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Polymethoxylated Flavones and Other Phenolic Derivates from Citrus in Their Inhibitory Effects on P-Glycoprotein-Mediated Transport of Talinolol in Caco-2 Cells

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Many studies investigating drug interactions with citrus compounds focus on the major grapefruit furanocoumarins bergamottin, dihydroxybergamottin, and the flavonoid naringenin. This study evaluated the influence of polymethoxylated flavones (PMFs), tangeretin, nobiletin, 3,5,6,7,8,3,4′-heptamethoxyflavone, and sinensetin, as well as other minor occurring citrus phenols, hesperetin, limettin, 7-OH-coumarin, 7-geranyloxycoumarin, and eriodictyol, on P-glycoprotein-mediated transport of the β-blocker talinolol using the Caco-2 cell monolayer model and was used to determine the structure−function aspects of the interaction. The transport of talinolol across Caco-2 cells monolayers was determined in the absence and presence of distinct concentrations of the calcium-channel blocker verapamil (a known inhibitor of P-glycoprotein) and citrus compounds. A sigmoid dose−response model was used to fit the data and to estimate the IC_{50} values of the potential inhibitors. Results from this study show that PMFs significantly decreased talinolol transport from the basolateral to apical side, where tangeretin had the lowest IC_{50} of 3.2 μmol/L, followed by nobiletin, heptamethoxyflavone, and sinensetin with IC_{50} values of 3.5, 3.8, and 3.9 μmol/L, respectively. However, the efficacy of the compounds did not appear to be dependent on the number of methoxy groups. Other citrus compounds did not have any significant effect on the transport of talinolol. This study suggests that PMFs have a high potential in the interaction with P-gp-mediated talinolol transport in Caco-2 cells. Based on their relatively low concentrations ($\leq$3 μg/mL) in citrus, the clinical relevance of these interactions needs to be further elucidated in in vivo studies.

KEYWORDS: Polymethoxylated flavones (PMF); coumarins; grapefruit−drug interaction; P-glycoprotein; drug-transport

INTRODUCTION

Citrus and citrus-based products are rich in flavonoids (*1*), which have been inversely correlated to the occurrence of certain chronic diseases, such as several types of cancer and cardiovascular disease as demonstrated in several cohort and case-control studies (*2, 3*). Overall, consumers have developed an increased interest in health benefits of antioxidant, phytochemical-rich fruits and vegetables. In 1997, grapefruit juice (GFJ) was consumed by 21% of all American households, mostly preferred by the elderly as a popular antioxidant breakfast juice (*4*). Yet, recent studies have indicated that a class of grapefruit phenols (furanocoumarins) has a high potential of interacting with the metabolism and bioavailability of certain prescription drugs, which involve mechanism-based irreversible inhibition of intestinal CYP 3A4 (*5, 6*).

In addition to these initially detected CYP3A4-based interactions of grapefruit juice with drugs, the involvement of transporters including P-glycoprotein (P-gp) and organic anion transporting polypeptides (OATP) in grapefruit drug interactions has been described in more recent reports (*7−9*). The inhibition of P-gp by GFJ was first demonstrated in Caco-2 cells (*10*), where tangeretin was determined to be more potent than the other tested compounds. Further in vitro studies corroborate the findings that GFJ inhibits the efflux of P-gp substrates (*11−13*). Overall, the clinical significance of P-gp-related interactions between drugs and GFJ will have to be clarified in further clinical studies.

While most in vitro experiments have been performed with grapefruit components occurring in higher quantities including bergamottin, dihydroxybergamottin, naringin, and naringenin (*14−16*), less information is available regarding the drug

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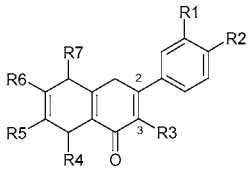
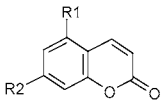


A

Compound	R1	R2	R3	R4	R5	R6	R7
Heptamethoxyflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
Tangeretin	H	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3
Nobiletin	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3
Sinensetin	OCH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	H
Eriodictyol*	OH	OH	H	OH	H	OH	H
Hesperetin*	OH	OCH_3	H	OH	H	OH	H

* The bond between C2 and C3 is a single bond



B

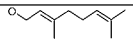
Compound	R1	R2
Geranosyloxycoumarin	H	
7-OH-Coumarin	H	OH
Limettin	OCH_3	OCH_3

Figure 1. Structures of citrus compounds. (**A**) Flavonoids, (**B**) coumarins.

interactive potential of compounds occurring in lower concentrations in grapefruit and other varieties of citrus. Of particular interest are the polymethoxylated flavones (PMFs), which, in orange juice, occur in concentrations of 0.41−2.94 ppm (*17*). In fresh squeezed orange juice, the concentrations of methoxylated flavones were determined as 0.37−11.94 mg/kg (*18*). These compounds have been shown to exhibit numerous biological activities, and in preclinical trials have been demonstrated to significantly modify blood serum lipid profiles (*19*, *20*). Hence, there is considerable interest in these compounds as possible specialty food ingredients and nutraceuticals targeted at cholesterol and triglyceride reduction in humans.

In addition to the PMFs, numerous other phenols occur in citrus, including several classes of flavonoids and coumarins (*21*). The polyphenolics hesperetin, limettin, 7-hydroxycoumarin, eriodictyol, and 7-geranosyloxycoumarin have been identified in different citrus varieties (*22*−*25*); however, only few scientific reports describe the quantification of these compounds in citrus, and also their potential involvement in drug interactions is largely unknown. Hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone) occurs mainly as its glycoside, hesperidin, at concentrations within 200 ppm (*26*). Concentrations of limettin were determined in essential citrus lemon oil as 192 ppm (*27*). Eriodictyol occurs at high concentrations (200−1400 ppm) as the disaccharide, eriocitrin, in lemon peel (*28*). 7-Geranosyloxycoumarin was detected at concentrations of 5.2 ppm of unprocessed grapefruit juice (*23*). For this study, the β-blocker talinolol was used as probe based on its high specificity to be transported by P-gp. However, recent results from a human clinical trial with talinolol and grapefruit juice indicated that potentially influx transporters such as OATP might be involved (*29*). In this study, four PMFs, tangeretin, nobiletin, heptamethoxyflavone, and sinensetin, were evaluated regarding their potency to inhibit the transport of talinolol in a Caco-2 cell monolayer to determine whether the number of methoxy residues has a significant influence on the IC_{50} in the inhibition of P-gp. Moreover, a number of other minor citrus phenols, which to date are less investigated regarding their drug-interaction potential, have been included in this study. This study was designed to determine the potential for inhibition of P-gp in vitro by PMFs and other phenolic derivates from citrus. Data from this study will significantly contribute to the growing knowledge in the area of food−drug interactions.

MATERIALS AND METHODS

Citrus Compounds. Hesperetin and 7-hydroxycoumarin were purchased from Sigma-Aldrich (St. Louis, MO). Limettin and 7-geranyloxycoumarin were isolated as previously described (*30*). Eriodictyol was isolated as described by Horowitz and Gentili (*31*). The PMFs, tangeretin, nobiletin, sinensetin, and heptamethoxyflavone ,were isolated from orange oil using methods previously described by Swift (*32*, *33*), and Tatum and Berry (*34*). Upon isolation, compound identification and purity were measured by analytical thin layer chromatography (*34*), HPLC−MS, and melting point comparisons (*35*) with authentic standards. Each compound was ≥98% pure.

Cell Culture. Caco-2 coloncarcinoma cells (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 10% of fetal bovine serum, 1% of nonessential aminoacids, 100 U/mL penicillin G, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 10 mM sodium pyruvate incubating at 37 °C, and 5% CO_2 (all chemicals were obtained from Gibco BRL Life Technology, Grand Island, NY).

Transport Study. The P-gp transporter is located on the apical side of the Caco-2 monolayer. P-gp, if not inhibited, transports specific drugs including talinolol from the basolateral (BL) to the apical (AP) side

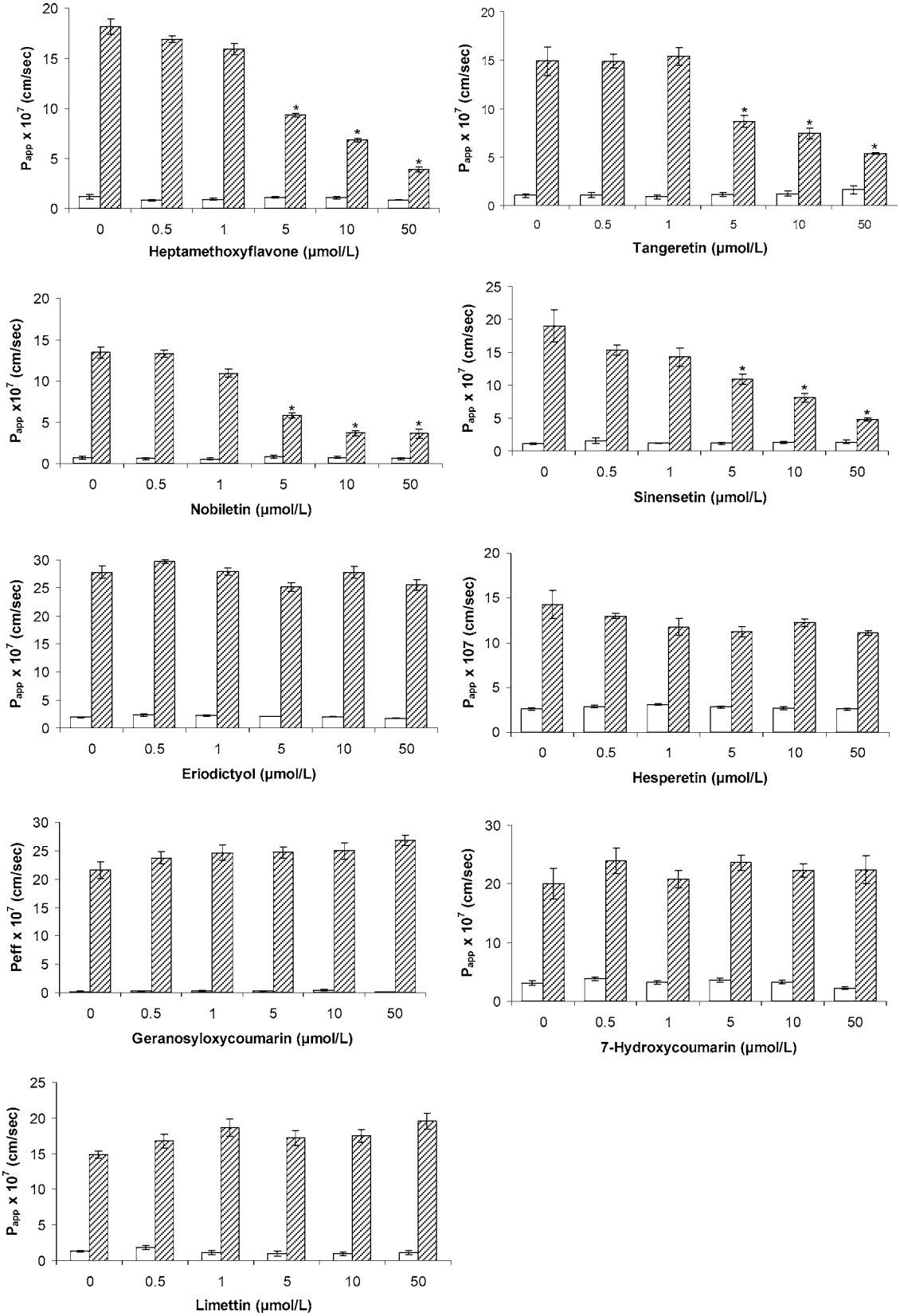


Figure 2. Effects of polymethoxylated flavones and other phenolic derivates from citrus on BL/AP transport of talinolol in Caco-2 cell monolayer after 2 h. Values are means ± SEM, $n = 3–6$; values significantly different from the control are marked with an asterisk, $p \leq 0.05$.

and also reduces the transport of drugs from the AP to the BL side across the monolayer by transporting it back into the apical lumen (efflux transport). Talinolol was placed either in the AP or in the BL chamber to assess the transport in both directions.

Cells were passaged and seeded as previously described into 12-mm transparent polyester cell culture insert well plates (Transwell, Corning Costar Corp., Cambridge, MA) and used for transport studies after 18−21 days as previously described (*36*, *37*). Transepithelial electrical resistance values (TEER) were monitored with an EndOhm Voltohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL) and used for transport studies at TEER values >350 Ω cm^2 after correction for the resistance in control wells.

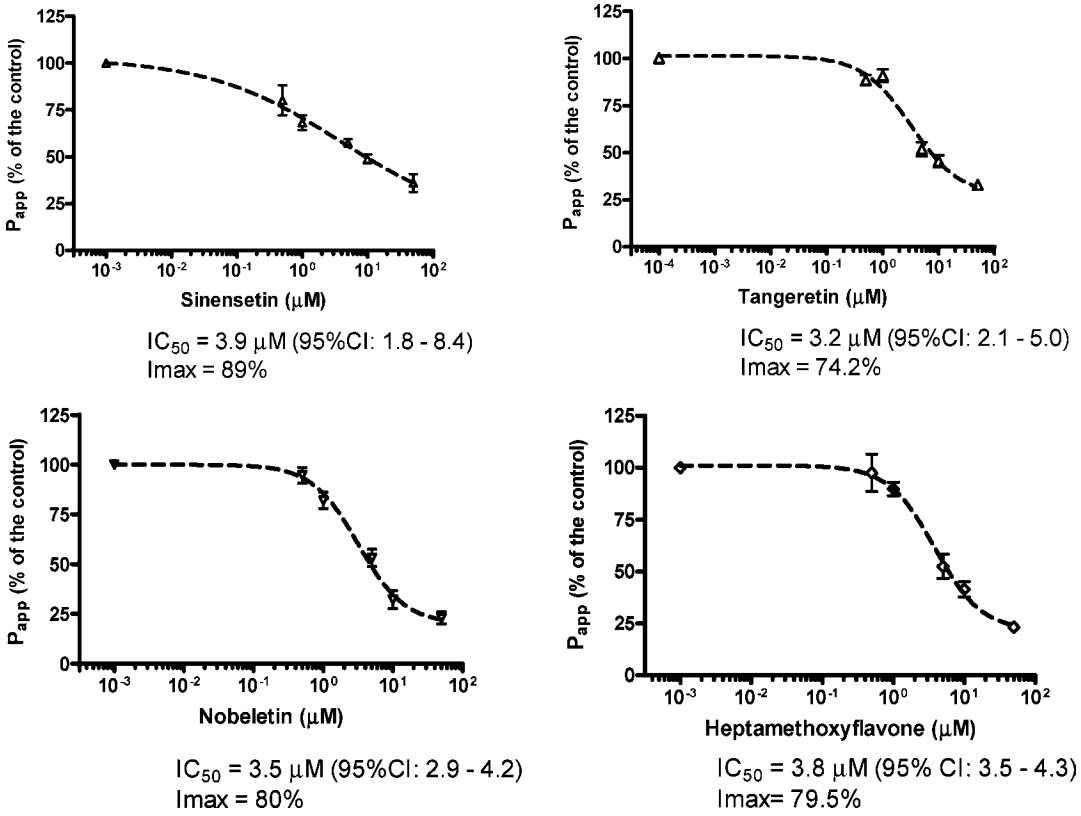


Figure 3. IC_{50} and I_{max} for polymethoxylated flavones given as percentage of the control. Effects of non-polymethoxylated flavones. Values are means ± SEM, $n = 3–6$.

pH was adjusted to 6.0 and 7.4 on the apical (AP) and basolateral (BL) side, with pH-adjusted Hank's balanced salt solution (HBSS) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid solution (MES), and HBSS containing *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES) (all chemicals obtained from Gibco BRL Life Technology, Grand Island, NY) to create a pH gradient similar to the absorption sites on the small intestine environment (all chemicals obtained from Gibco BRL Life Technology, Grand Island, NY) (*38*, *39*).

Grapefruit compounds were pre-solubilized in dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO) and diluted in pH 6.0 HBSS buffer to a final concentration range of 0.5−50 μmol/L. Verapamil hydrochloride (Mediatech Inc., Herdon, VA) and talinolol (kindly provided by AWD-Pharma GmbH & Co. KG, Dresden, Germany) were pre-solubilized in ethanol. Final DMSO and ethanol concentrations were below 0.5% and did not have any significant effect on the transport of talinolol (data not shown). Sample aliquots (200 μL) were taken at 20, 40, 60, 90, and 120 min. Integrity of the monolayer was confirmed using lucifer yellow dipotassium salt (Sigma Chemical Co., St. Louis, MO) after performing the transport study as previously described (*36*).

Sample Analysis. A reversed phase-HPLC method previously described (*40*) was optimized to enable talinolol quantification on a Shimadzu VP series HPLC system (Kyoto, Japan) equipped with a RF-10AXL fluorescence detector, a LC-10ATvp solvent delivery unit, an SIL-10AF autosampler, a CTO-10Avp column oven, a SCL-10Avp system controller, a DGU-14A on-line degasser, a FCV-10ALvp low-pressure gradient unit, and Class VP 7.2 SP1 chromatographic software, at excitation and emission wavelengths of 252 and 332 nm with an analytical column Lichrospher 60RP-Select B column, 250 × 4.6 mm i.d., 5 μm, preceded by a Lichrospher 60RP-Select B guard column (Merck KGaA, Darmstadt, Germany). Talinolol was eluted isocratically using a mobile phase consisting of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0) and acetonitrile (77:23). The range of validation (5−2000 ng/mL) was linear with coefficients of correlation more than 0.998, using $1/X^2$ as weighting factor. Samples were stored at −20 °C until analysis.

Data Analysis. The values of coefficient of permeability (P_{app}) talinolol were calculated using eq 1:

$$P_{app} = \frac{\Delta Q}{\Delta T \times A \times C_o} \quad (1)$$

where $\Delta Q/\Delta T$ is the amount of drug (ng/min) appearing in the acceptor compartment as a function of time obtained from the slope of the linear portion of the amount transported versus time plot, C_o is the initial concentration of talinolol in the donor compartment (ng/mL), and A is the surface area of the semipermeable membrane (cm^2) (*13*). The IC_{50} values of verapamil and grapefruit components were assessed using nonlinear regression according to the hill eq 2, where E_c is the % inhibited observed, E_o is the % effect without inhibitor, E is the maximum inhibition observed (%), IC_{50} is the concentration to reach 50% of maximum inhibition, C is the concentration of the inhibitor, and η is the hill factor.

$$E_c = E_o + \left(\frac{(E - E_o)}{(1 + 10^{(\log IC_{50} - \log[c])^h})}\right) \quad (2)$$

Values are means ± SEM ($n \geq 3$). The confidence interval of each IC_{50} value was determined. Statistical analyses were performed with one-way ANOVA followed by Dunnet's multiple comparison tests as post-hoc analysis using GraphPad Prism v.4.0 (GraphPad Software Inc, San Diego, CA). A probability of less than 0.05 ($p < 0.05$) was deemed statistically significant.

RESULTS AND DISCUSSION

Neither citrus compounds nor the positive control verapamil demonstrated a significant influence on the AP-BL transport. When preincubated for 30 min, verapamil at a concentration of 500 μmol/L caused a decrease in the BL-AP transport by 94%, which demonstrated the high specificity of talinolol to P-gp as a major transporter (data not shown). PMFs (**Figure 1A**,**B**)

decreased the BL-AP transport in a concentration-dependent manner (**Figure 2**), with an IC_{50} of 3.2−3.9 μmol/L, where a maximum inhibition of 100% was not reached at the used concentrations (**Figure 3**). Tangeretin, followed by nobiletin, heptamethoxyflavone, and sinensetin, had the highest IC_{50} of 3.2 μmol/L. Sinensetin induced the highest I_{max} of 89%. The IC_{50} values of PMF did not differ significantly from each other. None of the other citrus flavonoids and coumarins altered the transport of talinolol significantly.

In this study, only PMFs decreased the P-gp-mediated transport of the betaadrenoceptor-blocker talinolol, whereas all other tested compounds did not have a significant effect. The number of methoxy residues as well as their position did not appear to affect the magnitude of effect of the PMF. Moreover, hesperetin and eriodictyol are flavanones, whereas PMF are flavones, which are more lipophilic due to fewer hydroxyl groups. In previous reports, the inhibitory effects of PMF on the efflux transporter P-gp have been discussed. Tangeretin, hepatomethoxyflavone, and nobiletin have been demonstrated to have an inhibitory effect on P-gp- and MRP2-mediated efflux in human colon carcinoma Caco-2 cells and in porcine kidney epithelial cells using vinblastine and saquinavir as probe (*39*). This work also demonstrated the substrate dependency of the inhibitory effect. Nobelitin and tangeretin inhibited OATP-B-mediated uptake of estrone-3-sulfate into human embryonic kidney cells (*41*), which demonstrates that these compounds also have an inhibitory influence on influx transporters. Heptamethoxyflavone, nobiletin, and tangeretin have been shown to decrease the activity of P-gp in adriamycin-resistant human myelogenous leukemia cells where the uptake of vincristine was increased (*42*). Hesperetin, which did not show an inhibitory effect on P-gp in this previously, was tested at much higher nonphysiological concentrations and was demonstrated to have an IC_{50} of more than 3000 μmol/L (*43*). Takanaga and co-workers previously investigated the inhibitory effects of PMF on P-gp as compared to CYP3A4 in vitro using vinblastine and testosterone as probes. It was found that tangeretin, nobiletin, and heptamethoxyflavone increased the steady-state uptake of [^{3}H]vinblastine by Caco-2 cells in a concentration-dependent manner, where at a concentration of 50 μM tangeretin was more potent than heptamethoxyflavone and nobiletin (*44*). In our study, tangeretin as well showed a trend to be the most potent among the PMFs; however, there was no statistically significant difference between the determined IC_{50} values. In the study performed by Takanaga et al. (*44*) and our study, the degree of methoxylation did not appear to have a significant influence on the potency of PMFs in the inhibition of P-gp. In our study, the overall concentration range was kept $\leq$50 μmol/L for all compounds, as the purpose of this study was to determine the potential contribution of different citrus components to P-gp-mediated drug interactions, when consumed from citrus products such as orange juice.

In conclusion, PMFs appeared to have a high potency in the inhibition of P-gp, whereas the other tested flavonoids and coumarins did not have any significant effect on the transport of talinolol in Caco-2 cells. PMFs occur in low concentrations in citrus; for this reason, the clinical relevance of drug interactions with PMF needs to be further investigated. Potentially, polymethoxylated flavones may have a future in the targeted use in the inhibition of P-gp in the treatment of multidrug-resistance in cancer treatment.

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