Screening for Chemosensitizers from Natural Plant Extracts through the Inhibition Mechanism of P-glycoprotein

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ABSTRACT − P-gp plays a critical role in drug disposition and represents a mechanism for the development of multidrug resistance. Flavonoids, a major class of natural compounds widely present in foods and herbal products, have been shown to inhibit P-gp. Therefore, the aim of this study was to identify new candidate chemosensitizers by screening various plant extracts. The ability of natural plant extracts to inhibit P-gp activity was assessed by measuring cellular accumulation of calcein AM, daunorubicin and vincristine in P-gp overexpressing MDCKII-MDR1 cells. Among more than 800 plant extracts, eight were found to inhibit P-gp activity. *Curcuma aromatica* extract produced greatest inhibition, followed by *Curcuma longa* and *Dalbergia odorifera* extracts. Extracts of *Aloe ferox*, *Curcuma zedoariae rhizome*, *Zanthoxylum planispinum*, and *Ageratum conyzoides* showed moderate inhibitory effects. Curcumin and quercetin exhibited similar inhibition of P-gp-mediated efflux of daunorubicin and vincristine, and flavones had a lesser effect. When chemosensitizing effect was evaluated by measuring daunorubicin sensitivity to MDCKII-MDR1 cells in the presence of natural plant extracts, *Curcuma aromatica* showed the most potent chemosensitizing effect based on daunorubicin cytotoxicity. In conclusion, natural plant extracts such as *Curcuma aromatica* can potently inhibit P-gp activity and may have potential as a novel chemosensitizers.

Key words − Natural plant extracts, *Curcuma aromatica*, P-gp inhibitors, Chemosensitizer

Multidrug resistance (MDR) can develop in tumor cells that have been exposed to a cytotoxic agent. This cross-resistance of tumor cells to a range of structurally and functionally unrelated compounds present a significant obstacle to successful chemotherapy for many cancers (Thomas and Coley, 2003). Strategies for circumventing MDR include the administration of combination therapy with MDR inhibitors and the development of new anticancer drugs that are not susceptible to MDR (Tan et al., 2000; Efferth et al., 2002).

P-glycoprotein (P-gp) is an ATP-dependent efflux pump responsible for the efflux of anticancer drugs from inside of the cells, and P-gp overexpression is reportedly a major molecular mechanism underlying the development of MDR (Bansal et al., 2009). Accordingly, many P-gp inhibitors such as valspodar (PSC833), tariquidar (XR9576), zosuquidar (LY335979) and elacridar (GF120918) have been extensively studied as MDR modulators.

Three generations of P-gp inhibitors have been developed, and they vary in specificity or affinity for P-gp (Bansal et al., 2009; Krishna and Mayer, 2000). First generation P-gp inhibitors are pharmacological compounds, which have been used originally for other indications but shown to inhibit P-gp function. These compounds, including verapamil, quinidine and cyclosporin A (CsA), tend to be non-selective and less potent, and their use is generally limited by toxicity at the high serum concentrations required to effectively inhibit P-gp (Dantzig et al., 2003). Second generation P-gp inhibitors have higher affinities for P-gp and thus avoid the non-specific effects of the first-generation compounds. Examples of these compounds include valspodar (PSC833) and dexverapamil. Although these compounds were developed with the aim of being less toxic than the first generation drugs, most second generation modulators are also cytochrome P450 3A4 (CYP3A4) substrates and often result in unpredictable pharmacokinetic interactions affecting the metabolism and/or clearance of coadministered drugs (Liscovitch and Lavie, 2002). Structure-activity relationships and combinational chemistry approaches have guided the development of novel third-generation P-gp inhibitors. These compounds have high affinity for P-gp, are not metabolized by CYP3A4, and do not alter the pharmacokinetics of co-administered anticancer drugs (Bansal et al., 2009). Clinical trials using third-generation P-gp inhibitors such as tariquidar, alacridar and zosuquidar have produced encouraging results in phase I and II studies with anticancer drugs. However, phase III trials of some of these agents have been unsuccessful, and no significant survival benefit of P-gp

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inhibition has yet been demonstrated (Liscovitch and Lavie, 2002; Ozben, 2006).

The first report documenting the effects of P-gp inhibition described the interactions of grapefruit juice with a variety of drugs (Bailey et al., 1991). Since then, both beneficial and harmful pharmacokinetic interactions have been attributed to herbal supplements (Marchetti et al., 2007; Zhou et al., 2004). Among the potentially beneficial effects is the improvement of pharmacokinetic profiles of coadministered drugs through enhanced bioavailability, increased tissue accumulation, decreased excretion clearance, or modulation of MDR. In recent years, various pharmaceutical excipients and herbal supplements have emerged as potential alternatives to currently used P-gp inhibitors (Deferme and Augustijns, 2003; Deferme et al., 2002; Zhou et al., 2004). The safety of many herbs has been demonstrated by a long and continuous history of dietary use, making some herbal supplements ideal candidates for MDR modulation.

Thus, to identify new candidate chemosensitizers, we screened more than 800 natural plant extracts used in Korea and China for P-gp inhibition by examining cellular accumulation of calcein AM in P-gp overexpressing cells and evaluated chemosensitizing effect of natural plant extracts using daunorubicin sensitivity to P-gp overexpressing cells.

Materials and Methods

Materials

Natural plant extracts were provided from Plant Extract Bank (http://extract.pdrc.re.kr, Daejeon, Korea). Natural plant materials were washed, shade dried and crushed. The crushed materials were extracted with 95% ethanol at 50°C. The extracts were concentrated under reduced pressure at 45°C and kept in the refrigerator.

Mardin-Darby canine kidney II (MDCKII)-mock and MDCKII-MDR1 cells were obtained from Dr. A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). Cyclosporin A (CsA), para-aminohippuric acid (PAH), quercetin, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) and calcein AM were purchased from Invitrogen (Carlsbad, CA). Curcumin and flavone were purchased from ICC Chemical Co. (New York, NY). [³H]Vincristine (9.6 Ci/mmol) and [³H]daunorubicin (4.4 Ci/mmol) were purchased from Perkin Elmer Inc. (Boston, MA).

Western blot analysis

MDCKII-mock and MDCKII-MDR1 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin–streptomycin. The cells were harvested by centrifugation at 6,000 rpm for 3 min at 4°C and cell pellets were swollen in one volume of lysis buffer [50 mM Tris-Cl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and protease inhibitor] for 10 min. Aliquots that contained 30 µg of protein were separated by SDS-polyacrylamide gel electrophoresis in a 4-12% gradient gel (Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% nonfat milk and probed with anti-P-gp (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-actin antibodies (Cell Signaling Technology, Beverly, MA). The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using the enhanced chemiluminescence system (Santa Cruz Biotechnology).

Intracellular calcein accumulation assay in MDCKII-MDR1 cells

The intracellular accumulation of calcein was measured using the cell marker reagent calcein acetoxymethyl ester (calcein AM, Sigma Aldrich). The cells were incubated with calcein AM (0.5 µM) at 37°C for 30 min in the presence of 10 µg/mL natural plant extracts, and accumulation was halted after 30 min by rapidly washing the cells three times with ice-cold PBS. The cells were then lysed by incubation with 250 µL of 1% Triton X-100 (Sigma Aldrich) in PBS for 1 h. A 200 µL aliquot of cell lysate was transferred into a 96-well plate, and the fluorescence due to intracellular calcein was measured using a Victor3 plate reader (Perkin-Elmer LAS, Waltham, MA) with excitation and emission wavelengths of 485 and 535 nm, respectively. A Bradford protein assay kit (Bio-Rad) was used to determine the amount of cellular protein in each lysate. CsA and PAH were used as a positive and a negative control, respectively, for this assay.

The effect of natural plant extracts on the cellular accumulation of daunorubicin and vincristine in MDCKII-MDR1 cells

To screen the effect of natural plant extracts on the cellular accumulation of P-gp substrate anti-cancer drugs, daunorubicin and vincristine, 10^4 cells were seeded in 96-well plates. After the cells reached 95% confluence, the growth medium was discarded and the attached cells were washed and preincubated with Dulbecco's phosphate buffered saline (DPBS) supplemented with 20 mM glucose, 9 mM sodium bicarbon-

ate, and 25 mM Hepes for 30 min at 37°C. The uptake of [^{3}H]daunorubicin (0.1 and 1 μM) and [^{3}H]vincristine (0.1 and 1 μM) was initiated by the addition, to the cells, of medium containing 10 μg/mL natural plant extract, followed by incubation at 37°C for 30 min. The cells were washed three times with 1 mL of ice-cold DPBS immediately after placing the plates on ice. The radioactivity of [^{3}H]daunorubicin and [^{3}H]vincristine in the cells were measured by liquid scintillation counting after incubating the cells in 50 μL of cell lysis reagent (100 mM KH$_2$PO$_4$, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1% Triton X-100, and 10% glycerol, pH 7.8).

Cell viability test with natural plant extracts

Cells grown in 96 well plates (10^4 cells/well) were incubated in the absence and the presence of 5 and 10 μg/mL herbal constituents or natural plant extracts for 48 h. Then, 200 μL of MTT (0.5 mg/mL) was added to each well, and the plate was incubated for 4 h. The medium was removed and the purple formazan products were solubilized with 120 μL dimethyl sulfoxide. The cell contents were measured by the absorbance at 570 nm. Cell viability was indicated as a percentage of live cells compared with control group after the background correction.

Determination of daunorubicin sensitivity in the presence of natural plant extracts

Cells grown in 96 well plates (10^4 cells/well) were continuously exposed to various concentrations of daunorubicin (0.01-50 μM) with or without 10 μg/mL herbal constituents or natural plant extracts for 48 h. After the 48 h incubation, 200 μL of MTT (0.5 mg/mL) was added to each well, and the plate was incubated for 4 h. The medium was removed and the formazan products were solubilized with 120 μL dimethyl sulfoxide. The cell contents were measured by the absorbance at 570 nm. The IC$_{50}$ value (μM) was calculated by the Hill plot method using WinNonlin software (ver 5.1, Pharsight, Mountain View, CA).

Results

Inhibitory effect of natural plant extracts on P-gp mediated efflux

First, we characterized the expression and functionality of P-gp transporters in MDCKII-MDR1 cells. P-gp overexpression in MDCKII-MDR1 cells was confirmed by Western blot analysis as described in Methods. The expression level of P-gp in the cells was much greater than that in MDCKII-mock cells (Fig. 1). To evaluate the functionality of P-gp in MDCKII-
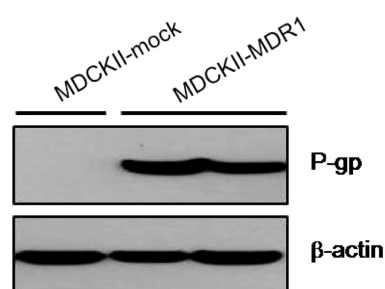

Figure 1. Western blot analysis of MDCK cells that stably express MDR1. Lane 1 represents vector-transfected control cells (MDCKII-mock). Lane 2 and 3 represent cells overexpressing P-gp (MDCKII-MDR1) and β-actin was used as a loading control.

MDR1 cells, the inhibitory effect of CsA on the P-gp mediated efflux of calcein, a representative substrate for P-gp, was measured. CsA significantly inhibited the calcein efflux and increased the cellular accumulation of calcein, whereas PAH had no effect on cellular calcein accumulation. Taken together, our MDCKII-MDR1 cell system was confirmed as appropriate for screening natural plant extracts with the potential to modulate P-gp function.

Using our system, we screened more than 800 natural plant extracts, in duplicate, for P-gp inhibition. Among these, we selected eight extracts that produced a greater than 3-fold increase in cellular calcein accumulation: *Aloe ferox, Caesalpinia sappon, Dalbergia odorifera, Curcuma zedoariae rhizome, Zanthoxylum planisinum, Ageratum conyzoides, Curcuma aromatica,* and *Culcuma longa* (Fig. 2).

We also screened the effects of three herbal constituents, curcumin, quercetin and flavones, and eight natural plant extracts on the P-gp mediated efflux of daunorubicin and vincristine, both of which are commonly prescribed anti-cancer drugs and representative P-gp substrates. As shown in Fig. 2, the most profound inhibition of P-gp activity was produced by the *Curcuma aromatica* extract, followed by *Curcuma longa* and *Dalbergia odorifera* extracts. Extracts of *Aloe ferox, Curcuma zedoariae rhizome, Zanthoxylum planispinum* and *Ageratum conyzoides* showed moderate inhibitory effects. Curcumin and quercetin exhibited similar inhibitory effects on P-gp mediated efflux, and flavones had a lesser effect compared with curcumin and quercetin (Fig. 2).

Effect of herbal constituents and plant extracts on cell viability

To confirm the safety of the herbal constituents and plant extracts in our screening system, we measured cell viability after incubation with the extracts. Cell viability was decreased in a concentration dependent manner after 48 h incubation
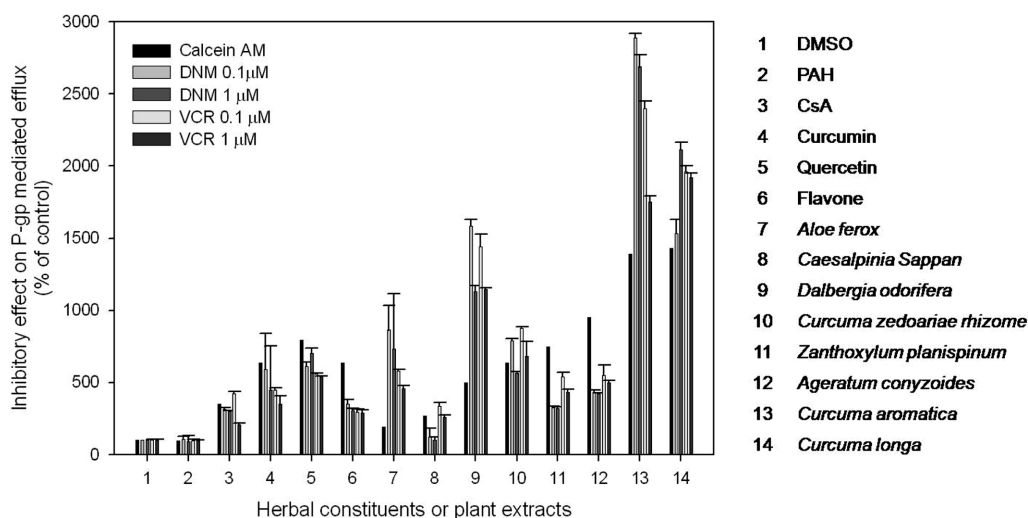
Figure 2. Cellular accumulation of 1 μM calcein AM, [³H]daunorubicin (0.1 and 1 μM), and [³H]vincristine (0.1 and 1 μM) after incubation for 30 min in the presence of 10 μg/mL 3 herbal constituents and 8 natural plant extracts. Each data point represents the mean±S.D. of three determinations.
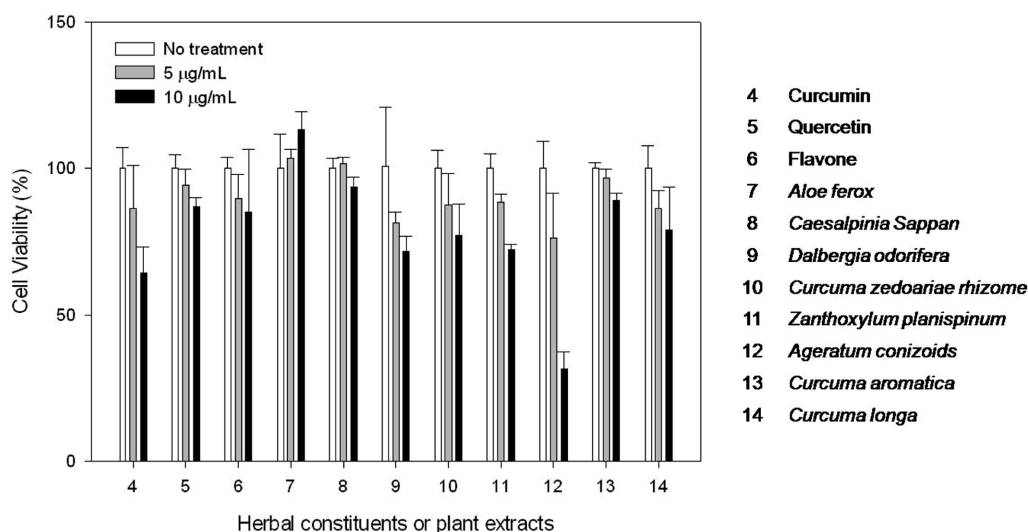

Figure 3. The effect of herbal constituent or natural plant extracts on cell viability in MDCKII-MDR1 cells. MTT assay was performed after the 48 h incubation of MDCKII-MDR1 cells (10^4 cells/well) in the absence and the presence of 5 and 10 μg/mL herb constituents or natural plant extracts.

with 5 and 10 μg/mL curcumin or *Ageratum conyzoides* extracts. Incubation with *Darbergia odorifera*, *Curcumin zedoariae rhizome* and *Zanthoxylum planispinum* extracts (10 μg/mL each) resulted in 71.6%, 77.1%, and 72.2% viability, respectively, compared with controls. Quercetin, flavone and the remaining extracts did not reduce viability by more than 20% (Fig. 3).

Taken together, these findings suggest that *Curcuma aromatica* is a promising MDR modulator, as it produced the greatest inhibition of P-gp activity on 3 different substrates, calcein, daunorubicin, and vincristine, with no significant cytotoxic effects at concentrations up to 10 μg/mL. *Dalbergia*

odorifera and *Curcuma longa* also showed significant P-gp inhibition and relatively low cytotoxicity.

Curcuma aromatica sensitizes MDCKII-MDR1 cells to daunorubicin cytotoxicity

We defined the resistance factor for daunorubicin as the ratio between IC_{50} value in P-gp overexpressing MDCKII-MDR1 cells and IC_{50} value in MDCKII-mock cells. IC_{50} values were calculated from the data in Fig. 4 by non-linear regression analysis using an inhibitory effect E_{max} model. The resistance factor for daunorubicin alone was 39.5 but decreased to 3.3 when CsA was added. These results indicated that dauno-
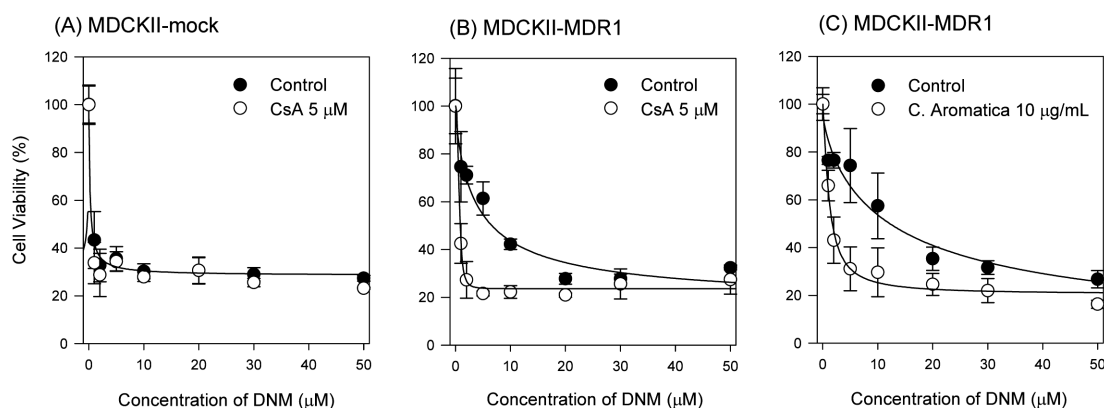
Figure 4. Chemosensitizing effect of *Curcuma aromatica* on the cytotoxicity of daunorubicin in MDCKII-mock and MDCKII-MDR1 cells. (A) Cytotoxicity of daunorubicin was measured by MTT assay in the presence and absence of 5 μM CsA in MDCKII-mock cells with a concentration range of 0.01-50 μM daunorubicin. (B) Cytotoxicity of daunorubicin was measured by MTT assay in the presence and absence of 5 μM CsA in MDCKII-MDR1 cells with a concentration range of 0.01-50 μM daunorubicin. (C) Cytotoxicity of daunorubicin was measured by MTT assay in the presence and absence of 10 μg/mL *Curcuma aromatica* in MDCKII-MDR1 cells with a concentration range of 0.01-50 μM daunorubicin.

Table I. *Changes in the Daunorubicin Sensitivity (IC_{50} values) to MDCKII-mock and MDCKII-MDR1 Cells in the Presence of P-gp Inhibitor, 5 μM CsA and 10 μg/mL Curcuma Aromatica with a Concentration Range of 0.01 - 50 μM Daunorubicin*

	IC_{50} (μM)		
	MDCKII-mock	MDCKII-MDR1	Resistant factor
Control	0.206	8.17	39.5
CsA	0.202	0.683	3.38
Fold change (sensitivity increase)	1.01	11.9	
Control		13.5	
Curcuma aromatica		1.13	
Fold change (sensitivity increase)		11.9	

Table II. *Chemosensitizing Effect of Three Herbal Constituents and Eight Natural Plant Extracts (10 μg/mL each) on the Daunorubicin Sensitivity (IC_{50} values) to MDCKII-MDR1 Cells with a Concentration Range of 0.01 - 50 μM Daunorubicin*

	IC_{50} (μM)	fold change (sensitivity increase)
Control	10.4	
Curcumin	2.48	4.19
Quercetin	5.54	1.88
Flavone	26.6	0.39
Aloe ferox	52.0	0.20
Caesalpinia sappan	14.6	0.71
Dalbergia odorifera	19.3	0.54
Curcuma zedoariae rhizome	6.65	1.57
Zanthoxylum planispin	2.33	4.48
Ageratum conyzoides	1.87	5.56
Curcuma aromatica	0.922	11.2
Curcuma longa	2.57	4.05

rubicin resistance had occurred through P-gp overexpression and that the inhibition of P-gp by CsA chemosensitized the cells to daunorubicin (Table I).

Then we determined the daunorubicin cytotoxicity in the presence of 10 μg/mL *Curcuma aromatica*, as this extracts had produced the greatest P-gp inhibition without significant cytotoxicity. We compared the effect of *Curcuma aromatica* and that of CsA, which is one of the most commonly used P-gp inhibitors (Muzi et al., 2009; Liow et al., 2007). The sensitivity of P-gp overexpressing MDCKII-MDR1 cells to daunorubicin was increased 12-fold in the presence of CsA, because the inhibitory effect of CsA on P-gp function resulted in increased intracellular accumulation of daunorubicin, thereby enhancing drug sensitivity (Fig. 4A and B). The drug sensitivity of MDCKII-mock cells to daunorubicin was not changed by

CsA. Similarly, *Curcuma aromatica* decreased the IC_{50} value for daunorubicin in MDCKII-MDR1 cells by 11.9-fold, indicating that the inhibition of P-gp by *Curcuma aromatica* sensitized MDCKII-MDR1 cells to daunorubicin by increasing cellular accumulation of this drug as a result of P-gp inhibition (Fig. 4C).

Chemosensitizing effects of herbal constituents and plant extracts

Next, we measured daunorubicin cytotoxicity in MDCKII-MDR1 cells in the presence and absence of three herbal constituents and eight plant extracts. As Shown in Table II, treat-

ment with curcumin, quercetin, and the extracts of *Xanthoxylum planispinum, Ageratum conyzoides, Curcuma aromatica* and *Curcuma longa* sensitized MDCKII-MDR1 cells to daunorubicin cytotoxicity. In contrast, flavone and extracts of *Aloe ferox* and *Dalbergia odorifera* increased resistance to daunorubicin by more than 2-fold.

Discussion

Drug resistance is a major obstacle in anti-cancer chemotherapy. Some cancers such as non-small cell lung cancer and rectal cancer are particularly resistant to initial drug treatment. On the other hand, many types of sensitive tumors respond well to chemotherapy early in treatment, but subsequently develop resistance (Choi, 2005). The transporter P-gp is thought to be responsible for 30% of MDR, making P-gp inhibition a promising strategy for overcoming MDR in cancer chemotherapy (Bansal et al., 2009).

Some flavonoids are reported to be specific inhibitors of P-gp. Flavonoids are particularly abundant in vegetables, fruits, and plant-derived beverages. Moreover, the average daily intake of total flavonoids was estimated to be more than 1 g per person in US (Zhang et al., 2004; Bansal et al., 2009). To search for similar plant products, we screened natural plant extracts for the ability to inhibit P-gp activity. Using intracellular accumulation of calcein, daunorubicin and vincristine as a marker for P-gp inhibition, we screened more than 800 plant extracts that are available in Korea and China and selected 8 for further investigation: *Aloe ferox, Caesalpinia sappon, Dalbergia odorifera, Curcuma zedoariae rhizome, Zanthoxylum planisinum, Ageratum conyzoides, Curcuma aromatica* and *Culcuma longa*. We also examined the effect of 3 herbal constituents, curcumin, quercetin and flavones, which are abundant in the extracts (Pilarski et al., 1998) and are reported to inhibit P-gp (Choi et al., 2004a; Choi et al., 2004b; Shin et al., 2006; Limtrakul, 2007; Pilarski et al., 1998). Since calcein, daunorubicin and vincristine, P-gp substrates used in the present study, are also substrates for MRP1 (Essodaigui et al., 1998; Renes et al., 1999; Versantvoort et al., 1994), the involvement of endogenous transporters (e.g. MRP1) could not be excluded although its contribution should be minimal.

Curcumin was identified from *Curcuma zedoariae rhizome, Curcuma aromatica* and *Curcuma longa* extracts using the LC/MS/MS analysis (Lee et al., 2005; Ahn et al., 2008). *Caesalpinia sappon, Dalbergia odorifera* and *Zanthoxylum planisinum* were reported to contain high content of flavonoids, based on LC/MS/MS analysis and a flavonoid database search (Lee et al., 2005; Ahn et al., 2008). Using the similarity search

of the flavonoid database, 3'-methoxy-4',5,7-trihydroxyflavone, 7,4'-dihydroxy-6-methoxyflavone, 3'-methoxyquercetin, and 5,7,4'-trihydroxy-3'-methoxyflavone were identified in *Dalbergia odorifera* extract; 2',4',6-trihydroxyflavanone and 3,3',4',7-tetrahydroxyflavone in *Caesalpinia sappon* extract; and 6,7-dimethoxy-5,3',4'-trihydroxyflavone in *Zanthoxylum planisinum* extract. *Dalbergia odorifera* extract also contained quercetin derivatives (Lee et al., 2005; Ahn et al., 2008).

Additional screening using daunorubicin and vincristine revealed that *Curcuma aromatica* extract produced the greatest P-gp inhibition and increased daunorubicin sensitivity by 11.2-fold. This chemosensitizing effect was also produced by curcumin, quercetin and extracts of *Zanthoxylum planispin, Ageratum conyzoides* and *Curcuma longa*. Surprisingly, the presence of flavone, *Aloe ferox* and *Dalbergia odorifera* increased resistance to daunorubicin more than 2-fold, which are inconsistent with intracellular accumulation data. The reason for this discrepancy is currently unknown. The increased IC_{50} values of daunorubicin produced by flavone, *Aloe ferox* and *Dalbergia odorifera* may be a result of the antioxidant and anti-carcinogenic effects of these compounds overcoming the P-gp inhibitory effect (Fawole et al., 2010; Havsteen, 2002; Zhang et al., 2004).

Notably, the chemosensitizing effect of *Curcuma aromatica* was greater than that of curcumin, even though curcumin is a major component of *Curcuma aromatica*. *Curcuma aromatica* oil was reported to show chemoprotective effects in esophageal carcinogenesis and to reduce tumor size in hepatic carcinoma (Deng et al., 2004; Li et al., 2009; Wu et al., 2000). Therefore, synergism among the components of *Curcuma aromatica* may explain the greater effect of the extract compared with purified curcumin.

In conclusion, *Curcuma aromatica* extract was the most potent P-gp inhibitor among more than 800 natural plant extracts screened in this study and suggested to be further developed as a chemosensitizer through the in vivo experiments.

Acknowledgments

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