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Inhibitory Effects of Luteolin Isolated from *Ixeris sonchifolia* Hance on the Proliferation of HepG2 Human Hepatocellular Carcinoma Cells

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We investigated the anti-proliferative effects of luteolin and apigenin, isolated from *Ixeris sonchifolia* Hance, on HepG2 human hepatocellular carcinoma cells. In MTT assay luteolin showed more efficient anti-proliferative effects on cells than apigenin did. According to propidium iodide staining and flow cytometry studies, we postulated that these effects might be a result of cell cycle arrest. Hence we examined the changes of protein expressions related to cell cycle arrest. Western blotting data demonstrated that the down-regulated expression of CDK4 was correlated to the increase of p53 and CDK inhibitor $p21^{WAF1/CIP1}$ protein. These data suggest that luteolin may have potential as an anti-cancer agent.

Key words: Luteolin, Apigenin, *Ixeris sonchifolia* H., HepG2 cells, Cell cycle arrest

INTRODUCTION

Ixeris sonchifolia Hance, one of the family *Compositae*, is a bitter perennial herb distributed and cultivated widely in Korea (Shin, 1988). Its components have been used as a food and herb folk medicine for suppurative inflammations and pain symptoms. It was previously reported that the whole plants contain free sugars, amino acids, fatty acid, minerals, polyphenols (Shin, 1988), flavonoids (Young *et al.*, 1992a,b,c), and triterpenes (Shin, 1993).

The extracts treated on the liver injury in CCl_4-treated rats had lower values of glutamic pyruvic transaminase and malondialdehyde than the control group and revealed less of necrosis in histological findings of liver tissue (Bae *et al.*, 1997a,b). Species of the genus *Ixeris* showed hepatoprotective activity upon estimating serum transaminases concentrations and histopathologic change in liver injured rats (Chiu *et al.*, 1989; Lin *et al.*, 1994; Lu *et al.*, 2000). The hexane extracts enhanced the stability and fluidity of biological liposomal membranes (Bae *et al.*, 1998a,b).

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From methanol extracts of the leaves of *Ixeris sonchifolia*, luteolin and its glucoside and apigenin 7-*O*-glucuronide were isolated and cynaroside (luteolin 7-*O*-glucoside) caused a significant hypocholesterolemic activity in the hyperlipidemic rats (Young *et al.*, 1992a).

Few studies on their anticancer effects have been reported. Water extracts of the plant's root increased the survival period by 55% and alcohol extracts by 38% on Ehrlich carcinoma (Park, 1977). The triterpene compounds (α-amyrin, lupeol) isolated from the whole plant's $CHCl_3$-soluble fraction, and the guaianolide sesquiterpene lactone isolated from the ethyl acetate (EtOAc) soluble fraction, showed cytotoxic activity against the human stomach cancer cell line (SNU-1) and human colon cancer cell line (SNU-C4) on MTT assay (Chung, 2000; Chung, 2001). However, confirmation of the effective component and establishment of the anti-proliferative effects on human cancer have not been carried out yet.

Luteolin and apigenin are classes of flavone, and the former has been shown to possess potent antioxidant and anti-inflammatory/anti-allergic (Shimoi *et al.*, 2000), antibacterial (Pettit *et al.*, 1996), and antineoplastic activities (Li *et al.*, 2001). Luteolin inhibits the growth of human leukemia cell (HL-60) (Post and Varma, 1992; Li *et al.*,

2001), thyroid cancer cell (ARO, NPA, WRO) (Yin *et al.*, 1999a,b), prostate tumor cell (PC-3) (Knowles *et al.*, 2000), and melanoma cell (OCM-1) (Casagrande and Darbon, 2000). Apigenin shows anticancer effects and inhibits the growth of human prostate tumor cell (PC-3, CA-HPV-10) (Knowles *et al.*, 2000; Gupta *et al.*, 2001), leukemia cell (HL-60) (Wang *et al.*, 1999), melanoma cell (OCM-1) (Casagrande and Darbon, 2000), thyroid cancer cell (ARO, NPA, WRO) (Yin *et al.*, 1999a,b), colon carcinoma cell (SW480, HT-29, Caco-2) (Wang *et al.*, 2000), and breast carcinoma cell (MCF-7, MDA-MB-468, ZR-75-1) (Hirano *et al.*, 1989; Yin *et al.*, 2001). However, the effect luteolin and apigenin on liver cancer cells has not been studied until now.

In this study, we investigated the anti-proliferative effects of luteolin and apigenin, isolated from *Ixeris sonchifolia* Hance, on HepG2 human hepatocellular carcinoma cells.

MATERIALS AND METHODS

Luteolin and apigenin

The luteolin and apigenin isolated from *Ixeris sonchifolia* H. roots were isolated by a previously reported method (Suh *et al.*, 2002). Each compound was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) to 20-200 μg/mL stock solutions for further experiments.

Cell line and cell culture

HepG2 human hepatocellular carcinoma cells were obtained from American Type Culture Collection (Rockville, MD, USA). They were grown in minimum essential medium (MEM) with 50 μg/mL gentamicin.

MTT assay

The anti-proliferative effect of luteolin and apigenin was tested by MTT assay, which measures mitochondrial activity in viable cells. This method is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to MTT-formazan crystal by mitochondrial enzyme as previously described (Tada *et al.*, 1986). Cells, at 0.96×10^4 cells/well seeded in Falcon 96 well plate, were allowed to adhere overnight, and then the culture medium was replaced with fresh MEM. Cells were exposed to each compound at proper concentration for 48 h. Control groups were treated with DMSO equal to the highest percentage (0.1%) of solvent used in the experimental conditions. After 48 h the medium was replaced with serum free medium. MTT was freshly prepared at 5 mg/mL in phosphate buffered saline (PBS, Sigma) and passed through a 0.2 μm-pore-size filter. An aliquot of 100 μL of MTT stock solution was added to each well, and the plate was incubated at 37°C for 4 h in humidified 5% CO_2 incubator. After 4 h the medium was removed. Aliquots of 200 μL of EtOH-DMSO (1:1 mixture solution) were added per one well in order to solubilize the formazan. After 10 min the optical density of each well was measured with a spectrophotometer equipped with a 560 nm filter. The proliferation rate was calculated from 4 wells using percentage of control.

Cell growth curve

HepG2 cells were grown over a 5-day period, day 1 to day 6, changing medium supplemented with luteolin (7 and 12.5 μg/mL) every other day. Each properly treated dish was trypsinized, and the cell pellets were spun down and then resuspended with an equal volume of medium and trypan blue (GibcoBRL) for the counting of survived cells. Living cells were counted on a hemocytometer.

Observation of cell morphology and propidium iodide staining for observing nuclear structure

HepG2 cells were incubated either with or without luteolin (7 and 12.5 μg/mL) for 6 h, 12 h, 24 h, 48 h, or 72 h for the same period. Then cells were washed twice with PBS containing 1% bovine serum albumin (PBS-B) and fixed with 70% ethanol containing 0.5% Tween 20 at 4°C for 30 min. Fixed cells were washed with PBS-B, and stained with propidium iodide (PI, Sigma) solution (50 μg/mL in PBS) for 30 min at room temperature. Stained cells were washed twice with PBS-B and observed via a fluorescence microscope at ×100 and 320 magnification.

Flow cytometry analysis

Treated cells were detached using trypsin/EDTA (GibcoBRL), washed with PBS and fixed in 75% ethanol at 4°C for 30 min. Prior to analyses, cells were washed again with PBS, resuspended in cold PI solution (PI in PBS, 50 μg/mL) and incubated at room temperature in the dark for 30 min. Flow cytometry analyses were performed on a flow cytometry system (Becton Dickinson, San Jose, CA, USA).

Protein preparation and western blotting

Cells were harvested and washed twice in PBS at 4°C. Total cell lysates were lysed in lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride (PMSF)]. The supernatant was collected and protein concentrations were then measured with protein assay reagents (Pierce, Rockford, IL, USA). An equal amount of proteins was boiled for 3 min and chilled on ice, subjected to 12.5% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Monoclonal antibodies to $p21^{WAF1/CIP1}$, p53, cyclin B1, cyclin D1 and polyclonal antibody to CDK4 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz,

CA, USA). Monoclonal antibody to β-actin (Sigma) was used as an internal control. Peroxidase-labeled, donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Life Science (Arlington Heights, IL, USA). The proteins were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham Life Science).

RESULTS

Anti-proliferative effect of luteolin and apigenin

IC_{50} values of luteolin and apigenin on HepG2 cells were 8 and 13 mg/mL, respectively (Fig. 1). Therefore, luteolin was used for further experiments. HepG2 cells were cultured in the absence or presence of luteolin (7 and 12.5 μg/mL) for different time periods. As shown in Fig. 2, untreated control HepG2 cells displayed exponential growth during the 6-day incubation period, whereas luteolin (7 and 12.5 μg/mL-treated cells showed prominent reductions in proliferation.

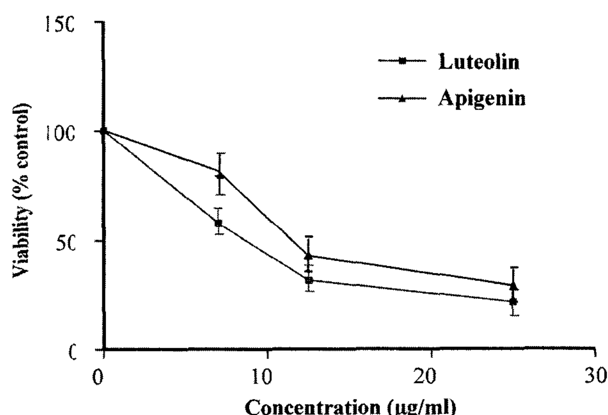

Fig. 1. Effects of luteolin and apigenin on the proliferation of HepG2 cells. Results are mean ± S.D. of three experiments.

Cell cycle arrest

After PI staining apoptotic bodies were not detected on HepG2 cells treated with luteolin (7 and 12.5 μg/mL) (Fig. 3). Moreover, DNA ladder formation was not detected at the DNA fragmentation assay (data not shown). These results indicate that the growth inhibition of luteolin was due to cytostatic, but not cytotoxic, effects. To determine whether this growth inhibitory effect was caused by specifically perturbing, cell cycle-related events, a set of experiment was performed to measure DNA content and the cell cycle distribution of luteolin (7 μg/mL) -treated or -untreated cells by flow cytometry after staining with PI. The results showed that luteolin-treated HepG2 cells led to a marked accumulation of cells in the G1 phase, suggesting that the growth inhibition was the result of a block during this G1 phase and that such cells did not enter the S phase, whereas the effect was not observed in control cells treated with vehicle (Fig. 4).

Down-regulation of CDK4 and up-regulations of p53 and p21

To understand the mechanism of cell growth inhibition,

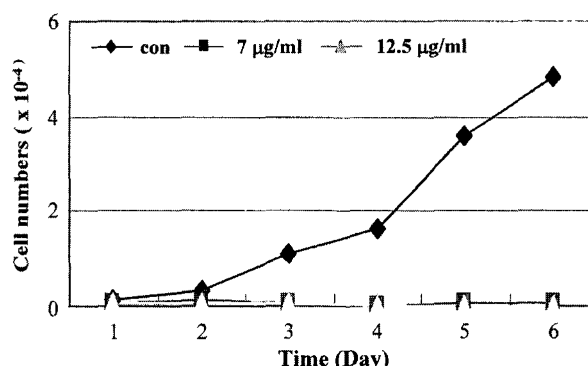

Fig. 2. Effects of luteolin on the growth of HepG2 cells. Results are mean ± S.D. of three experiments. Con, control.

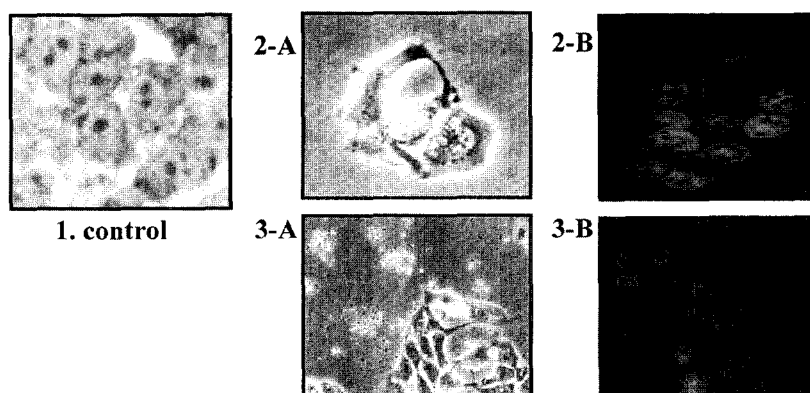

Fig. 3 Morphological changes of HepG2 cells treated with luteolin. Control, untreated HepG2 cells; 2-A, HepG2 cells treated with luteolin (7.0 μg/mL); 2-B, HepG2 cells treated with luteolin (7.0 μg/mL) and PI staining; 3-A, HepG2 cells treated with luteolin (12.5 μg/mL); 3-B, HepG2 cells treated with luteolin (12.5 μg/mL) and PI staining.

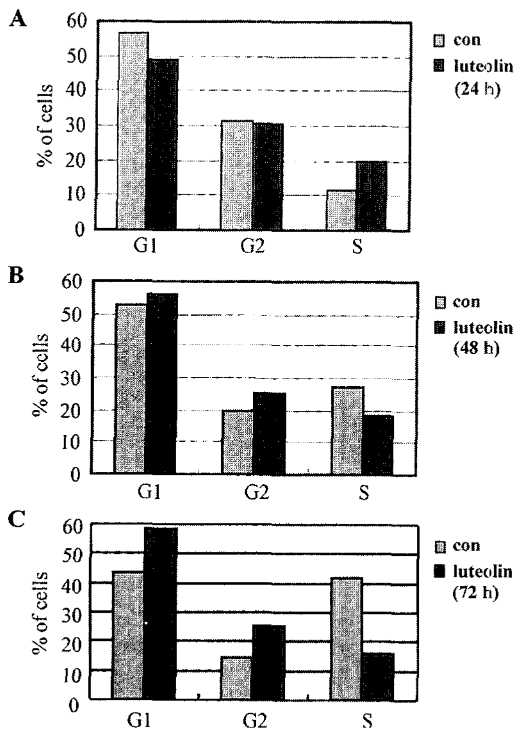

Fig. 4. Cell cycle analysis of HepG2 cells treated with luteolin. Cells treated with luteolin (7 μg/mL) for 24 h (A), 48 h (B), and 72 h (C). Con, control.

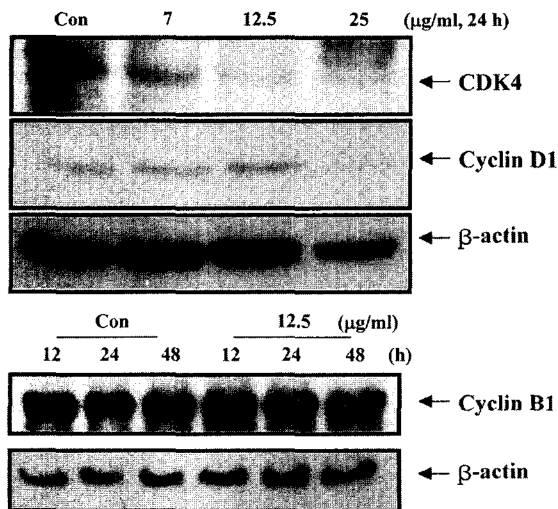

Fig. 5. Effects of luteolin on the cell cycle regulators, CDK4, cyclin D1, and cyclin B1 in HepG2 cells. HepG2 cells were treated with luteolin for 24 h. Total cell lysates were prepared and immunoblotted. Western blots were detected with antibodies against CDK, cyclin D1, and cyclin B1, and with ECL detection. Con, control.

asynchronous HepG2 cells were treated with or without luteolin (7, 12.5, and 25 μg/mL) for 24 h and the expression of cell cycle regulating proteins (i.e., cyclin D1 and CDK4) was compared by Western blot analysis. The intracellular protein level of CDK4 was down-regulated by luteolin at increasing concentration (Fig. 5). The protein level of cyclin D1 was up-regulated at 7 and 12.5 μg/mL, but down-regulated at 25 μg/mL, in HepG2 cells. In addition, the protein level of cyclin B1 in HepG2 cells treated with luteolin (12.5 μg/mL) for 12, 24, and 48 h was not changed with time.

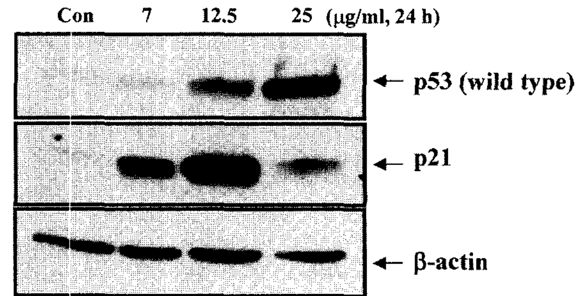

Fig. 6. Effects of luteolin on the cell cycle regulatory proteins, p53 and $p21^{WAF1/CIP1}$, in HepG2 cells. HepG2 cells were treated with luteolin for 24 h. Total cell lysates were prepared and immunoblotted. Western blots were detected with antibodies against p53 and $p21^{WAF1/CIP1}$, and with ECL cetection. Con, control.

It has been shown that the tumor suppressor p53 regulates a DNA damage-triggered G1 checkpoint by up-regulation of $p21^{WAF1/CIP1}$ expression (Hartwell and Kastan, 1994). Experiments were carried out to examine whether CDK inhibitors were induced by luteolin. The results showed that treatment with luteolin (7, 12.5, and 25 μg/mL) resulted in a marked increase in the level of the p53 and CDK inhibitor $p21^{WAF1/CIP1}$ protein in HepG2 cells (Fig. 6).

DISCUSSION

This study investigated the anti-proliferative effects of two active flavonoid components, luteolin and apigenin, on HepG2 human cancer cells. Furthermore, the mechanisms of luteolin's antiproliferative effects on HepG2 cells were studied. The results of PI staining and DNA fragmentation assay on luteolin-treated HepG2 cells indicate that the growth inhibition of luteolin was a result of cytostatic, but not cytotoxic, effects. By flow cytometry analysis, luteolin led to a marked accumulation of cells in the G1 phase. The level of CDK4 involved in G1 progression was down-regulated, and the levels of tumor suppressor, p53, and CDK inhibitor, $p21^{WAF1/CIP1}$, were up-regulated by luteolin in HepG2 cells. Therefore, luteolin induces G1 arrest on HepG2 cells via up-regulation of the p53-dependent $p21^{WAF1/CIP1}$ induction pathway.

Even though further experiments with apigenin, except MTT assay, were not performed in this study, several reports have shown that apigenin induces G2/M arrest on several cancer cell lines, such as human colon carcinoma cells (SW480, HT-29, Caco-2), and human melanoma cells (OCM-1) by the increase of $p34^{cdc2}$ cyclin-dependent

kinase or CDK2 (Wang *et al.*, 2000; Casagrande and Darbon, 2001).

In human melanoma cells OCM-1, the presence of a hydroxyl group at the 3'-position of the ring B in luteolin correlated to a G1 cell cycle arrest by the decrease of CDK2 expression while its absence in apigenin correlated to a G2/M block via down-regulation of CDK1 (Casagrande and Darbon, 2001). Little research has been conducted into chemotherapeutic mechanisms from the presence or absence of a hydroxyl group at the 3'-position of the ring B but we suspect that on many cancer cell lines, its absence might induce G2 arrest and its presence might result in G1 arrest.

Various phases of cell cycle in eukaryotic cells are governed by a series of cyclins and cyclin dependent kinases (CDKs) (Sherr, 1996). The catalytic partners of D-type cyclins are CDK4 and CDK6 and that of cyclin E is CDK2 (Pike *et al.*, 1998). It is already recognized that cyclin D/CDK4, 6 complexes are involved in G1 progression, cyclin E/CDK2 acts as the G1 to S transition, cyclin A/CDK2 operates during the S stage, cyclin A/CDK1 functions in late S and G2 stages and cyclin B/CDK1 functions during G2-M stages (Morgan, 1995; Elledge, 1996). There are well-known CDK inhibitors, such as p16^{INK4a}, p21$^{WAF1/CIP1}$, and p27^{KIP1}, which bind to and inhibit the CDK-cyclin complexes (Sherr and Roberts, 1995). It has been reported that p53 regulates a DNA damage-triggered G1 checkpoint by up-regulation of p21$^{WAF1/CIP1}$ (Hartwell and Kastan, 1994). p21$^{WAF1/CIP1}$ is not only activated by the p53 protein, but it also blocks the cell cycle at the G1 phase (Im *et al.*, 2001). In addition, an increased level of p21$^{WAF1/CIP1}$ is associated in cyclin-containing complexes with decreasing cyclin-dependent activity in damaged cells destined to apoptosis (El-Deiry *et al.*, 1993).

In conclusion, these data suggest that luteolin, one of the active components of *Ixeris sonchifolia* Hance, shows G1 cell cycle arrest effects on the growth of HepG2 cells. Therefore, luteolin could be used as a leading compound for further studies on the development of a chemotherapeutic agent.

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