Supporting Information to:

*In vitro* Interactions with Repeated Grapefruit Juice Administration – to Peel or not to Peel?

Shlomo Brill¹,², Christian Zimmermann¹, Karin Berger³, Juergen Drewe¹, Heike Gutmann¹

Affiliation

¹ Department of Clinical Pharmacology & Toxicology, University Clinic Basel, Basel, Switzerland
² Department of Pharmacology; School of Pharmacy, University of London, London, UK
³ Vitaplant AG, Witterswil, Switzerland

Correspondence

Prof. Dr. Juergen Drewe
Department of Clinical Pharmacology and Toxicology
University Clinic Basel
Petersgraben 4
4031 Basel
Switzerland
Tel.: +41/61/265 3848
Fax: +41/61/265 8581
juergen.drewe@unibas.ch
Material and Methods

The amounts of the flavonoids naringenin and naringin and of the furanocoumarin bergamottin were determined by HPLC exactly as described by De Castro et al. (De Castro et al., 2006).

For naringin, naringenin and bergamottin in GFJ, only single determinations have been performed. Coefficient of correlation $R^2$ of calibration curve was above 0.98. For each of the peaks, peak purity was assessed and peak identification was done by comparison with UV-spectra and retention times.

Determination of naringin and naringenin

200 μL of the GPJ were mixed with cold methanol, vortexed and centrifuged at 2500 rpm. The supernatant was filtered through an RC membrane (0.45 μm) and analysed in a Waters HPLC system (equipped with a pump controller 600 Series, autosampler 717, diode array detector 996) using a LiChrospsphere 100 RP 18-5µm (250 x 4.6 mm) column with a precolumn of the same material (both Macherey Nagel) as stationary phase. The mobile phase consisted of two solvent systems (A: water at pH 2.4, adjusted with $H_3PO_4$; and B: methanol/water at pH 2.4; 60:40) in a linear gradient (0 – 5 min 100% A, 5 – 55 min 100 % A to 100% B, 55 – 70 min 100% B). The flow rate was set to 0.5 mL/min and the column temperature to 35 °C. The detection was carried out at 285 nm and quantification of the flavonoids was performed by the external standard method using naringin from Sigma and naringenin from Sigma as reference substance. The flavonoids in the samples were identified according to their retention time and UV spectra. HPLC fingerprint is given in Figs. 1S A and 1S B.
The concentrations of major constituents were higher in GFJP+ than in GFJP-. In GFJP+ and GFJP-, naringin concentrations were 6535.5 µM and 1040.5 µM [reported natural concentration 218 – 2062 µM (Ho et al., 2000)], naringenin concentrations were 1023.3 µM and 172.5 µM [reported natural concentration 19.5–595 µM (De Castro et al., 2006)], respectively.

Fig. 1S A HPLC fingerprint of naringin and naringenin in GFJP-.

Fig. 1S B HPLC fingerprint of naringin and naringenin in GFJP+.
**Determination of bergamottin**

1 mL of the GFJ was mixed with 670 µL of ethyl acetate, vortexed and centrifuged at 3200 rpm. The ethyl acetate phase was evaporated, the residue was dissolved in 200 µL DMSO, filtered through an RC membrane (0.45 µm) and analysed in a Waters HPLC system using a LiChrosphere 100 RP 18-5 µm (250 x 4.6 mm) column with a precolumn of the same material (both Macherey Nagel) as stationary phase. The mobile phase consisted of two solvent systems (A: water; and B: methanol) in a linear gradient (0 – 20 min 45% A, 20 – 40 min 45 % A to 100% B, 4 – 75 min 100% B). The flow rate was set to 1.0 mL/min and the column temperature to 35°C. The detection was carried out at 310 nm and quantification of bergamottin was performed by the external standard method using bergamottin from Sigma as reference substance. Bergamottin in the samples were identified according to the retention time and UV spectra. HPLC fingerprint is given in Figs. 1S C and 1S D.

The concentrations of major constituents were higher in GFJP+ than in GFJP-. In GFJP+ and GFJP-, bergamottin concentrations were 22.4 µM and 4.6 µM (reported natural concentration 2.0–28.3 µM (De Castro et al., 2006)), respectively.

![HPLC fingerprint of bergamottin and 6,7-dihydroxybergamottin in GFJP-](image_url)

**Fig. 1S C** HPLC fingerprint of bergamottin and 6,7-dihydroxybergamottin in GFJP-.
Cell cultures and treatment

The LS180 cell line (between passages 48 and 53) was purchased from ATCC. It was cultured in Dulbecco’s MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum, 1% non essential amino acids, 1% sodium pyruvate, 50 µg/mL gentamycin. Cells were seeded onto 6 well plastic culture dishes (9.2 cm²/well). After the cells had reached confluence they were treated with the compound of interest for 72 h [e.g., 10 µM rifampicin, 2mM naringin, 10 µM bergamottin, 200 µM naringenin, GFJP- 1% (v/v), GFJP+ 1% (v/v)]. Medium containing the compound of interest was freshly prepared and changed every 24 hours. The compounds and extracts were dissolved in dimethyl sulfoxide (DMSO). As reported in literature, 10 µM rifampicin was used as the control compound for CYP3A4 and MDR1 mRNA induction (Schuett et al., 1996). The final DMSO concentration did not exceed 0.5%. Up to this concentration, the solvent did not significantly change the expression level of any gene of interest compared to control without DMSO.

Sulforhodamine B assay

LS 180 cells were seeded in 96 well plates. After 72 hours, cells were incubated with the substances of interest and the GFJ extracts for 72 hours. Media with and without treatment were changed daily. Cells were controlled by microscopy to make sure,
that they did not detach during the three day incubation period. With the exception of different concentrations of compounds of interest, all cells were treated identically.

The supernatant was removed and the cells were washed twice with 200 µL culture medium. To fix the cells to the plate, 180 µL culture medium and 20 µL of 50% TCA (final concentration in the well was 5%) were added to each well. The plate was put on ice for 1 hour. The plate was rinsed 5 times with milliQ water and was allowed to air-dry for two hours at room temperature. Then the cells were stained with 100 µL of 0.4 % SRB in 1% acetic acid for 15 minutes. After removing the solution and rinsing four times with 1% acetic acid, the plates were again allowed to air-dry at room temperature. The stained cells were lysed with 100 µL of 10 mM Tris at pH 10.5 and put on a shaker (Polymix; Kinematica) for 15 minutes. The absorption was detected at 540 nm on a plate reader (Spectra Max 250; Bucher Biotec AG).

As shown in Fig. 2S A for GFJP+ and GFJP-, cytotoxic effects appeared at concentrations above 1%. Therefore, GFJP+ and GFJP- were used in a concentration of 1% for all subsequent induction experiments. With the exception of naringenin, all individual constituents showed cytotoxicity (Fig. 2S B). Bergamottin showed toxicity at concentrations above 10 µM, naringenin at concentrations above 200 µM, whereas naringin showed no signs of cytotoxicity at any of the concentrations tested (0 – 5 mM). Based on these results, the following concentrations were used for each substance in subsequent incubations: GFJP+ and GFJP- at 1% (v/v), naringin 2 mM, bergamottin 10 mM and naringenin 200 µM. These concentrations reflect the natural concentrations (see above). Non-linear regression analysis was done by Origin Software, version 8.0.

**Western blot analysis**

Proteins were isolated in protein extraction buffer and protein concentration was determined using the BCA protein assay reagent kit (PIERCE) according to the manufacturer’s protocol.

The isolated proteins were electrophoretically separated by SDS page. The protein was then transferred to a nitrocellulose membrane (2 h at a constant amperage of 250 mA) using a Mini Trans-Blot cell (Bio-Rad).
The nitrocellulose membrane was washed with 0.05% Tween-20 in PBS for 15 min (3 times) at room temperature. Non-specific binding sites on the membrane were then blocked overnight at 4 °C with PBS containing 5% milk powder and 0.05% Tween-20. After washing (0.05% Tween-20 in PBS), the membrane was incubated for 2 hours at 37 °C in a humid chamber with the primary, mouse anti-human antibody C219 against P-gp, 0.1 mg/mL (Alexis Corporation) diluted 1:100 in PBS containing 0.05 % Tween-20, 1% bovine serum albumin (BSA) and 1% milk powder. Then the membrane was washed 3 times (with PBS containing 0.05% Tween-20) and incubated with the secondary horseradish peroxidase-conjugated, rabbit anti-mouse IgG (Amersham) diluted 1:500. Secondary antibody incubation was carried out for 1 hour at room temperature. The membrane was washed as described above (with 0.05% Tween-20 in PBS) and Pgp protein detection was performed with the enhanced chemiluminescence system (ECL-Detection-Kit; Amersham). The molecular weight of the protein(s) was identified by using molecular weight Kaleidoscope™ Standard (Bio Rad).
**Fig. 2S A** Cytotoxic effects of GFJP+ and GFJP- on LS180 cells determined by the sulforhodamine assay. Data are means ± SEM (n = 3).

**Fig. 2S B** Cytotoxic effects of different GFJ constituents on LS180 cells determined by the sulforhodamine assay. Data are means ± SEM (n = 3).
References

