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Antioxidant and Antiproliferative Activities of Methanolic Extract from Celandine

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Abstract Celandine (*Chelidonium majus*, family Papaveraceae) is an herb used extensively in traditional Korean medicine. To investigate its antioxidant and antiproliferative activities, the methanolic extract of celandine was introduced. The antioxidant properties of the extract were tested using various *in vitro* systems, including hydroxyl radical scavenging assay, DNA damage protection assay, 1,1-diphenyll-2-2-pricylhydrazyl (DPPH) free radical scavenging activity, metal chelating activity, and reducing power assay. The extract exhibited stronger antioxidant activity (IC_{50}=7.92 μg/mL) against hydroxyl radicals in the Fenton system than butylated hydroxyanisole (IC_{50}=51.46 μg/mL) and α-tocopherol (IC_{50}=67.48 μg/mL). Likewise, damage to the plasmid pBR 322 induced by hydroxyl radicals was found to be protected by the extract at a concentration of 400 μg/mL. Cellular proliferation and the induction of apoptosis were also examined by a cellular proliferation assay, flow cytometry, and mRNA expression analysis. Taken together, the extract significantly inhibited the growth of HT-29 cells in a concentration- and time-dependent manner, and gradually increased both the proportion of apoptotic cells and the expression of *caspase-3*. Overall, our research suggests that celandine possesses antioxidant and antiproliferative properties.

Keywords: celandine (*Chelidonium majus*), antioxidant, antiproliferation, flow cytometry, reverse transcription-polymerase chain reaction (RT-PCR)

Introduction

Reactive oxygen species (ROS), including hydroxyl radicals, superoxide anions, hydrogen peroxide, and singlet oxygen, are a major cause of oxidative damage to biological molecules in the human body (1). These molecules are unstable and highly reactive; thus, they can induce cellular damage by initiating chemical chain reactions such as lipid peroxidation or the oxidation of DNA and proteins (2). Moreover, ROS are known to cause coronary heart disease, aging, and cancer (3). Antioxidants have diverse functions in biological systems, including defense against oxidative damage and participation in all of the major signaling pathways in cells. One of the chief functions of antioxidants in cells is to prevent damage caused by the action of ROS (4,5). Plants contain a wide variety of free radical scavenging molecules such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and other endogenous metabolites that are rich in antioxidant activity. Many of these antioxidant compounds possess anti-inflammatory, antimutagenic, anticancer, antibacterial, or antiviral activities (6,7).

Cancer is one of the most formidable causes of human suffering, with more than 10 million new cases of cancer each year and over 6 million cancer-related deaths (8). Cancer, a neoplastic disorder caused by excessive cellular proliferation, can be treated via chemotherapy, surgery, or radiation therapy (9,10). However, an increasing amount of cancer research is being directed toward the investigation of plant-derived anticancer compounds, many of which have been used in traditional herbal treatments for centuries (11). Apoptosis, an intrinsic cell death program, is a key regulator of tissue homeostasis, and imbalances between cell death and proliferation may result in tumor formation. The aim of anticancer agents is to induce apoptosis-related signaling in cancer cells while disrupting their proliferation (12,13).

Celandine (*Chelidonium majus*) is widely distributed across Southern and Central Europe as well as parts of Asia and North America. It belongs to the family Papaveraceae and is rich in biologically active substances. Celandine has also been found to exhibit a variety of biological activities, including anti-inflammatory, antimicrobial, antiviral, and antitumor effects (14). To date, several reports on isoquinoline alkaloids and their biological activities have been published (15,16).

The purpose of the present study was to investigate the antioxidant and antiproliferative bioactivities of celandine by means of antioxidant tests, including hydroxyl radical scavenging assay, DNA damage protection assay, DPPH free radical scavenging activity, metal chelating activity and reducing power assay. Cellular proliferation and the induction of apoptosis were assessed by MTT assay, flow cytometry, and mRNA expression analysis.

Materials and Methods

Chemicals 1,1-Diphenyll-2-2-pricylhydrazyl (DPPH), 2-deoxy-D-ribose, α-tocopherol, ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-Thiobarbituric acid (TBA) was purchased from Alfa Aesar (Karlsruhe, Germany). RPMI medium

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1640, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA were acquired from Gibco BRL (Grand Island, NY, USA). The culture supplies (e.g., 96-well plates) were obtained from SPL Brand Products (SPL, Suwon, Korea). All other chemicals were of analytical grade.

Preparation of the extract Celandine was dried in the shade at room temperature and powdered. Fifty g of powder were then extracted with 1,000 mL of absolute methanol at room temperature for 72 hr. The extract was filtered through filter paper (100-mm; Whatman, Maidstone, UK) and evaporated under reduced pressure using a vacuum rotary evaporator (CCA-1110; Eyela, Tokyo, Japan). The dried sample was weighed and kept in a refrigerator until further analysis.

Hydroxyl radical scavenging activity The scavenging ability of the test extract toward ·OH was determined using the deoxyribose assay (17). The reaction mixture [$FeSO_4$ (10 mM, 0.2 mL), EDTA (10 mM, 0.2 mL), H_2O_2 (10 mM, 0.2 mL), and 2-deoxy-D-ribose (10 mM, 0.2 mL)] was mixed with or without extract to make a final volume of 1 mL, then mixed with 1 mL of phosphate buffer (0.1 M, pH 7.4). The mixture was then incubated at 37°C for 4 hr. After incubation, 1 mL each of 2.8% TCA and 1% TBA were added and the mixture was placed in boiling water for 10 min. Finally, the reaction mixture was cooled and centrifuged at 800×g for 10 min. The absorbance of the supernatant was measured at 532 nm.

DNA damage protection assay To test for DNA damage induced by hydroxyl radicals, 2 μL of celandine extract and 0.5 μg of the plasmid pBR 322 were mixed and incubated for 10 min at room temperature followed by the addition of 7 μL of Fenton's reagent (3 μL of 30% H_2O_2, 2 μL of 5 mM $FeSO_4$, and 2 μL of 50 mM PB buffer). The resulting mixture (final volume 20 μL) was incubated for 30 min at 37°C. The DNA was analyzed on 1% agarose gels and visualized using ethidium bromide staining and a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Kiryat Anavim, Israel). Densitometric analysis was done with image analysis software (Quantity one; Bio-Rad, Hercules, CA, USA). The percentage of DNA damage protection was calculated by comparing the proportion of each supecoiled DNA with Fenton's reagent intensity to the supercoiled DNA without Fenton's reagent.

DPPH radical scavenging activity The free radical scavenging activity of the extract was determined by the DPPH test as previously described (18). Briefly, 0.5 mL of 0.1 mM DPPH (in methanol) was added to a test tube containing 0.5 mL of the extract at various concentrations (0.003125-0.4 mg/mL). The mixture was then shaken vigorously for 1 min and kept at room temperature for 30 min in the dark. The absorbance of each sample solution was subsequently measured at 517 nm. The experiment was carried out in triplicate and the results were averaged. The ability to scavenge DPPH radicals was calculated using the following equation: $I\ (\%)=[1\times(A_i\times A_j)/A_c]\times 100$. In the equation, A_c is the absorbance of the DPPH solution without sample (0.5 mL DPPH solution+0.5 mL methanol), A_i is the absorbance of the test sample mixed with DPPH solution (0.5 mL sample+0.5 mL DPPH solution), and A_j is the absorbance of the sample without DPPH solution (0.5 mL sample+0.5 mL methanol).

Metal chelating activity The chelation of ferrous ions by the extract was estimated as described previously (19). In brief, 1 mL of celandine extract at varying concentrations was mixed with 3.7 mL of absolute methanol and 0.1 mL of 1 mM $FeCl_2$. The reaction was initiated by adding 0.2 mL of 5 mM ferrozine followed by vigorous shaking; the mixture was then left to react at room temperature for 10 min. Each test was repeated 3 times. The absorbance was measured at 562 nm.

Reducing power assay The reducing power was determined as described previously (20). Various concentrations of extract (1 mL) were mixed with 2.5 mL sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 0.1% potassium ferricyanide. The mixture was incubated at 50°C for 30 min. After adding 2.5 mL of 10% TCA, the mixture was centrifuged at 3,000×g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% of ferric chloride, and the absorbance was measured at 700 nm.

Cell line and cell culture HT-29 cells (human colon cancer cell line) and HEK 293 cells (HEK293 embryonic kidney cell line) were purchased from the Korean Cell Line Bank (Seoul, Korea). HT-29 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. HEK 293 cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cellular proliferation assay HT-29 cancer cells were plated at a density of 1×10^4 cells/well in 96-well plates with 200 μg/mL culture medium for 24 hr and then exposed to 25, 50, 100, 200, or 400 μg/mL celandine extract for 24, 48, or 72 hr. The HEK 293 cells were also exposed to 25, 50, 100, 200, or 400 μg/mL celandine extract for 72 hr. A total of 20 μL of MTT solution [2 mg/mL in phosphate-buffered saline (PBS)] was added to each well at the time of incubation. After 4 hr of incubation, the supernatant was discarded and 200 μL of dimethyl sulfoxide (DMSO) was added to each well to terminate the reaction. The absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek, Winooski, VT, USA).

Assay of the apoptotic rate by Annexin V-FITC staining The proportion of HT-29 cancer cells undergoing apoptosis was measured using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) as described by the manufacturer. Cells (1×10^6) were seeded into 6-well plates and treated with various concentrations of extract (25-200 μg/mL) for 24 hr. Following digestion with 0.25% trypsin-EDTA for 3 min, the cells were collected and centrifuged at 13,000×g for 15 min. The pellets were then washed twice with PBS and re-suspended in 1×binding buffer at a concentration of

1×10^6 cells/mL. Subsequently, 5 μL of Annexin V-FITC conjugate and 10 μL of propidium iodide were added, and the cells were kept in the dark at room temperature for 10 min. The Annexin V-FITC-/PI-stained cells were analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

Determination of the expression level of *caspase-3* Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze gene expression in the cells. HT-29 cancer cells were grown in 6-well plates for 24 hr and then treated with various concentrations of extract for 24 hr. Total RNA was then isolated from the cells using a Trizol RNA isolation kit (Invitrogen, Carlsbad, CA, USA). Subsequently, 1 μg of the RNA was reverse-transcribed into cDNA and used as the template for PCR amplification. The forward and reverse primers were as follows: sense, 5'-TCACAGCAAAAGGAGCAGTTT-3' and antisense, 5'-CGTCAAAGGAAAAGGACTCAA-3' for *capspase-3* and 5'-TCACCCTGAAGTACCCCATC-3' and 5'-CCATCTCT TGCTGCAAGTCC-3' for *β-actin*. The conditions for *caspase-3* and *β-actin* were 94°C for 5 min followed by 30 cycles at 94°C for 60 sec, 55°C for 60 sec, and 72°C for 90 sec, with a final extension at 72°C for 7 min. The products were separated by 1% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed. The level of *caspase-3* expression was calculated as the proportion of the *caspase-3* PCR product intensity to that of the *β-actin* product from the same RNA sample.

Statistical analysis All tests were carried out in independent triplicate (n=3) and data was expressed as mean±standard derivation (SD). Analyses were performed using SPSS 7.5 (SPSS Institute, Cary, NC, USA).

Results and Discussion

Hydroxyl radical scavenging activity The effect of the extract on hydroxyl radicals generated from Fe^{3+} ions was measured based on the extent of deoxyribose oxidation, which is an indicator of TBA-malondialdehyde (MDA) adduct formation. The extract and positive controls (BHA and α-tocopherol) exhibited dose-dependent hydroxyl radical scavenging ability (Fig. 1). The extract exhibited strong inhibition of the radicals at a much lower concentration than BHA or α-tocopherol: the IC_{50} of the extract was 7.92 μg/mL, while those for BHA and α-tocopherol were 51.46 and 67.68 μg/mL, respectively. Thus, the scavenging abilities were as follows: celandine extract>BHA>α-tocopherol.

Hydroxyl radicals are the most reactive of all free radicals and can be formed from superoxide anions and hydrogen peroxide in the presence of metal ions (21). Hydroxyl radicals have the capacity to bond with the nucleotides in DNA, causing strand breakage that ultimately results in carcinogenesis, mutagenesis, and cytotoxicity. In addition, these species are believed to initiate the lipid peroxidation process, whereby hydrogen atoms are extracted from unsaturated fatty acids (22).

DNA damage protection assay Oxidative DNA damage has been implicated in various degenerative diseases (23).

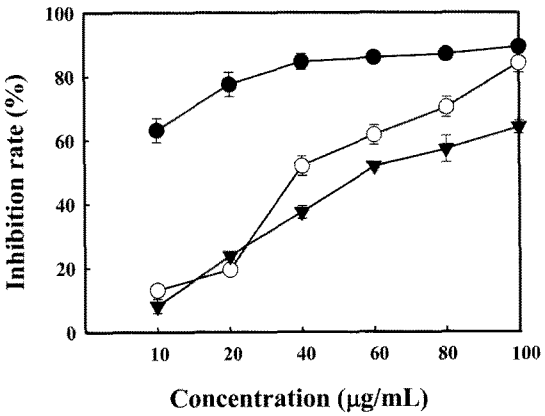

Fig. 1. Hydroxyl radical scavenging activity of celandine extract. (●) Celandine extract, (○) BHA, (▼) α-tocopherol. BHA and α-tocopherol were used as positive controls. Each value is expressed as the mean±SD (n=3).

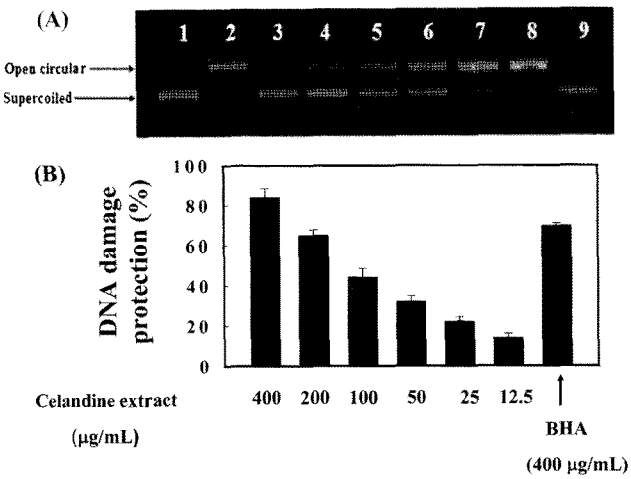

Fig. 2. (A) Visualization of the damage induced by hydroxyl radicals on plasmid DNA in the presence of celandine extract by agarose gel electrophoresis. (B) Histogram showing the protective effect of the extract against DNA damage based on densitometric measurements. Line 1, DNA incubated without Fenton's reagent; Line 2, DNA incubated with Fenton's reagent; Line 3-8, DNA incubated with Fenton's reagent in the presence of 400, 200, 100, 50, 25, and 12.5 μg/mL celandine extract, respectively. Line 9, DNA incubated with Fenton's reagent and 400 μg/mL BHA.

The antioxidant effect of the extract was evaluated based on its ability to protect against hydroxyl radical-induced DNA damage in the plasmid pBR 322. pBR 322 exists in 3 forms, supercoiled, open circular, and linear. When the plasmid was subjected to the Fenton reaction for 30 min, the intact supercoiled DNA was broken into the open circular form compared to the untreated plasmid (Fig. 2). However, at concentrations ranging from 50 to 400 μg/mL, celandine extract protected against hydroxyl radical-induced DNA damage in a dose-dependent manner (lane 3-8); in particular, it exhibited 82.5% protective activity at 400 μg/mL. In comparison, 400 μg/mL BHA (lane 9) produced a smaller protective effect on the supercoiled DNA. These results are consistent with our hydroxyl radical scavenging results.

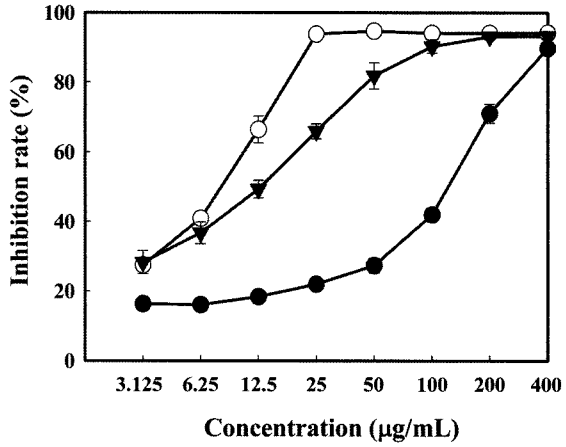

Fig. 3. DPPH radical scavenging activity of celandine extract. (●) Celandine extract, (○) α-tocopherol, (▼) BHT. BHT and α-tocopherol were used as positive controls. Each value is expressed as the mean±SD (n=3).

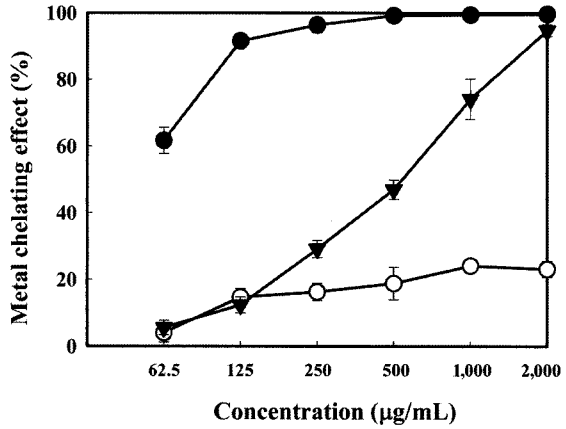

Fig. 4. Metal chelating activity of celandine extract. (●) EDTA, (○) α-tocopherol, (▼) celandine extract. EDTA and α-tocopherol were used as positive controls. Each value is expressed as the mean±SD (n=3).

DPPH radical scavenging activity DPPH has been used extensively as a free radical to evaluate reducing substances. The DPPH radical scavenging activity of the extract has been attributed to its ability to pair with the odd electron in the DPPH radical (24). As shown in Fig. 3, celandine extract exhibited dose-dependent DPPH radical scavenging activity. Based on the calculated IC_{50} values, the order of activity was as follows: α-tocopherol (8.52 μg/mL)>BHT (14.67 μg/mL)>celandine extract (126.69 μg/mL). The result showed that celandine extract had weak DPPH free radical scavenging activity compared to the standards used.

Metal chelating activity An important feature of antioxidants is their ability to chelate metals. Chelating agents have been reported as being effective as secondary antioxidants because they reduce oxidized forms of the metal iron (25). The metal chelating ability of celandine extract was tested against that of EDTA and α-tocopherol by evaluating their capacity to complex with Fe^{2+}. As shown in Fig. 4, EDTA exhibited a stronger inhibitory effect, followed by celandine extract and α-tocopherol. Although the iron chelating ability of celandine extract was moderate, it was stronger than that of α-tocopherol.

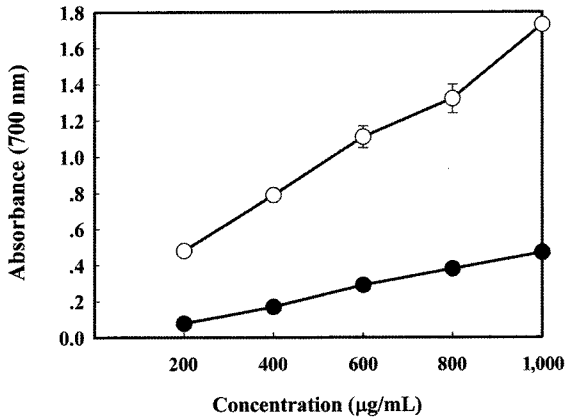

Fig. 5. Reducing power activity of celandine extract. (●) Celandine extract, (○) α-tocopherol. α-Tocopherol was used as positive control. Each value is expressed as the mean±SD (n=3).

Reducing power assay It is reported that reducing activity is generally associated with the presence of reductones, which have been shown to exert an antioxidant effect by donating a hydrogen atom and thereby breaking the free radical chain (26). The reducing power of celandine extract revealed that the reducing power was directly proportional to the concentration (200 to 1,000 μg/mL) used. The reducing power ability of celandine extract is much lower than α-tocopherol (Fig. 5).

Cellular proliferation assay Cellular proliferation was estimated by the MTT assay using cells treated with varying concentrations of celandine extract for 24, 48, or 72 hr. As shown in Fig. 6, the extract inhibited cellular proliferation in a time- and dose-dependent manner. The proliferation of HT-29 cells was reduced by 50% following 24 hr of exposure to 496.2 μg/mL extract, 48 hr of exposure to 288.9 μg/mL extract, and 72 hr of exposure to 87.2 μg/mL extract. These data provide evidence for the antiproliferative effect of celandine extract. The celandine extract at doses up to 400 μg/mL and a treatment time of 72 hr caused no more than 30% growth inhibition of the HEK 293 cells. This cytotoxicity level was much lower than that against HT-29 cancer cells.

Assessment of the apoptotic rate by Annexin V-FITC staining To estimate the degree of apoptosis induced by celandine extract, we utilized Annexin V-FITC and propidium iodide to distinguish between apoptotic and necrotic cells. Annexin V is a binding protein with high affinity and selectivity for phosphatidylserine (27). The appearance of phosphatidylserine on the cell surface is a general indicator of apoptosis; however, the translocation of phosphatidylserine to the cell surface also occurs during necrosis. Therefore, the measurement of Annexin V binding to the cell surface was performed in conjunction with propidium iodide (28). The results, shown in Fig. 7, indicate that celandine extract induced apoptosis in a dose-dependent manner. The proportion

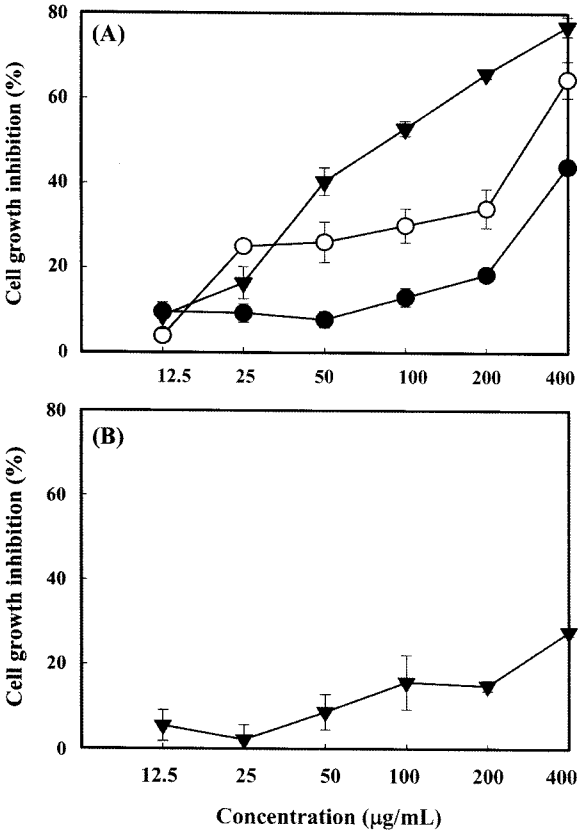

Fig. 6. (A) HT-29 cancer cells treated with various concentrations of celandine extract for 24, 48, or 72 hr. (B) HEK 293 cells treated with various concentrations of celandine extract for 72 hr. (●) 24 hr, (○) 48 hr, (▼) 72 hr. Each value is expressed as the mean±SD (n=3).

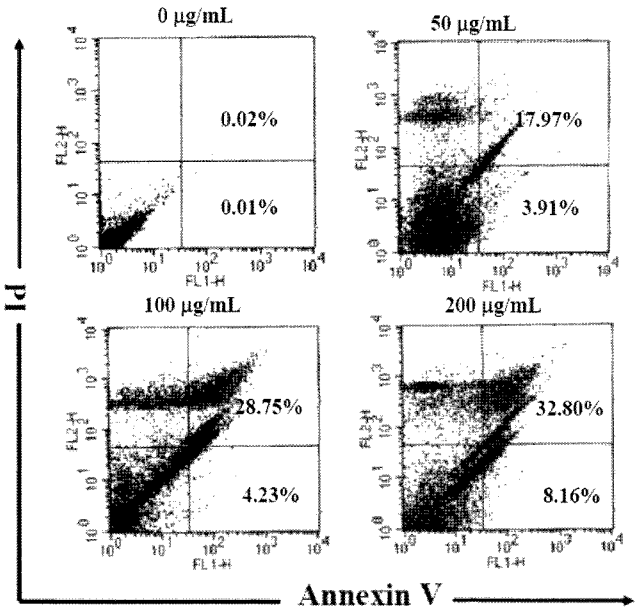

Fig. 7. Flow cytometric analysis of HT-29 cancer cells incubated with varying concentrations of celandine extract for 24 hr. The right bottom quadrant represents the Annexin V-stained cells (early-phase apoptotic cells). The top right quadrant represents PI- and Annexin V-stained cells (late-phase apoptotic/ necrotic cells).

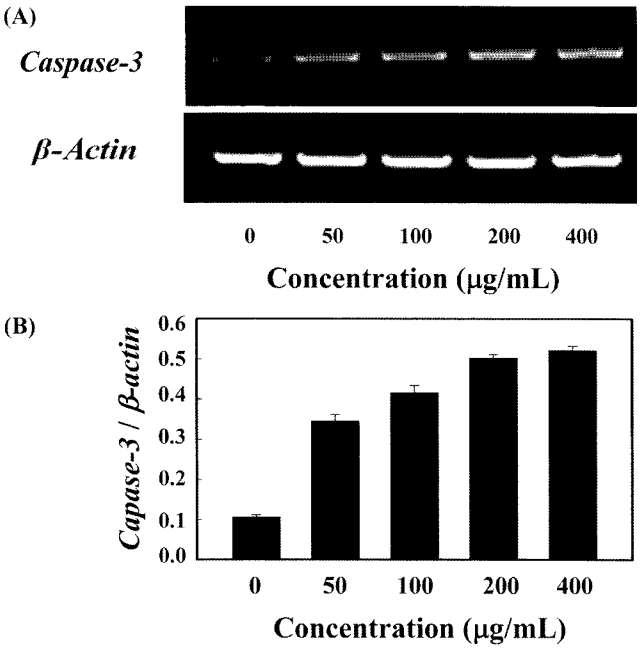

Fig. 8. Induction of *caspase-3* mRNA expression in HT-29 cancer cells. HT-29 cells were cultured in the absence (control) or presence of celandine extract for 24 hr; β-actin was used as an internal control. (A) Typical results of agarose gel electrophoresis. (B) Quantification of the *caspase-3* expression level was achieved by densitometric measurement.

of early-stage apoptotic cells increased significantly from 0.01 to 8.16%, while the proportion of late-stage apoptotic/ necrotic cells increased from 0.02 to 32.8%. Thus, celandine extract can induce apoptosis leading to the subsequent inhibition of cellular proliferation.

Determination of the expression level of *caspase-3*

Caspase-3 is the final executor of apoptotic DNA damage and cleaves several cellular proteins (29). Based on the increase in apoptosis among the celandine extract-treated cells, we examined whether *caspase-3* plays a role in the execution of apoptotic events. Following treatment with the extract at 25-400 μg/mL for 24 hr, the mRNA expression of *caspase-3* increased significantly in HT-29 cancer cells in a concentration-dependent manner (Fig. 8).

Summarizing the above result, it is conformed that the celandine extract exhibits antioxidant and antiproliferative activities. It has been stated that ROS in moderate concentrations exerts some functions necessary for cell homeostasis maintenance. However, when produced in excess they play a role in the causation of cancer (30). Early administration of antioxidants could prevent the initiation and progression of cancer by quenching the action of potentially mutagenic reactive free radicals (31). Therefore, additional studies are needed to identify the active compounds that confer the antioxidant and/or antiproliferative activities of celandine extract.

Acknowledgments

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