



Effect of acidless orange on P-glycoprotein function

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Keywords: Sweet orange; Polymethoxyflavones; P-glycoprotein; Drug efflux

Objectives: This study was performed to evaluate the potential role of green acidless orange as a P-glycoprotein (P-gp) modulator.

Methods: Squeezed juice from green acidless orange was extracted by subjecting to amberlite XAD-16 column and eluted with methanol and acetone. Methanol and acetone extracts were combined and evaporated to dryness to obtain crude extract. The dried crude extract was then partitioned between dichloromethane and water. The dried dichloromethane extract was applied to silica gel PF254 column chromatography to isolate active ingredients. The effect of crude extract on digoxin transport was studied in Caco-2. The effects of isolated polymethoxyflavones were examined in overexpressed human P-gp cell line, LLC-GA5-COL300, compared with that of LLC-PK₁.

Results: Crude extract of green acidless orange significantly decreased the efflux ratio of digoxin transport across Caco-2 monolayer from 25.2 to 6.1 times, suggesting that this citrus may inhibit P-gp function. The active ingredients were elucidated as polymethoxyflavones including 5,6,7,3',4'-pentamethoxyflavone (sinensetin), 3,5,6,7,3',4'-hexamethoxyflavone (quercetagenin hexamethyl ether), and 3,5,6,7,8,3',4'-heptamethoxyflavone. All three polymethoxyflavones obviously increased CAM accumulation in LLC-GA5-COL300 in a concentration-dependent manner. 3,5,6,7,8,3',4'-heptamethoxyflavone found in this citrus fruit demonstrated the most potent effect on human P-gp function.

Conclusion: Taken together, the results from this study indicate that acidless orange could inhibit P-gp function. The active ingredients were elucidated to be polymethoxyflavones.

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Introduction

Overexpression of P-glycoprotein (P-gp, encoded by the ABCB1 (or MDR-1) gene) on the surface of cancer cells is one of the most common causes of multidrug resistance (MDR), leading to resistance to anti-cancer drugs. Moreover, P-gp is the major transporter related to the drug excretion in the intestine, liver, brain, and other epithelial tissues.^{1,2} This transporter is highly expressed in enterocytes of the small intestine contributing to a reduced bioavailability of multiple drugs and clinically significant drug interactions.³ Plant-derived dietary compounds modulating P-gp function have attracted attention as putative MDR modulating agents because of being non-toxic natural products. Grapefruit (*Citrus paradisi*) is the most well-known citrus that can inhibit both CYP3A4 and P-gp, influencing the pharmacokinetics of many oral administered drugs.⁴ A number of citrus are widely grown in Thailand. Green acidless orange (*Citrus sinensis*) is one of the citrus commonly consumed as fruit and juice, however, the information of this citrus on P-gp function has not been clarified. This study was carried out to investigate the effect of acidless orange on P-gp function and the active ingredients were isolated and identified.

Methods

Extraction and isolation of active compound from acidless orange

Freshly squeezed juice from green acidless orange was extracted by subjecting to amberlite XAD-16 column (Fluka Chemie GmbH, Switzerland) and eluted with methanol and acetone. Methanol and acetone extracts were combined and evaporated to dryness to obtain crude extract. The dried crude extract was then partitioned between dichloromethane and water and dichloromethane layer was concentrated to dryness. The dried dichloromethane extract was applied to silica gel PF254 column chromatography, eluted with gradients of toluene-acetone (10:1), toluene-acetone-ethylacetate (10:1:0.5 to 10:1:1), chloroform-methanol (30:1 to 1:1). Fractions were collected and pooled on the basis of their TLC profiles to yield 10 fractions.

Fraction 2-7 was further purified by preparative TLC, yielding 3 polymethoxyflavones. Their structures were determined by analyses and comparison of the MS and NMR spectra with those described in the literature.

Digoxin transport study

Caco-2 (ATCC, passage number 42-50) cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% penicillin-streptomycin, and 2 mM glutamine was seeded on Transwell® insert (80,000 cells/cm²) and grown for 21 days in a humidified atmosphere of 5% CO₂ at 37°C.

The cell monolayer was preincubated in HBSS in the absence (control) and presence of test compounds for 30 min. To start experiments, 50 µM digoxin (final concentration) was added to the donor side. The test compound was added to both donor and receiver sides. The sample was withdrawn from the receiver side and the amount of digoxin permeated across the monolayer was quantified by HPLC method as a function of time.⁵

The apparent permeability coefficient (P_{app}, cm/s) is calculated based on the following equation:

$$P_{app} = (dQ/dt)/(A \times C_0 \times 60)$$

where dQ/dt = cumulative transport rate (µmol/min), A = surface area of the cell monolayer (cm²), and C₀ = initial concentration in the donor compartment (µmol/ml).

An efflux ratio is calculated from the apparent permeability coefficients as follows:

$$\text{Efflux ratio} = P_{app,B \rightarrow A} / P_{app,A \rightarrow B}$$

Calcein-AM uptake experiment

LLC-PK₁ (ATCC) and LLC-GA5-COL300 (Riken cell bank) were grown in M199 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin. LLC-GA5-COL300, which was derived by transfecting LLC-PK₁ with human MDR1 cDNA, was cultured in the presence of 300 ng/ml colchicine, as previously reported.^{6,7} Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. LLC-PK₁ and LLC-GA5-COL300 cells were respectively seeded at 120,000 and 360,000 cells/cm² onto 24-well plates and cultured for 3 days. The media were changed every day and replaced for fresh and colchicine-free media 6 h before experiments.

The cells were preincubated in HBSS in the absence (control) and presence of the test compounds for 30 min. To initiate the experiment, the final concentration of 1 µM calcein-AM (CAM) was added to the cells and incubated at 37°C. The reaction was stopped by adding ice-cold HBSS. The cells were carefully washed 3 times and then lysed with 0.1% Triton X-100. The amount of the fluorescent calcein was determined by reading the fluorescence intensity on Packard Fusion® Universal Microplate Reader at the excitation and emission wavelength of 485 and 535 nm, respectively.

Results

Three compounds in flavonoid family were isolated as active substances in green acidless orange, and identified by NMR and MS analysis as 1) 5,6,7,3',4'-pentamethoxyflavone (sinensetin, C₂₀H₂₀O₇, MW 372) 2) 3,5,6,7,3',4'-hexamethoxyflavone (quercetagenin hexamethyl ether, C₂₁H₂₂O₈, MW 402), and 3) 3,5,6,7,8,3',4'-heptamethoxyflavone (C₂₂H₂₄O₉, MW 432).^{8,9} (Figure 1)

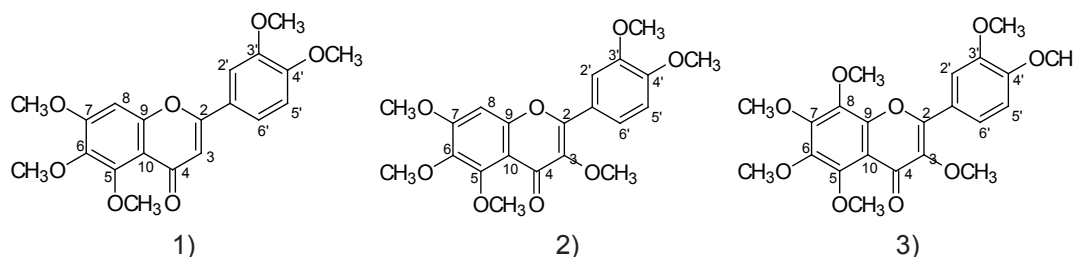


Figure 1. Active polymethoxyflavones isolated from green acidless orange

The cytotoxicity of crude extract and all three polymethoxyflavones was checked with trypan blue dye exclusion and lactate dehydrogenase assay to ensure that all concentrations used in this study showed no toxic effect to the cells.

As seen in Figure 2, digoxin transport from apical side to basolateral side was significantly increased and permeability from basolateral side to apical side was significantly decreased in the presence of 650 mg/ml of crude extract. As the result, the efflux ratio of digoxin transport across Caco-2 cell monolayer was reduced from 25.2 to 6.1 times.

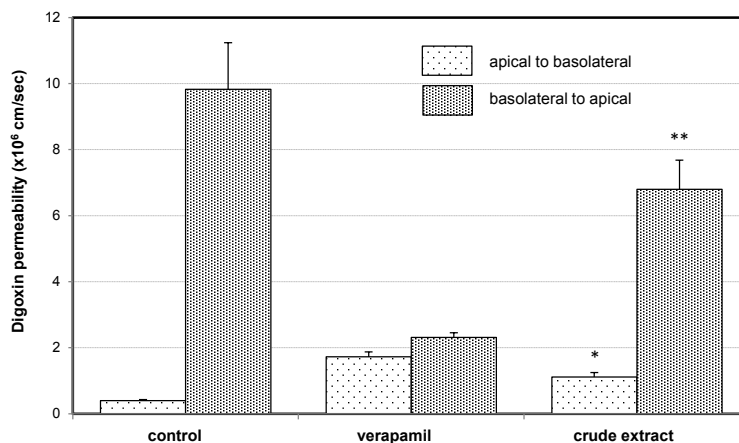


Figure 2. Effects of verapamil and crude extract prepared from green acidless orange on digoxin permeability across Caco-2. 100 mM verapamil was used as a positive control. **p*<0.05, ***p*<0.01

Figure 3 demonstrates that polymethoxyflavones enhanced CAM uptake in the concentration-dependent manner. As shown, the amount of CAM uptake in LLC-GA5-COL300 was obviously higher than that of LLC-PK₁, indicating that polymethoxyflavones can inhibit function of human P-gp. Verapamil (100 μ M) was used as a positive control in this study. Verapamil significantly increased CAM uptake in LLC-GA5-COL300 to 1155.25 ± 195.76 % of control, confirming the expression of human P-gp in this cell line.

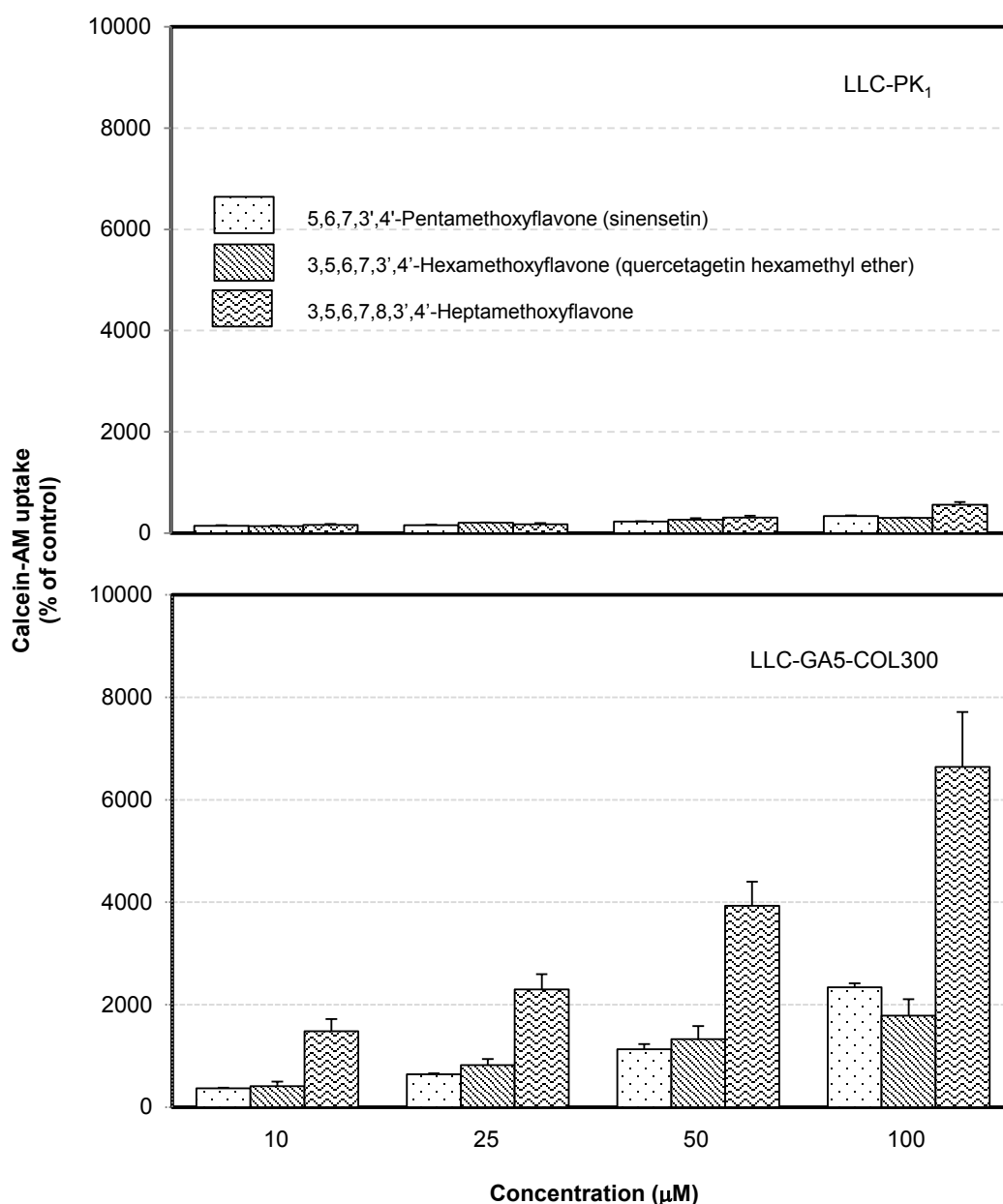


Figure 3. The effects of isolated polymethoxyflavone on CAM uptake in LLC-PK₁ and LLC-GA5-COL300

Discussion

Acidless orange is a common citrus fruit grown and consumed in Thailand. To evaluate the role of this orange as a natural P-gp modulator, the changes in accumulation and efflux of P-gp substrates were studied in the presence of orange extract or isolated pure substances. Digoxin and CAM were used as P-gp substrates and verapamil was used as a positive control.¹⁰ As can be seen, the crude extract of green acidless orange was able to lower the efflux ratio of digoxin transport across Caco-2. On the basis of significant expression of P-gp in Caco-2 cells, these data suggest the inhibitory potency of acidless orange on P-gp function. In order to confirm that the effect is specific to P-gp, LLC-PK₁ and overexpressed human P-gp LLC-GA5-COL300 cell line^{6,7} were used in the study. The uptake of CAM by LLC-GA5-COL300 was about 5 times lower than that of the parental LLC-PK₁ (data not shown). Remarkably, the uptake of CAM by LLC-GA5-COL300 was obviously sensitive to verapamil (increasing about 11 times), which confirmed the existence of human P-gp in this cell line. The results performed in LLC-GA5-COL300 show that all three isolated polymethoxyflavones significantly increased CAM uptake in the concentration-dependent manner (Figure 3). These results substantiate the role of this citrus as a natural P-gp inhibitor.

Conclusion

The results from this study indicate that green acidless orange could inhibit P-gp function. The active ingredients were elucidated to be three polymethoxyflavones including 5,6,7,3',4'-pentamethoxyflavone (sinensetin), 3,5,6,7,3',4'-hexamethoxyflavone (quercetagenin hexamethyl ether), and 3,5,6,7,8,3',4'-heptamethoxyflavone. As shown, 3,5,6,7,8,3',4'-heptamethoxyflavone found in this citrus fruit is likely to be the most potent inhibitor of human P-gp.

Acknowledgements

The authors thank Research and Development Institute, Silpakorn University for the financial support, and Faculty of Pharmacy, Silpakorn University for all facilities used in this study.

References

1. Thiebaut F, Tsurno T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci. USA* 1987;84:7735-8.
2. Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev.* 2003;55:3-29.
3. König J, Müller F, Fromm MF. Transporters and drug-drug interactions: important determinants of drug disposition and effects. *Pharmacol Rev.* 2013;65:944-66.
4. Kirby BJ, Unadkat JD. Grapefruit juice, a glass full of drug interactions? *Clin Pharmacol Ther.* 2007;81(5):631-3.
5. Jedlička A, Grafnetterová T, Miller V. HPLC method with UV detection for evaluation of digoxin tablet dissolution in acidic medium after solid-phase extraction. *J Pharm Biomed Anal.* 2003;33:109-15.
6. Tanigawara Y, Okamura N, Hirai M, Yasuhara M, Ueda K, Kioka N, Komano T, Hori R. Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *J Pharmacol Exp Ther.* 1992;263:840-5.
7. Ueda K, Okamura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T, Hori R. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J Biol Chem.* 1992;267: 24248-52.
8. Chen J, Montanari AM, Widmer WW. Two new polymethoxylated flavones, a class of compounds with potential anticancer activity, isolated from cold pressed dancy tangerine peel oil solids. *J Agric Food Chem.* 1997;45:364-8.
9. Ahmed AA, Ali AA, Mabry TJ. Flavonoid aglycones from *Jasonia montana*. *Phytochemistry.* 1989;28(2): 665-7.
10. Rautio J, Humphreys JE, Webster LO, Balakrishnan A, Keogh JP, Kunta JR, Serabjit-Singh CJ, Polli JW. In vitro p-glycoprotein inhibition assays for assessment of clinical drug interaction potential of new drug candidates: a recommendation for probe substrates. *Drug Metab Dispos.* 2006;34:786-92.
11. Troutman MD, Thakker DR. Novel experimental parameters to quantify the modulation of absorptive and secretory transport of compounds by P-glycoprotein in cell culture models of intestinal epithelium. *Pharm Res.* 2003;20:1210-24.