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ORIGINAL ARTICLE

Liquiritin Enhancing Intestinal Absorption of Paeoniflorin in *in situ* Single-pass Intestinal Perfusion and *in vitro* Caco-2 Cell Monolayer Absorption Models

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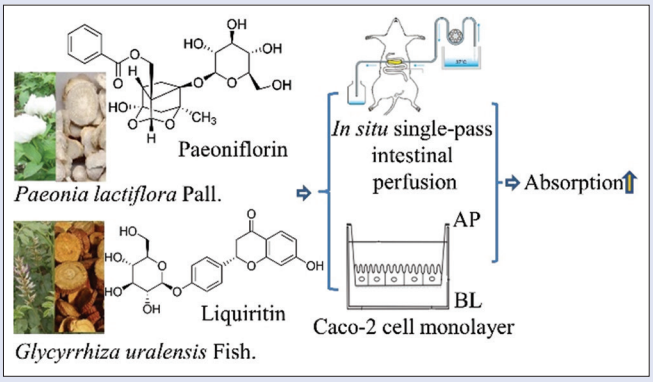
ABSTRACT

Background: Paeoniflorin and liquiritin are the primary active components of Shaoyao-Gancao-tang (SGT), a classical prescription for reducing pains. However, the interaction of paeoniflorin and liquiritin during intestinal absorption needs to be further studied. **Objectives:** In this study, we aimed to determine the interaction of paeoniflorin and liquiritin during intestinal absorption. **Materials and Methods:** The interaction between paeoniflorin and liquiritin (100 μM) was studied using *in situ* single-pass intestinal perfusion (SPIP) model use the whole small intestine and *in vitro* Caco-2 cell monolayer bidirectional transport model. **Results:** *In situ* SPIP research demonstrated that liquiritin significantly increased the K_a, P_{app}, absorption rate, and cumulative amount of paeoniflorin up to 7.97, 8.98, 7.07, and 10.71 folds, respectively, even higher than that of verapamil, a specific P-gp inhibitor, and control. Furthermore, 18 β-glycyrrhetinic acid (18 β-GA) markedly increased the K_a, P_{app}, absorption rate, and cumulative amount of paeoniflorin up to 3.30, 3.27, 3.42, and 4.04 folds, respectively. Bidirectional transport studies indicated that liquiritin and paeoniflorin could prompt the absorption of each other by increasing the $P_{app\ (AP\text{-}BL)}$ of paeoniflorin and liquiritin from (3.83 ± 0.51) ×10^{-7} to (5.60 ± 0.51) ×10^{-7} cm/s and (3.86 ± 0.34) ×10^{-7} to (8.26 ± 0.51) ×10^{-7} cm/s, respectively. The 18 β-GA significantly prompted the $P_{app\ (AP\text{-}BL)}$ of paeoniflorin to (5.54 ± 0.92) ×10^{-7} cm/s. **Conclusion:** Liquiritin and paeoniflorin increased the absorption of each other. This could provide essential reference to predict the oral bioavailability, the pharmacokinetics, and the clinical application of coadministration of liquiritin-and paeoniflorin-containing SGT and other herbal formulas.

Key words: Flavonoid, *Glycyrrhiza uralensis* Fish, *Paeonia lactiflora* Pall, saponins, Shaoyao-Gancao-tang

SUMMARY

- There is a scarcity of information regarding the absorptive interaction of liquiritin and paeoniflorin, the main constituents of Shaoyao-Gancao-tang (SGT). Liquiritin and paeoniflorin might prompt intestinal absorption of each other, which we studied by conducting deep and intensive study through *in situ* single-pass intestinal perfusion and *in vitro* Caco-2 cell monolayer absorption models.
- The results of this study might help to understand the implied mechanism of synergistic therapeutic effect of SGT to some extent and provide essential information for predicting the oral bioavailability and pharmacokinetics of coadministration of liquiritin-and paeoniflorin-containing prescriptions and herbal formulas.



Abbreviations used: PF: Paeoniflorin; SGT: Shaoyao-Gancao-tang; 18 β-GA: 18 β-glycyrrhetinic acid; TCM: Traditional Chinese Medicine; TFA: Trifluoroacetic acid; DMEM: Hyclone Dulbecco's Modified Eagle Medium; PBS: Phosphate buffered solution; HBSS: Hanks' Balanced Salt Solution; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; FBS: Fetal bovine serum; NEAA: Nonessential amino acid; K–R: Krebs-Ringer; P_{app}: Permeability coefficient; AR: Absorption rate; TEER: Transepithelial Electrical Resistance; AP-BL: Apical-to-basolateral; BL-AP: Basolateral-to-apical; ER: Efflux ratio.

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INTRODUCTION

Shaoyao-Gancao-tang (SGT) is a classical prescription composed of equal proportions of Baishao (the dry root of *Paeonia lactiflora* Pall. from *Ranunculaceae*) and Gancao (radix of *Glycyrrhiza uralensis* Fish. from *Leguminosae*). It has been widely used in China,[1] Korea,[2] and Japan[3-5] for reducing pains, owing to its anti-inflammatory and anti-spasmodic effects. It is the representative prescription to relieve pain and spasm in Traditional Chinese Medicine (TCM). In addition, the Shaoyao-Gancao drug pair is used extensively in

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25 prescriptions in Zhang Zhongjing's Treatise on Febrile Diseases (containing 115 prescriptions in total) and in 5866 prescriptions in database of Chinese medicine prescriptions (containing 96,593 TCM prescriptions in total) with the proportion of 6.1%.[6] It is well known that the combination of Shaoyao and Gancao has synergistic effect. The therapeutic effects of SGT result from the primary constituents of these two herbs, such as paeoniflorin, glycyrrhizin, and liquiritin. Interestingly, paeoniflorin, glycyrrhizin, and liquiritin are poorly absorbed.[7,8] Our previous study[9] demonstrated that the absorption of paeoniflorin was affected by the main ingredients of Gancao which is glycyrrhizin, and its dominant metabolite, namely 18 β-glycyrrhetinic acid (18 β-GA). Literature demonstrates the interaction between the constituents of Shaoyao and Gancao during intestinal absorption.[1,8,10,11] However, the absorptive interaction of liquiritin and paeoniflorin has been little reported.

Liquiritin is the primary flavonoid of Gancao. Paeoniflorin is the main saponin of Shaoyao. Although liquiritin and paeoniflorin are poorly absorbed,[7-9] they have many pharmacological activities, such as antinociceptive,[3,12] anti-inflammatory,[1] antioxidant,[13,14] antidiabetic,[15] antiasthma,[16] antirheumatoid arthritis,[17,18] immunoregulatory,[19] and preventing rat hearts from ischemia/reperfusion injury effects.[20] Coadministration of liquiritin and paeoniflorin has synergistic pharmacological effect.[21] Therefore, the absorptive interaction of liquiritin and paeoniflorin needs to be further studied.

Therefore, in this study, we focused on the absorptive interaction of liquiritin and paeoniflorin using two absorption models, *in situ* single-pass intestinal perfusion (SPIP) model and *in vitro* Caco-2 cell monolayer model. In addition, we further confirmed the absorption effect of glycyrrhizin and 18 β-GA on paeoniflorin by performing the aforementioned two methods as previously we only performed the everted rat gut sac study.[9] Samples were analyzed for paeoniflorin, liquiritin, 18 β-GA, and glycyrrhizin concentration using reversed-phase high-performance liquid chromatography (HPLC) with diode array detector. This might help to ascertain the mechanism of synergistic therapeutic effect of SGT to some extent, provide useful information for predicting the oral bioavailability, pharmacokinetics of coadministration of liquiritin-and paeoniflorin-containing herbal formulas and prescriptions, and offer a reference for clinical application of SGT and liquiritin-and paeoniflorin-containing herbal formulas or prescriptions.

MATERIALS AND METHODS

Chemicals and reagents

Reference substance of paeoniflorin (98%), 18 β-GA (98%), verapamil hydrochloride (98%), and ammonium glycyrrhizate (98%) was obtained from Sigma-Aldrich (Beijing, China). Paeoniflorin (98%) and liquiritin (98%) were supplied by Chengdu Push Bio-Technology Co., Ltd. (Sichuan, China). Trifluoroacetic acid (TFA) and acetonitrile (both HPLC-grade) were provided by Fisher Scientific agented by Beijing Honghu Lianhe Huagong Chanpin co., Ltd (Beijing, China). Deionized Milli-Q (Millipore, Bedford, MA, USA) water was employed. Other reagents were purchased from Beijing Chemical Company (Beijing, China).

High glucose Dulbecco's Modified Eagle Medium (DMEM) and penicillin and streptomycin solution (10,000 units/mL and 10,000 μg/mL, respectively) were provided by HyClone Company (Logan, Utah, USA). Moreover, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT); Hanks' balanced salt solution (with Ca^{2+} and Mg^{2+}) (HBSS); phosphate-buffered solution; and dimethyl sulfoxide (DMSO) were purchased from Beyotime (Shanghai, China). Fetal bovine serum (FBS), ×100 MEM non-essential amino acid (NEAA) solution, 0.25% trypsin-EDTA (×1), and other reagents were purchased from Gibco Life Technologies Corporation (Grand Island, NY, USA). Tissue culture plastics and 12-well (pore size 0.4 μm, diameter12 mm) Transwell™ were purchased from Corning Costar. Corp. (Cambridge, MA, USA).

Animals

Healthy male Sprague-Dawley rats (190 ± 20 g) were provided from Weitonglihua Laboratory Animal Services Center (Changping, Beijing, China) and were kept in standard conditions with free access to food and water and a 12 h light/dark cycle. The animals were adapted to the conditions about 1 week before the experiment. The rats suffered no <12 h starvation before the experiment, and water was available *ad libitum*. Animal studies were performed according to rules and guidelines ratified by the Institutional Animal Care and Use Committee of Capital Medical University, and all animals used received humane care.

In situ single-pass intestinal perfusion research

In situ SPIP was performed according to the previous experimental design with slight modification.[22,23] Briefly, rats were intraperitoneally injected with urethane (1g/kg) for anesthetization. The intestinal segment was exposed through a midline incision. An inflow cannula was inserted in the duodenum approximately 1 cm below the pylorus. An outflow cannula was set up at a place 10 cm upward the terminal ileum. The whole small intestinal segment including the duodenum, the jejunum, and the ileum was then flushed with 37°C warmed saline solution and then was perfused with Krebs–Ringer (K–R) buffer (133 mM NaCl, 3.33 mM $CaCl_2$, 4.69 mM KCl, 0.210 mM $MgCl_2$, 2.67 mM NaH_2PO_4, 16.3 mM $NaHCO_3$, and 7.78 mM glucose, pH 7.4) first using 1.0 mL/min for 10 min and then using 0.2 mL/min for 20 min for equilibration using a BT100-1 L longer peristaltic pump (longer precision pump Co., Ltd, Baoding, Hebei, China). Subsequently, the intestinal segment was perfused with K–R buffer containing 100 μM paeoniflorin with or without 100 μM liquiritin/18 β-GA/glycyrrhizin/verapamil using 0.2 mL/min constantly for 60 min. Perfusate sample was collected every 15 min. Finally, the length and width of the intestinal segment were determined after being cut open.

The test and collection samples were lyophilized and dissolved well in 500 μL HPLC grade methanol. After filtration, the samples were analyzed via HPLC.

Intestinal permeability coefficient P_{app} was calculated by using the formula as follows: $P_{app} = v\ln (C_{out\ c}/C_{in})/(2\ \pi rl)$.

Intestinal absorption rate constant K_a was calculated by using the equation as follows: $K_a = (1 - [C_{out\ c}/C_{in}] \times [v/[\pi r2l])$.

Total absorption amount (μmol/cm^2) was calculated as $Q = 15\ v\ (C_{in} - C_{out\ c})/(2\ \pi rl)$.

Absorption rate (%) was calculated as $AR = (1 - C_{out\ c}/C_{in}) \times 100\%$.

Where *v* is the perfusion flow rate of the intestinal segment (0.2 mL/min), $C_{out\ c}$ is the outlet concentration (μmol/mL) of the perfused compound corrected by weight or density, C_{in} is the inlet concentration (μmol/mL) of the perfused compound, *l* and *r* were the lengths and the radius of the perfused segment.

Paeoniflorin and glycyrrhizin were freshly prepared in 50% and 10% ethanol, respectively. Liquiritin, 18 β-GA, and verapamil were freshly prepared in ethanol. Solutions were further diluted in K–R solution with a final ethanol concentration of less than 1% (v/v).

Cell culture

Caco-2 cell line was bought from the Chinese Academy of Medical Sciences, Peking Union Medical College (Beijing, China), and passage 30 and 40 were used. Cells were incubated in high glucose DMEM containing 15% FBS, penicillin–streptomycin (100 units/mL and 100 μg/mL), and 1% NEAA at 37°C in 5% CO_2 air atmosphere. Medium was replaced every 2 days. In transport study, cells were seeded on insert in 12-well Transwell® with 2×10^5 cells/cm^2. The fresh medium (1.5 mL in the well and 0.5 mL in the insert) was refreshed on the 2nd day and then every 2 days for the first 7 days and every day thereafter. Transepithelial Electrical Resistance (TEER), determined by Millipore Millicell ERS2 volt-ohm meter (Merck Millipore, Bedford, MA, USA), was employed to monitor the integrity of the monolayers.

Cellular viability study using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium br omide assay

The cytotoxic effects of compounds under investigation were examined by performing the MTT assay. Cells were seeded on 96-well plate (1×10^4 cells/well) and incubated for 24 h. Subsequently, they were treated with paeoniflorin, liquiritin, glycyrrhizin, and 18 β-GA (1000, 500, 250, 125, 62.5, 31.2, and 15.6 μM) for 24 h. Then, 0.05 mg MTT in phenol red-free DMEM was added to cells and incubated for 4 h at 37°C. The medium was discarded and 100 μL of DMSO (100%) was added per well to dissolve the crystals formed. After dissolution at 37°C for 10 min, the absorbance was measured at 490 nm on a SPECTRA MAX PLUS 384 microplate reader (Thermo Scientific, Waltham, USA). Cell viability was calculated using the formula as follows: cellular viability (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank})$

Where OD_{sample} is absorption of sample, $OD_{control}$ is absorption of control, and OD_{blank} is absorption of blank.

Paeoniflorin, liquiritin, 18 β-GA, glycyrrhizin, and verapamil were freshly prepared in DMSO. The solutions were further diluted in DMEM, and the concentration of DMSO was always ≤ 0.5% (v/v).

Bidirectional transport studies

Monolayer cells with TEER >500 Ωcm^2[24] were used for the transport experiment at 16–21 days after seeding. After washing the monolayer twice with HBSS (37°C), the cells were preincubated in HBSS for 30 min at 37°C. Then, HBSS buffer in the well and the insert was discarded. In apical-to-basolateral (AP-BL) transport experiment, the drug solution in HBSS buffer was put into the insert and HBSS buffer was added to the well. In basolateral-to-apical (BL-AP) transport study, the drug was put into the well and HBSS was added to the insert. The donor compartment was referred to the drug-containing compartment (paeoniflorin, liquiritin, 18 β-GA, glycyrrhizin, paeoniflorin-liquiritin, paeoniflorin-18 β-GA, and paeoniflorin-glycyrrhizin, 100 μM) and the acceptor compartment was referred to the sample withdrawing compartment. A 200 μL sample was withdrawn from the acceptor compartment at 20, 40, 60, 80, and 120 min, respectively, and 200 μL of fresh prewarmed HBSS buffer was replaced. The inhibitory effects of verapamil on paeoniflorin flux were investigated by adding verapamil (100 μM) to both sides. All experiments were conducted in triplicates at 37°C. Paeoniflorin, liquiritin, 18 β-GA, glycyrrhizin, and verapamil were prepared freshly in DMSO before the experiment. The solutions were further diluted in HBSS buffer, and the concentration of DMSO was always ≤0.5% (v/v).

The collected samples were lyophilized and dissolved in 100 μL methanol. After filtration, the samples were used for HPLC analysis of paeoniflorin, liquiritin, 18 β-GA, and glycyrrhizin.

The P_{app}, apparent permeability coefficient in cm/s and the efflux ratio (ER) were calculated as follows:

$P_{app} = (\Delta Q/\Delta t) \times (1/[A \times C_0])$

Where $\Delta Q/\Delta t$ is cumulative transport rate of the constituent on the acceptor compartment (μmol/s), A is the superficial area of the insert or cell monolayer (1.13 cm^2), and C_0 is the initial concentration of the constituent in the donor compartment (μmol/L).

$ER = P_{app\ (BL\text{-}AP)}/P_{app\ (AP\text{-}BL)}$

Where $P_{app\ (BL\text{-}AP)}$ was the P_{app} from BL-AP side and $P_{app\ (AP\text{-}BL)}$ was the P_{app} from AP-BL side.

Determination of paeoniflorin, liquiritin, 18 β-GA, and glycyrrhizin via high-performance liquid chromatography

The content of paeoniflorin, liquiritin, 18 β-GA, and glycyrrhizin were determined using Agilent 1100 HPLC with a diode-array UV-Vs detector. The experiment was conducted on a Kromasil 100-5 C_{18} column (250 mm × 4.6 mm, 5 μm) with a flow rate of 1 mL/min with the detection wavelength of 230 nm, 276 nm, 250 nm, and 250 nm, respectively.

For the intestinal perfusion samples, the mobile phase was 14% acetonitrile in water containing 0.05% TFA. The calibration curve was linear from 5 to 200 μM of paeoniflorin ($Y = 6.3678X - 13.676$, $R^2 = 0.9996$). Y is the area of the peak; X is the paeoniflorin concentration. The samples were diluted appropriately to accommodate the calibration curve.

For the transport samples, the mobile phase consisted of 0.05% (v/v) TFA in acetonitrile (A) and water (B). A gradient elution was used: 20% A for 0–12 min, 20%–90% A for 12–15 min, 90% A for 15–18 min, 90%–100% A for 18–20 min, and 100% A for 20–22 min. Each calibration curve was established by using a mixture of four standards in seven concentration ranges. The calibration curve was linear from 5 to 100 μM of paeoniflorin ($Y = 11.155X+11.447$, $R^2 = 0.9985$), liquiritin ($Y = 13.758X-18.467$, $R^2 = 0.9993$), 18 β-GA ($Y = 13.886X-20.443$, $R^2 = 0.9987$), and glycyrrhizin ($Y = 10.224X-0.19528$, $R^2 = 0.9990$). Y is the area of the peak; X is the concentration of the constituent.

Statistical analysis

The data were analyzed through one-way analysis of variance using SPSS (IBM, Armonk, New York, USA) software version 19.0, and the results are expressed as mean ± standard deviation ($n = 3$). P values <0.05 were considered as statistically significant.

RESULTS

In situ single-pass intestinal perfusion studies

Effects of liquiritin, 18 β-glycyrrhetinic acid, glycyrrhizin, and verapamil on K_a of paeoniflorin

As shown in Figure 1, liquiritin enhanced the intestinal absorption rate constant (K_a) of paeoniflorin, by up to 7.97 folds in perfused entire small intestine segment including the duodenum, the jejunum, and the ileum. The enhancement was even higher than that of verapamil, a specific P-gp inhibitor, which increased K_a of paeoniflorin by up to 3.02 folds. Furthermore, 18 β-GA also markedly ($P < 0.05$) increased the K_a of paeoniflorin, by up to about 3.30 folds. However, glycyrrhizin had no effect on the K_a of paeoniflorin.

Effects of liquiritin, 18 β-glycyrrhetinic acid, glycyrrhizin, and verapamil on P_{app} of paeoniflorin

Figure 2 shows that liquiritin significantly ($P < 0.01$) improved the intestinal permeability coefficient (P_{app}) of paeoniflorin, by up to

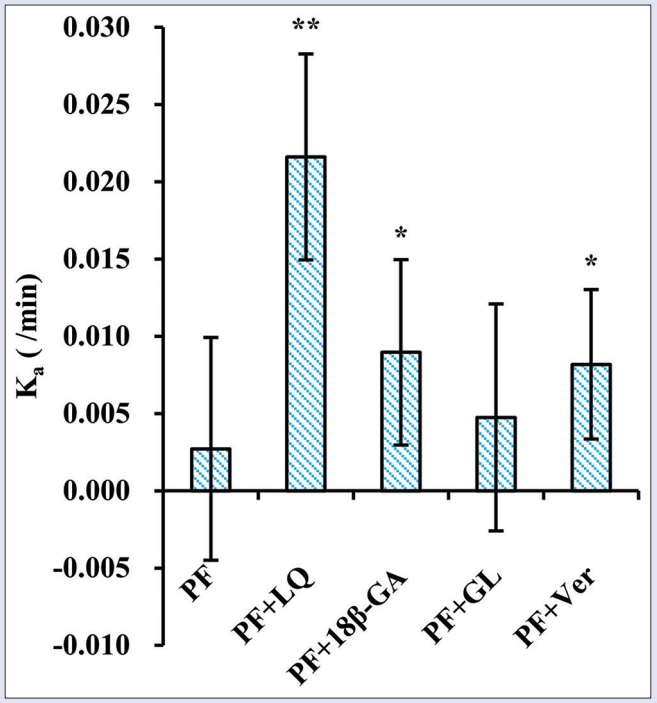

Figure 1: Effects of LQ, 18 β-GA, glycyrrhizin, and verapamil on K_a of paeoniflorin *in situ* single-pass intestinal perfusion studies. LQ, 18 β-GA, and verapamil significantly enhanced (**P < 0.01, *P < 0.05), the K_a of paeoniflorin in perfused segments. Date was shown as mean ± SD (n = 3). PF: Paeoniflorin; LQ: Liquiritin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: Glycyrrhizin; Ver: Verapamil; SD: Standard deviation

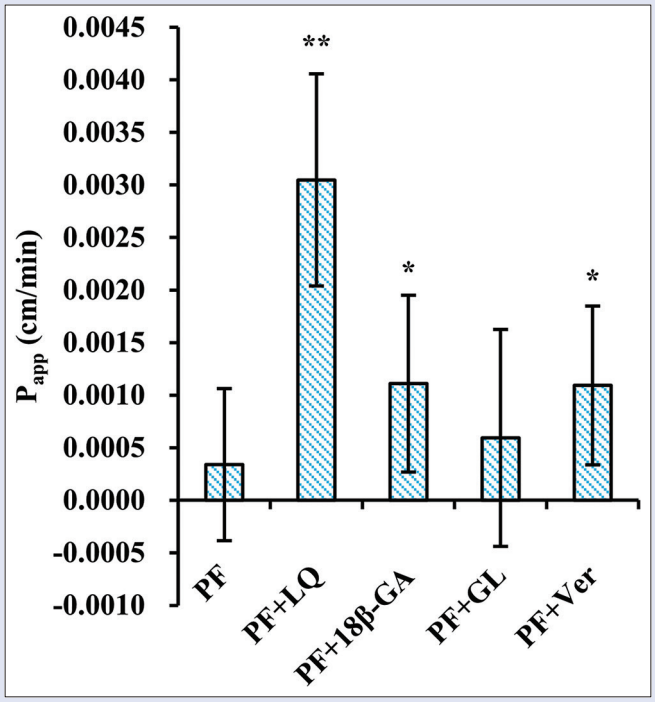

Figure 2: Effects of LQ, 18 β-GA, glycyrrhizin, and verapamil on P_{app} of paeoniflorin *in situ* single-pass intestinal perfusion studies. LQ, 18 β-GA, and verapamil significantly enhanced (**P < 0.01, *P < 0.05), the P_{app} of paeoniflorin in perfused segments. Date was shown as mean ± SD (n = 3). PF: Paeoniflorin; LQ: Liquiritin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: Glycyrrhizin; Ver: Verapamil; SD: Standard deviation

8.98-fold in perfused entire small intestine. The enhancement was even higher than that of verapamil, which increased (P < 0.05) the P_{app} of paeoniflorin by up to 3.22-fold. Furthermore, 18 β-GA also improved the P_{app} of paeoniflorin significantly (P < 0.05), by up to 3.27 folds, about the same as verapamil. Glycyrrhizin had no effect on the P_{app} of paeoniflorin.

Effects of liquiritin, 18 β-glycyrrhetinic acid, glycyrrhizin, and verapamil on the absorptivity of paeoniflorin

As illustrated in Figure 3, during the *in situ* SPIP studies, liquiritin, 18 β-GA, and verapamil increased the intestinal absorption rate of paeoniflorin by up to 7.07-, 3.42-, and 3.69-fold, respectively. The increase in the rate of absorption of paeoniflorin by liquiritin was higher than that of verapamil. However, glycyrrhizin exhibited no enhancement to absorptivity of paeoniflorin when co-administered with paeoniflorin.

Effects of liquiritin, 18 β-glycyrrhetinic acid, glycyrrhizin, and verapamil on total absorption of paeoniflorin

Figure 4 shows the cumulative amount of paeoniflorin absorbed in perfused entire small intestine when co-administered with liquiritin, 18 β-GA, glycyrrhizin, and verapamil. The figure shows that total absorption of paeoniflorin increased significantly when it was co-administered with liquiritin (P < 0.01), 18 β-GA (P < 0.05), and verapamil (P < 0.01) by up to 10.71-, 4.04-, and 4.74-fold, respectively. Nevertheless, the increase in the total absorption of paeoniflorin co-administered with glycyrrhizin was not significant.

In situ SPIP studies [Figures 1-4] demonstrated that liquiritin significantly increased (P < 0.01) the K_a, P_{app}, absorption rate, and cumulative amount of paeoniflorin by up to 7.97-, 8.98-, 7.07-, and 10.71-fold, respectively. The increase was even higher than that of verapamil, a known P-gp inhibitor. Furthermore, 18 β-GA also markedly enhanced (P < 0.05) the K_a, P_{app}, absorption rate, and cumulative amount of paeoniflorin by up to 3.30-, 3.27-, 3.42-, and 4.04-fold, respectively, and the enhancement was similar to verapamil (3.02-, 3.22-, 3.69-, and 4.74-fold, respectively). Glycyrrhizin showed no obvious influence on the K_a, P_{app}, absorption rate, and cumulative amount of paeoniflorin in perfused entire small intestine including the duodenum, the jejunum, and the ileum.

Caco-2 cell monolayer studies

Cell viability of test compounds

As shown in Figure 5, paeoniflorin, liquiritin, 18 β-GA, and glycyrrhizin (125, 250, 125, and 250 μM, respectively) showed no toxic effects on Caco-2 cells and over 90% of the cells were viable. According to these results, a noncytotoxic concentration (100 μM) was selected for further experiments to maintain cell viability.

Bidirectional transport interaction of paeoniflorin and liquiritin by Caco-2 cells

Co-administered with liquiritin, the transport of paeoniflorin from AP-BL (absorption direction) significantly increased and the apparent permeability coefficient, $P_{app\ (AP-BL)}$ of paeoniflorin improved from (3.83 ± 0.51) ×10^{-7} cm/s to (5.60 ± 0.51) ×10^{-7} cm/s. However, the transport of paeoniflorin from BL-AP (secretory direction) did not decrease [Figure 6]. These results demonstrated that liquiritin improved the absorption of paeoniflorin via increase of the transport of paeoniflorin from AP-BL and not decrease of BL-AP transport. Co-administered with paeoniflorin, the AP-BL transport of liquiritin was increased markedly, and the $P_{app\ (AP-BL)}$ of liquiritin increased from (3.86 ± 0.34) ×10^{-7} cm/s to (8.26 ± 0.51)

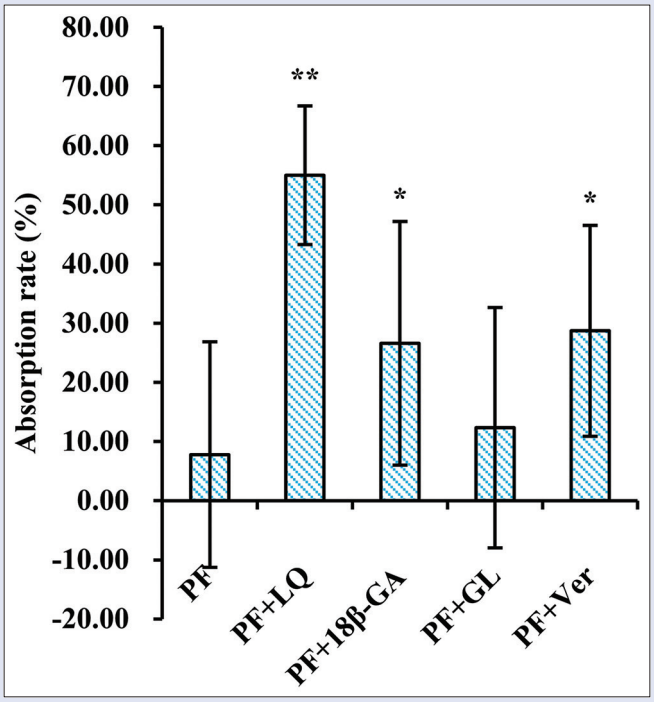

Figure 3: Effects of LQ, 18 β-GA, glycyrrhizin, and verapamil on absorptivity of paeoniflorin *in situ* single-pass intestinal perfusion studies. LQ, 18 β-GA, and verapamil significantly improved (**P < 0.01, *P < 0.05) the absorptivity of paeoniflorin in perfused segments. Date was shown as mean ± SD (n = 3). PF: Paeoniflorin; LQ: Liquiritin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: Glycyrrhizin; Ver: Verapamil; SD: Standard deviation

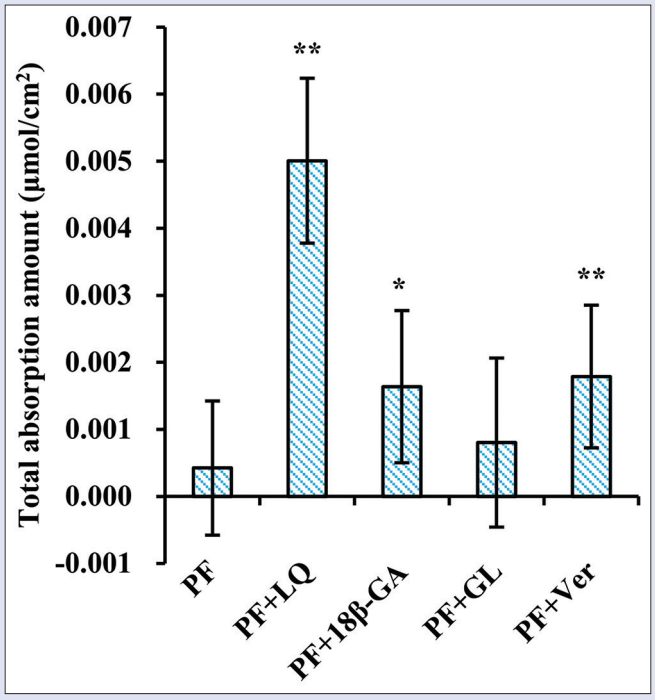

Figure 4: Effects of LQ, 18 β-GA, glycyrrhizin, and verapamil on total absorption amount of paeoniflorin *in situ* single-pass intestinal perfusion studies. LQ, 18 β-GA, and verapamil significantly increased (**P < 0.01, *P < 0.05) total absorption amount of paeoniflorin in perfused segments. Date was shown as mean ± SD (n = 3). PF: Paeoniflorin; LQ: Liquiritin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: Glycyrrhizin; Ver: Verapamil; SD: Standard deviation

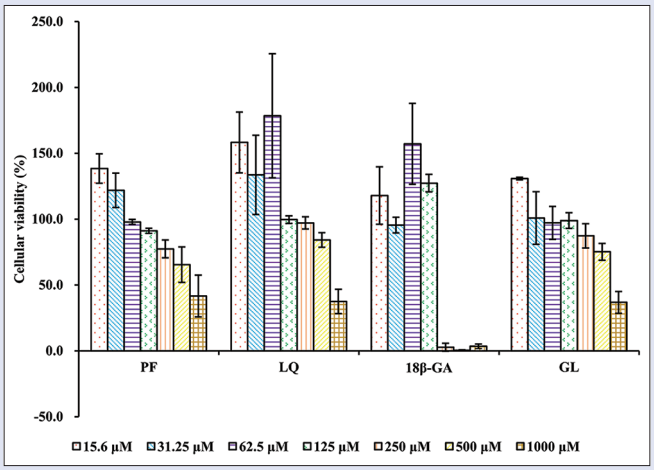

Figure 5: Cell viability of test compounds at different concentrations by MTT assay. Date was shown as mean ± SD (n = 3). PF: Paeoniflorin; LQ: Liquiritin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: Glycyrrhizin; SD: Standard deviation; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

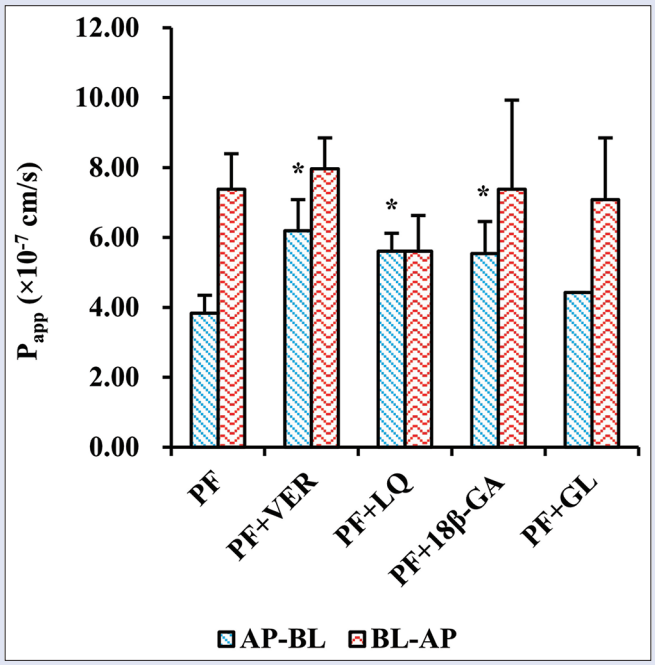

Figure 6: Effects of LQ, 18 β-GA, glycyrrhizin, and verapamil on bidirectional transports of paeoniflorin on the Caco-2 cell monolayer. Data were presented as the mean ± SD (n = 3). *P < 0.05, $P_{app\ (AP-BL)}$ of those groups versus $P_{app\ (AP-BL)}$ of group paeoniflorin. PF: Paeoniflorin; LQ: Liquiritin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: glycyrrhizin; Ver: Verapamil; SD: Standard deviation

$\times 10^{-7}$ cm/s, whereas the BL-AP transport of liquiritin had no obvious change [Figure 7]. These results suggested that the absorption of liquiritin was improved by paeoniflorin through increase of the AP-BL transport. Commonly, the absorption of combinational drugs can be prompted by increasing the AP-BL transport, or decreasing the BL-AP transport, or both. Here, results indicate that liquiritin

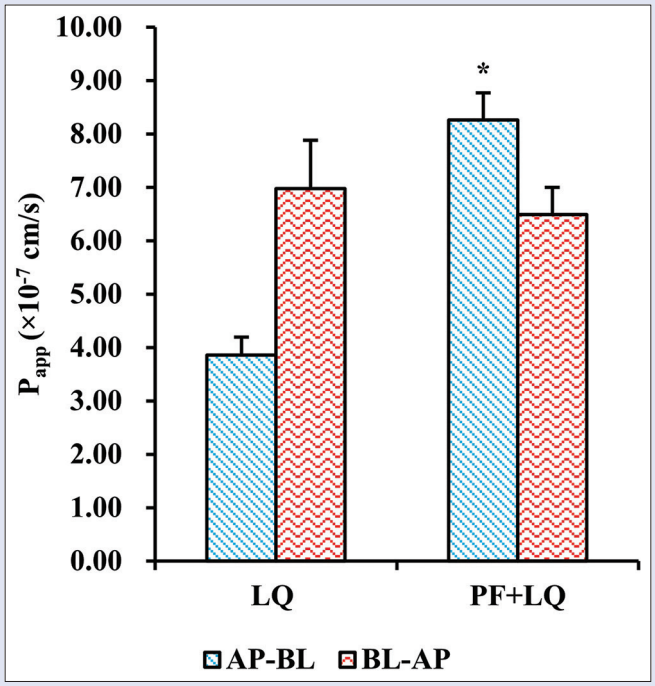

Figure 7: Effects of paeoniflorin on bidirectional transports of LQ on the Caco-2 cell monolayer. Data were presented as the mean ± SD (n = 3). *P < 0.05, $P_{app\ (AP-BL)}$ of group PF + LQ *vs.* $P_{app\ (AP-BL)}$ of group LQ. PF: Paeoniflorin; LQ: Liquiritin; SD: Standard deviation

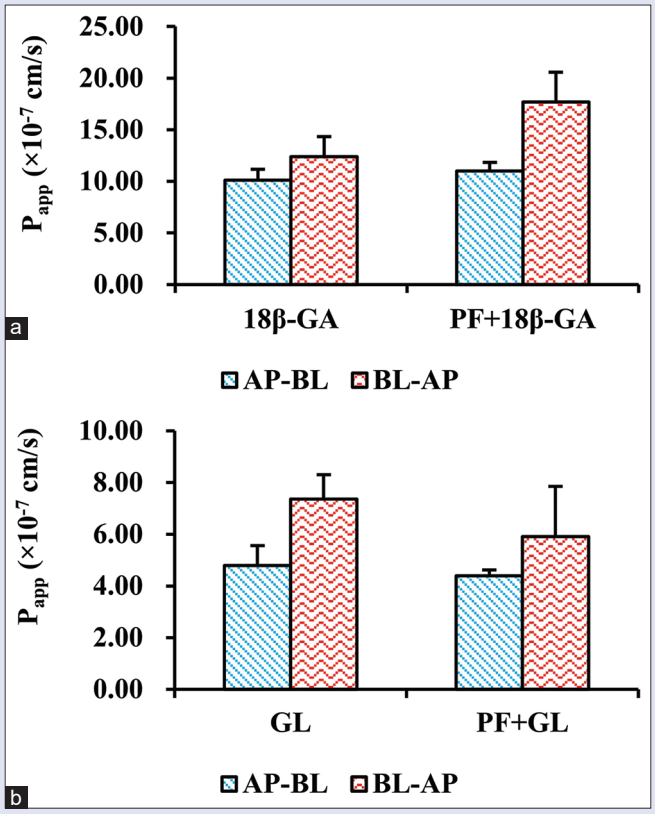

Figure 8: Effects of PF on bidirectional transports of 18 β-GA (a) and glycyrrhizin (b) on the Caco-2 cell monolayers. Data were expressed as the mean ± SD (n = 3). PF: Paeoniflorin; 18 β-GA: 18 β-glycyrrhetinic acid; GL: Glycyrrhizin; SD: Standard deviation

and paeoniflorin prompt absorption of each other only by increasing the AP-BL transport.

Bidirectional transport interaction of paeoniflorin and 18 β-GA and paeoniflorin and glycyrrhizin by Caco-2 cells

The 18 β-GA significantly prompted the AP-BL transport of paeoniflorin and increased the $P_{app\ (AP-BL)}$ of paeoniflorin to (5.54 ± 0.92) $\times10^{-7}$ cm/s; however, it did not influence on the BL-AP transport of paeoniflorin [Figure 6]. This shows that 18 β-GA increased paeoniflorin absorption by increasing the AP-BL absorption. However, paeoniflorin had no effect on the transport of 18 β-GA on both sides [Figure 8a].

As shown in Figures 6 and 8b, paeoniflorin and glycyrrhizin showed no influence on each other's bidirectional transport. This agrees with the results of intestinal perfusion studies.

Bidirectional transport studies indicated that liquiritin and paeoniflorin could prompt absorption of each other. Moreover, 18 β-GA significantly prompted the absorption of paeoniflorin.

DISCUSSION

Bidirectional transport [Table 1] of paeoniflorin, liquiritin, 18 β-GA, and glycyrrhizin through the Caco-2 cell monolayer indicated that for AP-BL transport, the $P_{app\ (AP-BL)}$ value of paeoniflorin, liquiritin, and glycyrrhizin was (3.83 ± 0.51) $\times10^{-7}$ cm/s, (3.86 ± 0.34) $\times10^{-7}$ cm/s, and (4.80 ± 0.77) $\times10^{-7}$ cm/s, respectively, suggesting that the absorption of paeoniflorin, liquiritin, and glycyrrhizin was poor. Moreover, 18 β-GA, the main hydrolysate of glycyrrhizin, had higher $P_{app\ (AP-BL)}$ ((1.01 ± 1.06) $\times10^{-6}$ cm/s), indicating that 18 β-GA was well absorbed across the Caco-2 cells. These results are consistent with the previous reports,[7,8] which show that paeoniflorin, liquiritin, and glycyrrhizin also have poor absorption. According to Yee,[25] paeoniflorin, liquiritin, and glycyrrhizin with

Table 1: The P_{app} ($\times10^{-7}$ cm/s) of paeoniflorin, liquiritin, 18β-glycyrrhetinic acid, and glycyrrhizin by caco-2 cells

Title 1	Paeoniflorin	Liquiritin	18β-GA	Glycyrrhizin
$P_{app\ (AP-BL)}$	3.83±0.51	3.86±0.34	10.12±1.06	4.80±0.77
$P_{app\ (BL-AP)}$	7.37±1.02**	6.97±0.91**	12.39±1.95	7.36±0.94*
ER	1.92	1.81	1.22	1.54

*P<0.05; $P_{app\ (AP-BL)}$ versus. $P_{app\ (BL-AP)}$; **P<0.01; Data were expressed as mean±SD (n=3). ER=$P_{app\ (BL-AP)}$/$P_{app\ (AP-BL)}$; $P_{app\ (BL-AP)}$ is Papp from the apical to basolateral (BL-AP) side and $P_{app\ (AP-BL)}$ is P_{app} from the apical to basolateral (AP-BL) side; BL: Basolateral; AP: Apical; GA: Glycyrrhetinic acid; ER: Efflux ratio; SD: Standard deviation

P_{app} < 1 × 10^{-6} cm/s only 20% or less were absorbed in humans. Moreover, 18 β-GA was absorbed moderately (20-70%) in human. BL-AP transports of paeoniflorin (P < 0.01), liquiritin (P < 0.01), and glycyrrhizin (P < 0.05) were dramatically higher than that of AP-BL transports. The ER of paeoniflorin, liquiritin, and glycyrrhizin was 1.92, 1.81, and 1.54, respectively, which is higher than 1.5, suggesting that the transport of these compounds might be mediated by efflux transporters.

Bidirectional transport studies indicated that paeoniflorin, liquiritin, and glycyrrhizin were poorly absorbed (the P_{app} < 1.0 × 10^{-6} cm/s), whereas 18 β-GA had higher absorption (the P_{app} >1.0 × 10^{-6} cm/s). The transport of paeoniflorin, liquiritin, and glycyrrhizin might be mediated by efflux transporters. Liquiritin and paeoniflorin might prompt the absorption of each other. Moreover, 18 β-GA significantly prompted the absorption of paeoniflorin.

In situ SPIP and cell-based assay using Caco-2 are the most commonly used absorption models. The *in situ* SPIP model[26] is recognized by the United States Food and Drug Administration and is useful for assessing a chemical's absorption characteristics. Human colonic adenocarcinoma Caco-2 cells are widely accepted for evaluating human intestinal absorption because they share similar morphological and functional characteristics with human small intestinal mature enterocytes. Moreover, it is reported that some important transporter proteins for instance MRP2, P-gp, and BCRP, are functionally expressed in Caco-2 monolayer cells.[22] Caco-2 cell lines are extensively employed for predicting absorption *in vivo* in humans and to investigate transport mechanisms. However, results obtained from cell culture models are usually affected by the experimental conditions. Conversely, the *in situ* perfusion model forcefully reflects the *in vivo* intestinal absorption situation that compounds actually happened after oral dosing. Hence, here *in situ* perfusion model was utilized with Caco-2 cell model to mainly evaluate the influence of liquiritin on paeoniflorin absorption. In addition, we confirmed the effect of 18 β-GA and glycyrrhizin on paeoniflorin absorption and the participation of P-gp in the efflux transport of paeoniflorin during the intestinal absorption.

When paeoniflorin (100 μM) was co-perfused with verapamil (100 μM), a specific P-gp inhibitor, in the *in situ* intestinal perfusion model, the K_a, P_{app}, absorption rate, and cumulative amount of paeoniflorin were increased significantly [Figures 1-4] by up to 3.02, 3.22, 3.69, and 4.74 folds, respectively. In Caco-2 cell monolayer bidirectional transport, the $P_{app\ (AP\text{-}BL)}$ of paeoniflorin increased significantly ($P < 0.05$), whereas the $P_{app\ (BL\text{-}AP)}$ of paeoniflorin had no significant change [Figure 6] when coincubation of verapamil (100 μM). These results show that paeoniflorin is the substrate of P-gp. This agrees with our previous study in which we employed the everted rat gut sac model[9] and with the findings reported by Liu *et al.*[27] and Chen *et al.*[8]

Permeability coefficients of the effect of liquiritin on paeoniflorin determined by the human Caco-2 cell monolayer had been shown to correlate highly with *in situ* SPIP studies. Liquiritin could significantly prompt the absorption of paeoniflorin in both the studied absorption models [Figures 1-4 and 6]. Moreover, paeoniflorin also enhanced the absorption of liquiritin in Caco-2 cell monolayer transport [Figure 7]. One underlying mechanism might involve intestinal efflux transporters, which play a significant part in the absorption of relevant drugs. Paeoniflorin is the substrate of P-gp. Furthermore, liquiritin might be a P-gp substrate.[8] Downregulation of the expression of P-gp and/or suppression of the function by its substrates might be a unique mechanism to explain the increase in absorption interaction between liquiritin and paeoniflorin. Whether other efflux transporters (e.g., BCRP and MRP2), also expressed functionally in Caco-2 monolayer cells, are involved in the increase in the absorption of paeoniflorin and liquiritin needs to be further studied. In fact, the combination of paeoniflorin and other drugs is also reported. A previous study reported that the antihypertensive effect of the combination of paeoniflorin-enriched extract and metoprolol was notably increased, even though the underlying mechanism is uncertain.[28]

In addition, 18 β-GA significantly prompted the absorption of paeoniflorin [Figures 1-4 and 6]. This result is consistent with our previous study.[9] However, paeoniflorin had no influence on the absorption of 18 β-GA [Figure 8a]. Paeoniflorin and glycyrrhizin did not affect each other's absorption at 100 μM concentration [Figures 1-4 and 8b] using *in situ* SPIP and Caco-2 cell monolayer *in vitro* absorption models. There is no consistent report on the pharmacokinetic behavior of paeoniflorin and glycyrrhizin. Our previous study[9] and *in vivo* experiment of Sun *et al.*[10] demonstrate that glycyrrhizin influenced the paeoniflorin absorption in connection with concentrations and intestinal segments. According to *in vivo* studies,[10,29] low concentration (100, 300, or 900 mg/kg BW in male Sprague-Dawley rats) of glycyrrhizin inhibits the absorption of paeoniflorin (20 and 30 mg/kg, respectively), whereas high concentration (2700 mg/kg BW) increases the absorption. Furthermore, 100 μM of glycyrrhizin inhibits paeoniflorin absorption in the jejunum and has no effect before 60 min, and it increases the absorption of paeoniflorin after 90 min in the ileum.[9] Interestingly, this study showed that glycyrrhizin did not affect the absorption of paeoniflorin, which might be due to the different perfused segments in *in situ* SPIP studies. The entire perfused small intestine included the duodenum, the jejunum, and the ileum. Therefore, the effect of glycyrrhizin on paeoniflorin absorption in perfused entire small intestine reflected holistic effect.

Moreover, besides SGT, Shaoyao-Gancao drug pair is widely used in Chinese prescriptions. The intestinal absorption interaction of paeoniflorin and liquiritin, the main constituent of this drug pair, is useful to predict the oral bioavailability, pharmacokinetics of coadministration of Shaoyao-and Gancao-containing formulas or prescriptions.

It is noteworthy that poor absorption of paeoniflorin and liquiritin are incompatible with their specific pharmacological activities. Our previous study[23] shows that absorption of paeoniflorin and liquiritin in SGT is enhanced synergistically than that of its single component. The absorption increase of paeoniflorin and liquiritin by combination of Shaoyao and Gancao might be one mechanism to uncover the above contradiction. Otherwise, it is reported that poorly absorbed glycyrrhizin is metabolized to glycyrrhetinic acid, an easily absorbed molecule in the rat gastrointestinal tract by gut microbiota.[30] Chemical structure of paeoniflorin is modified in the gastrointestinal tract after oral administration.[31] A previous study demonstrates that gut microbiota plays a vital role in exerting pharmacological effects of some drugs.[32] Poorly absorbed paeoniflorin and liquiritin might interact with gut microbiota to exert their therapeutical effect.

CONCLUSION

Liquiritin and paeoniflorin might prompt intestinal absorption of each other. Furthermore, 18 β-GA significantly prompted the absorption of paeoniflorin. However, paeoniflorin had no effect on the absorption of 18 β-GA. Paeoniflorin and glycyrrhizin had no effect on each other's absorption at 100 μM. The results of this study might help to understand the implicit mechanism of synergistic therapeutic effect of SGT to some extent and provide essential information for predicting the oral bioavailability and pharmacokinetics of coadministration of liquiritin-and paeoniflorin-containing prescriptions and herbal formulas.

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Conflicts of interest

There are no conflicts of interest.

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