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Effects of 4',7-Dimethoxyflavanone on Cell Cycle Arrest and Apoptosis in Human Breast Cancer MCF-7 Cells

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The present study was designed to investigate the anticancer activity of 4,7-dimethoxyflavanone *in vitro*. When human breast cancer MCF-7 cells were treated with 4',7-dimethoxyflavanone at various concentrations (1-200 μM) for 24 h, antiproliferative effects were first observed at 1 μM and the IC_{50} was 115.62 μM. Conversely, 4',7-dimethoxyflavanone was not cytotoxic (measured as lactate dehydrogenase release in CHO-K1 cells) under the same conditions. MCF-7 cells exposed to the 4',7-dimethoxyflavanone at the IC_{50} concentration showed cell cycle arrest and apoptosis. Compared to the respective control level, exposure to 4',7-dimethoxyflavanone resulted in a remarkable increase of small DNA fragments at the sub-G1 phase and an increase in the G2/M phase cell population. Moreover, when 4',7-dimethoxyflavanone treatment caused G2/M phase arrest, an increase in CDK1 together with an increase in cyclin B was observed. Based on these results, 4',7-dimethoxyflavanone may be a useful anticancer agent.

Key words: 4',7-Dimethoxyflavanone, Anticancer, G2/M phase, CDK1, Cyclin B

INTRODUCTION

Natural antioxidants have antiinflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic effects (Hocman, 1989; Craig, 1997). Among these natural antioxidants, flavonoids are a large group of phytochemicals that include flavones, flavonols, flavanones, and isoflavones.

Flavanone is an important naturally occurring flavonoid compound and constitutes a relatively high proportion of the total daily flavonoid intake from citrus fruits and juices (Ameer et al., 1996; Pelt et al., 2003). The biological activities of flavonoids have been extensively examined in terms of their anti-inflammatory, antitumor, and antioxidant capacites (Kim et al., 2004; Walle, 2004; Cushnie and Lamb, 2005; Kanadaswami et al., 2005).

Recently, many of researches have attempted the synthesis of new flavanone analogues in order to increase the anticancer activity of flavanone. Moreover, the anticancer activity of these synthetic compounds has been observed in various cancer cell lines *in vitro* (Liu et al., 1992; Min et al., 1997; Choi et al., 2004; Ying et al., 2008). We previously reported the production of synthetic flavonoids (Lee and Jung, 2005; Lee et al., 2007; Lee, 2008) that exert various biological activities *in vitro* (Choi et al., 2010) and have since been examining the phytoceutical applications of synthetic flavanone derivatives. Our results indicate that 4',7-dimethoxyflavanone, a flavanone derivative, exhibits potent anticancer activity. This study is the first report on the ability of 4',7-dimethoxyflavanone *in vitro* to induce cell cycle arrest and apoptosis in human breast cancer MCF-7 cells.

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MATERIALS AND METHODS

Synthesis of 4',7-dimethoxyflavanone

4',7-dimethoxyflavanone (Fig. 1) used in the present study was synthesized from 2'-hydroxyacetophenones at the Department of Chemistry, Duksung Women's University (South Korea). The chemical structures of 4',7-dimethoxyflavanone and its related compounds are shown in Fig. 1. The subsequent reaction of lithium enolates, generated from 2'-hydroxyacetophenones

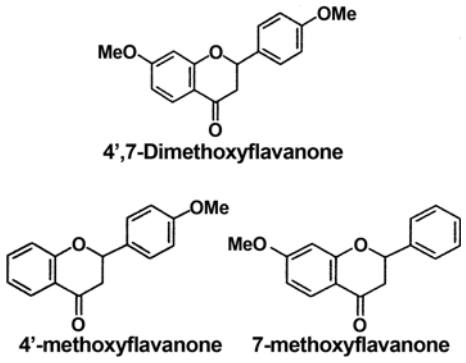

Fig. 1. The chemical structure of 4',7-dimethoxyflavanone

and LDA, with benzaldehydes in THF afforded 1-(2'-hydroxyphenyl)-1-oxo-propan-3-phenyl-3-ols, which were cyclodehydrated with diethyl azodicarboxylate and Ph_3P in CH_2Cl_2 to give flavanones in high yields. These compounds were purified by recrystallization, after by silicagel column chromatography when recrystallization was inefficient. Compound integrity and purity were confirmed by thin layer chromatography (TLC) and nuclear magnetic resonance (NMR, Bruker DPX-300), and gas chromatography (GC-mass, Agilent 6890N). All the compounds included in the study were at least 95% pure. 4',7-dimethoxyflavanone was dissolved in dimethyl sulfoxide (DMSO, final concentration 0.2% in medium).

Cells culture

Human breast cancer MCF-7 and chinese hamster ovary CHO-K1 cell lines were purchased from the KCLB (Korean Cell Line Bank). Cells were routinely maintained in RPMI 1640 (Invitrogen [Molecular Probes], Gibco), supplemented with 10% FBS and antibiotics (50 U/mL of penicillin and 50 μg/mL streptomycin, Gibco) at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

Human breast cancer MCF-7 cell lines were plated at a density of 1×10^5 cells/well in 96-well tissue culture plate (Corning), and incubated at 37°C for 24 h. Plated cells were treated with indicated concentrations of 4',7-dimethoxyflavanone for 24 h. After treatment, plated cell were incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma Chemical Company, 0.5 mg/mL final concentration) for 4 h at 37°C. After discarding all medium from the plates, 100 μL of dimethyl sulfoxide (DMSO) was added to the each well. The plates were placed for 5 min at room temperature with shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by a UV spectrophotometric plate reader (Emax; MolecularDevices). The value of IC_{50} (i.e., the concentration of the extract required to inhibit cancer cell growth by 50% of the control level, which is each cells treated with only compound solvent) was estimated from the plot. 4',7-dimethoxyflavanone on MDA-MA-453 cells was applied for the assay anticancer.

Lactate dehydrogenase (LDH) release assay

The cytotoxicity of 4',7-dimethoxyflavanone in CHO-K1 cells was assessed by measuring the release of the enzyme LDH using a LDH cytotoxicity Detection kit (Cat. No. 630117 Takara Korea Biomedical Inc.).

Cell cycle distribution

For cell cycle analysis, after human breast cancer MCF-7 cells were fixed in absolute ethanol, RNase A (20 μg/mL final concentration) and propidium iodide staining solution (50 μg/mL final concentration) was added to the cells and incubated for 30 min at 37°C in the dark. The cells were analyzed a FACS Calibur instrument (BD Biosciences) equipped with CellQuest 3.3 software. ModFit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

Immunoblotting assay

Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris, pH 7.5) containing protease inhibitor at 4°C for 1 h. The supernatant was separated by centrifugation, and protein concentration was determined by Bradford protein assay kit II (Bio-rad). Proteins (25 μg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45 μm). The membranes were blocked with a 1% BSA solution for 3 h and washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody at 4°C overnight. Antibodies against CDK4, CDK6, cyclin D, $p21^{Cip1}$, p53, Bcl-2, Bax, cytochrome c, precursor caspase-3, and β-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc.) and used to probe the separate membranes. On the next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase–conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (Bio-rad Laboratories).

Apoptosis assay

For annexin-based FACS analysis, human breast cancer MCF-7 cells were trypsinized, washed twice in

ice-cold PBS, and resuspended in 500 μL binding buffer (Sigma-Aldrich Inc.). Annexin V and propidium iodide solution were added to the cell preparations and incubated for 25 min in the dark. Binding buffer (400 μL) was then added to each tube and the samples were analyzed by a FACS Calibur instrument equipped with CellQuest 3.3 software. Additionally, the features of MCF-7 cells exposed to 4',7-dimethoxyflavanone were also observed using a Nikon inverse phase contrast microscope (Nikon TMS) equipped with an objective (Plan 10/0.30DL/Ph1) of 100× magnification.

Statistical analyses

All the data were expressed as percent compared with vehicle-treated control cells, which were arbitrarily assigned 100%. Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (Sigma Stat, Jandel). For all comparisons, differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of 4',7-dimethoxyflavanone on cell proliferation of human breast cancer MCF-7 cells

Antiproliferative and cytotoxic effects of 4',7-dimethoxyflavanone for human breast cancer MCF-7 cells were shown in Fig. 2. The effects of 4',7-dimethoxyflavanone on cell proliferation were measured with the MTT assay, using human breast cancer MCF-7 cells expos-

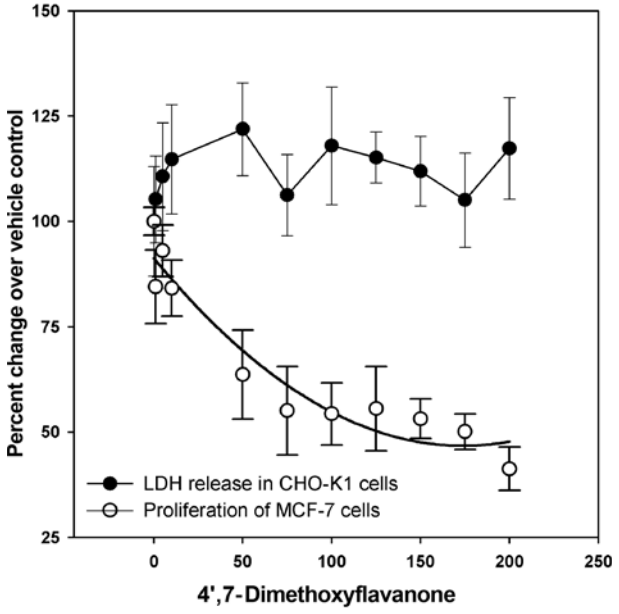

Fig. 2. Antiprolifeative activity and cytotoxicity of 4',7-dimethoxyflavanone. For MTT and LDH release assay, MCF-7 and CHO-K1 cell were exposed to either vehicle (0.1% DMSO in medium) or 4',7-dimethoxyflavanone at range from 1 to 200 μM for 24 h

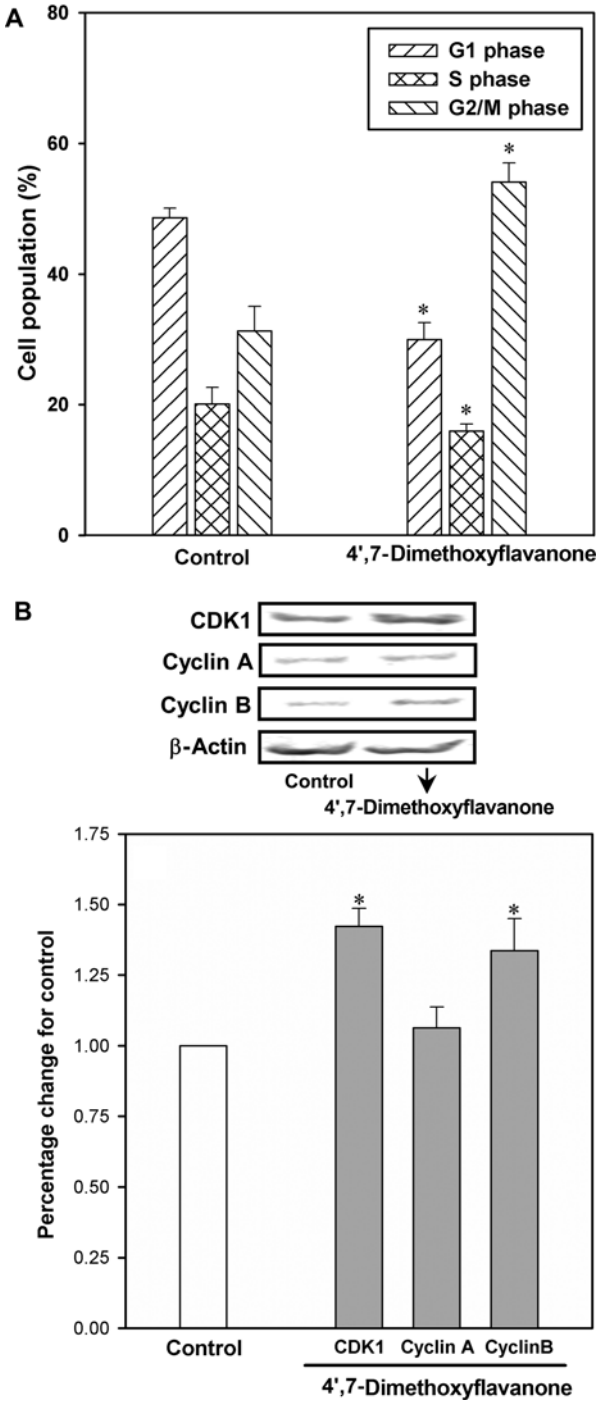

Fig. 3. Cell cycle distribution (**A**) and related regulator protein expression (**B**). MCF-7 cells was exposed to either vehicle (0.1% DMSO in medium) or 4',7-dimethoxyflavanone at IC_{50}. The protein expression levels were calculated relative to β-actin. Consequently, the value for the control group (vehicle-only group) was set as "1.0." Values are mean ± S. D. (n = 4-5). *$p < 0.05$, significantly different from the vehicle-control group.

ed to between 1 and 200 μM 4',7-dimethoxyflavanone for 24 h. 4',7-dimethoxyflavanone significantly decreased cell proliferation in a dose-dependent manner ($p < 0.05$). A significant antiproliferative effect of 4',7-dimethoxyflavanone appeared after treatment with 1 μM. In addition, 4',7-dimethoxyflavanone exhibited antiproliferative effects against human breast cancer MCF-7 cells with the IC_{50} values of 115.62 μM. Moreover, 4',7-dimethoxyflavanone. On the other hands, there was no evidence of cytotoxicity under the same conditions. Exposing with 4',7-dimethoxyflavanone for 24 h at concentrations up to 200 μM did not show significantly cytotoxicity in CHO-K1 cells.

Effect of 4',7-dimethoxyflavanone on cell cycle arrest of human breast cancer MCF-7 cells

Based on this results, the IC_{50} concentrations of 4',7-dimethoxyflavanone of were used for further DNA flow-cytometric analysis. Cell cycle arrest in MCF-7 cells 24 h after exposure to 4',7-dimethoxyflavanone was observed at 115 μM. After expose to 4',7-dimethoxyflavanone, the proportion of MCF-7 cells in the G1 phase decreased from 48.63% to 29.99%, and the proportion of the G2/M-phase cells significantly increased from 31.27% to 54.08% compared with control cells (Fig. 3A).

Moreover, when cell cycle arrest was induced in MCF-7 exposed to 4',7-dimethoxyflavanone at the IC_{50}, 4',7-dimethoxyflavanone increased CDK1 expression remarkably, by up to 42.25%, compared with the control level ($p < 0.05$, Fig. 3B). The expression of cyclin B, which combined with CDK1 to control the G2/M phase, was increased significantly, by 33.6%, compared with the control.

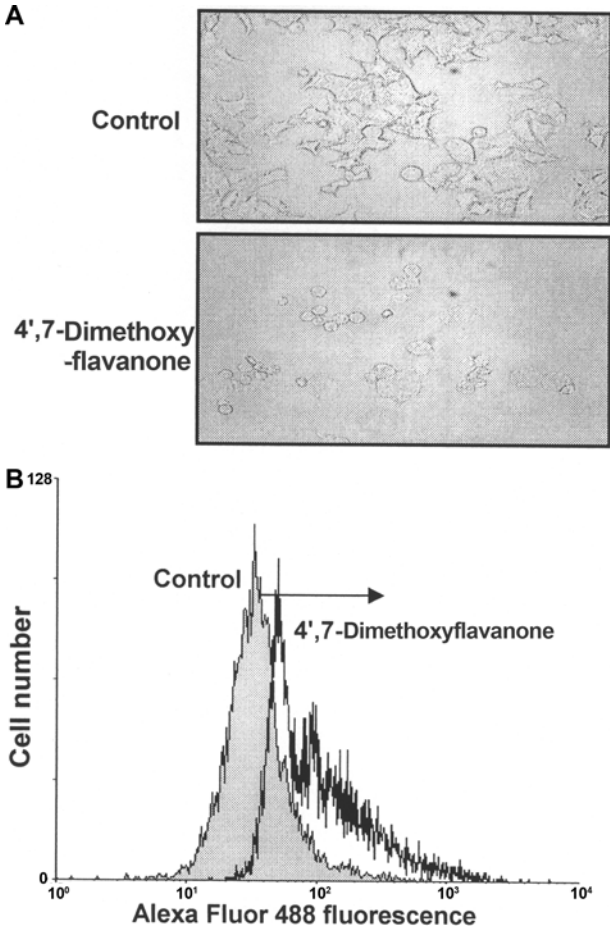

Fig. 4. Apoptosis induction by 4',7-dimethoxyflavanone. MCF-7 cells was exposed to either vehicle (0.1% DMSO in medium) or 4',7-dimethoxyflavanone at IC_{50}. Apoptotic population was determined by Annexin-V assay (**B**). Besides, human breast cancer MCF-7 exposed to 4',7-dimethoxyflavanone were observed using a Nikon inverse phase contrast microscope (**A**).

Effect of 4',7-dimethoxyflavanone on apoptosis of human breast cancer MCF-7 cells

To verify that anticancer activity of 4',7-dimethoxyflavanone in human breast cancer MCF-7 cells, we observed the apoptotic feature after exposing cells to 4',7-dimethoxyflavanone at the IC_{50} concentration. Apoptotic morphological features such as cell shrinkage and dot-shaped nuclear fragments were observed in exposing cells to 4',7-dimethoxyflavanone (Fig 4A). Furthermore, these results are supported by Annexin-based flow cytometry. 4',7-dimethoxyflavanone increased significantly the total number of apoptotic cells (34.89% in apoptotic cell population, $p < 0.05$, Fig 4B).

DISCUSSION

We examined the antiproliferative effect of 4',7-dimethoxyflavanone on human breast cancer MCF-7 cells to investigate its anticancer activity. Breast cancer is one of the most frequently diagnosed cancers in women, and its occurrence has been increasing in recent years. It is now the major cause of mortality and morbidity in Korean women, which may be attributable to changes from a traditional to Westernized lifestyle.

In the present study, 4',7-dimethoxyflavanone significantly inhibited cellular proliferation in a dose-dependent manner. The first significant antiproliferative effect of 4',7-dimethoxyflavanone was observed at a relatively low dose (1 μM) without cytotoxicity, and its IC_{50} was 115.62 μM. In preliminary experiments the IC_{50} of the structurally similar analogues 4'-methoxyflavanone and 7-methoxyflavanone was 158.22 and 132.11 μM, respectively. In addition, their initial significant antiproliferative effect was found at 10 μM (data not shown). Based on these results, the anti-

cancer activity of the methoxy group at C-7 was related to its position and number, that is, a methoxy group at C-7 had an effect and two had a greater effect than one. Thus, 4',7-dimethoxyflavanone may be a good candidate as an anticancer agent.

4',7-Dimethoxyflavanone at the IC_{50} concentration induced cell cycle arrest at the G2/M phase and caused apoptosis. MCF-7 cells exposed to 4',7-dimethoxyflavanone accumulated more than 1.6-folds in the G2/M phase compared with that in control value. Besides, apoptotic feature and the shift of cell populations were observed. Recently, interest is growing in inducing apoptosis as a new target for cancer chemotherapy (Sun et al., 2004; Fesik, 2005; Zhang et al., 2005). Apoptosis is an important series of events that leads to programmed cell death and is essential for tissue development and homeostasis. Several studies have indicated that anticancer drugs or cancer chemopreventive agents act by inducing apoptosis in various cancer cells. Additionally, the initiation of apoptosis appears to be a common mechanism of many new chemotherapeutic anticancer agents (Hersey and Zhang, 2003; Tsuruo et al., 2003)

In the present study, CDK1 (Cdc2) was down-regulated by 4',7-dimethoxyflavanone, which may have been the main cause for the G/M phase arrest. Cyclins A and B increased slightly with the increase in CDK1. The cell cycle is tightly mediated through a complex network of positive and negative cell-cycle regulatory molecules such as cyclin-dependent kinases (CDKs), CDK inhibitors (CKIs), and cyclins. CDK1 is a catalytic subunit of the M-phase promoting factor, which is activated at the G2/M transition and controls the onset of mitosis (Takizawa and Morgan, 2000). Several studies have shown that CDK1 in combination with cyclins A and B are critical in the G2/M phase transition (Porter and Donoghue, 2003).

In conclusion, our results suggest that 4',7-dimethoxyflavanone hindered cancer cell growth via cell cycle arrest and apoptosis. Moreover, 4',7-dimethoxyflavanone was not cytotoxic even at higher doses. The characteristics that we have described indicated that 4',7-dimethoxyflavanone is a promising anticancer drug candidate.

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REFERENCES

Ameer, B., Weintraub, R. A., Johnson, J. V., Yost, R. A., and Rouseff, R. L., Flavanone absorption after naringin, hesperidin, and citrus administration. *Clin. Pharmacol. Ther.*, 60, 34-40 (1996).

Choi, E. J., Lee J. I., and Kim, G. H., Anti-carcinogenic effect of a new analogue 4'-chloroflavanone from flavanone in human breast cancer cells. *Int. J. Mol. Med.*, 25, 293-298 (2010).

Choi, Y, J., Lee, M. K., Lee, Y. J., Jeong, Y. J., Yoon-Park, J. H., Sung-Lim, S., and Kang, Y. H., Inhibition of hydrogen peroxide-induced endothelial apoptosis by 2',4',7-trihydroxyflavanone, a flavonoid form. *J. Med. Food,* 7, 408-416 (2004).

Craig, W. J., Phytochemicals: guardians of our health. *J. Am. Diet. Assoc.*, 97, S199-S204 (1997).

Cushnie, T. P. and Lamb, A. J., Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, 26, 343-356 (2005).

Fesik, S. W., Promoting apoptosis as a strategy for cancer drug discovery. *Nat. Rev. Cancer*, 5, 876-885 (2005).

Hersey, P. and Zhang, X. D., Overcoming resistance of cancer cells to apoptosis. *J. Cell Physiol.*, 196, 9-18 (2003).

Hocman, G., Prevention of cancer: vegetables and plants. *Comp. Biochem. Physiol. B*, 93, 201-212 (1989).

Kanadaswami, C., Lee, L. T., Lee, P. P., Hwang, J. J., Ke, F. C., Huang, Y. T., and Lee, M. T., The antitumor activities of flavonoids. *In Vivo*, 19, 895-909 (2005).

Kim, H. P., Son, K. H., Chang, H. W., and Kang, S. S., Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.*, 96, 229-245 (2004).

Korhonen, R., Lathi, A., Kankaanranta, H., and Moilanen, E., Nitric oxide production and signaling in inflammation. Curr. Drug Targets Inflamm. *Allergy*, 4, 471-479 (2005).

Lee, J. H., Choon, S. J., and Kim, G. H., Antioxidant and suppressive effects of ethanolic extract fractions from *Carthamus tinctorius* L. (Safflower) flower on the biosynthesis of inflammatory mediators from LPS-stimulated RAW 264.7 cells. *Food Sci. Biotechnol.*, 18, 143-149 (2009).

Lee, J. I. and Jung, M. G., A New Route for the Synthesis of Flavanones from 2-Methoxybenzoic Acids. *Bull. Korean Chem. Soc.*, 26, 2044-2046 (2005).

Lee, J. I., Jung, M. G., and Jung, H. J., A Novel Synthesis of Flavanones from 2-Hydroxybenzoic Acids. *Bull. Korean Chem. Soc.*, 28, 859-862 (2007).

Lee, J. I., A new synthesis of thioflavanones from thiosalicylic acid. *Bull. Korean Chem. Soc.*, 29, 1263-1265 (2008).

Liu, Y. L., Ho, D. K., Cassady, J. M., Cook, V. M., and Baird, W. M., Isolation of potential cancer chemopreventive agents from Eriodictyon californicum. *J. Nat. Prod.*, 55, 357-363 (1992).

Luss, H., Nussler, N. C., Beger, H. G., and Nussler, A. K., Expression and detection of inducible nitric oxide synthase in experimental models of inflammation. *Methods*, 10, 51-60 (1996).

Min, B. S., Chung, K. S., and Bae, K. H., Antitumor activity

of 2(S)-5,2',5'-trihydroxy-7,8-dimethoxyflavanone and its analogues. *Arch. Pharm. Res*., 20, 368-371 (1997).

Pelt, J. L., Downer, W. A., Schoborg, R. V., and McIntosh, C. A., Flavanone 3-hydroxylase expression in Citrus paradisi and Petunia hybrida seedlings. *Phytochemistry*, 34, 435-444 (2003).

Porter, L. A. and Donoghue, D. J., Cyclin B1 and CDK1: nuclear localization and upstream regulators. *Prog. Cell Cycle Res*., 5, 335-347 (2003).

Sun, S. Y., Hail, N., Jr., and Lotan, R., Apoptosis as a novel target for cancer chemoprevention. *J. Natl. Cancer Inst*., 96, 662-672 (2004).

Sutherland, H., Khundkar, R., Zolle, O., McArdle, A., Simpson, A. W., Jarvis, J. C., and Salmons, S., A fluorescence-based method for measuring nitric oxide in extracts of skeletal muscle. *Nitric Oxide*, 5, 475-481 (2001).

Takizawa, C. G. and Morgan, D. O., Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. *Curr. Opin. Cell Biol*., 12, 658-665 (2000).

Tsuruo, T., Naito, M., Tomida, A., Fujita, N., Mashima, T., Sakamoto, H., and Haga, N., Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Sci*., 94, 15-21 (2003).

Walle, T., Absorption and metabolism of flavonoids. *Free Radic. Biol. Med.,* 36, 829-837 (2004).

Ying, M., Tu, C., Ying, H., Hu, Y., He, Q., and Yang, B., MSFTZ, a flavanone derivative, induces human hepatoma cell apoptosis via a reactive oxygen species- and caspase-dependent mitochondrial pathway. *J. Pharmacol. Exp. Ther*., 325, 758-765 (2008).

Zhang, Z., Li, M., Rayburn, E. R., Hill, D. L., Zhang, R., and Wang, H., Oncogenes as novel targets for cancer therapy (part IV): regulators of the cell cycle and apoptosis. *Am. J. Pharmacogenomics*, 5, 397-407 (2005).