Effects of *Kaempferia parviflora* extracts and their flavone constituents on P-glycoprotein function

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Shorten title: Effects of *K. parviflora* extracts on P-gp

ABSTRACT: The purpose of this study was to examine the effects of extracts and flavone derivatives from the rhizome of *Kaempferia parviflora* on P-glycoprotein (P-gp)-mediated transport in LLC-GA5-COL150, a transfectant cell line of a porcine kidney epithelial cell line LLC-PK_1 with human *MDR1* cDNA. Ethanol extract obtained from *Kaempferia parviflora* rhizome significantly increased the accumulation of rhodamine 123 and daunorubicin, P-gp substrates, in LLC-GA5-COL150 cells, but not in LLC-PK_1 cells. The aqueous extract also increased the accumulation in LLC-GA5-COL150 cells with lower potency than the ethanol extract. The effects of flavone derivatives isolated from the rhizome of *Kaempferia parviflora* on P-gp function were examined. Among six flavones tested, 3,5,7,3',4'-pentamethoxyflavone most potently increased the accumulation of rhodamine 123 and daunorubicin in LLC-GA5-COL150 cells in a concentration-dependent manner. In addition, 5,7-dimethoxyflavone to lesser degree increased rhodamine 123 accumulation in LLC-GA5-COL150 cells. In contrast, the other four flavone derivatives had no significant effect on the accumulation of rhodamine 123 in LLC-GA5-COL150 cells in a concentration range tested. These results indicate that extracts and flavone derivatives from the rhizome of *Kaempferia parviflora* can inhibit P-gp function, which may be useful for overcoming P-gp-mediated multidrug resistance and improving the oral bioavailability of anticancer agents.

Keywords: Multidrug resistance, P-glycoprotein, Cancer chemotherapy, Bioavailability, Cell culture

INTRODUCTION

Resistance of tumor cells to anticancer drugs is a serious problem in cancer chemotherapy. P-glycoprotein (P-gp), about 170 kDa in size, is a member of the ATP-binding cassette (ABC) superfamily of drug transporters, conferring multidrug resistance to cells by extruding a variety of structurally and pharmacologically diverse cancer chemotherapeutic agents using energy from ATP hydrolysis.[1] Such anticancer drugs include clinically important compounds such as vinca alkaloids, anthracyclines, etoposide and paclitaxel. Thus, human P-gp (ABCB1, MDR1) plays an important role in intrinsic or acquired resistance of the tumor cells, which represents a major obstacle for successful treatment of cancer.

A variety of agents were reported to reverse the P-gp-mediated multidrug resistance in tumor cells in vitro and are known as chemosensitizers or modulators. Such chemosensitizers include calcium channel blockers, immunosuppressants, steroidal agents.[2,3] However, most of these compounds have side effects at the doses required to obtain clinically effective concentrations in plasma or tumor cells. In addition, these chemosensitizers are actively effluxed from the cells because they are also substrates for P-gp. Thus, their clinical use is limited. Recently, a variety of flavonoids, which are natural products present in our diet, were reported to modulate P-gp-mediated transport in multidrug resistant cells, but it has remained controversial whether flavonoids activate or attenuate the P-gp-mediated transport. Some flavonols such as kaempferol and quercetin were

shown to be potent stimulators of the P-gp-mediated efflux in tumor cells.[4,5] On the other hand, it has been reported that flavonoids such as quercetin, genistein, biochanin A and morin inhibited the P-gp-mediated transport.[6-9] Mitsunaga et al.[10] showed that quercetin and kaempferol had a biphasic effect on uptake of [^{3}H]vincristine, a substrate of P-gp, in cultured mouse brain capillary endothelial cells. Based on structure activity relationships of flavonoids, there have been many efforts to develop flavonoid derivatives as a new class of P-gp modulators.[11]

Kaempferia parviflora Wall. ex Baker (*K. parviflora*) (local name in Thailand, Krachai Dum), a member of the family Zingiberaceae, is a perennial herb. The black rhizome has been considered to be of highest quality, compared to other different types: yellow, white and red ones. The fresh or dried rhizome has been used in Thai folk medicine as an aphrodisiac and for the treatments such as colic disorder and hypertension. However, the efficacy and safety of *K. parviflora* have not been scientifically elucidated. Recently, some flavonoids were isolated from the rhizome of *K. parviflora* and its flavonoid constituents have been reported to display various pharmacological effects. Among flavonoids isolated, 5,7-dimethoxyflavone showed an inhibitory effect on prostaglandin biosynthesis and possessed hypothermic activity.[12] In addition, 5,7,4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone exhibited antiplasmodial and antifungal activities.[13] Two chalcone derivatives, (-)-hydroxypanduratin A and (-)-panduratin A, were found to show anti-inflammatory activity and may be related to the use of the

rhizome for colic disorder in traditional medicine.[14] More recently, the ethanol extract of *K. parviflora* has been shown to possess gastroprotective effects.[15]

In this study, we first examined the effect of extract from *K. parviflora* tincture (called Ya-dong in Thailand), a typical dosage form for the rhizome, on P-gp function by employing LLC-GA5-COL150 cells, a porcine kidney epithelial cell line (LLC-PK_1) transfected with human *MDR1* cDNA. In addition, the effects of ethanol and aqueous extracts from the rhizome of *K. parviflora* were compared. Furthermore, the concentration-dependent effects of flavone derivatives isolated from *K. parviflora* rhizome on P-gp function were investigated.

MATERIALS AND METHODS

Materials

Rhodamine 123 was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Verapamil hydrochloride and daunorubicin hydrochloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used for the experiments were of the highest purity available.

Cell Culture

LLC-GA5-COL150 cells were obtained from Riken Cell Bank (Ibaraki, Japan). The cells were cultured with Medium 199 containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 150 ng/ml colchicine in an atmosphere of 5% CO_2-95% air at 37°C, and were subcultured every 6 or 7 days using 0.02% (w/v) EDTA and 0.05% (w/v) trypsin.[16-18] One day prior to accumulation studies, the cells were incubated with fresh Medium 199 to remove colchicine. The cell passages of LLC-GA5-COL150 cells used were between 15 and 26. LLC-PK_1 cells were cultured with Medium 199 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2-95% air at 37°C, and were subcultured every 6 or 7 days using 0.02% EDTA and 0.05% trypsin. The cell passages of LLC-PK_1 cells used were between 216 and 228.

Western blot analysis

For immunoblot analysis, the crude membrane fractions from LLC-GA5-COL150 cells and LLC-PK_1 cells 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. These samples were subjected to SDS-polyacrylamide gel electrophoresis with 8% polyacrylamide gels, and the proteins were transferred for 60 min to polyvinylidene difluoride (PVDF) membrane at 4°C. The membrane was blocked in 5% (w/v) non-fat dry milk in phosphate-buffered saline [PBS-T; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 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 C219 mouse monoclonal IgG_{2a} (Signet Laboratories, Inc., Dedham, MA) (1: 10 dilution). The membranes were washed three times in PBS-T, and were incubated with the horseradish peroxidase-labeled goat antibody to mouse

IgG (H+L) (1: 1,000 dilution), washed 3 times in PBS-T, and visualized with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Accumulation experiments

Accumulation of rhodamine 123 or daunorubicin in LLC-GA5-COL150 cells or LLC-PK_1 cells was measured in the 24-well plates (Asahi Techno Glass Corp., Tokyo, Japan). Cells were used at 7 days after seeding at a cell density of 5 x 10^4 cells/well. Fresh medium was replaced every 2 or 3 days. In the case of LLC-GA5-COL150 cells, culture medium was replaced with fresh medium without colchicine 1 day before transport experiments. Experiments were performed in Dulbecco's phosphate-buffered saline (PBS buffer containing in mM, 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$) supplemented with 5 mM D-glucose [PBS(G) buffer]. After removal of the culture medium, each well was washed twice with 1 ml of PBS(G) buffer and the cells were preincubated with 300 μl of PBS(G) buffer in the absence or presence of an extract fraction (1 - 100% of the original tincture for *K. parviflora* tincture, 1 - 300 μg/ml for ethanol and aqueous extracts of *K. parviflora* rhizome) or each flavone compound (1 - 300 μM for each compound) at 37°C for 15 min. Then, PBS(G) buffer containing 20 μM rhodamine 123 or 50 μM daunorubicin without or with an extract fraction or a flavone compound was added to each well and the cells were incubated at 37°C for a specified period. The *K. parviflora* tincture extract, ethanol extract

of *K. parviflora* rhizome and flavone compounds were prepared in dimethyl sulfoxide (DMSO). The aqueous extract of *K. parviflora* was dissolved in distilled water. In all uptake studies, the final concentration of DMSO was adjusted to 4% (v/v). The control cells were incubated with the same concentration of DMSO. At the end of incubation, the uptake buffer was aspirated and the wells were rinsed rapidly twice with 1 ml of ice-cold PBS(G) buffer. The cells were scraped with a rubber policeman into 200 μl of 0.1% (v/v) Triton X-100 (for rhodamine 123 uptake study) or 1% (w/v) SDS (for daunorubicin uptake study) in 1 mM HEPES/Tris (pH7.4) and the wells were rinsed again with 200 μl of 0.1% Triton X-100 (for rhodamine 123 uptake study) or 800 μl of 1% SDS (for daunorubicin uptake study) in 1 mM HEPES/Tris to improve the recovery of the cells. The cells were lysed by vortexing and the homogenate was allowed to stand for 1 h. After centrifugation at 10,000 rpm for 5 min, the supernatant was used for fluorescence and protein assays. The intracellular fluorescence of rhodamine 123 was measured by using a microplate fluorometer (Molecular Devices, CA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The intracellular fluorescence of daunorubicin was measured by using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. Protein concentration was measured by the methods of Bradford[19] for rhodamine 123 uptake study and of Lowry et al.[20] for daunorubicin uptake study with bovine γ-globulin as a standard.

Preparation of extracts from *Kaempferia parviflora* rhizome

Black rhizomes of *K. parviflora* were collected from Loei province, Thailand and a voucher specimen (BS-47-01) was deposited at Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University. The fresh rhizomes were washed, dried in an oven at 45°C until the weight remained constant, and then powdered. The following three kinds of extracts were prepared: *K. parviflora* tincture, ethanol and water extracts from powder of rhizome of *K. parviflora*. First, to prepare *K. parviflora* tincture according to a typical preparation, powdered rhizome of *K. parviflora* (30 g) was extracted with 40% (v/v) ethanol (750 ml) at room temperature. Then, the solvent was evaporated in vacuo and the extract fraction was dissolved in DMSO to obtain *K. parviflora* tincture extract fraction. Secondly, to make ethanol extract fraction of *K. parviflora* rhizome, powdered rhizome was extracted with ethanol [>99.5% (v/v)] at room temperature and evaporated under vacuum to yield 3.44% (w/w). After evaporation, the ethanol extract fraction was dissolved in DMSO. Thirdly, powder of the rhizome was extracted with boiling distilled water to obtain aqueous extract fraction of *K. parviflora* rhizome. The aqueous extract fraction was lyophilized to yield 0.50% (w/w) and dissolved in distilled water. These extract fractions were stored at -20^{o}C until use.

Data analysis

Statistical analysis was performed by Student's t-test, or by the one-way analysis of variance (ANOVA) with the Tukey’s post hoc analysis. A difference of $P<0.05$ was considered statistically significant.

RESULTS

Expression and function of P-gp in LLC-GA5-COL150 cells

Western blot analysis was performed to identify the expression of P-gp in LLC-GA5-COL150 cells. As shown in Figure 1A, P-gp expression was detected in LLC-GA5-COL150 cells, but not in the parental LLC-PK_1 cells. Next we examined the effect of verapamil, a typical inhibitor for P-gp, on the accumulation of rhodamine 123, a typical P-gp substrate,[21] in LLC-GA5-COL150 cells. Rhodamine 123 accumulation in the presence of 100 μM verapamil was significantly increased, compared with that in the absence of verapamil, indicating an inhibition of P-gp-mediated efflux (Figure 1B). On the other hand, 100 μM verapamil had no significant effect on rhodamine 123 accumulation in LLC-PK_1 cells (control, 0.62 ± 0.01; with verapamil, 0.59 ± 0.01 nmol/mg protein/30 min, n=3). Thus, we confirmed that it is possible to detect changes in P-gp function under our experimental conditions. In addition, 100 μM verapamil significantly increased the accumulation of the anticancer agent daunorubicin, another substrate for P-gp, in LLC-GA5-COL150 cells, but not in LLC-PK_1 cells (data not shown).

Effect of *K. parviflora* tincture extract on accumulation of rhodamine 123 and daunorubicin

In Thai traditional medicine, fresh or dried rhizome of *K. parviflora* is often used as a tincture, which is prepared by preserving the rhizome (400 - 500 g

for fresh ones or 30 g for dried ones) in 750 ml of whiskey including 28-40% ethanol. Therefore, we examined whether or not *K. parviflora* tincture extract has an effect on P-gp function. The tincture extract increased the accumulation of rhodamine 123 and daunorubicin in LLC-GA5-COL150 cells in a concentration-dependent manner, but not in LLC-PK_1 cells (Figures 2A and 2B). Significant effects of the tincture on the accumulation of rhodamine 123 and daunorubicin were observed at concentrations over 30% (v/v) and 3% (v/v) of the original tincture, respectively.

Effects of ethanol and aqueous extracts from *K. parviflora* rhizome on accumulation of rhodamine 123 and daunorubicin

We next examined the effects of ethanol and aqueous extracts from *K. parviflora* rhizome on the accumulation of P-gp substrates in LLC-GA5-COL150. As shown in Figures 3A and 3B, the ethanol extract increased the accumulation of both P-gp substrates, rhodamine 123 and daunorubicin, in LLC-GA5-COL150 cells in a concentration-dependent manner, which were significant at concentrations over 30 μg/ml and 10 μg/ml, respectively. The aqueous extract also caused an increase in the accumulation of rhodamine 123 and daunorubicin in LLC-GA5-COL150 cells, while the aqueous extract had lower potency than the ethanol extract (Figure 4). In LLC-PK_1 cells, there were no significant effects of the ethanol and aqueous extracts on the accumulation of rhodamine 123 and daunorubicin (Figures 3 and 4).

Effects of flavone constituents in *K. parviflora* rhizome on P-gp function

Some flavones have been isolated from the rhizome of *K. parviflora*.[13,22] Since several polymethoxyflavones were reported to potently inhibit P-gp-mediated transport,[23,24] we investigated the effects of flavone derivatives isolated from the rhizome of *K. parviflora* on rhodamine 123 accumulation in LLC-GA5-COL150 cells. The structures of six flavones tested in this study are shown in Figure 5. Among these flavones, 3,5,7,3',4'-pentamethoxyflavone most potently increased the accumulation of rhodamine 123 in LLC-GA5-COL150 cells and its stimulatory effect was significant at a concentration of 3 μM or higher (Figure 6A). In addition, 5,7-dimethoxyflavone increased the accumulation of rhodamine 123 in LLC-GA5-COL150 cells, which reached significance at 300 μM (Figure 6A). The other four flavones had no significant effect on the accumulation of rhodamine 123 in LLC-GA5-COL150 cells over the same concentration range (Figures 6A and 6B).

We further examined the effect of 3,5,7,3',4'-pentamethoxyflavone on the accumulation of daunorubicin in LLC-GA5-COL150 cells and LLC-PK_1 cells (Figure 7). The accumulation of daunorubicin in LLC-GA5-COL150 cells, but not in LLC-PK_1 cells, was increased in the presence of 3,5,7,3',4'-pentamethoxyflavone in a concentration-dependent fashion. A significant increase in the accumulation of daunorubicin in LLC-GA5-COL150 cells was observed at a concentration of 30 μM or higher.

DISCUSSION

The rhizome of *K. parviflora* has often been used in Thai traditional medicine for treatment of various disorders such as hypertension and colic. In addition, the medicinal plant has been known as a health-promoting herb for a long time. But so far, little scientific evidence for its clinical usefulness has been shown. It was reported that various flavonoids were isolated from the rhizome of *K. parviflora*.[12,22] Subsequently, 5,7-dimethoxyflavone, a flavone isolated from *K. parviflora*, was reported to possess a potent anti-inflammatory activity, which may provide an explanation for the traditional use of this herbal medicine. In addition, 5,7,4'-trimethoxyflavone was found to exhibit antiplasmoidal and antifungal activities.[13] In this study, we investigated *K. parviflora* extracts and their flavone constituents on P-gp function by employing in vitro transport study using LLC-GA5-COL150 cells, a cell line of porcine kidney epithelial cell line LLC-PK_1 transfected with human *MDR1* cDNA encoding P-gp. As shown in Figure 1A, expression of P-gp was detected in LLC-GA5-COL150 cells, but not in LLC-PK_1 cells. Verapamil (100 μM), a well characterized P-gp inhibitor, significantly increased the accumulation of rhodamine 123, a typical P-gp substrate, in LLC-GA5-COL150 cells, but not in LLC-PK_1 cells. Furthermore, verapamil significantly increased the accumulation of the anticancer drug daunorubicin, another substrate of P-gp, in LLC-GA5-COL150 cells without affecting that in LLC-PK_1 cells. Therefore, by comparing the effect of a test compound on accumulation of P-gp substrate in LLC-GA5-COL150 cells and LLC-PK_1 cells, it was

possible to examine a specific modulation of P-gp function, excluding involvement of other drug efflux transporters such as multidrug resistance-associated proteins (MRPs) and non-specific cytotoxicity.

K. parviflora tincture (local name, Ya-dong), a typical dosage form of *K. parviflora*, is prepared by immersing the rhizome in whiskey (28-40% ethanol) for 7-14 days. Therefore, we first examined the effect of 40% ethanol extract of *K. parviflora* rhizome, corresponding to *K. parviflora* tincture, on P-gp function. The extract of *K. parviflora* tincture increased the accumulation of rhodamine 123 and daunorubicin in LLC-GA5-COL150 cells in a concentration-dependent manner. In contrast, *K. parviflora* tincture extract had no effect on the accumulation of rhodamine 123 and daunorubicin in LLC-PK_1 cells in the same concentration range. Thus, the effect of *K. parviflora* tincture extract on the accumulation of P-gp substrates would be due to specific inhibition of P-gp function by component(s) in 40% ethanol extract, but not due to nonspecific cytotoxicity. To investigate whether such a component which inhibits P-gp function is hydrophobic or hydrophilic in nature, effects of pure ethanol and aqueous extracts from *K. parviflora* rhizome on the accumulation of P-gp substrates were compared. As shown in Figures 3 and 4, the ethanol extract significantly increased the accumulation of P-gp substrates in LLC-GA5-COL150 cells at lower concentrations as compared with the aqueous extract. These observations suggest that P-gp modulating compound(s) in *K. parviflora* rhizome may be relatively hydrophobic.

Various flavonoids have been isolated from the rhizome from *K. parviflora* as described above. Among those flavonoids, six flavone derivatives were used for this analysis (Figure 5). These six 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-hydroxy-3,7-dimethoxyflavone, 2.5%; 5,7-dimethoxyflavone, 21.0%; 5-hydroxy-3,7,4'-trimethoxyflavone, 2.7%; 5,7,4'-trimethoxyflavone, 29.8%; 3,5,7,4'-tetramethoxyflavone, 8.2%; 3,5,7,3',4'-pentamethoxyflavone, 18.1% of ethanol extract from *K. parviflora* rhizome (Sripanidkulchai B, Yenjai C, Sutthanut K, Weerapreeyakul N, unpublished data). Among these flavone derivatives, 3,5,7,3',4'-pentamethoxyflavone most potently inhibited P-gp-mediated transport of rhodamine 123 in LLC-GA5-COL150 cells (Figure 6). Ethanol extract from *K. parviflora* rhizome had a significant inhibitory effect on P-gp-mediated transport of rhodamine 123 at a concentration of 30 μg/ml or higher as shown in Figure 2A. According to the above-mentioned content, 30 μg/ml ethanol extract is expected to contain 5.4 μg/ml 3,5,7,3',4'-pentamethoxyflavone, which is relatively correlated with the lowest concentration (3 μM = 1.1 μg/ml) where a significant inhibitory effect on P-gp function was observed (Figure 6).

In the case of the aqueous extraction of *K. parviflora* rhizome, trace amounts of the six flavone derivatives were detected; 5-hydroxy-3,7-dimethoxyflavone, 0.06%; 5,7-dimethoxyflavone, 1.72%; 5-hydroxy-3,7,4'-trimethoxyflavone, not detectable; 5,7,4'-trimethoxyflavone, 3.21%; 3,5,7,4'-

tetramethoxyflavone, 0.59%; 3,5,7,3',4'-pentamethoxyflavone, 1.23% of aqueous extract from *K. parviflora* rhizome (Sripanidkulchai B, Yenjai C, Sutthanut K, Weerapreeyakul N, unpublished data). As shown in Figure 4A, the aqueous extract significantly increased the accumulation of rhodamine 123 in LLC-GA5-COL150 cells at concentrations over 100 μg/ml, which is estimated to contain 1.2 μg/ml 3,5,7,3',4'-pentamethoxyflavone based on the above-mentioned content. This estimated concentration value is also very close to the lowest concentration (3 μM = 1.1 μg/ml) at which 3,5,7,3',4'-pentamethoxyflavone significantly increased accumulation of rhodamine 123 in LLC-GA5-COL150 cells. Taken together, the significant inhibitory effects of extracts from *K. parviflora* rhizome on P-gp-meditated rhodamine 123 transport may be mainly attributed to 3,5,7,3',4'-pentamethoxyflavone.

In addition, the ethanol and aqueous extracts significantly increased the accumulation of daunorubicin in LLC-GA5-COL150 cells at concentrations of 10 and 300 μg/ml, respectively. These concentration values are estimated to contain 1.8 and 3.7 μg/ml 3,5,7,3',4'-pentamethoxyflavone, respectively. These estimated values are somewhat lower than the lowest concentration (30 μM = 11.0 μg/ml) at which 3,5,7,3',4'-pentamethoxyflavone significantly increased the accumulation of daunorubicin in LLC-GA5-COL150 cells. Therefore, other flavone derivatives in *K. parviflora* extracts may be involved in the inhibition of P-gp-mediated daunorubicin efflux.

Since P-gp is expressed not only in tumor cells but also in normal tissues including the luminal membrane of intestinal epithelial cells and

transports orally administered drugs out of the cells into the lumen, P-gp is considered to be responsible for a change in bioavailability of various oral drugs.[1,25] Therefore, *K. parviflora* tincture may be useful to improve oral bioavailability of drugs which are pumped out of the intestinal epithelial cells by P-gp. Recently, Choi et al.[26] found that coadministration of flavone with paclitaxel resulted in enhanced bioavailability of paclitaxel, possibly by inhibiting cytochrome P450 activity and P-gp function. In this study, 3,5,7,3',4'-pentamethoxyflavone most potently inhibited P-gp function among flavones tested, and therefore it may be useful to improve the bioavailability of P-gp substrate drugs.

A single oral dose of *K. parviflora* tincture is generally 30 ml in Thai folk medicine. Such an oral application of the tincture can be expected to inhibit P-gp function in the human intestine. On the other hand, *K. parviflora* tincture administered orally is probably not useful for overcoming P-gp-mediated multidrug resistance in cancer chemotherapy, since the plasma concentration of flavone constituents after oral administration of the tincture would not be high enough to interact with P-gp in cancer cells. Intravenous coadministration of a specific flavone such as 3,5,7,3',4'-pentamethoxyflavone with anticancer drugs may be an effective approach for overcoming P-gp-mediated multidrug resistance. In a preliminary experiment, we observed that the transport of rhodamine 123 from serosal to mucosal surfaces across everted rat intestine was significantly inhibited by *K. parviflora* rhizome tincture at a concentration of 30% of the original tincture (data not shown), indicating that the ethanol extract potently modulates P-gp function in the

intestine. Further studies are needed for the application of the extracts and flavone compounds to clinical pharmacotherapy.

In conclusion, our study demonstrated that the extracts of *K. parviflora* rhizome and their flavone constituents inhibited the P-gp function in LLC-GA5-COL150 cells in a concentration-dependent manner. In contrast, there were no effects on the accumulation of P-gp substrates in LLC-PK_1 cells, indicating that the extracts and their flavone constituents showed no toxicity in the concentration range studied. Furthermore, it was suggested that the inhibitory effects of the extracts may be mainly attributed to 3,5,7,3',4'-pentamethoxyflavone, a constituent of *K. parviflora* rhizome extract. Recently, in addition to P-gp function,[27] a variety of flavonoids has been reported to modulate MRPs-mediated transport.[28-30] Therefore, further studies are needed to clarify the effects of *K. parviflora* extracts and their constituents on MRPs-mediated drug transport.

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Figure legends

Figure 1. (A) Western blot analysis of crude membranes from LLC-GA5-COL150 cells and LLC-PK_1 cells. Four, eight and sixteen micrograms of crude membranes were subjected to 8% SDS-PAGE. The proteins were blotted on a PVDF membrane and detected with a monoclonal antibody for P-glycoprotein (C219). (B) Effect of verapamil on the accumulation of rhodamine 123 in LLC-GA5-COL150 cells. The accumulation of rhodamine 123 (20 μM) at 37°C for 30, 60, 90 and 120 min was measured in the absence (open circle) or presence (closed circle) of 100 μM verapamil. Each point is expressed as mean ± SE of results from three monolayers. When error bars are not shown, they are included within the symbols. **P<0.01, significantly different from the value in the absence of verapamil.

Figure 2. Effects of *Kaempferia parviflora* (*K. parviflora*) tincture extract on accumulation of rhodamine 123 (A) and daunorubicin (B) in LLC-GA5-COL150 cells and in LLC-PK_1 cells. Accumulation of rhodamine 123 (20 μM) and daunorubicin (50 μM) in LLC-GA5-COL150 cells (open circle) and LLC-PK_1 cells (open triangle) at 37°C for 30 min was measured in the absence (control) or presence of *K. parviflora* tincture extract diluted to be equivalent to 1, 3, 10, 30, 100 and 300% (v/v) of the original tincture. The unit of the x-axis represents the ratio of *K. parviflora* tincture extract concentration in the uptake buffer to that in the original tincture, which was prepared according to a

general formulation in Thai traditional medicine. Each point is expressed as mean ± SE of results from three monolayers. *P<0.05, **P<0.01, significantly different from each control.

Figure 3. Effects of ethanol extract of *K. parviflora* rhizome on accumulation of rhodamine 123 (A) and daunorubicin (B) in LLC-GA5-COL150 cells and in LLC-PK_1 cells. Accumulation of rhodamine 123 (20 μM) and daunorubicin (50 μM) in LLC-GA5-COL150 cells (open circle) and LLC-PK_1 cells (open triangle) at 37^{o}C for 30 min was measured in the absence (control) or presence of ethanol extract of *K. parviflora* rhizome (1-300 μg/ml). Each point is expressed as mean ± SE of results from three monolayers. *P<0.05, **P<0.01, significantly different from each control.

Figure 4. Effects of aqueous extract of *K. parviflora* rhizome on accumulation of rhodamine 123 (A) and daunorubicin (B) in LLC-GA5-COL150 cells and in LLC-PK_1 cells. Accumulation of rhodamine 123 (20 μM) and daunorubicin (50 μM) in LLC-GA5-COL150 cells (open circle) and LLC-PK_1 cells (open triangle) at 37^{o}C for 30 min was measured in the absence (control) or presence of aqueous extract of *K. parviflora* rhizome (1-300 μg/ml). Each point is expressed as mean ± SE of results from three monolayers. **P<0.01, significantly different from each control.

Figure 5. Chemical structures of flavone derivatives contained in *K. parviflora* rhizome.

Figure 6. Effects of flavone derivatives contained in *K. parviflora* rhizome on accumulation of rhodamine 123 in LLC-GA5-COL150 cells. Accumulation of rhodamine 123 (20 μM) in LLC-GA5-COL150 cells at 37^{o}C for 30 min was measured in the absence (control) or presence of a flavone derivative (1-300 μM). (A) Effects of 3,5,7,3',4'-pentamethoxyflavone (open circle), 3,5,7,4'-tetramethoxyflavone (open square), 5,7,4'-trimethoxyflavone (open diamond) and 5,7-dimethoxyflavone (open triangle). (B) Effects of 5-hydroxy-3,7,4'-trimethoxyflavone (open circle) and 5-hydroxy-3,7-dimethoxyflavone (open square). Each point is expressed as mean ± SE of results from three monolayers. *P<0.05, **P<0.01, significantly different from each control.

Figure 7. Effects of 3,5,7,3',4'-pentamethoxyflavone on accumulation of daunorubicin in LLC-GA5-COL150 cells and in LLC-PK$_1$ cells. Accumulation of daunorubicin (50 μM) in LLC-GA5-COL150 cells (open circle) and LLC-PK$_1$ cells (open triangle) at 37^{o}C for 30 min was measured in the absence (control) or presence of 3,5,7,3',4'-pentamethoxyflavone (1-300 μM). Each point is expressed as mean ± SE of results from three monolayers. **P<0.01, significantly different from each control.

Fig. 1

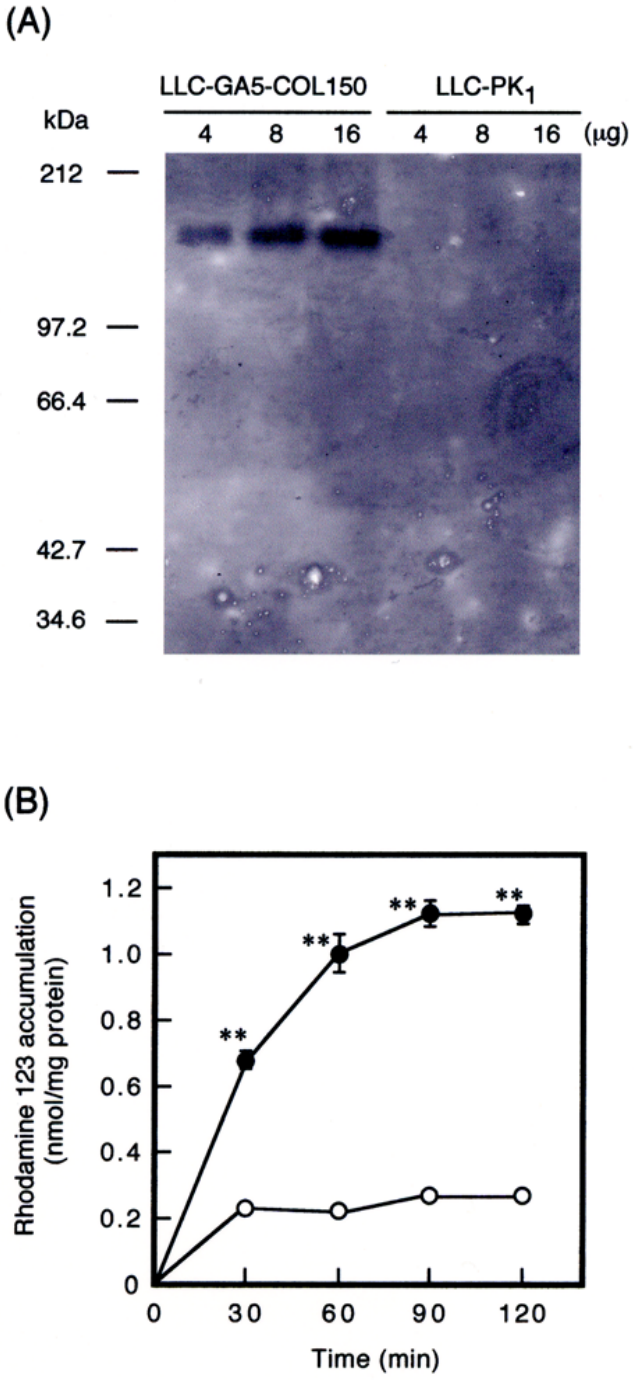

(A)
LLC-GA5-COL150
LLC-PK1
kDa
4 8 16 4 8 16 (μg)
212
97.2
66.4
42.7
34.6
(B)
Rhodamine 123 accumulation (nmol/mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
**
**
0 30 60 90 120
Time (min)

Fig. 2

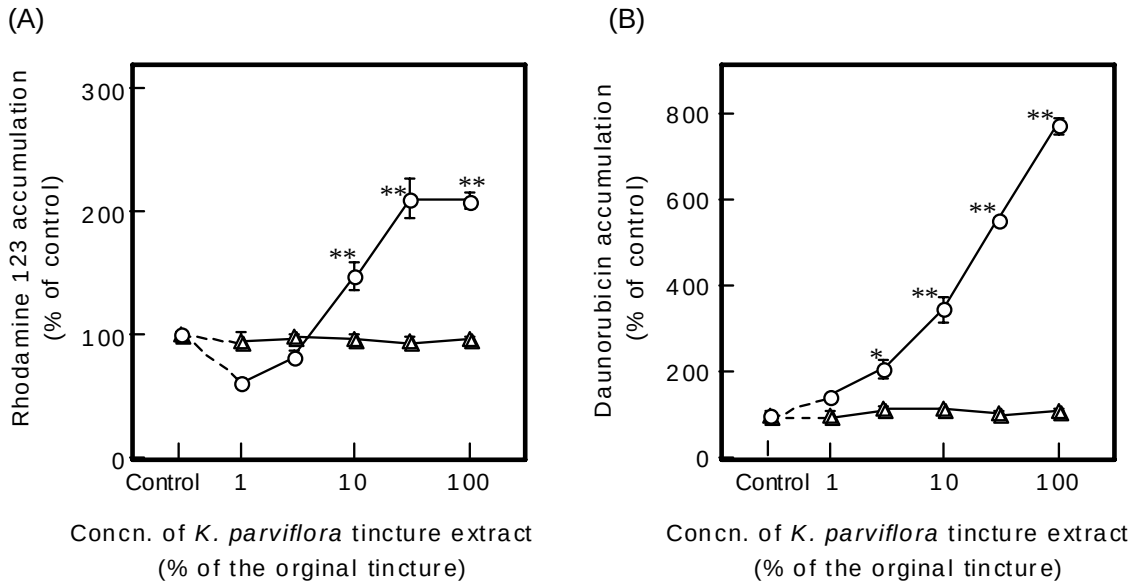

(A)
Rhodamine 123 accumulation
(% of control)
300
200
100
0
Control
1
10
100
**
**
**
Concn. of K. parviflora tincture extract
(% of the orginal tincture)
(B)
Daunorubicin accumulation
(% of control)
800
600
400
200
0
Control
1
10
100
*
**
**
**
Concn. of K. parviflora tincture extract
(% of the orginal tincture)

Fig. 3

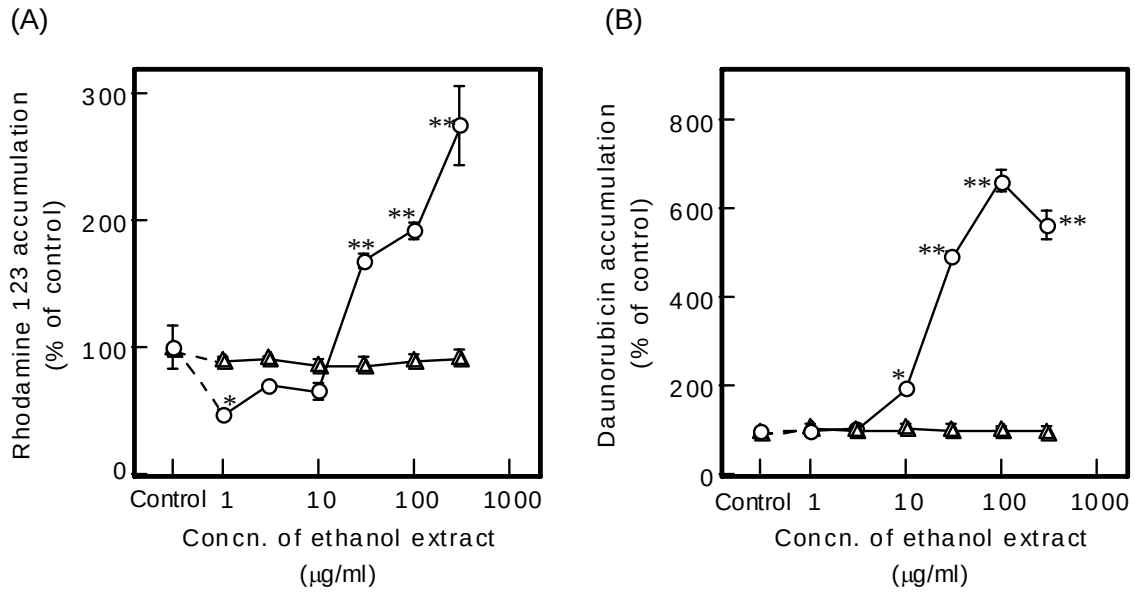

(A)
Rhodamine 123 accumulation
(% of control)
300
200
100
0
Control 1 10 100 1000
Concn. of ethanol extract
(μg/ml)
(B)
Daunorubicin accumulation
(% of control)
800
600
400
200
0
Control 1 10 100 1000
Concn. of ethanol extract
(μg/ml)

Fig. 4

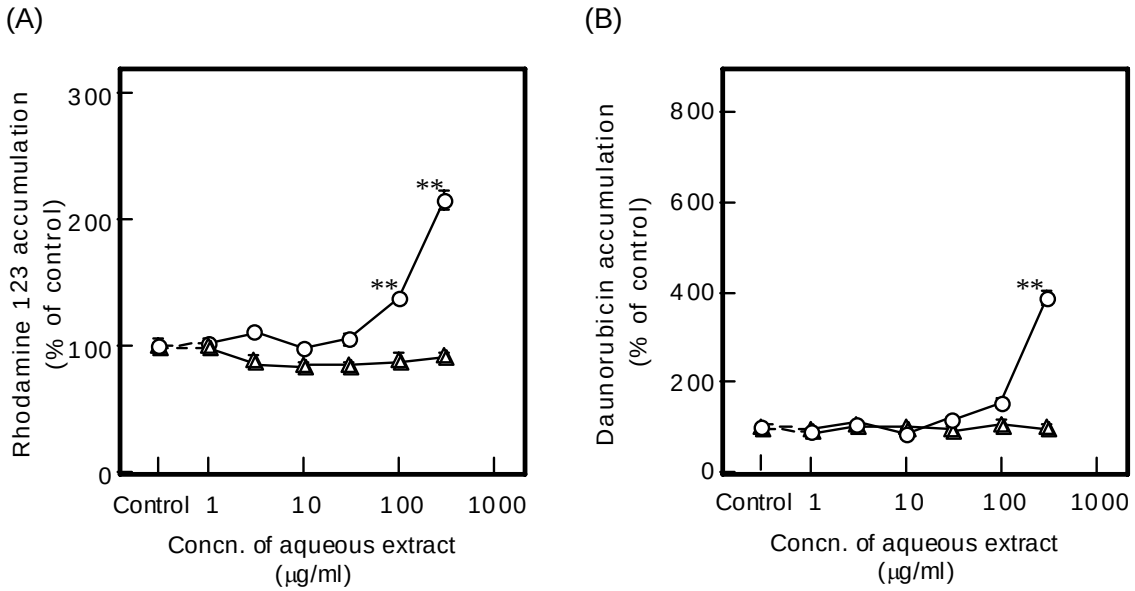
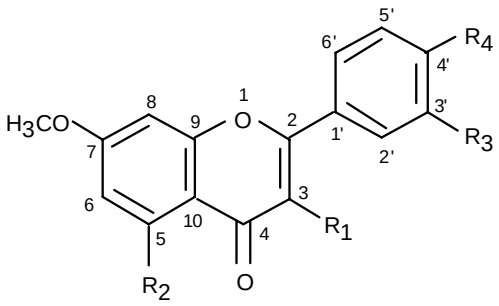

(A)
Rhodamine 123 accumulation
(% of control)
300
200
100
0
**
**
Control 1 10 100 1000
Concn. of aqueous extract
(μg/ml)
(B)
Daunorubicin accumulation
(% of control)
800
600
400
200
0
**
Control 1 10 100 1000
Concn. of aqueous extract
(μg/ml)

Fig. 5



	R_1	R_2	R_3	R_4
5,7-dimethoxyflavone	H	OCH_3	H	H
5,7,4'-trimethoxyflavone	H	OCH_3	H	OCH_3
3,5,7,4'-tetramethoxyflavone	OCH_3	OCH_3	H	OCH_3
3,5,7,3',4'-pentamethoxyflavone	OCH_3	OCH_3	OCH_3	OCH_3
5-hydroxy-3,7-dimethoxyflavone	OCH_3	OH	H	H
5-hydroxy-3,7,4'-trimethoxyflavone	OCH_3	OH	H	OCH_3

Fig. 6

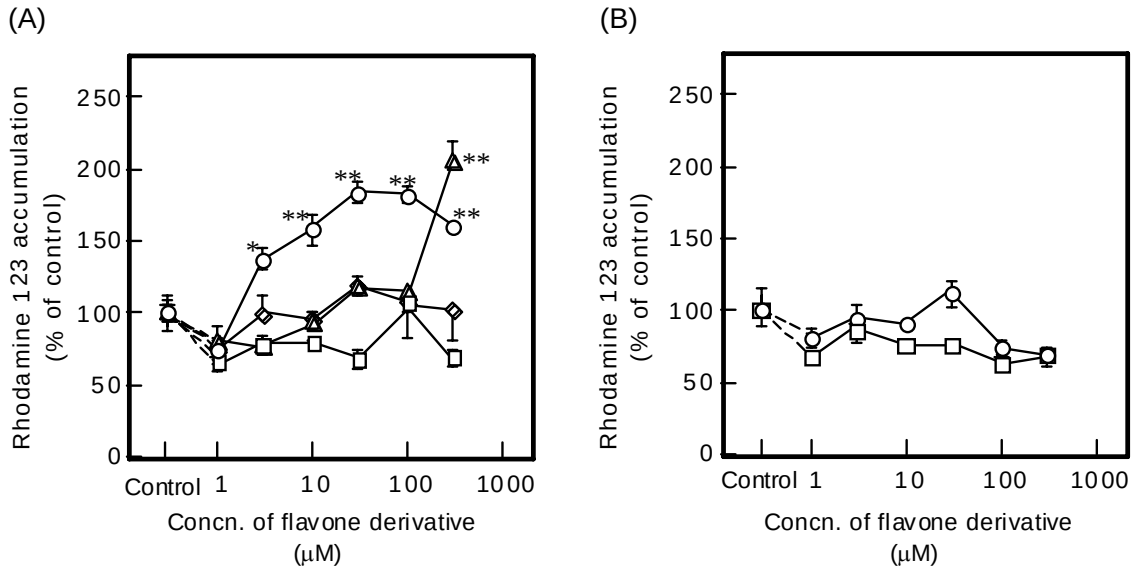
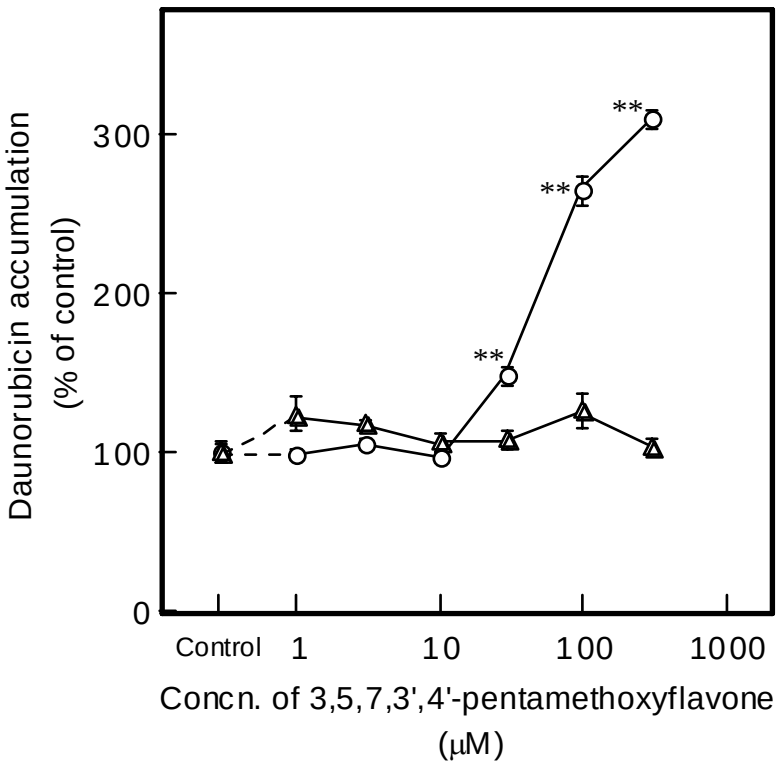

(A)
Rhodamine 123 accumulation
(% of control)
250
200
150
100
50
0
Control
1
10
100
1000
Concn. of flavone derivative
(μM)
*
**
**
**
**
**
(B)
Rhodamine 123 accumulation
(% of control)
250
200
150
100
50
0
Control
1
10
100
1000
Concn. of flavone derivative
(μM)

Fig. 7


Daunorubicin accumulation
(% of control)
300
200
100
0
**
**
**
Control
1
10
100
1000
Concn. of 3,5,7,3',4'-pentamethoxyflavone
(μM)