Cirsium japonicum extract induces apoptosis and anti-proliferation in the human breast cancer cell line MCF-7

DO-YEON KIM, SUNG-HAK KANG and SUNG-HO GHIL

Department of Life Science, Kyonggi University, Suwon 443-760, Korea

Abstract. *Cirsium japonicum* is a wild perennial herb that has been used as an anti-hemorrhagic, anti-hypertensive and uretic agent in traditional Chinese medicine. Recently, it was reported that *C. japonicum* inhibits the growth of implanted cancer cells. However, the molecular mechanisms underlying the anti-cancer properties of *C. japonicum* are not fully understood. In this study, we investigated the effect of a methanol extract of *C. japonicum* on cell growth in the human breast cancer cell line MCF-7. *C. japonicum* extract inhibited the cell proliferation of MCF-7 cells in a time- and dose-dependent manner, as evaluated by the MTT assay. Furthermore, *C. japonicum* extract induced an anti-proliferative effect by causing G_1 phase cell cycle arrest and also induced apoptosis by affecting mitochondrial apoptotic events, as determined by nuclear derangement, flow cytometry and Western blot analysis. Taken together, our findings indicate that *C. japonicum* extract induces the inhibition of MCF-7 cell growth at both the proliferation and apoptosis levels.

Introduction

Currently, many efforts are underway to develop new therapeutic anti-cancer drugs from natural sources. A major natural reservoir is plant material. Plant secondary metabolites have occasionally been used as chemotherapeutic agents to mediate cytostatic activity in cancer cells. Widely used anti-mitotic anti-cancer drugs including Vinca alkaloids and paclitaxel have been isolated from *Catharanthus roseus* and *Taxus baccata*, respectively (1). The DNA polymerase I inhibitor camptothecin was sourced from *Camptotheca acuminata* (2). Curcumin isolated from *Curcuma longa* has shown anti-cancer properties, such as the inhibition of tumor promotion and the induction of apoptosis in cancer cells (3-5).

Correspondence to: Dr Sung-Ho Ghil, Department of Life Science, Kyonggi University, Suwon 443-760, Korea
E-mail: shghil@kgu.ac.kr

Cirsium japonicum is a wild perennial herb found in many areas of Korea, Japan and China. It has been used as an anti-hemorrhagic, anti-hypertensive and uretic agent in traditional Chinese medicine (6). Recent studies have found that the water extracts of *C. japonicum* induce the activation of estrogen receptors and have estrogenic effects (7). *C. japonicum* also contains a vasorelaxant principle, mediating histamine H1-receptor activation (8). In traditional medicine, *C. japonicum* has sometimes been used for the management of different types of cancer, including liver and uterine cancer and leukemia (9).

Recently, it was reported that *C. japonicum* and its flavone components induce the inhibition of tumor formation in mice (9,10). However, the mechanism underlying the anti-cancer effects of these *C. japonicum* compounds are not fully understood.

In the present study, we observed that a methanol extract of *C. japonicum* significantly reduced cancer cell growth in a time- and dose-dependent manner, using the human breast cancer cell line MCF-7. Furthermore, we investigated the molecular mechanisms underlying the anti-proliferative activities of *C. japonicum*.

Materials and methods

Preparation of C. japonicum extract. Dried roots of *C. japonicum* from Korea were purchased from the Kyungdong oriental medicine market in Seoul, Korea. *C. japonicum* material (100 g) was extracted with 99.8% methanol (1 liter) for 72 h at room temperature. The extract was evaporated to dryness on a rotary evaporator and dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO).

Cell culture. The human breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Hyclone, South Logan, UT) at 37°C in a humidified incubator under 5% CO_2.

Cell proliferation assay. The effect of *C. japonicum* extract on MCF-7 cell proliferation was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the ability of live cells to cleave the tetrazolium ring to a molecule that absorbs at 570 nm. Cells ($3x10^3$/well) were plated on 96-well microplates.

Twenty-four hours later, the cells were incubated with various concentrations of *C. japonicum* extract (0-100 μg/ml) for the indicated time. After incubation, 10 μl MTT solution (5 mg/ml) (Sigma) was added to each well, and cells were further incubated at 37°C. After 4 h of incubation, 100 μl of isopropyl alcohol dissolved in 5% 1N HCl was added to solubilize the formazan crystals. Absorbance was measured using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Cells were never exposed to >0.5% DMSO concentrations.

Analysis of nuclear morphology. MCF-7 cells were plated on coverslips at a density of 5x10^3 cells/coverslip and exposed to *C. japonicum* extract at 30 μg/ml. After 72 h of incubation, the cells were fixed in 4% paraformaldehyde for 10 min, and nuclei were stained with 10 μg/ml Hoechst 33258 (Sigma) for 20 min. Nuclear morphology was observed by fluorescence microscopy (BX-50; Olympus, Tokyo, Japan).

DNA ladder formation assay. MCF-7 cells (2x10^5 cells/dish) were plated on 100-mm tissue culture dishes. The cells were treated with 100 μg/ml *C. japonicum* extract and incubated for the indicated times, then washed with PBS and lysed with the AccuPrep® Genomic DNA Extraction kit (Bioneer, Daejeon, Korea) as recommended by the manufacturer. DNA concentration was measured using a BioPhotometer (Eppendorf, Hamburg, Germany), then the DNA was separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Cell cycle and Annexin-V/propidium iodide flow cytometric analysis. MCF-7 cells (2x10^5 cells/dish) were plated on 100-mm tissue culture dishes. Cells were treated with 70 μg/ml *C. japonicum* extract for the indicated times. Cells were harvested by trypsinization, washed with ice-cold PBS and fixed in 70% ethanol for 20 min. For cell cycle analysis, fixed cells were stained with propidium iodide (PI) solution (PBS containing 50 μg/ml PI, 10 μg/ml RNase A and 3.8 mM sodium citrate) at 4°C for 20 min. For Annexin-V/PI flow cytometric analysis, fixed cells were stained using the FITC Annexin-V Apoptosis Detection kit I (BD Biosciences, San Diego, CA) as recommended by the manufacturer. Flow cytometric analysis used a FACSVantage SE (Becton-Dickinson, San Jose, CA). Data from 10,000 cells per sample were collected and analyzed.

Western blot analysis. MCF-7 cells (2x10^5 cells/dish) were plated on 100-mm tissue culture dishes. Cells were treated with 50 μg/ml of *C. japonicum* extract for the indicated times, then rinsed twice with ice-cold PBS, harvested and extracted with PBTX buffer (PBS containing 5 mM MgCl$_2$, 1 mM EDTA and 0.1% Triton X-100) containing protease inhibitors (5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A and 2 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C. Protein (50 μg) was subjected to 8-12% SDS-PAGE before electrotransfer to a PVDF membrane (Westran S; Whatman, Florham Park, NJ). Blots were blocked in 5% non-fat dry milk for 1 h at room temperature and incubated with antibodies against p53, Bax, Bcl-2, cyclin-dependent protein kinase 4 (cdk4), cyclin D1, E2F1, poly (ADP-ribose) polymerase (PARP)-1/2, caspase-7
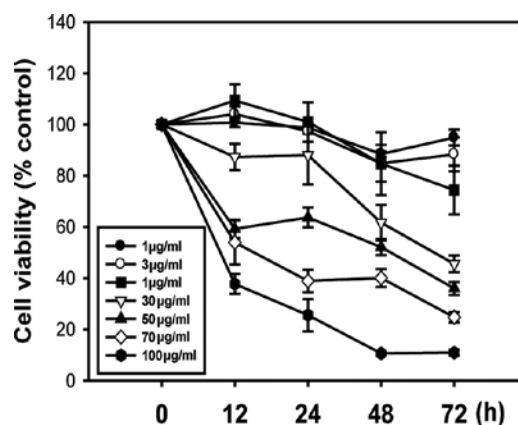

Figure 1. Effect of *C. japonicum* extract on the proliferation of MCF-7 cells. Cells were treated with a methanol extract of *C. japonicum* for various times and at different concentrations. Cell proliferation was measured using the MTT assay. Each point represents the mean of data from three independent experiments.

(all at 1:500 dilution) (all from Santa Cruz Biotechnology, Santa Cruz, CA), or α-tubulin (1:2,500 dilution) (Upstate Biotechnology, Temecula, CA). Membranes were then incubated with the corresponding secondary antibodies, either goat anti-mouse IgG HRP conjugate (1:5,000 dilution) (Zymed, Carlsbad, CA) or goat anti-rabbit IgG HRP conjugate (1:25,000 dilution) (Zymed). Transferred proteins were visualized using an enhanced chemiluminescence detection kit (West-Zol®; Intron Biotechnology, Sungnam, Korea).

Results

Effect of C. japonicum extract on the proliferation of the human breast cancer cell line MCF-7. To investigate the effects of *C. japonicum* extract on MCF-7 proliferation, we exposed cells to 0-100 μg/ml of *C. japonicum* extract for the indicated times and measured cell proliferation using the MTT assay. As shown in Fig. 1, *C. japonicum* extract significantly inhibited the proliferation of MCF-7 cells in a time- and dose-dependent manner. The median effective doses (EC$_{50}$ values) of *C. japonicum* extract were 78.7±8.8, 73.6±11.3, 51.8±10.0 and 44.2±12.1 μg/ml at 12, 24, 48 and 72 h, respectively.

Apoptotic activity of C. japonicum extract acting on MCF-7 cells. To determine whether *C. japonicum* extract causes the apoptotic cell death of MCF-7 cells, we treated the cells with the extract at 30 μg/ml for 72 h, then stained them with Hoechst 33258. As shown in Fig. 2A, condensed chromatin was observed by fluorescence microscopy in the test cells, but not in the controls. Furthermore, agarose gel electrophoresis of DNA from cells treated with *C. japonicum* extract showed a ladder-like pattern of DNA fragments. The apoptosis-inducing activity of the extract was time-dependent (Fig. 2B). To confirm the apoptosis-promoting potential of *C. japonicum* extract, we treated the cells with the extract at 70 μg/ml for the indicated times and performed flow cytometry after Annexin-V/PI dual staining. The dot plots show non-apoptotic live cells in the lower left quadrant (Annexin-V$^-$/PI$^-$), apoptotic cells in the lower right quadrant (Annexin-V$^+$/PI$^-$) and cells in late apoptosis or necrosis in the upper right quadrant
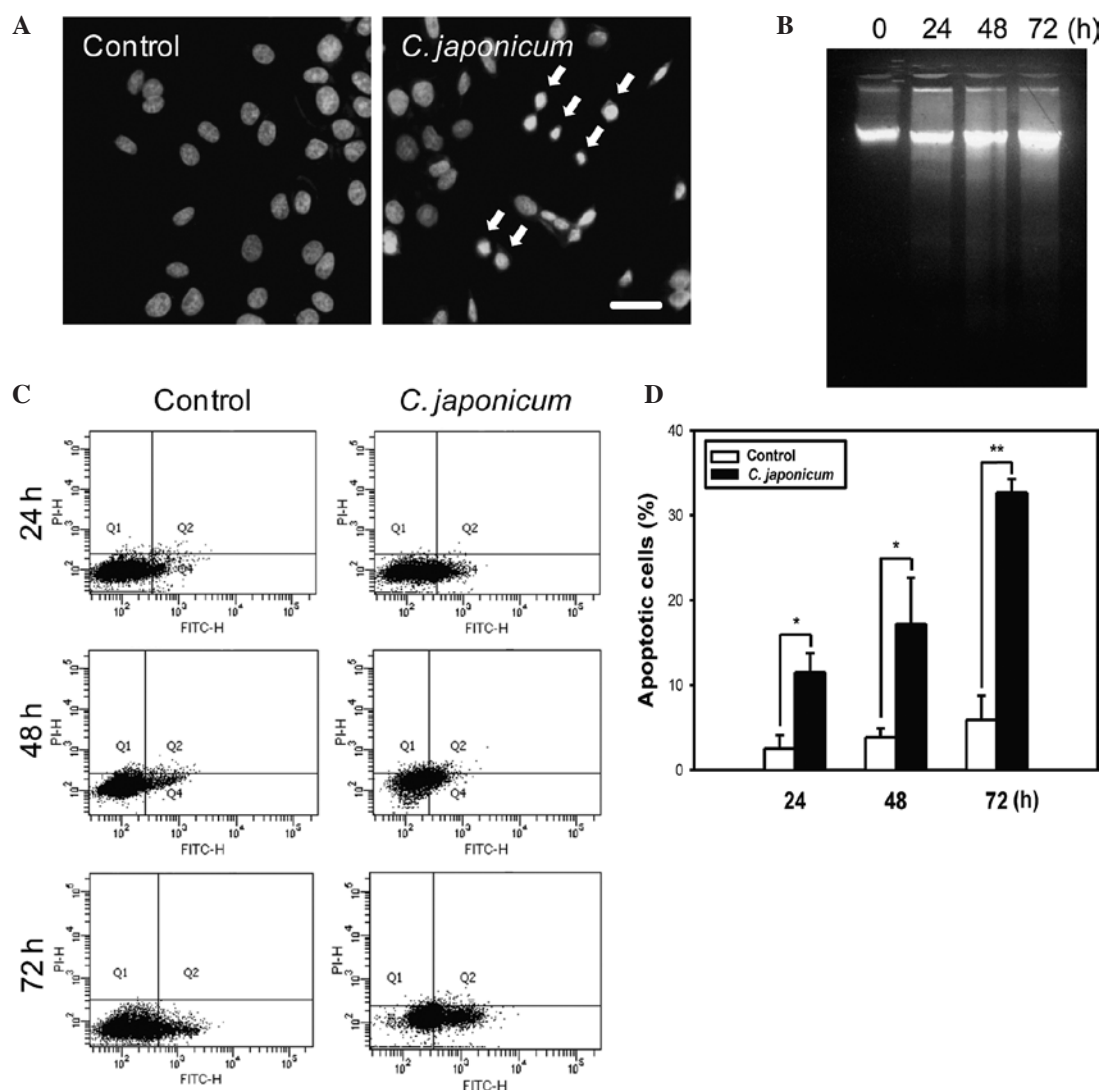
Figure 2. Apoptotic activity of *C. japonicum* extract acting on MCF-7 cells. (A) Cells were incubated with or without 30 µg/ml extract for 72 h and stained with Hoechst 33258 dye. Arrows indicate condensed chromatin in cell nuclei. Scale bar, 50 µm. (B) Cells were incubated with 100 µg/ml *C. japonicum* extract for the indicated times followed by the DNA ladder formation assay. (C) MCF-7 cells were incubated with or without 70 µg/ml *C. japonicum* extract for the indicated times, stained with Annexin-V/PI and analyzed by flow cytometry. (D) The percentages of apoptotic cells (Annexin-V+/PI- cells) are shown. Data are the means ± SE of three independent experiments. *P<0.05; **P<0.01.

(Annexin-V+/PI+). The addition of *C. japonicum* extract significantly increased the proportion of apoptotic cells at all time points (Fig. 2C and D). These results indicate that *C. japonicum* extract potentiated the apoptotic death of MCF-7 cells.

Effect of C. japonicum extract on cell cycle progression. To investigate the basis for the anti-proliferative properties of *C. japonicum* extract, MCF-7 cells were treated with the extract at 30 µg/ml for the indicated times, stained with PI and analyzed by flow cytometry. Incubation with *C. japonicum* extract significantly increased the proportions of cells in the sub-G_1 and G_1 phases at all time points, compared with the control cells (Fig. 3A and B). The number of cells in the G_2/M and S phases decreased or was similar to the levels noted in the controls (Fig. 3C and D). These results indicated that *C. japonicum* extract induced both apoptotic cell death and cell cycle arrest in the G_1 phase in MCF-7 cells.

Effect of C. japonicum extract on the expression of cell cycle- and apoptosis-related proteins. Western blot analysis was used to determine the expression levels of cell cycle- and apoptosis-related proteins. The levels of the G_1 phase cell cycle regulatory proteins, including cyclin D1, cdk4 and the transcription factor E2F1, significantly decreased in a time-dependent manner after exposure of the cells to the extract (Fig. 4A and B). The tumor suppressor gene p53 mediates either cell cycle arrest or apoptosis in response to DNA damage (11-13). Treatment with 50 µg/ml *C. japonicum* extract increased expression of p53 (Fig. 4C and D). Bcl-2 and Bax protein levels were also measured in cultured MCF-7 cells to investigate the involvement of these proteins in *C. japonicum* extract-mediated apoptosis. As shown in Fig. 4C, cells treated with *C. japonicum* extract showed an increase in Bax expression and a decrease in Bcl-2 in a time-dependent manner. Densitometry was used to determine Bax/Bcl-2 ratios. The ratio was significantly increased in a time-dependent
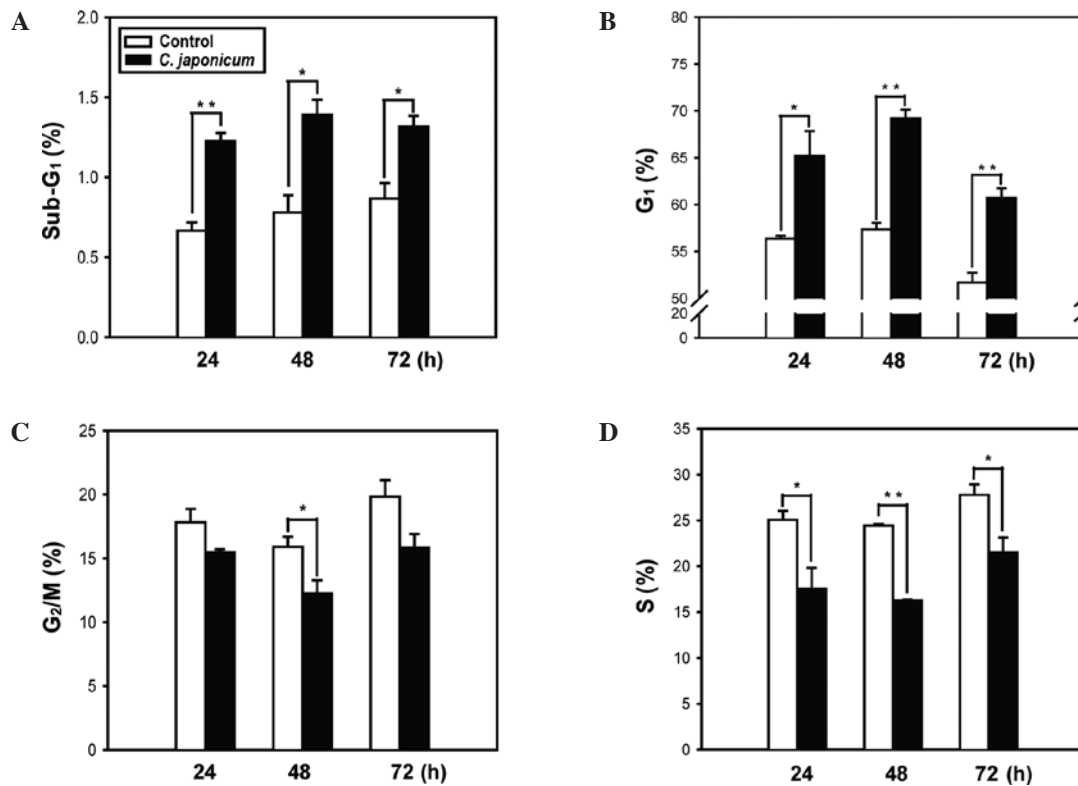
Figure 3. Effect of *C. japonicum* extract on cell cycle progression in MCF-7 cells. Cells were incubated with 70 μg/ml of *C. japonicum* extract for the indicated times, stained with PI and analyzed by flow cytometry. G_1 (B), G_2/M (C) and S (D) indicate the cell cycle phase, and sub-G_1 (A) DNA content refers to the proportion of apoptotic cells. Data are the means ± SE of three independent experiments. *P<0.05; **P<0.01.

manner after treatment with the extract (Fig. 4E). The final activators of apoptosis are the caspases, a family of cysteine proteases that are activated by mitochondrial cytochrome *c* release (14,15). Our Western blotting results indicated that the specific immunoreactive band intensity of procaspases-7 decreased in the cells treated with *C. japonicum* extract in a time-dependent manner (Fig. 4C and D). Additional evidence of caspase activation was provided by the cleavage of PARP, as shown by Western blot analysis. The incubation of MCF-7 cells with extract resulted in the formation of 85-kDa protein fragments in a time-dependent manner (Fig. 4C and D).

Discussion

In the present study, *C. japonicum* extract potently inhibited the proliferation of MCF-7 cells in a time- and dose-dependent manner (Fig. 1). Treatment with the *C. japonicum* extract induced nuclear derangement such as nuclear condensation and DNA ladder formation, caused an increase in the Annexin-V$^+$/PI$^-$ cell population and resulted in cell accumulation in the sub-G_1 phase, indicating that *C. japonicum* extract induces apoptotic cell death in MCF-7 cells (Figs. 2 and 3). Morphological changes such as condensed and fragmented chromatin are associated with apoptotic cell death. An increase in Annexin-V reactivity and sub-G_1 phase cell accumulation are also usually considered to reflect an apoptotic cell death profile (16).

To determine the precise mechanism of *C. japonicum* extract-induced apoptotic cell death in MCF-7 cells, we examined the expression of apoptosis-related proteins by Western blot analysis. Bcl-2 family proteins regulate the progression of cells to apoptosis via activation of the caspase cascade (17). Increased expression of pro-apoptotic proteins (such as Bax and Bak) and decreased levels of anti-apoptotic proteins (such as Bcl-2 and Bcl-xL) activate the mitochondrial release of pro-apoptotic molecules, including cytochrome *c* and apoptosis-inducing factor, into the cytosol (18-20). It has been reported that an increase in the Bax/Bcl-2 ratio triggers mitochondria-mediated cell death in MCF-7 cells (21,22). Cytochrome *c* released from mitochondria induces the conversion of procaspases to caspases (17,23,24). Activated caspases cause the cleavage of various cellular substrates, and induce the morphological and biochemical characteristics of apoptosis, including cell shrinkage, DNA fragmentation, chromatin condensation and membrane blebbing (19,25). Activated caspases-3 and -7 cleave PARP, a nuclear protein involved in the maintenance of DNA stability, the modification of many nuclear proteins and post-translational modification, thus triggering apoptosis (26). Caspase-3, however, is not expressed in MCF-7 cells (27). In the present study, increases in the Bax/Bcl-2 ratio, decreases in the level of procaspase-7, and PARP cleavage resulting from treatment with an extract of *C. japonicum* all indicate the involvement of mitochondrial events in the apoptosis of MCF-7 cells.

The growth of normal cells is regulated by a balance between cell proliferation and apoptosis. One of the characteristics of cancer cells is the derangement of this control system (28). In the present study, *C. japonicum* extract not only
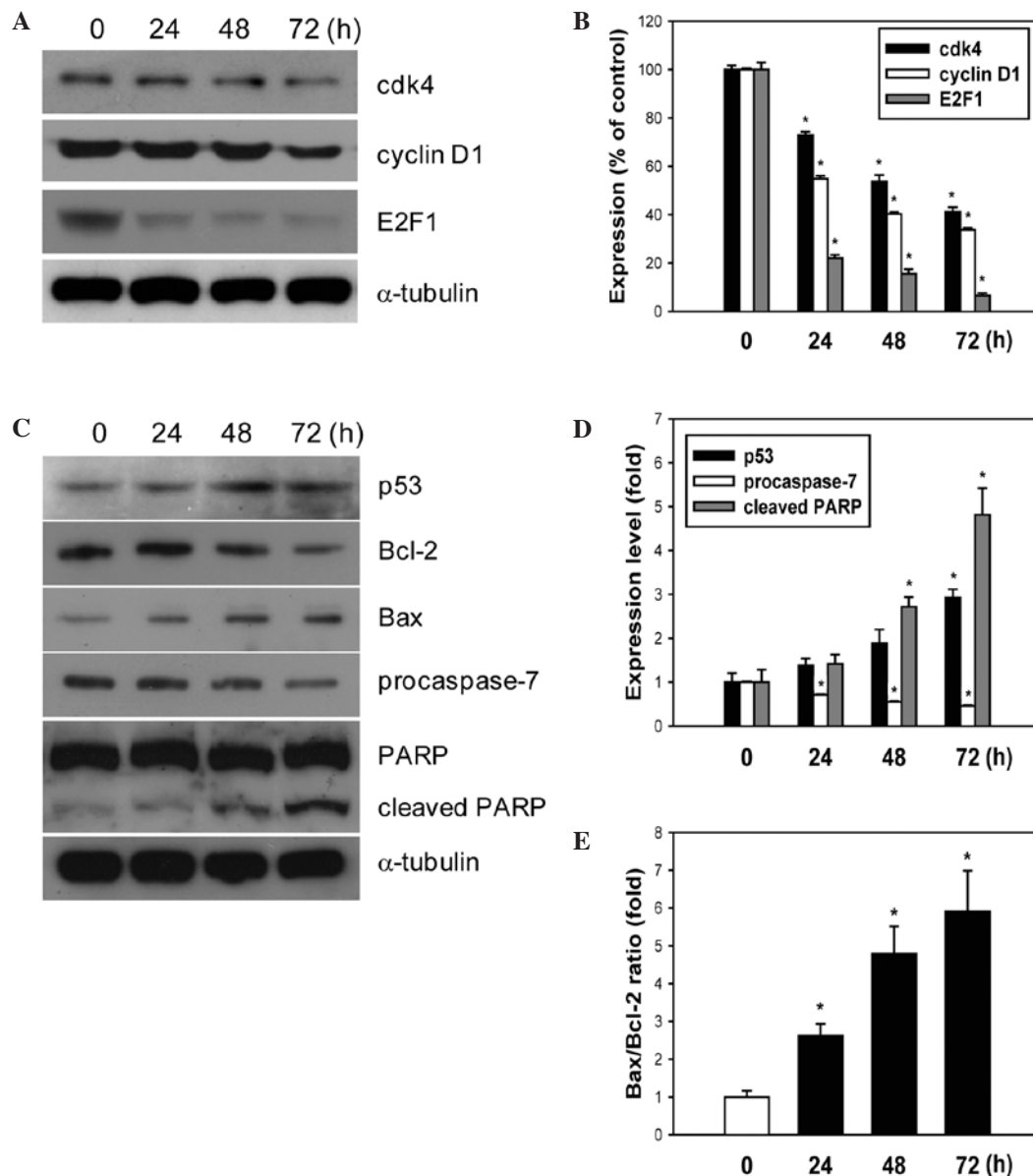
Figure 4. Effects of *C. japonicum* extract on cell cycle- and apoptosis-related protein expression. MCF-7 cells were treated with 50 µg/ml *C. japonicum* extract for the indicated times and subjected to Western blot analysis. (A) Expression levels of cell cycle-related proteins were measured by using antibodies against cdk4, cyclin D1 and E2F1. α-tubulin was used as an internal control. (B and D) Relative band intensities of each protein were quantified by densitometry. (C) Expression levels of apoptosis-related proteins were measured by using antibodies against p53, Bax, Bcl-2, caspase-7 and PARP. (E) The Bax/Bcl-2 ratios are shown. Data are the means ± SE of three independent experiments. *P<0.01, with respect to the corresponding control.

induced apoptosis, but repressed proliferation, thereby leading to the significant accumulation of cells in the G_1 phase. As shown in Fig. 3, after a 48-h incubation with MCF-7 cells, the extract increased the cell population in the G_1 phase from 57.4 to 69.2%. Our data clearly show that this substantial increase in the proportion of cells in the G_1 phase was accompanied by a decrease in cells in the G_2/M and S phases at this time point.

The cell cycle is regulated by cyclin and cdk complexes (29). In the G_1 phase, cyclinD/cdk4 or cdk6 phosphorylate retinoblastoma protein (pRb), resulting in the inactivation of pRb. This in turn allows for the expression of the E2F1 transcription factor, which controls the gene expression necessary for cell cycle progression (30,31). As shown in Fig. 4, treatment with *C. japonicum* extract significantly down-regulated the

expression of cdk4, cyclin D1 and E2F1 in a time-dependent manner, suggesting a possible explanation for the G_1 phase cell cycle arrest noted here.

In conclusion, we present a possible mechanism by which *C. japonicum* extract induces apoptosis as well as anti-proliferative activity in the human breast cancer cell line MCF-7. *C. japonicum* extract induces apoptosis by affecting mitochondrial apoptotic events such as the increase in Bax/Bcl-2 ratio, caspase activity and PARP cleavage. *C. japonicum* extract also exerts an anti-proliferative effect through the down-regulation of cdk4, cyclin D1 and E2F1, which leads to cell cycle arrest in the G_1 phase. If these anti-tumor effects are supported by additional *in vivo* experiments, it may be possible to use *C. japonicum* to mediate chemotherapeutic and cytostatic activity in human breast cancer.

Acknowledgements

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