Possible Intestinal Absorption Enhancers from Citrus hystrix

May Phyu Thein Maw, Panadda Phattanawisin, Chanokporn Sukpan, and Nusara Piyapolrungroj

1Department of Biopharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand
2Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand

Abstract. Bioavailability of orally administered drugs is regulated by P-gp, a member of the ATP binding cassette transporter families. It expresses at the apical surface of epithelial cells and effluxes out several clinically important drugs resulting in decreased absorption and bioavailability. In recent years, the utilization of bioenhancer to increase the bioavailability of drugs has extensively studied. The objective of this study was to evaluate the potential of the compounds found in Citrus hystrix as a bioenhancer for orally administered drugs by modulation of P-gp function. The modulation effects of fruit extracts and isolated pure compounds on P-gp were investigated by uptake assay of the P-gp substrate calcein-AM in Caco-2, LLC-PK1 and LLC-GA5-COL300 cell lines. The results show that the extract from the flavedo part remarkably increased calcein-AM uptake in Caco-2 and LLC-GA5-COL300 cell lines. Among five furanocoumarins identified, 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin and oxyypeucedanin significantly enhanced calcein-AM uptake in LLC-GA5-COL300 in a concentration-dependent manner, indicating strongly inhibition effects on P-gp function. Taken together, 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin and oxyypeucedanin could be employed as the potential intestinal bioenhancer to improve the bioavailability of P-gp substrate drugs. However, further studies including in vivo studies should be performed to confirm these findings.

1 Introduction

The oral route of drug administration is the most commonly used since it is convenient, relatively safe and economical. The orally administered drug must pass through the gastrointestinal epithelia to be absorbed into the systemic circulation and exert its biological effect. Various factors are affecting the rate and extent of absorption of orally administered drugs which in turn governed their bioavailability. Absorption from the GI tract is regulated by physicochemical properties of drugs such as lipid solubility, intestinal permeability, particle size, degradation in the gastrointestinal tract, physical state of the drug (solution, suspension, or solid dosage form) and physiological factors such as gastrointestinal pH, alteration of gut flora, altering motility, and transporter proteins [1].

Transporter proteins that are expressed in the intestine mediate the selective absorption and excretion of both endogenous compounds and xenobiotics. Transporters are generally classified into two major superfamilies, the ATP binding cassette (ABC) and solute carrier (SLC) transporters. They play a critical role in the pharmacokinetics and pharmacodynamics processes of drugs which in turn regulate the therapeutic and adverse effects [2]. Among the ABC transporters, P-glycoprotein (P-gp), an efflux transporter, has been recognized as one of the important transporters that regulate the intestinal absorption of orally administered drugs. P-gp located at the apical surface of epithelial cells exports a variety of orally administered drugs, such as cyclosporin, into the intestinal lumen by utilizing ATP thereby serve as the intestinal barrier [3].

Bioenhancer is an agent which enhances the bioavailability of concurrently administered drug without having the pharmacological activity of their own. They increase the bioavailability of drugs by various mechanisms such as by increasing the absorption of the drug across the membrane, function as receptors for xenobiotics, potentiate drug by conformational interaction and making target cells susceptible to drug molecules [4]. There has been an increasing interest in the utilization of bioenhancer in clinical application as it has many potential advantages. Concomitant administration with a bioenhancer would result in a reduction of dosage, toxicity and minimization of drug resistance [5].

Nowadays medicinal plants are widely used as a supplement and in treating disease. Medicinal plants such as Vernonia amygdalina, Carica papaya and Vernonia amygdalina which are used in the treatment of malaria have been reported to enhance digoxin absorptive transport in Caco-2 cells [6]. It has been reported that quercetin, genistein, naringin, sinomenine, piperine, glycyrrhizin and nitrile glycoside improve the bioavailability of concomitant administered drugs such as antibiotics, antituberculosis, anticancer and cardiovascular drugs [4, 7]. Naringin was reported to enhance the absorption of antiplatelet drug clopidogrel, by inhibiting P-gp efflux pump [8]. It has been described that quercetin increased the AUC and Cmax of diltiazem by inhibition of intestinal
P-gp efflux pump. Natural compound genistein was reported to inhibit efflux transporter P-gp thereby increasing the intestinal absorption of paclitaxel [9].

P-gp plays an important role in inhibiting optimal drug delivery of orally administered drugs since it is located at the apical surface of the intestinal cells. Modulation of P-gp function would affect the absorption of many P-gp substrate drugs. The purpose of this study was to investigate the potential role of compounds isolated from the fruits of Citrus hystrix as the bioenhancer. The effects of the active compounds on the function of the efflux transporter P-gp were investigated.

2 Materials and methods

2.1 Chemicals and Reagents

Dulbecco’s modified eagle medium (DMEM), Medium 199 (M199), Hank’s balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, non-essential amino acids, penicillin (10.000 units/mL)-streptomycin (10 mg/mL), and 0.25% trypsin-1mM EDTA were obtained from Gibco BRL (Grand Island, NY, USA). Verapamil and 0.4% trypan blue were purchased from Sigma Chemical Company (St. Louis, MO, USA). Calcein acetoxy methyl ester (calcein-AM), was acquired from Fluka Chemie GmbH (Switzerland). Silica gel 60 (0.015-0.040 mm mesh, Merck, Germany) and silica gel 60 F254 aluminium plates (Merck, Germany) were used for isolation of the active compound. All other chemicals used were of reagent grade.

The Caco-2, a human colon adenocarcinoma cell line (passage 35-50, ATCC, sale order no. S0249019) was grown in heat-inactivated FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, 1% non-essential amino acids. LLC-PK1, a porcine kidney epithelial cell line (passage 30-60) was routinely cultured in M199 supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. LLC-GA5-COL300, a human P-gp overexpressed-LLC-PK1 cell line (passage 20-45, Riken Cell Bank, air waybill no: 8462-5956-2849) was cultured in M199 supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 300 ng/mL colchicine. All of the cells were maintained in a humidified 5% CO2 atmosphere at 37°C.

2.2 Preparation of crude extracts from Citrus hystrix fruits

The fruits were purchased from a fresh market in Nakhon Pathom, Thailand. The fresh fruits were separated into four parts (flavedo, albedo, segment membrane and juice). Flavedo, albedo, and segment membrane were dried at 50°C for 2 days, ground, and then macerated with methanol room temperature (3 days, 3x) with frequent agitation. The macerate was filtered, evaporated and dried to obtain crude extracts. The juice was extracted with methanol (3x) and the organic layer was evaporated by employing a rotary evaporator and dried at 40°C on a water bath. All the dried extracts were kept at -20°C until used. The fingerprints of all methanic extracts were analyzed by HPLC. An Agilent 1260 infinity liquid chromatography system, provided with a quaternary solvent delivery system, an autosampler and a DAD detector, was used. A Zorbax SB-C18 column (4.6 x 150 mm, 3.5 µm) was utilized and detection wavelength was set at 310 nm. The mobile phase was composed of water-acetonitrile-tetrahydrofuran (THF) (85:10:5) (Solvent A) and acetonitrile-methanol-THF (65:30:5) (Solvent B) (v/v) using gradient elution with a flow rate of 0.3 mL/min. The gradient profile was as follows: 0-5 min, 0% of B; 5-20 min, 0-32% of B; 20-24 min, 32% of B; 24-38 min, 32-55% of B; 38-40 min, 55-90% of B; 40-50 min; 90% of B and 50-60 min; 0% of B.

2.3 Isolation of active compounds from flavedo by chromatographic technique

The dried methanic extract of flavedo (21.05 g) was partitioned between EtOAc and deionized water (1:1). The EtOAc layer was evaporated by a rotary evaporator to obtain greenish residue as EtOAc extract (8.92 g, 6.99% of dried weight). EtOAc extract (8 g) was loaded into the glass column (3 x 60 cm) which contains silica gel 60 (200 g) as the stationary phase. The gradient elution of n-hexane: ethyl acetate (EtOAc), starting with 10% EtOAc in n-hexane followed by 20% of EtOAc and finally 80% of EtOAc in n-hexane was used as mobile phase. The fractions were collected and combined based on TLC profiles. The fractions were further isolated by PTLC to obtain pure compounds. Five furanocoumarins were elucidated and identified by LC-MS and 1H NMR as follows: bergamottin (C21H22O6, MW 338), 6',7'-dihydroxybergamottin (C21H22O6, MW 372), 6',7'-epoxybergamottin (C21H22O6, MW 354) oxypeucedanin hydrate (C16H16O6, MW 286), oxypeucedanin hydrate (C16H16O6, MW 304) [10-14].

2.4 Calcein-AM uptake study in cell culture

Caco-2 cells were seeded at a cell density of 150,000 cells/well onto 24-well plates and cultured for 21 days for the experiment. LLC-PK1 (80,000 cells/well) and LLC-GA5-COL300 (130,000 cells/well) were seeded onto 24-well plates and grown for 3 days for the studies. To start the experiments, cells were preincubated in HBSS in the absence or presence of tested extracts or compounds for 30 min, then P-gp substrate calcein-AM (1 µM final concentration) was added and further incubated for 30 minutes. Following this, the solution was removed and washed carefully with ice-cold HBSS. After washing, the cells were lysed with 0.1% Triton X-100 and the amount of calcein accumulated in the cells was measured directly on a microplate reader (VictorNivo, PerkinElmer, USA) at an excitation and emission wavelength of 485 and 535 nm, respectively. The amount of calcein retained in the cells was normalized by the protein concentration. All experiments were done in triplicate and presented as mean ± S.E.M. All the tested extracts or compounds were dissolved in methanol and diluted in HBSS to receive the final methanol concentrations not exceeding 1%. HBSS
containing 1% methanol was used as vehicle control. Verapamil which is an already acknowledged P-gp inhibitor was used as the positive control [15] at concentration of 100 µm, a concentration that was reported to inhibit P-gp [16, 17]. The cytotoxicity of the tested extracts or compounds was investigated by checking cell morphology under a microscope and analyzed by trypan blue dye exclusion method.

3 Results

The effects of methanolic (flavedo, albedo, segment membrane and juice) extracts of *Citrus hystrix* fruits on the P-gp function were firstly screened in the Caco-2 cell line. As seen in Table 1, the extracts remarkably increased calcein accumulation in Caco-2 cells by approximately 1.9 (flavedo), 1.4 (albedo), 1.2 (segment membrane) and 1.6 (juice) folds, respectively. The extract from the flavedo notably increased calcein-AM uptake, suggesting the inhibition effect on the function of multidrug resistance protein including P-gp. To confirm the effects of the extracts on the P-gp function, the uptake of calcein-AM in LLC-PK1 and LLC-GA5-COL300 cell lines were performed. The accumulation of calcein in LLC-GA5-COL300 was about 14 times lower than that in LLC-PK1, indicating that higher expression of P-gp in LLC-GA5-COL300. As noticed, the flavedo extract remarkably increased calcein-AM uptake in LLC-GA5-COL300 by 11 folds (176.03 ± 12.40 % of control in LLC-PK1 compared with 1967.34 ±159.13 % of control in LLC-GA5-COL300), suggesting that the compounds in flavedo could potentially inhibit the function of P-gp. While the other three parts of fruits did not show the difference in calcein-AM uptake between these two cell lines. Moreover, the flavedo extracts significantly increased calcein accumulation in the concentration-dependent manner (Table 2).

Because the flavedo part of *Citrus hystrix* exhibited a noticeable effect on P-gp function, its active compounds were isolated and identified. Five furanocoumarins were elucidated as shown in Fig. 1. The fingerprint profile of the methanolic extract of flavedo is depicted in Fig. 2.

The effect of bergamottin, 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin, oxypeucedanin and oxypeucedanin hydrate on P-gp function was performed by calcein-AM uptake in LLC-PK1 and LLC-GA5-COL300 cell monolayers (Fig. 3). As shown, the accumulation of calcein in LLC-GA5-COL300 was remarkably higher in 6',7'-epoxybergamottin and 6',7'-dihydroxybergamottin treated cells indicating that these compounds could inhibit P-gp function. The inhibition activity was followed by oxypeucedanin. Bergamottin weakly inhibited P-gp function and oxypeucedanin showed the weakest effect on P-gp inhibition. Besides, 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin and oxypeucedanin increased calcein accumulation in a concentration-dependent manner (Fig. 4).

### Table 1. Effects of the methanolic extracts obtained from *Citrus hystrix* fruits on calcein-AM uptake in Caco-2, LLC-PK1 and LLC-GA5-COL300 cell monolayers.

| Cell lines         | Calcein-AM uptake (% of control) | Flavedo Extract (80 µg/mL) of | | |
|--------------------|---------------------------------|-------------------------------|----------------|----------------|
|                    | Verapamil (100 µm)              |                                |                |                |
| Caco-2 (passage 35-50) LLC-PK1 | 161.71 ± 7.33 | 199.25 ± 1.93 | 141.94 | 126.35 | 162.41 |
| LLC-GA5-COL300 (passage 20-34) | 144.72 ± 5.60 | 176.03 ± 2.40 | 99.80 | 115.62 | 120.60 |
|                    |                                 | 894.05 ± 69.52 | 1967.34 ± 59.13 | 92.71 | 98.14 | 106.35 |

Data are presented as mean ± SEM, n = 5. Verapamil - the positive control. *Data are presented as an average from 2 cell passages.

### Table 2. Concentration-dependent effect of the flavedo extract on calcein-AM uptake in Caco-2, LLC-PK1 and LLC-GA5-COL300 cell monolayers.

| Cell lines         | Calcein-AM uptake (% of control) | Flavedo extract (µg/mL) | 8  | 40  | 80  |
|--------------------|---------------------------------|-------------------------|----|-----|-----|
| Caco-2 (passage 35-50) LLC-PK1 | 152.34 ± 8.19 | 124.08 ± 3.90 | 148.42 ± 3.78 | 176.14 ± 3.53 |
| LLC-GA5-COL300 (passage 25-35) | 1075.44 ± 17.76 | 2384.55 ± 49.69 |

Data are presented as the mean ± SEM, n = 3.
Fig. 1. Structure of furanocoumarins isolated from the flavedo of *Citrus hystrix* (1) Bergamottin (2) 6',7'-Epoxybergamottin (3) 6',7'-Dihydroxybergamottin (4) Oxypeucedanin (5) Oxypeucedanin hydrate.

Fig. 2. HPLC fingerprint profile of the flavedo extract of *Citrus hystrix*. Five furanocoumarins were isolated from column chromatography and identified by LC-MS and 1H NMR as follows (1) Oxypeucedanin hydrate, (2) 6',7'-Epoxybergamottin, (3) 6',7'-Dihydroxybergamottin, (4) Oxypeucedanin, (5) Bergamottin.

Fig. 3. Effects of furanocoumarins (100 µm) isolated from the flavedo of *Citrus hystrix* on calcine-AM uptake in LLC-PK₁ (passage 40-60) and LLC-GA5-COL300 (passage 25-45) cell monolayers.

Fig. 4. Concentration-dependent effects of 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin and oxypeucedanin on calcine-AM uptake in LLC-GA5-COL300 (passage 25-45) cell monolayers.

4 Discussion

P-gp plays a vital role in pharmacokinetic mechanisms of several clinically important drugs because of its localization as a barrier to important organs and its wide substrate spectrum [18]. P-gp transports vinca alkaloids (vinblastine, vincristine), camptothecin derivatives (topotecan), tubulin polymerizing drugs (paclitaxel, docetaxel), anthracyclines (doxorubicin, idarubicin), epipodophyllotoxins (etoposide), tyrosine kinase inhibitors (e.g., erlotinib and imatinib), antibiotics (e.g., grepafloxacin), anticonvulsants (e.g., dabigatran, rivaroxaban, and apixaban), dihydropyridines (e.g., nicardipine and talinolol), digitalis glycosides (e.g., digoxin), immunosuppressive agents (e.g., ciclosporin), anticoagulants (e.g., ximelagatran), steroids (e.g., cortisol, aldosterone, and dexamethasone) and HIV protease inhibitors (e.g., saquinavir, ritonavir, and nelfinavir) [19].

P-gp localized at the apical membrane of intestinal cells pumps out the therapeutically important drugs, playing a pharmaceutical challenge in clinical application because of low bioavailability [20-21]. In recent years, the use of bioenhancers to increase the bioavailability of drugs are of interest in research fields. To discover P-gp inhibitors which can improve the bioavailabilities for P-gp substrate drugs would be beneficial for oral route delivery.

The increased concentration of P-gp substrate drugs in patients having phytochemical-rich food and drinks concurrently with conventional medicine has been reported. It was reported that plant secondary metabolites such as anthraquinone, emodin, carnosic acid from Rosemary, polyphenols derived from *Mangifera indica* such as mangiferin, norathyriol, quercetin and naphthoquinone ester rhinacanthin-c inhibit P-gp activity [22-24]. Quercetin inhibited digoxin efflux which in turn increased the absorption of digoxin [25]. Popular beverage green tea constituent, epigallocatechin-3-gallate inhibits P-gp mediated transport of digoxin in human adenocarcinoma cell line has been reported [26].
Citrus hystrix, a citrus fruit native to tropical Southeast Asia and southern China, contains significant quantities of furanocoumarins and other constituents. This study aimed to study the potential of Citrus hystrix fruits on P-gp function using a P-gp substrate calcine-AM uptake assay. The investigation was performed in Caco-2, a human colon carcinoma derived cell lines, which widely utilized as an in vitro culture model for absorption study because of the expression of several multidrug proteins including P-gp [27]. The effects on P-gp were further confirmed in LLC-PK1 and LLC-GA5-COL300, which overexpresses human P-gp [28-29].

From the results, the flavedo extract gave the optimal inhibition effect on P-gp and exhibited a concentration-dependent manner (Table 1 and 2). To identify the active compounds in flavedo, column chromatography and PTLC techniques were employed. Consequently, five furanocoumarins, namely bergamottin, 6',7'-dihydroxybergamottin, 6',7'-epoxybergamottin, oxypeucedanin and oxypeucedanin hydrate were isolated (Fig. 1 and 2). Among 5 compounds isolated, 6',7'-epoxybergamottin, 6',7'-dihydroxybergamottin and oxypeucedanin significantly increased calcein accumulation in vitro study should be performed to substantiate these results. However, further evaluation in the in vivo study should be performed to substantiate these results.

The authors would like to thank the Research and creative Fund, Faculty of Pharmacy, Silpakorn University for financial support in part.

References

1. I. L. O. Buxton, L. Z. Benet, Goodman & Gilman’s The Pharmacological Basis of Therapeutics (The McGraw-Hill, New York, 2011)
2. K. M., Giacomini, Y. Sugiyama, Goodman & Gilman’s The Pharmacological Basis of Therapeutics (The McGraw-Hill, New York, 2011)
3. J. Yu, P. Zhou, J. Asenso, X. D., Yang, C. Wang, W. Wei, J Enzyme Inhib Med Chem. 31, 867 (2016)
4. R. Gupta, K. Kesarwani, Asian Pac J Trop Biomed. 3, 253 (2013)
5. G. Jain, U. K. Patil, IJPSR. 6, 5315 (2015)
6. E.F. Oga, S. Sekine, Y. Shitara, T. Horie, J Ethnopharmacol. 144, 612 (2012)
7. S. Chavhan, S. Shinde, H. Gupta, Int J Pharmacogn Chinese Med. 2, 1 (2018)
8. M.A. Lassoued, S. Sfar, A. Bouraoui, F. Khemiss, J Pharm Pharmacol. 64, 541 (2012)
9. X. Li, J.S. Choi, Int J Pharm. 337, 188 (2007)
10. H. Wangensteen, E. Molden, H. Christensen, K.E. Malterud, Eur J Clin Pharmacol. 58, 663 (2003)
11. O. Gökay, D. Kühner, M. Los, F. Götz, U. Bertsche, K. Albert, Anal Bioanal Chem. 398, 2039 (2010)
12. J.A. Manthey, K. Myung, S. Merten-Talcott, H. Derendorf, V. Butterweck, W.W. Widmer, Proc Fla State Hort Soc. 119, 361 (2006)
13. E. Frearot, E. Decorzant, J Agric Food Chem. 52, 6879 (2004)
14. K. Myung, J.A. Manthey, J.A. Narciso, Bioorg Med Chem Lett. 22, 2279 (2012)
15. T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, Cancer Res. 42, 4730 (1982)
16. M. Sababi, O. Borga, U. Hultkvist-Bengtsson, Eur J Pharm Sci. 14, 21 (2001)
17. R. Wongwanakul, N. Vardhanabhuti, P. Siripong, S. Jianmongkol, Fitoterapia. 89, 80 (2013)
18. E.L.M. Jouan, A. Mayati, C. Denizot, Y. Parmentier, O. Fardel, Pharmaceutics. 8, (2016)
19. Y. Li, J. Revalde, J.W. Paxton, Adv Drug Deliv Rev. 116, 45 (2017)
20. K. Ueda, Biosci Biotechnol Biochem. 75, 401 (2011)
21. R. Krishna, L. D. Mayer, Eur J Pharm Sci. 11, 265 (2000)
22. E. Chieli, N. Romiti, I. Rodeiro, G. Garrido, Food Chem Toxicol. 47, 2703 (2009)
23. R. J. Choi, T. M. Ngoc, H. J. Cho, D.-D. Kim, J. Chun, S. Khan, Y. S. Kim, Eur J Pharm Sci. 48, 272 (2013)
24. T. Nabekura, T. Yamaki, T. Hiroi, K. Ueno, S. Kitagawa, Pharmacol Res. 61, 259 (2010)
25. R. Wongwanakul, N. Vardhanabhuti, P. Siripong, S. Jianmongkol, Fitoterapia. 89, 80 (2013)
26. J. Knop, S. Misaka, K. Singer, E. Hoier, F. Müller, H. Glaeser, J. Konig, M. F. Fromm, PLoS ONE. 10, (2015)
27. E.F. Oga, S. Sekine, Y. Shitara, T. Horie, J Ethnopharmacol. 144, 612 (2012)
28. K. Ueda, N. Okamura, M. Hirai, Y. Tanigawara, N. Okamura, M. Hirai, Y. Tanigawara, T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, Cancer Res. 42, 4730 (1982)
29. Y. Tanigawara, N. Okamura, M. Hirai, M. Shibata, T. Hiroi, K. Ueno, S. Kitagawa, Pharmacol Res. 61, 259 (2010)