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Title page

Citrus grandis Peel Increases the Bioavailability of Cyclosporine and Tacrolimus, Two Important Immunosuppressants, in Rats

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running title: DRUG INTERACTIONS of *Citrus grandis*

ABSTRACT

Citrus grandis peel is a beverage ingredient and a medicinal herb in oriental countries. Cyclosporine and tacrolimus, important immunosuppressants with narrow therapeutic windows, are widely used in transplant patients. This study investigated the effects of coadministering *Citrus grandis* peel (CGP) on the bioavailability of cyclosporine and tacrolimus. Male Sprague-Dawley rats were orally administered tacrolimus or cyclosporine with and without CGP. The concentrations of cyclosporine and tacrolimus in blood were assayed by monoclonal fluorescence polarization immunoassay and microparticle enzyme immunoassay, respectively. P-gp- and CYP3A4- associated mechanisms were investigated by using everted rat gut sac and recombinant CYP3A4 isozyme. The results showed that CGP significantly increased the bioavailability of cyclosporine and tacrolimus by 100.0 and 234.7%, respectively. *Ex-vivo* studies indicated that the interaction was mediated by the inhibition of CYP 3A4. We suggest that CGP is contraindicated for transplant patients treated with cyclosporine or tacrolimus to minimize the risk of intoxication.

KEY WORDS: ● *Citrus grandis* ● *cyclosporine* ● *CYP3A4* ● *drug interaction* ● *P-glycoprotein* ● *tacrolimus*

INTRODUCTION

Citrus grandis, also known as pomelo, is a common food worldwide and the peel is used as beverage ingredient and also a medicinal herb in oriental countries.[1] The chemical constituents of the fruit of *Citrus grandis* include naringin, naringenin, neohesperidin, bergamottin, 6,7-dihydroxybergamottin, geraniol, limomene, cadinene, citral, aurapten and lycopene etc.[2-3] Chemotaxonomically, *Citrus grandis* belongs to the same genus as grapefruit and shares many common constituents such as naringin, naringenin, neohesperidin, bergamottin and 6,7-dihydroxybergamottin etc.[4] Several clinical studies have reported that grapefruit juice and pomelo juice significantly increased the blood levels of a variety of important drugs such as cyclosporine, tacrolimus, pitavastatin, atorvastatin and acebutalol etc.[5-7] Therefore, we hypothesize that the peel of *Citrus grandis* is likely to exert relevant pharmacokinetic interaction with the above-mentioned drugs as did grapefruit juice and pomelo.

Cyclosporine and tacrolimus, calcineurin inhibitors, which modulate the function of T cells, are important immunosuppressants prescribed for transplant patients to prevent allograft rejection. Due to the narrow therapeutic windows, the blood levels of cyclosporine and tacrolimus are routinely monitored in clinical practice. Supratherapeutic levels of cyclosporine may lead to adverse effects including nephrotoxicity, thrombotic-microangiopathy and hypertension.[8-9] Toxicological concentrations of tacrolimus may result in neurotoxicity, nephrotoxicity, gastrointestinal toxicity, hyperkalaemia, hypertension and myocardial hypertrophy.[10-13] On the other hand, subtherapeutic levels of cyclosporine and tacrolimus induced acute rejection of allografts including kidney, liver and heart.[14-16]

The oral bioavailabilities of cyclosporine and tacrolimus have been known to be associated with P-glycoprotein (P-gp) and cytochrome 3A4 (CYP3A4).[17-18] The interaction mechanisms of cyclosporine or tacrolimus with grapefruit juice and pomelo are ~~is~~ through inhibition of P-gp and CYP3A4.[5-6, 19-20] This study aimed to investigate the effects of coadministration of the peel extract of *Citrus grandis* (CGP) on the pharmacokinetics of cyclosporine and tacrolimus in rats. Furthermore, P-gp- and CYP3A4- associated mechanisms were investigated by using everted gut sac and recombinant CYP3A4 isozyme, respectively.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 300-400 g were supplied by National Laboratory Animal Center (Taipei, Taiwan) and kept in controlled environment with a 12 h light-dark cycle and constant temperature at the Animal Center of China Medical University (Taichung, Taiwan). The animal study adhered to "The Guidebook for the Care and Use of Laboratory Animals" published by the Chinese Society of Animal Science, Taiwan, ROC. The animal protocol was approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan.

Plant material, chemicals and reagents

The peel of *Citrus grandis* was purchased from a Chinese drugstore in Taichung and identified by Dr, Yu-Chi Hou. Neoral® (containing 100 mg/mL of cyclosporine and excipients including castor oil and alcohol) was a gift kindly provided by Novartis Co. Ltd. (Taiwan). Prograf®

(containing 5.0 mg/mL of tacrolimus and excipients including castor oil, corn oil, propylene glycol and alcohol) was purchased from Fujisawa Pharmaceutical Company (Osaka, Japan). TDx and IMx kit were supplied by Abbott Laboratories (Abbott Park, IL, USA). Rhodamine 123 was purchased from Aldrich Chemical Company (Milwakee, WI, USA). Acetonitrile was LC grade and purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Milli-Q plus water (Millipore, Bedford, MA, USA) was used for all preparations.

Instrumentation

The HPLC apparatus included a pump (LC-10AS, Shimadzu, Japan) and an UV/VIS detector (SPD-10A, Shimadzu, Japan). The RP-18e column (Apollo®, 5 μm, 250 × 4.6 mm) was equipped with a guard column (LiChrospher 100, 5 μm).

Preparation and characterization of CGP

To prepare the CGP aqueous extract, 25.0 g dry peel was cut into small pieces and macerated in 500 mL of water for 4 h, then heated on a gas stove. After boiling, gentle heating was continued until the volume was reduced to less than 100 mL. The mixture was filtered while hot and sufficient hot water was added to make 100 mL.

For the characterization of CGP, a previous HPLC method was adopted with minor modification.[21] Briefly, 3.0 mL of the CGP were added with 7.0 mL of methanol, vortexed and centrifuged at 10,000 *g* for 15 min. The supernatant was diluted with an equal volume of methanol (containing 40.0 μg/mL of 6,7-dimethoxycoumarin as internal standard). After filtering through a 0.45 μm filter, the sample was analyzed by

HPLC to determine naringin and naringenin concentrations.

Drug administrations and blood collection

Neoral® and Prograf® were diluted with distilled water to afford 1.25 and 1.0 mg/mL, respectively. After an overnight fast, eight male rats were orally administered 2.5 mg/2.0 mL/kg of cyclosporine solution with and without CGP (2 g/8.0 mL/kg) in a crossover design. Similarly, 1.5 mg/1.5 mL/kg of tacrolimus solution was orally administered to another ~~two groups~~ group of six rats ~~each~~ with and without CGP (2 g/8.0 mL/kg) in a crossover design. Drug administration was carried out via gastric gavage and CGP was given 10 min before cyclosporine or tacrolimus. Equal volumes of water as the extract were administered to control rats as blank vehicle. In another experiment, an intravenous bolus of cyclosporine (0.8 mg/kg) was injected into eight rats with and without CGP (2 g/8.0 mL/kg) in a crossover design. Food was withheld for another 3 h after dosing. One-week was allowed for washout between the two treatments in each crossover study.

Rats were anesthetized with isoflurane and blood samples (0.3 mL) were drawn at 20, 40, 60, 180, 300, 540, 1440 and 2880 min after oral dose of cyclosporine, and at 5, 15, 30, 60, 120, 240 and 480 min after oral dose of tacrolimus. Following intravenous bolus of cyclosporine, blood samples were drawn at 5, 10, 20, 40, 60, 180, 300 and 540 min after dosing. Blood samples were drawn via cardiopuncture and collected in vacutainer tubes containing EDTA (Becton Dickinson), which were stored at 4℃and analyzed within 24 h.

Determination of blood concentrations of cyclosporine and tacrolimus

Blood cyclosporine concentrations were determined by a specific monoclonal fluorescence polarization immunoassay (FPIA). The assay was calibrated for concentrations from 0 to 1500.0 ng/mL. Blood tacrolimus concentration was determined by microparticle enzyme immunoassay (MEIA). The assay was calibrated from 3.0 to 30.0 ng/mL. All procedures followed the manuals provided by the manufacturer.

Everted rat gut sac study

Everted gut sacs of rats were used to measure the effect of CGP on P-gp function.[22] After an overnight fast, the jejunum and ileum of six rats were immediately isolated after sacrifice. Following flushing with ice-cold saline, each segment was everted and both ends were ligated tightly to prepare a 25-cm long gut sac. All the procedures were carried out on ice.

For the controls, the sacs were immersed in 50 mL of medium TC 199 in a beaker prewarmed at 37℃ and preoxygenated with a mixture gas of 95% O_2/5% CO_2, and incubated for 20 min. For treatment groups, CGP was added to medium TC 199 to make concentrations of 5 and 10 mg/mL. After 20-min incubation, 3 mL of rhodamine 123 solution (20.0 μg/mL in medium TC 199) was introduced into the everted sac (serosal side) and then the medium was sampled every 20 min from the mucosal medium until 100 min. The transport of rhodamine 123 solution from the serosal to mucosal surfaces across the intestine was then measured fluorometrically by using Luminescence Spectrometer LS-50B (Perkin Elmer).

Preparation of serum metabolite of CGP

In order to mimic the molecules interacting with CYP 3A4 in the enterocytes, the serum metabolites of CGP were prepared from rats to evaluate the effect on CYP 3A4. CGP was orally administrated to rats fasted overnight at a dose of 2 g/8.0 mL/kg. Blood was collected via cardiopuncture at 1 h after dosing. After coagulation, the serum was vortexed with two-fold methanol. After centrifuging at 10,000 *g* for 15 min, the supernatant was concentrated in a rotatory evaporator under vacuum to dryness. To the residue, appropriate amount of water was added to prepare a solution, which represented 10-fold serum concentration of metabolites and was divided into 1-mL aliquots, stored at -30℃ for later use.

Effects of serum metabolite of CGP on CYP3A4 activity

Vivid® CYP 450 screening kits (Invitrogen) was used to evaluate the effect of serum metabolite of CGP on the activity of CYP3A4. All the procedures were performed according the manual provided by the manufacturer. Briefly, after incubating serum metabolite of CGP (0.5- and 1- fold of serum concentration) with CYP 450 recombinant BACULOSOMES®, glucose-6-phosphate and glucose-6-phosphate dehydrogenase in 96-well black plate at room temperature for 20 min, benzyloxymethyl resorufin, a specific CYP3A4 substrate (Vivid® BOMR), and $NADP^+$ were then added and incubated at room temperature for another 30 min. At the end of incubation, ketoconazole was added to stop the reaction and the fluorescence was measured with excitation at 530 nm and emission at 590 nm.

Data analysis

Noncompartment model of WinNonlin (version 1.1, SCI software, Statistical Consulting Inc.) was used for the computation of pharmacokinetic parameters of cyclosporine and tacrolimus. The area under the blood concentration - time curve (AUC_{0-t}) was calculated by the trapezoidal rule to the last point. The C_{max} was obtained from experimental measurement. Pharmacokinetic parameters were compared using paired Student's *t*- test with SPSS version 12.0 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Characterization of CGP

The characterization of CGP followed that reported in a previous study with minor modification ~~was consistent with a previous study with~~ [21]. The concentrations of naringin and naringenin in the extract were determined being 434.8 and 5.1 μg/mL, respectively. ~~with the exception that the concentrations of naringin and naringenin were 434.8 and 5.1 μg/mL, respectively.~~

Effects of CGP on the pharmacokinetics of oral cyclosporine and tacrolimus

The blood cyclosporine profiles after oral administration of cyclosporine alone and coadministered with CGP are shown in Figure 1. The pharmacokinetic parameters of cyclosporine are listed in Table 1. Coadministration of CGP significantly increased the AUC_{0-t} and C_{max} of cyclosporine by 100.0 % and 80.1 %, respectively. Coadministration with

CGP significantly increased the AUC_{0-t} and C_{max} of tacrolimus by 234.7 % and 130.2 %, respectively (Fig.2); pharmacokinetic parameters are shown in Table 1.

Effect of CGP on the pharmacokinetics of intravenous cyclosporine

Figure 3 depicts the blood cyclosporine profiles after administering an intravenous bolus of cyclosporine with and without oral coadministration of CGP. Two blood profiles were essentially superposable, indicating no significant difference in intravenous pharmacokinetics of cyclosporine between two treatments.

Everted gut sac study

Figure 4 shows the effects of CGP on the efflux of rhodamine 123 across the jejunum and ileum of rats. The results revealed that CGP at 5 and 10 mg/mL did not significantly affect the efflux of rhodamine 123 from serosal side to mucosal side in either jejunum or ileum.

Effects of serum metabolites of CGP on CYP3A4 activity

The effects of the serum metabolites of CGP on CYP3A4 activity are shown in Figure 5. The positive control ketoconazole at 10 μM significantly decreased CYP3A4 activity by 87.2 %. The serum metabolite of CGP at 0.5- and 1- fold serum concentration significantly decreased CYP3A4 activity by 54.3 % and 89.2 %, respectively, when compared to blank serum at a correspondent concentration.

DISCUSSION

This study employed a rat model to evaluate the effects of CGP on the pharmacokinetics of cyclosporine and tacrolimus. The results showed that coadministration of CGP markedly increased the C_{max} and AUC_{0-t} of cyclosporine and tacrolimus, suggesting that oral bioavailabilities of cyclosporine and tacrolimus were significantly increased. In contrast, the intravenous pharmacokinetics of cyclosporine was not altered by oral coadministration of CGP; therefore, it becomes evident that the interaction between CGP and oral cyclosporine must occur at the absorption site. We can thus infer that the absorptions of cyclosporine and tacrolimus at intestine were significantly enhanced by CGP. This CGP - cyclosporine interaction in rats echoes our previous finding that CGP brought about acute intoxication of cyclosporine in pigs.[21]

Two mechanisms affecting the fate of cyclosporine and tacrolimus at absorption site have been identified: pumping out by P-gp in the intestinal surface first, and subsequently the residuals are metabolized by CYP3A4 in intestine and liver.[17-18] Accordingly, it is likely that the increased bioavailabilities of cyclosporine and tacrolimus stemmed from inhibition of P-gp and/or CYP3A4.

The fact that CGP did not significantly affect the function of intestinal P-gp as demonstrated by the everted gut sac study (Fig. 4) suggested that these interactions might not be associated with intestinal P-gp. In regard to whether CYP3A was affected, given that various constituents in CGP, such as flavonoids, were extensively metabolized during the first pass, we thus prepared the serum metabolite from rats administered CGP in order to mimic the virtual molecules interacting with intestinal CYP 3A.[23] Although rats do not express CYP3A4 [24-25], in order to mimic the metabolism of cyclosporine and tacrolimus in humans,

an *in vitro* model employing microsomes expressing human CYP3A4 was used in this study. This *ex-vivo* approach was distinct from most previous *in vitro* studies reporting the modulation of herbal extract or natural compounds on CYP 3A by targeting their parent forms without considering their intestinal metabolism. The *in vitro* inhibition of CYP3A4 activity by CGP at 0.5- and 1.0-fold serum concentrations clearly suggested that the enhanced absorption of cyclosporine and tacrolimus in rats might be in part resulted from the modulation on enteric CYP3A. Given many unmanageable drug-drug interactions involve inhibition of CYP 3A4, combination therapy of CGP with critical western medicines, which are substrates of CYP 3A4, should be approached with caution.

Grapefruit juice is well known to elevate the blood levels of tacrolimus and cyclosporine.[19, 26] So far, many constituents have been reported as possible causes for the interaction between grapefruit juice and western medicines which were substrates of CYP3A4 and/or P-gp. Among the putative causative agents, 6',7'-dihydroxybergamottin and bergamottin, two minor furanocoumarins, have been demonstrated to inhibit CYP3A4 and P-gp.[27-30] However, most of these conclusions were drawn essentially from in vitro studies. Until a human study reporting that lack of interaction with felodipine was caused by furanocoumarins - free grapefruit juice,[31] 6',7'-dihydroxybergamottin and bergamottin were believed to be responsible for grapefruit juice - drug interactions.

Citrus grandis has been reported to contain bergamottin and 6,7-dihydroxybergamottin,[4] which may be responsible for the increased bioavailabilities of tacrolimus and cyclosporine. However, HPLC/UV analysis of the CGP prepared in this study detected no trace of bergamottin. As for 6,7-dihydroxybergamottin, the analysis of this putative causative agent was not attempted in this study owing to the

unavailability of authentic compound. Therefore, the chemical constituent in CGP responsible for the interactions observed in present study remained to be clarified in the future. Although other constituents in grapefruit juice such as quercetin, hesperetin and naringenin have been suspected to be the causative agents to inhibit CYP 3A4 based on in vitro studies [29], several pharmacokinetic studies have reported that these compounds virtually existed in the bloodstream as glucuronides/sulfates during the first pass following oral intake [23, 32-34]. Therefore, the *in vitro* inhibition on CYP 3A4 by these flavonoids should be not associated with the *in vivo* pharmacokinetic interactions. Furthermore, quercetin was recently reported to reduce the bioavailability of cyclosporine, a clear indication that quercetin was not an in vivo inhibitor of CYP 3A4 [35].

In conclusion, CGP markedly increased the blood levels of cyclosporine and tacrolimus in rats through inhibition of intestinal CYP 3A. We suggest that CGP is contraindicated for transplant patients treated with cyclosporine or tacrolimus to minimize the risk of intoxication.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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FIGURES

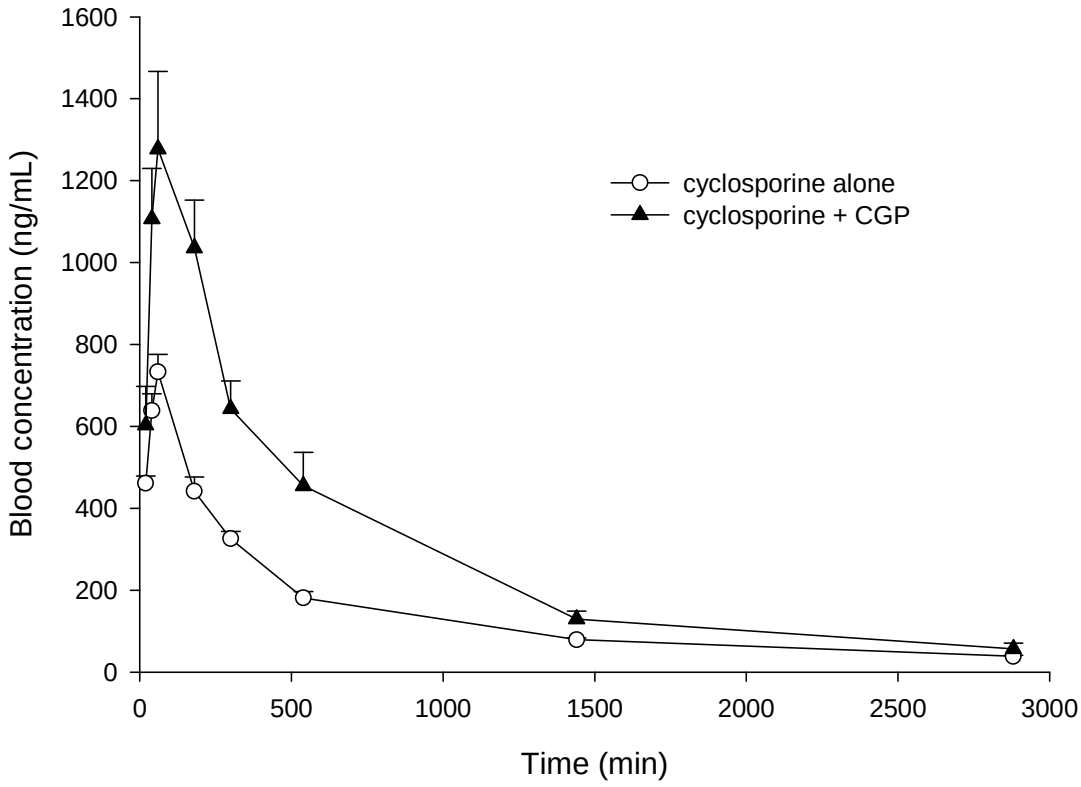

FIG. 1. Mean (±S.E.) blood concentration - time profiles of cyclosporine after oral administration of cyclosporine alone (2.5 mg/kg, ○) and coadministration with 2 g/kg of *Citrus grandis* peel (CGP, ▲) in eight rats.

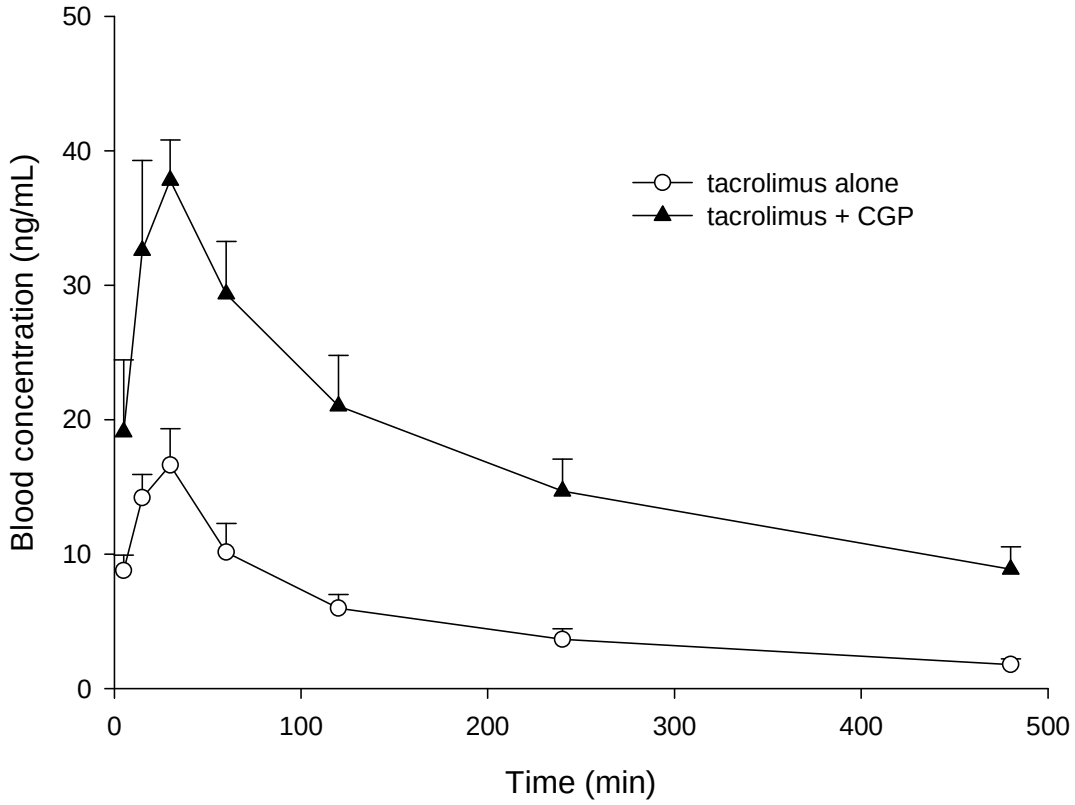

FIG. 2. Mean (±S.E.) blood concentration - time profiles of tacrolimus after oral administration of tacrolimus alone (1.5 mg/kg, ○) and coadministration with 2 g/kg of *Citrus grandis* peel (CGP, ▲) in six rats.

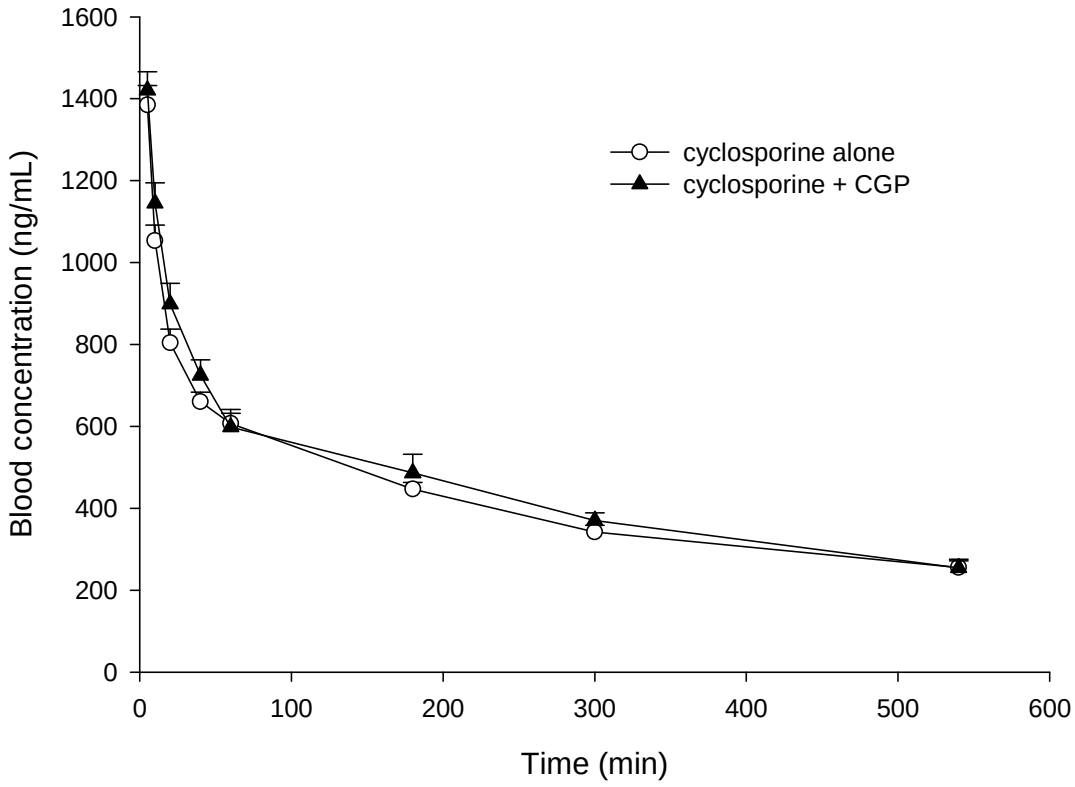

FIG. 3. Mean (±S.E.) blood concentration - time profiles of cyclosporine after intravenous bolus of cyclosporine alone (0.8 mg/kg, ○) and oral coadministration with 2 g/kg of *Citrus grandis* peel (CGP, ▲) in eight rats.

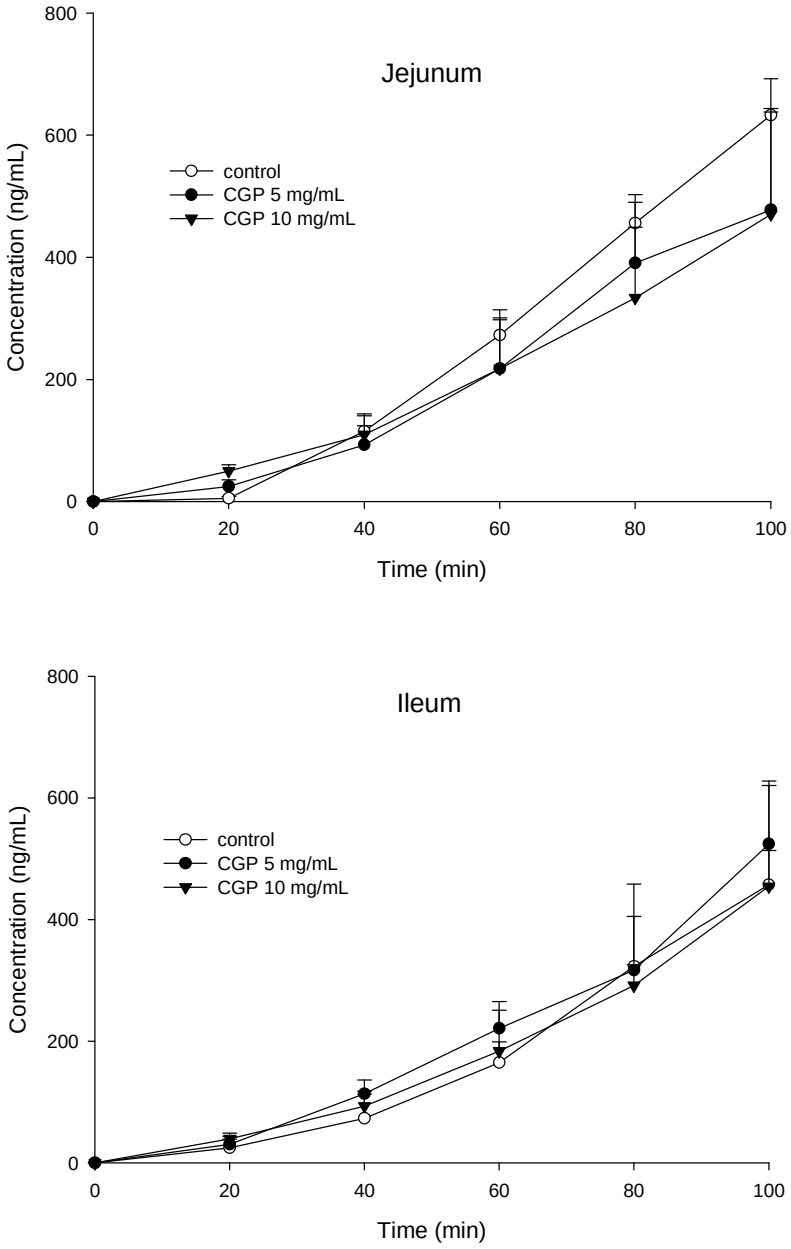

FIG. 4. Average transport of rhodamine 123 (ng/mL) across jejunum and ileum in the absence (○) and presence of of *Citrus grandis* peel (CGP) at concentrations of 5 mg/mL (●) and 10 mg/mL (▼).

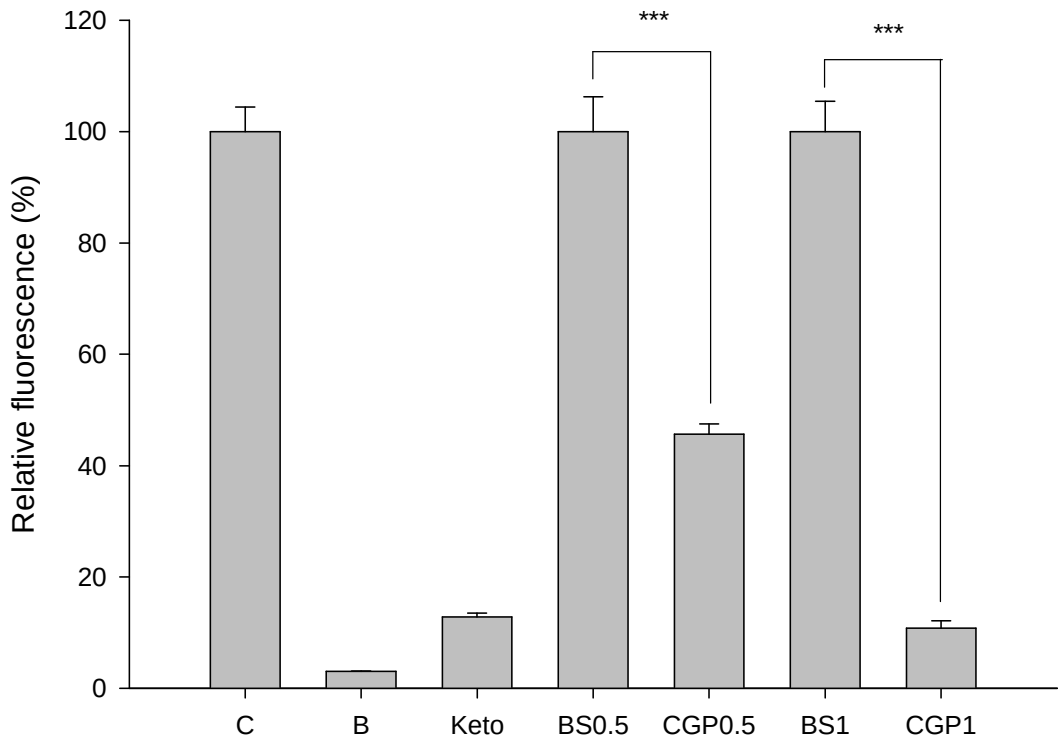

FIG. 5. Effects of serum metabolite of *Citrus grandis* peel (CGP, 0.5- and 1-fold serum concentration), blank serum (BS, 0.5- and 1-fold serum concentration) and ketoconazole (Keto, 10 μM) on the activity of CYP3A4. C: control, B: background.

$^{***}P < 0.001$, compared to blank serum at correspondent concentration.

TABLES

TABLE 1 PHARMACOKINETIC PARAMETERS OF CYCLOSPORINE AND TACROLIMUS IN RATS GIVEN CYCLOSPORINE OR TACROLIMUS ALONE AND COADMINISTRATED WITH PEEL EXTRACT OF *CITRUS GRANDIS* (CGP).

Parameters / Treatments	C_{max} (ng/mL)	AUC_{0-t} (μg・min/mL)	MRT (min)
cyclosporine alone[a]	745.1 ± 41.5	407.5 ± 20.2	736.3 ± 21.5
cyclosporine + CGP[b]	1341.6 ± 154.8**	815.0 ± 104.3**	664.8 ± 43.7
tacrolimus alone[c]	19.2 ± 1.7	2.5 ± 0.4	144.3 ± 6.2
tacrolimus + CGP	44.2 ± 4.5**	8.3 ± 1.1**	174.8 ± 8.5*

Data expressed as Mean ± S.E.

C_{max} : the maximum blood concentration

$AUC_{0\sim t}$: the area under concentration - time curve to the last time

$MRT_{0\sim t}$: the mean residence time

[a] dosage of cyclosporine: 2.5 mg/kg

[b] dosage of CGP: 2 g/kg

[c] dosage of tacrolimus: 1.5 mg/kg

*$P < 0.05$, **$P < 0.01$, compared to correspondent control

FIGURE LEGENDS

FIG. 1. Mean (±S.E.) blood concentration - time profiles of cyclosporine after oral administration of cyclosporine alone (2.5 mg/kg, ○) and coadministration with 2 g/kg of *Citrus grandis* peel (CGP, ▲) in eight rats.

FIG. 2. Mean (±S.E.) blood concentration - time profiles of tacrolimus after oral administration of tacrolimus alone (1.5 mg/kg, ○) and coadministration with 2 g/kg of *Citrus grandis* peel (CGP, ▲) in six rats.

FIG. 3. Mean (±S.E.) blood concentration - time profiles of cyclosporine after intravenous bolus of cyclosporine alone (0.8 mg/kg, ○) and oral coadministration with 2 g/kg of *Citrus grandis* peel (CGP, ▲) in eight rats.

FIG. 4. Average transport of rhodamine 123 (ng/mL) across jejunum and ileum in the absence (○) and presence of *Citrus grandis* peel (CGP) at concentrations of 5 mg/mL (●) and 10 mg/mL (▼).

FIG. 5. Effects of serum metabolite of peel extract of *Citrus grandis* (CGP, 0.5- and 1-fold serum concentration), blank serum (BS, 0.5- and 1-fold serum concentration) and ketoconazole (Keto, 10 μM) on the activity of CYP3A4. C: control, B: background.

$^{***}P < 0.001$, compared to blank serum at correspondent concentration.

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