Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components

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Abstract

In this study, the effects of extracts and flavone derivatives from the rhizome of *Kaempferia parviflora* on multidrug resistance associated-proteins (MRP)-mediated transport in A549 cells were examined. The cells employed express MRP1 and MRP2, but not P-glycoprotein. The cellular accumulation of calcein, an MRP substrate, was significantly increased by various MRP inhibitors without being affected by verapamil, a typical P-glycoprotein inhibitor. Ethanol and aqueous extracts from *Kaempferia parviflora* rhizome increased the accumulation of calcein and doxorubicin in A549 cells in a concentration-dependent manner. The inhibitory potency of the ethanol extract for MRP function was greater than that of the aqueous extract. Among six flavone derivatives isolated from *Kaempferia parviflora* rhizome, 5,7-dimethoxyflavone exhibited a maximal stimulatory effect on the accumulation of doxorubicin in A549 cells. The accumulation of doxorubicin was increased by four flavone derivatives without 5-hydroxy group, but not by the other two flavone derivatives with 5-hydroxy group. In addition, 5,7-dimethoxyflavone and 3,5,7,3’,4’-pentamethoxyflavone decreased resistance to doxorubicin in A549 cells. These findings indicate that extracts and flavone derivatives from the rhizome of *Kaempferia parviflora* suppress MRP function, and therefore may be useful as modulators of multidrug resistance in cancer cells.

**Keywords:** Multidrug resistance associated-protein; *Kaempferia parviflora*; Flavones; Multidrug resistance; A549 cells

1. Introduction
Resistance of cancer cells to chemotherapy is a serious problem of cancer treatment. P-glycoprotein, a member of the ATP-binding cassette (ABC) superfamily of drug transporters, has been reported to confer multidrug resistance to cells. Besides, the multidrug resistance-associated proteins (MRP) including MRP1, 2 and 3 (ABCC1, 2 and 3, respectively) are also involved in multidrug resistance in cancer chemotherapy (Borst et al., 1999), providing complementary and overlapping functions as drug efflux pump.

MRP1 shows relatively ubiquitous expression, whereas MRP2 and 3 are restricted mainly to the renal, intestinal and hepatic epithelia (Borst et al., 1999; Schinkel and Jonker, 2003). Since there is extensive overlap in the tissue distribution between P-glycoprotein and MRP2, it is likely that these two proteins play a cooperative role in pharmacological and toxicological protective functions, by mediating efflux of different (but partially overlapping) sets of substrates (Schinkel and Jonker, 2003; Chan et al., 2004). In view of the general properties of substrates, P-glycoprotein extrudes large hydrophobic molecules that are uncharged or positively charged, while the members of the MRP family tend to pump out both hydrophobic uncharged molecules and water-soluble anionic compounds. So far, many drugs and compounds have been reported to be substrates and/or inhibitors of MRPs. Not only many of the well-known drugs (for example, probenecid, cyclosporin A, sulfinpyrazone, etc.), but also natural products, especially flavonoids from medicinal plants, have been shown to modulate MRP-mediated transport (Nguyen et al., 2003; Schinkel and Jonker, 2003; Boumendjel et al., 2005; Morris and Zhang, 2005; van Zanden et al., 2005; Takano et al., 2006).

In Thailand, herbal medicines have been used for centuries as an integral part of Thai culture. Due to an increased public interest in alternative medicine and disease prevention, the use of herbal preparations for health maintenance has become more popular. There are so many plants that have a great potential to be used as a source of new drugs. However, many of the
possible curative properties have neither been scientifically proved nor properly investigated. *Kaempferia parviflora* Wall. ex Baker (*K. parviflora*) (local name, Krachai Dum, a member of the family Zingiberaceae) has been long used in Thai traditional medicine. The alcoholic infusion (tincture) of its rhizome has been used as a tonic drink for rectifying male impotence, body pains, relief symptoms of colic and gastrointestinal upset. Recently, it was reported that the ethanol extracts and some isolated compounds including flavonoids from this plant exert various pharmacological activities such as anti-inflammatory effects, antimicrobial activities and gastroprotective effects (Panthong et al., 1989; Panthong et al., 1994; Yenjai et al., 2004; Rujjanawate et al., 2005).

Our previous study found that ethanol extracts and several compounds from *K. parviflora* rhizome including 3,5,7,3’,4’-pentamethoxyflavone showed the inhibitory effects on P-glycoprotein function (Patanasethanont et al., 2006). Since some compounds have partial overlap in inhibitory effects on P-glycoprotein and MRP, our interest is to determine whether extracts and flavone compounds from *K. parviflora* rhizome modulate the function of MRP, in addition to P-glycoprotein.

In this study, ethanol and aqueous extracts and flavone compounds isolated from *K. parviflora* were investigated for their ability to modulate MRP function by employing A549 cells, a human lung carcinoma cell line. In addition, the effects of the flavone components on doxorubicin toxicity in A549 cells were evaluated by the XTT assay.
2. Materials and methods

2.1. Chemicals

Calcein acetoxymethyl (calcein-AM), doxorubicin hydrochloride and glibenclamide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Indomethacin, probenecid and XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide inner salt] were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Rhodamine 123 was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). All other chemicals used in the experiments were commercial products of the highest purity available.

2.2. Cell culture

A549 cells, the human lung carcinoma cell line, were obtained from Riken Cell bank (Ibaraki, Japan). A594 cells were cultured in 100 mm dish with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂-95% air. Fresh medium was replaced every 2-3 days and the cells reached confluence on the 4th day after seeding. A549 cells were subcultured every 7 days using 1 mM EDTA and 0.25% trypsin. A549 cells were used between passages 94 and 125.

2.3. Western blot analysis

For immunoblot analysis, the crude membrane fractions from A549 cells and LLC-GA5-COL150 cells (a positive control for P-glycoprotein) (Tanigawara et al., 1992) were prepared at 7 days after seeding. Briefly, after removal of the culture medium, each dish was washed with ice-
cold PBS buffer and the cells were collected with rubber policeman. The cell suspension was homogenized for 2 min with an IKA T25 Basic disperser (IKA® LABORTECHNIK, Germany) in an ice-cold buffer (150 mM NaCl, 1 mM EDTA, 1 mM PMSF with 20 mM Tris, pH 7.4), and was subsequently homogenized with a glass/Teflon Potter homogenizer with 10 strokes at 1,000 rpm. The homogenate was centrifuged at 3,000 g for 10 min at 4°C in an Avanti 30 Compact Centrifuge with rotor F0630. The supernatant was centrifuged at 40,000 g for 30 min at 4°C. The pellet was resuspended in the ice-cold buffer containing 1% (v/v) Triton X-100, and centrifuged at 14,000 g for 15 min at 4°C. The supernatant, which contains crude membrane fractions, was mixed with a loading buffer. The samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 8% polyacrylamide gels, and the proteins were transferred for 60 min to polyvinylidene difluoride membrane at 4°C. The membrane was blocked in 5% (w/v) non-fat dry milk in phosphate-buffered saline [PBS-T; 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% (v/v) Tween 20, pH7.5] overnight at 4°C. The membranes were washed three times for 10 min in PBS-T, and were incubated with MRPr1 rat monoclonal IgG₂a (for MRP1, 1:40 dilution) (PROGEN Biotechnik GmbH, Heidelberg, Germany), M₂III-6 mouse monoclonal IgG₂a (for MRP2, 1:50 dilution) (Alexis Biochemicals, San Diego, CA, USA) or C219 mouse monoclonal IgG₂a, (for P-glycoprotein, 1: 10 dilution) (Signet Laboratories, Inc., Dedham, MA, USA). The membranes were washed three times in PBS-T, and were incubated with the secondary antibody [rabbit anti-rat IgG horseradish peroxidase conjugate (dilution 1:10,000) for MRPr1; horseradish peroxidase-labeled goat antibody to mouse IgG (H+L) (1:1,000 dilution) for M₂III-6 and C219]. After the membranes were washed 3 times in PBS-T, enhanced chemiluminescence assay was performed as described by the manufacture’s protocol (ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).
2.4. Intracellular accumulation studies

Calcein-AM is a nonfluorescence and lipophilic acetoxymethyl ester of calcein that rapidly diffuses through the plasma membrane into the cells. In the cytosol, calcein-AM is metabolized by esterase to be calcein, the hydrophilic and highly fluorescent which is effluxed by MRP1 and MRP2 but not P-glycoprotein (Essodaigui et al., 1998; Evers et al., 2000). Thus, modulation of MRP-mediated efflux activity can be evaluated by measuring changes in the cellular accumulation of calcein fluorescence.

A549 cells were used at the 7th day after seeding in 24 well-plate at a density of $5 \times 10^4$ cells/well. Fresh medium was replaced every 2-3 days. Experiments were performed in Dulbecco’s phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, pH 7.4) supplemented with 5 mM D-glucose [PBS(G)]. In accumulation studies using MRP and/or P-glycoprotein inhibitors, the cells were preincubated without or with 1 mM probenecid, 100 μM indomethacin, 20 μM cyclosporin A, 100 μM glibenclamide or 100 μM verapamil for 15 min at 37°C. Then, 2 μM calcein-AM or 20 μM doxorubicin without or with each inhibitor was added, and the accumulation of calcein or doxorubicin was measured after 3 h incubation at 37°C.

The K. parviflora tincture extract, ethanol extract of K. parviflora rhizome and flavone compounds were dissolved in dimethyl sulfoxide (DMSO). The aqueous extract of K. parviflora was prepared in distilled water. The final concentration of DMSO was adjusted to 4% (v/v). After removal of the culture medium, the cells were washed twice with PBS(G) and then were preincubated with 300 μl of buffer in the absence or presence of an extract fraction (1 - 3% of the original tincture for K. parviflora tincture, 0.3 - 100 μg/ml for ethanol and aqueous extracts of K. parviflora rhizome) or each flavone compound (1 - 300 μM) at 37°C for 15 min. Then, 300 μl
of the loading solution containing either 2 μM calcein-AM or 20 μM doxorubicin without or with an extract fraction or a flavone compound was added to each well and the cells were incubated at 37°C for 1 h and 3 h, respectively. The incubation times for calcein (1 h) and doxorubicin (3 h) were determined based on the preliminary time-course studies, in which a significant increase in the accumulation of calcein was observed by coincubation with 1 mM probenecid for 1 h or longer, whereas doxorubicin accumulation was significantly increased by coincubation with 1 mM probenecid for 2 h or longer (data not shown). The transport was stopped by rinsing the cells with 1 ml of ice-cold PBS(G) twice. Cells were then solubilized with 0.1% Triton X-100 in 1 mM HEPES/Tris buffer for calcein-AM or 1% SDS in 1 mM HEPES/Tris buffer for doxorubicin. The aliquots were used to determine concentration of calcein and doxorubicin. Fluorescence of calcein and doxorubicin samples was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm by a microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). Protein content was analyzed by Bradford method (Bradford, 1976) for calcein transport study and Lowry method (Lowry et al., 1951) for doxorubicin transport study with bovine γ-globulin as a standard. The accumulations of these fluorescent probes were normalized for the protein content of the cells in each well.

2.5. High performance liquid chromatography (HPLC) analysis of flavonoid lipophilicity

To determine the lipophilicity of flavone derivatives from K. parviflora rhizome, HPLC analysis was carried out using a Jasco PU-980 Intelligent HPLC system (Jasco, Tokyo, Japan). The flavone derivatives were prepared in a concentration of 30 μM in DMSO and 50 μl was injected onto a reverse-phase TSKgel ODS-80TM column (4.6 x 100 mm, I.D.) (Tosoh, Tokyo, Japan). The isocratic mobile phase consisted of 0.1% (v/v) trichloroacetic acid and methanol.
(45:55, v/v) and the flow rate was 1 ml/min. Detection was performed by measuring absorbance at 254 nm using a Jasco UV-970 Intelligent UV/VIS detector. The lipophilicity of flavone derivatives was determined by the retention time.

2.6. Cytotoxicity assay

Cytotoxicity was determined by the XTT tetrazolium/formazan assay. This method is based on the cleavage of XTT by metabolic active cells. Briefly, A549 cells were seeded at a density of 30 x 10^4 cells in 35 mm dish, and were incubated for 24 h. Then, medium containing various concentrations of doxorubicin (0, 0.3, 1, 2, 3, 4 and 10 µM) in the absence or presence of a flavone derivative (30 µM) was replaced. The control cells were incubated with the same concentration of DMSO in each experiment (0.4-0.8%). The cells were further incubated for 48 h. After the cells were washed twice with PBS(G), 1 ml of 250 µM XTT solution containing 10 µM phenazine methosulfate (PMS) was added to each dish, and then the cells were incubated for 30 min at 37°C. After transfer of the reaction solution to a microplate well, the amount of orange formazan dye produced was quantitated using a spectrophotometric plate reader to measure absorbance at a wavelength of 490 nm. The reference absorbance (nonspecific readings) was measured at a wavelength of 650 nm. In study evaluating the concentration-dependent effect of 3,5,7,3’,4’-pentamethoxyflavone on doxorubicin-induced cytotoxicity, A549 cells were treated with various concentrations of the flavone (0, 1, 3, 10, 30 µM) in the absence or presence of 3 µM doxorubicin for 48 h, and then the XTT assay was performed as described above. The percentage of viability was calculated as follows: Viability (%) = \( \frac{(A_{490} - A_{650})_{\text{treated}}}{(A_{490} - A_{650})_{\text{control}}} \times 100 \). The half-maximal inhibitory concentration (IC\(_{50}\)) value of doxorubicin was determined by the following Hill equation,
\[ V = \frac{100}{100 + ([I]/IC_{50})^n} \]

where \([I]\) is the concentration of doxorubicin, \(V\) is the viability in the presence of doxorubicin and \(n\) is the Hill coefficient. The KaleidaGraph\textsuperscript{TM} program (Version 3.08, Synergy Software, PA, USA) was used for the curve-fitting. The IC\textsubscript{50} value was assessed from the curve-fitting to the above-mentioned equation.

2.7. Preparations of extract and isolated compounds from K. Parviflora rhizome

Black rhizomes of \textit{K. parviflora} were collected from Loei province, Thailand. The plant was authenticated by the Center of Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University. A voucher specimen (BS-47-01) of the plant material has been lodged at the Herbarium, Khon Kaen University. The fresh rhizomes were washed, dried in an oven at 45ºC until the weight remained constant, and then powdered. A tincture from rhizome of \textit{K. parviflora} was prepared as the commercial products available in Thailand. Briefly, 30 g of dried rhizome was macerated in 750 ml of 40% (v/v) ethanol and was kept for 7 days in a tight, amber container. Then, solvent of \textit{K. parviflora} tincture was evaporated to dryness in a rotary evaporator. The extract fraction was dissolved in DMSO to obtain \textit{K. parviflora} tincture extract fraction. For preparing the ethanol extract and water extract of \textit{K. parviflora} rhizome, powdered rhizome was extracted with ethanol and boiling water by Soxhlet method. The extract fractions from \textit{K. parviflora} rhizome were evaporated to dryness in a rotary evaporator and were freeze-dried. The yields of ethanol and water extract fraction were 3.44% and 0.5%, respectively. These extract fractions were stored at -20ºC until use. Six flavonoids tested in this study (5,7-dimethoxyflavone, 5,7,4’-trimethoxyflavone, 3,5,7,4’-
tetramethoxyflavone, 3,5,7,3’,4’-pentamethoxyflavone  5-hydroxy-3,7-dimethoxyflavone and 5-hydroxy-3,7,4’-trimethoxyflavone, ) were isolated from the portion of dichloromethane extract from rhizome of *K. parviflora*. These flavone derivatives were chromatographed on silica gel and were confirmed by HPLC, IR and NMR spectroscopy as described previously (Jaipetch et al., 1983; Panthong et al., 1994).

2.8. *Statistical analysis*

Statistically significant differences were determined by Student’s *t*-test, or one way analysis of variance (ANOVA) with the Tukey’s or the Scheffe’s test for post hoc analysis. A *P* value less than 0.05 was considered statistically significant.
3. Results

3.1. Immunoblotting for MRP1, MRP2 and P-glycoprotein in A549 cells

Expression of MRP 1, MRP2 and P-glycoprotein in A549 cells employed in this study was analyzed by Western blot analysis. As shown in Fig. 1, expression of MRP1 and MRP2 was observed in the cells (Fig. 1). In contrast, P-glycoprotein was not detected in A549 cells even though its protein amount for loading was more than 10 times as many as that of LLC-GA5-COL150 cells expressing P-glycoprotein (Fig. 1).

3.2. Effects of various inhibitors on accumulation of substrates for MRP and P-glycoprotein

To confirm whether MRP, but not P-glycoprotein, are functioning in A549 cells, the effects of various inhibitors of MRP and/or P-glycoprotein on the accumulation of calcein (MRP substrate) and rhodamine 123 (P-glycoprotein substrate) were examined and compared. As shown in Fig. 2A, the accumulation of calcein after 3 h incubation was significantly increased by all MRP inhibitors examined, including probenecid, indomethacin, cyclosporin A and glibenclamide, but not by a P-glycoprotein inhibitor verapamil. Like calcein, these MRP inhibitors significantly increased the accumulation of doxorubicin, another substrate for MRP, in A549 cells (1 mM probenecid, 194.1 ± 5.3; 100 μM indomethacin, 205.8 ± 4.9; 20 μM cyclosporin A, 255.6 ± 7.1; 100 μM glibenclamide, 190.6 ± 3.5 % of control; n=3). In contrast, no significant effect of verapamil on rhodamine 123 accumulation was observed (Fig. 2B), which was compatible with the above-mentioned result that no P-glycoprotein was expressed in A549 cells. Thus, it is possible to specifically analyze MRP-mediated transport by employing A549 cells used in this study.
3.3. *Effect of* *K. parviflora* tincture extract *on accumulation of calcein and doxorubicin in A549 cells*

First, the effect of *K. parviflora* extract tincture on MRP function was investigated in A549 cells. The tincture from the rhizome of *K. parviflora* was prepared as described in *Materials and methods*. The tincture extract increased the cellular accumulation of calcein and doxorubicin in A549 cell in a concentration-dependent manner (Fig. 3). Statistically significant effects on calcein and doxorubicin accumulation were observed at concentrations over 0.1 and 1% of the original tincture preparation, respectively. Thus, MRP function is more sensitive than P-glycoprotein function to *K. parviflora* tincture, which was previously shown to significantly increase the accumulation of P-glycoprotein substrates in LLC-GA5-COL150 cells at concentrations higher than 3% of the original tincture (Patanasethanont et al., 2006).

3.4. *Effects of ethanol and aqueous extracts from* *K. parviflora* rhizome *on MRP function*

We next examined the effects of ethanol and aqueous extracts from *K. parviflora* rhizome on cellular accumulation of calcein and doxorubicin in A549 cells. As shown in Fig. 4, both ethanol and aqueous extracts of *K. parviflora* increased the cellular accumulation of calcein and doxorubicin in a concentration-dependent manner. Significant effects of ethanol and aqueous extracts on calcein accumulation were observed at concentrations over 1 and 10 µg/ml, respectively. In the case of doxorubicin accumulation assay, significant effects of ethanol and aqueous extracts were observed at concentrations over 10 and 30 µg/ml, respectively. Thus, the inhibitory potency of the ethanol extract on MRP function was greater than that of the aqueous extract, as it was observed in the case of P-glycoprotein function (Patanasethanont et al., 2006).
3.5. Effects of flavone derivatives from K. parviflora rhizome on MRP function

Since many of flavonoids have been reported to potently inhibit MRP-mediated transport, especially MRP1 and MRP2 (Leslie et al., 2001; van Zanden et al., 2005; Morris and Zheng, 2006), the effects of flavone derivatives from K. parviflora rhizome on MRP function were investigated (Fig. 5). The isolated flavone derivatives examined in this study are shown in Fig. 6. These six flavone derivatives were classified into two groups according to the presence of hydroxy group at position 5, polymethoxyflavones and 5-hydroxy-polymethoxyflavones. All of four flavones in polymethoxyflavone group significantly increased the accumulation of calcein and doxorubicin in A549 cells (Figs. 5A-D). Among this group, 5,7-dimethoxyflavone most potently increased the accumulation of both MRP substrates and significant effects were observed at concentrations over 1 and 3 µM, respectively. In contrast, two flavones in 5-hydroxy-polymethoxyflavone group (Figs. 5E and F), 5-hydroxy-3,7-dimethoxyflavone and 5-hydroxyl-3,7,4’-trimethoxyflavone, had no effects on the accumulation of calcein and doxorubicin in A549 cells.

3.6. Lipophillicity of flavone derivatives from K. parviflora rhizome

The flavone derivatives from K. parviflora tested in this study were analyzed by HPLC to estimate the lipophilicity. Their lipophilicities were compared by measuring retention time of each flavone by a reverse phase column (Fig. 6). The retention times of 5,7-dimethoxyflavone, 5,7,4’-trimethoxyflavone, 3,5,7,4’-trimethoxyflavone, 3,5,7,3’,4’-pentamethoxyflavone, 5-hydroxy-3,7-dimethoxyflavone and 5-hydroxy-3,7,4’-trimethoxyflavone were 20.5 ± 1.2, 26.5 ± 1.0, 30.3 ± 0.4, 17.5 ± 0.2, 62.1 ± 1.3 and 69.1 ± 1.4 min (n=3), respectively. The 5-hydroxy-polymethoxyflavone group (5-hydroxy-3,7-dimethoxyflavone and 5-hydroxy-3,7,4’-
trimethoxyflavone) was much more lipophilic than the polymethoxyflavone group (5,7-dimethoxyflavone, 5,7,4’-trimethoxyflavone, 3,5,7,4’-trimethoxyflavone, 3,5,7,3’,4’-pentamethoxyflavone). Among these flavone derivatives, the most and the second most hydrophilic flavones were 3,5,7,3’,4’-pentamethoxyflavone and 5,7-dimethoxyflavone, respectively, which more potently increased the accumulation of MRP substrates than other flavones tested (Fig. 5).

3.7. Effects of flavone derivatives from K. parviflora rhizome on doxorubicin toxicity in A549 cells

The cytotoxic effects of doxorubicin on A549 cells in the absence or presence of flavone derivatives from K. parviflora rhizome were analyzed by the XTT assay. As shown in Fig. 7, 5,7-dimethoxyflavone (30 μM) and 3,5,7,3’,4’-pentamethoxyflavone (30 μM) increased the sensitivity of A549 cells to doxorubicin. Significant enhancements of the doxorubicin-induced cytotoxic effects by these flavones were observed at concentrations of doxorubicin over 3 μM. The IC$_{50}$ values of doxorubicin in the presence of 5,7-dimethoxyflavone and 3,5,7,3’,4’-pentamethoxyflavone were 2.66 and 2.85 μM, respectively. These IC$_{50}$ values were lower than that in the absence of the flavone (control, 9.60 μM). Moreover, 3,5,7,3’,4’-pentamethoxyflavone concentration-dependently sensitized A549 cells to the cytotoxic effect of 3 μM doxorubicin, whereas the flavone itself did not affect the viability of A549 cells at the concentration range of 1 to 30 μM (Fig. 8).
4. Discussion

In Thai traditional medicine, the rhizome of *K. parviflora*, known as the health-promoting herbs, has often been used in treatment of colic disorder and gastric ulcer. However, the scientific evidence for clinical usefulness of this plant is still limited. In a previous study, we reported the inhibitory effects of *K. parviflora* extracts and its flavone components on P-glycoprotein function. In the present study, we investigated the effects of *K. parviflora* extracts and its components on MRP–mediated transport by employing in vitro transport study using A549 cells. The protein expression of MRP1 and MRP2 was detected in A549 cells employed in this study. In contrast, no expression of P-glycoprotein was observed, consistent with some previous reports (Berger et al., 1997; Courage et al., 1997; Regina et al., 1998; Yang et al., 1998; Campbell et al., 2003). These observations allowed detailed examination of a specific modulation for MRP function, excluding involvement of P-glycoprotein.

As shown in Figs. 3 and 4, exposure to those extracts from *K parviflora* rhizome significantly increased the cellular accumulation of MRP substrates, calcein and doxorubicin, in a concentration–dependent manner. The ethanol extract decreased the MRP-mediated efflux more potently than the aqueous extract. These findings indicated that MRP-modulating compounds in the rhizome of *K parviflora* are relatively hydrophobic, which are more easily partitioned into ethanol solvent than into water solvent. As described previously, we observed that the ethanol extract significantly increased the accumulation of P-glycoprotein substrates at lower concentrations as compared with the aqueous extract (Patanasethanont et al., 2006). Therefore, it was expected that the ethanol extract from *K. parviflora* rhizome may contain components which inhibit both MRP and P-glycoprotein.
In this study, the effects of six flavone derivatives isolated from the rhizomes of *K. parviflora* on MRP function were compared. These flavone derivatives constitute a large portion of the ethanol extract from *K. parviflora* rhizome, accounting for 82.3% of the ethanol extract. The content of each flavone derivative in ethanol extract was as follows: 5,7-dimethoxyflavone, 21.0%; 5,7,4’-trimethoxyflavone, 29.8%; 3,5,7,4’-tetramethoxyflavone, 8.2%; 3,5,7,3’,4’-pentamethoxyflavone, 18.1%; 5-hydroxy-3,7-dimethoxyflavone, 2.5%; 5-hydroxy-3,7,4’-trimethoxyflavone, 2.7% of ethanol extract from *K. parviflora* rhizome. According to the presence of 5-hydroxy group, these flavone derivatives are classified into two groups; polymethoxyflavones (3,5,7,3’,4’-pentamethoxyflavone, 3,5,7,4’-tetramethoxyflavone, 5,7,4’-trimethoxyflavone, and 5,7-dimethoxyflavone) and 5-hydroxy-polymethoxyflavones (5-hydroxy-3,7,4’-trimethoxyflavone and 5-hydroxy-3,7-dimethoxyflavone). Thus, the polymethoxyflavone group consisting of four flavones constitutes a large portion of the ethanol extract (77.1%). Therefore, the four flavones in the polymethoxyflavone group may be mainly responsible for the inhibitory effect of the ethanol extract on MRP function.

From the point of view of structure-activity relationships, the structural requirements of flavonoids for MRP inhibition were investigated by van Zanden et al. (2005). They suggested that the total numbers of methoxy moieties and hydroxyl groups as well as the presence of 2,3-double bond in ring C are important factors to modulate MRP1 function, whereas the presence of a flavonol B-ring pyrogallol group is essential for modulation of MRP2 function. In our investigation, all four compounds classified in the polymethoxyflavone group showed the inhibitory effect on MRP function. On the other hand, there were no inhibitory effects of 5-hydroxy-polymethoxyflavone group on MRP function. Like on MRP, 5-hydroxy-polymethoxyflavone group had no effect on P-glycoprotein function as reported in the previous paper (Patanasethanont et al., 2006). Therefore, the absence of 5-hydroxy group in the A-ring
seems to be an important structural characteristic to modulate the functions of MRP and P-glycoprotein.

We further investigated the lipophilicity of flavone compounds from *K. parviflora* rhizome. From the result, it is likely that the lipophilicities are not related to the total number of methoxy moieties in the polymethoxyflavone group. In contrast, the presence of hydroxy group at position 5 in flavone structure dramatically increased lipophilicity (retention time > 60 min) when compared to the flavone compounds without the hydroxy group (17~30 min). Such differences in lipophilicity between polymethoxyflavone and 5-hydroxy-polymethoxyflavone groups may be related to their potencies to modulate MRP functions since most substrates and inhibitors of MRP are relatively hydrophilic.

*K. parviflora* has been used as tonic drink, a tincture formulation (local name, Ya-dong) in Thailand. Since the usual dose of *K. parviflora* tincture is 30 ml once a day before meal, intestinal concentration is estimated to be around 2% of the tincture prepared as described above, assuming human intestinal volume of 1.65 l/70 kg (Davies and Morris, 1993). The present results showed that the cellular accumulation of calcein and doxorubicin was significantly increased by *K. parviflora* tincture at a concentration of 1% or higher. In addition, experiments with in-vitro everted sac method demonstrated that *Kaempferia parviflora* tincture extract significantly decreased the efflux of an MRP2 substrate, 2,4-dinitrophenyl-S-glutathione, in rat intestine to a similar extent to 1 mM probenecid, indicating almost complete inhibition of MRP2 function (Patanasethanont et al., unpublished observation). These observations suggest that *K. parviflora* tincture may modulate MRP2 function in the intestine, leading to possible changes in oral bioavailability of drugs that are MRP2 substrates. This information should be taken into account in clinical setting, especially when patients take some drugs with *K. parviflora* tincture preparation as a dietary supplement.
As described above, *K. parviflora* tincture administered orally may have the ability to modulate MRP function in the intestine. However, the plasma concentration of flavone constituents after oral administration of the tincture would not be high enough to interact with MRP in cancer cells. Therefore, the tincture administered orally is probably not useful for overcoming MRP-mediated multidrug resistant cancer. On the other hand, an intravenous administration of a specific flavone such as 5,7-dimethoxyflavone with anticancer drugs may be the preferred way of overcoming MRP-mediated multidrug resistance. In this study, the effects of *K. parviflora* ethanol extract and its flavone components on cytotoxicity of the chemotherapeutic agent doxorubicin were assessed using XTT assay. As shown in Fig. 7, 5,7-dimethoxyflavone most potently enhanced the sensitivity of A549 cells to doxorubicin. In addition, 3,5,7,3′,4′-pentamethoxyflavone enhanced the doxorubicin-induced cytotoxicity of A549 cells in a concentration-dependent manner, without change in cell viability by the flavone alone. These findings indicate that several flavone derivatives from *K. parviflora* may be clinically useful for reversal of the MRP-mediated multidrug resistance in cancer treatment.

In conclusion, our present studies demonstrated that the ethanol extract of *K. parviflora* and some of its flavone derivatives can modulate MRP function. Furthermore, our previous studies showed that 3,5,7,3′,4′-pentamethoxyflavone may be useful for P-glycoprotein mediated multidrug resistance. Taken together, these observations suggest that the flavone derivatives from *K. parviflora* might be valuable in clinical situations as an adjunct to chemotherapy for multidrug resistant cancer abundantly expressing P-glycoprotein and MRP. However, these possible efficacies need to be examined under in vivo conditions in detail.


References


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Figure legends

**Figure 1.** Western blot analysis for MRP1 (A), MRP2 (B) and P-glycoprotein (C) in A549 cells. The indicated protein amounts of crude membranes from A549 cells and LLC-GA5-COL150 cells (a positive control for P-glycoprotein) were subjected to 8% SDS-PAGE. The proteins were blotted on a polyvinylidene difluoride membrane and detected with each primary antibody as described in *Materials and methods*.

**Figure 2.** (A) Effects of various MRP inhibitors and verapamil on accumulation of calcein in A549 cells. A549 cells were preincubated without (control) or with 1 mM probenecid (Pro), 100 μM indomethacin (IND), 20 μM cyclosporin A (CsA), 100 μM glibenclamide (Glib) or 100 μM verapamil (VRP) for 15 min at 37°C. Then, 2 μM calcein-AM in the absence or presence of each compound was added and the accumulation was measured after 3 h incubation at 37°C. Each column represents the mean ± S.E.M. of results from three to six monolayers. (B) Effect of verapamil on accumulation of rhodamine 123 in A549 cells. A549 cells were preincubated without (control, open circle) or with (closed circle) 100 μM verapamil for 15 min at 37°C. Then, 20 μM rhodamine 123 in the absence or presence of 100 μM verapamil was added and the accumulation was measured after incubation for 30, 60, 90, and 120 min at 37°C. Each symbol represents the mean ± S.E.M. of results from three monolayers. *P<0.05, significantly different from the value of control.

**Figure 3.** Effects of *Kaempferia parviflora* (*K. parviflora*) tincture extract on accumulation of calcein and doxorubicin in A549 cells. Accumulation of calcein (open circle) and doxorubicin
(closed circle) for 1 and 3 h, respectively, at 37°C was measured in the absence (control) or presence of *K. parviflora* tincture extract diluted to be equivalent to 0.1, 0.3, 1 and 3% (v/v) of the original tincture. Each symbol represents the mean ± S.E.M. of results from three monolayers. *P*<0.05, significantly different from the value of each control.

**Figure 4.** Effects of ethanol and aqueous extracts of *K. parviflora* rhizome on accumulation of calcein and doxorubicin in A549 cells. Accumulation of calcein (open circle) and doxorubicin (closed circle) for 1 and 3 h, respectively, at 37°C was measured in the absence (control) or presence of ethanol (0.3 -100 μg/ml) or aqueous (1 -100 μg/ml) extract from *K. parviflora* rhizome. Each symbol represents the mean ± S.E.M. of results from three monolayers. *P*<0.05, significantly different from the value of each control.

**Figure 5.** Effects of flavone derivatives contained in *K. parviflora* rhizome on accumulation of calcein and doxorubicin in A549 cells. Accumulation of calcein (open circle) and doxorubicin (closed circle) for 1 and 3 h, respectively, at 37°C was measured in the absence (control) or presence of a flavone derivative (1-300 μM) consisting of 5,7-dimethoxyflavone (A), 5,7,4’-trimethoxyflavone (B), 3,5,7,4’-tetramethoxyflavone (C), 3,5,7,3’,4’-pentamethoxyflavone (D), 5-hydroxy-3,7-dimethoxyflavone (E) and 5-hydroxy-3,7,4’-trimethoxyflavone (F). Each symbol represents the mean ± S.E.M. of results from three monolayers. *P*<0.05, significantly different from the value of each control.

**Figure 6.** Chemical structures of flavone derivatives contained in *K. parviflora* rhizome and their HPLC retention times. The retention times of flavone derivatives were measured as
described in Material and methods. Each value represents the mean ± S.E.M. of results from three determinations.

**Figure 7.** Effects of 5,7-dimethoxyflavone (DMF) and 3,5,7,3’,4’-pentamethoxyflavone (PMF) on viability of A549 cells determined by XTT assay. A549 cells were incubated without (control) or with various concentrations of doxorubicin (1, 2, 3, 4 and 10 μM) in the absence (open circle) or presence of 30 μM 5,7-dimethoxyflavone (closed circle) and 30 μM 3,5,7,3’,4’-pentamethoxyflavone (closed square) for 48 h. Each symbol represents the mean ± S.E.M. of results from six experiments. *P<0.05, significantly different from the value in the absence of DMF or PMF at the indicated concentration of doxorubicin.

**Figure 8.** Concentration-dependent effect of 3,5,7,3’,4’-pentamethoxyflavone on doxorubicin-induced toxicity in A549 cells. A549 cells were incubated without (control) or with various concentrations of 3,5,7,3’,4’-pentamethoxyflavone (1 - 30 μM) in the absence (open circle) or presence (closed circle) of doxorubicin (3 μM) for 48 h. Then, viability of A549 cells was determined by the XTT assay. Each symbol represents the mean ± S.E.M. of results from three monolayers. *P<0.05, significantly different from the value of each control.
Fig. 1

A

B

C

MRP1

MRP2

P-glycoprotein

A549

LLC-GA5-COL150

10 20 30 (μg)

1 3 5 (μg)

10 30 50 (μg)

4 8 16 (μg)
Fig. 2

A

Calcine accumulation in A549 cells (% of control)

B

Rhodamine 123 accumulation in A549 cells (nmol/mg protein)

* Statistically significant difference compared to control
Accumulation in A549 cells (% of control)

Concn. of *K. parviflora* tincture extract (% of the original tincture)

- **Calcein**
- **Doxorubicin**

*Fig. 3*
Fig. 4

A

Accumulation in A549 cells (% of control)

Concn. of ethanol extract (μg/ml)

B

Accumulation in A549 cells (% of control)

Concn. of aqueous extract (μg/ml)
Fig. 5

A  
[Graph showing accumulation of 5,7-dimethoxyflavone in 549 cells (% of control)]

B  
[Graph showing accumulation of 5,7,4'-trimethoxyflavone in 549 cells (% of control)]

C  
[Graph showing accumulation of 3,5,7,4'-tetramethoxyflavone in 549 cells (% of control)]

D  
[Graph showing accumulation of 3,5,7,3',4'-pentamethoxyflavone in 549 cells (% of control)]

E  
[Graph showing accumulation of 5-hydroxy-3,7-dimethoxyflavone in 549 cells (% of control)]

F  
[Graph showing accumulation of 5-hydroxy-3,7,4'-trimethoxyflavone in 549 cells (% of control)]
### Table 1: Retention times of flavones

<table>
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<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>Retention time (min)</th>
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<tr>
<td>5,7-dimethoxyflavone</td>
<td>H</td>
<td>OCH₃</td>
<td>H</td>
<td>H</td>
<td>20.5 ± 1.2</td>
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<tr>
<td>5,7,4’-trimethoxyflavone</td>
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<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>26.5 ± 1.0</td>
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<tr>
<td>3,5,7,4’-tetramethoxyflavone</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>H</td>
<td>OCH₃</td>
<td>30.3 ± 0.4</td>
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<tr>
<td>3,5,7,3’,4’-pentamethoxyflavone</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>17.5 ± 0.2</td>
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<tr>
<td>5-hydroxy-3,7-dimethoxyflavone</td>
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<td>OH</td>
<td>H</td>
<td>H</td>
<td>62.1 ± 1.3</td>
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<tr>
<td>5-hydroxy-3,7,4’-trimethoxyflavone</td>
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<td>OH</td>
<td>H</td>
<td>OCH₃</td>
<td>69.1 ± 1.4</td>
</tr>
</tbody>
</table>

Fig. 6
Fig. 8

Viability (% of control) vs. Concentration of 3,5,7,3',4'-pentamethoxyflavone (μM)

- Open circles: without doxorubicin
- Filled circles: with doxorubicin

* indicates statistical significance