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Radical scavenging and cytochrome P450 3A4 inhibitory activity of bergaptol and geranylcoumarin from grapefruit

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Abstract—Grapefruit juice has been shown to increase the oral bioavailability of several clinically important drugs by inhibiting first pass metabolism. Several compounds in grapefruit juice have shown different biological activities. Unique among them are furocoumarins with potent inhibitory activity against cytochrome P450 enzymes. In the present study, two bioactive compounds were isolated from grapefruit juice and grapefruit peel oil. The purity of the isolated compounds has been analyzed by HPLC. Structures of the compounds were elucidated by extensive NMR and mass spectral studies and identified as bergaptol and geranylcoumarin. The isolated compounds were tested for their radical scavenging activity using 2,2′-azobis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazil (DPPH) methods at different concentrations. Bergaptol showed very good radical scavenging activity at all the tested concentrations. Furthermore, these compounds were evaluated for their inhibitory activity against CYP3A4 enzyme. Bergaptol and geranylcoumarin were found to be potent inhibitors of debenzylation activity of CYP3A4 enzyme with an IC_{50} value of 24.92 and 42.93 μM, respectively.

1. Introduction

Furocoumarins are widely distributed in plants and found in commonly used food plants such as parsnips, parsley, celery, and grapefruit.[1–4] Grapefruit juice furocoumarins are responsible for marked increase in the oral bioavailability of many drugs,[5–13] with unintended consequences and risk of toxicity.[5] Furocoumarins increase the bioavailability of drugs mainly by reducing the first pass metabolism of drugs by inhibiting the CYP3A4 enzyme and modulating the activity of transporter proteins in the gut.[14–17] CYP3A4 is the most abundant enzyme among all the cytochrome P450 enzymes, expressed in the human liver and small intestine and also in some extra-hepatic tissues such as lungs, stomach, and colon.[18] In humans, members of the CYP3A subfamily account for 30% of total cytochrome in liver and 70% of those in the gut.[18] CYP3A4 contributes to the metabolism of approximately 50% of the drugs marketed or under development.[19] Many of these are important drugs such as lovastatin, statins, prostate hypertrophy inhibitor, immunosuppressants, protease inhibitors, and sildenafil.[20] Inhibition and induction of CYP3A4 affects drug development process and clinical use related to the role of drug disposition, bioavailability, and drug interactions such as drug–drug and food–drug interactions. Bergamottin is the major furocoumarin in fresh grapefruit and present in similar levels in juice and segment membranes while to a lesser degree in the peels.[3] Studies in our laboratory[1] and elsewhere[4,21–23] showed that furocoumarins isolated from grapefruit juice such as 6′,7′-dihydroxybergamottin, bergamottin, paradisin A, paradisin B, and paradisin C are potent CYP3A4 inhibitors when tested in vitro.

Several reports from our laboratory[24–26] and elsewhere[27–29] have shown that citrus fruits possess high antioxidant potential and studies with whole fruit extracts or their parts have shown to influence plasma lipids.[29] Citrus secondary metabolites such as ascorbic

Abbreviations: HPLC, high-performance liquid chromatography; CYP P450, cytochrome P450; NMR, nuclear magnetic resonance; MS, mass spectrometry.

Keywords: Grapefruit juice–drug interaction; Furocoumarin; Coumarin; Antioxidant activity; CYP3A4.

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acid, flavonones, phenolics possess antioxidant property and have shown several health benefits. Our lab recently showed inhibition of human cancer cell proliferation and induction of apoptosis in human breast cancer cell lines and colon carcinogenesis in rat models by citrus limonoids.[26,30,31] Several antioxidant compounds have been identified from citrus[29] however, there is no report of antioxidant potential of coumarins and furocoumarins from grapefruits. To the best of our knowledge this is the first report on isolation, characterization and evaluation of radical scavenging activity and P450 3A4 inhibition properties of bergaptol and geranylcoumarin from grapefruit.

2. Results and discussion

2.1. Isolation and identification

Concentrated grapefruit juice was extracted with ethyl acetate. Extract was purified on a silica column by a gradient of hexane and ethyl acetate. Elution was carried out using ethyl acetate in hexane to obtain 395 mg of compound **1**. Grapefruit peel oil was injected onto preparative HPLC column and eluted with gradient aqueous methanol as mentioned in methods section. The peak containing compound **2** was pooled from several runs. The aqueous methanol fraction was rotary evaporated to remove the organic phase and freeze-dried to obtain 280 mg of compound **2**. TLC of the isolated compounds showed single spot after spraying with 10% sulfuric acid in methanol followed by heating at 110 °C for 10 min. Further, purity of the compounds has been analyzed by HPLC and the chromatograms have been presented in Figure 1. UV absorption, HPLC retention times, and molecular mass of isolated compounds have been presented in Table 1. Moreover, the isolated compounds have been analyzed for mass, and compounds **1** and **2** showed [M+1] ions at *m/z* 203.2 and 299.0, respectively (Fig. 2).

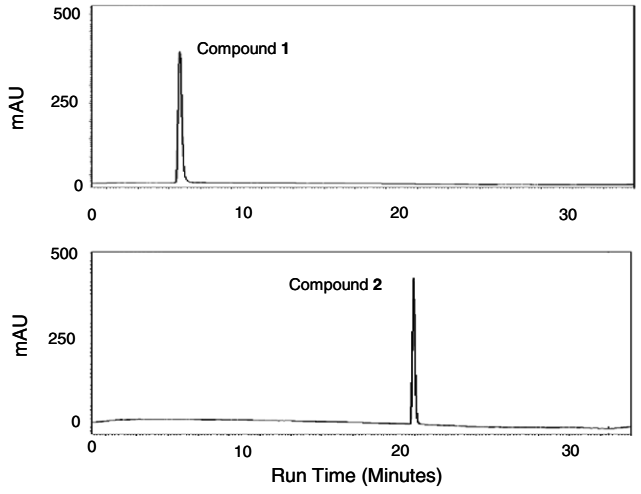

Figure 1. HPLC chromatograms of isolated compounds **1** and **2**, elution was carried out with aqueous methanol using C-18, luna column.

Table 1. Molecular mass, retention time, absorption maxima, and IC_{50} values for CYP3A4 inhibition of bergaptol and geranylcoumarin

Compound	Molecular mass	Retention time	Absorption maxima	IC_{50} values CYP3A4 inhibition (μM)
1	202	5.61	268, 248, 313	24.92
2	298	21.1	218, 316, 310	42.93

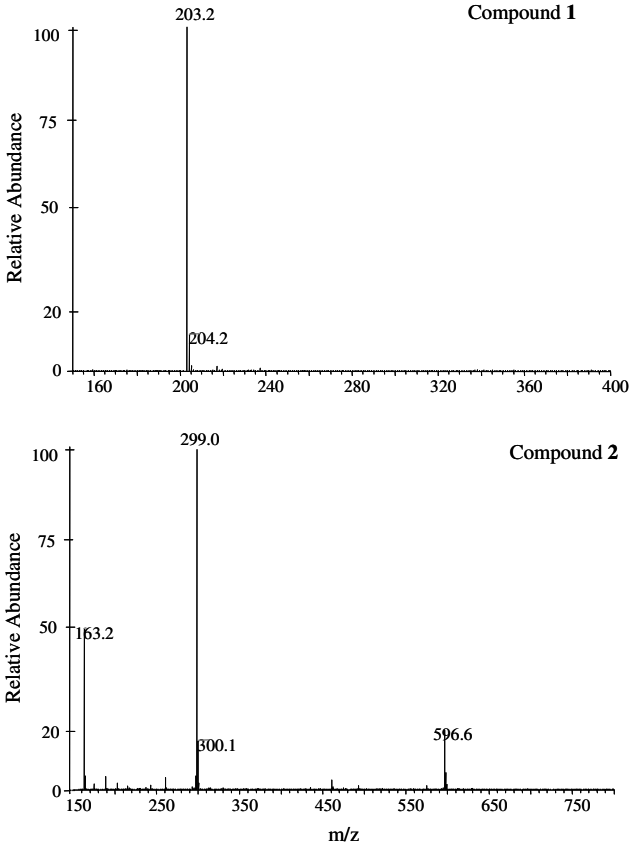

Figure 2. Mass spectra of compounds **1** and **2** using APCI mode.

Derivation of the chemical structures of the isolated compounds was based on the use of several 1D and 2D NMR experiments. DQF-COSY spectra showed cross peaks due to geminal proton–proton (2 bond couplings in methylene protons) as well as vicinal (3 bond) proton–proton *J* couplings. By tracing the cross peaks in DQF-COSY spectra along both the frequency dimensions, connectivity network of protons was made. Carbon NMR signals were grouped according to their multiplicity using SEFT spectra (which show positive signals for quaternary and CH_2 carbons; negative signals for CH and CH_3 carbons) and edited HSQC spectra (positive cross peaks for CH_2 carbons; negative cross peaks for CH and CH_3 carbons). Individual assignment of the carbons and the attached protons was then made from the combination of multiplicity edited HSQC and HMBC spectra. Each proton in HMBC spectra showed cross peaks to carbons which are up to three bonds away. Finally, the derived chemical structures of both the natural products were confirmed using

the carbon–carbon connectivity based 1, 1-ADEQUATE experiments. Typical 1D ^{1}H and ^{13}C spectra of compound **1** are shown in Figure 3 along with the assignments of all the protons and carbons. Figure 4 shows 2D spectrum of HMBC for the compound **2** along with the assignments of all the protons and carbons. Complete ^{1}H and ^{13}C chemical shifts determined for both the compounds are given in Table 2. Based on different spectral studies compound **1** was identified as bergaptol and compound **2** was identified as geranylcoumarin.

2.2. Free radical scavenging activity

Free radical scavenging potentials of bergaptol, geranylcoumarin, and ascorbic acid at different concentrations were tested by ABTS and DPPH methods. The results of ABTS and DPPH methods are presented in Figures 5 and 6, respectively. At 200 ppm, compound **1** and **2** and ascorbic acid showed 29.7%, 1.3%, and 89%, respectively, using ABTS method. Conversely, DPPH method showed 42.5%, 3.3%, and 88%, respectively. Compound **1** showed 91.1 and 82.9 radical scavenging activity at 800 ppm using ABTS and DPPH methods, respectively. Generally antioxidants will react with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm and converted into 1,1,-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate.[32] The degree of discoloration indicates the scavenging potentials of the antioxidant extracts. It is known that free radicals cause auto-oxidation of unsaturated lipids in food.[33] On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid.[34] The data obtained revealed that the isolated compounds are free radical scavengers and primary antioxidants that react with DPPH radical, which may be attributed to its proton donating ability. The antioxidant activity of the citrus fractions was ascribed to their hydrogen donating ability.[33]

2.3. CYP3A4 inhibition activity

Results of CYP3A4 enzyme inhibition by bergaptol and geranylcoumarin at various micromolar concentrations

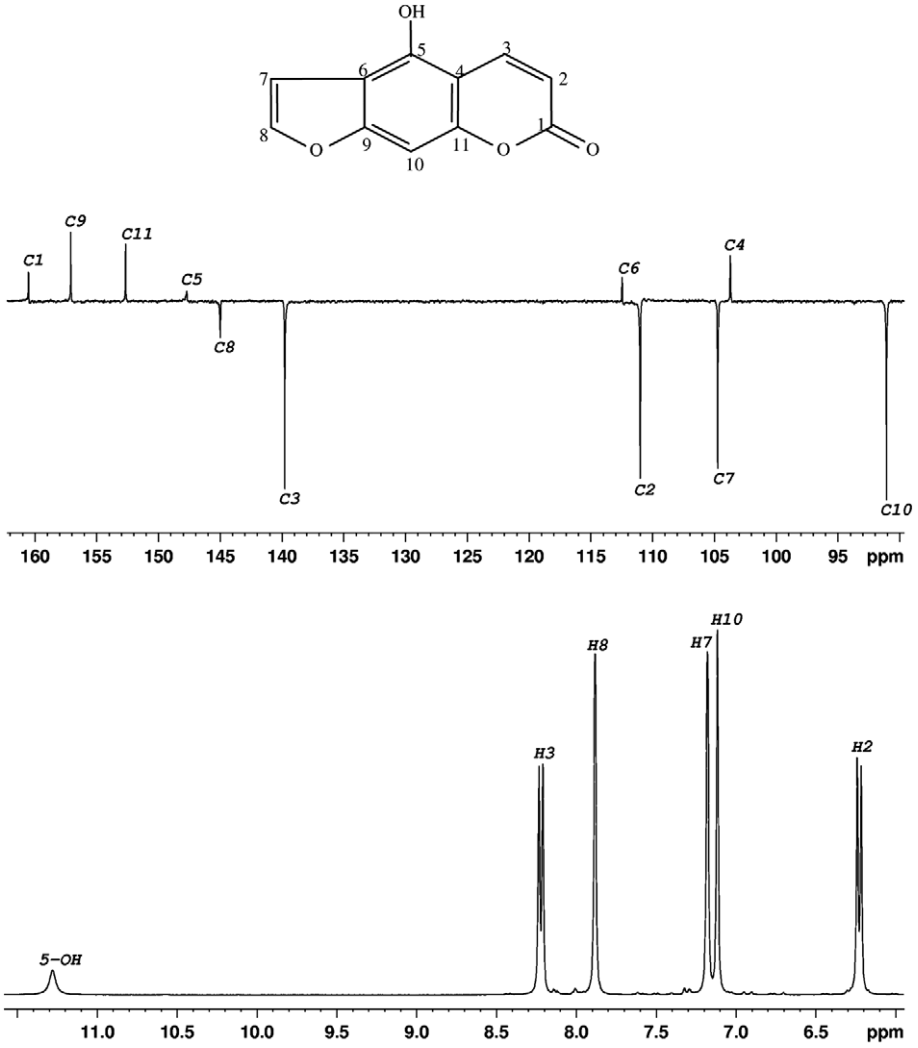

Figure 3. Structure of compound **1** (bergaptol), and ^{1}H (bottom) and ^{13}C SEFT (top) spectra obtained at 400 MHz. Assignments of various protons and carbons are made on the respective spectrum. In the SEFT spectrum, the CH carbons are negative, while the quaternary carbons are positive in intensity.

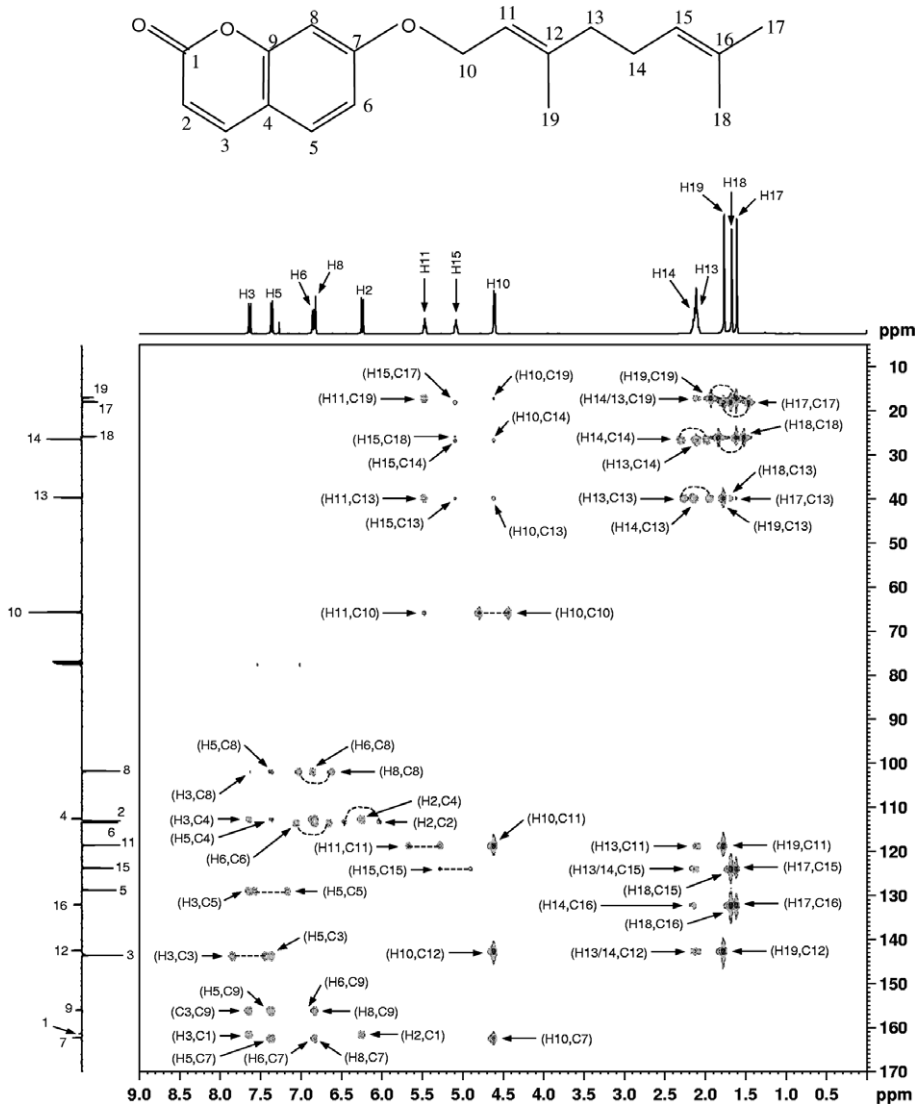

Figure 4. Structure of compound **2** (geranylcoumarin) and ^{1}H–^{13}C HMBC spectrum obtained at 400 MHz. Assignments of the entire cross peaks in the 2D spectrum are made. One-dimensional ^{1}H and ^{13}C SEFT spectra are also shown on the top and left of the 2D spectrum along with the assignments of various proton and carbon resonances respectively. In the ^{13}C SEFT spectra, quaternary and CH_2 carbons are positive and, CH and CH_3 carbons are negative in intensity.

are summarized in Figure 7. Among the two compounds, bergaptol was potent inhibitor of debenzylation activity of CYP3A4 enzyme. Inhibition of 98.9 ± 0.75 and 96.5 ± 1.25 was observed for bergaptol and geranylcoumarin at 0.1 mM concentration, respectively. IC_{50} values for bergaptol and geranylcoumarin are presented in Table 1. Among the two compounds the lowest IC_{50} was observed for bergaptol.

Identification of the grapefruit juice bioactive compounds will help to understand the mechanism of grapefruit juice–drug interaction and other food–drug interaction. Initial investigations considered flavonoids as potential inhibitors of CYP3A4, but subsequent studies showed furocoumarin monomers and dimers as major inhibitors of cytochrome P450 and transporter proteins.[2,16] Bergamottin and dihydroxybergamottin are two abundant furocoumarins present in the juice and play a significant role in grapefruit juice induced drug interaction, while monomers are distributed far more scarcely in the juice but are more potent inhibitors of CYP3A4 enzyme.[4] In our previous study,[1] we reported dihydroxybergamottin, paradisin A, and bergamottin as potent inhibitors of human CYP3A4 and CYP 1B1 isozymes. Several other furocoumarins such as epoxybergamottin, paradisin B, and paradisin C have also shown to inhibit the CYP3A4 enzyme.[4,23]

Based on the available research information, considerable inconsistencies in the outcome of drug interaction studies were reported over the last decade. These inconsistencies stem from the characteristics of 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.[13] It is possible that wide variability in the grapefruit juice–drug interaction studies may be attributed to variation of pharmacokinetics and pharmacodynamics of the drugs.[13]

Table 2. ^{1}H and ^{13}C NMR chemical shifts for compound **1** (solvent: acetone-d_6 + 2 drops of DMSO-d_6) and compound **2** (solvent: $CDCl_3$)

Number[a]	Compound **1**			Compound **2**		
	Carbon type	^{1}H	^{13}C	Carbon type	^{1}H	^{13}C
1	C	—	160.5	C	—	161.41
2	CH	6.22	111.01	CH	6.24	113.05
3	CH	8.22	139.79	CH	7.63	143.57
4	C	—	103.718	C	—	112.54
5	C–OH	11.28	147.71	CH	7.36	128.79
6	C	—	112.468	CH	6.848	113.35
7	CH	7.18	104.73	C	—	162.27
8	CH	7.88	145.02	CH	6.818	101.71
9	C	—	157.08	C	—	155.98
10	CH	7.11	91.07	CH_2	4.6	65.61
11	C	—	152.68	CH	5.46	118.54
12				C	—	142.46
13				CH_2	2.08	39.62
14				CH_2	2.12	26.35
15				CH	5.08	123.73
16				C	—	132.06
17				CH_3	1.6	17.82
18				CH_3	1.66	25.76
19				CH_3	1.76	16.87

[a] Refer to the structures for the numbering of carbon/proton.

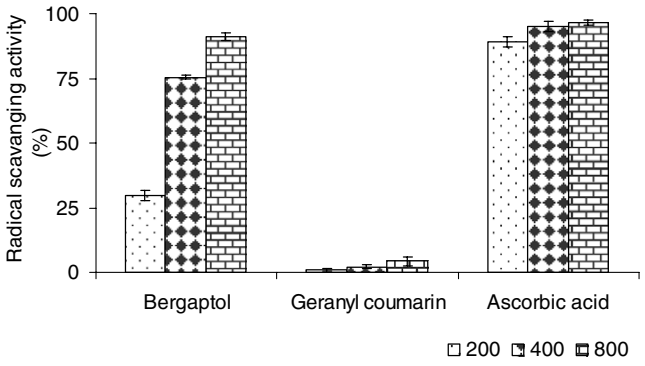

Figure 5. Radical scavenging activity of isolated compounds and ascorbic acid using ABTS method at different concentrations.

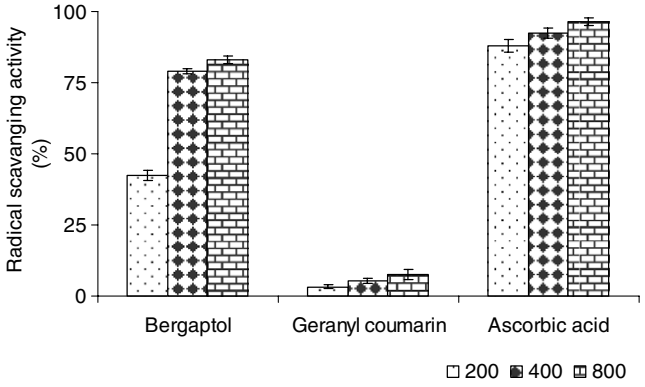

Figure 6. Radical scavenging activity of isolated compounds and ascorbic acid using DPPH method at different concentrations.

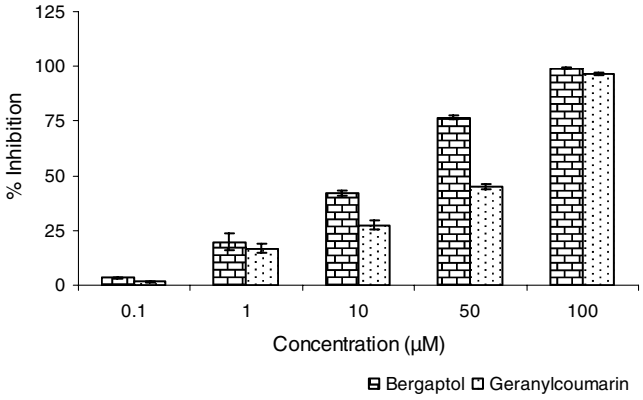

Figure 7. Inhibitory effects of bergaptol and geranylcoumarin on debenzylation activity of CYP3A4. Values are means ± SD, $n = 3$.

This study shows that grapefruit juice and grapefruit peel oil contain bergaptol and geranylcoumarin, which probably add to the list of CYP3A4 inhibitors present in the grapefruit juice. Inhibitory potency of grapefruit juice tends to be higher with higher furocoumarin content in the juice, while such clear correlation could not be made for any of the specific compound(s).[4] The potent inhibitory effect seen for the grapefruit juice could be due to synergistic effect of all furocoumarin and other bioactive components in the grapefruit juice. The differential inhibition of CYP3A4 for bergaptol and geranylcoumarin could be due to structural features of the compounds. Bergaptol is furocoumarin with furan moiety, while geranylcoumarin lacks the furan moiety. Row et al. postulated the possible sites of interaction of furocoumarins with CYP3A4 enzyme. The essential sites of furocoumarins interacting with enzyme are olefin region, 6′,7′-position,2,3-furan moiety, and lactone ring.[21] Furan part of the molecule is involved in the bio-activation and covalent interaction with cytochrome P450 enzymes. Bergamottin, a major component of grapefruit juice shown to inhibit the activities of several cytochrome isoenzymes inactivated CYP3A4 in a time- and concentration-dependent manner, via reactive furano-epoxide, that covalently binds to CYP3A4.[35] In

another study by our group on bacterial auto-inducer signaling, bergaptol showed potent inhibition, while geranylcoumarin showed moderate activity. This potent activity of the bergaptol is due to the presence of furan moiety.[36]

3. Conclusion

Bergaptol and geranylcoumarin are found to be potent inhibitors of P450 enzyme. Along with other bioactive compounds such as flavonoids and limonoids these compounds may significantly alter the bioavailability of clinically important drugs.

4. Experimental

4.1. Materials

Concentrated white grapefruit juice and grapefruit peel oil were obtained from Texas Citrus Exchange (Mission, Texas). The concentrated grapefruit juice was stored at 5 °C and 95% of relative humidity. All the solvents and chemicals used were of GR/HPLC grade and were obtained from EMD chemicals Inc. (Gibbstown, NJ). Silica gel of 200–400 mesh size 60 Å for column chromatography was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI).

4.2. Extraction and purification of compound 1

Concentrated grapefruit juice (12 L) was diluted with 30 L of distilled water. The diluted juice was extracted with ethyl acetate in 1:1 ratio for three times consecutively. Organic layer was separated and concentrated to obtain crude extract. Dried ethyl acetate extract (16 g) was impregnated with 16 g of silica gel and loaded onto silica gel (320 g) column chromatography. The column was eluted with hexane, mixtures of hexane/ethyl acetate and ethyl acetate. All the fractions were analyzed by TLC and HPLC. Fractions containing similar peaks were pooled and concentrated under vacuum. Fractions eluted with hexane/ethyl acetate showed compound of interest and these fractions were concentrated under vacuum to obtain compound **1**.

4.3. Purification of compound 2 by preparative HPLC

The preparative HPLC run was performed using Waters prep HPLC system (Waters Corporation, Milford, Massachusetts, USA). Chromatographic separations were accomplished on Alltech Econosil C18 column (250×22 mm, 10 μm particle size) (Alltech, Deerfield, IL, USA). Two milliliter of grapefruit peel oil was filtered and injected onto the column and eluted with aqueous methanol. Mobile phase used was as follows, Solvent A (methanol) and solvent B (water), 0 min, 40% A; 30 min, 65% A; 90 min, 80% A; 120 min, 99% A; 130 min, 100% A. The flow rate was set 10 ml/min and detection was carried out at 240 nm. Different fractions were collected as per the peaks, retention time and fractions were analyzed with TLC and HPLC. Fractions containing compound **2** were pooled, evaporated under vacuum, and freeze-dried. A total 60 ml of grapefruit peel oil was used for preparative HPLC run to obtain 280 mg of compound **2**.

4.4. TLC analysis

Purified compounds were dissolved in methanol, spotted on TLC plates, and developed using hexane/ethyl acetate (2:1) 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.

4.5. HPLC analysis

The HPLC system consisted of a Thermo Electron Corporation P-400 quaternary HPLC pump (Thermo Electron Corporation USA), Membrane degasser LDC analytical, and Spectra system AS3000 auto sampler (Thermo separation products). Peaks were analyzed with Thermo separation products PDA detector. Chromatographic separations were accomplished on luna C-18 column with particle size of 3 μm and dimensions of 150×4.60 mm (Phenomenex Inc., Torrance, CA, USA). A volume of 25 μl of isolated compounds dissolved in methanol at 0.25 mg/ml was injected onto HPLC column; the elution was carried out at room temperature under aqueous methanol gradient conditions. Starting with 0 min, 55% methanol and 45% water; 10 min, 75% methanol and 25% water; 20 min, 95% methanol and 5% water; 30 min, 100% methanol and 0% water, at 35 min initial conditions of 55% methanol and 45% of water were maintained. The flow rate was set at 0.8 ml/min and elution was monitored at 240 nm with a photodiode array detector.

4.6. Mass spectrometry

Mass spectrometric analyses were performed using ThermoFinnigan LCQ-DECA instrument (Thermo, San Jose, CA, USA). Conditions were 450 °C vaporizer with 300 μl of flow of methanol with 5 μA current and 30 PSI sheath and 15 PSI aux.

4.7. NMR equipment and experiments

^{1}H and ^{13}C NMR experiments for the compounds **1** and **2** were performed on Bruker Biospin Avance 400 or 800 NMR spectrometers at 298 K using 5-mm broad band inverse room-temperature probehead or cryo-probehead, respectively. One-dimensional ^{1}H and ^{13}C spectra were obtained using one pulse sequences. One-dimensional ^{13}C spectra using Spin Echo Fourier Transform (SEFT) were also obtained to distinguish carbon multiplicity.

4.8. 2D experiments at 400 MHz

Homonuclear and heteronuclear two-dimensional (2D) NMR experiments such as ^{1}H–^{1}H double quantum filtered correlation (DQF-COSY), sensitivity enhanced and multiplicity edited ^{1}H–^{13}C heteronuclear single quantum correlation (edited HSQC), and ^{1}H–^{13}C

gradient enhanced heteronuclear multiple bond correlation (HMBC) experiments were performed. For DQF-COSY experiment, sweep width of 4800 Hz was used in both the dimensions and 512 FIDs were obtained with t_1 increments, each of 2048 complex data points. Number of transients and the relaxation delay used were 32 and 2.5 s. Phase sensitive data were obtained using TPPI method. For ^{1}H–^{13}C multiplicity edited HSQC and HMBC experiments, spectral widths of 4800 and 24,000 Hz were used in ^{1}H and ^{13}C dimensions, respectively. 512 FIDs were collected with t_1 increments each of 2048 data points, 32 transients, and 2 s recycle delay. Phase-sensitive data for edited HSQC experiment were obtained using echo-anti echo mode, whereas for HMBC, magnitude mode data were obtained. Resulting 2D data were zero filled to 1024 points in t_1 dimension and double Fourier transformed after multiplying by a squared sine-bell window function shifted by $\pi/2$ along both the dimensions.

4.9. 2D experiments at 800 MHz

For further confirming the structure of compound **2** based on carbon–carbon connectivity, 1, 1-ADEQUATE experiments were performed on 800 MHz spectrometer. Spectral widths of 8800 and 42,000 Hz were used in ^{1}H and ^{13}C dimensions, respectively. Three hundred FIDs were collected with t_1 increments each of 2048 data points, 160 transients, and 1 s recycle delay. The delays for proton–carbon and carbon–carbon magnetization transfer were set equivalent to the J coupling of 165 and 80 Hz, respectively. Phase sensitive data were obtained using echo-anti echo mode. Resulting 2D data were zero filled to 1024 points in t_1 dimension and double Fourier transformed after multiplying by a squared sine-bell window function shifted by $\pi/2$ along both the dimensions.

4.10. $ABTS^{\cdot+}$ assay

The $ABTS^{\cdot+}$ was prepared by reaction of 7 mM aqueous ABTS solution and 2.45 mM potassium persulfate solution as proposed.[37] The mixed solution was stored in the dark for 16 h, the radical cation solution was further diluted in methanol until the initial absorbance value of 0.7 at 734 nm. Solutions of isolated compounds and ascorbic acid were prepared in methanol, and 10, 20, and 30 μl (equivalent to 200, 400, and 800 ppm) were pipetted into 96-well plates. The total volume of samples and ascorbic acid was adjusted to 40 μl using methanol. Diluted ABTS radical solution (200 μl) was added to each well and the readings were recorded after 5 min at 734 nm using KC4 plate reader (BioTek Instruments, Inc., Winooski, VT, USA). The radical scavenging activity was calculated using the formula mentioned in the DPPH method. For each molecule and each concentration, measurements were made in triplicate. All tests were performed in triplicate.

4.11. Radical scavenging activity using DPPH method

Different range of concentrations such as 10, 20 and 30 μl (equivalent to 200, 400 and 800 ppm) of isolated compounds and ascorbic acid were pipetted into 96-well plates. The volume of the samples and ascorbic acid was adjusted to 40 μl by adding methanol. Two hundred microliters of methanolic solution of DPPH (100 μM) was added, shaken vigorously, and the plate was allowed to stand at 27 °C for 20 min.[38] A control was prepared as described above without samples or standards. Methanol was used for the baseline correction. The changes in the absorbance of all the samples and standards were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula, % radical scavenging activity = (control optical density − sample optical density/control optical density) × 100.

4.12. CYP3A4 enzyme and substrates

Membrane preparation containing recombinant human CYP3A4 (expressed from cDNA using a baculovirus expression system), specific cytochrome enzyme substrate luciferin 6′ benzyl ether, assay mixtures, and luciferin were purchased from Promega (Promega Inc., Madison WI, USA). Ketoconazol was purchased from Sigma–Aldrich (St. Louis, MO, USA).

4.13. CYP3A4 enzyme inhibition assay

To determine the effect of compound **1** and compound **2** on inhibition of CYP3A4 activity, assays were performed as follows. To a 96-well microtiter plate, 12.5 μl of luciferin-free water (positive control), ketoconazol (positive control for inhibition), and test compound were added to the appropriate wells at 4× concentrations. Thawed, 12.5 μl of control reaction mixture and membrane preparations containing CYP3A4 were added at 4× concentration to the respective wells. The reaction mixture was mixed by shaking the plate and plate was pre-incubated at 37 °C for 10 min. CYP3A4 assay reaction was started by adding 25 μl of 2× NADPH regeneration systems to all the wells. The reaction mixture was mixed by shaking the plate and plate was incubated at 37 °C for 30 min. After 30 min, 50 μl of reconstituted luciferin detection reagent was added to all wells. Reaction mixture was mixed briefly on a plate shaker. The plate was incubated at 37 °C for 20 min. The luminescence was recorded using Wallac Victor 2 luminometer (Perkin-Elmer, Boston, MA) in terms of relative light units (RLU). The inhibition was calculated on the basis of the relative activity of the positive control. The positive control was chosen as 100%. Mathematically, the percent inhibition was calculated from the formula 100 − [(relative CYP activity/relative activity of positive control) × 100]. IC_{50} values were calculated using GraphPad Prism version 4.03 for Windows (GraphePad Software, San Diego California, USA).

Acknowledgments

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