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# Effects of Diosmin, a Flavonoid Glycoside in Citrus Fruits, on P-Glycoprotein-Mediated Drug Efflux in Human Intestinal Caco-2 Cells

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The effects of citrus flavonoids on P-glycoprotein (P-gp)-mediated drug efflux were examined in human intestinal Caco-2 cells. The cellular accumulation of rhodamine-123 was measured using 10 citrus flavonoids for preliminary screening. Among the flavonoids tested, diosmin significantly increased the accumulation of rhodamine-123 in Caco-2 cells. In the bidirectional transport of digoxin, diosmin increased the apical-to-basal (A-to-B) transport but decreased the basal-to-apical (B-to-A) transport in both concentration- and time-dependent manners. The digoxin transport ratio (B−A/A−B) was estimated to be 2.3 at a concentration of 50 $\mu$M of diosmin, which was significantly lower than the 15.2 found in the control. The apparent $K_i$ values for $P_{app,A-B}$ and $P_{app,B-A}$ were 16.1 and 5.7 $\mu$M, respectively. These results demonstrated that diosmin effectively inhibited the P-gp-mediated efflux in Caco-2 cells. Diosmin is one of the main components in citrus fruits, and the intake of food supplements containing this compound may potentially increase the absorption of drugs able to act as P-gp substrates. The clinical relevance of this interaction should be further evaluated using *in vivo* experiments.

**KEYWORDS: P-glycoprotein; diosmin; flavonoid; citrus fruit; Caco-2 cells**

## INTRODUCTION

Recently, the interactions between drugs and beverages have drawn clinical attention, and the effects of citrus fruit juices, including grapefruit and orange juices, on drug absorption and metabolism has been extensively investigated. Grapefruit juice is known to increase the bioavailability of a wide variety of drugs that can serve as substrates of cytochrome P450 (CYP) 3A4, including dihydropyridine calcium channel antagonists, terfenadine, saquinavir, cyclosporine, and midazolam (*1*−*6*). This effect has been attributed to the selective down-regulation of the expression of CYP3A4 protein in the small intestine (*1*, *7*). Unlike grapefruit juice, orange juice is not known to affect the CYP3A4 protein activity (*2*, *8*, *9*). However, both grapefruit juice and orange juice have been shown to exert inhibitory effects on P-glycoprotein (P-gp)-mediated drug efflux (*9*−*15*). Therefore, the intake of citrus fruit juices, including grapefruit and orange juices, would be expected to enhance the bioavailability of drugs, which might lead to an adverse drug reaction.

Flavonoids are widely distributed throughout plants and contained in citrus fruits, such as grapefruit and orange, as well as many vegetables and other fruits (*16*, *17*). Flavonoids are known to have a wide range of biochemical and pharmacological effects; for example, they inhibit oxidative drug metabolism (*18*−*20*), exhibit anti-inflammatory and antiallergic effects (*21*), and inhibit chemical carcinogenesis (*22*, *23*). In relation to the effects on P-gp function, it has been reported that aglycones of several flavonoids inhibited P-gp in a concentration-dependent manner, whereas their glycosides were found to be inactive (*24*). Flavonols, such as quercetin and kaempferol, have shown a biphasic effect on vincristine efflux, causing inhibition or enhancement depending on their concentration (*24*). The effect of flavonoids on the drug efflux pump seems to be complicated. Nevertheless, flavonoids are considered to play a major role in the P-gp inhibition of citrus fruits; thus, the effect of citrus flavonoids on P-gp was investigated to identify the constituents involved in the P-gp inhibition caused by citrus fruits.

The human colon adenocarcinoma line Caco-2 is the most common cell-based model for assessing drug interaction related to intestinal epithelial transport (*25*). Rhodamine-123 or digoxin is generally used as an established substrate for the P-gp pump (*26*, *27*). In this study, the P-gp inhibitory effect of 10 citrus flavonoids, apigenin, hesperetin, hesperidin, kaempferol, luteolin, quercetin, rutin, diosmin, naringenin and naringin, were investigated with the effect of diosmin on digoxin transport in Caco-2 cells subsequently demonstrated.

## MATERIALS AND METHODS

**Materials.** The apigenin, hesperetin, hesperidin, kaempferol, luteolin, quercetin, and rutin were kindly provided by Dr. Chen Po (Hunan Univ.,

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PR of China). The diosmin, (±)-naringenin, naringin, vinblastine, nicardipine, digoxin, rhodamine-123, verapamil, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), 100× minimum essential medium (MEM) nonessential amino acids solution, 100× antibiotic−antimycotic solution and L-glutamine were from Gibco BRL (Buffalo, NY). BIOCOAT poly(D-lysine)-coated 96-well black/clear plates were purchased from Becton Dickinson (Franklin Lakes, NJ) and Transwells (12-well, polycarbonate membrane, 0.4 μm pore size) from Corning Costar (Cambridge, MA).

**Preparation of Caco-2 Differentiated Monolayer.** Caco-2 cells (HTB-37) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM media with high glucose and L-glutamine, supplemented with 10% FBS, 100× antibiotic− antimycotic solution, and 1% nonessential amino acids. P-gp induction was performed by subculturing in the above media, supplemented with 20 nM vinblastine. Down regulation of P-gp during cell culture was overcome by inducing its expression with vinblastine (*28*). For the uptake studies, cells were harvested 96 h prior to the experiments, washed, and then resuspended in the above media without the vinblastine at a density of $1.5 \times 10^5$ cells/mL. BIOCOAT poly-(D-lysine)-coated 96-well black/clear plates were seeded with 200 μL of the cell suspension and maintained at 37 °C in a 5% $CO_2$/95% air atmosphere for 96 h to allow complete washout of the vinblastine and reconditioning of the cells. For the transport experiments, Caco-2 cells (passage number 39) were seeded in Transwell inserts (polycarbonate membrane, 1 $cm^2$ growth area, 0.4 μm pore size) in 12-well plates at a density of $5 \times 10^4$ cells/$cm^2$. The culture medium was replaced every other day for 18−24 days until analysis. The integrity of the cell monolayer was confirmed by the transephitherial electric resistance (TEER) values measured using a Millicell-ERS voltohmmeter (Millipore Corp). Confluent Caco-2 monolayers with TEER values greater than 550 Ω·$cm^2$, after correction for the resistance obtained in the control blank wells, were used in the transport experiments.

**Evaluation of P-gp Inhibitory Activity.** Caco-2 cells were washed, and the media was replaced by DMEM containing 5% FBS. Stock solutions of test compounds (apigenin, hesperetin, hesperidin, kaempferol, luteolin, quercetin, rutin, diosmin, naringenin, and naringin) were prepared at a concentration of 10 mM in dimethylsulfoxide (DMSO). One microliter of sample solution was added to each cell well of 96 well plates, to a final concentration of 50 μM, and the cells were incubated for 15 min at 37 °C in a 5% $CO_2$/95% air atmosphere. Rhodamine-123 was then added to a final concentration of 2.6 μM, and the cells were further incubated for 4 h at 37 °C. After incubation, the cells were washed twice with ice-cold PBS and scanned using a CytoFlour fluorescence multiwell plate reader (PerSeptive Biosystems by Applied Biosystems, Foster City, CA) at excitation and emission wavelengths of 450 and 530 nm, respectively. The results were expressed as the percentage of the control value for rhodamine-123 accumulation. The accumulation of rhodamine-123 was further tested over a range of diosmin concentrations. Nicardipine was used as a positive control.

**Cell Viability Test.** An MTT assay (*29*) was employed to check the cell viability in parallel to the P-gp inhibition assay. Caco-2 cells were incubated under the same conditions stated above. The MTT solution (0.5 mg/mL) was added, followed by further incubation for 4 h. The medium was then removed, and the wells were washed with PBS. DMSO (200 μL) was added to each well, and the absorbance of each well was measured at 570 nm using a microplate reader.

**Bidirectional Transport of Digoxin in Presence of Diosmin.** The integrity of the Caco-2 cell monolayers was checked, and the cells with TEER values of 550−750 Ω·$cm^2$ were used in the experiment. The Caco-2 cells were washed twice with HBSS and preincubated for 20 min with prewarmed HBSS transport medium (A, 0.5 mL; B, 1.5 mL) at 37 °C in a 5% $CO_2$/95% air atmosphere. The transport assay was initiated by exchanging the transport medium in either the A or B chamber with an equal volume of HBSS containing 30 μM of the P-gp substrate, digoxin. After incubation of the cell monolayers at 37 °C at each time point (0, 30, 60, and 90 min), 50 μL of the medium was taken from the receiver chamber, and the receiver chamber was replenished with 50 μL of fresh transport medium. The collected samples were analyzed using LC/MS. Both the apical to basolateral (A-to-B) and basolateral to apical (B-to-A) directions were tested in triplicate, parallel experiments. At the end of the transport experiment, the cell monolayers were replaced with fresh transport medium and incubated for 30 min at 37 °C, and the TEER values were measured.

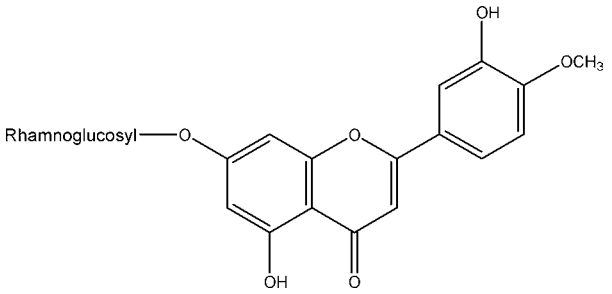


**Figure 1.** Chemical structure of diosmin.

**HPLC/MS Analysis.** The digoxin samples from the transport assay were analyzed using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). Prior to the LC/MS analysis, the collected samples were spiked with an internal standard (10 μL of 5 μM vinblastine) and then passed through the Sep-Pak $C_{18}$ cartridges, which were activated with MeOH and equilibrated with 1 mL of water. After sample loading, the Sep-Pak $C_{18}$ cartridges were washed twice with 1 mL of water, and then samples were eluted with 1 mL of methanol. The eluate was dried under a stream of nitrogen gas, the residue was dissolved in 50 μL methanol, and a 20 μL sample was injected onto the HPLC column for the LC/MS/MS analysis.

The HPLC system consisted of an LC-10ADvp binary pump, SIL-10ADvp autosampler, and CTO-10ASvp oven (Shimadzu, Kyoto, Japan). The analytical column was a Capcell Pak $C_{18}$ (2.0 mm i.d. × 150 mm, 5 μm). The oven temperature was set at 40 °C. The mobile phases were as follows: A = 5 mM ammonium formate (pH 6.0) and B = 90% acetonitrile containing 5 mM ammonium formate (pH 6.0). Samples were eluted with an isocratic mobile phase consisting of 65% solvent B at a flow rate of 0.2 mL/min with a total run time of 4.5 min. Vinblastine was used as an internal standard. The HPLC system was coupled on-line to an SCIEX API2000 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, Canada), equipped with a Turbo Ion Spray source. The analytes were ionized using positive electrospray ionization (ESI), and then detected by multiple reaction monitoring (MRM). The *m*/*z* transitions (798 → 97 (digoxin) and 811 → 224 (vinblastine)) were used for quantification. Nitrogen gas was used as the nebulizing, turbo spray, and curtain gas, with optimum values set at 40, 75, and 30, respectively (arbitrary values). The heated nebulizer temperature was set at 400 °C. The data acquisition was ascertained using the Analyst 1.3.1 software.

The calibration standards were prepared by the addition of various amounts of digoxin to 50 μL of the media and then analyzed as described above. The calibration curves were prepared by plotting the peak area ratios of digoxin/vinblastine against the digoxin concentration, which was analyzed using a linear least-squares regression analysis. The calibration curve for digoxin was linear over the concentration range 0.3−10 μM, with correlation coefficients ($r^2$) of more than 0.99. For validation of the LC/MS/MS method, and the precision and accuracy were determined by repeated analysis at three concentration levels for QC samples (0.3, 1, and 10 μM, $n = 5$) on three separate days. The limit of quantification was 0.3 μM. The accuracy of the method was more than 90%, and the precision (as a percent relative standard deviation) did not exceed 10% for all the QC samples tested.

**Data Analysis.** The apparent permeability coefficients ($P_{app}$) across the Caco-2 cell monolayers in both the A-to-B ($P_{app,A-B}$) and B-to-A ($P_{app,B-A}$) directions were calculated as follows:

$$P_{app} = (dQ/dt)/(AC_0)$$

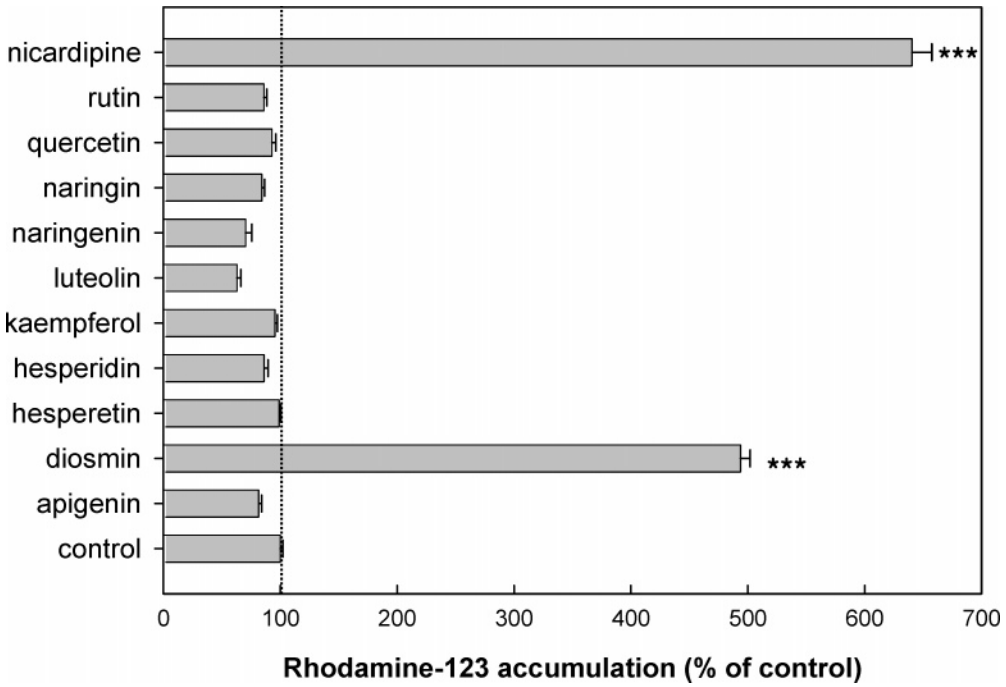


**Figure 2.** The effects of the flavonoids from citrus fruits on the cellular accumulation of rhodamine-123 in Caco-2 cells. Each flavonoid was added to Caco-2 cells to a final concentration of 50 $\mu$M, and the cells were incubated for 15 min at 37 °C. Rhodamine-123 was then added to a final concentration of 2.6 $\mu$M, and the cells were further incubated for 4 h at 37 °C. After incubation, the cellular accumulation of rhodamine-123 was measured using a fluorescence detector with nicardipine used as a positive control. Data are expressed as a percentage of the control and presented as the mean ± SEM ($n = 3$); (***) $P < 0.001$ vs control.

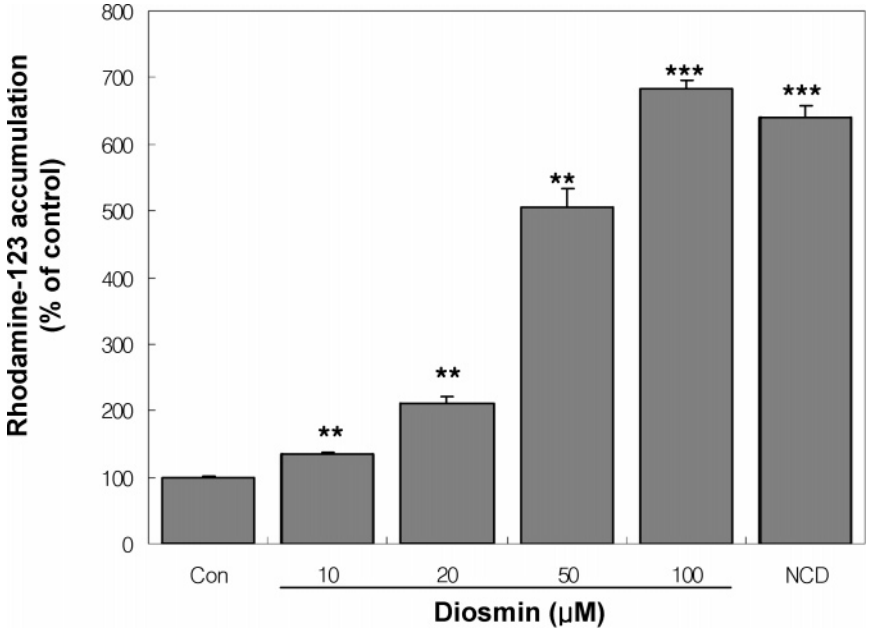


**Figure 3.** Concentration-dependent effects of diosmin on rhodamine-123 accumulation in Caco-2 cells. Sample solutions were added to Caco-2 cells to final concentrations of 10, 20, 50, and 100 $\mu$M, and cells were incubated for 15 min at 37 °C. Rhodamine-123 was then added to a final concentration of 2.6 $\mu$M, and the cells were further incubated for 4 h at 37 °C. After incubation, the cellular accumulation of rhodamine-123 was measured using a fluorescence detector. Data are expressed as a percentage of the control and presented as the mean ± SEM ($n = 3$); (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ vs control.

where d$Q$/d$t$ (nmol/min) is the rate of the compounds (digoxin) appearing in the receiver chamber, $C_0$ is the initial concentration of the compound in the donor chamber, and $A$ is the cell monolayer surface area. The transport ratio (B−A/A−B) was expressed as the ratio of $P_{app,B-A}$ to $P_{app,A-B}$. To calculate the $K_i$ value for inhibition of drug efflux, the results were normalized to the maximal P-gp inhibition value and then plotted and fitted to the Hill equation for allosteric interactions, using the SigmaPlot 2000 software: $i = (I_{max}S^n)/(K_i + S^n)$, where $i$ is the inhibition of drug efflux at a given diosmin concentration, $I_{max}$ is the maximal inhibition caused by 10 $\mu$M verapamil, $S$ is the concentration of diosmin, and $n$ is the Hill coefficient.

**Statistical Analysis.** Data are given as mean ± SEM. An analysis of variance with Tukey's post hoc test was performed using the SAS version 8.02 software (SAS Institute Inc., Cary, NC). A $p$ value $<0.05$ was considered statistically significant.

## RESULTS

**Effects on Cellular Accumulation of Rhodamine-123.** Ten flavonoids were evaluated for their inhibitory activities against the P-gp by rhodamine-123 uptake assay. The cellular fluorescence in Caco-2 cells was determined after incubation for 4 h in the presence of rhodamine-123 with a single concentration (50 $\mu$M) of each test compound. Nicardipine (50 $\mu$M) was used as a positive control. Of the 10 flavonoids tested, diosmin (**Figure 1**) markedly increased the cellular accumulation of rhodamine-123, but the effects of the other compounds were insignificant (**Figure 2**). The accumulation of rhodamine-123 was increased to 494% ± 8.4% and 640% ± 17% of the control value by diosmin and nicardipine, respectively. Diosmin showed a concentration-dependent effect on the accumulation of rhodamine-123 within the range 10−100 $\mu$M (**Figure 3**). No cytotoxicity was observed at any of the concentrations tested in a parallel cell viability test using the MTT assay (data not shown).

**Effects of Diosmin on the Transport of Digoxin across Caco-2 Cell Monolayers.** As indicated in the above result, diosmin elevated the cellular level of rhodamine-123 in Caco-2 cells, suggesting possible inhibitory effects on P-gp-mediated efflux. As a consequence, this possibility was further tested by examining the effects of diosmin on the transport of digoxin, a well-known P-gp substrate, across Caco-2 cell monolayers in both the A-to-B and B-to-A directions.

As shown in **Figure 4**, both the A-to-B and B-to-A transports of digoxin across Caco-2 cell monolayers were approximately proportional to the incubation time up to 1.5 h under all the tested conditions. As shown in **Table 1**, the apparent permeability coefficient for the B-to-A transport of digoxin ($P_{app,B-A}$ (41.1 ± 1.20) × $10^{-6}$ cm/s) was much higher than that for the A-to-B transport ($P_{app,A-B}$ (2.8 ± 0.07) × $10^{-6}$ cm/s), with a mean transport ratio ($P_{app,B-A}/P_{app,A-B}$) of 15.2, which was consistent with the involvement of P-gp-mediated efflux of digoxin in these cells. Diosmin increased the A-to-B transport of digoxin but decreased its B-to-A transport, which as a consequence decreased the mean transport ratio, in a concentration-dependent manner (**Table 1**). In the presence of 50 $\mu$M diosmin, the $P_{app,A-B}$ was significantly increased (from (2.8 ± 0.07) × $10^{-6}$ cm/s to (7.6 ± 0.06) × $10^{-6}$ cm/s) and the $P_{app,B-A}$ significantly decreased (from (41.1 ± 1.20) × $10^{-6}$ cm/s to (17.1 ± 0.10) × $10^{-6}$ cm/s), resulting in a mean transport ratio of 2.3. The positive control, verapamil (100 $\mu$M), increased the $P_{app,A-B}$ (from (2.8 ± 0.07) × $10^{-6}$ cm/s to (9.5 ± 0.06) × $10^{-6}$ cm/s) and decreased the $P_{app,B-A}$ (from (41.1 ± 1.20) × $10^{-6}$ cm/s to (10.4 ± 0.42) × $10^{-6}$ cm/s) with a mean transport ratio of 1.1, indicating the complete inhibition of P-gp. The calculated $K_i$ values of diosmin were 16.1 and 5.7 for the A-to-B and B-to-A transports, respectively, indicating that diosmin apparently affected both the A-to-B and B-to-A transports of digoxin by inhibiting the P-gp-mediated cellular efflux. The decrease in the TEER of the Caco-2 cell monolayers in this study was less than 20%, even at the highest concentration of diosmin, indicating that the increased transport of digoxin was not due to cell death or cell damage.

## DISCUSSION

There have been many reports on the effects of citrus fruit juices, including grapefruit and orange juices, on the P-gp-mediated drug efflux using both *in vitro* and *in vivo* models (*9*−*15*). Polymethoxylated flavones, such as heptamethoxyflavone, tangeretin, nobiletin, dihydroxybergamottin, and bergamottin, have been characterized as being responsible for the

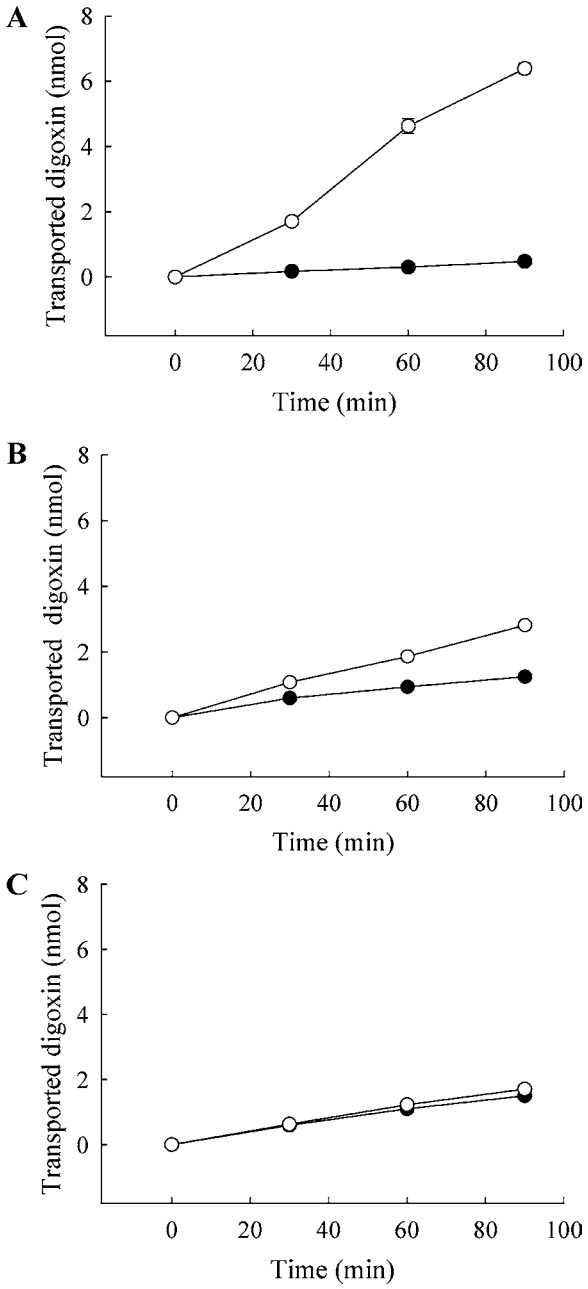


**Figure 4.** The time profiles of the A-to-B (●) and B-to-A (○) transports of digoxin across Caco-2 cell monolayers: (A) control; (B) 50 $\mu$M diosmin; (C) 100 $\mu$M verapamil. The transport assay was initiated by exchanging the transport medium in the apical or basolateral chamber with an equal volume of HBSS containing 30 $\mu$M digoxin. After incubation of the cell monolayers at 37 °C, at each time point (0, 30, 60 and 90 min) 50 $\mu$L of the medium was taken from the receiver chamber and analyzed using LC/MS/MS. Data are expressed as the mean ± SEM ($n$ = 3).

P-gp inhibitory effects of citrus fruits (*9*, *12*, *14*). Nevertheless, the possibility that there might be more active components in citrus fruits cannot be ruled out. In an attempt to investigate the P-gp inhibitory constituents of citrus fruits, several flavonoids found in citrus fruits were evaluated for their P-gp inhibitory effects. As a result, diosmin was identified to significantly increase the cellular accumulation of rhodamine-123 and transport of digoxin in Caco-2 cells, indicating its inhibitory effect on P-gp-mediated efflux.

Diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rutinoside) is known to possess both antioxidant (*30*) and anticarcinogenic activities (*31*) and has also been shown to improve venous tone,

**Table 1.** Concentration-Dependent Effects of Diosmin on the Transport of Digoxin across Caco-2 Cell Monolayers[a]

| concentration ($\mu$M) | $P_{app,A-B}$[b] | $P_{app,B-A}$[c] | transport ratio (B–A/A–B) |
|---|---|---|---|
| 0 | 2.8 ± 0.07 | 41.1 ± 1.20 | 15.2 |
| 10 | 3.9 ± 0.04 | 19.7 ± 0.31 | 5.1 |
| 20 | 6.9 ± 0.08 | 17.4 ± 0.21* | 2.5 |
| 50 | 7.6 ± 0.06 | 17.1 ± 0.10* | 2.3 |
| verapamil | 9.5 ± 0.06 | 10.4 ± 0.42 | 1.1 |
| apparent $K_i$ ($\mu$M) | 16.1 | 5.7 | |

[a] Data are expressed as the mean ± SEM ($n = 3$). An analysis of variance, followed by Tukey's post hoc test, was used for the statistical analysis. [b] Each group was statistically different at a significance level of 0.05. [c] Each group was statistically different at a significance level of 0.05, with the exception of two groups (20 and 50 $\mu$M). [d] No statistical significance was noted between the groups.

enhance microcirculation, and assist the healing of venous ulcers (*32*−*34*). Therefore, diosmin *per se* is used as a health supplement or alternative medicine, such as Daflon tablets (Les Laboratoires Servier, Gidy, France), which contain 450 mg of diosmin and 50 mg of hesperidin. Diosmin is also a common constituent found in many citrus species (*17*), and substantial amounts of this compound are found in most citrus species. Kanaze and colleagues estimated the concentrations of diosmin in orange, tangerine, lemon, and grapefruit juices to be 7.9−72.3, 6.7−21.2, 1.6−17.8, and 1.9−13.7 $\mu$g/mL, respectively (*35*). Therefore, the possibility exists for the interactions of diosmin with co-administrated drugs in humans.

The *in vivo* interaction of diosmin with other P-gp substrates is not easy to predict due to the lack of pharmacokinetic data. Local drug concentrations in the intestinal lumen tend to be higher than those in the systemic circulation. Therefore, the possibility of diosmin−P-gp substrate interactions cannot be excluded when diosmin tablets are taken orally. This speculation is further supported by the fact that pretreatment of diosmin has been shown to result in elevated plasma concentrations of metronidazole in humans (*36*).

A number of studies have looked at the effects of some naturally occurring flavonoids on the P-gp-mediated drug efflux, but conflicting results have been reported. Quercetin, kaempferol, and galangin were found to decrease the accumulation of adriamycin in HCT-15 colon cells due to stimulation of P-gp (*37*), whereas quercetin inhibited rhodamine-123 efflux and reverted multidrug resistance in MCF-7 cells (*38*), and biochanin A and silymarin were reported to inhibit P-gp-mediated transport of digoxin and vinblastine in Caco-2 cells (*39*). In another study, kaempferol and naringenin, which are related to grapefruit juice, inhibited P-gp activity in HK-2 cells (*11*). Herein, several flavonoids were also tested, including apigenin, hesperetin, hesperidin, luteolin, quercetin, rutin, diosmin, naringenin, naringin, and kaemperol, but only diosmin exhibited any inhibitory effect on P-gp. Although most flavonoids have a similar backbone, each seems to act differently on P-gp. The specific actions of flavonoids toward P-gp are considered to depend on their hydrophobicity. It has been reported that flavonoids behave as bifunctional modulators at the vicinal ATP- and steroid-binding sites on mouse P-gp, and their hydrophobicity influences the binding affinity (*40*). According to the same report, the hydroxyl groups at positions 3 and 5 and the hydrophobic methoxy group at position 4′ played key roles in the binding affinity. Diosmin also has a hydroxyl group at position 5 and a methoxy group at position 4′, which are consistent with the above, but this is still not yet enough to explain the P-gp modulation by flavonoids. The molecular mechanism of the interaction between P-gp and flavonoids, through both structural and functional approaches, should be investigated further.

## CONCLUSION

Diosmin, a citrus flavonoid, significantly increased both the cellular accumulation of rhodamine-123 and transport of digoxin within Caco-2 cells, indicating the inhibitory effect on P-gp-mediated efflux. This result shows that diosmin, as well as polymethoxylated flavones, is an active ingredient related to the P-gp inhibitory activity of citrus fruits. In addition, the intake of health supplements containing diosmin may increase the absorption or bioavailability of co-administered drugs able to serve as P-gp substrates, and some caution may be required with its clinical use.

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