February 2008

Doctor's Thesis

Effects of Flavonoid Substituents on Antioxidant, Chemosensitizing and Bioavailability-Enhancing Activities

Graduate School of Chosun University

Department of Bio New Drug Development

Ju-Mi Jeong

Effects of Flavonoid Substituents on Antioxidant, Chemosensitizing and Bioavailability-Enhancing Activities

플라보노이드 치환체의 항산화, 화학감작 및 생체 이용율 증진 활성에 대한 효과

February 2008

Graduate School of Chosun University

Department of Bio New Drug Development

Ju-Mi Jeong

Effects of Flavonoid Substituents on Antioxidant, Chemosensitizing and Bioavailability-Enhancing Activities

Advisor: Prof. Cheol-Hee Choi

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy

October 2007

Graduate School of Chosun University

Department of Bio New Drug Development

Ju-Mi Jeong

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy

Approved by the Guidance Committee:

Prof. In-Hwa Lee
Chosun University _____

Prof. Hoon Cho
Chosun University _____

Prof. Ji-Soo Yun
Chosun University _____

Prof. Su-Gyung Kang
Kwangju Woman's University _____

Prof. Cheol-Hee Choi
Chosun University _____

December 2007

Graduate School of Chosun University

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

국문초록

플라보노이드의 치환체의 항산화, 화학감작 및 생체 이용율 증진 활성에 대한 효과

정 주 미
지도교수: 최 철 희
조선대학교 대학원
바이오신약개발학과

플라보노이드는 항산화제, 화학예방제와 화학감작제 등으로 이용되고 있다. 화학감작제는 항암제 투여 시 동반되는 다약물 내성을 피하기 위해 개발된 것으로, 플라보노이드는 P-glycoprotein의 ATP 결합 부위에 상호 작용하는 새로운 화학감작제의 종류로 여겨지고 있다. 항암제 중 택솔(paclitaxel)은 위장 점막에 존재하는 P-glycoprotein의 기질로 내성뿐만 아니라 낮은 생체이용율이 문제되고 있다.

본 연구는 다양한 hydroxy (OH) 및/또는 methoxy (OCH_3, OMe) 그룹을 가진 11개 플라보노이드의 구조에 기초하여, 1,1-diphenyl-2-picrylhydrazyl를 이용한 플라보노이드 자체의 항산화 작용과 2',7'-dichlorofluorescein diacetate를 이용한 세포내에 항산화 작용을 확인하였으며, P-glycoprotein 과발현 내성세포인 AML-2/D100에서 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide 실험

을 이용하여 항암제인 vincristin의 존재 유·무의 상태로 세포독성과 화학감작 효과를 측정하였다. 또한 낮은 독성과 화학감작 효과를 보이는 플라보노이드를 선별하여 Caco-2 세포에서 paclitaxel을 HPLC 방법으로 측정하였다.

3,5,7,3',4'-hydroxyflavone의 항산화 효과는 5,7,3',4'-hydroxyflavone보다 더 높은 효과를 보였으며, 다양한 methoxy 그룹으로 치환된 플라보노이드는 항산화 효과가 감소되었다. 7-hydroxy 혹은 5,7-hydroxy 그룹을 가진 플라보노이드는 가장 높은 세포 독성을 보였고, 5,7-methoxy 그룹을 가진 플라보노이드가 세포 독성을 초래하는 것을 알 수 있었다. 5,7,3',4',5'-methoxyflavone과 5,7,4'-methoxyflavone의 세포 성장을 50% 억제하는 IC_{50}값은 0.4 μM과 1.4 μM, 5,6,7,3',4'-methoxyflavone과 5,6,7,3',4',5'-methoxyflavone의 IC_{50}값은 3.2 μM과 0.9 μM이었고, 5,6,7,3',4',5'-methoxyflavone과 5,7,3',4',5'-methoxyflavone의 IC_{50}값은 0.9 μM과 3.2 μM이었다. 플라보노이드 중 높은 화학감작 지수를 보인 7,3',4'-methoxyflavone (TMF)을 선택한 후 P-glycoprotein 억제제인 verapamil과 paclitaxel의 생체이용율 증진 활성을 비교하였다. Paclitaxel은 basolateral (BL)에서 apical (AP)로의 이동이 AP에서 BL로의 이동보다 10배 더 높았다. TMF나 verapamil 처치 시 AP에서 BL로의 이동이 증가되었으며, BL에서 AP로의 이동은 감소되어, 결국 50 μM TMF의 paclitaxel 이동의 순수효과는 50 μM verapamil과 비슷하였다. 또한 TMF와 paclitaxel을 AP에 투여 후 BL로 이동한 paclitaxel이 P-glycoprotein를 과발현하고 있는 paclitaxel 내성세포 SK-MES-1/PT4000의 감수성을 증가시켰다.

이상의 결과는 3-hydroxy 그룹을 가진 플라보노이드는 항산화 효

과가 있었고, 5-hydroxy 및/또는 7-hydroxy 그룹을 가진 플라보노이드는 높은 세포독성을 보였다. 3'-methoxy 및/또는 5'-methoxy 그룹을 가진 플라보노이드는 P-glycoprotein 억제효과를 보였으나, 6-methoxy 그룹을 가진 플라보노이드는 P-glycoprotein 억제 효과를 보이지 않았다. 또한, 항산화 작용과 P-glycoprotein 억제작용 사이에 역비례 관계가 있었다. 그러므로 낮은 독성을 가진 플라보노이드 TMF는 P-glycoprotein 억제 뿐만 아니라 paclitaxel의 생체 이용율을 증가시키는데 이용될 수 있을 것으로 사료된다.

I. INTRODUCTION

Flavonoids are ubiquitous in photosynthesizing cells and therefore occur widely in the plant kingdom (Havsteen, 1983). Flavonoids are constituents of fruits, vegetables, nuts, plant-derived beverages, traditional eastern medicines and herb-containing dietary supplements. Human have consumed flavonoids and other dietary phenolics since the arrival of human life on earth. Over 4000 different naturally occurring flavonoids have been described (Middleton and Kandaswami, 1994) and the list is still growing. Flavonoids have been known as plant pigments for over a century and belong to a vast group of phenolic compounds that are widely distributed in all foods of plant origin (Galati and O'Brien, 2004). The basic structural feature of flavonoid compounds is the 2-phenyl-benzopyrane or flavane nucleus, which consists of two benzene rings (A and B) linked through a heterocyclic pyrane ring (C) (Brown, 1980; Figure 1). Flavonoids constitute, one of the most characteristic classes of compounds in higher plants, are frequently components of the human diet including aurone, isoplavones, chalcone, flavanone, flavone, and anthocyanindin (Cushine and Andrew, 2005; Figure 2). Flavonoids have been used as antioxidants, chemopreventive or anticancer drugs and chemosensitizers (Choi, 2005; Duraj, *et al.*, 2005; Middleton and

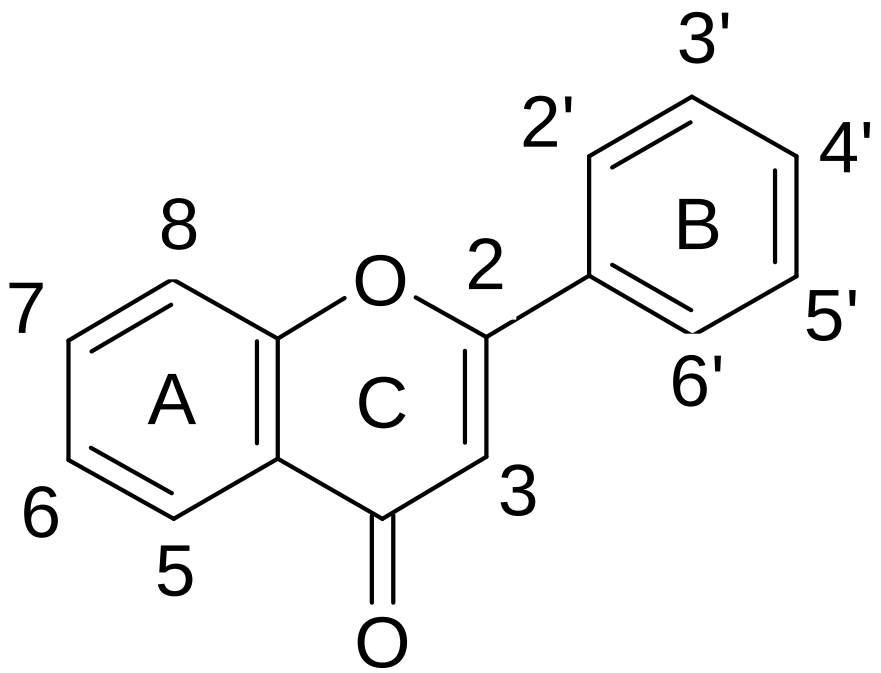
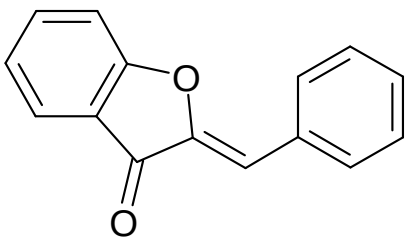
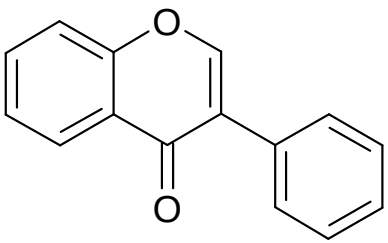


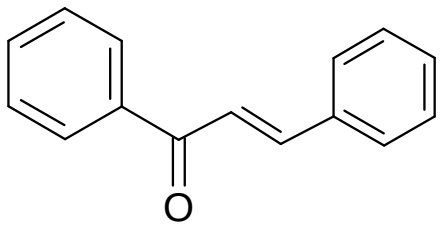
Figure 1. The skeleton structure of the flavones (a class of flavonoids) with rings named and positions numbered.



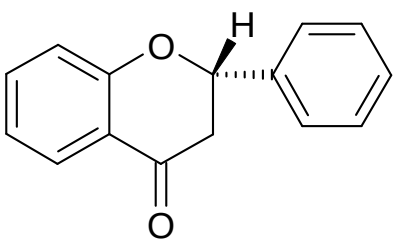
Aurone



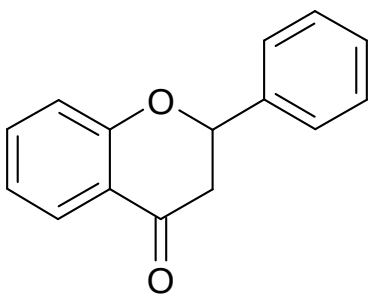
Isoflavone



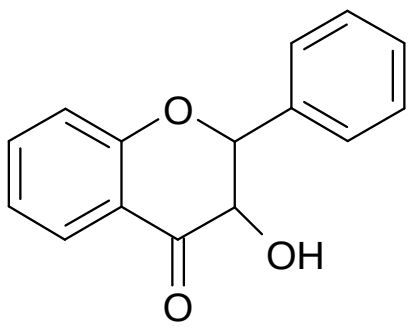
Chalcone



Flavanone



Flavone



Anthocyanidin

Figure 2. The skeleton structures of the main classes of flavonoids.

Chithan, 1993; Harborne and Williams, 2000).

Flavonoids have been shown to be effective scavengers of reactive oxygen species (ROS) (Rusak, *et al.*, 2005). Oxygen free radicals and their by-products, which are capable of causing oxidative damage, are collectively referred to as ROS. As shown in Figure 3, a small amount of reactive intermediates, such as superoxide radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are formed (Harborne and Williams, 2000; Halliwell and Gutteridge, 1984; Michikatsu, *et al.*, 1996; Barry, *et al.*, 1995). Superoxide radicals ($O_2^{-}\cdot$) are general from molecular oxygen or hydrogen peroxide by an one-electron transfer reaction (Keiicchi, *et al.*, 1990). ROS have been implicated in the pathogenesis of a wide variety of human diseases when produced in excess quantities (Sen and Packer, 1996). Cells possess antioxidant systems to protect themselves against dangerous ROS. Therefore, a delicate balance between intracellular oxidants and antioxidants can influence health and aging (Bonnefoy, *et al.*, 2002). Because ROS are produced continually, antioxidants must be continually present at their sites of production at high concentrations. A temporary decrease in the antioxidant concentration at a susceptible location, results in the accumulation of oxidative damage, which cannot be prevented or repaired by the late addition of the antioxidants (Kohen and Nyska, 2002). Flavonoids have been suggested that flavonoid anticancer activities depend heavily on their antioxidant

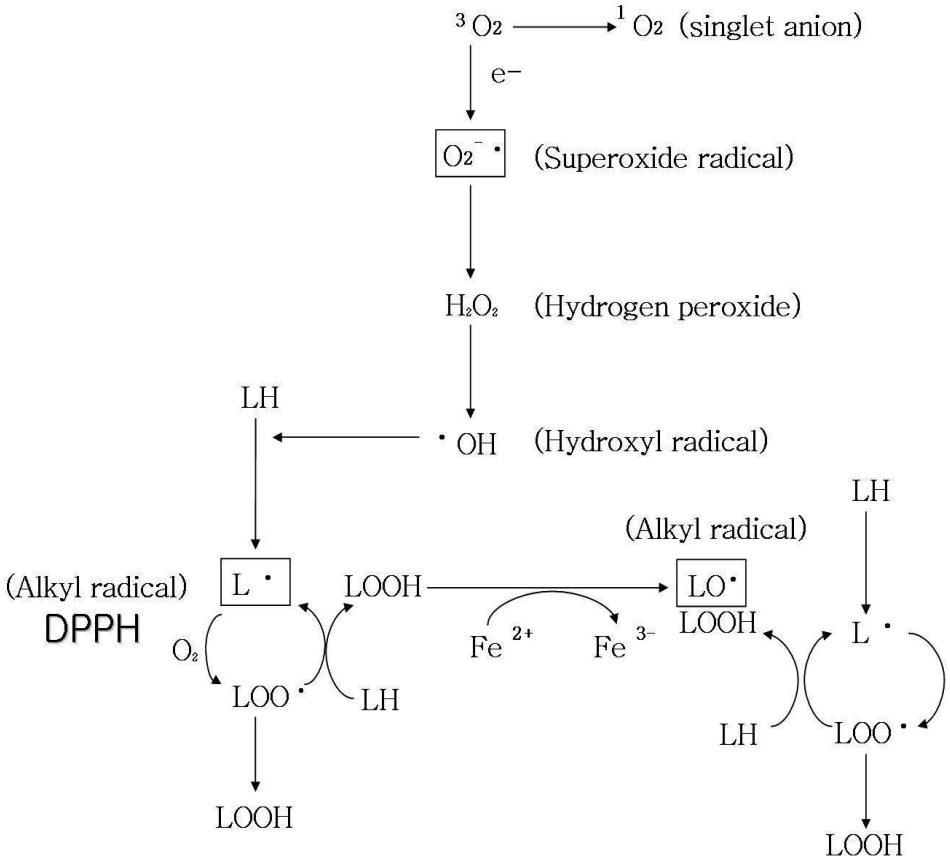


Figure 3. Generation of peroxides from radical chain reaction (Lee, 1998).

and chelating properties (Duthie and Dobson, 1999; Korkina, 1997). The chemopreventive effects of flavonoids are believed to result from their ability to scavenge ROS whereas the pro-oxidant effect of flavonoids has been shown to be responsible for their anticancer and apoptosis-inducing effects (Galati and O'Brien, 2004; Srinivasan, *et al.*, 2002; Tobi, *et al.*, 2002). Accordingly, exogenous antioxidants have been used for long time.

The oxidation of lipids, DNA, proteins, and other biologic molecules by ROS may cause DNA mutation or/and serve to damage target cells, and this may result in various pathological cellular disturbances (Rusak, *et al.*, 2005). The property of many flavonoids to alter the expression and activities of numerous enzymes involved in the regulation of cell cycle (Casgrande and Darbon, 2000; Gerritsen, 1998; Agullo, *et al.*, 1997) and of apoptosis (Wang, *et al.*, 1999; Constantinou, *et al.*, 1998) has been studied by several authors. Mitogenic signals commit cells to entry into a series of regulated steps allowing traverse of the cell cycle. Synthesis of DNA (S phase) and separation of two daughter cells (M phase) are the main features of cell cycle progression. The time between the S and M phases is known as G2 phase. This phase is important to allow cells to repair errors that occur during DNA duplication, preventing the propagation of these errors to daughter cells. In contrast, the G1 phase represents the period of commitment to cell cycle progression that separates M and S phases as cells prepare for DNA

duplication upon mitogenic signals (Ren, *et al.*, 2003; Zi, *et al.*, 1998; Choi, *et al.*, 2001; Casagrande and Darbon, 2001). Flavonoids have been found to arrest cell cycle progression either at G1/S or at G2/M boundaries, but conflicting results have been reported with regard to the stage-specific cell cycle arrest caused by the flavonoid quercetin (Casgrande and Darbon, 2000).

Flavonoids may exert their effects is through their interaction with phase I metabolizing enzymes (cytochrome P450), which metabolically activate a large number of procarcinogens to reactive intermediates that can interact with cellular nucleophiles and ultimately trigger carcinogenesis. Flavonoids are demonstrated to inhibit the activities of certain P450 isozymes such as CYP1A1 and CYP1A2 (Le Marchand, *et al.*, 2000; Moushumi, *et al.*, 1999; Tsyrlov, *et al.*, 1994). Thus, they are likely to have a protective role against the induction of cellular damage by the activation of carcinogens (Ren, *et al.*, 2003).

The development of multidrug resistance (MDR) by tumor cells is a major impediment to the success of cancer chemotherapy. Overexpression of membrane transporters have been believed as major mechanisms of MDR. These include the P-glycoprotein (Pgp, Riordan and Ling, 1979), the multidrug resistance-associated protein (MRP, Cole, *et al.*, 1994), and the breast cancer resistance protein (BCRP, Maliepaard, *et al.*, 1999) that are members of the ATP binding-cassette (ABC) superfamily of membrane transporters (Figure 4). The MDR phenotype is characterized by decreased

accumulation and enhanced efflux of anticancer drugs, which is reversible upon treatment with P-glycoprotein inhibitors such as verapamil, and cyclosporine A. (Choi, *et al.*, 2002). Structure and function of P-glycoprotein are shown in Figure 5-8, P-glycoprotein consists of two homologous halves each containing a transmembrane domain (TMD) involved in drug binding and efflux, and a cytosolic nucleotide-binding domain (NBD) involved in ATP binding and hydrolysis, with an overall (TMD-NBD) 2 domain topology. Certain flavonoids have been reported to possess potent inhibitory activity against the drug exporting function of P-glycoprotein, a plasma membrane ATP-binding cassette transporter that extrudes cytotoxic drugs at the expense of ATP hydrolysis (Conseil, *et al.*, 1998; Di pietro, *et al.*, 1999). Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein may be through (1) inhibiting the overexpression of multidrug resistance gene-1 (MDR1, Kioka, *et al.*, 1992), (2) direct binding to NBDs with high affinity (Di Pietro, *et al.*, 1999), (3) inhibiting ATPase activity, nucleotide hydrolysis and energy-dependent drug interaction with transporter-enriched membranes (Shapiro and Ling, 1997; Di Pietro, *et al.*, 2002; Figure 9). Chemosensitizers have been screened and developed in order to circumvent MDR by concomitant administration with anticancer drugs. Flavonoid considered to be a new class of chemosensitizers.

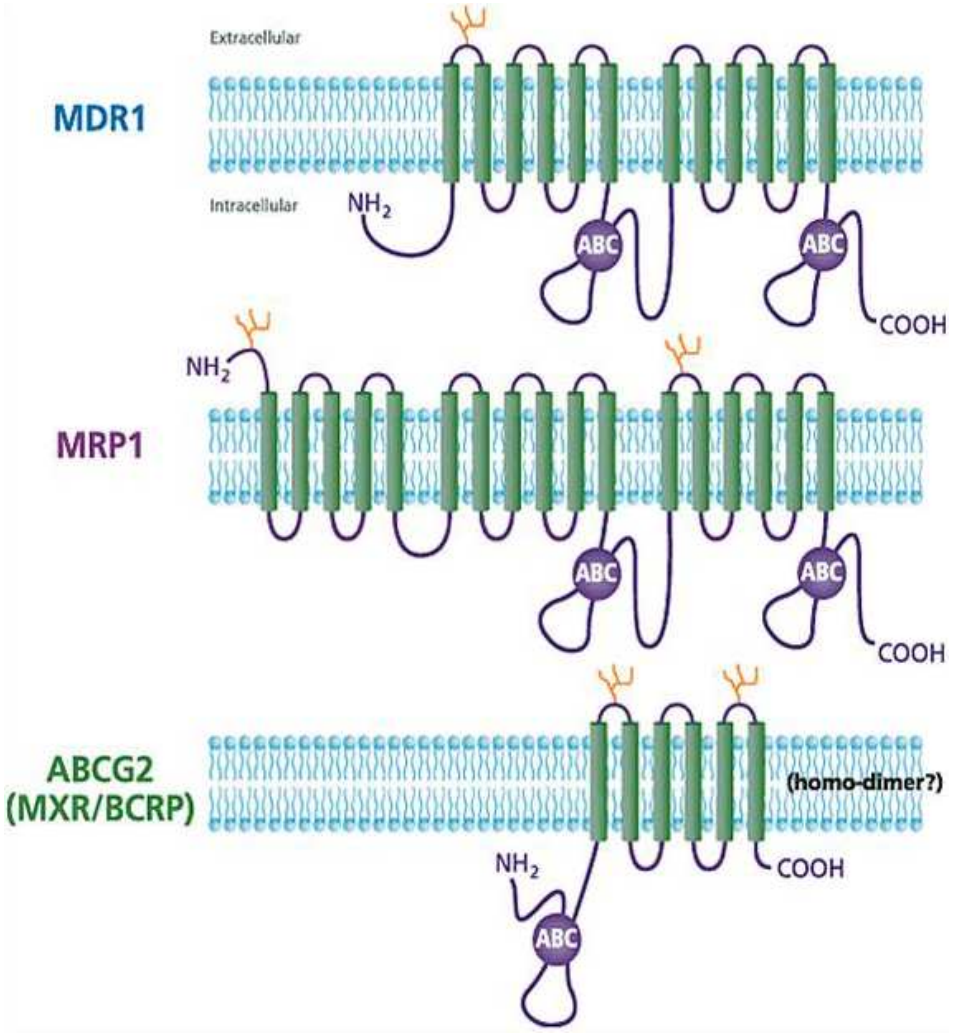


Figure 4. Structure of representative ABC transporters.

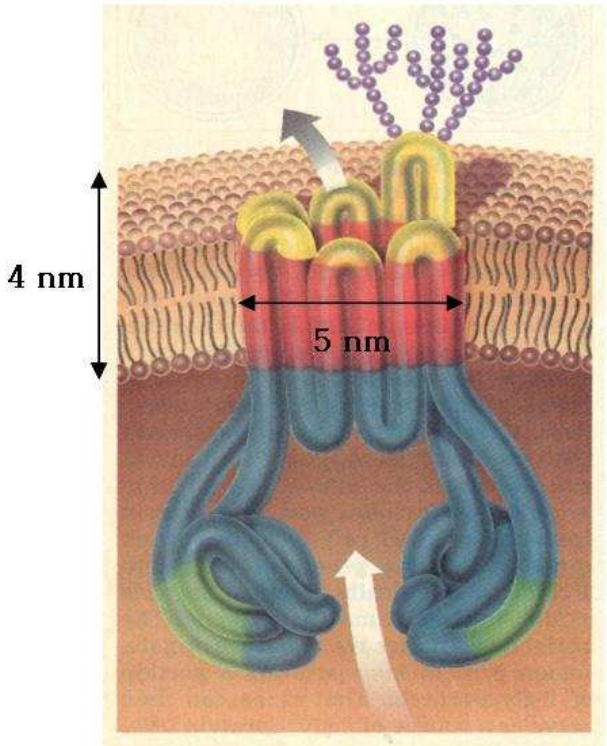


Figure 5. 3D structure of P-glycoprotein (*Pgp*, ABCB1;*MDR1*).

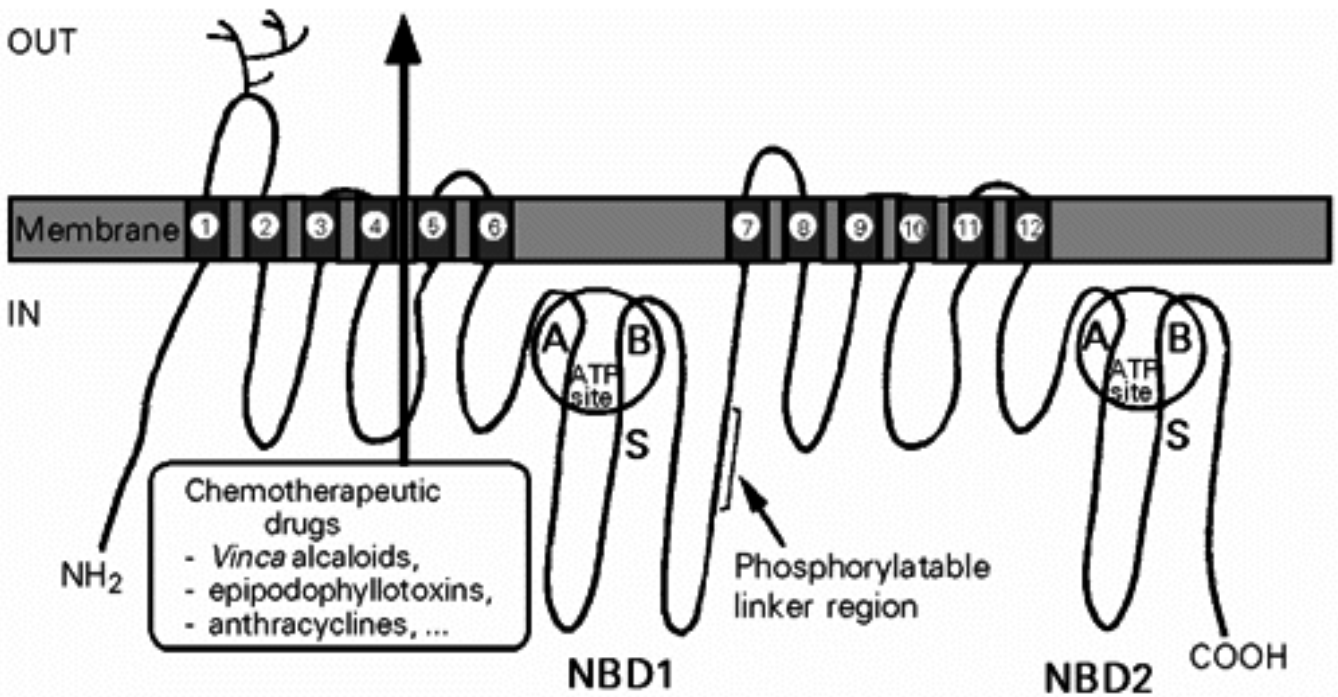


Figure 6. P-glycoprotein structure (Di Pietro, *et al.*, 1999).

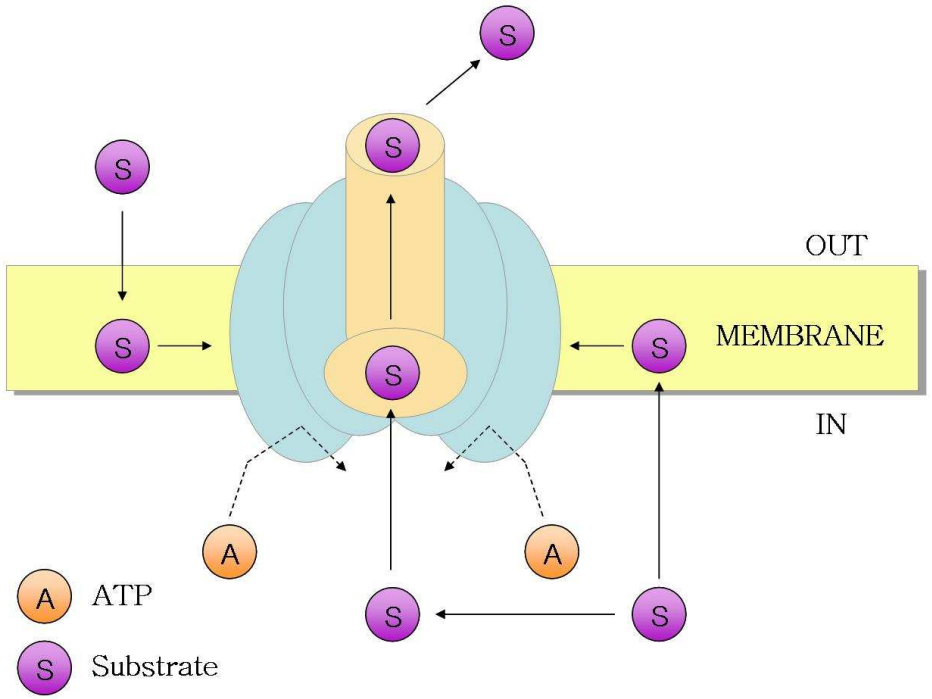


Figure 7. Simplified cartoon of P-glycoprotein structure and function (www.cyto.purdue.edu/class/bms524/524lect11.ppt).

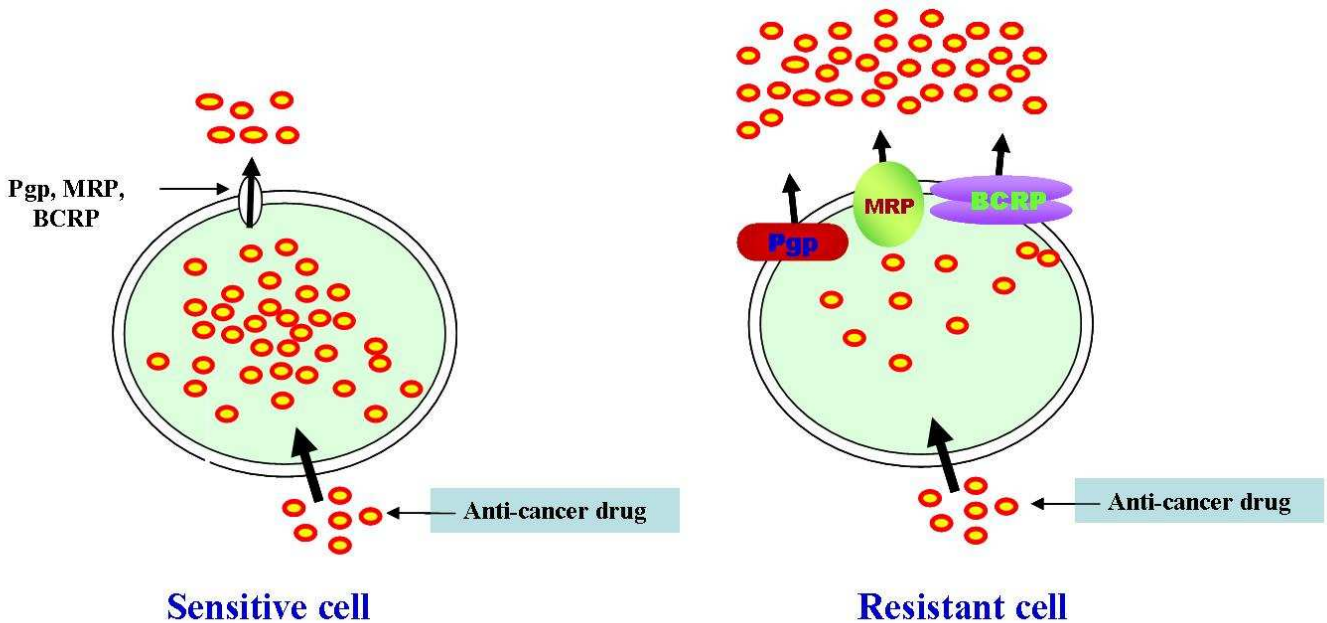


Figure 8. Effect of MDR transporters on drug accumulation.

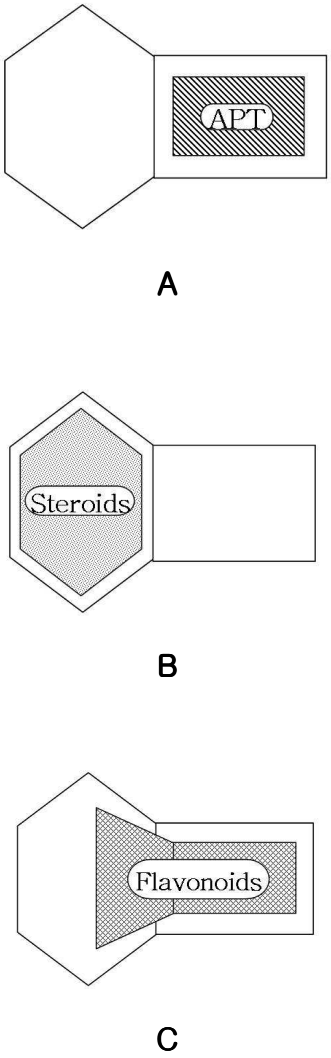


Figure 9. Tentative model for binding of steroids, ATP, and flavonoids to adjacent nucleotide- and modulator-binding sites within the cytosolic NBD domain of P-glycoprotein. A, ATP binding; B, interaction with drugs or modulators; C, overlapping of both sites by flavonoids (Conseil, *et al.*, 1998).

Taxol (paclitaxel) is a naturally occurring diterpenoid (originally isolated from the Pacific yew tree) that belongs to a new class of antimicrotubule anticancer drugs (Rowinsky and Donehower, 1995; Rowinsky, *et al.*, 1992).

The current clinical formulation of paclitaxel is an important anti-cancer agent against breast, ovary and non-small cell lung cancers (Rowinsky, *et al.*, 1992). However, paclitaxel chemotherapy presents two major problems: its administration route and drug resistance. The current clinical paclitaxel formulation has significant side effects, including allergic shock, that are related to the use of Cremophor EL/ethanol as co-solvents in the intravenous formulation; these co-solvents are necessary due to its poor aqueous solubility (Dorr, 1994). To solve this problem, water-soluble paclitaxel prodrugs have been prepared and then tested in breast xenograft assays (Figure 10) (Deutsch, *et al.*, 1989). Paclitaxel liposomal formulations were much better tolerated than paclitaxel after i.v. or i.p. administration in both healthy and tumor-bearing mice (Sharma, *et al.*, 1996). Recently, phase I clinical and pharmacokinetic studies of a novel water-soluble polymer-conjugated paclitaxel prodrug were performed (Meerum, *et al.*, 2001). Moreover, paclitaxel also has poor bioavailability because of its high affinity for the membrane transporter P-glycoprotein in the gastrointestinal tract (Wacher, *et al.*, 1998; Van Asperen, *et al.*, 1997; Sparreboom, *et al.*, 1997). *Mdr1a* P-glycoprotein knock-out mice, which lack functional

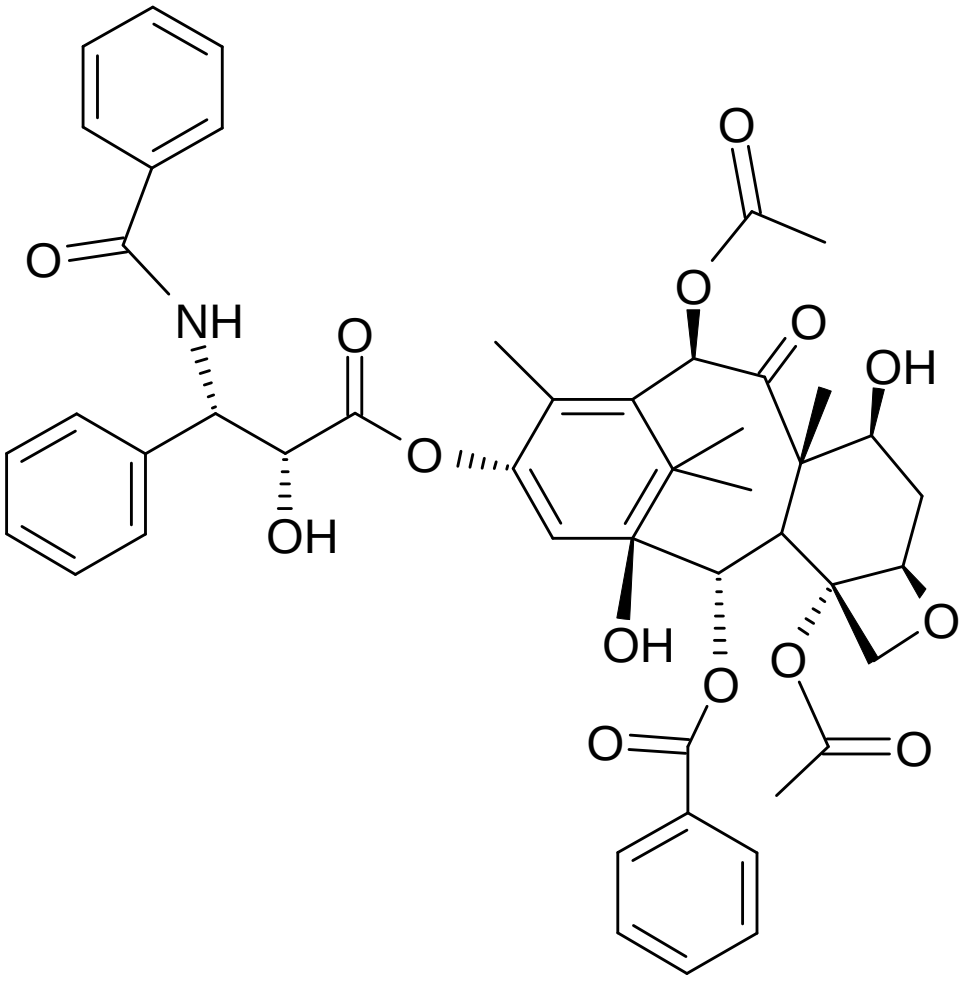


Figure 10. Structure of paclitaxel (Lowe, *et al.*, 2001).

P-glycoprotein activity in the gut, have shown significant bioavailability of orally administered paclitaxel (Malingre, *et al.*, 2001). P-glycoprotein blockers such as cyclosporin A or PSC 833 can drastically improve oral bioavailability of paclitaxel in mice and humans (Van Asperen, *et al.*, 1997; Sparreboom, *et al.*, 1997; Schellens, *et al.*, 2000). Emergence of resistance to paclitaxel is one of the major obstacles in clinical cancer chemotherapy. Paclitaxel is a substrate for P-glycoprotein whose overexpression is mainly responsible for paclitaxel resistance (Oza, 2002). Therefore, if a substance could not only reverse paclitaxel resistance in P-glycoprotein-overexpressing cancer cells, but also enhance paclitaxel bioavailability through P-glycoprotein inhibition, it would increase the efficiency of paclitaxel chemotherapy.

In this study, eleven flavonoids containing hydroxyl (OH) and/or methoxy (OMe) groups (Table 1) along with four well-known antioxidants were compared for their *in vitro* antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical solution-based chemical assay and 2',7'-dichlorofluorescein diacetate (DCFH-DA) cellular-based assay, cytotoxic and chemosensitizing effects and then their structure-activity relationships (SAR) were discussed. Thus, I screened flavonoids for P-glycoprotein inhibition and then tested whether they could not only enhance paclitaxel bioavailability in the Caco-2 system (monolayers of cultured epithelial cells in a two-compartment cell culture

Table 1. Flavonoids

Flavonoid	Substituents													
	1	2	3	4	5	6	7	8	1'	2'	3'	4'	5'	6'
3,6,3',4'-tetramethoxyflavone			OCH_3			OCH_3					OCH_3	OCH_3		
5,7,3',4',5'-pentametoxyflavone					OCH_3		OCH_3				OCH_3	OCH_3	OCH_3	
5,6,7,3',4'-pentamethoxyflavone					OCH_3	OCH_3	OCH_3				OCH_3	OCH_3		
3,7-dihydroxy-3',4'-dimethoxyflavone			OH				OH				OCH_3	OCH_3		
7,3',4'-trimethoxyflavone							OCH_3				OCH_3	OCH_3		
5,6,7,3',4',5'-hexamethoxyflavone					OCH_3	OCH_3	OCH_3				OCH_3	OCH_3	OCH_3	
5,7,4'-trimethoxyflavone					OCH_3		OCH_3					OCH_3		
3',4'-dimethoxyflavone											OCH_3	OCH_3		
3,5,7-trihydroxy-3',4',5'-trimethoxyflavone			OH		OH		OH				OCH_3	OCH_3	OCH_3	
5,7,3',4'-tetrahydroxyflavone					OH		OH				OH	OH		
3,5,7,3',4'-pentamethoxyflavone			OH		OH		OH				OH	OH		

system), but also increase the sensitivity of P-glycoprotein-over expressing cancer cells to paclitaxel. Here, I report that 7,3',4'-trimethoxyflavone (TMF) can serve as an enhancer of paclitaxel bioavailability and as a P-glycoprotein inhibitor.

II. MATERIALS AND METHODS

1. Flavonoids

5,7,3',4',5'-pentamethoxyflavone, 7,3',4'-trimethoxyflavone, 3',4'-dimethoxyflavone, 3,6,3',4'-tetramethoxyflavone, 5,6,7,3',4'-pentamethoxyflavone, 5,6,7,3',4',5'-hexamethoxyflavone, 5,7,4'-trimethoxyflavone, 3,5,7-trihydroxy-3',4',5'-trimethoxyflavone, 5,7,3',4'-tetrahydroxyflavone, 3,7-dihydroxy-3',4'-dimethoxyflavone and 3,5,7,3',4'-pentahydroxyflavone were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA) (Table 1 and Figure 11).

2. Materials

The Enhanced chemiluminescence (ECL^{R}) detection kit from Amersham (Piscataway, NJ, USA), the TRI reagent LS™ from Molecular Research Center (Cincinnati, OH, USA), the sheep polyclonal antibody for catalase from Biodesign International (Saco, ME, USA), the mouse C219 antibody for P-glycoprotein from Signet (Dedham, MA, USA), and the anti-mouse IgG horseradish peroxidase linked whole antibody from Amersham. *N*-acetyl-L-cysteine (NAC), pyrrolidine dithiocarbamate (PDTC), vitamin C (Vit C), vitamin E (Vit E) and others were obtained from Sigma Chemical Co. (ST. Louis, MO, USA).

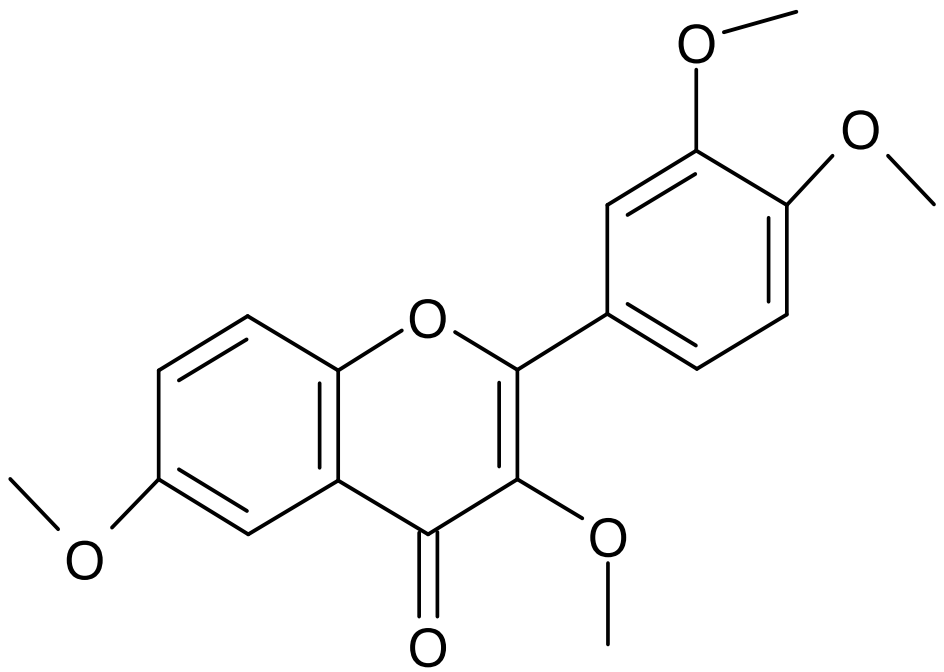


Figure 11-1. Chemical structure of 3,6,3',4'-tetramethoxyflavone.

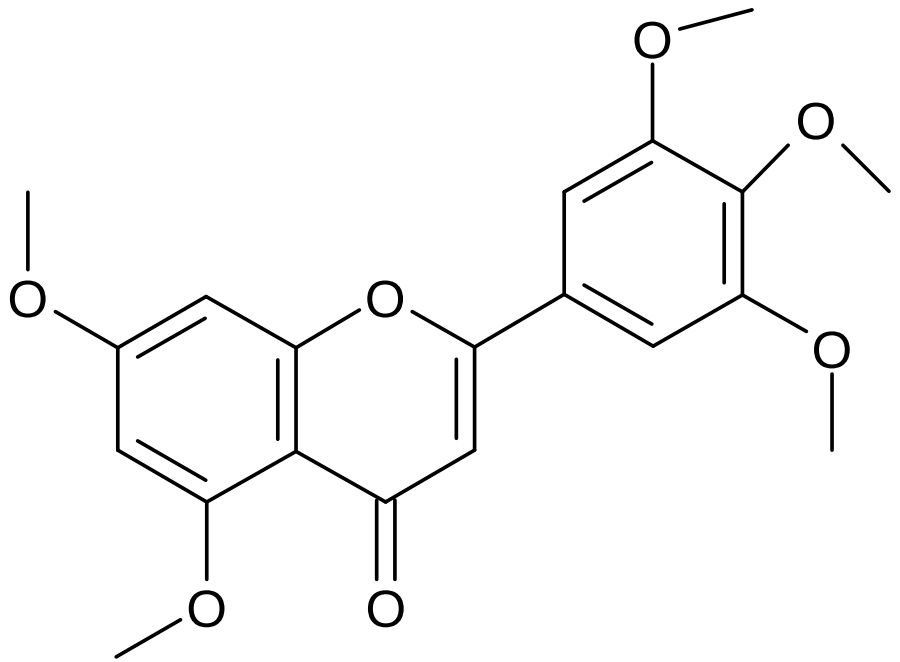


Figure 11-2. Chemical structure of 5,7,3',4',5'-pentamethoxyflavone.

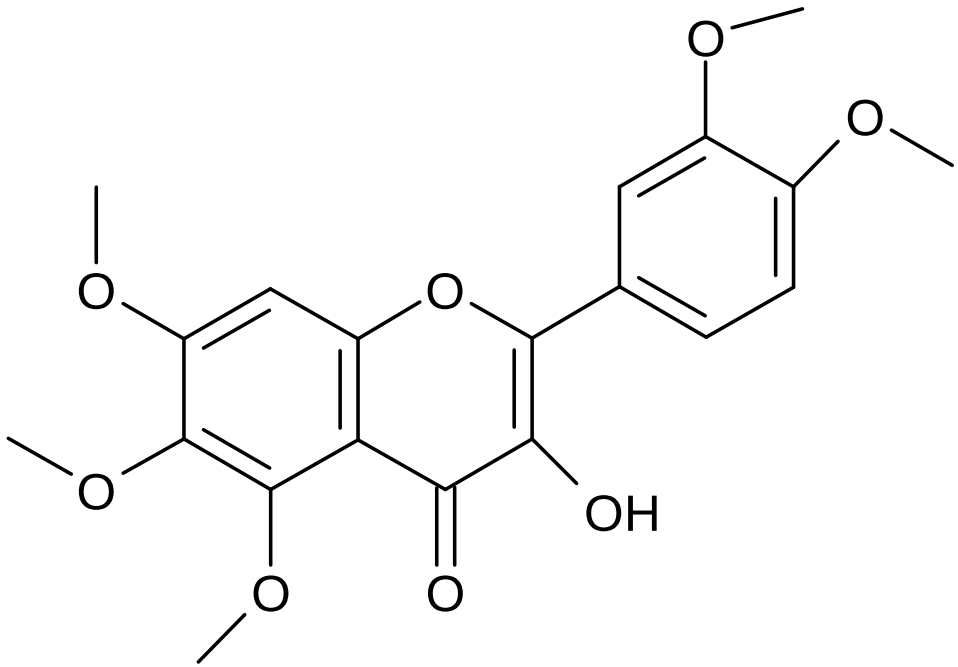


Figure 11-3. Chemical structure of 5,6,7,3',4'-pentamethoxyflavone.

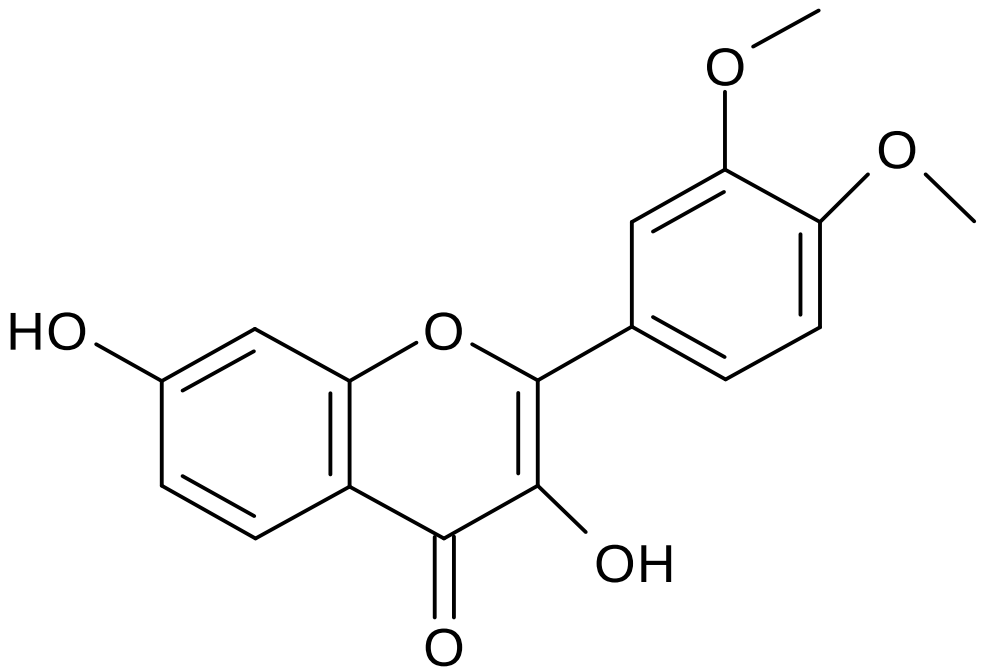


Figure 11-4. Chemical structure of 3,7-dihydroxy-3',4'-dimethoxyflavone.

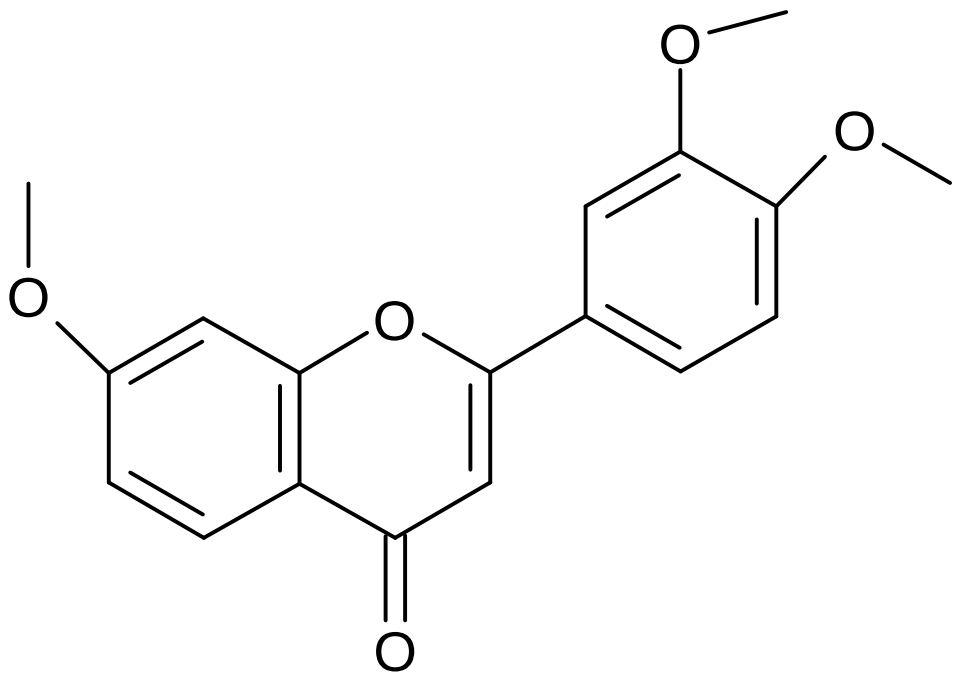


Figure 11-5. Chemical structure of 7,3',4'-trimethoxyflavone.

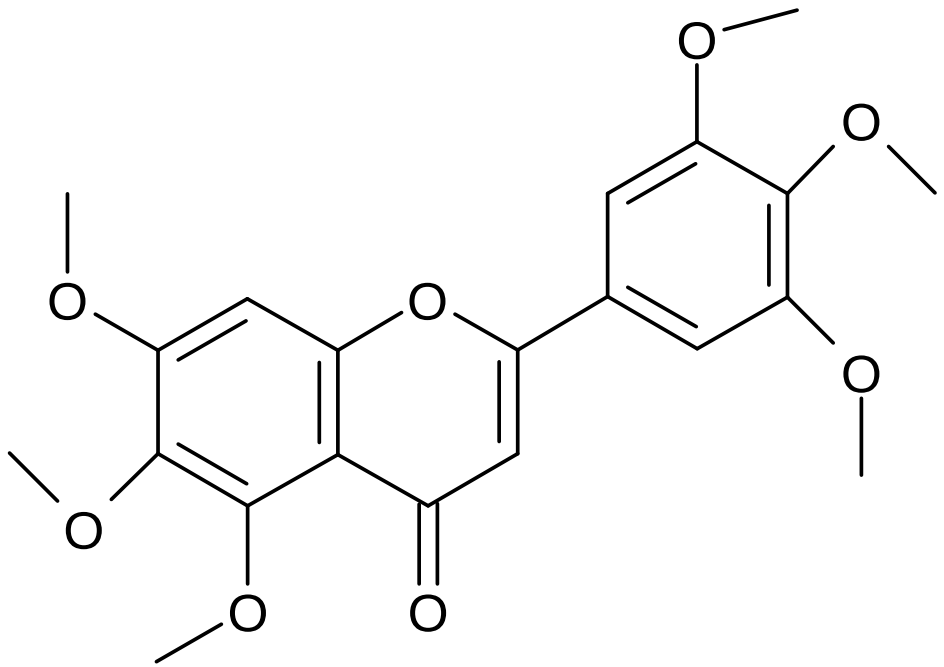


Figure 11-6. Chemical structure of 5,6,7,3',4',5'-hexamethoxyflavone.

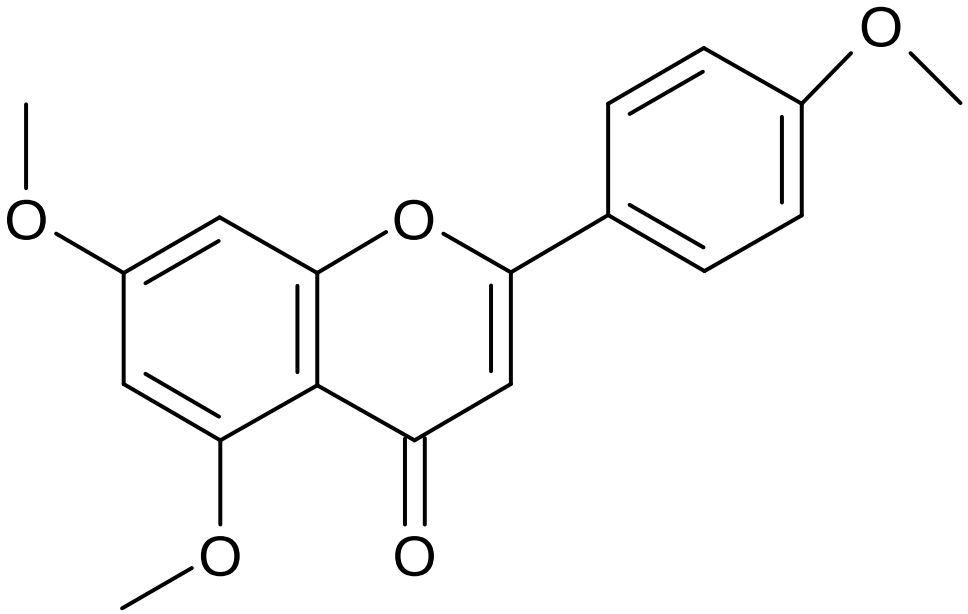


Figure 11-7. Chemical structure of 5,7,4'-trimethoxyflavone.

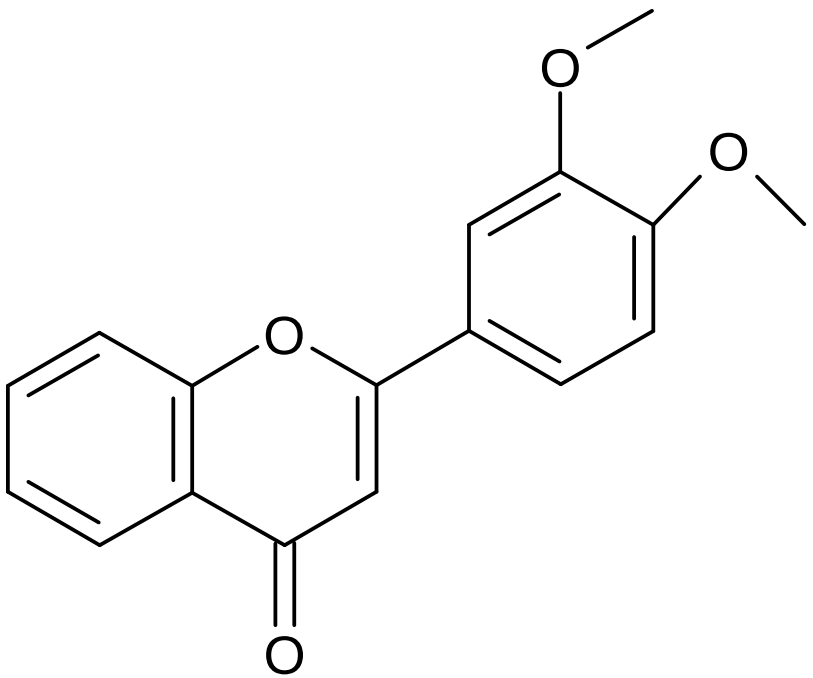


Figure 11-8. Chemical structure of 3',4'-dimethoxyflavone.

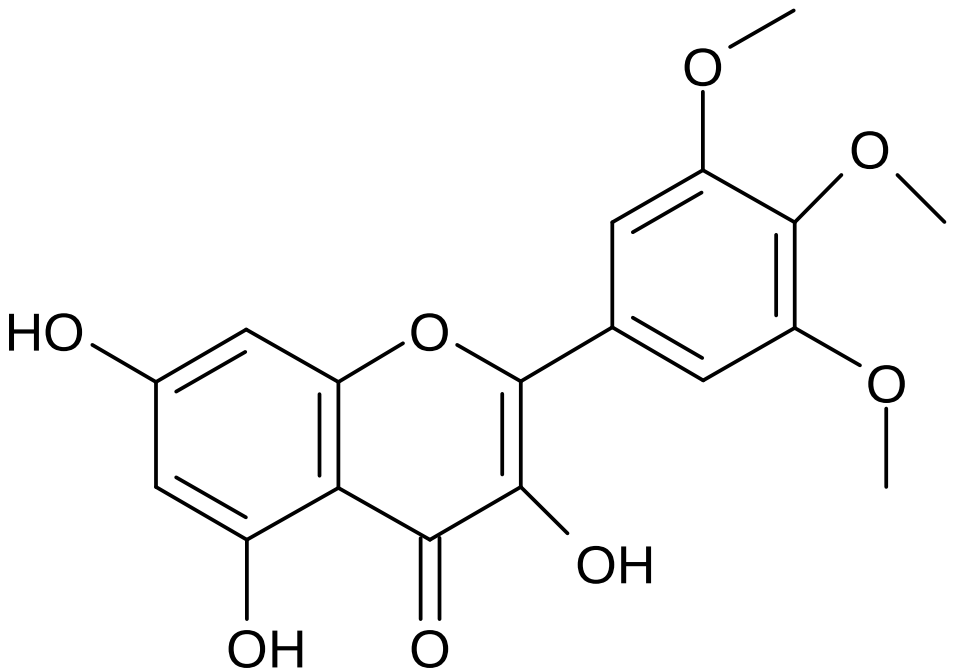


Figure 11-9. Chemical structure of 3,5,7-trihydroxy-3',4',5'-trimethoxyflavone.

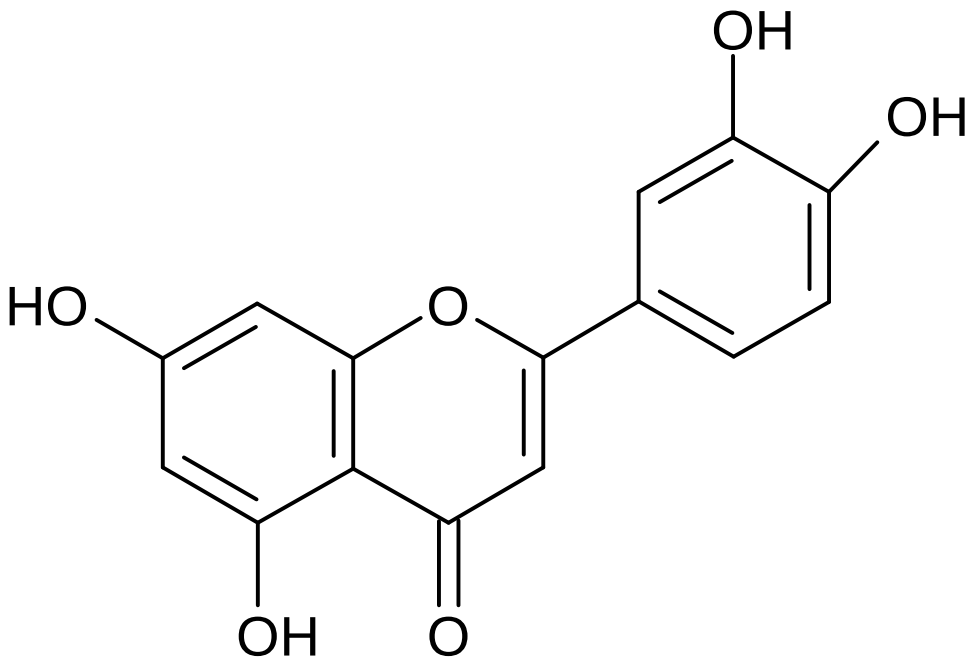


Figure 11-10. Chemical structure of 5,7,3',4'-tetrahydroxyflavone.

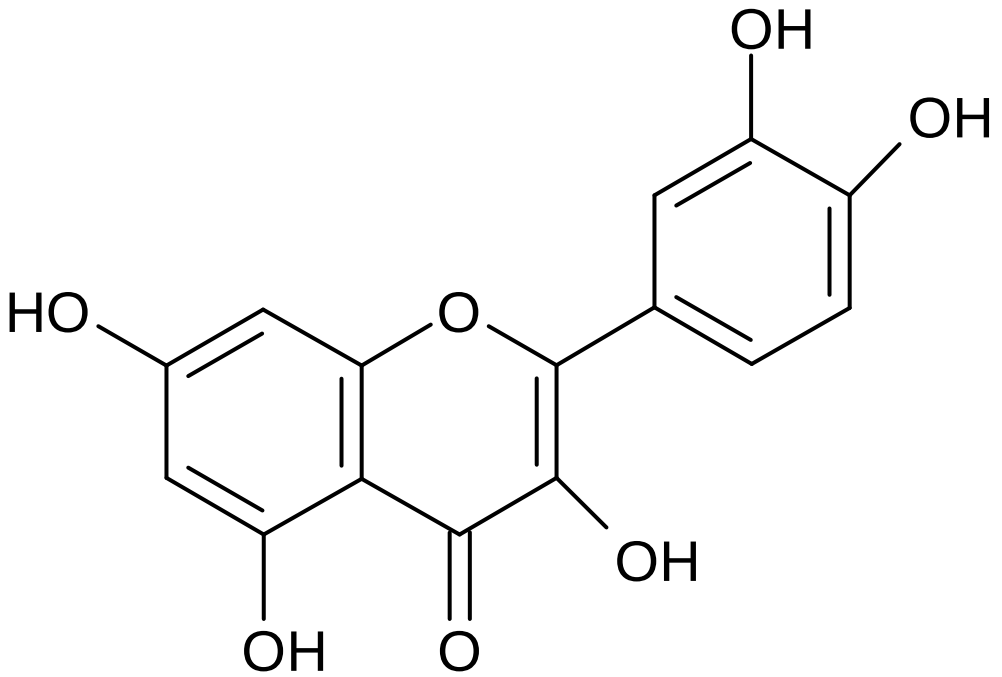


Figure 11-11. Chemical structure of 3,5,7,3',4'-pentahydroxyflavone.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), daunorubicin, vincristine, dimethyl sulfoxide (DMSO), triethylamine and ammonium acetate were obtained from Sigma Chemical Co.. Paclitaxel and docetaxel were obtained from Korea United Pharm Co. (Seoul, Korea). Cell culture media, minimum essential medium alpha (α-MEM) and Dulbecco's modified eagle medium alpha medium (DMEM), were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) and penicillin were obtained from Cambrex (San Diego, CA, USA). Moloney murine leukemia virus (MMLV) reverse transcriptase was obtained from Invitrogen (Carlsbad, CA, USA). Taq polymerase was purchased from Perkin-Elmer/Cetus (Norwalk, CT, USA). [α-^{32}P]dCTP was obtained from AgenBio Ltd. (Seoul, Korea). Methanol, hexane and acetonitrile were obtained from Merck Co. (Darmstadt, Germany). All other reagents were of analytical grade.

3. Cell culture

The acute myelogenous leukemia cell line OCI-AML-2 (AML-2) from the Ontario Cancer Institute (Toronto, Canada) was cultured at 37°C in a 5% (v/v) CO_2 atmosphere using α-MEM medium with 10% (v/v) heat inactivated FBS and penicillin.

The human colon carcinoma cell line Caco-2 and lung cancer cell SK-MES-1 line from the American Type Culture Collection were cultured in the same culture conditions using DMEM. Caco-2 cells were used between passage number 24 and 32.

The drug-resistant AML-2 sublines were selected from the parental cell line (AML-2/WT) after chronic exposure to either doxorubicin or daunorubicin. The cells were finally cultured in a fixed concentration (100 ng/mL) of each selecting drug.

The paclitaxel-resistant SK-MES-1 subline, SK-MES-1/PT4000, was selected from SK-MES-1/WT cells after chronic exposure to paclitaxel on an intermittent dosage schedule at sufficient time intervals to permit expression of the resistance phenotype. Paclitaxel was initially administered from 1 × IC_{50}, increased at 50% increments, and then finally cultured at a fixed concentration of paclitaxel (4000 ng/mL).

4. Determination of the scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The antioxidant activity was assessed according to the radical-scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Kim, *et al.*, 2001). A 100 μM DPPH radical solution was dissolved in 100% ethanol. The mixture was shaken vigorously and allowed to stand for 10 min in the dark. The test materials (100 μL each) were added to 900 μL of the resulting dark-blue DPPH radical solution in a cuvette. The absorbance at 517 nm was measured. The DPPH radicals-scavenging activity (%) was calculated with using the equation ($A_{517,\ control}$ - $A_{517,\ sample}$) × 100 $A_{517,\ control}$.

5. Determination of antioxidant activity using 2',7'-dichloro-dihydrofluorescein-diacetate (DCFH-DA)

DCFH was used to measure the ROS concentration (Le Bel, *et al.*, 1992). After 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) diacetate crossed the membrane, it was de-esterified to DCFH, which was oxidized to fluorescent DCF by the ROS (Figure 12). The reaction took place with 1×10^5 of the AML-2/DX100 cells and 1 μM DCFH-DA diacetate in 2 mL PBS. The cells were exposed to 700 μM H_2O_2 for 2 hours after preincubation with the flavonoids and the known antioxidants for 30 min. The fluorescence intensity was determined using a spectrofluorometer (Perkinelmer, Boston, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The DCFH-DA fluorescence-scavenging activity (%) was calculated with using the equation (Fluorescence $_{control}$ - Fluorescence $_{sample}$) × 100 / Fluorescence $_{control}$.

6. Cytotoxicity assay

The *in vitro* cytotoxicity of drugs was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Pieters, *et al.*, 1988). 90 μL aliquots of cells were seeded into a 96-well microplate which had already contained 10 μL of drug. Wells containing no drugs were used for control cell viability and wells containing no cells for blanking the spectro-photometer. A stock solution of 5 mg/mL MTT was prepared in

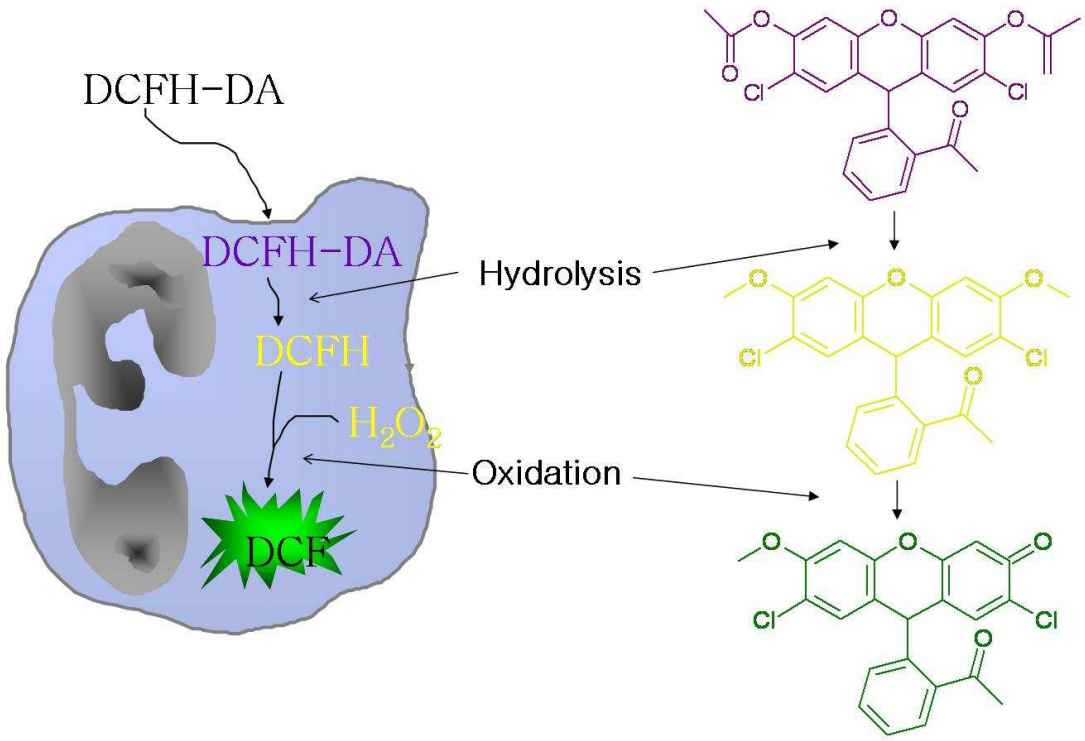


Figure 12. Oxidative process of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for H_2O_2 in cells (www.cyto.purdue.edu/class/bms524/524lect11.ppt).

saline and stored at -20℃. After the cells were incubated at 37℃ for 3 days, an aliquot of 10 μL of MTT solution was added to each well and shaking for 1 min the palate was incubation for 4 hours. Formazan crystals were dissolved with 100 μL of 0.04 N HCl-isopropanol or 150 μL DMSO. The optical density of the wells was measured with a microplate reader (Bio-Tek Isdtruments, Winooski, UT, USA) at 540 nm. The IC_{50} was defined as the drug concentration that resulted in a 50% reduction in the number of cells compared with untreated control cells, after 3 days treatment. The IC_{50} values were determined directly from the semi-logarithmic dose-response curves.

7. Chemosensitization assays

Chemosensitization is well known that microtubule-disrupting drugs such as vincristine can induce have antimitotic and apoptosis activity (Donaldson, *et al.*, 1994; Deutsch, *et al.*, 1989). The *in vitro* chemosensitizing activities of the flavonoids and verapamil were determined by MTT assays with AML-2/D100 cells in the presence or absence of vincristine (100 ng/mL) (Pieters, *et al.*, 1988). The chemosensitizing index (CI) was defined as the ratio of the IC_{50} value in the absence of vincristine to the IC_{50} value in the presence of vincristine.

8. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) assay

Total cellular RNA was isolated from cells using TRIzol® according to the manufacturer's protocol. RT-PCR was used to analyze the expression of mRNA for P-glycoprotein, catalase and β-actin (internal control). Table 2 shows the nucleotide sequences for sense and antisense primers used in this study. RNAs from each sample were reverse-transcribed using 200 units of Moloney murine leukemia virus (MMLV) and oligo (dT_{18}) primer for 90 min at 37°C.

The resulting cDNA was diluted 1 : 5 with water and then amplified using 2.5 units of Taq polymerase and 10 pM of each primer. PCR products were quantitated by incorporation of 5 μCi of [α-^{32}P] dCTP in each PCR reaction mixture in a GeneAmp 2720 Thermal Cycler (Applied Biosystems, Boston, MA, USA; Figure 13) for 21 cycles (but 16 cycles for β-actin) of sequential denaturation (at 95℃ for 30 sec), annealing (at 65℃ for P-glycoprotein, at 53℃ for catalase), and extansion (at 72℃ for 30 sec). The PCR products were electrophoresed on 7% non-denaturing polyacrylamide gels. The amount of each mRNA transcript was then normalized against that of β-actin mRNA. Auto radiographic films of the RT-PCR assay were subjected to densitometri can alyses using the Kodak Image Station 4000MM (Eastman Kodak, Rochester, NY, USA).

Table 2. PCR primers

Gene	S&AS[a]	Nucleotide sequences	Sequences region[b]	Length of PCR products	Reference[c]
Catalase	S	5'-TTTGGCTACTTTGAGGTCAC-3'	314-333	389 bp	Bell, *et al.*, 1986
	AS	3'-TCCCCATTTGCATTAACCAG-5'	734-753		
Pgp	S	5'-CTGGTTTGATGTGCACGATGTTGG-3'	907-930	446 bp	Chen, *et al.*, 1986
	AS	3'-GTCATCGACTTCTCCAGAACCGT-5'	1179-1201		
β-actin	S	5'-GACTATGACTTAGTTGCGTTA-3'	1912-1932	501 bp	Nakajima, *et al.*, 1985
	AS	5'-GTTGAACTCTCTACATACTTCCG-3'	2392-2412		

[a] Sense and antisense.

[b] The oligonucleotide primers constructed for PCR correspond to the sense and antisence bases within these reported sequences.

[c] References for gene sequences.

Pgp, P-glycoprotein.

β-actin, PCR control (house-keeping gene).

Figure 13. PCR cycler.

9. Western blotting analysis

Western blotting analysis was performed using a slight modification of the method described previously (Towbin, *et al.*, 1979). The proteins transferred onto a polyvinylidene fluoride (PVDF) transfer membrane (Pall Gelman, Ann Arobor, MI, USA) were incubated with the primary antibodies (diluted 1 : 1000) and the sheep polyclonal antibody for catalase and the mouse C219 antibody for P-glycoprotein. The membrane was incubated with the horseradish peroxidase-conjugated secondary antibodies (diluted 1 : 1000) against each IgG of the hosts of the primary antibodies for 1 hour. The membrane was then stained using the detection reagent of the ECL detection kit. For activity staining of catalase, enzyme samples were separated on a 7% nondenaturing polyacrylamide gel, and the catalase activity was visualized on the gel using an *in situ* staining technique (Clare, *et al.*, 1984).

10. Functional drug accumulation assay

Cell suspensions (1×10^6 cells/mL) in phosphatebuffered saline (PBS) were exposed to daunorubicin with or without verapamil (10 μM), at 37°C for 30 min. Cells were subsequently analyzed for their cellular drug fluorescence by a flow cytometer (Becton Dickinson, San Jose, CA, USA, Figure 14), in which a focused argon laser beam (488 nm) excited cells in a laminar sheath flow and their fluorescence emissions (585 nm) were collected to generate a histogram.

Figure 14. Flow cytometer.

11. Bi-directional transport experiment using Caco-2 cells

Caco-2 cells (Figure 15) were seeded at a density of 4×10^4 cells/cm^2 on top of Transwell® polycarbonate filters (pore size, 0.4 μm; diameter, 24.5 mm; growth area, 4.71 cm^2; Corning Costar Corp., Cambridge, MA, USA) and grown in a CO_2 incubator (37℃, 5% CO_2); the media were changed every 3 to 5 days (Figure 16). Experiments were conducted 16 to 20 days after the cells were seeded according to a previous report (Bromberg and Alakhov, 2003). Transepithelial electrical resistance (TEER) was measured using a Millicell-ERS device (Millipore, Bedford, MA, USA) equipped with rod-shaped electrodes. The TEER data were corrected for background readings from the blank filter and culture medium. The experiments were started when TEER values reached 800-850 Ω/cm^2. Paclitaxel was added to the apical (AP) or basolateral (BL) chamber at a final concentration of 100 μM with verapamil or flavonoids. Cells were incubated for 3 hours to assure measurable concentrations in the receiving chamber.

12. Determinati on of paclitaxel by high performance liquid chromatography (HPLC)

Paclitaxel concentration in media was determined using modified slightly from the previous high performance liquid

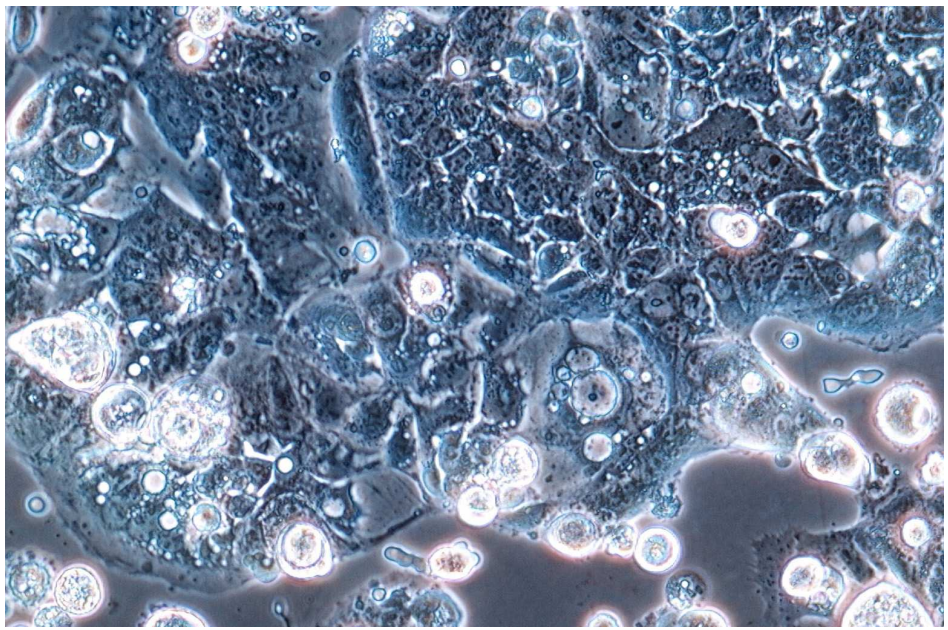


Figure 15. Caco-2 cell monolayers.

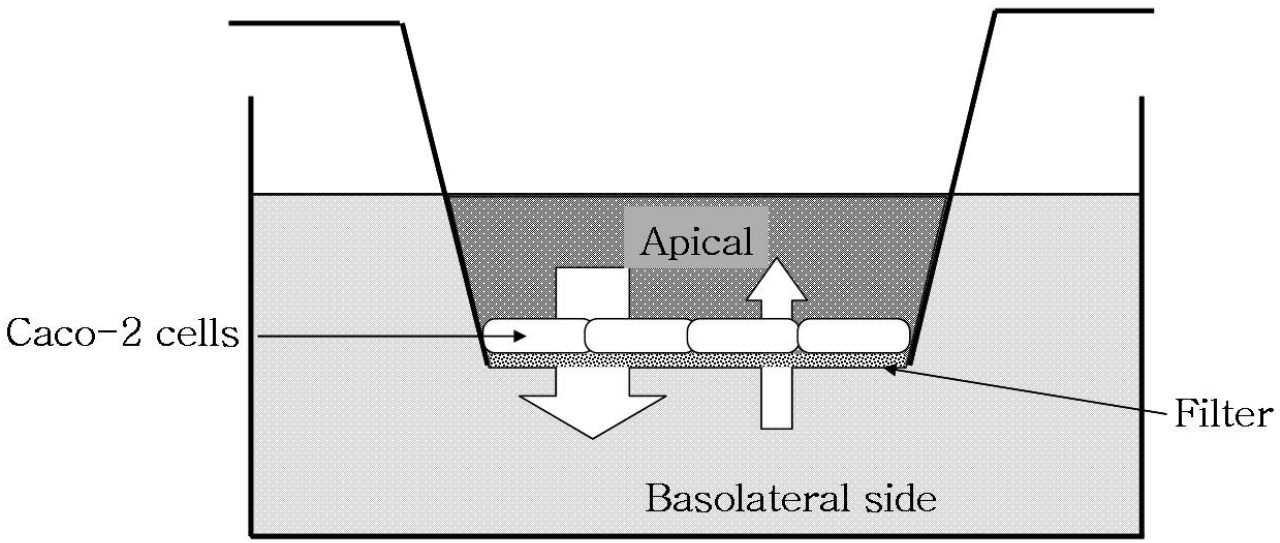


Figure 16. Schematic representation of a Caco-2 system permeability assay.

chromatography (HPLC; LC-20A, Shimazu, Kyoto, Japan; Figure 17) method (Willey, *et al.*, 1993). As shown in Figure 18, briefly, 0.4 mL of 0.2 M ammonium acetate buffer solution (pH 5.0) was added to Eppendorf tubes containing 0.4 mL samples. Solid-phase extraction (SPE) was used for purification. First, Cyano Bond Elut columns (1 mL, Analytichem International, Harbor, CA, USA) were conditioned by consecutive washes with 2.0 mL of methanol and 1.0 mL of 0.01 M ammonium acetate buffer solution. The sample-buffer mixtures were then loaded onto the columns. Next, the columns were consecutively washed with 2 mL of 0.01 M ammonium acetate buffer solution (pH 5.0), 1 mL of methanol/0.01 M ammonium acetate buffer solution (2 : 8, v/v), and 1 mL of hexane. The columns were dried in the operating clean bench for 15 min. Finally, the analytes were eluted from the columns into Eppendorf tubes with 1 mL of acetonitrile/triethylamine (1000 : 1, v/v). The eluate was evaporated to dryness using a centrifuge vacuum concentrator (Ecospin 314, Kyunggido, Korea) at ambient temperature. The residue was reconstituted with 100 μL of the HPLC mobile phase [acetonitrile, methanol and 20 mM ammonium acetate buffer solution (pH 5.0, 20 : 55 : 25)]. The reconstituted samples were then transferred to auto-sampler vials (250 μL) containing limited-volume inserts, and 50 μL was injected from each sample onto the ODS C18 column (4.6 × 250 mm, 5 μm; Shimazu). The mobile phase was passed through a 0.22 μm membrane filter

Figure 17. High performance liquid chromatography (HPLC) for determination of paclitaxel.

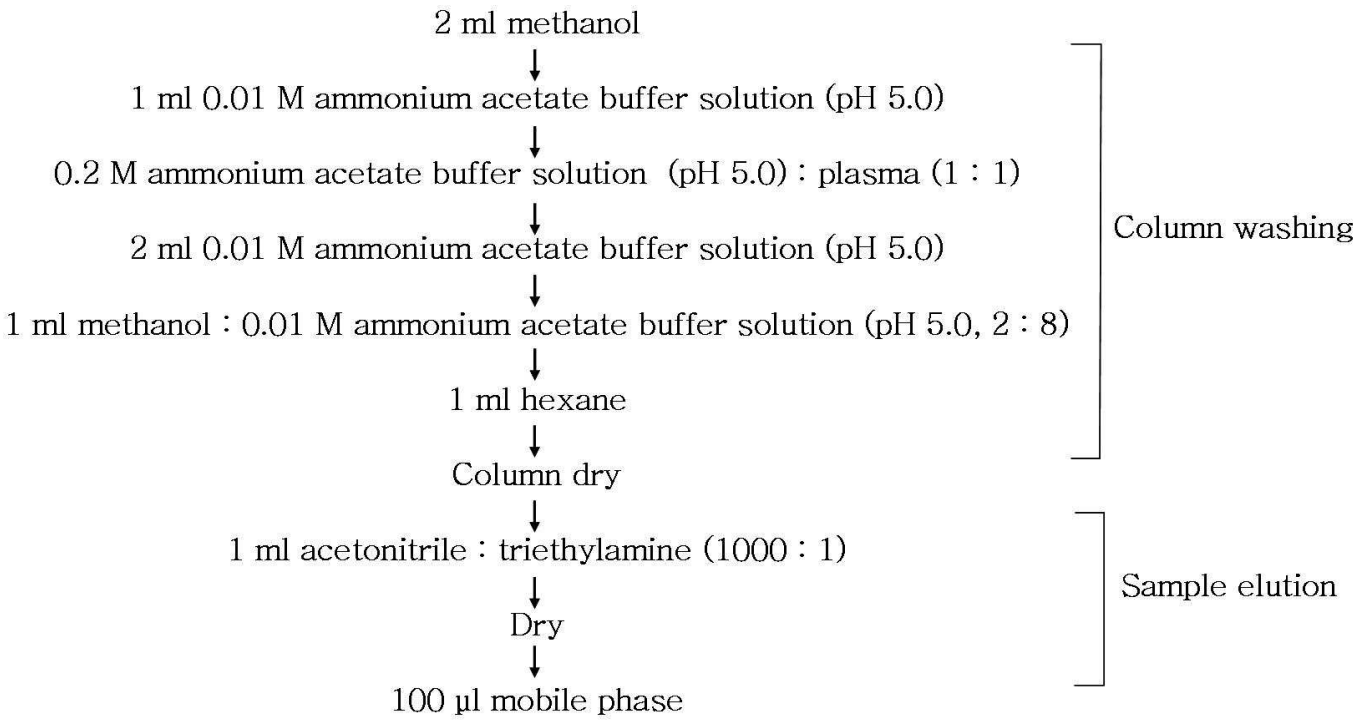
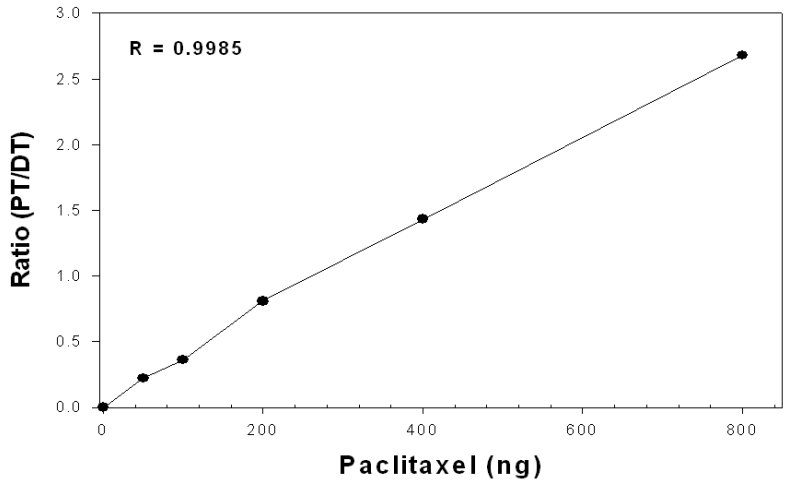


Figure 18. Extraction procedure of taxenes.

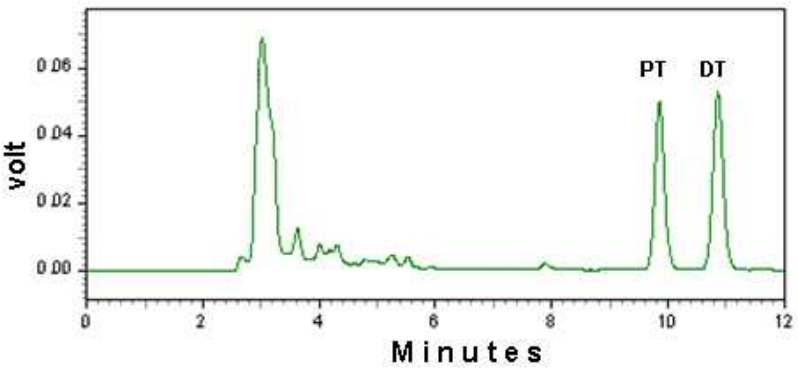
and degassed by ultrasonication under vacuum before use. The flow rate was 1 mL/min, and the effluent was monitored for UV absorption at 230 nm. Extraction procedures for the determination of taxenes are summarized in Figure 19. Stock solutions of paclitaxel and docetaxel (internal control) were prepared in methanol and kept at -20℃ until use.

13. Statistical analysis

The results are expressed as mean ± standard error of the mean. All statistical analyses were performed using SigmaPlot 2001 (Systat softwere Inc., Caliponia USA). Statistical comparisons were made using Student's t - test. P - value < 0.05 was considered significant.



(A)



(B)

Figure 19. Standard curve and chromatogram. (A) paclitaxel concentration standard curve. Paclitaxel standards were prepared in methanol. A 50 μL sample was injected and area count recorded at 230 nm. The standard curve was derived from a mean of values (n = 3) for each paclitaxel concentration determined

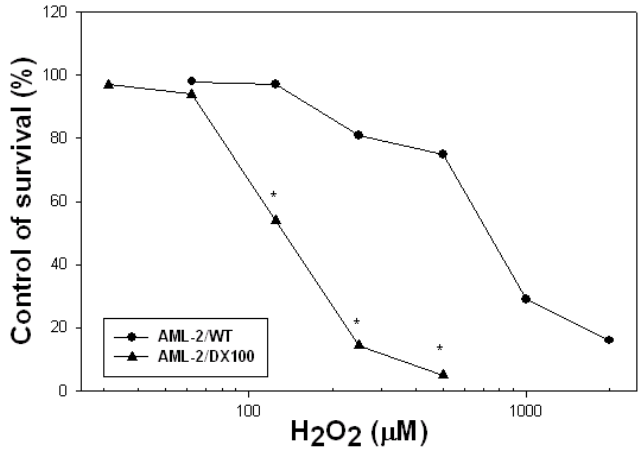
(R = 0.9985). (B) chromatogram of a mixture of paclitaxel (PT) and docetaxel (DT). Retention times as follows: PT; 9.853 min, DT; 10.854 min.

III. RESULTS

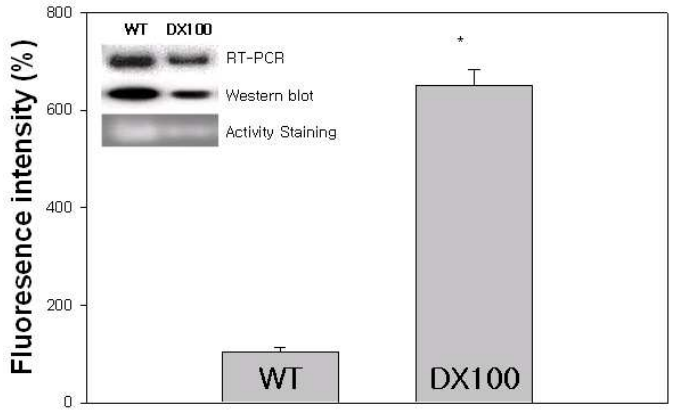
1. Antioxidant activities of flavonoids and structure-activity relationships

Two methods using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA) were used to compare the free-radical-scavenging activity on the same molar concentration (100 μM) basis using the AML-2/DX100 cells which are characterized by the down-regulated expression of catalase and resulting supersensitivity to H_2O_2 (Figure 20).

In the DPPH assay, flavonoids showed various antioxidant activities according to chemical structures. In advance, the numbers of flavonoids (F1-F11) used in this study were designated from the lowest F1 to the highest F11 antioxidant activities obtained from the DPPH assay (Table 3 and Figure 21). As shown in Table 3 and Figure 21, 3,5,7,3',4'-hydroxyl flavone (F11) and 5,7,3',4'-hydroxylflavone (F10) showed highest, 3,5,7-hydroxyl-3',4',5'-methoxyflavone (F9) intermediate and other flavonoids (F2-F8) low antioxidant activities in the DPPH assay. In the DPPH assay, the antioxidant activities of 3,5,7,3',4'-hydroxylflavone (F11) and 5,7,3',4'-hydroxylflavone (F10) were higher than four positive control antioxidants, *N*-acetyl-L-cysteine, pyrrolidine dithiocarbamate, vitamin C and



(A)



(B)

Figure 20. Supersensitivity to hydrogen peroxide of the AML-2/DX100 characterized by the down-regulated catalase expression. (A) Sensitivity to H_2O_2 was determined using the MTT assay, (B) ROS generation after treatment with 700 μM H_2O_2 was determined using the DCFH assay. Inserts of (B) show catalase expression and activity in the AML-2/WT and AML-2/DX100. *P < 0.05.

Table 3. Antioxidant, cytotoxic and chemosensitizing activities of the flavonoids

Abb[a]	Drug[b]	DPPH[c] (%)	DCFH[d] (%)	IC_{50}[e] (M)		CI[g]
				VCR−[f]	VCR+[f]	
F1	3,6,3',4'-tetramethoxyflavone	9.6	45.5	>400.0	1.9	>210.5
F2	5,7,3',4',5'-pentametoxyflavone	9.9	-5.0	>400.0	0.4	>1000.0
F3	5,6,7,3',4'-pentamethoxyflavone	10.1	26.2	>400.0	3.2	>125.0
F4	3,7-dihydroxy-3',4'-dimethoxyflavone	10.4	30.3	7.4	6.3	1.2
F5	7,3',4'-trimethoxyflavone	10.4	37.1	40.0	1.2	333.3
F6	5,6,7,3',4',5'-hexamethoxyflavone	10.8	36.0	64.7	0.9	71.9
F7	5,7,4'-trimethoxyflavone	10.9	19.6	72.6	1.4	51.9
F8	3',4'-dimethoxyflavone	11.0	24.4	386.0	1.2	321.7
F9	3,5,7-trihydroxy-3',4',5'-trimethoxy-flavone	24.5	69.0	12.6	3.2	3.9
F10	5,7,3',4'-tetrahydroxyflavone	43.2	83.7	32.7	19.6	1.7
F11	3,5,7,3',4'-pentamethoxyflavone	50.2	85.4	61.0	52.5	1.2
NAC	*N*-acetyl-L-cysteine	13.2	-125.7			
PDTC	Pyrrolidine dithiocarbamate	15.2	82.5			
Vit C	Vitamin C	29.9	-20.8			
Vit E	Vitamin E	32.4	33.4			
VP	Verapamil			61.0	0.4	152.5

[a] Abbreviation.

[b] Number of flavonoids was designated from the lowest to the highest antioxidant activities on the basis of the DPPH assay.

[c] The DPPH radicals-scavenging activity (%) was calculated with using the equation $(A_{517,\ control} - A_{517,\ sample}) \times 100\ A_{517,\ control}$.

[d] The DCFH fluorescence-scavenging activity (%) was calculated with using the equation (Fluorescence $_{control}$ - Fluorescence $_{sample}$) × 100 / Fluorescence $_{control}$.

[e] Drug concentrations with inhibit 50% growth of the cells.

[f] In the presence (+) or absence (-) of vincristine (VCR).

[g] Chemosensitizing index = IC_{50} (VCR-)/ IC_{50} (VCR+).

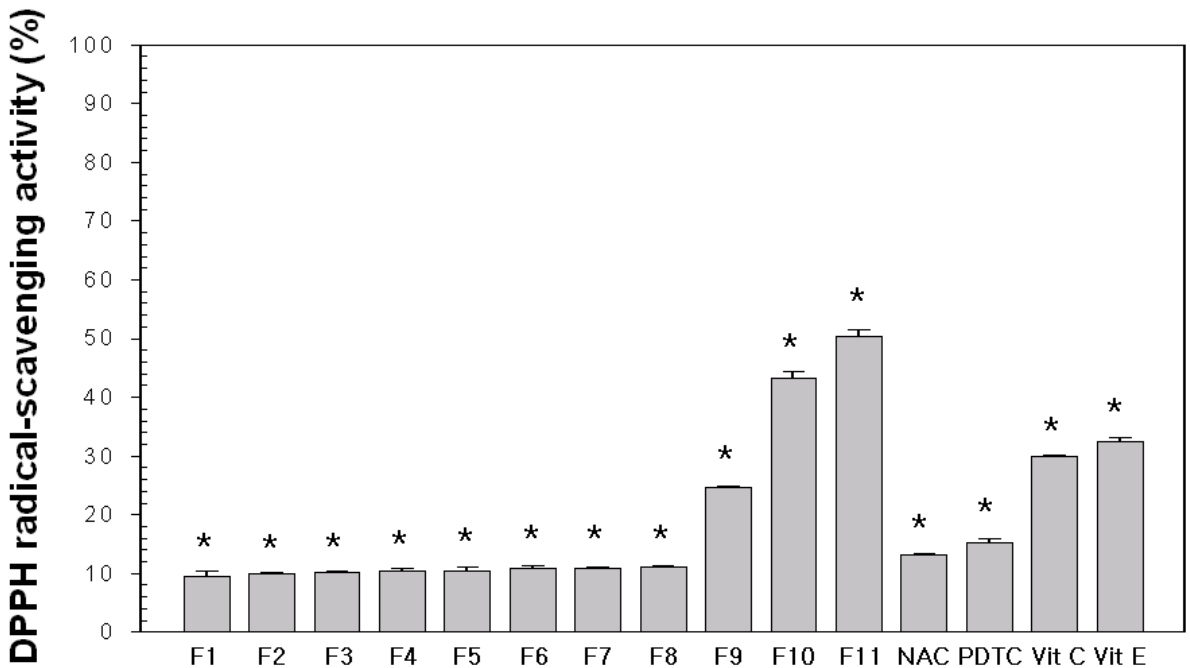


Figure 21. DPPH free radical-scavenging activity of flavonoids and antioxidants. Antioxidant activities of flavonoids and antioxidants were compared on the basis of the same molar concentration of 100 μM. Means ± SE (n = 3). *P < 0.05.

vitamin E. The antioxidant activities of 3,5,7-hydroxyl-3',4',5'-methoxyflavone (F9) were comparable to that of vitamin C and vitamin E whereas antioxidant activities of other flavonoids (F1-F8) were comparable to those of the two chemically distinct antioxidants, *N*-acetyl-L-cysteine, pyrrolidine dithiocarbamate (Figure 21).

In the DCFH assay, the antioxidant activities of 3,5,7,3',4'-hydroxylflavone (F11), 5,7,3',4'-hydroxylflavone (F10) and 3,5,7-hydroxyl-3',4',5'-methoxyflavone (F9) were similar to the results obtained using the DPPH assay whereas other flavonoids 3,6,3',4'-methoxyflavone (F1) and 5,7,3',4',5'-methoxyflavone (F2), *N*-acetyl-L-cysteine and vitamin C showed quite different results (Figure 22). As shown in Figure 22, the antioxidant activities of 3,5,7,3',4'-hydroxylflavone (F11) and 5,7,3',4'-hydroxylflavone (F10) were comparable with that of pyrrolidine dithiocarbamate, and those of F3-F8 with that of vitamin E. The antioxidant activity of 3,6,3',4'-methoxyflavone (F1) increased to the comparable level with that of 3,5,7-trihydroxy-3',4',5'-methoxyflavone (F9). However, 5,7,3',4',5'-methoxyflavone (F2), *N*-acetyl-L-cysteine and vitamin C even increased the level of ROS generation in the DCFH assay, suggesting their possible roles as pro-oxidants.

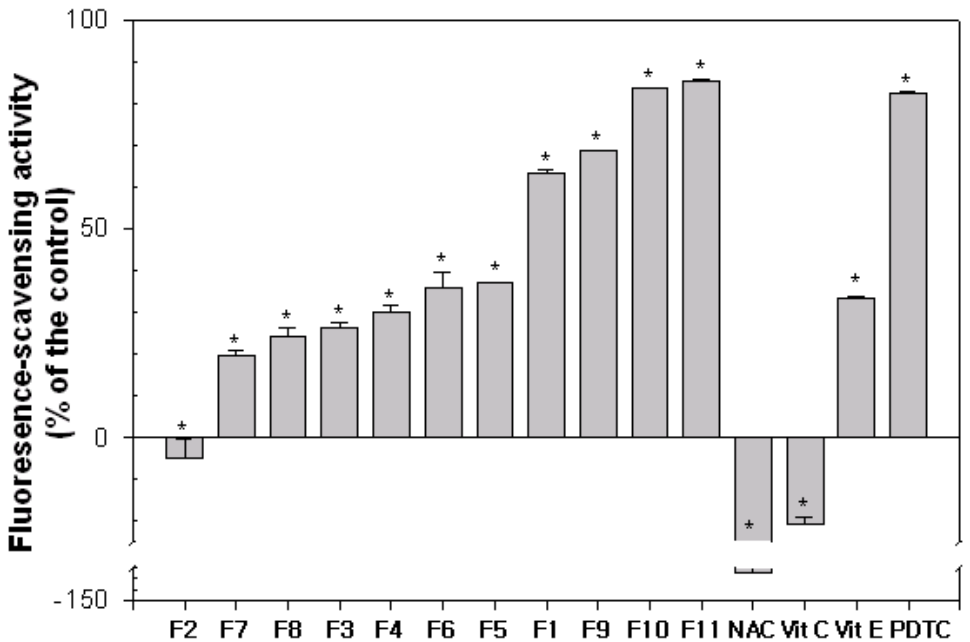
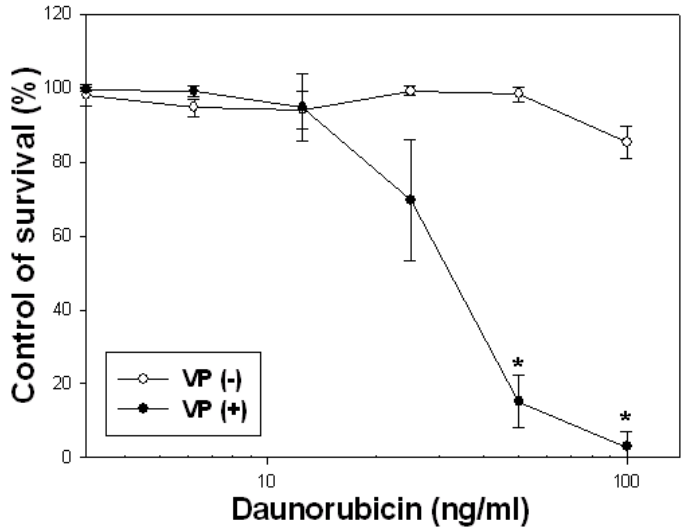


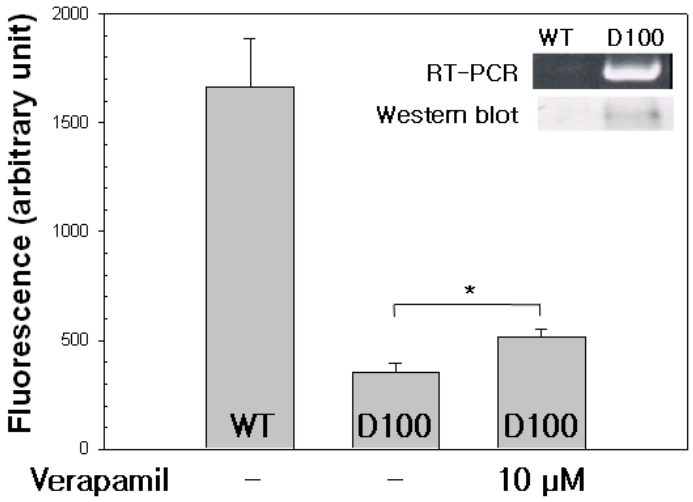
Figure 22. DCFH fluorescence-scavenging activity of flavonoids and antioxidants. The scavenging activity of DCFH fluorescence (%) was calculated using the equation (Fluorescence $_{control}$ - Fluorescence $_{sample}$) x 100 / Fluorescence $_{control}$. Means ± SE (n = 3). *P < 0.05. Other legends are the same as in Figure 18.

2. Cytotoxic and chemosensitizing activities of flavonoids and structure-activity relationships

The chemosensitizing and cytotoxic activities were determined by the MTT assay in the presence or absence of vincristine, a good substrate for P-glycoprotein, respectively, using the AML-2/D100 cells. The AML-2/D100 cells overexpress *MDR1* mRNA and P-glycoprotein, confirmed by RT-PCR and Western blot analyses, which was responsible for the resistance to daunorubicin and decreased intracellular accumulation that could be inhibited by a P-glycoprotein inhibitor verapamil (Figure 23). Four flavonoids 3,6,3',4'-tetramethoxyflavone (F1), 5,7,3',4',5'-pentametoxyflavone (F2), 5,6,7,3',4'-pentamethoxyflavone (F3) and 7,3',4'-trimethoxyflavone (F5) less than 400 μM did not show cytotoxicity and others showed differential cytotoxic activities (Table 3 and Figure 24). As shown in Table 3 and Figure 24, four flavonoids containing hydroxyl groups (F4 and F9-F11) have higher cytotoxic activity than others, suggesting an important role of the number of hydroxyl groups in cytotoxicity. However, two flavonoids with methoxy groups 5,6,7,3',4',5'-hexamethoxyflavone (F6) and 5,7,4'-trimethoxyflavone (F7) showed relatively high cytotoxicity. These results suggested the number and/or site of methoxy groups could be responsible for cytotoxicity, whose structure-activity relationships was not clear in this study. Flavonoids have been shown to have bifunctional interactions of their A and C rings at the ATP-binding site and the hydrophobic



(A)



(B)

Figure 23. Reversal of resistance (A) and accumulation (B) of daunorubicin in AML-2/D100 cells by a P-glycoprotein inhibitor verapamil. (A) MTT assay using AML-2/D100 cells was performed

or absence of verapamil (10 μM) as the function of danourubicin concentrations. (B) fluoresent intensity of intracellular danourubicin was determined by flow cytometry. Inserts show the profiles of P-glycoprotein expression in the AML-2/WT and AML-2/D100. Level of P-glycoprotein and its mRNA were determined using RT-PCR method and Western blot analysis, respectively. VP, verapamil. $^{*}P < 0.05$.

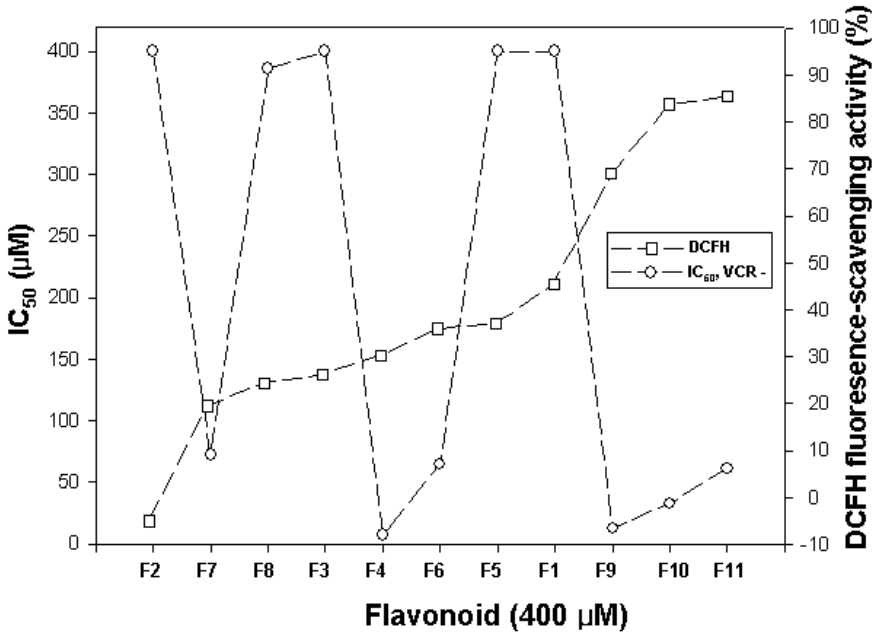


Figure 24. Comparison between IC_{50} values in the absence of vincristine and DCFH fluorescence-scavenging activity of the flavonoids. IC_{50} values were obtained in the absence of vincristine using P-glycoprotein-overexpressing AML-2/D100 cells. DCFH fluorescence-scavenging activity was determined using AML-2/DX100 cells as described in "Materials and Methods." VCR, vincristine.

B-ring at a steroid-interacting hydrophobic sequence of P-glycoprotein (Conseil, *et al.*, 1998). The chemosensitizing activity of flavonoids was determined using the AML-2/D100 cells in the presence of vincristine.

As shown in Table 3 and Figure 25, the IC_{50} values of 5,7,3',4',5'-methoxyflavone (F2) and 5,7,4'-methoxyflavone (F7), 0.4 μM and 1.4 μM, respectively. The IC_{50} values of 5,6,7,3',4'-methoxyflavone (F3) and 5,6,7,3',4',5'-methoxyflavone (F6), 3.2 μM and 0.9 μM, respectively. The IC_{50} values of 5,6,7,3',4',5'-methoxyflavone (F6) and 5,7,3',4',5'-methoxyflavone (F2) were 0.9 μM and 0.4 μM, respectively. In addition, IC_{50} values of flavonoids in the presence of vincristine were positively related to their DCFH scavenging activities (Table 3 and Figure 25).

3. Screening flavonoids for P-glycoprotein inhibition using MTT assay

Flavonoids interact bifunctionally with the ATP-binding site and a steroid-interacting hydrophobic sequence of P-glycoprotein (Wacher, *et al.*, 1998). The cytotoxicity and chemosensitizing activities of various hydroxy and/or methoxy flavonoids were determined by MTT assay in the presence or absence of vincristine in AML-2/D100 cells overexpressing P-glycoprotein (Figure 25). Eleven flavonoids with various methoxy and/or hydroxyl groups had already been screened for P-glycoprotein inhibition (Van Asperen, *et al.*, 1997). Among the flavonoids

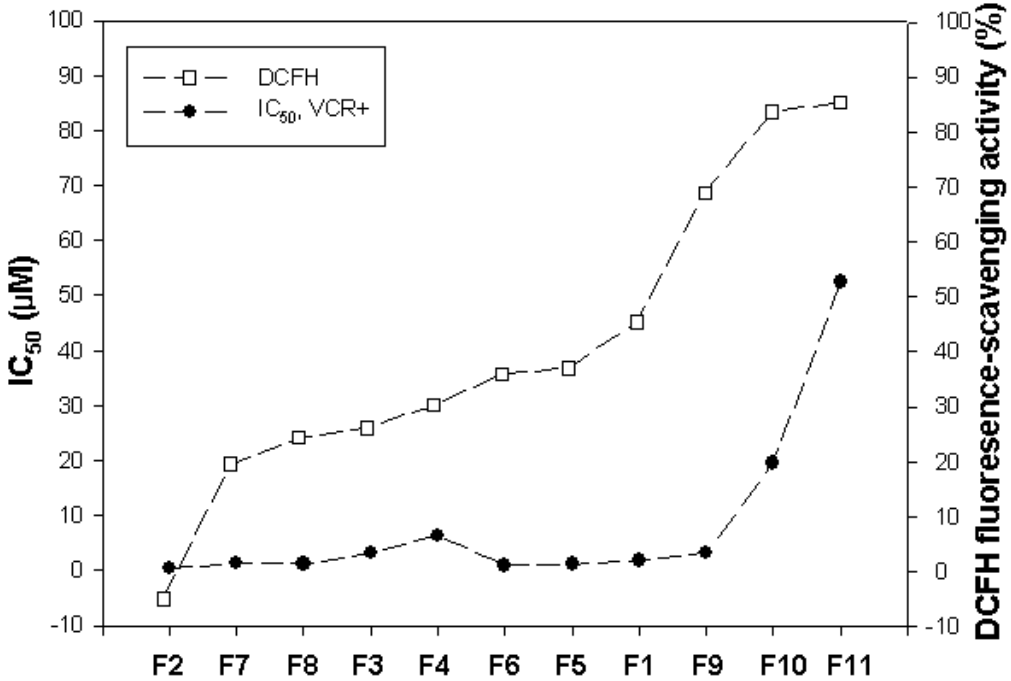


Figure 25. Comparison between IC_{50} values in the presence of vincristine and DCFH fluorescence-scavenging activity of the flavonoids. IC_{50} values were obtained in the presence of vincristine (100 ng/mL) using AML-2/D100 cells.

screened for P-glycoprotein inhibition, 5,7,3',4',5'-methoxyflavone and 7,3',4'-methoxyflavone exhibited the highest chemosensitizing indexes, > 1000 and > 333, respectively. In contrast, the chemo-sensitizing index of the wellknown P-glycoprotein inhibitor verapamil was 152 (Figure 26). Thus, on the basis of their high chemosensitizing indexes, 5,7,3',4',5'-methoxyflavone and 7,3',4'-methoxyflavone were selected and further tested in paclitaxel transport in Caco-2 cells.

4. Extraction and determination of paclitaxel

The extraction ratio of paclitaxel using the Cyano Bond Elut column was approximately 61%. The amount of paclitaxel was determined by high speed liquid chromatography (HPLC) using docetaxel as an internal standard (Figure 19). The standard curve was linear (R = 0.9985) and a chromatogram revealed good separation between paclitaxel (retention time = 9.867 min) and docetaxel (retention time = 10.867 min). The average coefficient of variance (CV) for triplicate samples was 15% for paclitaxel at 250 pM. The detection limit was 50 ng of paclitaxel. These parameters guaranteed a good quantitative assay of paclitaxel.

5. Effect of 5,7,4'-methoxyflavone on bi-directional transport of paclitaxel in the Caco-2 system

The transport of paclitaxel in the Caco-2 cell monolayer was linear over 3 hours (data not shown). This result is consistent

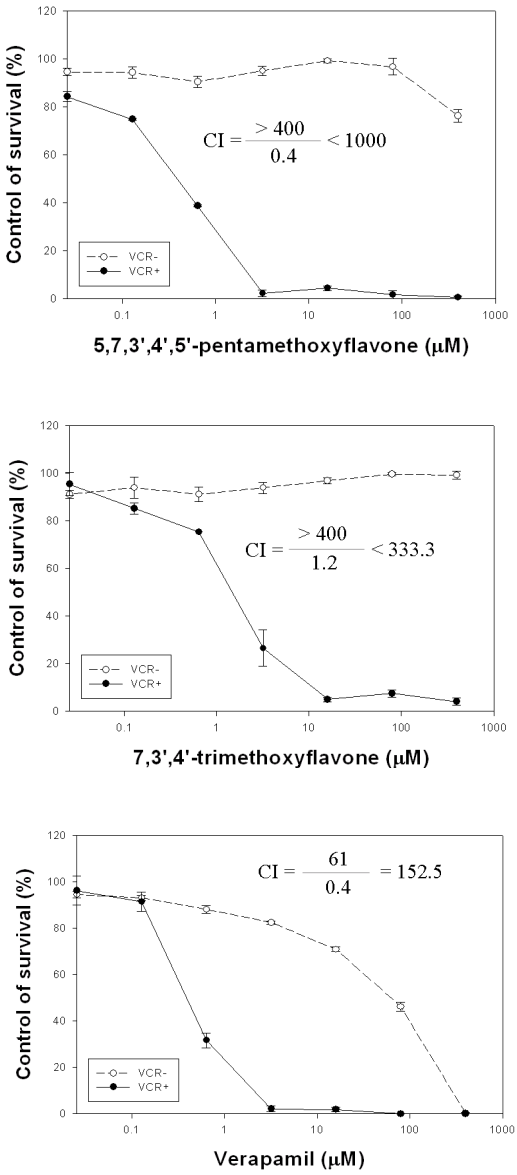


Figure 26. Cytotoxicity and chemosensitization of flavonoids and verapamil in AML-2/D100 cells. CI, chemosensitizing index = IC_{50} (VCR-) / IC_{50} (VCR+) in AML-2/D100 cells VCR, vincristine (100 ng/mL).

with a report that paclitaxel flux across the Caco-2 cell monolayer was linear with time for up to 3 hours (Sparreboom, *et al.*, 1997). In this study, paclitaxel transport across the Caco-2 cell monolayer was determined after incubation for 3 hours in the presence and absence of 5,7,3',4',5'-methoxyflavone, 5,7,4'-methoxyflavone, and verapamil in both AP-to-BL and BL-to-AP directions.

Since 5,7,3',4',5'-methoxyflavone unexpectedly decreased AP-to-BL paclitaxel transport, only 5,7,4'-methoxyflavone was compared to verapamil in terms of paclitaxel transport in this study. The BL-to-AP transport of paclitaxel was more than 10-fold greater than its AP-to-BL transport (Figure 27). 5,7,4'-methoxyflavone and verapamil increased the AP-to-BL transport of paclitaxel and decreased its BL-to-AP transport in a concentration-dependent manner (Figure 27).

However, the effect of 5,7,4'-methoxyflavone on absorptive (AP-to-BL) or secretory (BL-to-AP) paclitaxel transport was lower than that of verapamil. The net effect (BL-to-AP - AP-to-BL) of 50 μM 5,7,4'-methoxyflavone on paclitaxel transport was comparable to that of 50 μM verapamil, although the net effect of 400 μM 5,7,4'-methoxyflavone was equivalent to that of 100 μM verapamil.

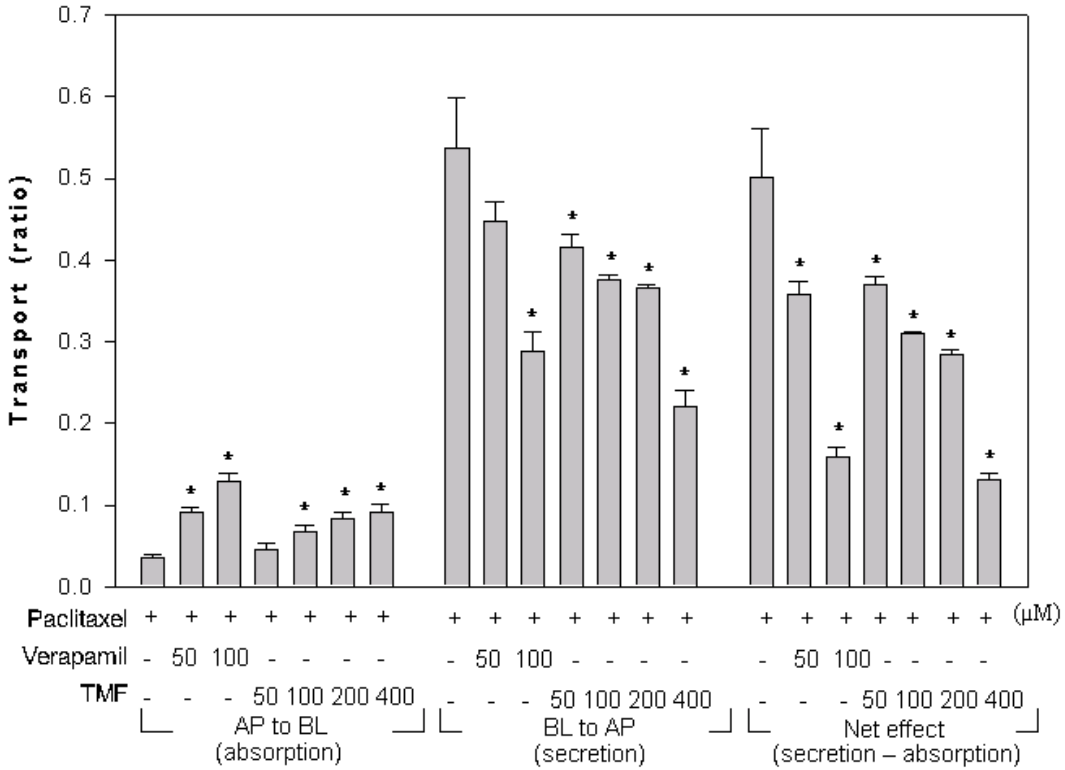


Figure 27. Effect of 5,7,4'-methoxyflavone (TMF) on paclitaxel transport across the Caco-2 monolayer. Paclitaxel (50 μM) with and without P-glycoprotein inhibitors was added to the AP or BL chamber in

DMEM (without serum). After incubating the cells with drugs for 3 hours, paclitaxel in the opposite chamber (chamber not initially loaded with paclitaxel) was extracted, and the amount was determined by HPLC. AP, Apical load; BL, Basolateral load. $^{*}P < 0.05$.

6. Effect of 5,7,4'-methoxyflavone on the sensitivity of SK-MES-1/PT4000 to paclitaxel in the Caco-2 system

To further demonstrate the usefulness of 5,7,4'-methoxyflavone in increasing the chemosensitivity of paclitaxel resistant cancer cells, we selected a paclitaxel resistant lung cancer cell subline. As shown in Figure 28, SK-MES-1/PT4000 cells overexpressing P-glycoprotein are 87-fold more resistant to paclitaxel than SK-MES-1/WT that does not express P-glycoprotein (Figure 28, insert).

I tested whether 5,7,4'-methoxyflavone could enhance the sensitivity of SK-MES-1/PT4000 cells to paclitaxel in the Caco-2 system. As shown in Figure 29A, aliquots of media were obtained from the BL chamber after both 50 μM paclitaxel and 5,7,4'-methoxyflavone were incubated in the AP chamber in order to simulate oral administration for 3 hours; these aliquots were then loaded on SK-MES-1/WT or /PT4000 cells in 96-well plates containing complete media. After a 3-day incubation, paclitaxel sensitivity was determined by MTT assay. 5,7,4'-methoxyflavone and verapamil both enhanced the sensitivity of SK-MES-1/PT4000 cells to paclitaxel compared with control (Figure 29B).

5,7,4'-methoxyflavone (200 μM) loaded in the AP chamber for 3 hours increased the sensitivity of SK-MES-1/PT4000 cells to paclitaxel to that of SK-MES-1/WT cells. Since 50 μM paclitaxel decreased 50% in the growth of SK-MES-1/WT cells (Figure 29B), the concentration of paclitaxel in the BL chamber could be

approximately estimated as 71 nM (IC_{50} value) (Figure 28).

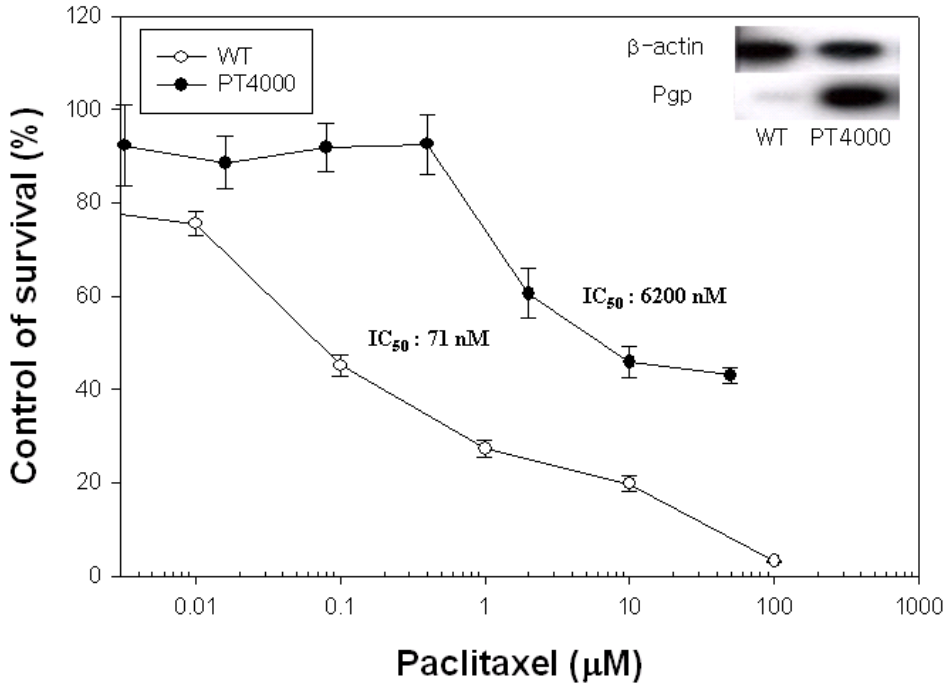


Figure 28. Sensitivity of paclitaxel in SK-MES-1 and SK-MES-1/PT4000 cells. Insert, mRNA levels; IC_{50}, Concentration of paclitaxel required for 50% inhibition of cell growth.

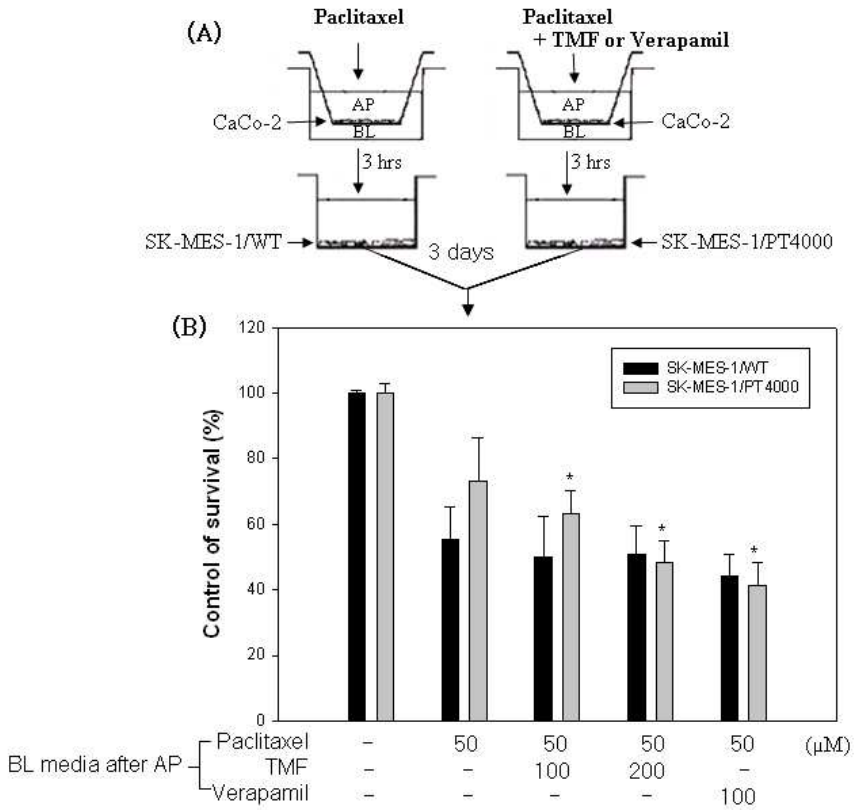


Figure 29. Effects of 5,7,4'-methoxyflavone (TMF) and verapamil on the sensitivity of SK-MES-1/PT4000 to paclitaxel in the Caco-2 system. (A) Experimental procedure: Aliquots of media were obtained from

the BL chamber after incubation with 50 μM paclitaxel and 5,7,4'-methoxyflavone (TMF) in the AP chamber for 3 hours. (B) MTT assay data: Media samples were taken from the BL chamber after the AP loading and then loaded on SK-MES-1/WT or /PT4000 cells seeded in a 96-well plate containing complete media. After 3-day incubation, paclitaxel sensitivity was determined by MTT assay. The data are means ± SD, n = 3. *P < 0.05, vs. 50 μM paclitaxel control.

Ⅳ. DISCUSSION

Flavonoids/phenolics can not only be antioxidative but also pro-oxidative, which suggests that the flavonoids can be potentially more of an oxidative risk than a benefit (Decker, 1997; Figure 30). However, the pro-oxidant effect of flavonoids may be a more important mechanism for their anticancer and apoptosis-inducing properties than their antioxidant effect (Rahman, *et al.*, 1990; Hadi, *et al.*, 2000). The antioxidant activities of *N*-acetyl-L-cysteine (NAC) and vitamin C (Vit C) showed pro-oxidant activities in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) were comparable to those of pyrrolidine dithiocarbamate (PDTC) and vitamin E (Vit E), respectively, in the DPPH assay. The former result is consistent with reports showing that Vit C or NAC behave as a prooxidant rather than an anti-oxidant (D'Agostini, 2000; Kleinveld, 1992). Since the big difference between the both assays is with or without cells, the 2',7'-dichlorofluorescein diacetate (DCFH) assay has the advantage in being able to interpret the permeability of the compounds through the plasma membrane of the cells. Thus, the DCFH assay represents the antioxidant activity of intracellular amounts permeable across the membrane whereas the DPPH assay shows antioxidant activity itself. It is therefore thought that a combination of the DPPH and

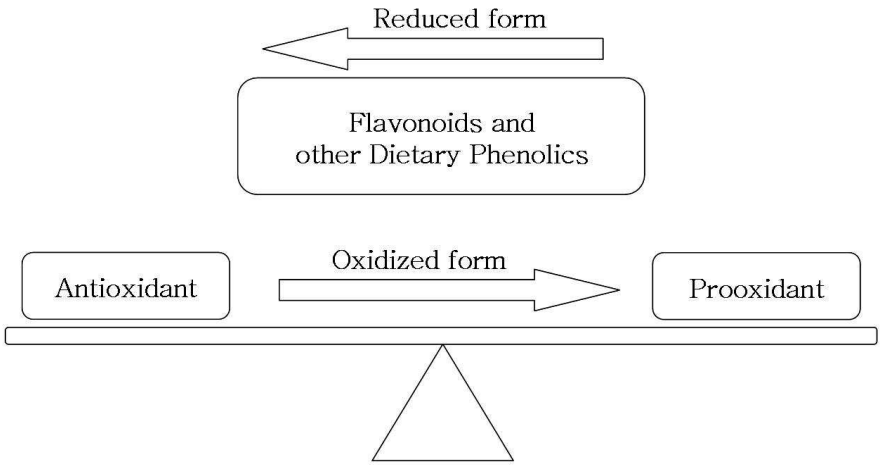


Figure 30. Diagram representing the balance between antioxidant and pro-oxidant characteristics of flavonoids and other dietary phenolics. The reduced forms of flavonoids or other dietary phenolics act as antioxidants; however, the oxidized forms (phenoxyl radicals or quinone/quinone methide intermediates) can have pro-oxidant activities (Galati and O'Brien, 2004).

DCFH methods is more effective in elucidating the differences in the antioxidant activity than that alone. The propensity of a flavonoid to inhibit free-radical mediated events is governed by its chemical structure. The number and positions of the substitutes of the flavone-based compounds can influence the radical-scavenging activity (Heim, *et al.*, 2002).

Flavonoids used in this study are characterized by various combinations of hydroxyl and/or methoxy groups. 3,5,7,3',4'-hydroxylflavone (F11) showing the highest antioxidant activity was a just addition of 3-hydroxyl to 5,7,3',4'-hydroxylflavone (F10), suggesting an important role of the number of hydroxyl and/or 3-hydroxyl groups of the ring B in the antioxidant activity of flavonoid. With the exception of 3,7-hydroxyl-3',4'-methoxy-flavone (F4) with two hydroxyl groups, antioxidant activities of flavonoids (F9-F11) with more than three hydroxyl groups showed higher than those of other flavonoids, suggesting more positive roles of hydroxyl groups in the antioxidant activity than methoxy groups. These results are consistent with reports showing that multiple hydroxyl groups on the molecule have a substantial antioxidant activity and that methoxy groups introduce unfavorable steric effects and increased lipophilicity and membrane partitioning (Heim, *et al.*, 2002). However, 3,7-hydroxyl-3',4'-methoxyflavone (F4) with 3,7-hydroxyl and 3',4'-methoxy groups showed lower antioxidant activity than two analogues, 7,3',4'-methoxyflavone (F5) and 5,6,7,3',4',5'-methoxyflvone (F6)

without hydroxyl groups did.

These results suggest that hydroxyl groups are not always related to the favorable antioxidant activity. The structure-activity relationships combining cytotoxicity with chemosensitization was created as follows. Firstly, flavonoids with the increased number of hydroxyl groups show the higher cytotoxicity. Secondarily, flavonoids with 3'-methoxy and/or 5'-methoxy groups play positive but 6-methoxy group negative roles in the P-glycoprotein inhibition. The latter suggests that 6-methoxy group might provide steric hindrance or steric repulsion by the walls of the P-gp pocket. This hypothesis could be supported not only by the finding that the CI values of three flavonoids (3,6,3',4'-methoxyflavone (F1), 5,6,7,3',4'-methoxyflavone (F3) and 5,6,7,3',4',5'-methoxyflvone (F6) with 6-methoxy group are less than those of tree flavonoids (5,7,3',4',5'-methoxyflavone (F2), 7,3',4'-methoxyflavone (F5), and 3',4'-methoxyflavone (F8)) without 6-methoxy group but also by a report that extensions at position 6 of ring A prevent the flavonoid from binding to the ATP site of P-glycoprotein (De Wet, *et al.*, 2001). On the basis of the above structure-activity relationships, flavonoids containing 3'-methoxy and/or 5'-methoxy groups without hydroxyl and 6-methoxy group include 5,7,3',4',5'-methoxyflavone (F2), 7,3',4'-methoxyflavone (F5), and 3',4'-methoxyflavone (F8), which are consistent with their high chemosensitization indexes. Finally, there was inverse relation between P-glycoprotein

inhibition and antioxidant activity (Figure 8).

Taken together, these structure-activity relationships studies indicate that the hydroxyl derivatives of the flavonoids are more likely to have higher antioxidant and cytotoxic effects than their methoxy counterparts, and vise versa with respect to the chemosensitizing effect although there are a few exceptions. It is believed that these structure-activity relationships results can be taken into account for the development of flavonoids with high therapeutic index.

Oral paclitaxel administration is preferred because this administration route is convenient to patients, reduces administration costs and facilitates the use of more chronic treatment regimens (Malingre, *et al.*, 2001). Nevertheless, oral paclitaxel administration is not recommended due to low oral bioavailability. I searched for an ideal enhancer of paclitaxel bioavailability. Flavonoids are of interest in this regard because of their pharmacological advantages including their P-glycoprotein inhibitory activity and low toxicity. I screened various hydroxyl and/or methoxy flavonoids for P-glycoprotein inhibition before selecting 7,3',4'-methoxyflavone as an enhancer of paclitaxel bioavailability. In this study, the BL-to-AP transport of paclitaxel was more than 10-fold greater than its AP-to-BL transport. This finding is consistent with the previous report that paclitaxel transport from the BL to the AP side was 4-10 times greater than that from the AP to the BL side (Walle and Walle, 1998).

We observed that both 7,3',4'-methoxyflavone and verapamil increased AP-to-BL paclitaxel transport, and decreased its BL-to-AP transport; this decreased the net effect (secretory - absorptive transport) of paclitaxel transport. P-glycoprotein inhibition had previously been shown to decrease the BL-to-AP paclitaxel transport and increase the AP-to-BL transport (Xiao, *et al.*, 2005).

Therefore enhances paclitaxel transport to the BL side, at least in part, by inhibiting P-glycoprotein, which is located in the brush border membrane. Although involvement of other transporters and cytochrome P-450 was not ruled out in this study, it is demonstrated that methylated flavones are superior to unmethylated analogues with respect to metabolic stability and transport (Wen and Walle, 2006). The net effect of 7,3',4'-methoxy-flavone on paclitaxel transport was less than that of verapamil. However, considering the low toxicity of 7,3',4'-methoxyflavone compared with that of verapamil, we could increase 7,3',4'-methoxy-flavone dose to further enhance paclitaxel biovailability. Unexpectedly, 5,7,3',4',5'-methoxyflavone, which had the highest chemosensitizing effect, did not inhibit BL-to-AP paclitaxel transport (data not shown), whose mechanisms remain to be determined.

Taken together, these results indicate that 7,3',4'-methoxyflavone, with its low toxicity, can be used not only as a P-glycoprotein inhibitor in patients with cancer cells that overexpress

P-glycoprotein, but also as an enhancer of oral paclitaxel bioavailability.

V. SUMMERY

Flavonoids have been used as antioxidants, chemopreventive or anticancer drugs and chemosensitizers. Flavonoids are considered to be a new class of chemosensitizers that interact with the cytosolic domains of P-glycoprotein as well as its ATP binding site. The current clinical formulation of paclitaxel is an important anti-cancer agent. Paclitaxel has poor bioavailability because of its high affinity for the membrane transporter P-glycoprotein (P-gp) in the gastrointestinal tract. In this study, eleven flavonoids containing hydroxyl (OH) and/or methoxy (OMe) groups along with four well-known antioxidants were compared for their *in vitro* antioxidant activity, cytotoxic and chemosensitizing effects and then their structure-activity relationships (SAR) were discussed. Thus, I screened flavonoids for P-glycoprotein inhibition and then tested whether they could not only enhance paclitaxel bioavailability in the Caco-2 system, but also increase the sensitivity of P-glycoprotein-overexpressing cancer cells to paclitaxel.

The antioxidant activity of 3,5,7,3',4'-hydroxylflavone was higher than that of 5,7,3',4'-hydroxylflavone. Flavonoids substituted with the various number of methoxy group decreased antioxidant activity. Flavonoids with 7-hydroxyl or 5,7-hydroxyl groups have

the highest cytotoxicity, and flavonoids with 5,7-methoxy group intermediate cytotoxicity. The IC_{50} values of 5,7,3',4',5'-methoxy-flavone and 5,7,4'-methoxyflavone, 0.4 μM and 1.4 μM. The IC_{50} values of 5,6,7,3',4'-methoxyflavone and 5,6,7,3',4',5'-methoxy-flavone, 3.2 μM and 0.9 μM, respectively, and those of 5,6,7,3',4',5'-methoxyflavone and 5,7,3',4',5'-methoxyflavone were 0.9 μM and 3.2 μM, respectively. Chemosensitizing indeces of 7,3',4'-trimethoxyflavone and verapamil was > 333 and 152, respectively. The basolateral (BL)-to-apical (AP) transport of paclitaxel was more than 10-fold greater than its AP-to-BL transport. 7,3',4'-trimethoxyflavone and verapamil increased the AP-to-BL transport of paclitaxel but decreased its BL-to-AP transport in a concentration-dependent manner. The net absorptive effect of 50 μM 7,3',4'-trimethoxyflavone on paclitaxel transport was comparable to that of 50 μM verapamil. In addition, AP loading of 7,3',4'-trimethoxyflavone increased the paclitaxel sensitivity of paclitaxel resistant SK-MES-1/PT4000 cells overexpressing P-glycoprotein on the BL side.

These results suggest that flavonoids with 3-hydroxyl group play a positive role in antioxidant activities, flavonoids with 5-hydroxyl and/or 7-hydroxyl groups show the higher cytotoxicity, and flavonoids with 3'-methoxy and/or 5'-methoxy groups play positive but 6-methoxy group negative roles in the P-glycoprotein inhibition. 7,3',4'-trimethoxyflavone with low toxicity can be used as an enhancer of oral paclitaxel bioavailability and as a

P-glycoprotein inhibitor.

REFERENCES

Agullo, G., Gamet-Payrastre, L., Maneti, S., Viala, C., Remesy, C., and Chap, H. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. Biochem. Pharmacol., **53**: 1649-1657, 1997.

Barry, H., Antonia, M., Chirico, S., and Okezie, L. A. Free radicals and antioxidants in food and in vitro: What tey do and how they work. Critical Reviews in food Science and Nutrition, **45**: 7-20 1995.

Bell, G. I., Najarian, R. C., Mullenbach, G. T., and Hallewell, R. A. cDNA sequence coding for human kidney catalase. Nucleic Acids Res., **14**: 5561-5562, 1986.

Bonnefoy, M., Drai, J., and Kostka, T. Antioxidants to slow aging, facts and perspectives. Presse. Med., **31**: 1174-1184, 2002.

Bromberg, L. and Alakhov, V. Effects of polyether-modified poly (acrylic acid) microgels on doxorubicin transport in human intestinal epithelial Caco-2 cell layers. J. Control Release, **88**: 11-22, 2003.

Brown, J. P. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. Mutat. Res., **75**: 243-277, 1980.

Casagrande, F and Darbon, J. M. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependent kinases CDK2 and CDK1. Biochem. Pharmacol., **61**: 1205-1215, 2001.

Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell, **47**: 381-389, 1986.

Choi, C. H. ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. Cancer Cell Int., **5**: 30, 2005.

Choi, C. H., Sun, K. H., An, C. S., Yoo, J. C., Hahm, K. S., Lee, I. H., Sohng, J. K., and Kim, Y. C. Reversal of P-glycoprotein-mediated multidrug resistance by 5,6,7,3',4'-pentamethoxyflavone (Sinensetin). Biochem. Biophys. Res. Commun., **295**: 832-840, 2002.

Choi, J. A., Kim, J. Y., Lee, J. Y., Kang, C. M., Kwon, H. J., Yoo, Y. D., Kim, T. W., Lee, Y. S., and Lee, S. J. Induction of

cell cycle arrest and apoptosis in human breast cancer cells by quercetin. Int. J. Oncol., **19**: 837-844, 2001.

Clare, D. A., Duong, M. N., Darr, D., Archibald, F., and Fridovich, I. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem., **140**: 532-537, 1984.

Cole, S. P., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M., and Deeley, R. G. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res., **54**: 5902-5910, 1994.

Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J. M., Barron, D., and Di Pietro, A. Flavonoids: A class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. Proc. Natl. Acad. Sci. USA, **95**: 9831-9836, 1998.

Constantinou, A. I., Kamath, N., and Murley, J. S. Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. Eur. J. Cancer, **34**: 1927-1934, 1998.

Cushnie, T. P. and Andrew, J. L. Antimicrobial activity of flavonoids. Int. J. Antimicrobial Agents, **26**: 343-356, 2005.

D'Agostini, F., Balansky, R. M., Camoirano, A., and De Flora, S. Interactions between Nacetylcysteine and ascorbic acid in modulating mutagenesis and carcinogenesis. Int, J, Cancer, **88**: 702-707, 2000.

De Wet, H., McIntosh, D. B., Conseil, G., Baubichon-Cortay, H., Krell, T., Jault, J. M., Daskiewicz, J. B., Barron, D., and Di Pietro, A. Sequence requirements of the ATP-binding site within the C-terminal nucleotide-binding domain of mouse P-glycoprotein: Structureactivity relationships for flavonoid binding. Biochemistry, **40**: 10382-10391, 2001.

Decker, E. A. Phenolics: Prooxidants or antioxidants? Nutr. Rev., **55**: 396-398, 1997.

Deutsch, H. M., Glinski, J. A., Hernandez, M., Haugwitz, R. D., Narayanan, V. L., Suffness, M., and Zalkow, L. H. Synthesis of congeners and prodrugs. 3. Water-soluble prodrugs of taxol with potent antitumor activity. J. Med. Chem., **32**: 788-792, 1989.

Di Pietro, A., Conseil, G., Perez-Victoria, J. M., Dayan, G., Baubichon-Cortaya, H., Trompiera, D., Steinfels, E., Jault, J. M., De Wet, H., Maitrejean, M., Comte, G., Boumendjel, A., Mariotte, A. M., Dumontet, C., McIntosh, D. B., Goffeau, A., Castanys, S., Gamarro, F., and Barron, D. Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. Cell Mol. Life Sci., **59**: 307-322, 2002.

Di Pietro, A., Dayan, G., Conseil, G., Steinfels, E., Krell, T., Trompier, D., Baubichon-Cortay, H., and Jault, J. P-glycoprotein-mediated resistance to chemotherapy in cancer cells: Using recombinant cytosolic domains to establish structure-function relationships. Braz. J. Med. Biol. Res., **32**: 925-939, 1999.

Donaldson, K. L., Goolsby G. L., KienerP. A., and Wahl. A. F. Activation of p34cdc2 coincident with taxol-induced apoptosis. Cell Growth Differ., **5**: 1041-1050, 1994.

Dorr, R. T. Pharmacology and toxicology of Cremophor EL diluent. Ann. Pharmacother, **28**: 11-14, 1994.

Duraj, J., Zazrivcova, K., Bodo, J., Sulikova, M., and Sedlak, J. Flavonoid quercetin, but not apigenin or luteolin, induced apoptosis in human myeloid leukemia cells and their resistant variants. Neoplasma, **52**: 273-279, 2005.

Duthie, S. J. and Dobson, V. L. Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro. Eur. J. Nutr., **38**: 28-34, 1999.

Galati, G. and O'Brien, P. J. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. Free Radic. Biol. Med., **37**: 287-303, 2004.

Gerritsen, M. E. Flavonoids: Inhibitors of cytokine induced gene expression. Adv. Exp. Med. Biol., **439**: 183-190, 1998.

Hadi, S. M., Asad, S. F., Singh, S., and Ahmad, A. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. IUBMB Life, **50**: 167-171, 2000.

Halliwell, B., and Gutteridge, J. M. C. Oxygen toxicity oxygen radicals, transition metals and disease. Biochem. J., **219**: 1-14, 1984.

Harborne, J. B. and Williams, C. A. Advances in flavonoid research since 1992. Phytochemistry, **55**: 481-504, 2000.

Havsteen, B. Flavonoids, a class of natural products of high pharmacological potency. Biochem. Pharmacol., **32**: 1141-1148, 1983.

Heim, K. E., Tagliaferro, A. R., and Bobilya, D. J. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem., **13**: 572-584, 2002.

Keiicchi, M., Yukio, M., Masahiro, K., Midori, H., and Akitane, M. The application of ESR sin-trapping thechnique to the evaluation of SOD-like activity of biological substances. Bull. Chem. Soc. Jpn., **63**: 187-191, 1990.

Kim, H. S., Lee, T. B., and Choi, C. H. Downregulation of catalase gene expression in the doxorubicin-resistant AML subline AML-2/DX100. Biochem. Biophys. Res. Commun., **281**: 109-114, 2001.

Kioka, M., Hosokawa, N., Komano, T., Hirayoshi, K., Nagata, K., and Ueda, K. Quercetin, a bioflavonoid, inhibits the increase of human multidrug resistance gene (MDR1) expression caused by arsenite. FEBS Lett., **301**: 307-309, 1992.

Kleinveld, H. A., Demacker, P. N., and Stalenhoef, A. F. Failure of N-acetylcysteine to reduce low-density lipoprotein oxidizability in healthy subjects. Eur. J. Clin. Pharmacol., **43**: 639-642, 1992.

Kohen, R. and Nyska, A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol., **30**: 620-650, 2002.

Korkina, L. G. and Afanas'ev, I. B. Antioxidant and chelating properties of flavonoids. Adv. Pharmacol., **38**: 151-163, 1997.

Le Marchand, L., Murphy, S. P., Hankin, J. H., Wilkens, L. R., and Kolonel, L. N. Intake of flavonoids and lung cancer. J. Natl. Cancer Inst., **92**: 154-160, 2000.

LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. Evaluation of

the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol., **5**: 227-231, 1992.

Lee, H. J. Antioxidant activity of flavonoids and wine on free radicals. The Catholic University, 5-7, 1998.

Lowe, J., Li, H., Downing, K. H., and Nogales, E. Refined structure of αβ-tubulin at 3.5A resolution. J. Mol. Biol., **313**: 1045-1057, 2001.

Maliepaard, M., A. F. Van Gastelen, M. A., De Jong, L. A., Pluim, D., Van Waardenburg, R. C., Ruevekamp-Helmers, M. C., Floot, B. G., and Schellens, J. H. Overexpression of the BCRP/MXR/ABCP gene in a topotecanselected ovarian tumor cell line. Cancer Res., **59**: 4559-4563, 1999.

Malingre, M. M., Beijnen, J. H., and Schellens, J. H. Oral delivery of taxanes. Invest New Drugs, **19**: 155-162, 2001.

Meerum Terwogt, J. M., Ten Bokkel Huinink, W. W., Schellens, J. H., Schot, M., Mandjes, I. A., Zurlo, M. G., Rocchetti, M., Rosing, H., Koopman, F. J., and Beijnen, J. H. Phase I clinical and pharmacokinetic study of PNU166945, a novel water-soluble polymerconjugated prodrug of paclitaxel. Anticancer Drugs, **12**: 315-323, 2001.

Michikatsu, S., Narashimhan, R., Yumiko, S., Tetsuro, O., Masao, T., and Hirotomo O. Varietal differences in the phenolic content and superoxide raducal scavenging potential of wines from different sources. J. Agri. Food Chem., **44**: 37-41, 1996.

Middleton, J. E. and Kandaswami, C. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. Chapman & Hall, 619-652, 1994.

Middleton, J. E. and Chithan, K. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: Harborne, J. B. editor. The flavonoids: Advances in research since 1986. London, UK: Chapman and Hall; 1993.

Moushumi, L. C., Katiyar, S. K., Mohan, R. R., and Agarwal, R. A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in the SENCAR mouse skin tumorigenesis model. Cancer Res., **59**: 622-632, 1999.

Nakajima-Iijima, S., Hamada, H., Reddy, P., and Kakunaga, T. Molecular structure of the human cytoplasmic β-actin gene: Interspecies homology of sequences in the introns. Proc. Natl. Acad. Sci. USA, **82**:6133-6137, 1985.

Oza, A. M. Clinical development of P-glycoprotein modulators in oncology. Novartis Found Symp., **243**: 103-118, 2002.

Pieters, R., Huismans, D. R., Leyva, A., and Veerman, A. J. Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. Cancer Lett., **41**: 323-332, 1988.

Rahman, A., Shahabuddin, Hadi S. M., and Parish, J. H. Complexes involving quercetin, DNA and Cu (II). Carcinogenesis, **11**: 2001-2003, 1990.

Ren, W., Qiao, Z., Wang, H., Zhu, L., and Zhang, L. Flavonoids: Promising anticancer agents. Med. Res. Rev., **23**: 519-534, 2003.

Riordan, J. R. and Ling, V. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. J. Biol. Chem., **254**: 12701-12705, 1979.

Rowinsky, E. K. and Donehower, R. C. Paclitaxel (Taxol). N. Engl. J. Med., **332**: 1004-1014, 1995.

Rowinsky, E. K., Onetto, N., Canetta, R. M., and Arbuck, S. G. Taxol: The first of the taxanes, an important new class of antitumor agents. Semin Oncol., **19**: 646-662, 1992.

Rusak, G., Gutzeit, H. O., and Muller, J. L. Structurally related flavonoids with antioxidative properties differentially affect cell cycle progression and apoptosis of human acute leukemia cells.

Nutrition Research, **25**: 143-155, 2005.

Schellens, J. H., Malingre, M. M., Kruijtzer, C. M., Bardelmeijer, H. A., van Tellingen, O., Schinkel, A. H., and Beijnen, J. H. Modulation of oral bioavailability of anticancer drugs: from mouse to man. Eur. J. Pharm. Sci., **12**: 103-110, 2000.

Sen, C. K. and Packer, L. Antioxidant and redox regulation of gene transcription. FASEB J., **10**: 709-720, 1996.

Shapiro, A. B. and Ling, V. Effect of quercetin on Hoechst 33342 transport by purified and reconstituted P-glycoprotein. Biochem. Pharmacol., **53**: 587-596, 1997.

Sharma, A., Sharma, U. S., and Straubinger, R. M. Paclitaxel-liposomes for intracavitary therapy of intraperitoneal P388 leukemia. Cancer Lett., **107**: 265-272, 1996.

Sparreboom, A., van Asperen, J., Mayer, U., Schinkel, A. H., Smit, J. W., Meijer, D. K., Borst, P., Nooijen, W. J., Beijnen, J. H., and van Tellingen, O. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc. Natl. Acad. Sci. USA, **94**: 2031-2035, 1997.

Srinivasan, P., Vadhanam, M. V., Arif, J. M., and Gupta, R. C. A rapid screening assay for antioxidant potential of natural and

synthetic agents in vitro. Int. J. Oncol., **20**: 983-986, 2002.

Tobi, S. E., Gilbert, M., Paul, N., and McMillan, T. J. The green tea polyphenol, epigallocatechin-3-gallate, protects against the oxidative cellular and genotoxic damage of UVA radiation. Int. J. Cancer, **102**: 439-444, 2002.

Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA, **76**: 4350-4354, 1979.

Tsyrlov, I. B., Mikhailenko, V. M., and Gelboin, H. V. Isozyme- and species-specific susceptibility of cDNA-expressed CYP1A P-450s to different flavonoids. Biochim. Biophys. Acta., **1205**: 325-335, 1994.

Van Asperen, J., Van Tellingen, O., Sparreboom, A., Schinkel, A. H., Borst, P., Nooijen, W. J., and Beijnen, J. H. Enhanced oral bioavailability of paclitaxel in mice treated with the P-glycoprotein blocker SDZ PSC 833. Br. J. Cancer, **76**: 1181-1183, 1997.

Wacher, V. J., Silverman, J. A., Zhang, Y., and Benet, L. Z. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. J. Pharm. Sci., **87**: 1322-1330, 1998.

Walle, U. K. and Walle, T. Taxol transport by human intestinal epithelial Caco-2 cells. Drug Metab. Dispos., **26**: 343-346, 1998.

Wang, I. K., Lin-Shiau, S. Y., and Lin, J. K. Induction of apoptosis by apigenin related flavonoids trough cytochrome crelease and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur. J. Cancer **35**: 1517-1525, 1999.

Wen X. and Walle T. Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. Drug Metab. Dispos., **34**: 1786-1792, 2006.

Willey, T. A., Bekos, E. J., Gaver, R. C., Duncan, G. F., Tay, L. K., Beijnen, J. H., and Farmen, R. H. High-performance liquid chromatographic procedure for the quantitative determination of paclitaxel (Taxol) in human plasma. J. Chromatogr., **621**: 231-238, 1993.

Xiao, J. J., Foraker, A. B., Swaan, P. W., Liu, S., Huang, Y., Dai, Z., Chen, J., Sadee, W., Byrd, J., Marcucci, G., and Chan, K. K. Efflux of depsipeptide FK228 (FR901228, NSC-630176) is mediated by P-glycoprotein and multidrug resistance-associated protein 1. J. Pharmacol. Exp. Ther., **313**: 268-276, 2005.

Zi, X., Feyes, D. K., and Agarwal, R. Anticarcinogenic effect of a flavonoid antioxidant, silymarin, in human breast cancer cells MDA-MB 468: Induction of G1 arrest through an increase in

Cip1/p21 concomitant with a decrease in kinase activity of cyclin-dependent kinases and associated cyclins. Clin. Cancer Res., **4**: 1055-1064, 1998.

감사의 글

박사과정 동안 부족한 저에게 많은 배움과 삶의 지식을 일깨워주신 최철희 지도교수님께 진심으로 감사드립니다. 학위과정동안 많은 도움을 주신 이인화 교수님께 진심으로 감사드립니다. 저에게 항상 많은 도움과 조언을 해주신 강수경 교수께 감사드립니다. 바쁘신 와중에도 논문 지도를 통해 좋은 지식과 정보를 주신 조훈 교수님께 감사드립니다. 실험을 하면서 저에게 많은 지식과 방법을 가르쳐주신 윤지수 교수님께 감사드립니다.

박사과정동안 많은 지식과 도움을 주신 여러 내성세포 연구센터 교수님과 약리학교실 임동윤 교수님, 경북대학교 김상현 교수님께 감사드립니다. 옆에서 많은 조언과 큰 힘이 되어준 분자약리학 실험실의 이태범 실장님, 박현 선생님, 이지윤 선생님, 김용일 선생님, 장미선 조교 선생님, 내성세포연구센터 최정은 선생님 감사합니다. 약리학 교실에서부터 인연을 맺어 항상 지치고 힘들 때마다 위로해주고 도와준 서윤경, 신혜경, 조현주, 김수현 선생님, 이도영, 박아연, 이민영 님께 감사드립니다.

항상 격려해주시고 힘이 되어 주시는 광주여자대학교 김한식 교수님, 나명석 교수님께 감사드립니다. 미용과학과 대학원 선생님들께 감사드립니다. 조우아, 송지원, 이정미, 윤서영, 최슬기, 김인아, 고은숙 님께 감사드립니다.

삶을 살면서 늘 행복지만은 않듯이 저에게도 가끔은 힘들고 고통스러운 날들이 있었습니다. 그때마다 좌절하거나 포기하지 않았던 것은 많은 사람들의 위로와 격려, 사랑 때문 이었던 것 같습니다.

내가 어떤 선택을 하든 항상 나를 믿어주고 힘이 되어주는 나의 가족에게 감사의 마음을 전합니다.

저작물 이용 허락서

학 과	바이오신약 개발학과	학 번	20057615	과 정	석사, 박사
성 명	한글: 정 주 미 한문: 鄭 珠 美 영문: Jeong Ju-Mi				
주 소	경기도 안산시 상록구 수암동 491-17				
연락처	E-MAIL : viyella@nate.com				
논문제목	한글 : 플라보노이드 치환체의 항산화, 화학감작 및 생체 이용율 증진 활성에 대한 효과 영문 : Effects of Flavonoid Substituents on Antioxidant, Chemosensitizing and Bioavailability-Enhancing Activities				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 조건부 동의( ) 반대( )

2008 년 2 월 일

저작자: 정 주 미 (서명 또는 인)

조선대학교 총장 귀하