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Bioorganic & Medicinal Chemistry 14 (2006) 2606–2612
Bioorganic & Medicinal Chemistry

Furocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes

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Received 26 October 2005; revised 17 November 2005; accepted 21 November 2005
Available online 9 December 2005

Abstract—Bioactive compounds present in grapefruit juice are known to increase the bioavailability of certain medications by acting as potent CYP 3A4 inhibitors. An efficient technique has been developed for isolation and purification of three furocoumarins. The isolated compounds have been tested for the inhibition of human CYP 1B1 isoform using specific substrates. Grapefruit juice was extracted with ethyl acetate (EtOAc) and the dried extract was loaded onto silica gel column chromatography. Further, column fractions were subjected to preparative HPLC to obtain three compounds. The purity of these compounds was analyzed by HPLC and structures were determined by NMR studies. The identified compounds, bergamottin, 6′,7′-dihydroxybergamottin (DHB), and paradisin-A, were tested for their inhibitory effects on hydroxylase and *O*-dealkylase activities of human cytochrome P450 isoenzymes CYP 3A4 and CYP 1B1. Paradisin-A was found to be a potent CYP 3A4 inhibitor with an IC_{50} of 1.2 μM followed by DHB and bergamottin. All three compounds showed a substantial inhibitory effect on CYP 3A4 below 10 μM. Inhibitory effects on CYP 1B1 exhibited a greater variation due to the specificity of substrates. Paradisin A showed an IC_{50} of 3.56 ± 0.12 μM for the ethoxy resorufin *O*-dealkylase (EROD) activity and 33.56 ± 0.72 μM for the benzyloxy resorufin (BROD). DHB and bergamottin showed considerable variations for EROD and BROD activities with an IC_{50} of 7.17 μM and 13.86 μM, respectively.
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1. Introduction

Grapefruit juice contain several potential health-promoting bioactive compounds such as lyocopene, beta carotene, limonoids, pectin, flavonoids. Our lab[1,2] and others[3] have been isolating some of these bioactive compounds to understand their biological activities.[4,5] Recent studies have also provided evidence of the health-promoting properties of certain bioactive compounds for the prevention of cancer and reduction of cholesterol in animal and cell culture studies.[6–10] Despite their potential health benefits, some bioactive compounds present in grapefruit juice have shown to increase the bioavailability of orally administered drugs such as cholesterol-lowering statins,[11–14] calcium channel blockers,[15–17] immunosuppressants,[18,19] antihistamines[20,21], and many other medications.[22–26] Increased plasma concentration of orally administered drugs is caused by inhibition of CYP 3A4 enzymes, which plays a pivotal role in the oxidative biotransformation of these drugs.[27,28] CYP 3A4 is an abundant subfamily of CYP 450 enzyme which metabolizes many lipophilic drugs delivered by P-glycoprotein through hydroxylation and other oxidative mechanisms.[22] The inadvertent discovery of grapefruit juice interaction with drug was reported nearly a decade ago, while investigating the ethanol masking effect of grapefruit juice.[29] Contradicting reports in drug interaction literature[30,31] indicate a need for further studies involving purified compounds.

Initial investigations considered naringin and its aglycone naringenin as possible putative compounds responsible for drug interactions. However, co-administration of naringin with medication in vivo had minimal effects on bioavailability of the drug.[32] Based on the observations of varied levels of furocoumarins inducing different

Abbreviations: HPLC, High-performance liquid chromatography; CYP 450, Cytochrome P450; NMR, nuclear magnetic resonance; EROD, ethoxy reosrufin *O*-dealkylase; BROD, benzyloxy resorufin *O*-dealkylase; DBF, dibenzyl fluorescein; DHB, dihydoxybergamottin.

Keywords: Grapefruit; Furocoumarins; Bergamottin; 6′,7′-Dihydroxybergamottin; Paradisin-A; Drug interaction; Cytochrome P450 inhibitors; EROD; BROD.

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doi:10.1016/j.bmc.2005.11.039

degrees of interaction, it was determined that furocoumarins play a significant role in grapefruit juice drug interactions.[33,34] Thus, research on furocoumarins or psoralens, known photosensitizers in cosmetics,[35] gained considerable attention for CYP 3A4 inhibitory effects. Furocoumarins are polyphenolic compounds, synthesized from L-phenylalanine, which may occur in a linear form with the furan ring attached to the 6, 7 position of the benzo-2-pyrene nucleus. These compounds are toxic in nature, acting as prooxidants by covalently binding to DNA and proteins in the presence of ultraviolet light.[36] Bergamottin is the major furocoumarin in fresh grapefruit and present in similar levels in juice and segment membranes, while to a lesser degree in peel extracts.[37,38] Some studies indicated that 6′,7′-hydroxybergamottin[39] and paradisin A[40] are the major CYP 3A4 inhibitors in vivo system.

Positive aspects of grapefruit juice induced drug interactions are related to the reduction of costs incurred in clinical and pharmacological applications to treat different illnesses. The juice contains innumerable number of health-promoting compounds, which are known to reduce atherosclerotic plaque formation,[41] inhibition of cancer cell proliferation,[42,43] and as antioxidants.[44] Furocoumarin derivatives and flavonoids of grapefruit have shown the capacity to inhibit the activity of certain other human cytochrome P450 enzymes[45] including CYP 1B1 which is a target for inhibition in anticarcinogenesis strategies.[46] CYP 1B1 is expressed at high frequency in various human cancerous but not in normal tissues.[47] Hence, much emphasis is on the inhibition of this enzyme to develop potential cancer therapeutic drugs. Naturally occurring isopimpinellin was shown to inhibit CYP 1B1 in 7,12 dimethylbenza[*a*]anthracene (DMBA) induced skin cancer.[48] Assessing the alkoxyresorufin dealkylase activity of CYP 450 isoforms is reported to be a good assay system to test the inhibitory effects of naturally occurring compounds.[45] While there are different reports of various magnitudes of inhibition with different grapefruits, like red and white cultivars,[49] most of the available reports were based on juice or total fruit extracts. The studies on individual furocoumarins are limited by the non-availability of purified compounds.[50] In the present study, we report the isolation, purification, and characterization of three furocoumarins from grapefruit juice. Furthermore, the isolated compounds have been tested for the inhibition of human CYP 1B1 isoform using specific substrates.

2. Results and discussion

Concentrated grapefruit juice was diluted and extracted with ethyl acetate. The crude extract was purified using silica gel column chromatography to obtain partially purified fractions (Fig. 1). Fraction 4 showed compounds of our interest and it was purified using preparative HPLC with gradient mobile phase as mentioned in Table 1 to obtain three compounds. The purity of compounds **1**–**3** was analyzed by analytical HPLC. Figure 2 depicts HPLC chromatograms of crude ethyl acetate extract and compounds (**1**–**3**). The relative retention times of compounds **1**, **2** and **3** were found to be 12.6 ± 0.12, 37.7 ± 0.32, and 43.6 ± 0.36 min, respectively. All three compounds were observed as a bluish-white fluorescent spot under UV light on the TLC plates. In addition, compounds **1**–**3** showed UV absorption maxima at 321 and 231 nm under UV light (366 nm), indicating the presence of coumarin nuclei.[51] Compounds **1**–**3** were characterized and identified as bergamottin, dihydroxybergamottin, and paradisin-A, respectively, using ^{1}H and ^{13}C NMR spectra. Chemical shifts[51,52] of the furocoumarins were in accordance with reported values (Tables 2 and 3). NMR spectral assignments were confirmed with the help of 2D NMR such as HMBC, DEFT, DQFCOSY, and HSQC spectra (Fig. 3).

Fukuda et al.[49] reported the isolation of bergamottin, paradisin A, and paradisin B from grapefruit juice.

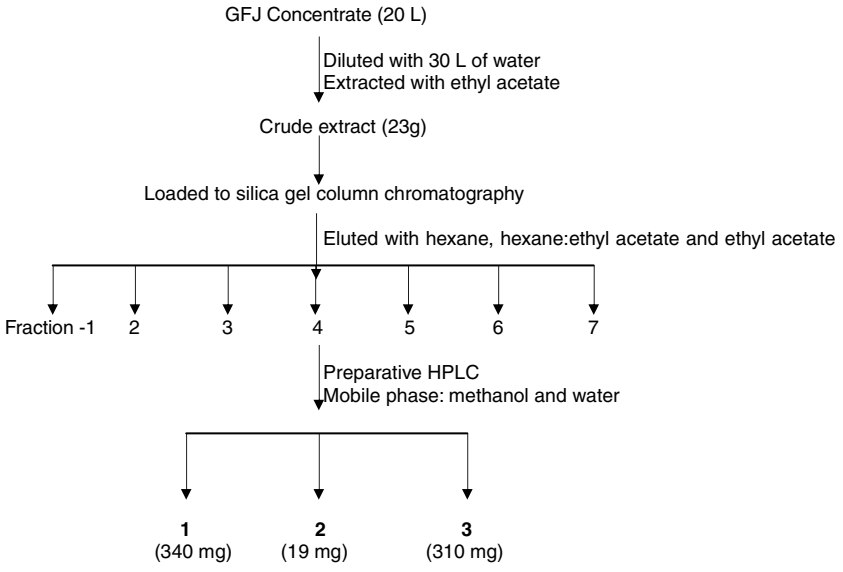


Figure 1. Isolation of furocoumarins from grapefruit juice concentrate (GFJ).

Table 1. Gradient mobile phase used for analytical and preparative high performance liquid chromatography

Time	Methanol (%)	Water (%)
Analytical HPLC		
0	60	40
20	80	20
25	80	20
45	85	15
50	90	10
55	95	5
60	100	0
65	100	0
Preparative HPLC		
0	40	60
45	45	55
90	60	40
120	90	10
130	95	5
140	100	0

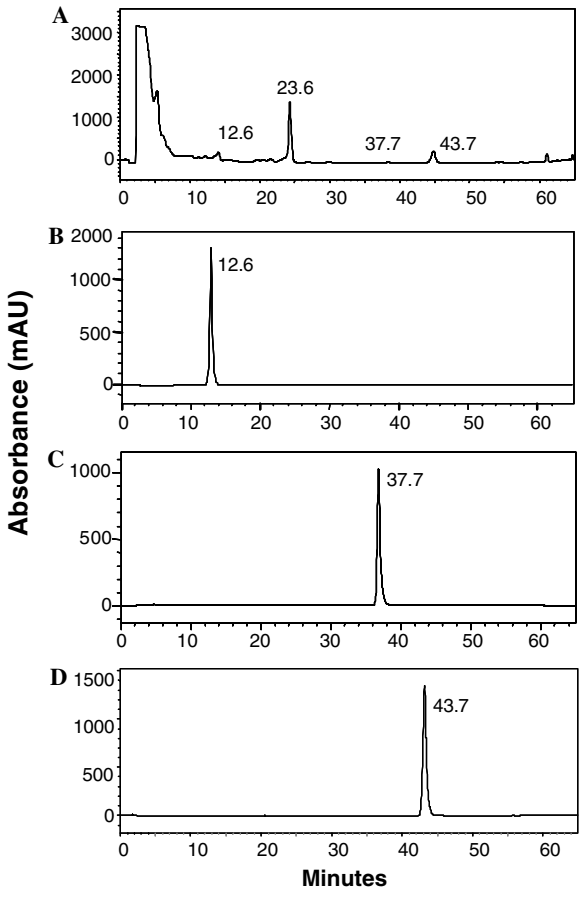


Figure 2. HPLC chromatograms of (A) crude extract, (B) dihydroxybergamottin, (C) paradisin A, and (D) bergamottin.

However, there is little or no information on isolation of furocoumarins. Recently, Manthey and Buslig[10] reported the composition of furocoumarins in different juice fractions by LC–MS.[10] Wangensteen et al.[52] reported the isolation of epoxybergamottin from peels of grapefruit using diethyl ether. Ohta et al.[40] reported isolation of paradisin C from grapefruit juice using hexane–ethyl acetate extract followed by column chromatography and HPLC separation. However, most of the published literature is not clear about the isolation procedures from grapefruit juice. In the present report, a systematic study has been conducted for the isolation and identification of three furocoumarins with yield.

CYP 3A4 activity was based on the hydroxylation of dibenzylfluorescein. A sharp decline in CYP 3A4 activity was observed for all the furocoumarins tested in a dose-dependent manner (Fig. 4A). Paradisin A strongly inhibited CYP 3A4 with an IC_{50} as low as 1.21 ± 0.08 μM, while bergamottin was the weakest among the three compounds tested with an IC_{50} of 6.78 ± 0.09 μM (Table 4). The activity of CYP 1B1, measured in terms of *O*-dealkylation of ethoxyresorufin (EROD) and benzyloxyresorufin (BROD) on the contrary, varied considerably with the use of different substrates (Table 3). It was observed that CYP 1B1 showed a higher affinity toward ethoxy resorufin as the inhibitory levels were found to be much lower than benzyloxy resorufin (Table 4). Similar to CYP 3A4 activity, paradisin A showed a greater inhibitory effect on CYP 1B1 when ethoxy resorufin was used as a substrate. However, bergamottin showed a greater inhibitory effect on CYP 1B1, when benzyloxy resorufin was used as a substrate. Despite low levels in grapefruit juice and the unstable nature of structural dimer,[39] paradisin A could be exerting an effect on CYP 3A4 inhibition, as could other major compounds such as bergamottin and DHB can exert inhibitory effects alone as their concentrations were found to be higher in grapefruit juice. Since CYP 1B1 plays a key role in anticancer drug research, the inhibition of this enzyme will lead to the enhanced pharmacological effects of anticancer agents, thereby lowering the dose of medication. More than 80% reduction in EROD activity at 50 μM levels (Fig. 4) of these major furocoumarins of grapefruit juice indicate the strong potential of these compounds as anticancer therapeutic agents. At the same time, the lower inhibitory effects of these compounds on BROD (Fig. 4) indicates the lower affinity of these isoenzymes for the biotransformation of benzyloxy derivatives. The inhibitory effect of these compounds was found to be considerably high within the 10 μM levels of the test dose and was found to reach a plateau effect of over 10–50 μM (Table 4). This indicates a specific number of substrate binding sites on this isoform. Since CYP 1B1 involved in the estrogen mediated hormonal carcinogenesis[46] and furocoumarins shares structural similarities with those of the cholesterol derivatives, the actual mechanisms involved in the inhibition of this enzyme and toxic effects related to the compounds need to be studied.

3. Materials and methods

3.1. Materials

All the solvents and chemicals used were of GR and HPLC grade, and were obtained from EMD chemicals Inc., Gibbstown, NJ. Silica gel of 200–400 mesh size

Table 2. ^{1}H NMR data of compounds **1**–**3**[a] ($CDCl_3$)

C	1	2	3
3	6.24 (1H d, 9.8 Hz)	6.24 (1H, d, 9.8 Hz); 6.26 (1H, d, 9.5 Hz)	6.26 (1H, d, 9.9 Hz)
4	8.12 (1H, d, 9.8 Hz)	8.05 (2H, d, 9.5 Hz)	8.05 (1H, d, 9.9 Hz)
8	7.10 (1H, s)	7.02 (2H, s)	6.74 (1H, s)
11	6.93 (1H, d, 2.0 Hz)	7.07 (2H, d, 2.3 Hz)	6.74 (1H, d, 2.5 Hz)
12	7.57 (1H, d, 2.0 Hz)	7.85 (1H, d, 2.3 Hz)	7.55 (1H, d, 2.2 Hz)
13	4.91 (2H, d, 6.5 Hz)	4.65 (2H, d, 6.5 Hz); 4.63 (2H, d, 2.3 Hz)	4.96 (2H, d, 6.6 Hz)
14	5.56 (1H, t, 6.5 Hz)	5.35 (1H, t); 5.38 (1H, t)	5.50 (1H, t, 6.8 Hz)
16	1.68 (3H, s)		1.67 (3H, s)
18	2.29 (2H, m)		2.07 (2H, m)
19	3.30 (lH, t)		1.95 (2H, m)
20	1.18 (3H, s)	1.18 (3H, s)	1.65 (3H, s)
21	1.14 (3H, s)	1.33 (3H, s)	1.58 (3H, s)

s, singlet; d, doublet; t, triplet; m, multiplet.
[a] Chemical shifts are followed by coupling constants *J* values in parentheses.

Table 3. ^{13}C NMR data of compounds **1**–**3** ($CDCl_3$)

C	1	2	3
2	161.6	160.5[a]	161.5
3	112.5	112.4[a]	112.5
4	139.9	138.6; 138.4[b]	139.7
5	148.9	149.0[a]	149.0
6	119.3	117.9; 118.4[b]	118.9
7	158.2	157.7[a]	158.2
8	93.3	93.8[a]	94.2
9	152.6	151.6[a]	152.7
10	107.5	106.9[a]	107.5
11	105.1	105.1[a]	105.2
12	145.1	144.6[a]	145.0
13	69.7	66.8; 66.7[b]	69.8
14	114.2	114.3[a]	114.3
15	143.0	142.8; 142.6[b]	143.1
16	16.7	17.01; 16.9[b]	16.8
17	36.5	34.5; 36.6[b]	39.6
18	29.5	29.3; 35.9[b]	26.3
19	73.1	82.5; 77.8[b]	123.6
20	77.9	71.6; 76.5[b]	132.1
21	23.3	25.8; 21.1[b]	17.8
22	26.6	27.7; 27.5[b]	25.8

[a] Showed one signal for two carbons.
[b] Two chemical shifts in the same row for two carbons in dimer unit for the corresponding carbon.

60 Å for column chromatography was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Concentrated grapefruit juice was obtained from Texas Citrus Exchange, Mission, Texas, USA. Juice was stored at 5 °C and 95% relative humidity. Human CYP 450 liver microsomes and ketoconazole were obtained from BD Gentest Inc. (Woburn, MA). Alkoxyresorufins and other chemicals from Sigma Aldrich Co. (St. Louis, MO, USA). For the activity studies stock solutions of 1 M alkoxyresorufins were prepared in DMSO and stored in dark.

3.2. Extraction

Concentrated grapefruit juice (20 L) was diluted by stirring with distilled water to attain the brix at around 30°. The diluted juice (50 L) was extracted with ethyl acetate in 1:1 ratio three times consecutively. Organic layer was separated and dried to obtain crude extract.

3.3. Purification of furocoumarins

Dried ethyl acetate extract (23 g) was impregnated with 23 g of silica gel and loaded onto silica gel (500 g) column chromatography. The column was eluted with hexane, hexane/ethyl acetate, and ethyl acetate with increasing polarity such as 95:5, 90:10, 85:15, 80:20, 60:40, 40:60 and 0:100. Fractions 1 through 7 were collected and analyzed by TLC and HPLC for furocoumarins. Fraction number four showed furocoumarins and it was subjected to concentration under vacuum.

3.4. Preparative HPLC

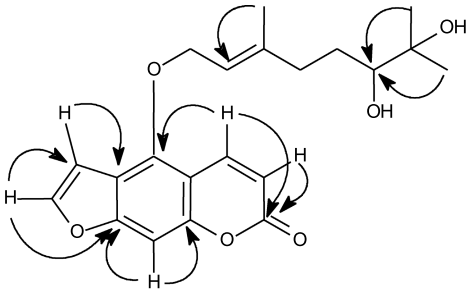
The preparative HPLC run was performed using Waters prep HPLC system (Waters Corporation, Milford, Massachusetts, USA). Mobile phase used was aqueous methanol as shown in Table 1. The flow rate was set 25 mL/min and detection was carried out at 240 nm. The vacuum concentrated fraction was reconstituted with methanol and injected into the preparative HPLC column. Different fractions were collected as per the peak retention time. All the fractions were analyzed with TLC and HPLC. Fractions having a similar spot/retention time were pooled, concentrated under vacuum, and freeze-dried. The yields of compounds **1**, **2**, and **3** were 340, 19, and 310 mg, respectively.

3.5. TLC analysis

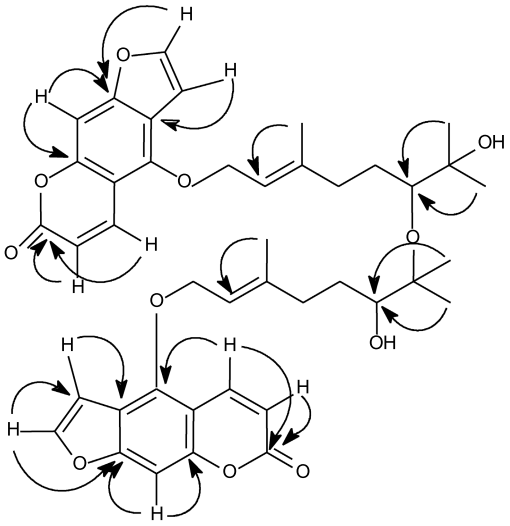
Purified compounds were dissolved in methanol, spotted on TLC plates, and separated using hexane/ethyl acetate (4:1) as mobile phase. The compounds were visualized as black spots when sprayed with 10% sulfuric acid in methanol followed by heating at 110 °C for 10 min.

3.6. HPLC analysis

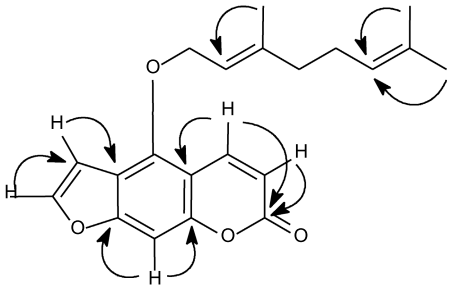
The HPLC system consisted of a Thermo Electron Corporation P-400 quaternary HPLC pump (Thermo Electron Corporation, CA, USA), Membrane degasser LDC analytical and Spectra system AS3000 autosampler (Thermo separation products, CA, USA). Peaks were analyzed with a photodiode array detector (Thermo separation products, CA, USA). Chromatographic



6', 7'-Dihydroxybergamottin (1)



Paradisin A (2)



Bergamottin (3)

Figure 3. HMBC correlations observed for isolated compounds (**1**–**3**).

separations were accomplished on Chemcosorb-5-ODS column (150 × 6.0 mm, 5 μm particle size) (ChemcoPak, Osaka, Japan). Elution was carried out at room temperature under water methanol gradient conditions as shown in Table 1. The flow rate was set at 1.1 mL/min and elution was monitored with UV at 240.

3.7. Identification

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on a JEOL AMX 300 FT instrument (JEOL-USA, Inc., Peabody, MA). TMS was used as internal standard. ^{13}C NMR assignments were given on the basis of FLOCK, DEFT spectra.

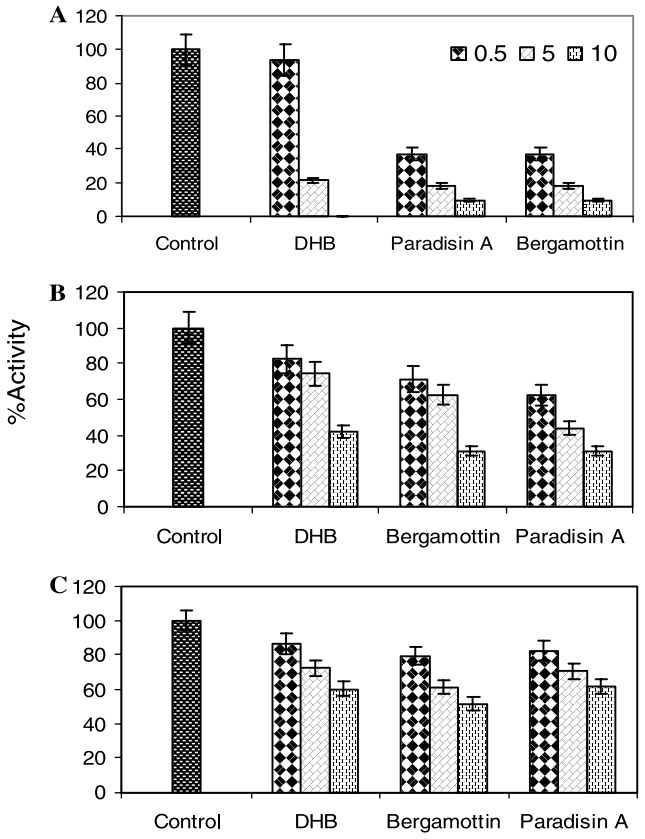


Figure 4. Inhibitory effects of furocoumarins on cytochrome P450 isoenzymes. (A) Hydroxylase activity of CYP 3A4; (B) benzyloxyresoruf in *O*-dealkylase activity of CYP 1B1 and (C) ethoxyresorufin *O*-dealkylase activity of CYP 1B1. Values are means ± SD, $n = 3$.

Table 4. IC_{50} values for the furocoumarins tested for the inhibitory effects on CYP isoforms, expressed as means (μM) ± SD, $n = 3$

	CYP 1B1		CYP 3A4
	ER	BR	DBF
Dihydroxybergamottin	8.89 ± 0.07	56.37 ± 0.69	3.26 ± 0.107
Paradisin A	3.56 ± 0.12	33.56 ± 0.72	1.21 ± 0.079
Bergamottin	7.17 ± 0.08	13.86 ± 0.24	6.78 ± 0.091

3.8. Ethoxy-, benzyloxy, -*O*-dealkylase activity of CYP 1B1

O-Dealkylase activity of CYP 1B1 isoforms was determined using ethoxy resorufin and benzyloxyresorufin substrates. The reactions were performed on a 96-well microtiter plate (BD falcon, Franklin Lakes, NJ, USA) using Synergy HT fluorescence plate reader (Bio-Tek Instruments Inc. Winoski, VA). Reaction buffer consisted of 1.3 mM NADP, 3.3 mM glucose 6-phosphate, 0.4 U/mL glucose 6-phosphate dehydrogenase, and 3.3 mM magnesium chloride in 0.1 M sodium phosphate buffer (pH 7.4). Furocoumarins ranging from 0.1 to 10 μM concentrations, along with 10 μM of substrates, were added to the reaction buffer, keeping the final volume at 200 μL. Ice-cold NADPH oxidoreductase and 5 pmol of CYP isoenzymes were added just before the start of the reaction. Fluorescence of resorufin formation was taken at an excitation of 530 ± 15 nm and

emission of 590 ± 15 nm. IC_{50} concentrations for different substrates and enzymes were calculated as described before.[6,53] Percent activity is compared against untreated controls, based on the picomoles of resorufin formed per picomoles of microsomal protein per minute at different levels of furocoumarins.

3.9. Assay of CYP 3A4 inhibition

Inhibition of CYP 3A4 by different furocoumarins was determined based on the reduction of dibenzylfluorescein by hydroxylase activity of CYP 3A4. The detailed procedure is described in the previous paper.[54,55] Ketoconazole was used as a positive control and the reaction was terminated with the addition of 2 N sodium hydroxide. An end point fluorescence reading of the fluorescein by CYP 3A4 hydroxylase activity was measured at an excitation of 485 ± 15 nm and emission of 538 ± 15 nm. The metabolite formation was compared using the standard fluorescein. The percent activity was based on the picomoles of fluorescein formed per picomoles of microsomal protein per minute at different levels of furocoumarins.

Acknowledgments

Funding for this project was provided by the Texas Citrus Board Producers 461555, USDA-IFAFS 2001-52102-11257 and USDA Food for Health 2004-34402-14768. We sincerely thank Mr. Manuel Ochoa, TCX, Mission, TX, and Dr. Gene Lester, USDA, Weslaco, TX, for their generous help during the course of this study. We wish to thank Prof. Jennifer S. Brodbelt, The University of Texas at Austin, Austin, TX, for the helpful suggestions during this research.

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