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Bioassay-guided isolation of potential intestinal absorption enhancers from *Citrus reticulata* Blanco

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ABSTRACT

Overcoming intestinal P-glycoprotein (P-gp) function is one strategy to enhance the oral bioavailability of P-gp substrate drugs. This study aimed to use bioassay-guided fractionation to isolate natural bio-enhancers from tangerine (*Citrus reticulata* Blanco). Isolation of bioactive compounds in cells based on P-gp inhibition activity was conducted. Rhodamine 123 uptake in Caco-2, and calcein acetoxymethyl ester uptake in LLC-PK$_1$ versus human P-gp overexpressed LLC-GA5-Col300, were employed to identify P-gp inhibitors. The investigation revealed four active polymethoxyflavones: 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone; 6,7,8,4′-tetramethoxyflavone; 5,6,7,8,4′-pentamethoxyflavone (tangeretin); and 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin). Nobiletin was found to be the most significant human P-gp inhibitor in tangerine juice. This study also demonstrated the roles of 6,7,8,4′-tetramethoxyflavone and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone as P-gp inhibitors, which had not been previously explored. In summary, all isolated polymethoxyflavones demonstrated their potential roles as absorption bio-enhancers. However, their effectiveness should be substantiated by conducting more mechanistic and clinical studies.

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Keywords: intestinal absorption enhancer; bioassay-guided isolation; *Citrus reticulata* Blanco; tangerine; P-gp; polymethoxyflavone

1. INTRODUCTION

The oral route is the most functional drug delivery method into the body. However, the absorption process of oral medications can be impaired by several factors related to the epithelial tissue of the small intestine, including the detoxifying systems, drug-metabolizing enzymes, and drug transporters (Shugarts and Benet, 2009; Gavhane and Yadav, 2012). Among the ATP-dependent efflux transporters, P-glycoprotein (P-gp) encoded by the multidrug-resistant gene (*MDR*1) is one of the most distinguished efflux transporters. P-gp expressed in many ordinary membranes of the body, including the blood–brain barrier, the gastrointestinal tract, kidney, liver, ovary, placenta, etc., extrudes a diverse range of substrates through the cells (Kim and Chen, 2018; Seelig, 2020). The characteristic properties of P-gp influence drug absorption and disposition in several organs. In the intestine, P-gp recognized in the apical membrane of enterocytes can impair the permeability of its substrates, thus preventing the optimal delivery of orally taken medicines. Overcoming P-gp function by co-administration of P-gp substrate drugs with inhibitors is one of the promising strategies to

improve oral drug bioavailability. So far, several generations of P-gp inhibitors have been discovered and some have been introduced in clinical trials. However, the search for P-gp modulators from natural resources is gaining more interest because they are safe and non-toxic (Marques et al., 2021).

Tangerine (*Citrus reticulata* Blanco, family Rutaceae) is known as "som khīo wān" in Thai. It is one of the most popular citrus fruits, characteristically known for its soft and easy-to-peel skin. It is regularly consumed as fresh fruit or juice. Citrus is a distinguished source of flavonoids, especially polymethoxyflavones. These exhibit a broad spectrum of biological activities, including anti-inflammatory, anti-cancer, and anti-aging. Recently, numerous flavonoids have been demonstrated as P-gp inhibitors, thereby making them candidates for natural bioavailability enhancement (Mohana et al., 2016; Marques et al., 2021). Grapefruit juice is a distinctive example of fruit strongly inhibiting cytochrome P450 3A and P-gp (Bailey and Dresser, 2004). Since both tangerine and grapefruit are members of the citrus family, our investigation focused on tangerine's effect on P-gp function. This study intended to utilize bioassay-guided fractionation to isolate and identify natural bio-enhancers from *C. reticulata* by searching for P-gp inhibitors.

2. MATERIALS AND METHODS

2.1 Chemicals, reagents, and instrumentation

Each chemical and solvent employed in bioassay-guided isolation and biological activity testing was of analytical grade. Silica gel 60 PF_{254}, silica gel TLC plate, toluene, acetone, and ethyl acetate were obtained from Merck (Germany). Amberlite® XAD-16, rhodamine 123 (R123), calcein acetoxymethyl ester (CAM), verapamil, and 0.4% trypan blue were acquired from Sigma-Aldrich (USA). Hanks' balanced salt solution (HBSS), Medium 199 (M199), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, non-essential amino acids, penicillin (10,000 units/mL)-streptomycin (10 mg/mL), and 0.25% trypsin-1 mM EDTA were received from Gibco BRL (USA). NMR spectra were recorded on Bruker Avance-III 300 and Varian Inova 500 NMR spectrometers, using $CDCl_3$ as solvent. EIMS were obtained at 70 eV using a VG 70 250S mass spectrometer and HP 5989B MS engine. The intensities of the fluorescent substrates were quantified using a microplate reader (Victor Nivo, PerkinElmer, USA). The Qubit® fluorometer (Thermo Fisher Scientific, USA) was utilized to quantify the protein concentration.

2.2 Cell culture and uptake study

All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Heat-inactivated FBS was applied in the study. The human colorectal adenocarcinoma cell line, Caco-2, (ATCC#S0249019) was routinely cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin-100 µg/mL streptomycin, 2 mM L-glutamine, and 1% non-essential amino acids. The porcine kidney epithelial cell line LLC-PK_1 and a human P-gp transfected LLC-PK_1 cell line, LLC-GA5-Col300 (Riken Cell Bank#8462-5956-2849), were respectively maintained in M199 supplemented with 10% FBS and 100 units/mL penicillin-100 µg/mL streptomycin in the presence and absence of colchicine (300 ng/mL), as per previous studies (Tanigawara et al., 1992; Ueda et al., 1992). The Caco-2 cells were harvested at 8×10^4 cells/cm^2 and grown for 21 d. The fresh media were replaced 6 h prior to the experiments. LLC-PK_1 and LLC-GA5-Col300 were respectively seeded at the densities of 8×10^4 and 1.3×10^5 cells/cm^2. Both LLC-PK_1 and LLC-GA5-COL300 were cultured for 3 d. The media were replaced for the fresh and colchicine-free media 6 h prior to analysis. The cytotoxicity of a tested substance was detected by the dye exclusion method and lactate dehydrogenase (LDH) test (Kaja et al., 2017).

Cells seeded onto a 24-well plate were utilized in the uptake study. On dedicated experimental days, cells were pre-incubated with HBSS in the absence or presence of a test compound at 37 °C for 30 min. Following which, the final concentration of 10 µM R123 or 1 µM CAM was added to initiate the experiments. The incubation periods for R123 and CAM were 60 min and 30 min, respectively. Less than 1% DMSO in HBSS was used as vehicle control. One hundred µM verapamil was utilized as the positive control in this study. To finalize the experiment, the cells were washed with an ice-cold HBSS and then lyzed with 0.1% Triton X-100. The fluorescence of cellular R123 and calcein were respectively analyzed at the excitation and emission wavelengths of 485 nm and 535 nm. The values were then normalized by protein concentrations. The uptake data were calculated using Equation 1.

$$\% \text{ of the control} = \frac{\text{Normalized fluorescence intensity of treated cells}}{\text{Normalized fluorescence intensity of the vehicle control}} \times 100 \quad (1)$$

2.3 Bioassay-guided isolation

Tangerines were bought from a local fresh-food market in Nakhon Pathom, Thailand. The juice (approximately 40 L) squeezed from the fruits was percolated through Amberlite® XAD-16 column chromatography, using water as the first eluent until the leaching was colorless and discarded. The column was subsequently washed out with ethanol. The ethanolic solution was then evaporated to dryness. The dried extract was re-dissolved in water and subsequently partitioned with dichloromethane, then ethyl acetate. The obtained dichloromethane, ethyl acetate, and aqueous extracts were collected individually and concentrated under reduced pressure to dryness. A small aliquot of each extract was subjected to R123 uptake to preliminarily determine P-gp inhibitory potential.

Further fractionation of the most active dichloromethane extract (1.8 g) was performed by using silica gel 60 PF_{254} column chromatography and eluted with the following mobile phases in increasing order of polarity: toluene-acetone, 10:1; toluene-acetone-ethyl acetate, 10:1:0.5–10:1:1.5; toluene-ethyl acetate, 10:2; and chloroform-methanol, 30:1–10:1. Fractions were collected and pooled in accordance with their TLC profiles to yield 9 fractions. Each combined fraction was dried and screened for the effects on P-gp function by CAM uptake, and the active fractions were further purified for active compounds.

TLC and recrystallization were employed to isolate compounds from fractions 2 and 5. Fraction 2 (30 mg) was subjected to two 20 × 20 cm^2 silica gel TLC plates (0.25-mm thickness), developed with toluene-acetone-ethyl acetate, 10:1:1 to yield T1 (16.4 mg). Silica gel preparative TLC of fraction 5 (16 mg) was also developed in duplicate with chloroform-hexane-methanol, 4:4:0.3, to yield T2 (2.9 mg). Fraction 4 (150 mg) was recrystallized with methanol to obtain T3 (26.3 mg). Fraction 6 (275 mg) was loaded onto a second column chromatography using silica gel 60 PF_{254}, eluted with toluene-acetone, 10:1, and toluene-acetone-ethyl acetate, 10:1:0.5–10:1:1.5, respectively. The fractions with similar TLC profiles were pooled to afford 4 fractions. The major fraction (130 mg) from a secondary column was then applied to a silica gel TLC plate and developed twice with toluene-acetone, 10:1, and chloroform-methanol, 30:1 to obtain T4 (106.4 mg). The structural identification of compounds T1–T4 were determined by comparative analyses of 1D NMR (^{1}H- and ^{13}C-NMR) and MS data with previous reports. The experimental spectra are as follows:

T1: 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, pale-yellow amorphous powder; ^{1}H-NMR at 300 MHz ($CDCl_3$) δ: 3.96 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 3.99 (6H, s, 2 × OCH_3), 4.12 (3H, s, OCH_3), 6.62 (1H, s, H-3), 7.01 (1H, d, J = 8.7 Hz; H-5′), 7.42 (1H, d, J = 2.1 Hz; H-2′), 7.59 (1H, dd, J = 8.7, 2.1 Hz; H-6′); ^{13}C-NMR at 75 MHz ($CDCl_3$) δ: 56.0, 56.1, 61.1, 61.7, 62.0 (5 × OCH_3), 103.9 (CH, C-3), 106.9 (C, C-10), 108.4 (CH, C-2′), 111.2 (CH, C-5′), 120.1 (CH, C-6′), 123.6 (C, C-1′), 132.9 (C, C-8), 136.5 (C, C-6), 145.7 (C, C-9), 149.3 (C, C-3′), 149.5 (C, C-5), 152.4 (C, C-4′), 153.0 (C, C-7), 163.9 (C, C-2), 182.9 (CO, C-4); EIMS (70 eV) at m/z 388 $[M]^+$ for $C_{20}H_{20}O_8$ (Wang et al., 2005; Dandan et al., 2007).

T2: 6,7,8,4′-tetramethoxyflavone, white amorphous powder; ^{1}H-NMR at 500 MHz ($CDCl_3$) δ: 3.89 (3H, s, OCH_3-4′), 3.92 (3H, s, OCH_3-7), 3.99 (3H, s, OCH_3-6), 3.99 (3H, s, OCH_3-8), 6.80 (1H, s, H-3), 6.82 (1H, s, H-5), 7.02 (2H, d, J = 8.9 Hz; H-3′, 5′), 7.86 (2H, d, J = 8.9 Hz; H-2′, 6′); 13C-NMR at 125 MHz ($CDCl_3$) δ: 55.5 (OCH_3,C-4′), 56.4 (OCH_3,C- 6), 61.6 (OCH_3,C-7), 62.3 (OCH_3,C-8), 96.2 (CH, C-5), 106.1 (CH, C-3), 114.5 (CH, C-3′), 114.5 (CH, C-5′), 123.4 (CH, C-1′), 128.0 (CH, C-2′), 128.0 (CH, C-6′), 140.7 (C, C-7), 152.5 (C, C-8), 114.5 (C, C-10), 154.6 (C, C-9), 158.3 (C, C-6), 162.3 (C, C-2), 162.6 (C, C-4′), 177.3 (CO, C-4); EIMS (70 eV) at m/z 342 $[M]^+$ for $C_{19}H_{18}O_6$ (Cao et al., 2020).

T3: 5,6,7,8,4′-pentamethoxyflavone (tangeretin), needle-like crystal; ^{1}H-NMR at 300 MHz ($CDCl_3$) δ: 3.90 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 4.03 (3H, s, OCH_3), 4.12 (3H, s, OCH_3), 6.89 (1H, s, H-3), 7.03 (2H, d, J = 8.9 Hz, H-3′, 5′), 7.91 (2H, d, J = 8.9 Hz, H-2′, 6′); ^{13}C-NMR at 75 MHz ($CDCl_3$) δ: 55.5, 61.7, 61.8, 62.1, 62.3 (5 × OCH_3), 105.9 (CH, C-3), 114.6 (C, C-10), 114.6 (CH, C-3′), 114.6 (CH, C-5′), 123.4 (CH, C-1′), 128.1 (CH, C-2′), 128.1 (CH, C-6′), 138.0 (C, C-6), 144.4 (C, C-5), 147.8 (C, C-9), 148.4 (C, C-8), 151.9 (C, C-7), 162.2 (C, C-4′), 162.7 (C, C-2), 177.5 (CO, C-4); EIMS (70 eV) at m/z 372 $[M]^+$ for $C_{20}H_{20}O_7$ (Chen et al., 1997).

T4: 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin), white amorphous powder; ^{1}H-NMR at 300 MHz ($CDCl_3$) δ: 3.96 (6H, s, 2 × OCH_3), 3.98 (3H, s, OCH_3), 3.99 (3H, s, OCH_3), 4.04 (3H, s, OCH_3), 4.13 (3H, s, OCH_3), 6.95 (1H, s, H-3), 7.01 (1H, d, J = 8.7 Hz, H-5′), 7.45 (1H, d, J = 2.1 Hz; H-2′), 7.62 (1H, d, J = 8.7 Hz; H-6′); ^{13}C-NMR at 75 MHz ($CDCl_3$) δ: 56.0, 56.1, 61.7, 61.8, 62.0, 62.3 (6 × OCH_3), 106.0 (CH, C-3), 108.8 (CH, C-2′), 111.3 (CH, C-5′), 114.0 (C, C-10), 120.2 (CH, C-6′), 123.6 (C, C-1′), 137.9 (C, C-8), 144.5 (C, C-6), 147.8 (C, C-9), 148.4 (C, C-5), 149.4 (C, C-3′), 152.0 (C, C-7), 152.5 (C, C-4′), 162.2 (C, C-2), 177.4 (CO, C-4); EIMS (70 eV) at m/z 402 $[M]^+$ for $C_{21}H_{22}O_8$ (Dandan, et al., 2007).

2.4 Data analysis

Data are presented as the mean ± SD of three distinct studies or otherwise stated. The statistically significant differences at p-values ≤0.05 were acceptable in the study.

3. RESULTS AND DISCUSSION

Verapamil was found to amplify both R123 uptake in Caco-2 and CAM uptake in LLC-GA5-Col300 by about 200% and 1,000% of the control, respectively. The results indicate that human P-gp adequately functions in both cell lines.

The toxicity of the compounds on cells was evaluated by the trypan blue dye exclusion method. Less than 1% of cells were damaged by all test compounds. The cytotoxicity was also evaluated by lactate dehydrogenase assay. Less than 5% of the cellular enzyme was released when a compound was added to the cell culture system. Consumed together, no test compound influenced cell viability in the study.

The bioassay-guided fractionation approach, a step-by-step separation process that assesses the biological activity of plant extracts, was implemented for this study. Initially, the uptake study of dried crude extract (200 µg/mL) was performed to identify the potential effect on P-gp function. The results demonstrated an increase of cellular R123 in Caco-2 (205.3% ± 38.2% of the control). Moreover, CAM uptake in LLC-GA5-Col300 (742.13% ± 191.59% of the control) was significantly enhanced in comparison to the values in LLC-PK_1 (191.57% ± 17.11% of the control). These results suggest the possible effect of tangerine on the P-gp function.

A simple solvent partitioning procedure of the crude extract was subsequently performed that yielded three extracts in accordance with their relative polarities. The dichloromethane, ethyl acetate, and aqueous extracts (150 µg/mL) were screened for their effects on P-gp function. The results are depicted in Table 1. As seen, the dichloromethane extract significantly augmented R123 uptake in Caco-2 cells and demonstrated the highest activity among all extracts. Confirmation was conducted by comparative analysis of CAM uptake in LLC-GA5-Col300 with that of wild-type LLC-PK_1. The results illustrated that the dichloromethane extract significantly elevated CAM uptake in LLC-GA5-Col300 cells, revealing its potential role in P-gp inhibition.

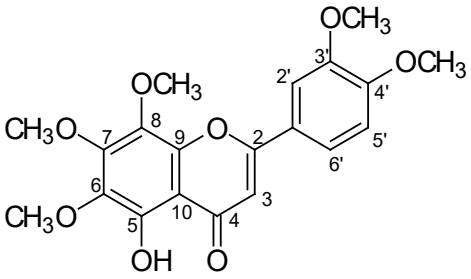
Table 1. Effects of *C. reticulata* fruit extracts on the uptake of P-gp substrates

Extract	Uptake (% of the control)		
	R123 uptake in Caco-2[#]	CAM uptake in	
		LLC-PK$_1$	LLC-GA5-Col300
Dichloromethane	307.8 (307.1–308.5)	243.0 ± 13.4	1,439.9 ± 82.5*
Ethyl acetate	167.7 (162.4–173.0)	ND	ND
Aqueous	93.1(88.4–97.7)	ND	ND

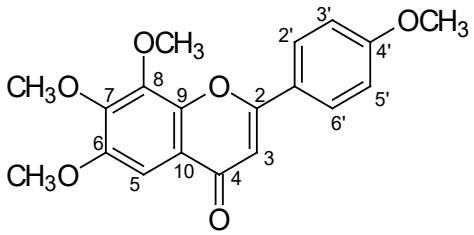
Note: R123 uptake in the presence of verapamil was 191.5% ± 28.1% of the control.
CAM uptake in the presence of verapamil in LLC-PK$_1$ and LLC-GA5-Col300 were 139.5% ± 24.3% and 1,061.9% ± 199.2% of the control, respectively.
[#]mean (range) from duplicate studies, *p < 0.001, ND: not determined

Table 2 depicts the mass of the nine fractions, the CAM uptakes in LLC-GA5-Col300, and number of prominent spots on TLC. Evidently, the presence of fractions 2–8 proliferated the CAM uptakes while fraction 5 had the greatest impact. Fractions 2, 4, 5, and 6 demonstrated one prominent spot, whereas fractions 3, 7, and 8 displayed several spots and small mass quantities. Consequently, the isolation of active compounds was attempted with fractions 2, 4, 5, and 6.

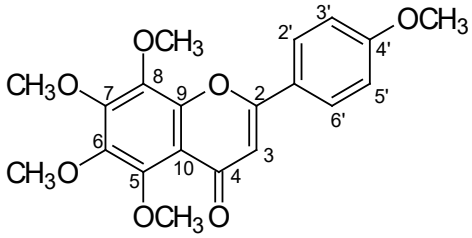
The separation of active chemicals from the selected fractions was accomplished using TLC and recrystallization techniques. TLC of fraction 2 and preparative TLC of fraction 5 provided T1 and T2. T3 was attained with recrystallization of fraction 4 in methanol. Additional column chromatography of fraction 6 resulted in the isolation of T4. Based on comparison of their NMR and MS spectral with the literature data (Chen et al., 1997; Wang et al., 2005; Dandan et al., 2007; Cao et al., 2020), compounds T1–T4 were identified as follows: T1, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone; T2, 6,7,8,4′-tetramethoxyflavone; T3, 5,6,7,8,4′-pentamethoxyflavone (tangeretin); and T4, 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin), The amount of polymethoxyflavones was in the order T4 > T3 > T1 > T2. Their structures are illustrated in Figure 1.



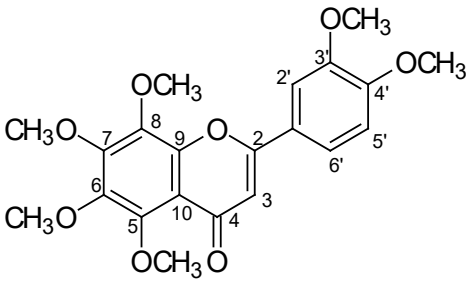
T1: 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone



T2: 6,7,8,4′-tetramethoxyflavone



T3: 5,6,7,8,4′-pentamethoxyflavone (tangeretin)



T4: 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin)

Figure 1. Chemical structures of polymethoxyflavones, T1–T4 from *C. reticulata*

Table 2. The characteristics of fractions 1–9 obtained from silica gel column chromatography of dichloromethane extract

Fraction	Mass weight (mg)	Spots on TLC	CAM uptake in LLC-GA5-Col300# (% of the control)
1	68.4	Several, tailing	239.4 (233.4–245.5)
2	32.2	1 major spot	522.7 (515.6–529.9)
3	47.9	Several	903.7 (891.0–916.4)
4	182.6	1 major spot	875.3 (790.3–960.2)
5	24.8	1 major spot	3,339.1 (3,283.2–3,394.9)
6	297.9	Several	2,174.7 (2,118.3–2,231.2)
7	80.5	Several	1,502.5 (1,452.5–1,552.6)
8	18.4	Several, tailing	428.4 (370.6–486.3)
9	690.1	Several, tailing	109.8 (71.7–147.9)

Note: #mean (range) from duplicate studies.
CAM uptake in the presence of verapamil was 993.1% (982.6%–1,003.4%) of the control.

In addition to elevating CAM uptake in LLC-GA5-Col300, increasing CAM uptakes in LLC-PK$_1$ were seen in the presence of polymethoxyflavones (Table 3). Kuteykin-Teplyakov, et al. (2010) reported the expressions of endogenous P-gp in transfected LLC-PK$_1$ and the expression of porcine P-gp were comparable in overexpressed versus wild-type cell lines. The enhancement of CAM uptakes in LLC-PK$_1$ suggested that the porcine P-gp was also influenced by the polymethoxyflavones. The findings suggest that when normalized by porcine P-gp, nobiletin exhibited the strongest P-gp inhibition while 6,7,8,4′-tetramethoxyflavone displayed the weakest P-gp inhibition.

Table 3. Effects of polymethoxyflavones on CAM uptake in LLC-PK$_1$ and LLC-GA5-Col300

Polymethoxyflavone	CAM uptake (% of the control) in	
	LLC-PK$_1$	LLC-GA5-Col300
T1 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone	146.26 ± 3.92	646.19 ± 112.06
T2 6,7,8,4′-tetramethoxyflavone	206.94 ± 10.34	341.29 ± 42.43
T3 5,6,7,8,4′-pentamethoxyflavone (tangeretin)	170.81 ± 1.98	1,125.66 ± 105.48
T4 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin)	184.79 ± 3.70	1,764.09 ± 618.83

Note: CAM uptakes in the presence of verapamil in LLC-PK$_1$ and LLC-GA5-Col300 were 160.9% ± 28.7% and 1,129.6% ± 346.8% of the control, respectively.

As illustrated in Figure 2, the P-gp inhibition effects of all polymethoxyflavones demonstrated concentration dependence. When normalized by porcine P-gp, all polymethoxyflavones still inhibited the human P-gp in a similar pattern. The P-gp inhibitions of nobiletin and tangeretin from this investigation are in line with the findings of previous studies (Ma et al., 2015; Feng et al., 2016; Liu et al., 2022). Moreover, the effects of 6,7,8,4′-tetramethoxyflavone and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone on P-gp inhibition had not been previously reported. Conclusively, it was discovered that nobiletin, in accordance with the quantity and activity, was the most significant human P-gp inhibitor in tangerine juice. All four polymethoxyflavones have the potential to be used as intestinal absorption enhancers. However, a crucial factor for polymethoxyflavone application is their hydrophilicity; hence, *in vivo* studies are integral to further elucidate their effectiveness.

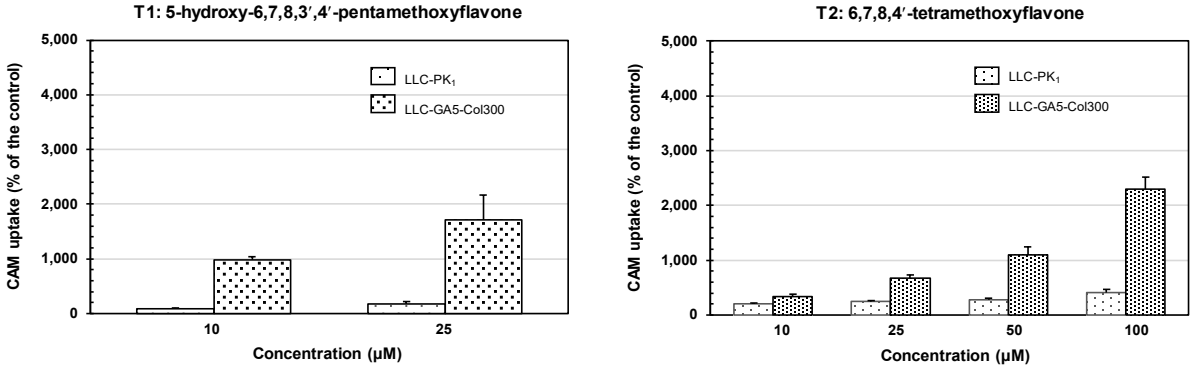


Figure 2. Concentration-dependent effects of polymethoxyflavones on CAM uptake in LLC-PK1 and LLC-GA5-Col300

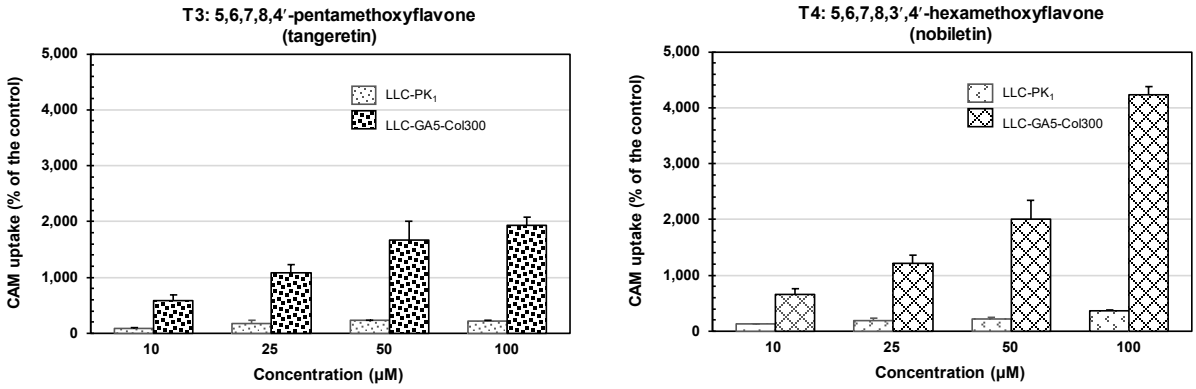


Figure 2. Concentration-dependent effects of polymethoxyflavones on CAM uptake in LLC-PK1 and LLC-GA5-Col300 (Continued)

4. CONCLUSION

Bioassay-guided fractionation and identification of *C. reticulata* for P-gp inhibitors were applied to explore four active polymethoxyflavones known as 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, 6,7,8,4′-tetramethoxyflavone, 5,6,7,8,4′-pentamethoxyflavone (tangeretin), and 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin). All polymethoxyflavones inhibited P-gp in a concentration-dependent manner. The results alluded that nobiletin appeared to be the most significant human P-gp inhibitor. In conclusion, the study indicated that all four polymethoxyflavones can potentially function as natural absorption enhancers for enhancing the oral bioavailability of P-gp substrate drugs. Nevertheless, due to food–drug interactions when taking P-gp substrate medications with tangerine fruit or juice, *in vivo* studies are required to assess the presumptive influences on the clinical effects.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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