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ORIGINAL PAPER

Variation of bioactive furocoumarins and flavonoids in different varieties of grapefruits and pummelo

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Abstract Grapefruit juice has been shown to increase the oral bioavailability of many therapeutic drugs. Certain citrus bioactive compounds such as furocoumarins and flavonoids have potent inhibitory effects on cytochrome P450 3A4 (CYP 3A4) enzyme and P-glycoprotein. The levels of these bioactive compounds in the grapefruit juice may affect the magnitude and mechanism of grapefruit juice-induced drug interaction. The levels of three furocoumarins such as dihydroxybergamottin (DHB), paradisin A and bergamottin and flavonoids have been separated and quantified using high-performance liquid chromatography (HPLC) in seven varieties of grapefruits and its parent pummelo. Considerable differences were observed in the levels of these bioactive compounds in different grapefruit varieties. Ray Red showed the lowest (0.492 ± 0.027 DHB μg/ml, 0.059 ± 0.001 μg/ml paradisin A and 0.344 ± 0.030 μg/ml bergamottin) levels of all three furocoumarins and Duncan contain the highest amount of DHB (2.587 ± 0.432 μg/ml) and bergamottin (1.004 ± 0.068 μg/ml), where as Star Ruby contain the highest levels of paradisin A. Pummelo contain the highest levels of naringin (4.587 ± 0.061 mg/ml), while Rio Red showed the lowest level (1.986 ± 0.145 mg/ml) of naringin.

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Abbreviations

HPLC	High-performance liquid chromatography
GFJ	Grapefruit juice
CYP P450	Cytochrome P450
P-gp	P-glycoprotein
LC/MS	Liquid chromatography/mass spectrometry
NMR	Nuclear magnetic resonance
EtOAc	Ethyl acetate

Introduction

Grapefruit is a rich source of health maintaining bioactive compounds. However, consumer concern has been increasing due to possible interaction of grapefruit juice with several medications resulting in the increased bioavailability of certain drugs and risk of toxicity [1, 2]. Several drugs such as felodipine [2], terfenadine [3], lovastatin [4] cyclosporine [5], and midazolam [6] and other categories [7] have shown to interact with grapefruit juice. The plausible mechanism of grapefruit juice-induced drug interaction is through inhibition of drug metabolizing enzyme cytochrome P450 3A4 (CYP 3A4) [8], intestinal membrane efflux transporter P-glycoprotein [9] and organic anion transporting polypeptides [10]. These mechanisms are involved in the drug metabolism, transportation of a wide variety of drugs from the enterocytes to the gut lumen and cellular uptake of a large number of compounds [11].

Previous studies indicated naringin, a grapefruit flavonoid, as the active CYP 3A4 inhibitor in grapefruit juice [12]. While naringin seems to have some inhibitory effect on CYP 3A4 in vitro, it has little effect in vivo [13].

Edwards and Bellevue [14] reported that DHB found in grapefruit juice was a potent CYP 3A4 inhibitor. Several furocoumarins such as bergamottin, epoxybergamottin, paradisin A, paradisin B and paradisin C have also shown to inhibit the CYP 3A4 enzyme [15–17]. Recently, our laboratory reported the isolation and characterization of specific furocoumarins and their differential inhibition of CYP 3A4 and CYP 1B1 [18]. Certain dimers are more effective inhibitors of CYP 3A4 enzyme compared with their respective monomers [16], but the dimer concentrations are very low in the juice which posses a challenge to isolate such compounds [19]. On the basis of available research information, considerable inconsistency in the outcome of drug interaction studies was reported over the last decade. These inconsistencies from the affected drugs, variation of the specific bioactive compounds, processing of grapefruit juice and differences in the timing of grapefruit juice ingestion relative to the intake of the medication under investigation [7].

The bioactive compounds profile of the citrus fruit varies due to variety, environmental conditions and the stage of fruit development and maturity [20]. Duncan and Marsh White grapefruit varieties showed significant variation in the naringenin [21] and limonin content [22]. The grapefruit trees exposed to freezing conditions showed higher levels of naringin and lower levels of limonin [23]. Other environmental stress parameters such as temperature, humidity and developmental stages of fruit also affected the levels of naringin [24, 25]. Furthermore, the highest concentration of naringin [25, 26], limonin [27] and nomilin [28] was observed at early developmental stages and progressively declined as fruit matured.

The content of certain bioactive furocoumarins in grapefruit juice has been shown to vary considerably. Tassaneeyakul et al. [29] reported several fold variations of bergamottin, DHB and dimers in the commercial grapefruit juice. Fukuda et al. [30] reported that furocoumarins levels in white colored commercial grapefruit juice were higher than in red commercial grapefruit juices. Furthermore, the highest levels of furocoumarins were found in the fruit meat. Fresh squeezed grapefruit juice has been shown to increase the bioavailability of terfanadine two fold higher compared to commercially processed juice [31]. While environmental factors may have influence on furocoumarins and flavonoids, genetic factors might play a major role in the variation of these bioactive compounds. Until now, studies related to variation of furocoumarins mainly involved commercially processed grapefruit juice. It is possible that during processing, juice undergoes high temperature treatment and some of the fruit components such as pulp are removed while essential oils are added back to the finished juice, this may change the quantity and quality of furocoumarins [32]. To the best of our knowledge, very little information is available on variation of furocoumarins in fresh grapefruits. Thus, the objective of this study was to determine the levels of specific furocoumarins and flavonoids content in fresh grapefruit varieties and pummelo.

Materials and methods

Plant materials

Seven grapefruit varieties Rio Red, Ruby Red, Ray Red, Star Ruby, Thompson Pink, Marsh White, Duncan and grapefruit parent Pummelo were harvested in the month of November 2005 from an orchard at the Texas A&M University-Kingsville, Citrus Center Weslaco, TX, USA.

Chemicals

All the solvents used for isolation were ACS grade and for quantitative analysis HPLC grade were obtained from EMD (Gibbstown, NJ, USA). Naringin and naringenin were obtained from Sigma-Aldrich (Sigma-Aldrich St. Louis, MO, USA).

Chromatographic system

The HPLC system consisted of a Thermo Electron Corporation P-400 quaternary HPLC pump (Thermo Electron Corporation, CA, USA), Membrane degasser LDC analytical and Spectra system AS3000 auto sampler (Thermo separation products, CA, USA), photodiode array detector (Thermo separation products, CA, USA). Chromatographic separations were accomplished on Chemcosorb-5–ODS column (150 × 6.0 mm, 5 µm particle size; ChemcoPak, Osaka, Japan) for furocoumarins where as Luna 3-ODS was used for flavonoids analysis (Phenomenex, Torrance, CA, USA). All the sample and standard stock solutions were filtered through 0.45 µm membrane filters (Pall Gelman Laboratory, Ann Arbor, MI, USA) and stored in brown vials at 4 °C until the analysis.

Purification of furocoumarins

DHB, paradisin A and bergamottin were isolated from grapefruit juice according to our published method [18]. Marsh white grapefruit juice was extracted with ethyl acetate (EtOAc) and concentrated under vacuum. The concentrated extract was broadly fractionated on silica column chromatography using different combinations of hexane and EtOAc as mobile phase. It was then subjected to preparative HPLC and eluted with aqueous methanol at a flow rate of 25 ml/min. Different fractions were collected and fractions from multiple runs were pooled and rotary

evaporated to remove the organic phase. The water portion was taken off by freeze drying. The structures of DHB, paradisin A and bergamottin were confirmed by ^{1}H and ^{13}C NMR and MS spectra [18].

Sample preparation and quantification of furocoumarins

Five pounds of each variety of fruits were juiced using home blender and stored at −80 °C until they were analyzed. Fifty milliliter of grapefruit juice was taken in a 250-ml of separating funnel and 50 ml of EtOAc was added, mixed well for 5 min. The organic layer was separated carefully and this extraction step was repeated twice. Extracts of each sample were pooled and the EtOAc was evaporated to dryness under vacuum using rotary evaporator (Buchi Analytical, New Castle, DE, USA). EtOAc extract residue was reconstituted in methanol and filtered through 0.45 μm membrane filter. Volumes of 95 μl of each samples were injected; elution was carried out at room temperature under methanol: water gradient conditions as per our previous published method [18].

Sample preparation and quantification of flavonoids

Flavonoid samples were prepared by treating 10 ml of grapefruit juice with 10 ml of methanol and mixed well for 5 min. The extract was separated from juice particles by filtration using a qualitative filter paper (VWR International, West Chester, PA, USA) and again filtered through 0.45 μm membrane for HPLC analysis. A volume of 10 μl of extract was injected into HPLC column; the elution was carried out at room temperature under aqueous methanol gradient conditions. Starting with 0 min, 35% methanol and 65% water; 5 min, 50% methanol and 50% water; 15 min, 75% methanol and 25% water; 20 min, 80% methanol and 20% water; 25 min, 100% methanol and 30 min initial conditions of 35% methanol and 65% of water were maintained. The flow rate was set at 0.6 ml/min and elution was monitored at 280 nm with a photodiode array detector. The column used was Luna C-18 with a particle size of 3 μm and dimensions of 150 × 4.60 mm (Phenomenex, Torrance, CA, USA).

Calibration curves for furocoumarins and flavonoids

Stock solutions of DHB, paradisin A, bergamottin, naringin and naringenin were prepared in methanol. Aliquots (25 μL) of six different concentrations (equivalent to 2.5, 5, 7.5, 10, 15 and 20 μg) of DHB, paradisin A, bergamottin, naringin and naringenin were injected into HPLC. Elution was carried out as discussed above to obtain peak area responses. The calibration curves for each compound were prepared by a plotting concentration of each compound versus peak area. For recovery study, grapefruit juice was divided into two groups; one group of juice samples were mixed with a known quantity of standard furocoumarins, flavonoids and samples were extracted as described above. The peak area corresponding to the DHB, paradisin A, bergamottin, naringin and naringenin were obtained from the spiked and non-spiked juices samples. The recovery percentage was calculated by comparing the difference in peak area between spiked and non-spiked samples.

Results and discussion

Citrus bioactive compounds

Health promoting properties of citrus are attributed to the wide array of bioactive compounds and their synergistic effects [32]. The major class of citrus bioactive compounds includes flavonoids, carotenoids, limonoids, folic acid, pectin, coumarins, vitamin C, soluble fiber and mineral potassium [33]. Our lab [34–36] and others [37, 38] have reported the anti-proliferative property of these bioactive compounds in several cancer cell lines. Unique among grapefruit bioactive compounds are furocoumarins, which have shown to increase the bioavailability of drugs by inhibiting the activity of CYP 3A4 and p-glycoprotein. These unique properties of furocoumarins lead to the consumer concern about toxicity, when grapefruit juice is consumed with certain medications [39].

Chromatographic profiles, calibration, percentage recovery and limit of quantification for furocoumarins and flavonoids

Figure 1 depicts the HPLC chromatograms and structures for dihydroxybergamottin, paradisin A and bergamottin. Figure 2 represents the HPLC chromatograms and structures of the flavonoids. The retention times for dihydroxybergamottin, paradisin A, bergamottin, naringin and naringenin were found to be 12.5, 37.8, 43.9, 9.4 and 15.6 min, respectively. Calibration curves for furocoumarins and flavonoids were derived from three injections of five different concentrations with good reproducibility and accuracy, as shown in Fig. 3. Regression equations were obtained with a correlation coefficient of ≥ 0.99. The limits of quantification were found to be 98, 79, 19, 38 and 19 ng for DHB, paradisin A, bergamottin, naringin and naringenin, respectively. The percentage recovery from spiked with non-spiked samples was found to be 99.3 ± 6.4, 97.2 ± 8.6, 98.3 ± 1.7, 99.5 ± 7.6, and 98.4 ± 3.1 for DHB, paradisin A, bergamottin, naringin and naringenin, respectively.

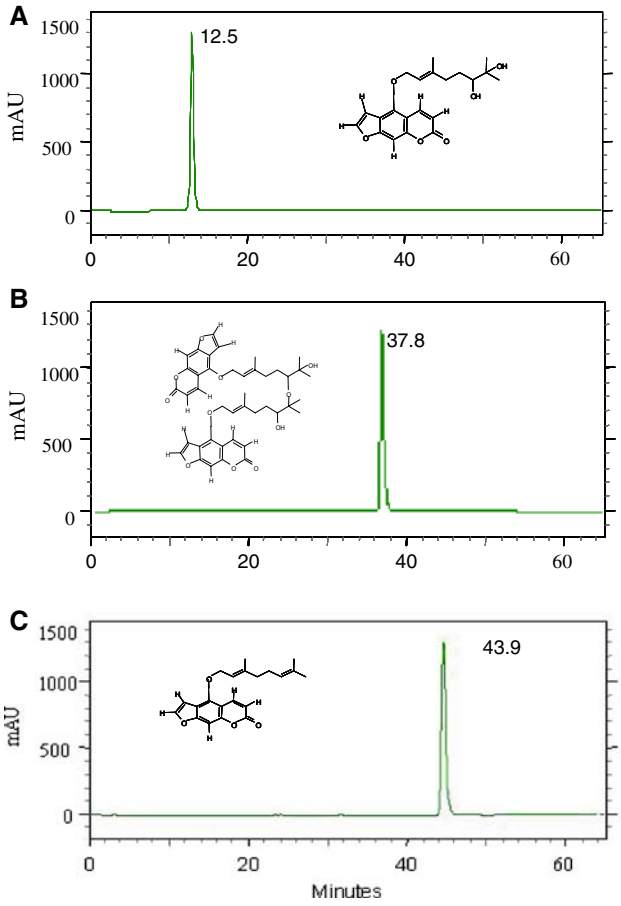

Fig. 1 Structures and HPLC chromatograms of furocoumarins found in grapefruit juice. **a** Dihydroxybergamottin, **b** paradisin A and **c** bergamottin

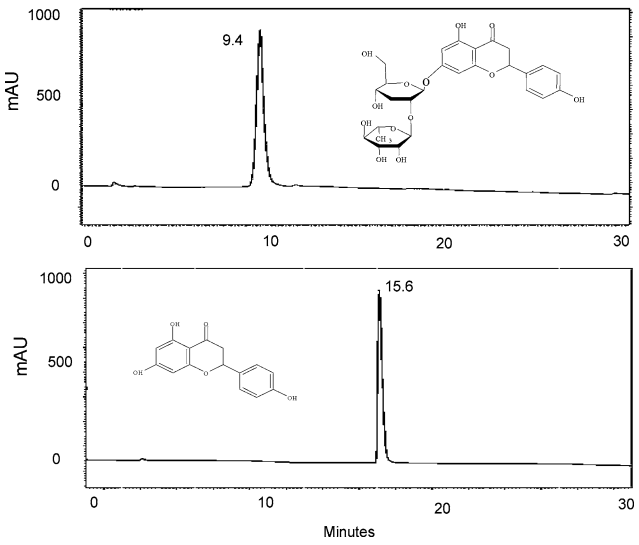

Fig. 2 Structures and HPLC chromatograms of standard naringin and naringenin. The elution was carried out on C-18 luna column with aqueous methanol

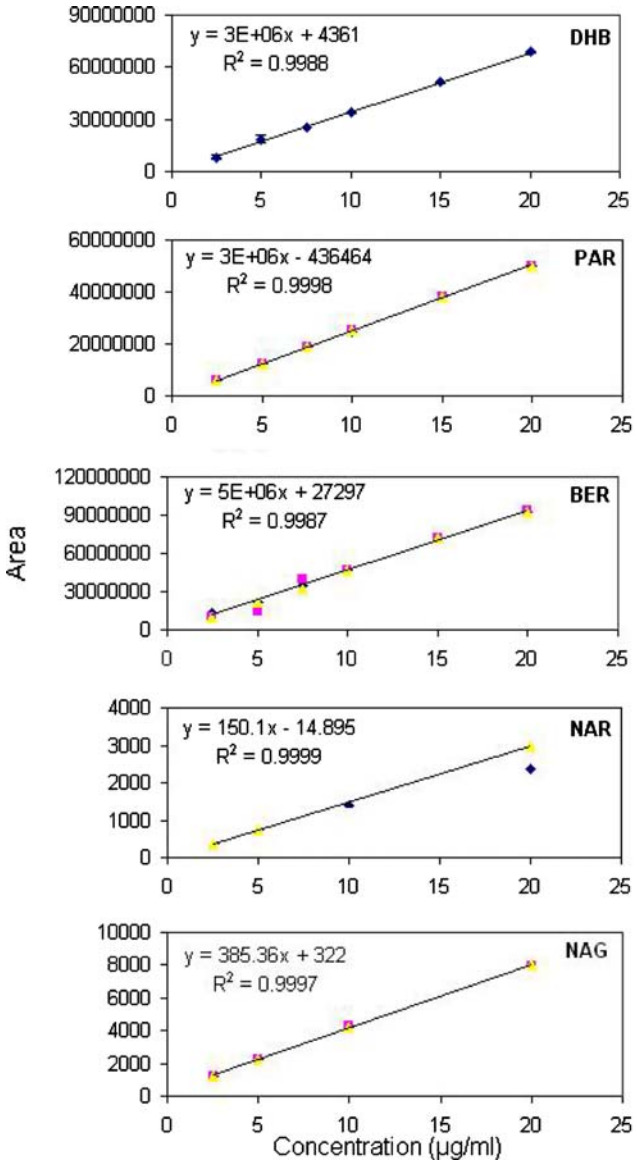

Fig. 3 Calibration curves and regression equations for standard furocoumarins (*DHB* dihydroxybergamottin, *PAR* paradisin A, *BEG* bergamottin) and flavonoids (*NAR* naringin and *NAG* naringenin) were eluted using the methods described in Materials and methods section. The standard curve was established with different concentrations ranging from 2.5 to 20 µg

Variation of furocoumarins and flavonoids

Furocoumarins were extracted with EtOAc from grapefruit juice, and the extract was concentrated under reduced pressure. The dried residue was dissolved in known quantity of methanol and injected to HPLC. Furocoumarins were resolved as a single peak in all the samples analyzed with no interference from other peaks/compounds. The identities of the peaks were confirmed by the determination of retention time and spiking with standards. Bergamottin and DHB levels showed more variation among grapefruit

varieties than paradisin A. Figure 4 demonstrates the levels of three furocoumarins in seven different grapefruit varieties and pummelo. The highest levels of DHB were found in Duncan (2.587 ± 0.092 μg/ml) while the lowest levels were found in Ray Red. In general, red and pink varieties contain higher levels of DHB than the white varieties while the level of DHB in pummelo was similar to colored varieties. The order of paradisin A content was found to be Star Ruby > Ruby Red > Marsh White > Pummelo > Duncan > Rio Red > Thompson Pink > Ray Red. There was no significant difference of paradisin A content between the colored and white varieties. The highest levels of bergamottin were found in Duncan and lowest levels were found in Ray Red.

A separate extraction and HPLC method were developed for the analysis of flavonoids. This method is modified over the existing methods to quantify naringin using small particle size column which reduced the run time by approximately 25 min. The extraction of both flavonoids varies, as naringin is more polar (due to glucosidal moiety) than its aglycones naringenin. Among the varieties tested, naringin in pummelo is higher as compared to other varieties. However, naringenin peak area could not be detected in any of the varieties using this extraction method. Naringin is a major flavonoid in all the varieties of the grapefruit juice, where as naringin content showed considerable variation among the varieties. These results are in agreement with the other reports [40]. The highest levels of naringin were found in pummelo and white varieties and the lowest levels were found in Rio Red (Fig. 5). Taxonomic studies showed that the grapefruit is a hybrid between pummelo and sweet orange [41]. Bitterness of grapefruit juice is mainly due to naringin. It is possible that higher levels of naringin in pummelo indicate the inheritance of a bitter property to grapefruits from pummelo.

Current available information on separation, quantification and variation of furocoumarins was on commercial grapefruit juices. It seems that marked differences exists

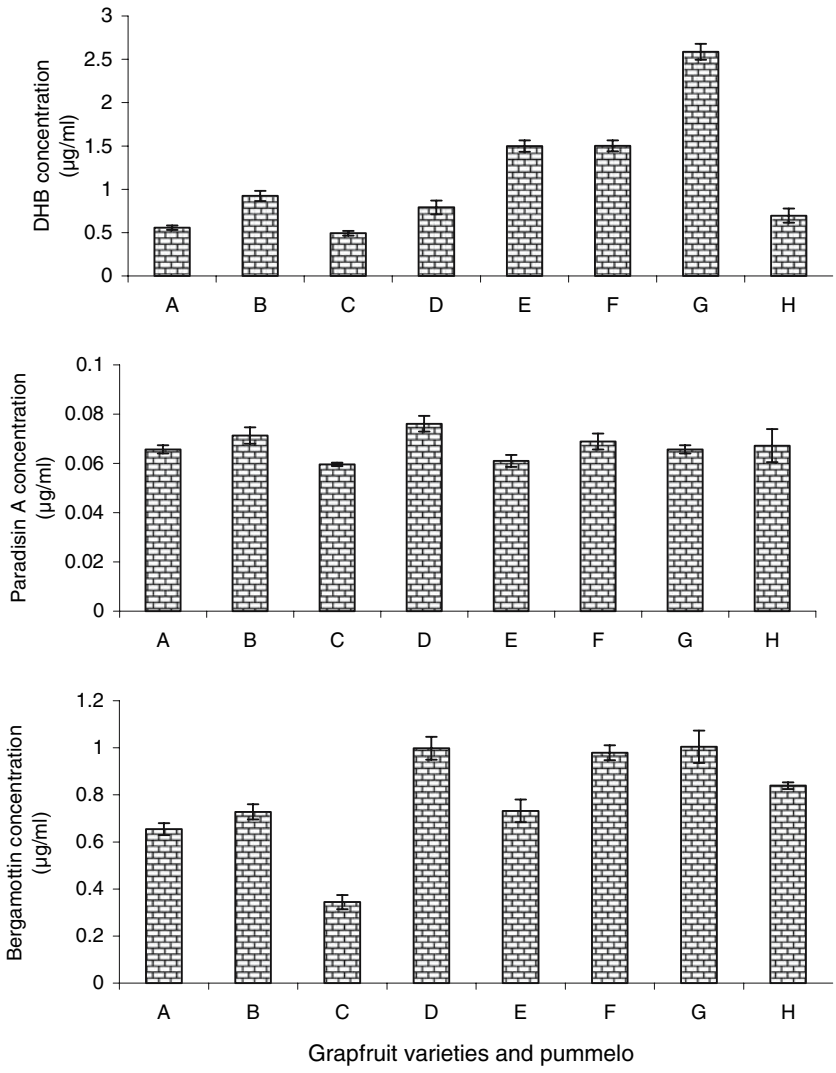

Fig. 4 Furocoumarins found in the seven varieties of grapefruits and pummelo. **a** DHB, **b** paradisin A and **c** bergamottin. Varieties used were *A* (*Rio Red*), *B* (*Ruby Red*), *C* (*Ray Red*), *D* (*Star Ruby*), *E* (*Thompson Pink*), *F* (*Marsh White*), *G* (*Duncan*) and *H* (*Pummelo*). The data are shown as the mean ± S.D, average of 15 samples.

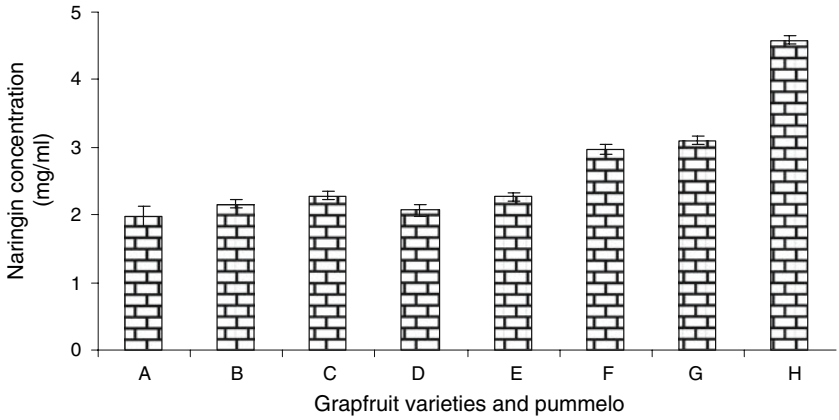

Fig. 5 Variation of naringin levels in seven varieties of grapefruits and its parent pummelo. Varieties used were *A* (*Rio Red*), *B* (*Ruby Red*), *C* (*Ray Red*), *D* (*Star Ruby*), *E* (*Thompson Pink*), *F* (*Marsh White*), *G* (*Duncan*) and *H* (*Pummelo*). The data are shown as the mean ± S.D, average of 15 samples

between fresh juice and commercially processed juice as some of the fruit components are removed, modified, added partially or fully back into the finished product [32]. Furthermore, juice undergoes physical treatments such as high temperature treatment and mechanical pressure which may influence these compounds.

When grapefruit juice is consumed, naringin is converted to naringenin by the intestinal gut bacteria, which is a key step in naringin metabolism [42]. The intestinal first-pass metabolism is exposed to both flavonoid glucosides and aglycones. Interestingly, the two flavonoids differ in their inhibitory effect on CYP 3A4 enzyme; naringenin is more potent than naringin. Naringin metabolism to naringenin differs among the individuals due to variation in the gut micro flora; this variation adds to the contradictory results of drug interaction with grapefruit juice and its bioactive components [43].

Co-consumption of grapefruit juice with certain medications (mainly metabolized by CYP 3A4) has substantial effect on oral bioavailability of drugs. This mainly occurs through the inhibition of first-pass metabolism of the drugs in the gut. In our earlier study, we reported dihydroxybergamottin, paradisin A and bergamottin as potent inhibitors of human CYP 3A4 and CYP 1B1 isoenzymes [18]. It is possible that wide variability in the grapefruit juice–drug interaction studies may be attributed to the variation of pharmacokinetics and pharmacodynamics [2, 7]. Two main causes are inter-individual variability in the CYP 3A4 enzyme levels and the variation of the bioactive compounds among grapefruit varieties. In comparison to experimental animal models, humans show large inter-individual variations in CYP 3A4 catalyzed drug oxidation reactions. These reactions sometimes may lead to different levels of susceptibility of humans to pharmacological and toxicological actions of drugs, xenobiotics and carcinogens [43].

It is evident from this study that grapefruit varieties differ in its bioactive furocoumarins and flavonoid contents. Studies related to the variation of other bioactive compounds, such as limonoids and carotenoids, due to different varieties, pre- and post-harvest effects may provide critical information on drug interaction. It is possible that some bioactive compounds may be low while other bioactive compounds might be higher in a specific variety. Thus, it is necessary to test grapefruit juice from different origins (cultivar, region, processing, fresh/stored) on the level of pharmacokinetics of drugs in vitro and in vivo to establish correlations between the levels of each compound and oral bioavailability. These compounds may act in synergistically making it more challenging to control the variability due to the bioactive compounds originating from the juice. It is also possible that hand-squeezed juice contain higher levels of specific furocoumarins than commercially processed juice [30].

Conclusion

This study demonstrates a wide variation of furocoumarins and flavonoids content among different varieties of grapefruits and pummelo. It provides a simple HPLC method for the separation and quantification of these compounds. Reported variation in the grapefruit juice-induced drug interaction stems for the fact that inhibitors of CYP3A4 enzyme varies considerably with juice preparation. There are exciting implications of this challenging problem of grapefruit juice–drug interactions. It may be possible to standardize the absorption of many medications with the identification of all the bioactive ingredients in grapefruit juice. Co-administration of grapefruit juice and/or purified bioactive compounds may inhibit CYP 3A4 and transporter proteins partially or fully. This type of strategy may reduce the dose of drugs and lead to a reduction in the cost of expensive medications by means of decreasing the first-pass metabolism.

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