumption of processed foods, alcohol and meat (1,2). To date, colorectal cancer treatment involves radiotherapy and traditional therapies, including surgery and chemotherapy (3). However, these treatments are limited by toxicity, adverse events and drug resistance (3). A number of studies have previously reported that colorectal and colon cancer is negatively associated with dietary factors, including plants, seaweeds, vegetables and fruits, which contain a variety of phytochemicals (4-6). These phytochemicals have been demonstrated to protect cells from damage that leads to cancer (7-13).

The halophyte *Calystegia soldanella* (Linnaeus) Roem. et Schult (Convolvulaceae) is a perennial herb that grows on coastal sand dunes worldwide (14). This plant has been extensively used in traditional medicine for general consumption and as a type of herbal treatment, since it is considered to confer bioactive effects against rheumatic arthritis, sore throat, dropsy, scurvy, fever and diarrhea (15-17). In particular, fractions of *C. soldanella* have been reported to exhibit anti-inflammatory (18-20), antifungal (21), antiviral (22-25), anticancer (26,27) and analgesic effects (28). Although the various bioactivities of *C. soldanella* have been assessed, its effects on colon cancer have not been explored.

In a previous study, the viability of numerous cancer cell lines was assessed, including the hepatocarcinoma HepG2, gastric cancer AGS, colorectal cancer HT-29 and the breast cancer cell line MCF-7, following treatment with the *C. soldanella* crude extract (27). Similar effects, including a decrease in cell viability, were observed in HT-29 and HepG2 cells (27). Therefore, the aim of the present study was to evaluate the mechanism underlying any changes in HT-29 cell physiology after treatment with *C. soldanella* extract fractions.

Materials and methods

Sample collection and preparation. Whole-plant *C. soldanella* samples were collected from Gijang, Busan, Korea. A voucher specimen was deposited at the Herbarium of the Division of Marine Environment and Bioscience, Korea Maritime and Ocean University (Busan, Korea). The entire plant samples were briefly air-dried at room temperature for 1 month, ground into a fine powder using a blender and stored at -20˚C.

Extraction and fractionation. The crude extract of the plant samples (500 g) was eluted in 99% ethanol for 3 h at room temperature before being filtered and concentrated three times. The concentrated crude extracts were evaporated under reduced pressure at 40˚C using rotary vacuum evaporator and partitioned between H_2O-methanol (9:1) and n-hexane (4.3 g). The organic layer was further partitioned into dichloromethane (DCM; 15.2 g) and ethyl acetate (2.3 g). The aqueous layer was also fractionated into n-butanol (14.6 g) and water (16.4 g). Each fraction used was completely removed using a reflux condenser and was subsequently freeze-dried for use in experiments. All solvent reagents used for extraction were of analytical grade. The DCM fraction was diluted to a final concentration of 0.2% DMSO so as not to induce toxicity. For the control, an equivalent volume of 0.2% DMSO was added to the culture medium.

Ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-Q-TOF-MS) analysis. The DCM fractions were analyzed using UPLC-ESI-Q-TOF-MS. The UPLC system (Agilent infinity 1260 series; Agilent Technologies Deutschland GmbH), with an incorporated photodiode array detector (DAD) and Impact II Q-TOF mass spectrometer (Bruker Corporation), was equipped with an ESI source that operated on the negative ion mode. A reverse phase Kintex core-shell C-18 column (100x2.1 mm, 1.7 μm, Phenomenex) was used at a flow rate of 0.5 ml/min. The mobile phase consisted of water containing 0.1% TFA (A) and 0.1% TFA containing acetonitrile (B) using the following gradient conditions: 0-1 min, 10% B; 1-4 min, 10-20% B; 4-6 min, 20-25% B; 6-8 min, 25% B; 8-9 min, 25-30% B; 9-11 min, 30% B; 11-12 min, 30-50% B; 12-14 min, 50-60% B; 14-15 min, 60-80% B; and 15-17 min, 80% B. The injection volume was 2 μl. Mass spectra in positive-ion or negative-ion mode were recorded within 20 min. The UPLC profiles of the extracts were measured using a DAD. The analyses were conducted in the negative ion mode in a mass range from m/z 50 to 1,000. The ESI source parameters were: Capillary voltage, 4.5 KV; nebulizing gas pressure, 1.5 bar; drying gas temperature, 200˚C, drying gas flow, 9.0 l/min; Funnel 1RF 250.0 Vpp; transfer time, 50.0 μs; and prepulse storage, 2.0 μs. The MS data were analyzed using Data Analysis 4.2 software (Bruker Corporation).

Cell culture. The human colorectal HT-29 cell line (cat. no. 30038) was purchased from the Korean Cell Line Bank, Korean Cell Line Research Foundation. The STR profile of the HT-29 cell line was as follows: D3S1358, 15/17; von Willebrand factor type A, 17/19; fibrinogen α chain, 20/22; amelogenin, X; tyrosine hydroxylase 1, 6/9; thyroid peroxidase, 8/9; CSF1P0, 11/12; D5S818, 11/12; D13S317, 11/12; and D7S820, 10. The cells were cultured at 37˚C with 5% CO_2 in RPMI-1640 medium (Welgene, Inc.) supplemented with 10% FBS (Welgene, Inc.) containing 100 U/ml penicillin and 100 μg/ml streptomycin (cat no. CA005-10; GenDEPOT, LLC). The culture medium was refreshed every 2 days and the cells were subcultured for use in subsequent experiments.

Cell viability assays. Cell viability was analyzed using a EZ-Cytox Kit (cat. no. EZ-1000; DoGenBio Co., Ltd.) according to the manufacturer's protocol. Cells were seeded into 96-well plates at $4x10^4$ cells/well and allowed to attach for 24 h. First, cell viability was examined for each fraction (hexane, DCM, ethyl acetate, butanol, water) at concentrations of 0, 25, 50 or 100 μg/ml for 24 h. Next, attached cells were treated with 0, 10, 20, 40, 60, 80 or 100 μg/ml of the DCM fraction in serum-free medium for 24 or 48 h. Subsequently, cells were incubated with the EZ-Cytox solution (100 μl/well) for 30 min at 37˚C before absorbance at 450 nm was quantified using the FilterMAX F5 microplate reader (Molecular Devices LLC.). In addition, morphological cell changes were subsequently observed using a light microscope (magnification, x200; Eclipse TS100-F; Nikon Corporation).

Apoptosis assay. Apoptosis was assessed using the Muse® Annexin V and Dead Cell Kit (cat. no. MCH100105; Luminex Corporation) according to the manufacturer's protocol. Cells were seeded into six-well plates at $1x10^5$ cells/well and treated with 20, 40 or 80 μg/ml concentrations of the DCM fraction for 20 h. The cells were then harvested at a density of $5x10^4$ cells/well and washed twice with PBS and stained with FITC-Annexin V and dead cell reagent for 20 min at room temperature in the dark. The percentage of apoptotic cells was determined using the Guava® Muse® Cell Analyzer (2012model; Luminex Corporation).

Assessment of mitochondrial membrane potential (MMP). The MMP was assessed using the Muse® MitoPotential Kit (cat. no. MCH100110; Luminex Corporation.) according to the manufacturer's protocol. Cells were seeded into six-well plates at $1x10^5$ cells/well and treated with 0, 20, 40 or 80 μg/ml concentrations of the DCM fraction for 20 h. The cells were harvested at a density of $5x10^4$ cells/well, washed twice with PBS, stained with MitoPotential working solution containing MitoPotential dye and incubated for 20 min in a 37˚C CO_2 incubator. The MMP was determined using the Guava Muse Cell Analyzer (2012 model).

Cell cycle assay. The cell cycle was assessed using the Muse® Cell Cycle Assay Kit (cat. no. MCH100106; Luminex Corporation) according to the manufacturer's protocol. Cells were seeded into six-well plates at $1x10^5$ cells/well and treated with 0, 20, 40 or 80 μg/ml concentrations of the DCM fraction for 20 h. The cells were harvested, washed twice with PBS, fixed in ice cold 70% ethanol and frozen at -20˚C for 3 h. The fixed cells were stained at a density of $5x10^4$ cells/ml with 200 μl Muse cell cycle reagent for 30 min at room temperature. The cell cycle phase was determined using the Guava Muse Cell Analyzer (2012 model).

Preparation of total cell lysate. HT-29 cells were treated with 0, 20, 40 or 80 μg/ml of the DCM fraction in serum-free medium for 24 h at 37˚C. The cells were washed with PBS and lysed in M-PER Mammalian Protein Extraction Reagent (cat. no. 78501; Thermo Fisher Scientific, Inc.) containing phosphate inhibitor cocktail (cat. no. 1862495; Thermo Fisher Scientific, Inc.) and ProteaseArrest™ protease inhibitor cocktail (cat. no. 786-108; G-Biosciences; Geno Technology, Inc.) on ice for 30 min. The extracts were centrifuged at 12,000 x g for 10 min at 4˚C and the supernatants were subsequently used for western blotting. The mitochondrial and cytosolic fractions were extracted using a Mitochondria Isolation Kit (cat. no. 89874; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Protein concentrations were measured using a BCA Protein Assay Kit (cat. no. 23225; Thermo Fisher Scientific, Inc.).

Western blotting. Total protein (20-40 μg protein/lane) was electrophoresed via SDS-PAGE on a 8-15% acrylamide gel and transferred onto polyvinylidene fluoride immobilon-P membranes (cat. no. MLP.IPVH00010; MilliporeSigma). The membranes were blocked with 1% bovine serum albumin (BSA; cat. no. A0100; GenDEPOT, LLC) in TBS with 0.1% Tween-20 (TBST; 5 mM Tris, 20 mM sodium chloride, pH 7.4) and incubated with primary antibodies (1:1,000) in 1% BSA/TBST with gentle agitation at 4˚C overnight. The membranes were then washed twice for 15 min in TBST each and incubated with the corresponding HRP-conjugated secondary antibodies (1:10,000) for 2 h at room temperature, before being washed again using TBST. Immunoreactive bands were detected using the WesternBright® ECL HRP Substrate (cat. no. K12045; Advansta, Inc.) and visualized using the GeneGnome 5 (model 75000; Syngene). Differences in protein levels were determined by semi-quantifying the western blotting band densities using ImageJ software version IJ.146r (National Institutes of Health).

The antibodies used were as follows: Anti-Fas (cat. no. sc-7886; rabbit; Santa Cruz Biotechnology, Inc.), anti-caspase-8 (cat. no. sc-7890; rabbit; Santa Cruz Biotechnology, Inc.), anti-Bcl-2 (cat. no. sc-7382; mouse; Santa Cruz Biotechnology, Inc.), anti-Bcl-extra-large (xL; cat. no. sc-7195; rabbit; Santa Cruz Biotechnology, Inc.), anti-Bad (cat. no. sc-8044; mouse; Santa Cruz Biotechnology, Inc.), anti-Bax (cat. no. sc-7480; mouse; Santa Cruz Biotechnology, Inc.), anti-caspase-9 (cat. no. sc-7885; rabbit; Santa Cruz Biotechnology, Inc.), anti-caspase-7 (cat. no. sc-6138; rabbit; Santa Cruz Biotechnology, Inc.), anti-caspase-3 (cat. no. sc-7148; rabbit; Santa Cruz Biotechnology, Inc.), anti-X-linked inhibitor of apoptosis protein (XIAP; cat. no. 2045; rabbit; Cell Signaling Technology, Inc.), anti-cellular inhibitor of apoptosis protein (cIAP)-1 (cat. no. 7065; rabbit; Cell Signaling Technology, Inc.), anti-cIAP-2 (cat. no. 3130; rabbit; Cell Signaling Technology, Inc.), anti-cytochrome *c* (cat. no. 4272; rabbit; Cell Signaling Technology, Inc.), anti-cytochrome *c* oxidase subunit IV (COX IV; cat. no. 4844; rabbit; Cell Signaling Technology, Inc.), anti-cyclin A (cat. no. BS-0571R; rabbit; BIOSS), anti-CDK2 (cat. no. sc-163; rabbit; Santa Cruz Biotechnology, Inc.), anti-cell division cycle 25 A (Cdc25A; cat. no. sc-7389; mouse; Santa Cruz Biotechnology, Inc.) and anti-cyclin dependent kinase inhibitor 1 (p21; cat. no. sc-271532; rabbit; Santa Cruz Biotechnology, Inc.). Anti-β-actin (cat. no. sc-47778; mouse; Santa Cruz Biotechnology, Inc.) antibody was used as the loading control. The secondary antibodies used were HRP-conjugated anti-mouse IgG (cat. no. 7076; Cell Signaling Technology, Inc.) and anti-rabbit IgG (cat. no. 7074; Cell Signaling Technology, Inc.).

Statistical analysis. All data are presented as the mean ± SD of three independent experiments. Means between >2 groups were compared using one-way or two-way ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism version 7 software (GraphPad Software, Inc). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Solvent fractions of C. soldanella reduce HT-29 cell viability. To determine the effects of the *C. soldanella* fractions on cell viability, HT-29 cells were treated with each of the five solvent fractions (n-hexane, DCM, ethyl acetate, n-butanol and water) at different concentrations (0, 25, 50 and 100 μg/ml) for 24 h, after which cell viability was examined. Cell viability was significantly decreased following treatment with the DCM fraction compared with that in the 0 μg/ml group (Fig. 1A). Therefore, the DCM fraction with the highest dose-dependent effect was selected for further study.

Reductions in the viability of HT-29 cells was confirmed following treatment with different concentrations (0-100 μg/ml) of DCM fraction for 24 and 48 h (Fig. 1B). HT-29 cell viability appeared to be decreased following DCM fraction treatment in a time- and dose-dependent manner compared with those in the 0 μg/ml group. After treatment with 0, 10, 20, 40, 60, 80 and 100 μg/ml DCM, cell viability was 100±4.7, 89.0±3.5, 74.6±6.7, 73.2±2.0, 53.5±7.1, 26.7±1.6 and 25.4±1.7% at 24 h, respectively, whereas it was 100±3.4, 92.6±8.3, 57.9±6.6, 35.4±3.6, 20.2±1.6, 9.3±0.5 and 8.7±0.2% at 48 h, respectively (Fig. 1B). In addition, it was observed that the morphological changes confirmed via microscope were reduced in the same way as the results of the cell viability assay (Fig. 1C).

DCM fraction from C. soldanella induces apoptosis in HT-29 cells. The Annexin V and Dead Cell Kit was used to determine whether this decrease in cell viability induced by DCM fraction treatment resulted from apoptosis. The rates of early and late apoptosis were significantly increased in a dose-dependent manner following treatment with the DCM fraction compared with those in the 0 μg/ml group (Fig. 2).

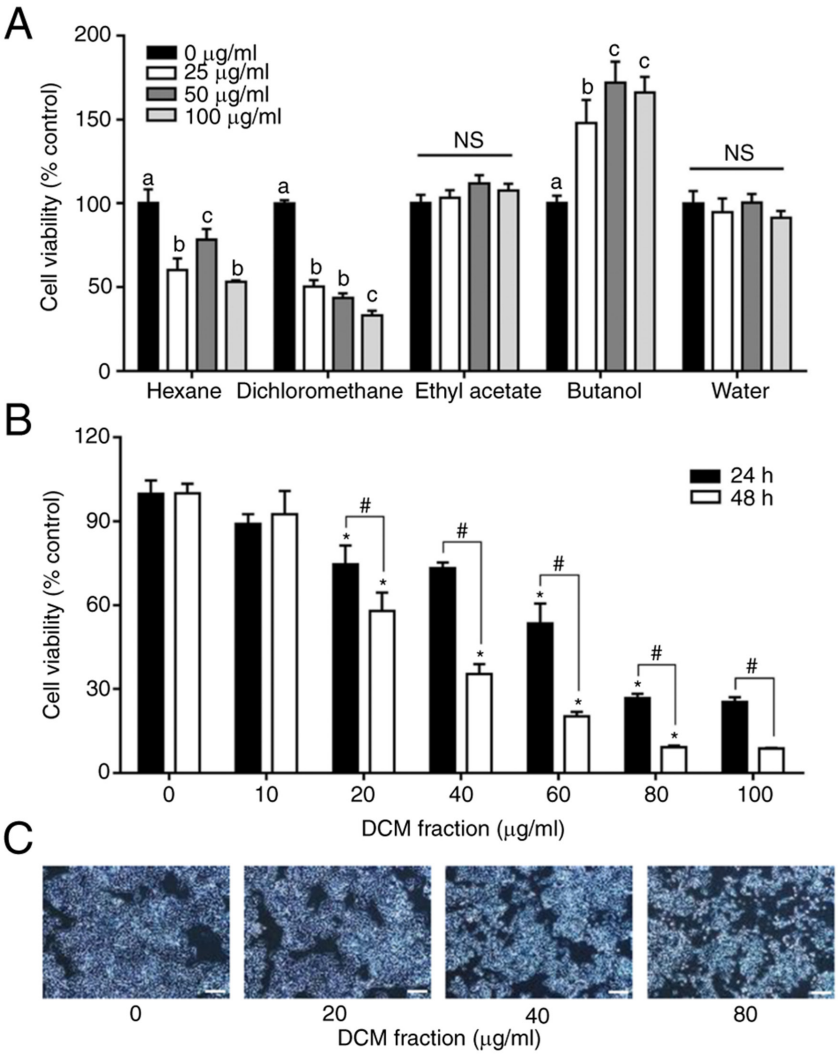

Figure 1. DCM from *Calystegia soldanella* reduces the viability of HT-29 colorectal cancer cells. (A) HT-29 cells were incubated with the five different solvent fractions at 0-100 μg/ml for 24 h. The letters displayed above the concentrations represent significant differences (P<0.05) as determined by Bonferroni's multiple comparisons test; groups with different letters are significantly different to one another, whereas those with the same letter are not. (B) HT-29 cells were incubated with the DCM fraction at 0-100 μg/ml for 24 and 48 h. Cell viability was examined using the EZ-Cytox proliferation assay. These data represent the percentage of surviving cells compared with the control (0 μg/ml). (C) Cell morphology changes were observed using light microscopy. Scale bar, 100 μm. Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. 0 μg/ml DCM; #P<0.05. DCM, dichloromethane; NS, not significant.

The proportions of early apoptotic cells were 0.7±0.67, 38.2±1.81, 23.0±3.07 and 1.0±0.38%, whilst those of late apoptotic cells were 0±0.05, 2.1±0.40, 12.9±7.12 and 35.1±0.58%, following DCM fraction treatment at concentrations of 0, 20, 40 and 80 μg/ml, respectively.

Regarding the protein expression levels of apoptosis-related proteins, DCM fraction treatment at 40 and 80 μg/ml significantly increased the protein expression levels of Fas protein whilst significantly decreasing those of pro-caspase-8, an extrinsic signaling pathway-related protein (Fig. 3), compared with those in the 0 μg/ml group. Other intrinsic apoptosis signaling pathway-related proteins that also showed significantly decreased protein expression levels after 40 and 80 μg/ml DMC treatment were Bcl-2 and Bcl-xL, whilst those that showed significantly increased protein expression levels following 40 and 80 μg/ml DCM fraction treatment were Bad and Bax (Fig. 3). Consequently, the Bax/Bcl-2 ratio was significantly increased in the 40 and 80 μg/ml DCM fraction treatment groups compared with that in the 0 μg/ml group. In addition, 40 and 80 μg/ml DCM fraction treatment also significantly decreased the expression of pro-caspase-9, pro-caspase-7 and pro-caspase-3 levels compared with those in the 0 μg/ml group. The protein expression levels of XIAP, cIAP-1 and cIAP-2, caspase inhibitors involved in apoptosis inhibition, were significantly decreased by 40 and 80 μg/ml DCM treatment compared with those in the 0 μg/ml group. These results suggest that the treatment of HT-29 cells with DCM from *C. soldanella* may induce apoptosis by regulating the expression of pro-apoptotic, pre-apoptotic and caspase inhibitor proteins.

DCM fraction from C. soldanella induces MMP changes in HT-29 cells. Since MMP changes are also associated with apoptosis (29-31), the effects of DCM fraction treatment on the MMP in HT-29 cells were investigated. The proportions of live and dead cells with depolarized mitochondria were markedly increased following DCM fraction treatment compared with those in the 0 μg/ml group. The proportions of depolarized

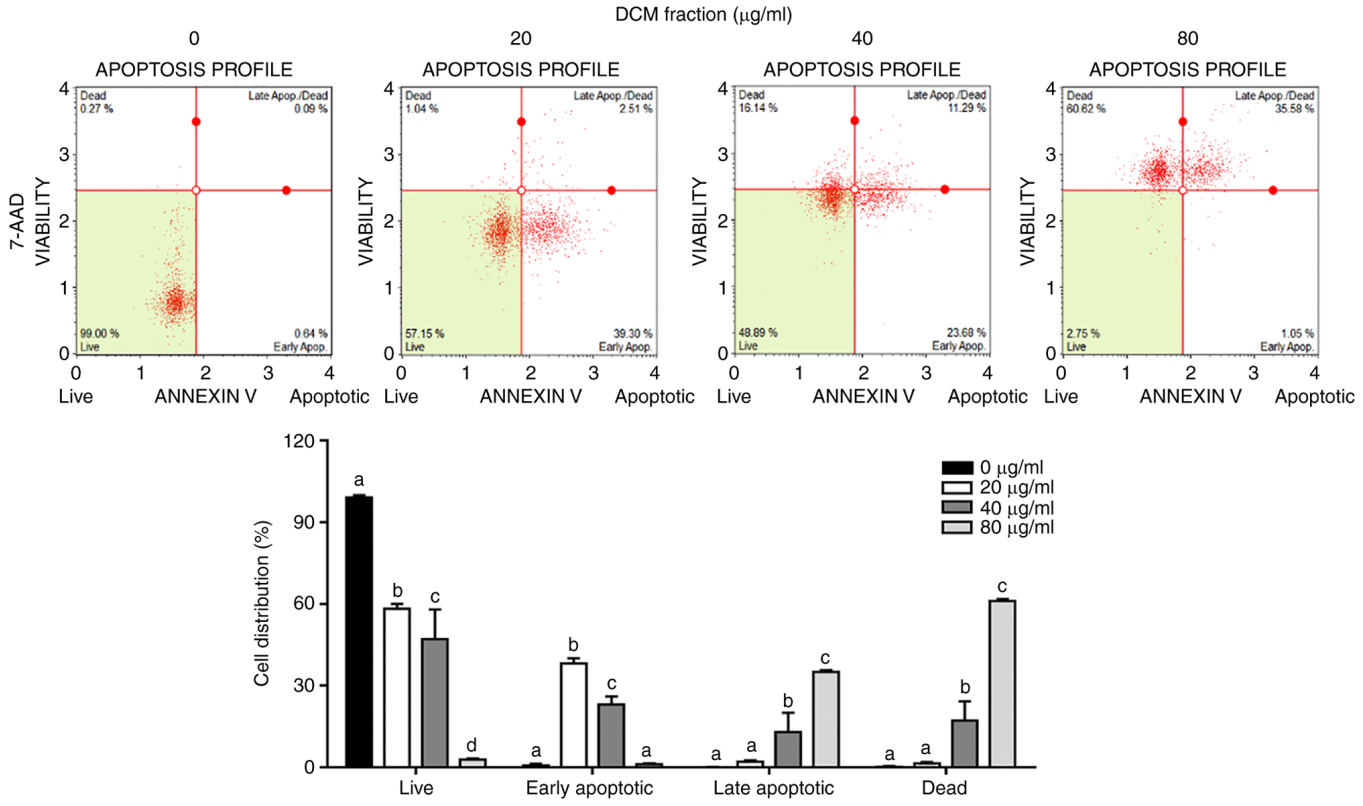

Figure 2. DCM fraction treatment induces apoptosis in HT-29 cells. HT-29 cells were incubated with various concentrations of the DCM fraction for 20 h. FITC-Annexin V staining followed by flow cytometry was used to determine the percentage of apoptotic cells. Data are presented as the mean ± SD of three independent experiments. The letters displayed above the concentrations represent significant differences ($P<0.05$) as determined by Bonferroni's multiple comparisons test; groups with different letters are significantly different to one another, whereas those with the same letter are not. DCM, dichloromethane; 7-AAD, 7-Aminoactinomycin D; apop, apoptotic.

live cells were 5.2±0.75, 43.6±1.49, 2.9±0.21 and 1.2±0.05%, whereas the proportions of depolarized dead cells were 0.8±0.24, 10.5±1.48, 89.7±0.33 and 95.8±0.35%, at concentrations of 0, 20, 40 and 80 μg/ml, respectively (Fig. 4A). The protein expression levels of MMP-related proteins were examined using western blotting. DCM fraction treatment at 40 and 80 μg/ml significantly increased the release of cytochrome *c* into the cytosol from the mitochondria compared with that in the 0 μg/ml group (Fig. 4B). In addition, DCM fraction treatment also resulted in the significantly increased translocation of Bax into the mitochondria from the cytosol in a dose-dependent manner compared with the 0 μg/ml group.

DCM from C. soldanella induces S-phase arrest in HT-29 cells. To determine whether decreased cell viability was associated with the cell cycle, HT-29 cell cycle progression was analyzed using a cell cycle kit following DCM fraction treatment. DCM significantly induced S-phase arrest in a dose-dependent manner compared with the 0 μg/ml group, with S-phase cell proportions of 20.7±1.8, 33.8±0.2, 39.9±5.6 and 45.3±0.2% at concentrations of 0, 20, 40 and 80 μg/ml of the DCM fraction, respectively (Fig. 5A). Subsequently, the relative protein expression levels of S-phase-related proteins were analyzed using western blotting. DCM fraction treatment at 40 and 80 μg/ml led to the significant downregulation of cyclin A, CDK2 and Cdc25A protein expression and the significant upregulation of p21 protein expression compared with those in the 0 μg/ml group (Fig. 5B).

ESI-Q-TOF-MS analysis of DCM fraction from C. soldanella. UPLC-ESI-Q-TOF-MS analysis was applied to analyze the polyphenolic compounds in the DCM fraction of *C. soldanella* to screen for any anticancer substances. The HPLC-UV chromatogram (350 nm) and total ion current chromatogram in the DCM fraction are presented in the Fig. 6A and B.

Molecular ion mass, MS/MS fragment ion mass and MS-based compound analysis data are all provided in Table I. UPLC-ESI-Q-TOF MS analysis demonstrated that the major compounds in the DCM fraction were hydroxybenzoic acid, hydrosinapinic acid and coumaric acid, which are phenolic acid derivatives, and quercetrin, which is a flavonoid quercetin derivative.

Discussion

The ultimate aim of discovering novel chemotherapeutic strategies is to overcome drug resistance (32). A number of studies have previously investigated the potential anticancer activity of natural compounds/products and suggested them to be promising sources of new anticancer drugs (4-13). For example, the natural compound S-adenosylmethionine has

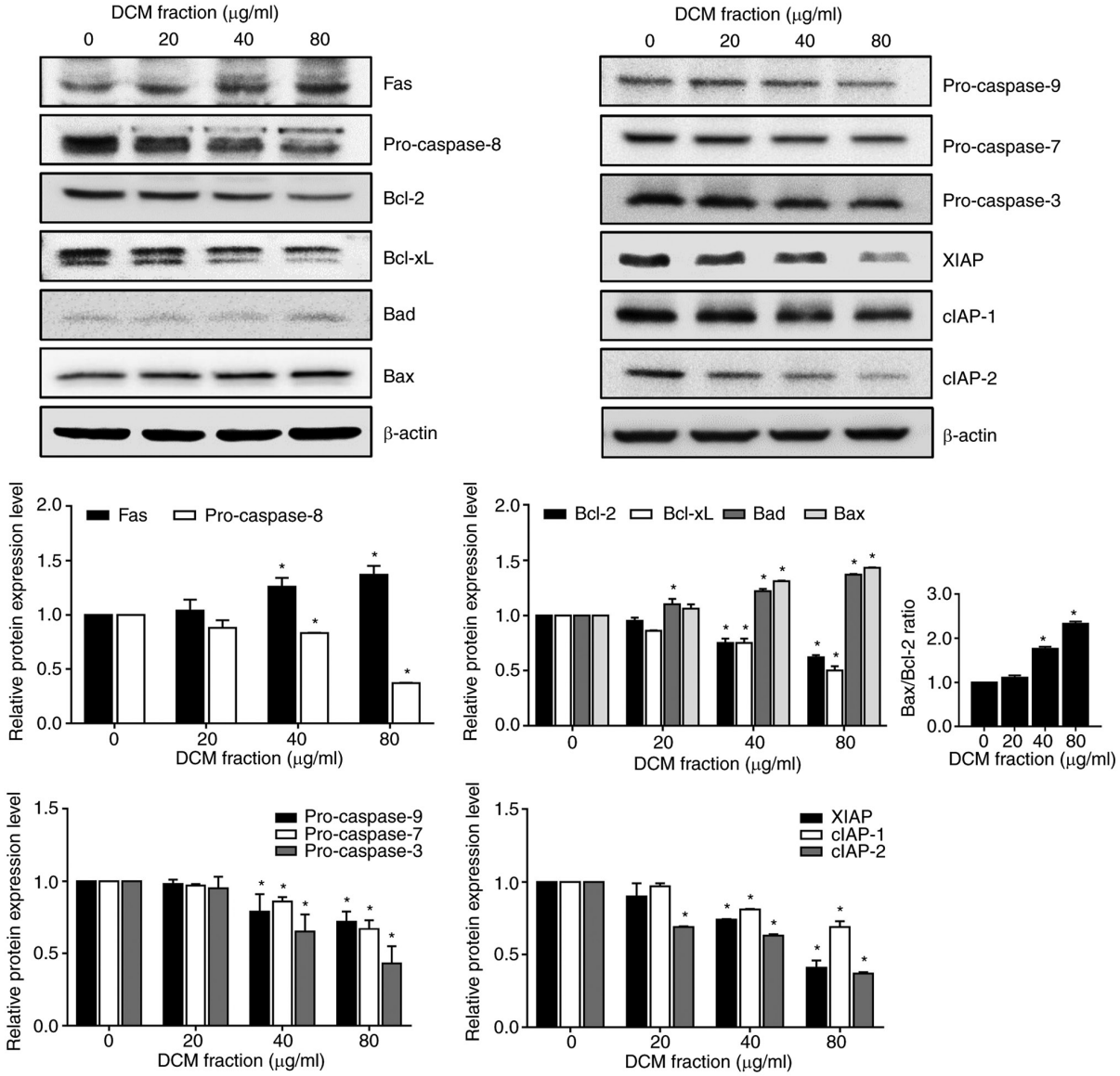

Figure 3. Effects of DCM fraction treatment on the expression of apoptosis-related proteins in HT-29 cells. HT-29 cells were incubated with various concentrations of the DCM fraction for 20 h. The expression levels apoptosis-related proteins Fas, pro-caspase-8, Bcl-2, Bcl-xL, Bad, Bax, pro-caspase-9, pro-caspase-7, pro-caspase-3, XIAP, cIAP-1 and cIAP-2 were then determined by western blotting. β-actin was used as the loading control. The bands were semi-quantitatively analyzed by ImageJ software and the relative protein expression levels are presented. All results were normalized to the untreated control (0 μg/ml). Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. 0 μg/ml DCM. DCM, dichloromethane; xL, extra-large; XIAP, X-linked inhibitor of apoptosis protein; cIAP-1, cellular inhibitor of apoptosis protein-1; cIAP-2, cellular inhibitor of apoptosis protein-2.

been found to exert anti-tumor properties, including reduction of cell proliferation, induction of apoptosis, autophagy and inhibition of invasion and metastasis, in various cancer cell types, such as human hepatocellular carcinoma, human breast cancer and head and neck squamous cancer cells, in previous *in vitro* and *in vivo* studies (33-36).

To date, anticancer-associated studies related to *C. soldanella* have revealed cytotoxic effects of methanol and chloroform extracts on the lung cancer A545 and colon cancer Col2 cell lines (26) and the anticancer effects of an 85% aqueous methanol fraction on the liver cancer cell line HepG2 (27). However, only a few studies have investigated the anticancer effects of *C. soldanella*.

In the present study, solvent fractions were obtained from *C. soldanella*, which have been previously reported to exert anticancer activity in human liver cancer cell line HepG2 (27), before an effective fraction that can exhibit anticancer effects on the colorectal cancer cell line HT-29 was selected. Following the crude extraction of *C. soldanella* with ethanol, the crude extracts were fractionated into n-hexane, DCM, ethyl acetate, n-butanol and water. The effects of these five fractions on HT-29 cell viability were examined before the DCM fraction was selected due to its significant time- and dose-dependent effects.

Apoptosis serves a critical role in the regulation of cell development and proliferation (37,38). This process has become a target of cancer treatments due to its association with a number of different types of cancer (39,40). Two major pathways of apoptosis have been identified: i) The extrinsic death receptor pathway; and ii) the endoplasmic reticulum stress pathway and intrinsic mitochondrial apoptosis (41). To determine the effect of DCM fraction treatment on cell

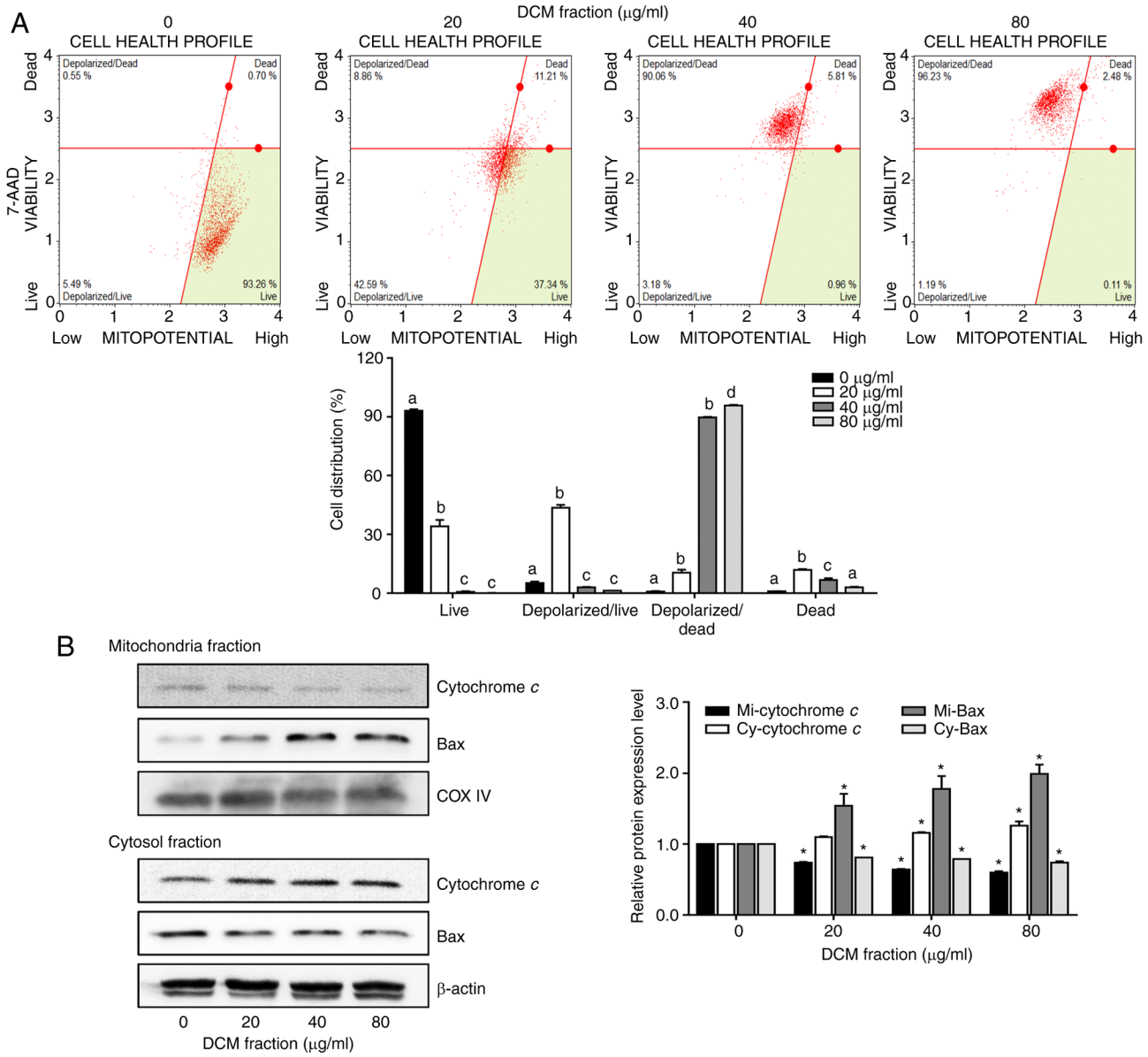

Figure 4. DCM fraction treatment induces depolarization of mitochondrial membrane potential in HT-29 cells. (A) HT-29 cells were incubated with various concentrations of the DCM fraction for 20 h. MitoPotential kit staining followed by flow cytometry was used to determine the percentages of depolarized cells. The letters displayed above the concentrations represent significant differences ($P<0.05$) as determined by Bonferroni's multiple comparisons test; groups with different letters are significantly different to one another, whereas those with the same letter are not. (B) Protein expression levels of MMP-related proteins cytochrome *c* and Bax were determined using western blotting analysis. β-actin and COX IV were used as loading controls for the cytosol and mitochondria, respectively. All results were normalized to the untreated control (0 μg/ml). Data are presented as the mean ± SD of three independent experiments. *$P<0.05$ vs. 0 μg/ml DCM. DCM, dichloromethane; 7-AAD, 7-Aminoactinomycin D; COX IV, cytochrome c oxidase subunit IV; mi, mitochondrial; cy, cytosol.

viability and apoptosis, apoptosis and apoptosis-related protein expression levels in HT-29 cells that were treated with the DCM fraction were investigated. The results demonstrated significantly increased apoptotic rates and marked changes in the expression levels of apoptosis-related proteins in both the extrinsic and intrinsic signaling pathways. Treatment with DCM fraction increased Fas and decreased pro-caspase-8, which corresponds to the extrinsic signaling pathway. In addition, treatment with DCM fraction increased Bad and Bax, and decreased Bcl-2, Bcl-xL, pro-caspase-9, pro-caspase-7 and pro-caspase-3, which corresponds to the intrinsic signaling pathway.

The mitochondrial apoptosis pathway involves a number of components, including pre-apoptotic proteins Bcl-2 and Bcl-xL and pro-apoptotic proteins Bax and Bak (42-44). The ratio of Bax/Bcl-2 determines the direction of apoptosis regulation. An increased Bax/Bcl-2 ratio can lead to the loss of mitochondrial membrane potentials, which is an important process in the initiation of apoptosis (45). Furthermore, it has been reported that an increased Bax/Bcl-2 ratio can activate caspase-3 to in turn activate apoptosis (46,47). In the present study, caspase-3 was also markedly activated following DCM fraction treatment. Since the Bax/Bcl-2 ratio was also markedly increased with DCM fraction treatment, it was hypothesized that DCM may activate apoptosis by altering the mitochondrial membrane potential.

Apoptosis involves the regulation of a series of proteins mainly in the mitochondrial signaling pathway (48). The

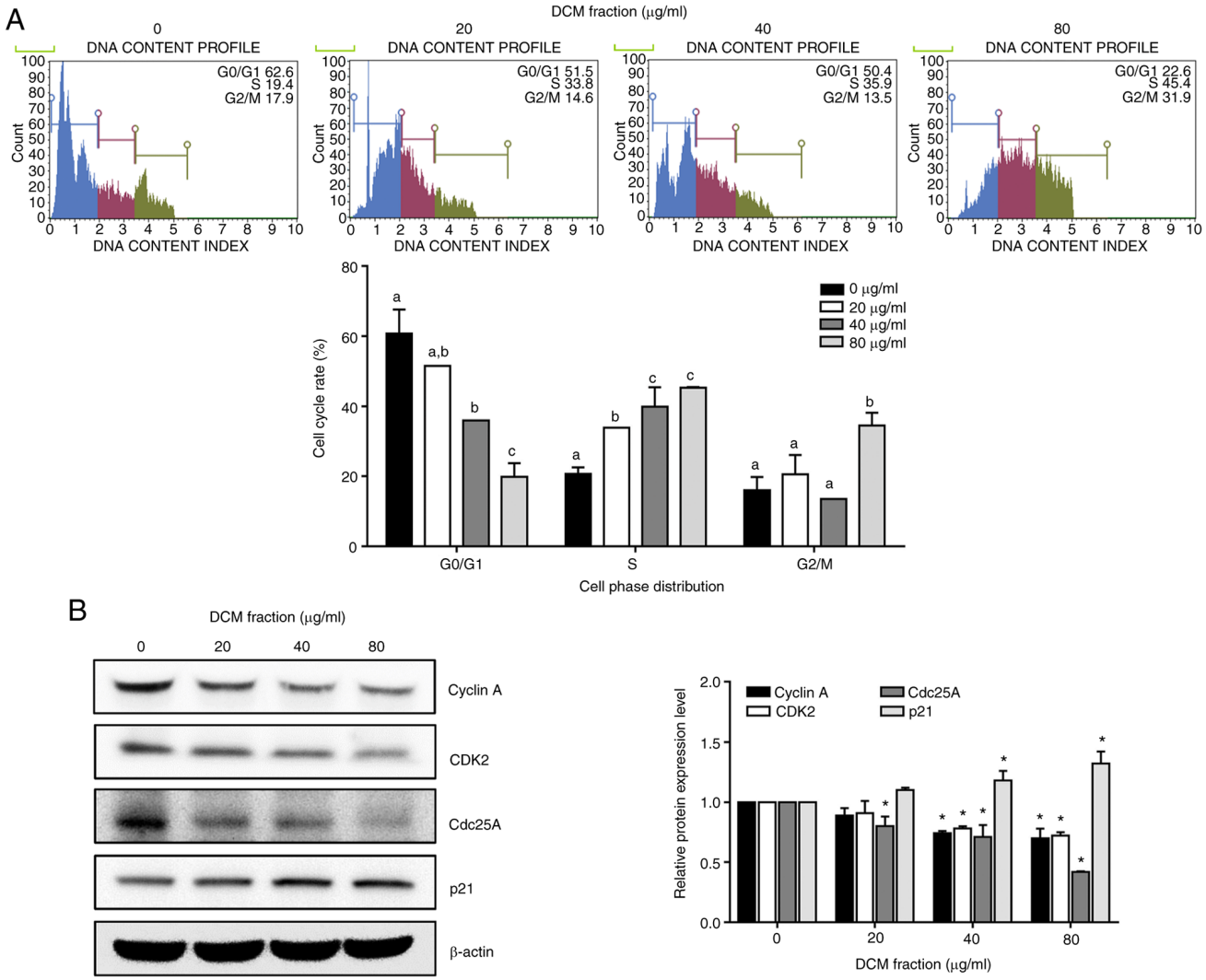

Figure 5. DCM fraction treatment induces S-phase arrest in HT-29 cells. (A) HT-29 cells were incubated with various concentrations of the DCM fraction for 20 h. Flow cytometry was used to determine the percentages of cells in each phase of the cell cycle. The letters displayed above the concentrations represent significant differences (P<0.05) as determined by Bonferroni's multiple comparisons test; groups with different letters are significantly different to one another, whereas those with the same letter are not. (B) Protein expression levels of S-phase-related proteins cyclin A, CDK2, Cdc25A and p21 were measured using western blotting. All results were normalized to the untreated control (0 μg/ml). Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. 0 μg/ml DCM. DCM, dichloromethane; CDK2, cyclin-dependent kinase 2; Cdc25A, cell division cycle 25 homolog A; p21, cyclin dependent kinase inhibitor 1.

mitochondria maintain the cellular energy balance and regulate cell death processes (49). Cellular energy generated during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane, which allows the mitochondria to induce ATP synthesis (49). Changes in the MMP are associated with apoptosis, necrosis and caspase-independent cell death processes in addition to the opening of mitochondrial transition pores, to release cytochrome *c* into the cytosol and initiate apoptotic and caspase cascades (31,49). In the present study, MMP changes following DCM fraction treatment led to matrix condensation and exposure of cytochrome *c* to the intermembrane space, which may have activated apoptosis (Fig. 4).

Numerous proteins associated with cell cycle regulation are known to be involved in apoptosis (50). In the present study, cell cycle assay was performed and the protein expression levels of several cell cycle-related proteins were investigated to determine whether apoptosis induced by DCM fraction treatment was due to cell cycle regulation in HT-29 cells. The results demonstrated that DCM fraction treatment significantly increased the proportion of cells in the S-phase. Cyclins and cyclin-dependent kinases serve important roles in cell cycle regulation, where changes in the composition of cyclin/CDK complexes can either increase or decrease cell proliferation and/or differentiation through apoptosis (51,52). Since cyclin A, CDK2 and Cdc25A serve critical roles in S-phase regulation (53,54), their protein expression levels following DCM fraction treatment were examined. The results showed marked downregulation of cyclin A, CDK2 and Cdc25A expression following DCM treatment. CDK1, p21 (Waf1/Cip1) have been previously shown to induce cell cycle arrest by inhibiting CDK activity (55,56). The results of the present study demonstrated a dose-dependent increase in p21 protein expression levels in response to DCM, which may have inhibited Cyclin A-CDK2 complex formation and contributed to cell cycle arrest between the S and G_2/M phases.

Table I. Identified compounds in the DCM fraction.

Peak no.	Compound	Retention time, min	Mass	m/z	MS fragment
1	Hydroxybenzoic acid	1.6	138.12	137.02	112.98
2	Hydrosinapinic acid	2.0	226.08	225.11	181.12, 121.02
3	Coumaric acid	2.2	164.04	163.03	119.05
4	Quercetrin	3.0	448.38	447.09	301.03, 146.93

Peaks correspond to peaks observed in Fig. 6B. DCM, dichloromethane; MS, mass spectrometry.

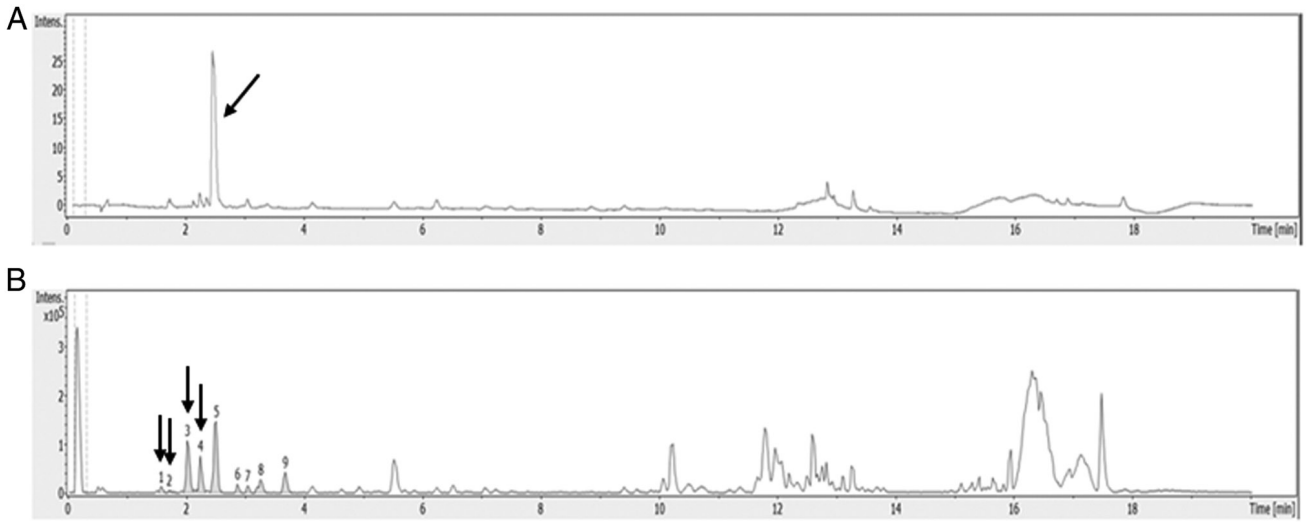

Figure 6. UPLC-ESI-Q-TOF-MS analysis of DCM fraction. (A) UV chromatogram at 350 nm and (B) UPLC-ESI-Q-TOF MS total ion chromatogram of the dichloromethane fraction. intens, intensity; UPLC-ESI-Q-TOF-MS, ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry.

It has been frequently reported that numerous compounds can exert anticancer activity independent of p53 (57-60). The tumor suppressor p53 has been found to be dysfunctional in ≥50% colorectal cancer cases (58,59). Colorectal cancer cells and tumors with p53 mutation are reported to be more aggressive and resistant to chemotherapy (60). In the present study, the HT-29 cell line is a p53 mutant cell line (genotype R273H), to which DCM exerted anticancer effects in the absence of p53. Although not conducted in the present study, anticancer effects following DCM fraction treatment are also hypothesized to be present in p53 wild-type cell lines and warrants further study.

Previous studies have reported that *C. soldanella* contains a variety of polyphenolic compounds, including flavonoids, flavonoid glycosides and phenolic acid derivatives (56,61).

Molecular ion mass, MS/MS fragment ion mass and MS-based compound analysis data are all provided in Table I. UPLC-ESI-Q-TOF MS analysis demonstrated that the major compounds in the DCM fraction were hydroxybenzoic acid, hydrosinapinic acid and coumaric acid, which are phenolic acid derivatives, and quercetrin, which is a flavonoid quercetin derivative. Various phenolic acids, such as coumaric acid, caffeic acid and ferulic acid, function as secondary plant metabolites (62). In particular, cinnamic acid-based phenoic acid compounds, such as coumaric acid, caffeic acid, ferulic acid and sinapinic acid, have been reported to exert anticancer activity in various colon cancer cell lines (63). Coumaric acid is reported to induce apoptosis in the colon cancer HCT-115 cell line and induce G_1/S arrest in the colon cancer cell line Caco-2 (64-66). These aforementioned phenolic acids also have inhibitory activities on the proliferation of various colorectal/colon cancer cell lines (67-69).

In conclusion, to the best of our knowledge, the present study was the first to report the anticancer effects of DCM from *C. soldanella* on HT-29 colorectal cancer cells, which possibly occurred by MMP alteration, S-phase arrest and induction of apoptosis through the intrinsic/extrinsic signaling pathways. The present study also demonstrated that phenolic acid derivatives are the main components of the DCM fraction, which exerted inhibitory activities on HT-29 colorectal cancer cell by apoptosis and cell cycle arrest.

Acknowledgements

Not applicable.

Funding

The present study was part of the Future Fisheries Food Research Center Project, funded by the Ministry of Oceans and Fisheries (grant no. 201803932).

Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

IHK, TE and TJN conceived and designed the experiments. IHK, JYP and HJK performed the experiments. IHK, TE and HJK analyzed and interpreted the results and wrote and revised the manuscript. IHK, TE and TJN confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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