GRAPEFRUIT-DRUG INTERACTION: ISOLATION, SYNTHESIS, AND BIOLOGICAL ACTIVITIES OF FUROCOUMARINS AND THEIR VARIATION DUE TO PRE- AND POST–HARVEST FACTORS

A Dissertation

by

BASAVARAJ GIRENNAVAR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Horticulture

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ABSTRACT

Grapefruit-Drug Interaction: Isolation, Synthesis and Biological Activities of Furocoumarins and Their Variation due to Pre- and Post–Harvest Factors. (August 2007)

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The health maintaining properties of citrus consumption are attributed to the wide assortment of bioactive compounds. Consumption of grapefruit along with certain medications, however, is posing a risk of drug toxicity and side reactions. The first study involved isolation of bioactive furocoumarins with a combination of chromatographic techniques and synthesis. Five furocoumarins namely, dihydroxybergamottin, paradisin A, bergamottin, bergaptol and geranylcoumarin were isolated from grapefruit and series of furocoumarin monomers and paradisin A were synthesized. The second study involved influence of pre- and post-harvest factors on the levels of furocoumarins in grapefruit juice. Considerable differences were observed in the levels of these compounds in different grapefruit cultivars. Ray Red showed the lowest levels of all three furocoumarins and Duncan contains the highest amount of DHB and bergamottin, where as the highest levels of paradisin A was observed in Star Ruby. The highest levels of DHB and bergamottin were found in Flame cultivar grown in California. The changes in the levels of these furocoumarins during the season in Rio Red and Marsh White grapefruit cultivars were evaluated.

The third study investigated biological activities of grapefruit juices and furocoumarins. Grapefruit and Pummelo juices were found to be potent inhibitors of cytochrome CYP3A4 and CYP2C9 isoenzymes at 5% concentration while CYP2D6 was less affected. Among the five furocoumarins tested, the inhibitory potency was in the order of paradisin A>dihydroxybergamottin>bergamottin>bergaptol>geranylcoumarin at 0.1 μM to 0.1 mM concentrations. A fourth study investigated the effect of furocoumarins on bacterial auto-inducer signaling, and found that furocoumarins are potent inhibitors of AI-1 and AI-2 activities at 0.01% concentration. In a fifth study, involving synthesized furocoumarin monomers and dimer on anti-proliferative activities on normal and cancer cell lines, furocoumarins found to be non-toxic to normal cells. However, bergamottin showed a significant anti-proliferative activity in HT-29 and MCF-7 cell lines.

This dissertation indicates that furocoumarins are bioactive compounds from grapefruit juice with potent inhibitory property of major drug metabolizing cytochrome P450 isoenzymes. Furocoumarins show a considerable variation between varieties, location and season. These results corroborate the involvement of furocoumarins in grapefruit drug interaction.

Dedicated

To

My Family and Friends for Their Unconditional, Support, Love and Inspiration

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CHAPTER I

INTRODUCTION

Background

Citrus is an important crop of subtropical areas, and red grapefruit (*Citrus paradisi* Macf.) is both a popular and state fruit of Texas *(1, 2)*. The United States is the world's largest producer and exporter of grapefruit; produced mainly in Florida, Texas, California, and Arizona states *(3)*. Purchased by 21% of United States households, grapefruit juice carries the American Heart Association's healthy "heart check" food mark *(4, 5)*. Grapefruit contains a cultivar of bioactive health maintaining compounds such as ascorbic acid, flavonoids, limonoids, folic acid, carotenoids, pectin, potassium, furocoumarins and related compounds. These compounds may serve as health maintaining and act as chemo-preventive bioactive functional components of citrus *(6)*. Citrus flavonoids, such as hesperidin and naringin, possess antioxidant activity *(7)* and naringin suppress high multiplicity aberrant crypt foci formation and cell proliferation in rat colon *(8)*. Carotenoids, such as lutein, showed antioxidant activity with its potential role in protection against prostate cancer and age-related macular degeneration *(9)*.

Grapefruit sales and crop value have declined since 1990's, due in part to consumer concerns about possible drug interactions and drug toxicity when consumed with common prescription medications *(10, 11)*. This is particularly critical in that juice

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This dissertation follows the style of *Journal of Agricultural and Food Chemistry*.

and medications are commonly consumed together at breakfast. Survey results show that multiple drug use is common in the United States. More than 50% of individuals in a given week would be taking at least one prescription drug and 7% would be taking at least five prescription drugs *(12)*. In AIDS and cancer patients, the use of multiple prescription drugs and dietary supplements is prevalent *(13)*. This presents a challenge in managing patient's health care in the context of common food-drug interaction.

Grapefruit juice has been reported to increase bioavailability of many drugs metabolized by cytochrome P4503A4 (CYP3A4) enzyme. The drugs affected include a number of dihydropyridine calcium channel antagonists *(14-16)*, triazolam, terfanidin, diltiazem, simvastatin, lovastatin, cyclosporine, sildenafil *(17-21)*. The possible mechanism of grapefruit juice induced-drug interactions is through inhibition of the drug-metabolizing enzyme cytochrome P4503A4 *(22-23)*, intestinal membrane efflux transporter P-glycoprotein *(24-28)* and the organic anion-transporting polypeptides *(29-32)*. These mechanisms may alter drug metabolism, transportation and cellular uptake of xenobiotic compounds *(33)*. This comprehensive effect of altered drug metabolism results in a significant reduction of the pre-systemic metabolism of drugs leading to a 1.5- to 15- fold increase in blood plasma concentration *(11)*.

Citrus Bioactive Compounds

Citrus is a subtropical, evergreen genus originating from Southeast Asia and belongs to the *Rutaceae* family. The plants are large shrubs or small trees, reaching 5-15 meters in height with spiny shoots and alternately-arranged leaves. Citrus fruits are notable for their fragrance, mainly due to terpenes and essential oils present in the rind.

Citrus fruits are consumed both as fresh and processed products. The fresh crop value of citrus in Florida and Texas alone was $493.2, million $50.0 million, respectively and the value of processed citrus products was more than $3.7 billion between 1999 and 2000 *(34)*. The most important citrus crops in the United States are oranges and grapefruits, and demand for grapefruit is declining as consumer concern about possible interaction of grapefruit juice with certain medication is increasing.

Consumption of fruits and vegetables, especially citrus, has long been known to prevent many human diseases, from scurvy to several types of cancers *(35)*. The health-maintaining properties of citrus fruits are attributed to the presence of several bioactive compounds *(35)*. Grapefruit juice has been shown to suppress carcinogen induced DNA damage *(36)*. Initially, it was assumed that vitamin C was the active agent with all the potential health-benefits. However, recent investigations showed several bioactive compounds with health maintaining properties in vivo and in vitro *(35-37)*.

Furocoumarins and Their Biosynthesis

Furocoumarins are minor constituent compounds present in species belonging to the *Umbelliferae, Rutaceae, Moraceae, Rosaceae* and *Leguminosae* families. Furocoumarins are also referred to as psoralens *(38)*. These naturally-occurring furocoumarins have been used clinically, coupled with ultraviolet light, to treat dermatological disorders for more than 2000 years *(38)*. Furocoumarins are known to posses photo-sensitizing properties and have been used extensively in photo-chemotherapy *(38)*. In vitro studies showed to inhibit the activity of several mammalian cytochrome P450 enzymes such as CYP3A4, CYP1A, CYP2D6 and CYP2B1 *(39)*. Other

studies reported the involvement of furocoumarins as the main components of grapefruit juice, in grapefruit-drug interaction by inhibiting the CYP3A4 enzymes metabolic activity *(40, 41)*. Grapefruit juice contains several furocoumarins and their derivatives, which are believed to be responsible for drug interactions. Until recently, six furocoumarins had been found in grapefruit juice with differential inhibitory effects on CYP P450 enzymes *(42)*. Bergamottin and 6', 7'–dihydroxybergamottin are the major furocoumarins, whereas paradisin A, paradisin B and paradisin C are strong inhibitors of microsomal CYP3A4 *(42)*.

Furocoumarins are phytoalexins produced by several plant species to protect against biotic and abiotic stresses *(43)*. Furocoumarins biosynthesis can be induced by herbivores, pathogens and environmental stresses leading to their accumulation of the furocoumarins in leaf tissue *(44)*. Furocoumarins in wild parsley and celery are thought to cause occupational "phyto-photo-dermatitis" in farm workers, when they are exposed to sunlight. Plants during the ecological war with herbivores vary their production of furocoumarins to maintain their defending ability. Cell suspension cultures have been used to study the induction of furocoumarins production in response to fungal elicitors and most of the elicited furocoumarins accumulated in the culture fluid *(45)*. Induction and accumulation of umbelliferone 7-*O*-prenyltransferease and a 6-*C*-prenyltransferase activity were reported in elicitor-treated *Ammi majus* cell suspension cultures *(45)*.

Mechanism of Grapefruit-Drug Interaction

Most lipophilic drugs are either metabolized by CYP3A4 or pumped back into the gut lumen by the P-glycoprotein transporter. Thus, CYP3A4 and P-glycoprotein act in

tandem as a barrier to the oral delivery of many drugs *(46)*. It seems that many bioactive compounds from grapefruit juice, such as furocoumarins and flavonoids, interfere with CYP3A4 activity. Interestingly, grapefruit juice has no effect on the bioavailability of drugs when they are administered intravenously *(47)*, suggesting the involvement of intestinal CYP3A4 inhibition, not the hepatic CYP3A4 inhibition, as the major cause for increased oral bioavailability *(48, 49)*. Bergamottin, a major furocoumarin in grapefruit juice, reversibly inhibits the activities of CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 in human liver microsomes *(50)*. Bergamottin also inactivates CYP3A4 after metabolic activation in a time- and concentration-dependent manner *(50)*. Bergamottin-induced inactivation led to the loss of up to 50% of CYP3A4 apoprotein, perhaps via apoprotein modification at the active site of the enzyme *(50)*. Metabolites of bergamottin may undergo oxidation to form a reactive furanoepoxide that covalently binds to CYP3A4 *(51)*. Grapefruit juice seems to interact with P-glycoprotein because P-glycoprotein and CYP3A4 have overlapping substrate specificities.

Factors Influencing Bioavailability of Drugs

The oral intake of a drug contemplates that the body must first absorb the active ingredient before it can reach the target organ. The efficacy of drug uptake depends on the chemical characteristics of the active substance such as its solubility and membrane permeability. However, absorption of the drug is also determined by the organism's ability to absorb pharmaceuticals through a specific transporter system, and later being able to excrete them. Since many pharmacologically active substances are poorly suited for oral intake, a decisive criterion for the efficacy of a medicine is its bioavailability.

Therefore, bioavailability is a measure the rate of the absorption of a drug as well as the extent to which this drug is absorbed *(52)*.

Multiple factors influence the absorption of a single drug. The external conditions that influence the bioavailability of drugs are related to the total patient–environment and modifiers at the time of the drug administration *(53)*. For example, the quality, quantity, temperature and food consumed along with drug, as well as the time taken relative to the intake of the drug, can have an effect on the degree of absorption. Other factors such as consuming liquids and social drinks coffee, tea and alcoholic substances influence the absorption of a drug *(53)*. It is also possible that the somatic and psychological characteristics of the patient have an effect on the extent of bioavailability of a drug. Specific anthropometric factors such as race, sex, age, height, and weight may also influence the extent of bioavailability of the drug. Furthermore, daily habits and living conditions may have an influence on drug bioavailability. The regular use of self-medicated drugs such as laxatives, antacids, vitamins and sedatives may interfere with absorption and metabolism of drugs *(53)*. In addition, the deliberate control and normal frequency of urination and defecation may influence the rate of elimination of a drug, thus changing the general pattern of the concentration-time curve expected for a particular drug *(53)*.

Food-Drug Interactions

The presence of food, solid or liquid, within the gastro-intestinal tract can markedly modify the oral availability of drugs via changes in the pre-systemic metabolism and systemic drug clearance *(54)*. These alterations may occur from a dietary

material effect on the saturation solubility of a drug in the gastro-intestinal tract. These include the crystalline form, lipophilicity and the ability of the drug to be solubilized by the native surfactants and co-ingested food stuffs, its aqueous solubility, pK and GI tract pH profile *(55)*. Direct interactions between drugs and dietary substance can also decrease or increase dramatically the absorption of susceptible xenobiotics. These interactions include the absorption of the drug to insoluble dietary components, chelation with metal ions and partitioning or solubilization in dietary fat, which is major constituent that can influence drug absorption *(54)*.

Cytochrome P450 Enzymes

Cytochrome P450 is a large multi gene family of heme-containing proteins found in the intestinal wall and liver, where they play an important role in the oxidative biotransformation of numerous drugs, endogenous substances and xenobiotics *(56, 57)*. Several isoforms have been characterized based on their structure, substrate specificity and response to various types of inducers *(58)*. Individual isoenzymes are thought to be responsible for the metabolism of specific substrates. The CYP3A subfamily is the most abundant enzyme; 70% of the enzyme is localized in the liver and 30% in the enterocytes. Furthermore, CYP3A4, CYP2C9 and CYP2D6 are major drug metabolizing enzymes (59). Certain cytochrome P450 isoenzymes such as, CYP3A4, CYP1A2, CYPB1 and CYP9 have a specific role in the onset of several types of cancers *(60-62)*.

P -glycoprotein

P-glycoprotein is an ATP-binding cassette (ABC) family membrane transporter, located in the intestinal gut wall. The function of the P-glycoprotein is to carry lipophilic molecules from intestinal epithelial cells back into the intestinal lumen *(63)*. After uptake of a drug by enterocytes, many lipophilic drugs are either metabolized by CYP3A4 or pumped back into the lumen by P-glycoprotein *(64)*. Therefore, CYP3A4 and P-glycoprotein act as tandem barriers to the oral delivery of many drugs *(65)*. Over-expression of certain ABC transporters occurs in cancer cell lines and tumors that are multi-drug resistant. Genetic variation in these genes contributes to a wide cultivar of human disorders such as cystic fibrosis, neurological disease, retinal degeneration, cholesterol and bile transport defects, anemia, and drug response phenotypes *(66)*. Inhibition of these transporter proteins in selected patient populations would offer a unique strategy to cure the above disorders and diseases.

Antimicrobial Activity of Bioactive Compounds

One of the properties of citrus flavonoids, which is related to their physiological action in plants, is their antifungal and antiviral activity. Grapefruit peel extracts showed varying degrees of antibacterial activity. Among the extracts, ethanol soluble fraction was most effective against Gram-positive bacteria *(67)*. Methoxyflavones, flavanones and coumarins are the main chemical constituents of grapefruit peel, and some coumarins have been reported to have anti-platelet aggregating and anti-mutagenic activity *(67)*. The antibacterial agents from grapefruit can be used as a bio-preservative in the food industries. It has been shown that grapefruit extract possess antibacterial and antifungal

activity *in vitro* and inhibits the proliferation of *Candida*, a yeast that can impinge upon probiotic bacteria and affect the gastrointestinal health. Heggers et al., reported the mechanism of action and in vitro toxicity of grapefruit extracts *(68)*. Synthetic and natural furanones are potential anti-quorum sensing agents in bacteria and have been studied for their antibacterial properties in food and medical microbiology *(69)*. Unfortunately, halogenated furanone compounds are unsuitable for human use due to unstable and toxicity. Furanones and furocoumarins share similarity in having a furan moiety in their structure. There is a need for identification and evaluation of naturally occurring anti-quorum sensing compounds from plants which are safe alternatives to the halogenated furanones and fungus derived mycotoxins.

Effects of Pre-Harvest Factors on Bioactive Compounds

The bioactive compound profile of citrus fruits varies with cultivar, environmental conditions and fruit development and maturity. Duncan seedy and marsh seedless grapefruit showed variation in naringenin and limonin content *(70, 71)*. Grapefruit trees exposed to freezing conditions showed more naringin and less limonin *(71)*. Other environmental stress parameters such as temperature and humidity affect the level of naringin *(72, 73)*. Grapefruit showed quantitative changes in the phytochemical levels of juices during developmental stages. The highest concentration of naringin *(74, 75)*, limonin *(76)* and nomilin *(77)* were observed at early developmental stage and progressively declined as the fruit matured.

Effects of Post-Harvest Factors on Bioactive Compounds

Several post-harvest factors such as temperature, relative humidity, atmosphere conditions and processing affect the quality of fruits. It is well known that in certain fruits, quality declines from the time of the harvest, particularly if the produce is not cooled effectively. The contents of bioactive furocoumarins in different lots of commercial grapefruit juices samples showed considerable variation *(10)*. A study conducted in Japan on commercial grapefruit juices showed four fold variation of bergamottin, 43- fold variation of dihydroxybergamottin (DHB) and 65- fold variations of dimers *(78, 79)*. Manually-squeezed grapefruit juice increased the bioavailability of terfanadine more than two-fold compared to the industrially processed juice *(17)*. It is possible that the hand-squeezed juice contain more drug-interaction causing bioactive components compared to commercially-processed juice. Commercially, grapefruit juice is manufactured in a multi-step process, which includes washing, puncturing of peel to obtain essential oils and squeezing of the whole fruit to juice. The juice is heated to175 ºF during pasteurization process and then essential oils, important constituents of grapefruit peel, are added as a flavor enhancer *(86)*.

Based on the research information on grapefruit-drug interaction, we hypothesized that furocoumarins are the bioactive agents responsible for grapefruit juice induced-drug interaction by inhibiting cytochrome P450 enzymes and P-glycoprotein activities and variability in the levels of the furocoumarins levels may be the prime reason for variation in the drug bioavailability. To test this hypothesis, we proposed the following objectives for the dissertation:

- Isolation and synthesis of furocoumarin monomers and dimer.
- To evaluate the effect of pre-harvest factors on the levels of furocoumarins in grapefruit.
- To evaluate the effect of post-harvest factors on the levels of furocoumarins in grapefruit and grapefruit juice.
- To evaluate the effect of grapefruit juice and furocoumarins on CYP P450 3A4, 2C9 and 2D6 isozymes and P-glycoprotein.
- To evaluate the effect of grapefruit juice and its furocoumarins on bacterial quorum sensing and biofilm formation.
- To evaluate the Anti-proliferative properties of furocoumarins on normal and cancer cell lines.

CHAPTER II

ISOLATION AND SYNTHESIS OF FUROCOUMARIN MONOMERS AND DIMER

Synopsis

Bioactive furocoumarins know to have drug-interaction properties were isolated with a combination of chromatographic techniques. An efficient and improved technique was developed for the isolation and purification of furocoumarins using an open and reverse phase column chromatography. Grapefruit juice was extracted with ethyl acetate and the dried extract was loaded on to a silica gel column. The column was eluted with a combination of solvents, ranging from non-polar (hexane) to polar (methanol) solvents. Furthermore, column fractions were subjected to preparative HPLC to obtain four compounds 6' 7'- dihydroxybergamottin, bergamottin, paradisin-A and bergaptol. Grapefruit peel oil was loaded on to preparative HPLC column and eluted with aqueous methanol. The fraction containing geranylcoumarin was rotary evaporated and freeze dried. The purity of these compounds was analyzed by HPLC and structures were determined by LC-MS and NMR studies. Series of monomers and a dimer were synthesized starting from bergapten.

Introduction

Phytochemicals are non-nutritive plant compounds that have disease protective or preventive properties. Plants produce these chemicals to protect themselves against a cultivar of stresses. Phytochemicals can range from medicinally useful agents to deadly poisons. Today a number of phytochemicals isolated from plants are used as drugs in the

pharmaceutical industry. Natural compounds from plants serve as a vital source for new drugs and model structures for synthetic formulations *(80)*. Citrus flavonoids, such as naringenin, apigenin, hesperetin and quercetin are potent inhibitors of cytochrome enzymes. Other compounds such as limonin glucoside and nomilin glucoside showed CYP 19 enzyme inhibition. Naringin and its aglycone, naringenin, were thought to be the grapefruit agents responsible for drug interactions. However, when naringin was co-administered with felodipine in vivo, no changes in bioavailability of felodipine was observed, thus indicating the involvement of non-flavonoid component of grapefruit juice in drug interaction *(40)*.

Furocoumarins play a significant role in the grapefruit juice drug interactions by inhibiting CYP3A4 enzyme and transporter proteins *(24, 25)*. Furocoumarins are polyphenolic compounds, synthesized from L-phenylalanine and may occur in linear form with a furan ring attached to the 6, 7, position of the benzo-2 pyrene nucleus. These compounds are toxic in nature, acting as pro-oxidants by covalently binding to DNA and proteins in the presence of ultraviolet light *(81)*. Bergamottin is the major furocoumarin in fresh grapefruit, present at similar levels in the juice and segment membranes but to a lesser extent in the peel extracts *(82, 83)*. Unlike flavonoids and limonoids, furocoumarins are not concentrated in the peel or peel oil but mainly distributed in the juice *(83)*. Since bioactive furocoumarins in grapefruit juice have been shown to interact with cytochrome enzymes, it is imperative to isolate and characterize them from grapefruit juice. Thus the objective of this study was to isolate furocoumarins from grapefruit and also to explore the possibility of synthesizing them for evaluating biological activity.

Materials and Methods

Chemicals. All the solvents and chemicals used were GR and HPLC grade and were obtained from EMD chemicals Inc., Gibbstown, NJ. Silica gel of 200-400 mesh size 60 A for column chromatography was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI).

Grapefruit Juice and Grapefruit Peel Oil. Concentrated grapefruit juice and grapefruit peel oil was donated by the Texas Citrus Exchange (Mission, TX). The concentrated grapefruit juice was stored at 5 °C and 95% relative humidity until further processing.

Extraction. Concentrated grapefruit juice (20L) was diluted by stirring with distilled water to attain a brix reading of 30%. The diluted juice (50 L) was extracted with ethyl acetate in 1:1 ratio three times, consecutively. The organic layer was separated and dried to obtain a crude extract.

Purification of Furocoumarins. Dried ethyl acetate extract (23 g) was impregnated with 23 g of silica gel and loaded on to a silica gel (500 g) column. The column was eluted with hexane:ethyl acetate with increasing polarity as follows 95:5, 90:10, 85:15, 80:20, 60:40, 40:60 0:100. 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 (80:20) showed compounds of interest and these fractions were concentrated under vacuum.

Preparative HPLC of Column Fractions. The preparative HPLC run was performed using a Waters prep HPLC system (Waters Corporation, Milford, MA). The mobile phase used was as follows, Solvent A (methanol) and solvent B (water), 0 min, 40% A; 45 min, 45% A; 90 min, 60% A; 120 min, 90% A; 130 min, 95% A. The flow rate was set at 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 according to the peak retention times and fractions were further analyzed with TLC and HPLC. Fractions having similar retention times were pooled, evaporated under vacuum and freeze-dried. The yields of compound **1**, **2, 3** and **4** were 340, 19, 310, and 451 mg, respectively.

Preparative HPLC for Grapefruit Peel Oil. The preperative HPLC run was performed with the above mentioned system and method. Five mL of grapefruit peel oil was filtered and injected on to the column and eluted with aqueous methanol. Different fractions were collected according to the peak retention times and fractions were analyzed with TLC and HPLC. Fractions having similar retention times were pooled, evaporated under vacuum and freeze-dried. The yield of compound **5** was 280 mg.

TLC Analysis. Purified compounds were dissolved in methanol, spotted on TLC plates and developed 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.

HPLC Analysis. The HPLC system consisted of a Thermo Electron Corporation P-400 quaternary HPLC pump (Thermo Electron Corporation San Jose, CA), Membrane degasser LDC analytical and Spectra system AS3000 auto sampler. Peaks were analyzed

with Thermo separation products PDA detector. Chromatographic 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 gradient conditions with a mobile phase starting from (A) methanol and (B) water, over a period of 65 min. Starting with 60% A; 20 min, 80% A; 25 min, 80% A; 45 min, 85% A; 50 min, 90% A; 55 min, 95% A; 60 min, 100% A; 65 min, 100% A. The flow rate was set at 1.1 mL / min and elution was monitored with UV detection at 240.

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.

Synthesis of Furocoumarins

Bergaptol: 4-Hydroxy-7*H*-furo-[3,2-*g*]chromen-7-one **[7]**. To a solution of bergapten [**1**] (1.8 g, 8 mmol) in dichloromethane (70 mL), BBr_3 (70 mL, 70 mmol, 1 M in DCM) was added drop wise and the mixture was stirred under argon at room temperature. After 4 hours the mixture was poured slowly into a solution of saturated sodium bicarbonate (400 mL) resulting in the precipitation of a yellowish solid. After 30 minutes of stirring, the product was recovered by filtration, washed with cold water and ether and dried under a high vacuum, yielding the compound [**7**] as an off-white solid (1.621 g, 7.9 mmol, 99%). ^{1}H NMR (d6 -acetone, 300 MHz): *d* 8.28 (d, 1H, *J* = 9.8 Hz), 7.8 (d, 1H, *J* = 2.3 Hz), 7.17 (d, 1H, *J* = 2.3 Hz), 7.06 (s, 1H), 6.24 (d, 1H, *J* = 9.8 Hz); *m*/z (EI) 202 (100%, M+).

Bergamottin. 4-(3, 7-Dimethylocta-2,6-dienyloxy)-furo[3,2-*g*]chromen-7-one **[8]**. Geranyl bromide (2.1 g, 9.6 mmol) was added drop wise to a stirred mixture of bergaptol [**7**] (1.571 g, 8 mmol) and potassium carbonate (1.656 g, 12 mmol) in acetone (100 mL) at RT, then the mixture was heated under reflux for 2 hour. Aqueous citric acid (5% w/v) was added to neutralize the potassium carbonate and the solution was extracted with DCM (2 × 100 mL). The combined organic layers were washed with water and brine and were dried (Na_2SO4). Removal of the solvent under reduced pressure furnished a pale yellow oil, which was purified by column chromatography eluting with ethyl acetate–hexane (1 : 4). Removal of the solvent under reduced pressure afforded a white crystals of the compound [**8**] (2.4943 g, 7.9 mmol, 95%). ^{1}H NMR ($CDCl_3$, 300 MHz): *d* 8.18 (d, 1H, *J* = 9.8 Hz), 7.61 (d, 1H, *J* = 2.1 Hz), 7.16 (s, 1H), 6.97 (d, 1H, *J* = 2.1 Hz), 6.29 (d, 1H, *J* = 9.8 Hz), 5.54 (t, 1H, *J* = 6.5 Hz), 5.07 (bs, 1H), 4.96 (d, 2H, *J* = 6.5 Hz), 2.09 (s, 4H), 1.69 (s, 6H), 1.60 (s, 3H); *m*/*z* (EI) 338 (17%, M+), 202 (100%).

Epoxybergamottin. 4-[5-(3,3-Dimethyloxiranyl)-3-methylpent-2-enyloxy]-furo[3,2-*g*]chromen-7-one (epoxybergamottin) **[9]**. 3-Chloroperoxybenzoic acid (776 mg, 3.15 mmol) was added to a stirred solution of bergamottin [**8**] (710 mg, 2.1 mmol) in DCM (25mL). The solution was stirred at −10 °C in an ice-methanol bath for 4 hours. The organic layer was washed with aqueous sodium sulfite (10% w/v) and sodium carbonate (5% w/v) (20 mL, 1:1), was dried and the solvent was evaporated under reduced pressure. The compound was obtained by column chromatography, eluting with ethyl acetate–hexane (1:3). Removal of the solvent under reduced pressure furnished white crystals (594 mg, 1.68 mmol, 80%). ^{1}H NMR ($CDCl_3$, 300 MHz): *d* 8.17 (d, 1H, *J*

= 9.8 Hz), 7.61 (d, 1H, *J* =2.3Hz), 7.16 (s, 1H), 6.95 (d, 1H, *J* =2.3Hz), 6.29 (d, 1H, *J* = 9.8 Hz), 5.6 (t, 1H, *J* = 6.4 Hz), 4.97 (d, 2H, *J* = 6.4 Hz), 2.73– 2.71 (m, 1H), 2.26–2.22 (m, 2H), 1.71 (s, 3H), 1.70–1.61 (m, 2H), 1.31 (s, 3H), 1.29 (s, 3H); *m*/*z* (EI) 354 (23%, M+), 202 (100%).

(R)-DHB. A mixture of $(DHQD)_2PHAL$ (35.0 mg, 0.044 mmol), $K_2OsO_4•2H_2O$ (8.1 mg, .022 mmol), $K_3Fe(CN)_6$ (4.38 g, 13.32 mmol), K_2CO_3 (1.84 g, 13.32 mmol), $CH_3SO_2NH_2$ (432 mg, 4.44 mmol), and bergamottin [**8**] (1.5 g, 4.44 mmol) in 50 mL of 1:1 *t*-BuOH-H_2O was stirred at 0 °C for 17 h. The reaction was quenched with 10 mL of saturated Na_2SO_3 and 10 mL of saturated $Na_2S_2O_3$ at 0 °C, and was then allowed to warm to 23 °C and stirred for 45 min. After removal of *t*-BuOH under reduced pressure, the reaction mixture was extracted with 3 X 50 mL of ethyl acetate. The combined extracts were washed with 25 mL of 1 N NaOH, followed by 25 mL of brine, dried over Na_2SO_4, and concentrated in vacuum. The residue was purified by silica gel chromatography using ethyl acetate–hexane (2:1) to give 1.289 g 78% of (R)-DHB (**10**), $[\alpha]_D^{20}$ 9.87 (c 1.00,CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 300 MHz): *d* 8.18 (d, 1H, *J* = 9.8 Hz), 7.61 (d, 1H, *J* = 2.1 Hz), 7.17 (s, 1H), 6.95 (d, 1H, *J* = 2.1 Hz), 6.3 (d, 1H, *J* = 9.8 Hz), 5.6 (t, 1H, *J* = 6.5 Hz), 4.97 (d, 1H, *J* = 6.5 Hz), 3.33 (m, 2H,), 2.37 (m, 1H), 2.16 (d, 2H, *J* = 3.1 Hz), 1.79 (s, 3H), 1.71 (s, 1H); 1.48 (s, 1H); 1.21 (s, 3H); 1.17 (s, 3H); *m*/*z* (EI) 372 (17%, M+), 202 (100%).

(S)-DHB. A mixture of $(DHQ)_2PHAL$ (35.0 mg, 0.044 mmol), $K_2OsO_4•2H_2O$ (8.1 mg, .022 mmol), $K_3Fe(CN)_6$ (4.38 g, 13.32 mmol), K_2CO_3 (1.84 g, 13.32 mmol), $CH_3SO_2NH_2$ (432 mg, 4.44 mmol), and bergamottin [**8**] (1.5 g, 4.44 mmol) in 50 mL of

1:1 *t*-BuOH-H_2O was stirred at 0 °C for 17 h. The reaction was quenched with 10 mL of saturated Na_2SO_3 and 10 mL of saturated $Na_2S_2O_3$ at 0 °C, and was then allowed to warm to 23 °C and stirred for 45 min. After removal of *t*-BuOH under reduced pressure, the reaction mixture was extracted with 3 X 50 mL of ethyl acetate. The combined extracts were washed with 25 mL of 1 N NaOH, followed by 25 mL of brine, dried over Na_2SO_4, and concentrated in vacuum. The residue was purified by silica gel chromatography using ethyl acetate–hexane (2:1) to give 1.36 g 82% of (S)-DHB (**11**), $[\alpha]_D^{20}$ -10.91 (c 1.00,CH_2Cl_2). ^{1}H NMR ($CDCl_3$, 300 MHz): *d* 8.18 (d, 1H, *J* = 9.8 Hz), 7.61 (d, 1H, *J* = 2.1 Hz), 7.17 (s, 1H), 6.95 (d, 1H, *J* = 2.1 Hz), 6.3 (d, 1H, *J* = 9.8 Hz), 5.6 (t, 1H, *J* = 6.5 Hz), 4.97 (d, 1H, *J* = 6.5 Hz), 3.33 (m, 2H,), 2.37 (m, 1H), 2.16 (d, 2H, *J* = 3.1 Hz), 1.79 (s, 3H), 1.71 (s, 1H); 1.48 (s, 1H); 1.21 (s, 3H); 1.17 (s, 3H); *m*/*z* (EI) 372 (17%, M+), 202 (100%).

Paradisin A. (1*R*)-(−)-10-Camphorsulfonic acid (232mg, 1 mmol) was added to a stirred solution of epoxybergamottin (**9**) (354 mg, 1 mmol) in 1,4-dioxane (25 mL) under argon and was stirred at rt for 15 min. After a solution of (R)-DHB (**10**) (372 mg, 1 mmol) in 1,4-dioxane (5 mL) was added and stirred at rt for 2 h. On completion of the reaction (TLC) a few drops of saturated aqueous sodium bicarbonate solution were added

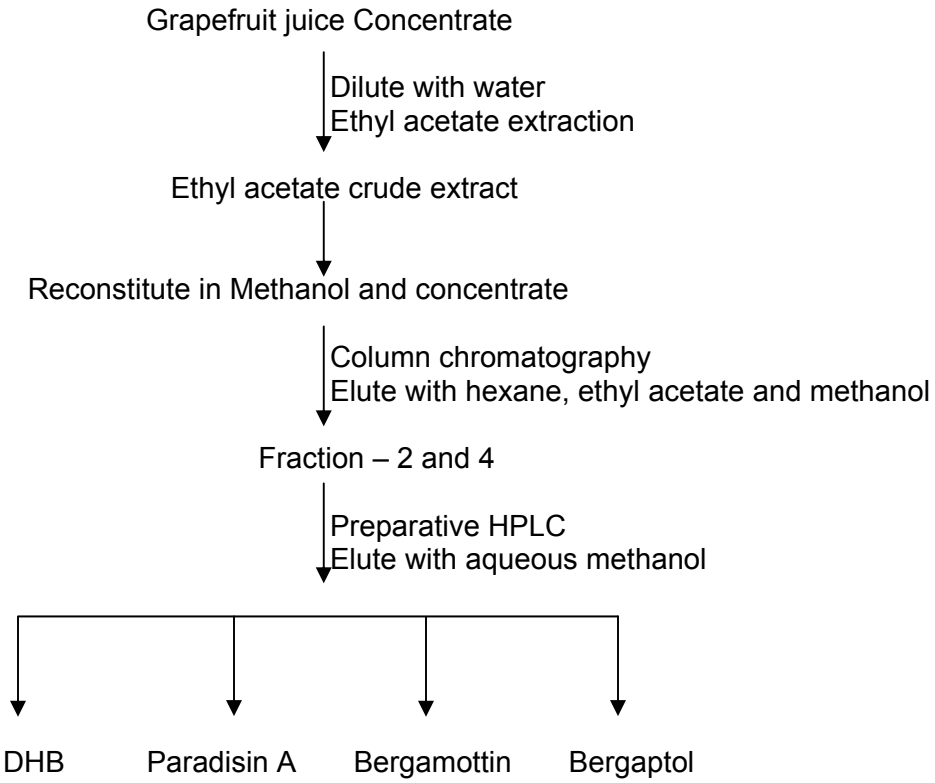
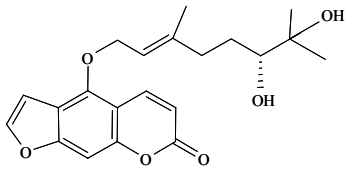
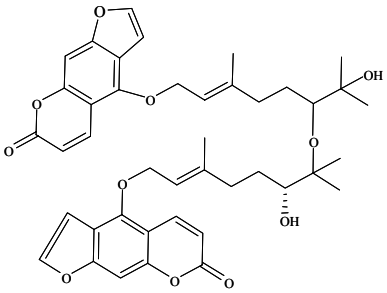


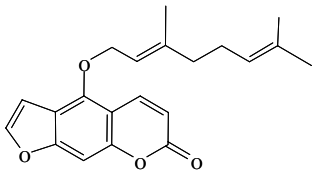
Figure 2.1. Isolation scheme of furocoumarins **1, 2, 3** and **4** from grapefruit juice concentrates.



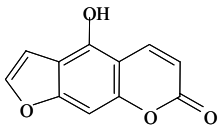
6'-7'-Dihydroxybergamottin (1)



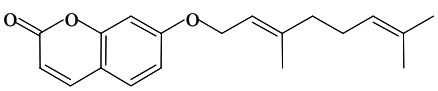
Paradisin A (2)



Bergamottin (3)



Bergaptol (4)



Geranylcoumarin (5)

Figure 2.2. Structure of isolated furocoumarins from grapefruit juice and grapefruit peel oil.

and the 1,4-dioxane was evaporated under reduced pressure. The residual oil was dissolved in DCM (20 mL), and then the organic layer washed with water (2 × 20 mL) and dried with sodium sulfate (Na_2SO_4). Removal of the solvent under vacuum yielded translucent oil. The oil was purified by silica gel chromatography using ethyl acetate–hexane (1:4) to give white crystals (**12**) (305 mg, 0.42 mmol, 42%). ^{1}H NMR ($CDCl_3$, 300 MHz): *d* 8.18 (d, 2H, *J* = 9.8 Hz), 7.61 (d, 2H, *J* = 2.1 Hz), 7.17 (s, 2H), 6.95 (d, 2H, *J* = 2.1 Hz), 6.3 (d, 2H, *J* = 9.8 Hz), 5.6 (t, 2H, *J* = 6.5 Hz), 4.97 (d, 2H, *J* = 6.5 Hz), 3.33 (m, 4H,), 2.37 (m, 2H), 2.16 (d, 4H, *J* = 3.1 Hz), 1.79 (s, 6H), 1.71 (s, 2H); 1.48 (s, 2H); 1.21 (s, 6H); 1.17 (s, 6H); *m*/*z* (EI) 726 (5%,M+), 202 (74%)

Results and Discussion

Concentrated grapefruit juice was diluted and extracted with ethyl acetate. The crude extract was purified using silica gel column chromatography as mentioned in **Figure 2.1**. Further, the compounds (**1-4**) (**Figure 2.2**) were obtained in pure form using preparative HPLC. The purity of these compounds was analyzed by HPLC. **Figure 2.3** depicts HPLC chromatograms of crude ethyl acetate extract and compounds (**1-4**). The relative retention times of DHB, paradisin A, bergamottin and bergaptol were found to be 12.16 ± 0.13, 29.38 ± 0.24, 37.44 ± 0.27 and 5.61 ± 0.19 minutes, respectively. The retention time for compound (**5**), which was isolated from grapefruit peel oil was found to be 21.11 ± 0.41. All five compounds observed on TLC as a bluish-white fluorescent spot under UV light. In addition, compounds (**1-5**) showed UV absorption maxima at 321 and 231 nm, indicating the presence of coumarin nuclei. Compounds (**1-5**) were characterized and identified as bergamottin, dihydroxybergamottin, paradisin-A,

bergaptol and geranylcoumarin respectively, using ^{1}H and ^{13}C NMR spectra. Chemical shifts of the furocoumarins *(23, 84, 86)* were in accordance with reported values (**Tables 2.1 and 2.2**). ^{13}C NMR spectral assignments were confirmed with the help 2D NMR such as FLOCK, DQFCOSY, HSQC spectra.

Furocoumarin derivatives were prepared using the methods outlined in **Figure 2.4**, where the initial step was the deprotection of the commercially available starting material bergapten [**6**]. Treatment with boron tribromide (BBr_3) in dichloromethane (DCM) afforded the phenolic derivative bergaptol [**7**] in excelent yields. Alkylation under conditions previously employed for the coumarins, with geranyl bromide resulted in the desired compound bergamottin [**8**]. Synthesis of the epoxybergamottin [**9**] was achieved by the treatment of [**8**] with mCPBA in DCM, the reaction was performed at−10 ˚C, resulting in the selective epoxidation of the terminal double bond. Generally epoxides are readily opened by nucleophiles under both acidic and basic conditions. This allowed the use of the epoxide intermediates as a diversification point, theoretically enabling the introduction of a cultivar of functional groups. The two enantiomers R and S 6', 7'-dihydroxybergamottin [**10**] and [**11**], respectively, were prepared from bergamottin [**8**] using the sharpless asymmetric dihydroxylation protocol, in good yields. Subsequent treatment of epoxybergamottin [**9**] and R-DHB [**10**] with dilute R-10-Camphorsulfonic acid resulted in the ring opening of the epoxybergamottin [**9**] to give Paradisin **A** [**12**] in moderate yields; the data was corroborated by NMR and MS studies.

The isolation of bergamottin, paradisin A and paradisin B from grapefruit juice was previously reported *(84)*. Wangensteen et al., reported the isolation of epoxy bergamottin from grapefruit peel using diethyl ether *(86)*. The dried extract was subjected

to flash chromatography to obtain epoxybergamottin along with other compounds. Ohta and co-workers reported isolation of paradisin C from grapefruit juice using hexane-ethyl acetate extract followed by column chromatography and ODS HPLC separation *(87)*. However there is no data on the levels of these compounds in the grapefruits as influenced by pre- and post-harvest factors.

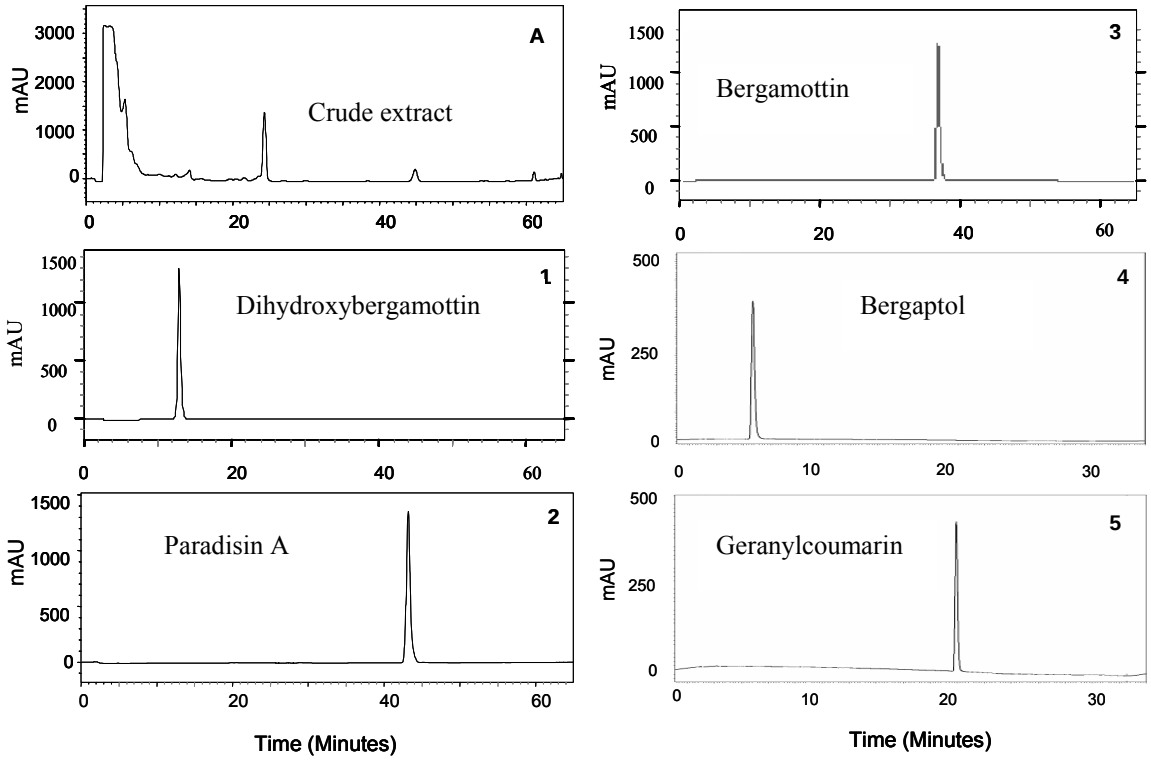


Figure 2.3. HPLC chromatograms of crude mixture and furocoumarin standards.

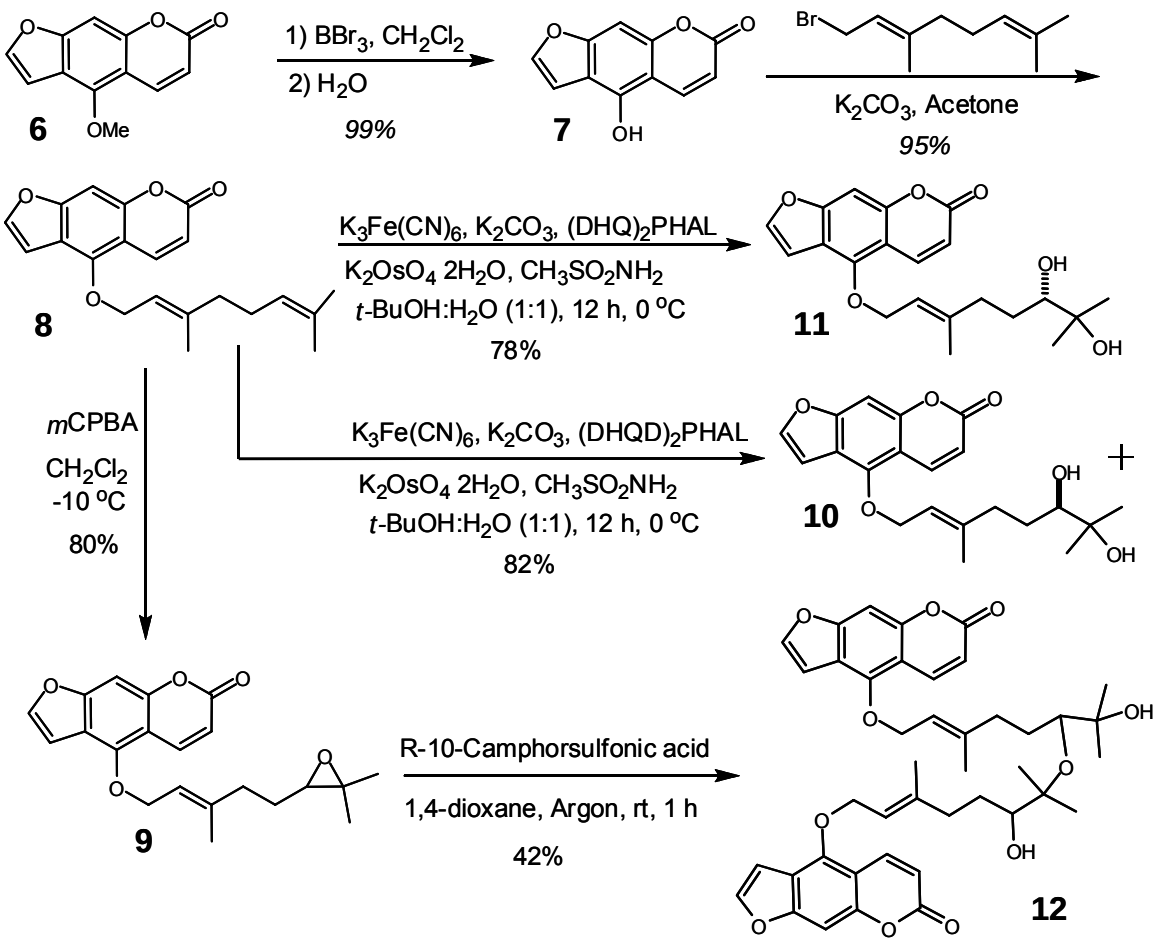


Figure 2.4. Reagents, reaction conditions and structural representation of compounds **6-12**.

Table 2.1 ^{1}H NMR data of compounds (1-5).

C	1	2	3	4	C	5
3	6.26 (1H, d, 9.9 Hz)	6.24 (1H d, 9.8 Hz)	6.24 (1H, d, 9.8 Hz); 6.26 (1H, d, 9.5 Hz)	6.22 (1H,)	3	6.24
4	8.05 (1H, d, 9.9 Hz)	8.12 (1H, d, 9.8 Hz)	8.05 (2H, d, 9.5 Hz)	8.22 (1H,)	4	7.63
5				11.28 (-OH,)	5	7.36
8	6.74 (1H, s)	7.10 (1H, s)	7.02 (2H, s)	7.11 (1H,)	8	
11	6.74 (1H, d, 2.5 Hz)	6.93 (1H, d, 2.0 Hz)	7.07 (2H, d, 2.3 Hz)	7.18 (1H,)	11	
12	7.55 (1H, d, 2.2 Hz)	7.57 (1H, d, 2.0 Hz)	7.85 (1H, d, 2.3 Hz)	7.88 (1H,)	12	
13	4.96 (2H, d, 6.6 Hz)	4.91 (2H, d, 6.5 Hz)	4.65 (2H, d, 6.5Hz); 4.63 (2H, d, 2.3 Hz)		13	
14	5.50 (1H, t, 6.8 Hz)	5.56 (1H, t, 6.5 Hz)	5.35 (1H, t); 5.38 (1H, t)		14	
16	1.67 (3H, s)	1.68 (3H, s)			16	
18	2.07 (2H,m)	2.29 (2H, m)			18	
19	1.95 (2H, m)	3.30 (1H, t)			19	
21	1.65 (3H, s)	1.18 (3H, s)	1.18 (3H, s)		21	
22	1.58 (3H, s)	1.14 (3H, s)	1.33 (3H, s)		22	

Table 2.2 ^{13}C NMR data of compounds (1-5).

C	1	2	3	4	C	5
2	161.5	161.6	160.5*	160.5	2	161.41
3	112.5	112.5	112.4*	111.01	3	113.05
4	139.7	139.9	138.6; 138.4**	139.79	4	143.57
5	149	148.9	149.0*	147.71	5	128.79
6	118.9	119.3	117.9; 118.4**	112.468	6	113.35
7	158.2	158.2	157.7*	157.08	7	162.27
8	94.2	93.3	93.8*	91.07	8	101.71
9	152.7	152.6	151.6*	152.68	9	155.98
10	107.5	107.5	106.9*	103.718	10	112.54
11	105.2	105.1	105.1*	104.73	11	65.61
12	145	145.1	144.6*	145.02	12	118.54
13	69.8	69.7	66.8; 66.7**		13	142.46
14	114.3	114.2	114.3*		14	39.62
15	143.1	143	142.8; 142.6**		15	26.35
16	16.8	16.7	17.01; 16.9**		16	123.73
17	39.6	36.5	34.5; 36.6**		17	132.06
18	26.3	29.5	29.3; 35.9**		18	17.82
19	123.6	73.1	82.5; 77.8**		19	25.76
20	132.1	77.9	71.6; 76.5**		20	16.87
21	17.8	23.3	25.8; 21.1**			
22	25.8	26.6	27.7; 27.5**			

* Showed one signal for two carbons

** Two chemical shifts in the same row for two carbons in dimer unit for the corresponding carbon

CHAPTER III

INFLUENCE OF PRE-HARVEST FACTORS ON FUROCOUMARIN LEVELS IN GRAPEFRUITS

Synopsis

Variation of three furocoumarins such as dihydroxybergamottin, paradisin A and bergamottin were quantified in seven cultivars of grapefruits and its parent Pummelo. The changes in the levels of these compounds due to season and location were also monitored during the season and the effect of location, using high performance liquid chromatography. Considerable differences were observed in the levels of these compounds in different grapefruit cultivars. Ray Red showed the lowest levels of all three furocoumarins (0.492 ± 0.027 µg/mL DHB, 0.059 ± 0.001 µg/mL paradisin A and 0.344 ± 0.030 µg/mL bergamottin) and Duncan showed the highest amount of DHB (2.587 ± 0.432 µg/mL) and bergamottin (1.004 ± 0.068 µg/mL), where as the highest levels of paradisin A was observed in Star Ruby. The levels of both DHB and bergamottin in both cultivars of grapefruit decreased as the season progresses except for the bergamottin in Marsh White grapefruit. Influence of growing location, processing and storage on the levels of these compounds was also evaluated. Among the cultivars the highest levels of DHB (2.266 µg/mL) and bergamottin (2.411 µg/mL) were found in Flame grapefruit. The highest level of paradisin A was found in Rio Red grapefruit grown in California and the lowest levels were observed in Rio Red grapefruit grown organically in Texas.

Introduction

The phytochemical profile of the citrus fruit varies with cultivar, environmental conditions and the stage of fruit development and maturity. Duncan and Marsh White showed variation in naringenin *(70)* and limonin content *(71)*. Grapefruit trees exposed to freezing conditions showed more naringin and less limonin *(72)*. Other environmental stress parameters such as temperature and humidity affected the levels of naringin *(73, 74)*. Bioactive compound levels changes during developmental stages. Furthermore, highest concentration of naringin *(74, 75)*, limonin *(76)* and nomilin *(77)* were observed at early developmental stages and progressively declined as fruit matured.

The content of certain bioactive furocoumarins in grapefruit juice can vary considerably. Tassanyakula and his group reported four fold variations of bergamottin, 43- fold variations of DHB and 65- fold variations of dimers in the commercial grapefruit juice *(79)*. Fresh squeezed grapefruit juice increased the bioavailability of terfanadine more than twofold compared to industrially-processed juice *(17)*. Fukuda et al., *(78)* reported that furocoumarins levels in white were higher than red commercial grapefruit juices. Furthermore, the highest levels of furocoumarins were found in the fruit meat *(78)*. While environmental factors may have influence on furocoumarins and flavonoids, genetic factors might play a major role in variation of these bioactive compounds. Variation and quantification of furocoumarins reported so far involve commercially processed juice. 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. Thus, the objective of this study was to determine the levels of specific

furocoumarins content in different fresh grapefruit cultivars and from different locations and also changes during season.

Materials and Methods

Cultivar Study. Seven grapefruit cultivars such as Rio Red, Ruby Red, Ray Red, Star Ruby, Thompson Pink, Marsh White, Duncan and grapefruit parent Pummelo were harvested in the month of February 2005 from an orchard at the Texas A&M University-Kingsville Citrus Center Weslaco, Texas.

Seasonal Study. Rio Red and Marsh White grapefruits were harvested from an orchard at the Texas A&M University-Kingsville Citrus Center, Weslaco, Texas. The fruits were harvested at 30 days interval starting form the month of November 2003 to May 2004. The fruits were juiced and stored at -80 ºC until analyzed for the furocoumarins levels.

Location Study. In the month of March, Rio Red grapefruits were collected from Texas, Florida and California while. Marsh White grapefruits were collected from only two states Florida and Texas. Fruits were shipped from Florida and California through overnight mail. Fruits were juiced and analyzed for the levels of furocoumarins immediately after receiving the shipment.

Chemicals and HPLC Analysis. All the solvents for extraction and isolation were ACS grade and for quantitative analysis HPLC grade were obtained from EMD (EMD Chemicals Inc., Gibbstown, NJ). HPLC analyses were performed as described in chapter II.

Standard Furocoumarins and Standard Curve. Furocoumarins were isolated and characterized as described in chapter II. Isolated compounds were used as standards for quantification. Standards of DHB, paradisin A and Bergamottin were weighed and dissolved in 1 mL of methanol to give serial concentrations. Three injections were performed for each dilution. The standard curve was by plotting concentration against the peak area.

Sample Preparation for Furocoumarins. Samples stored at -80 ºC were thawed to room temperature and 50 mL of juice was taken in a 250 mL separating funnel 50 mL of ethyl acetate was added and mixed well for 5 minutes. The organic layer was separated carefully, and then this step was repeated twice. Extracts of each sample were pooled and the ethyl acetate was evaporated to dryness under vacuum using Buchi rotary evaporators. Extract was reconstituted in HPLC grade methanol for HPLC analysis.

Statistical Analysis. The amounts listed are averages of 15 samples. Dunnett's multiple comparisons test was used to determine the significance at $P < 0.005$. Analysis was performed using GraphPad Instat version 3.00 for windows. Mean values and standard deviations (SD) were reported.

Results and Discussion

Calibration. A calibration curves for furocoumarins and flavonoids were derived from three injections of different concentrations with good reproducibility and accuracy. Regression equations were obtained with correlation coefficient of ≥ 0.99.

Variation of Furocoumarins in Different Cultivars of Grapefruits. Bergamottin and **Figures 3.1, 3.2 and 3.3** demonstrates the levels of three furocoumarins

in seven different cultivars of grapefruits and Pummelo. DHB levels showed more variation among grapefruit cultivars than paradisin A. 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 cultivars contain more DHB than the white cultivars while Pummelo was on par with the colored cultivars. 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 cultivars. The highest levels of bergamottin were found in Duncan and lowest levels were found in Ray Red. Pummelo showed higher bergamottin levels compared to different grapefruit cultivars.

Available information on separation, quantification and variation was on commercial grapefruit juices. There is vast difference 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. And also as juice undergoes physical treatments such as high temperature treatment and mechanical pressure which may influence these compounds. It is evident from this study that grapefruit cultivars differ in its bioactive furocoumarins contents.

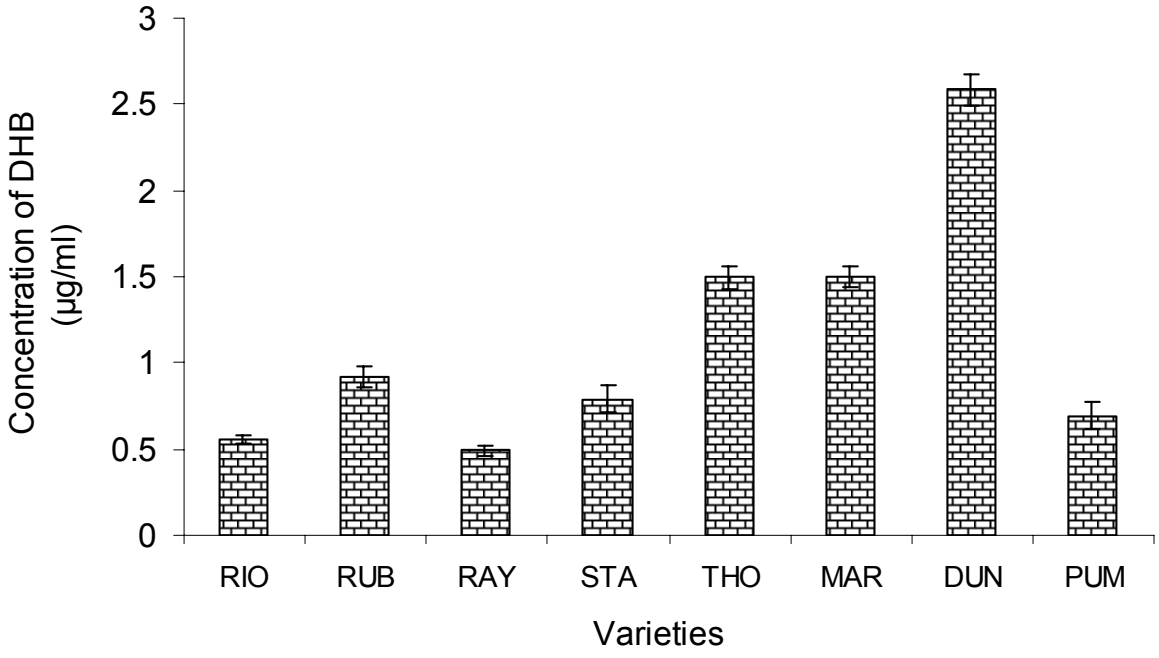


Figure 3.1. DHB found in the seven grapefruit cultivars and Pummelo. Cultivars used were RIO (Rio Red), RUB (Ruby Red), RAY (Ray Red), STA (Star Ruby), THO (Thompson Pink), MAR (Marsh White), DUN (Duncan) and PUM (Pummelo). The data are shown as the mean of ± S.D. n=15 samples.

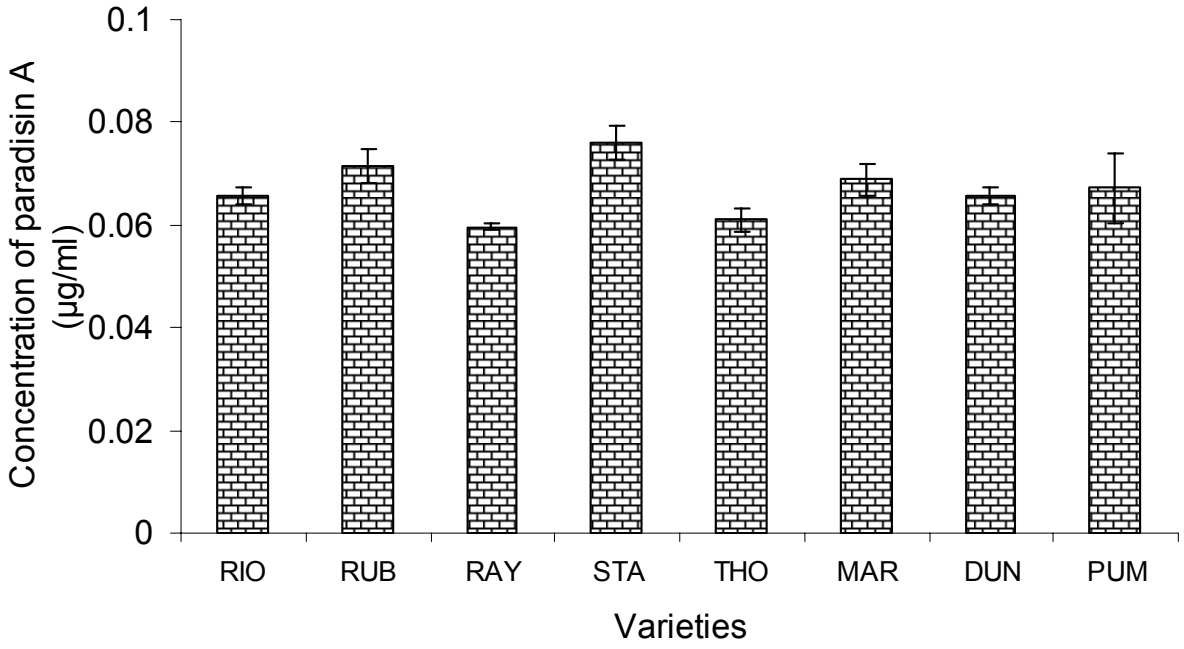


Figure 3.2. Paradisin A found in the seven grapefruit cultivars and Pummelo. Cultivars used were RIO (Rio Red), RUB (Ruby Red), RAY (Ray Red), STA (Star Ruby), THO (Thompson Pink), MAR (Marsh White), DUN (Duncan) and PUM (Pummelo). The data are shown as the mean of ± S.D. n=15 samples.

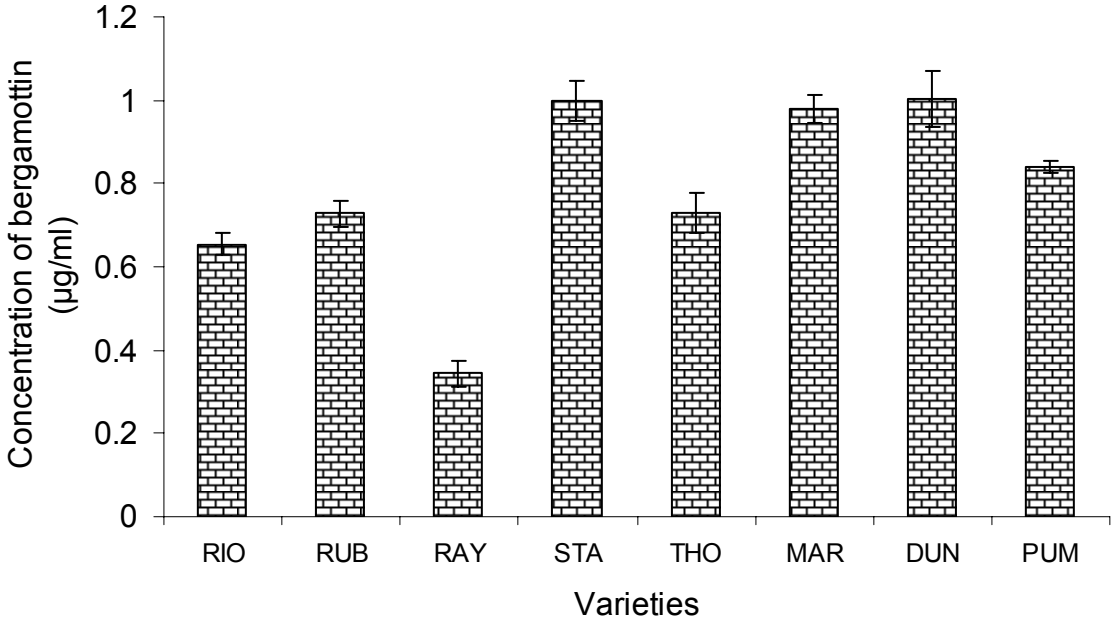


Figure 3.3. Bergamottin found in the seven grapefruit cultivars and Pummelo. Cultivars used were RIO (Rio Red), RUB (Ruby Red), RAY (Ray Red), STA (Star Ruby), THO (Thompson Pink), MAR (Marsh White), DUN (Duncan) and PUM (Pummelo). The data are shown as the mean of ± S.D. n=15 samples.

Seasonal Variation of DHB, Paradisin A and Bergamottin. Furocoumarins are phytoalexins produced perhaps in response to external stimuli such as stress or pathogenic infection. At any given time the level of these compounds reflect the physiological condition of the plant. DHB levels were highest in the beginning of the season and showed decreasing trend till the end of the season with 1.171 and 1.675 μg/mL in Rio Red and Marsh White cultivars, respectively (**Figure 3.4-3.6**). There was 43.21 % and 39.82% decrease in the concentration of DHB from the beginning of the season to the end of the season in Rio Red and Marsh White grapefruit. Throughout the season, Marsh White contains more DBH than Rio Red. Marsh White had 30.08% and 34.02% more DBH than Rio Red at the beginning and end of the season, respectively. Paradisin A, a dimer probably derived from DHB by a dehydration reaction, is distributed 10 to 15 times lower than DBH. Paradisin A concentrations were 91 and 87 ng/mL at the beginning of the season and 81 and 76 ng/mL at the end of the season in Rio Red and Marsh White, respectively. There is no particular trend in the distribution of the paradisin A during the season as seen in case of DHB. However, paradisin A is potent inhibitor of CYP3A4 than DHB and bergamottin *(42)*. The magnitude is several folds higher than both monomers together, indicating the potential of paradisin A to be highly active even in at low concentration. Bergamottin is probably precursor of DHB in the furocoumarins biosynthesis. The concentration of bergamottin showed decreasing trend from the beginning of the season to the end, among the two cultivars Marsh White contains average of 35.58% more bergamottin than Rio Red.

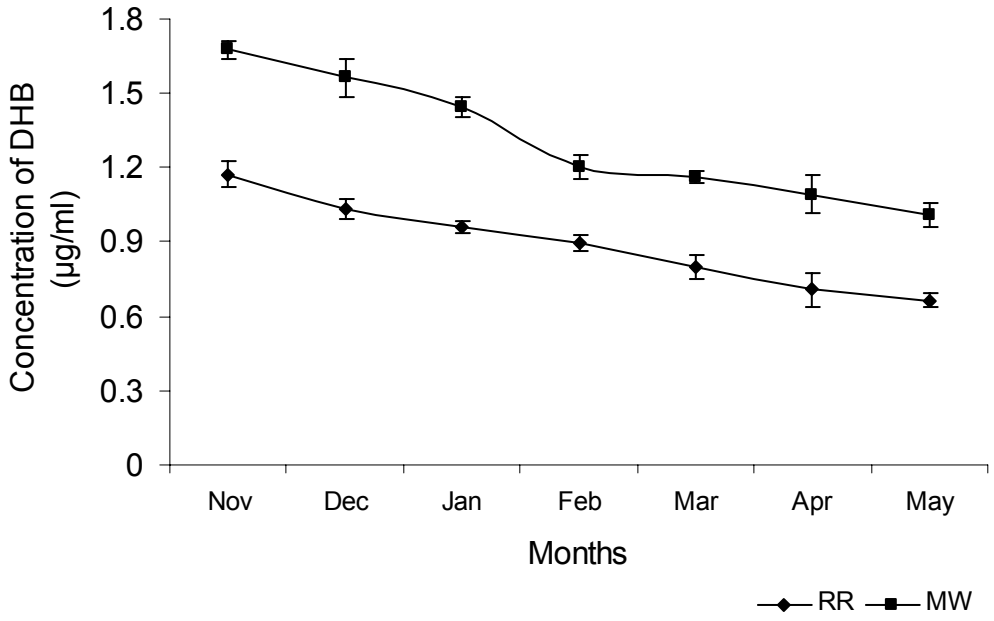


Figure 3.4. Influence of season on the level of DHB in Rio Red and Marsh White grapefruit juice. The data are shown as the mean of ± S.D. n=15 samples.

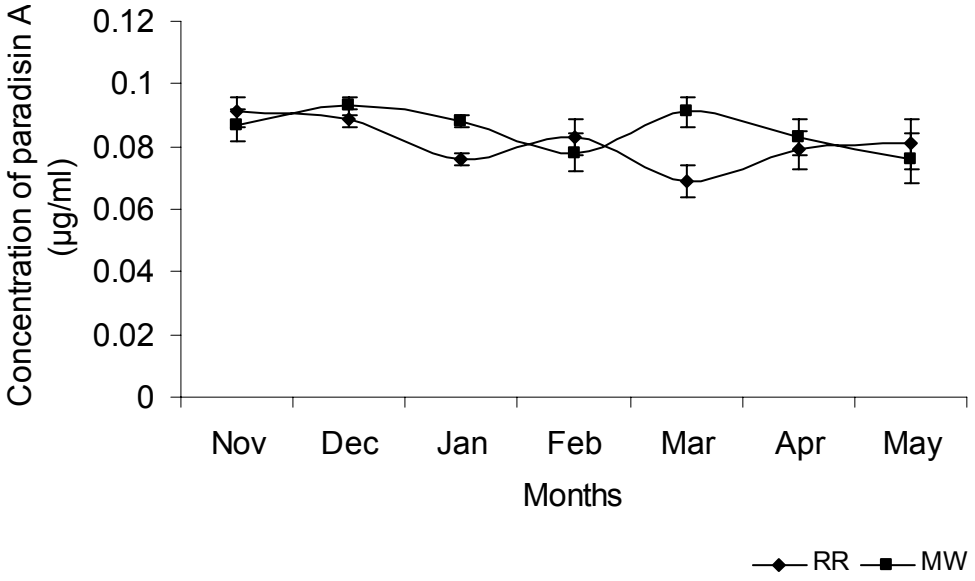


Figure 3.5. Influence of season on the level of paradisin A in Rio Red and Marsh White grapefruit juice. The data are shown as the mean of ± S.D. n=15 samples.

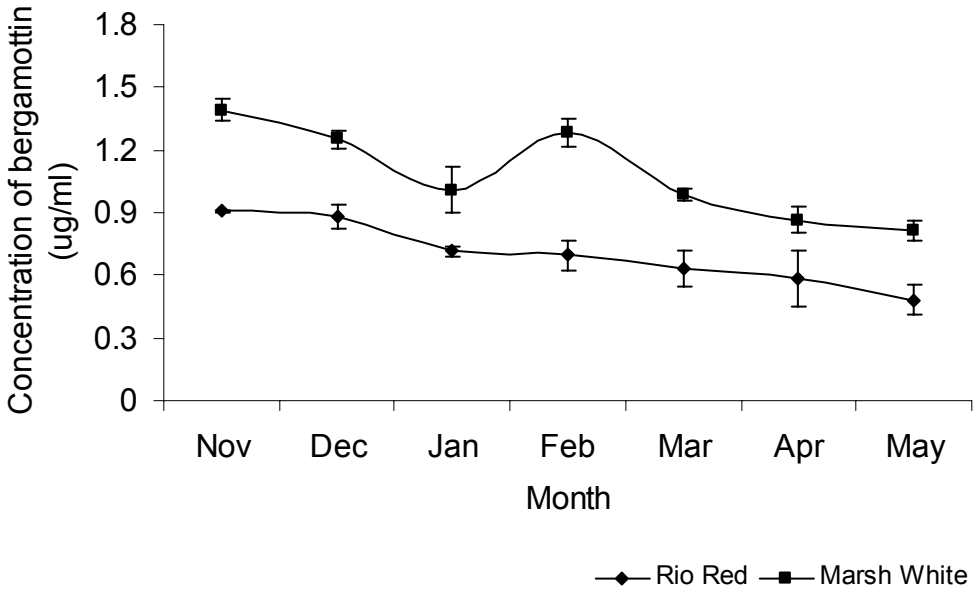


Figure 3.6. Influence of season on the level of bergamottin in Rio Red and Marsh White grapefruit juice. The data are shown as the mean of ± S.D. n=15 samples.

Influence of Location and Cultivation Method on the Levels of Furocoumarins. Due the lack of availability of the tested grapefruit cultivars in all three locations, only two cultivars were compared for growing location study. Marsh White grown in Texas contain 32.15% higher DBH compared to the same cultivar grown in Florida (**Figure 3.7**). While significantly higher levels of bergamottin were observed when Marsh White grapefruit was grown in Florida compared to the same cultivar grown in Texas, no differences of paradisin A were observed in between both locations. Interestingly, among the three growing locations, when Rio Red grown in California, Texas and Florida, DHB levels were significantly higher in fruits grown in California compared the same grapefruit grown in Texas and Florida. Furthermore, Rio Red grapefruit grown in Florida had significantly higher paradisin A compared to the same grapefruit grown in Texas and California while there is no differences in the levels of bergamottin were observed among the three locations (**Figure 3.8, 3.9**).

Rio Red grapefruit grown using conventional practices were compared with the same cultivar grown organically. DHB levels were higher in Rio Red grapefruit grown with conventional practices compared the same cultivar grown organically (**Table 3.1**), while paradisin A levels were more in organically grown grapefruit compared conventionally grown grapefruit. No significant differences were observed in the levels of bergamottin levels in Rio Red grapefruit grown with conventional and organic practices. Among the grapefruit cultivars grown at different locations, Flame grapefruit from Florida had the highest concentration of DHB, bergamottin and paradisin A, while lowest levels of DHB and paradisin A were observed in organically grown Rio Red grapefruits (**Table 3.1**).

Table 3.1. Influence of cultivation method and cultivar on the levels of furocoumarins in grapefruit.

Grapefruit	**Furocoumarins levels (µg/mL)**		
	DHB	**Paradisin A**	**Bergamottin**
Rio Red- Conventional	0.989 ± 0.115[a]	0.0910 ± 0.004	0.754 ± 0.057
Rio Red- Organic	0.627 ± 0.061	0.0669 ± 0.014	0.774 ± 0.074
Flame- Florida	2.266 ± 0.113	0.2089 ± 0.004	2.411 ± 0.116
Melgold- California	2.129 ± 0.068	0.0794 ± 0.012	1.451 ± 0.118

[a]Mean ± standard deviation, n=15.

Furocoumarins are phytoalexins produced perhaps in response to external stimuli such as stress or pathogenic infection *(43)*. At any given time the level of these compounds reflect the physiological condition of the plant. A considerable proportion of furocoumarins may be recovered from surface of plants *(44)*. The concentration of three furocoumarins such as xanthotoxin, bergapten and psoralen were measured by Zobel and Brown in whole leaves of *Heracleum lanatum* and in the extracts of leaf over vegetative season. Furocoumarins concentration in the whole leaf varied, with highest being in the month of April and sharp decline afterwards as leaf enlarged rapidly *(44)*. It has been previously shown that celery grown near urban centers in California was found to stimulate accumulation of psoralen, bergapten, xanthotoxin and isopimpinellin *(88)*. Genetic make up such as cultivar/cultivar, environmental conditions, developmental stage of plant also influence the content of plant secondary metabolites. De Castro et al., reported considerable variability of naringin, bergamottin and DHB in grapefruit juice with several fold variation between the juice different juice preparations *(89)*. In another study by Ho et al reported 1.5, 2.3 and 4.7 fold variation in the content of naringin, naringenin and bergapten, respectively in different lots of grapefruit juice preparations in New Zealand *(90)*. Guo et al reported the 43 fold variation of DHB, 14 fold variations for paradisin A and 4.1 fold variation for bergamottin in different juices *(42)*.

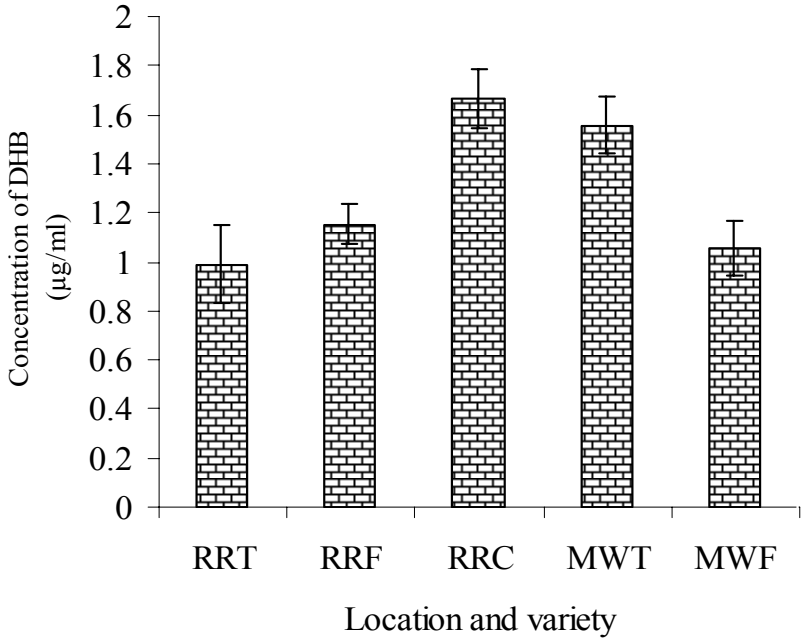


Figure 3.7. DHB levels in Rio Red and Marsh White grapefruits grown at different locations. The codes in the panel (RRT) Rio Red –Texas, (RRF) Rio Red –Florida, (RRC) Rio Red –California, (MWT) Marsh White –Texas and (MWF) Marsh White – Florida. Data shown are the mean ± SD for 15 samples.

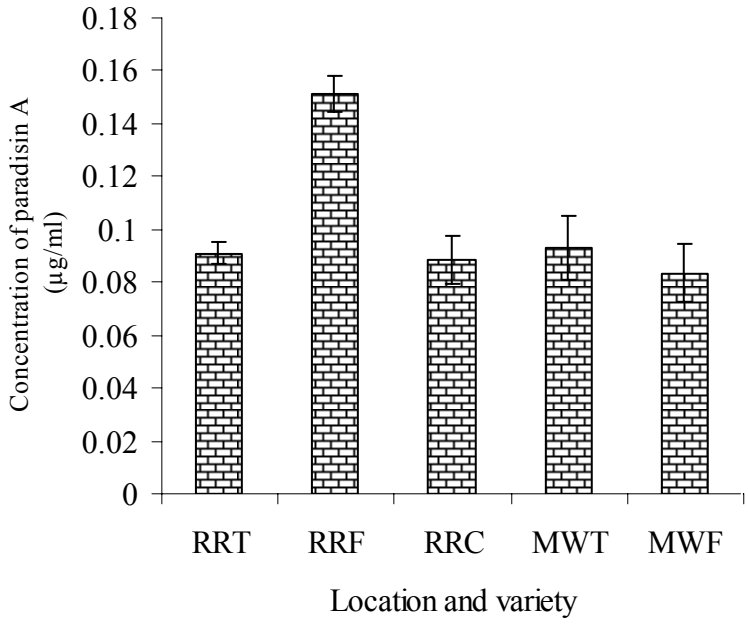


Figure 3.8. Paradisin A levels in Rio Red and Marsh White grapefruits grown at different locations. The codes in the panel (RRT) Rio Red –Texas, (RRF) Rio Red –Florida, (RRC) Rio Red –California, (MWT) Marsh White –Texas and (MWF) Marsh White – Florida. Data shown are the mean ± SD for 15 samples.

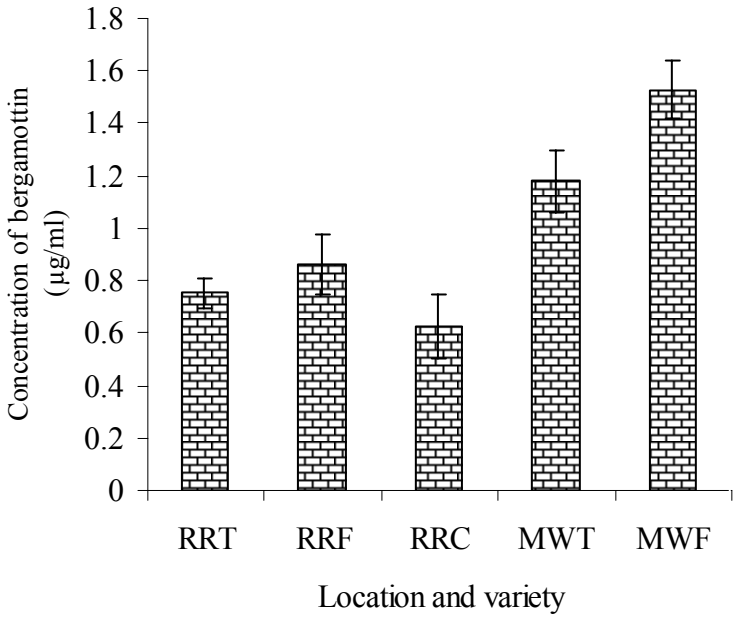


Figure 3.9. Bergamottin levels in Rio Red and Marsh White grapefruits grown at different locations. The codes in the panel (RRT) Rio Red –Texas, (RRF) Rio Red – Florida, (RRC) Rio Red –California, (MWT) Marsh White –Texas and (MWF) Marsh White –Florida. Data shown are the mean ± SD for 15 samples.

CHAPTER IV

INFLUENCE OF POST-HARVEST FACTORS ON FUROCOUMARIN AND OTHER BIOACTIVE COMPOUNDS OF GRAPEFRUIT

Synopsis

Several post-harvest factors such as processing, storage, quarantine treatments have potential impact on the levels of bioactive compounds of grapefruit. Rio Red and Marsh White grapefruits were irradiated with E-beam at 0, 1.0, 2.5, 5.0 and 10.0 KGys. The dose effect of E-beam on levels of various bioactive fruit components such as vitamin C, flavonoids, carotenoids, furocoumarins and limonoids, were monitored. Quality parameters such as appearance, taste and acceptability were evaluated. Results showed that the irradiation does not affect the vitamin C content at 1KGys. However, doses beyond 1 KGys drastically reduce the vitamin C. Both carotenoids (lycopene and β-carotene) respond differently to the irradiation. The levels of DHB showed decreasing trend while bergamottin content did not change. Naringin a major flavonoid of grapefruit showed significant increase over the control at 10KGys in Rio Red and Marsh White. Nomilin showed decreasing tend with increase in dose while limonin remained same in all the doses. Likeness of appearance and taste were influenced differently at different doses. One KGys was shown to improve the appearance and organoleptic quality while at 10KGys had significant decrease in the taste and organoletic qualities in both cultivars. Hand squeezed juice contained 1.98, 1.06 and 3.03 fold more DHB, paradisin A and bergamottin, respectively as compared to processed juice. The levels of furocoumarins showed a decreasing trend in all the juices with progress of storage. Levels of

furocoumarins were more in cartoon containers than the cans and cardboard container juices.

Introduction

Ionizing radiation is the most effective technique that inactivates human pathogens and reduces the spoilage of fruit juices and other foods *(91-93)*. The number of food items approved for irradiation is increasing at the global level. Food and Drug Administration in the United States approved low –level irradiation of food and food products to reduce the incidence of illness resulting from pathogenic microorganisms *(94)*. Food irradiation with doses even >10 kGys was endorsed by a joint FAO/IAEA/WHO study group *(95)*. In fact, irradiation, commonly referred to as cold pasteurization, is less environmentally and nutritionally harmful than most other traditional practices. However, irradiation can reduce the food quality by developing off-odors and flavors *(96)*.

The content of bioactive compounds in fruits and vegetables may be altered by post harvest treatments such as irradiation, storage and freeze drying conditions *(97)*. Thus the objective of this study was to evaluate the effect E-beam irradiation, γ-irradiation, processing and storage on furocoumarins found in grapefruit juice.

Material and Methods

Samples. Rio Red and Marsh White grapefruits were harvested from an orchard at Texas A&M University Kingsville Citrus Center, Weslaco Texas, during the month of

December 2005. Fruits were juiced using blenders after irradiation and stored at -80 ºC until analyzed for various parameters.

E-beam Source and Dose Calculations. Rio Red and Marsh White grapefruits were subjected to ionizing radiation ranging from 0 (non-irradiated control) to 10 kGys using a 2.0 MeV Van der Graff Electron Accelerator (High Voltage Engineering Cooperation) located at the National Center for Electron Beam Food Research, Texas A&M University, College Station, Texas. Absorbed dose was determined from the count of C × 10^{-8} coulombs in the Van der Graff and related with dose previously determined with a Farmer Dosimeter (J.L. and associates, Glendale CA, USA) placed on the top and bottom of the fruits when the fruits were irradiated (**Figure 4.1**). The time dose application was recorded using a stopwatch (Fisher Scientific, Canada). The dose was adjusted accordingly to treat the fruit samples with 1, 2.5, 5 and 10 KGys. Ten pounds (per treatment) of fruits were packed in 5 inch cardboard boxes and irradiated with above doses of ionizing radiation (E-beam).

γ-irradiation and Dose Calculations. Rio Red and Marsh White grapefruits were subjected to γ-irradiation ranging from 0 (non-irradiated control) to 2.4 KGys using self contained dry storage units of ^{137}Cs (Husman model 521A, Inc., Whippany, NJ) at the USDA facility Mission, Texas. The non-irradiated control fruits were also transported to the irradiation facility along with the samples to be irradiated to expose them to the similar conditions.

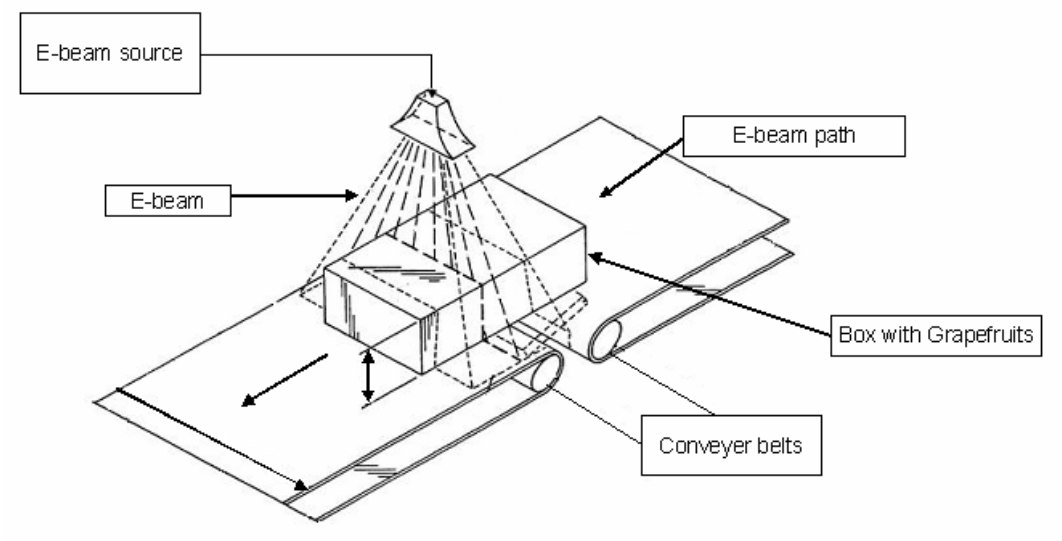


Figure 4.1. Design and placement of grapefruit boxes for irradiation treatments using a 2.0 MeV Van der Graff linear accelerator at room temperature.

Post-Harvest Storage Study. Rio Red and Marsh White grapefruits were packed in to boxes (total of 36 numbers), separately, each box contained 12-15 fruits. Boxes were stored at two storage conditions. One was at 24 ºC and another was at 9 ºC with relative humidity between 90-95%. Every two weeks, fruit samples were analyzed for furocoumarin levels. Total of four samplings were taken for the grapefruits stored at 9 ºC. However, for fruits stored at 24 ºC, only three samplings were taken as fruits desiccated after 30 days. Grapefruits were juiced using blender and juice was analyzed for furocoumarins content.

Juice Storage Study. Three single strength commercial Rio Red grapefruit juice products, such as cans (100% juice from concentrate), cartoons (100% pure fresh juice and not from concentrate, can be stored for 60 days at refrigerated condition) and cardboards (100% juice from concentrate) were obtained from Texas Citrus Exchange, Mission, TX, USA. Total of 25 cans and cardboard containers and 20 cartoon containers were used for the storage study. Cans and cardboard containers were stored at room temperature while cartoon containers were stored at refrigerated conditions, simulating the grocery store conditions. Cans and cardboard containers were stored for 75 days while cartoon containers were stored 60 days and every 15 days five containers were used for analysis.

Processing of Juice. In order to determine the effect of processing on furocoumarins, a separate experiment was conducted at industrial scale level. The Rio Red grapefruits were harvested from the Texas A&M University-Kingsville Citrus Center at Weslaco and grouped into two batches. One batch of fruits from two bins of fruits were juiced in the lab and the other batch containing 48 bins of fruits were processed at Texas

Citrus Exchange commercial juice plant under commercial processing conditions. Sequence of events in the processing of grapefruits include i) washing the fruits, ii) puncturing the fruits to obtain peel oil, iii) mechanical cutting of fruits into two halves, iv) juicing the halves by automated juicers, v) removing the pulp by straining the juice, vi) pasteurizing the juice and vii) automated packing of the juice into cans. Both types of juices were stored at room temperature until sampled for analysis.

Standards. Ascorbic acid, lycopene, β-carotene, naringin and naringenin were purchased from Sigma chemical Co. (St. Luis, MO, USA). Dihydroxybergamottin, bergamottin, limonin and nomilin were purified according to published method *(98)*.

Determination of Acidity and Total Soluble Solids. Acidity of juice samples were measured by an Acuumet pH reader (Fisher Scientific Pittsburgh PA, USA) and total soluble solids were measured using a hand refractometer (American Optical Corporation Buffalo NY, USA).

Standard Curve. Stock solutions (3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 PPM) of vitamin C and carotenoids (lycopene and β-carotene) were prepared in milli Q water and chloroform respectively. Stock solutions (3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 PPM) of limonin and nomilin were prepared in acetone. Elution was carried out to obtain peak area responses. The calibration curves for each compound were prepared by plotting concentration of each compound versus peak area.

Determination of Vitamin C. Two mL of grapefruit juice was mixed with 10 mL of 3% metaphosporic acid and homogenized for 5 minutes. The homogenate was filtered through filter paper. An aliquot of one mL was filtered through 0.45 μm membrane filter (Pall Corporation Ann Arbor, MI USA). Twenty microliters of one mL aliquot sample

was injected into HPLC system. Alltech Alphbonda Amino 10μ C-18 column (300 x 3.9) was used for the separation and quantification. The mobile phase used was acetonitrile:water (70:30 v/v) with 1.15 g/L of $(NH_4)H_2PO_4$ at a flow rate of 1 mL/min. Vitamin C peak was detected at 255 nm with retention time of 6.49 ± 0.04 min.

Determination of Carotenoids. Ten mL of grapefruit juice was mixed with 50 mL of acetone and mixed well. Five mL of hexane and 50 mL of water was added to the mixture. The hexane layer was separated and dried with nitrogen and a one mL sample was prepared with acetone and filtered with 0.45 μm membrane filter. Ten microliters of extract was loaded on to Waters Sperisorb ODS-2 5μm column (250 X 4.6 mm). Elution was carried out with mobile phase containing solvent A (35% of ethyl acetate in acetonitrile) and B (ethyl acetate with 1mL of TEA/liter). Lycopene and β-carotene were detected at 450 nm with peak retention times at 7.73 and 10.69 minutes respectively.

Determination of Furocoumarins and Flavonoids. Furocoumarins were determined according the methods as described in chapter II.

Determination of Limonoid Aglycones. Juice was extracted with ethyl acetate and 50 μL of extract was injected on to Geminai (Phenomenex Torrance CA, USA) column and eluted with acetnitrile (C) and water (D) as follows: 0 min A, C 20%; 20 min, C 35%; 40min, C 42%; 50 min, 55%; 55 min, A 100%; 57 min, A 100%; 60 min, A 20%. The flow rate was set at 1 mL/min and elution was monitored at 210 nm with a photodiode array detector.

Quality, Taste and Flavor Evaluation. Control and irradiated fruits were evaluated for consumer acceptability by 23 untrained panelists at Vegetable and Fruit Improvement Center Texas A&M University College Station. Preference evaluation was

conducted to determine the appearance, taste and flavor. Quantitative preference rating (American Society for Testing and Material, 1968) was used to evaluate ratings for appearance, taste, flavor and organoleptic properties. Five fruits of each Rio Red and Marsh White were presented for appearance evaluation. Eight slices were prepared from each fruit total of five fruits were presented for flavor and taste analysis. Panel was asked to mark on the hedonic scale ranging from extremely dislike to extremely like (1-10 cm scale line) according to their feel and preference. Results from 1 -10 scale were converted to percentile.

Results and Discussion

Influence of E-beam on Acidity and Total Soluble Solids. Figure 4.2 shows the effect of E-beam on acidity and total soluble solids of Rio Red and Marsh White grapefruit juice. As doses increased, acidity of the juice decreases and total soluble solids increased slightly, compared to control.

Influence of E-beam on Vitamin C Content. Figure 4.3 shows the levels of vitamin C in different treatments of E-beam irradiated Rio Red and Marsh White grapefruit juice. Vitamin C concentration in Rio Red at 1 KGys treatment showed 0.77% decrease in Marsh White and 1.26% in Rio Red. As the dose of the irradiation increased, the level of vitamin C decreased considerably. Remarkable decrease was observed at 10 KGys in both the cultivars of juices; Rio Red showed a 53.52% decrease while Marsh White showed a 50.03% decrease in total vitamin C concentration. Vitamin C in several

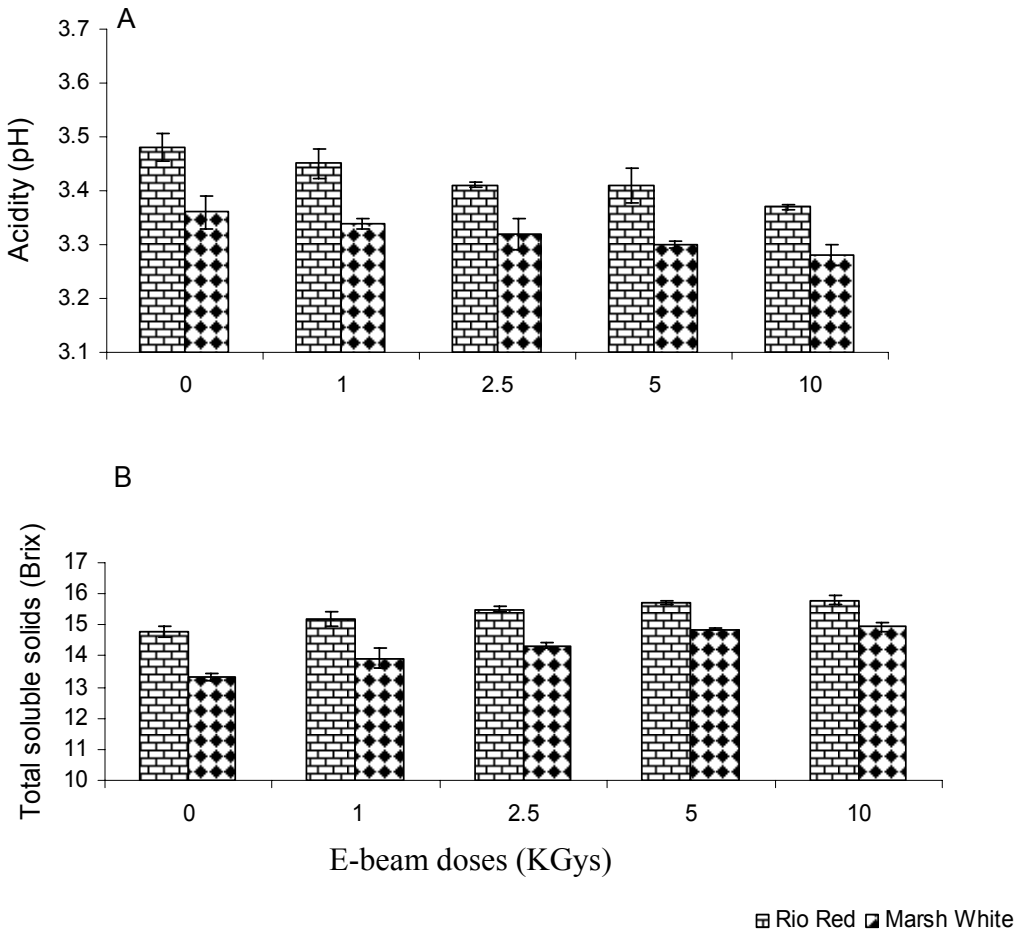


Figure 4.2. Effect of E-beam irradiation on A) acidity, B) total soluble solids of Rio Red and Marsh White grapefruit.

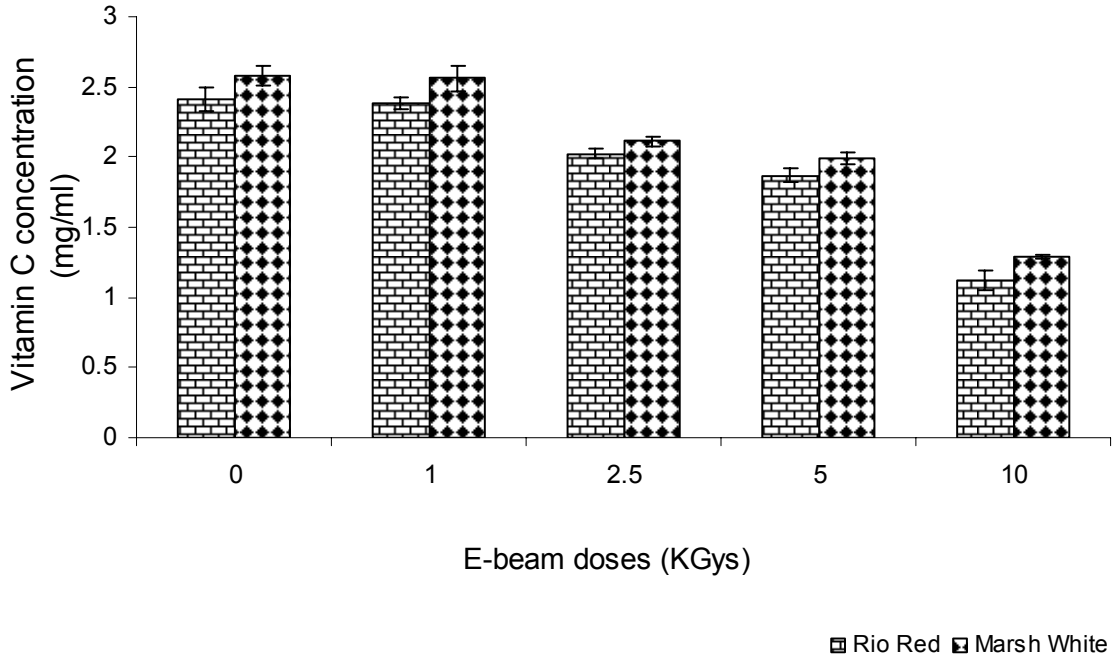


Figure 4.3. Effect of E-beam irradiation on vitamin C levels of Rio Red and Marsh White grapefruit.

citrus fruits undergoes degradation during processing, storage *(99, 100)* and is affected by storage length, conditions and temperature *(101, 102)*. Oxidation of ascorbic acid proceeds both aerobic and anaerobic pathways and depends upon several factors, including oxygen, heat and light *(103)*. Degradation products of vitamin C along with amino acids lead to the formation of brown pigments, which is another problem of quality loss in citrus juices during storage *(104)*.

Influence of E-beam on Carotenoids. Figure **4.4** shows the concentration of lycopene and β-carotene in the Rio Red and Marsh White grapefruit juices influenced by E-beam irradiation at different doses; both lycopene and β-carotene were not detected in Marsh White grapefruit juice. Lycopene concentrations decreased by 2.98% and 12.33% at 1KGy and 10 KGys respectively compared to those of untreated fruit. Conversely, β-carotene levels showed an increase in concentration. There was a 0.14% and 0.39 % increase in the β-carotene concentration at 1KGys and 10 KGys, respectively. Studies have shown that lycopene content of late season fruit was significantly lower than early season fruit *(105)*, especially from October to May *(106)*. Fruit pulp attains the highest color early in the season and decreases as the season progresses *(107)*. Gamma irradiation doses of 10 KGys and 20 KGys did not affect β-carotene levels *(108)*. Previous studies have demonstrated that, irradiation, freeze drying, season and storage affect the carotenoids content *(96)* and freeze drying alone can reduced the β-carotene up to 30%. It has been shown that plants respond to oxidative stress by increasing the levels of antioxidants such as carotenoids and also by increasing some antioxidant enzymes *(109)*.

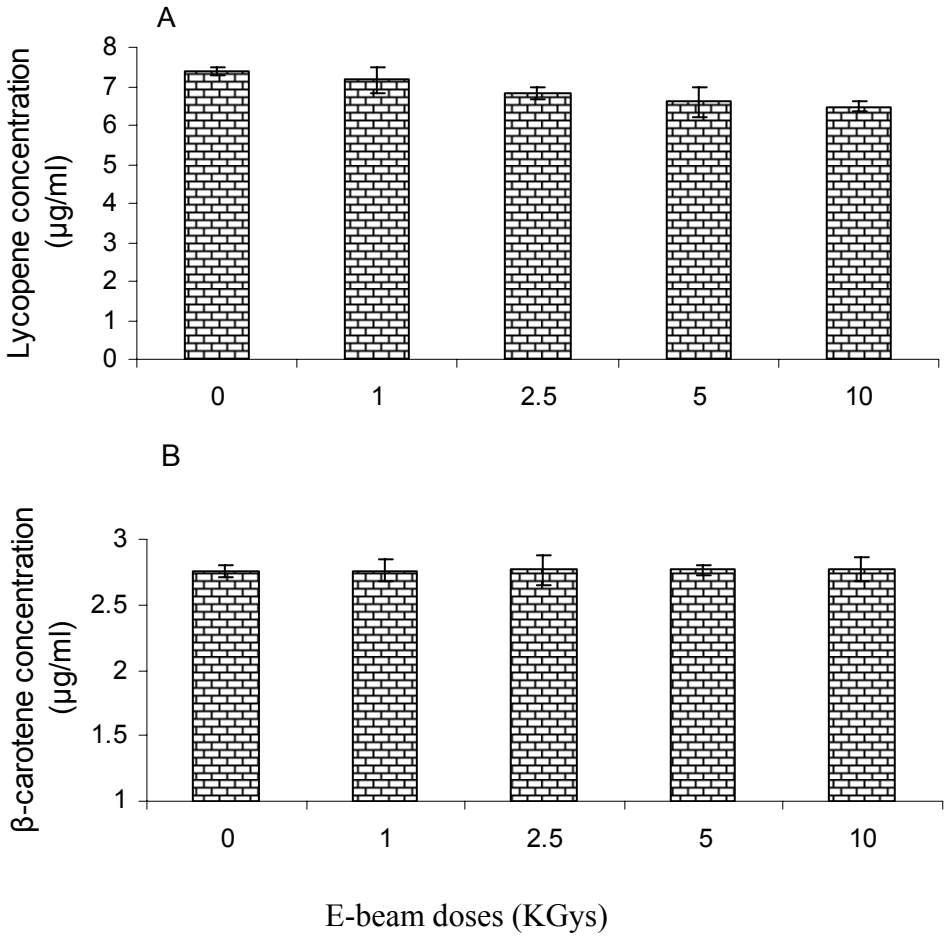


Figure 4.4. Effect of E-beam irradiation on A) lycopene, B) β-carotene levels of Rio Red grapefruit.

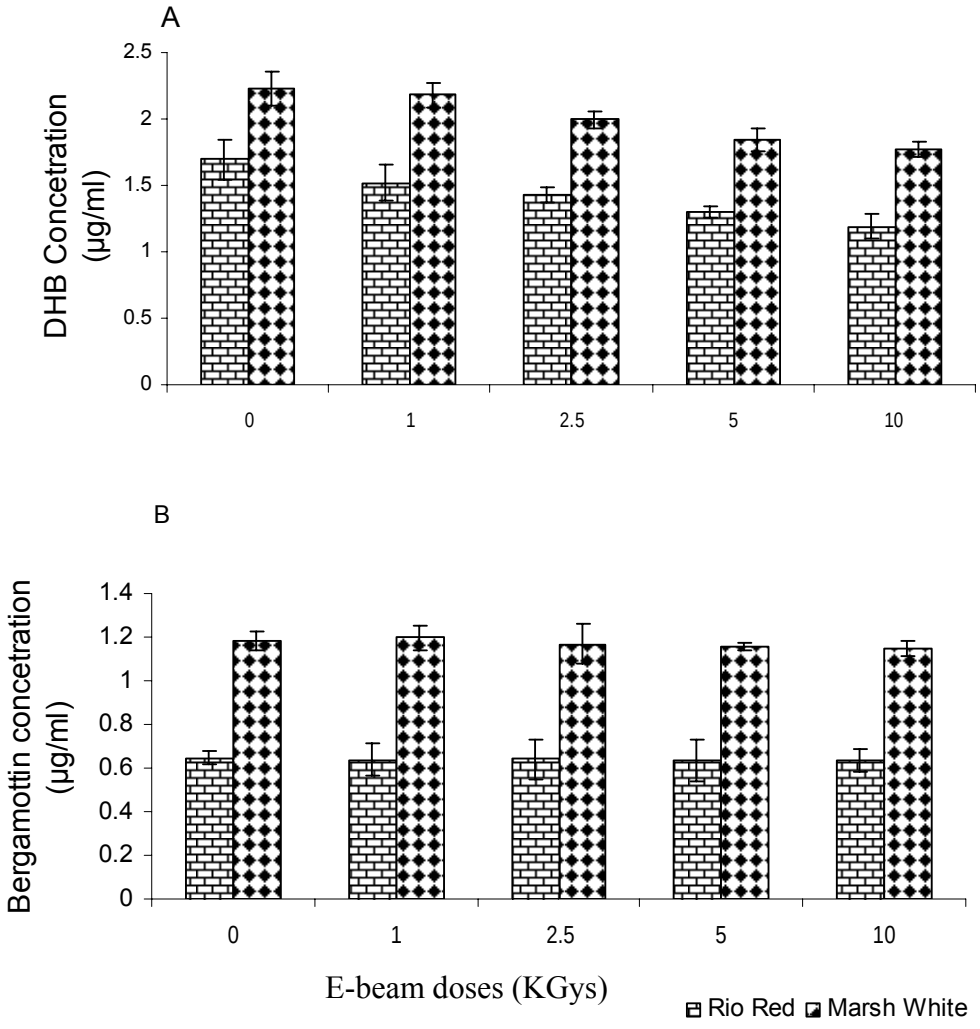


Figure 4.5. Effect of E-beam irradiation on A) DHB, B) Bergamottin levels of Rio Red and Marsh White grapefruit.

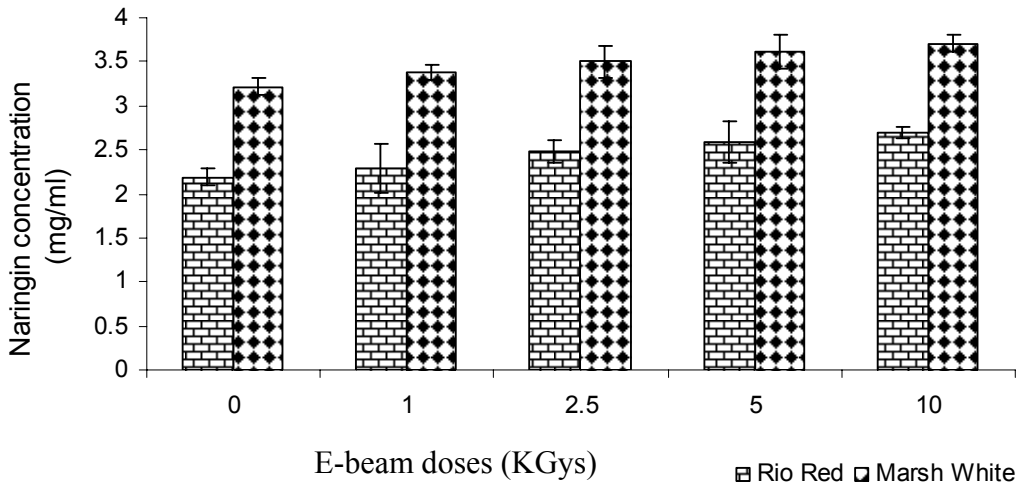


Figure 4.6. Effect of E-beam irradiation on naringin levels of Rio Red and Marsh White grapefruit.

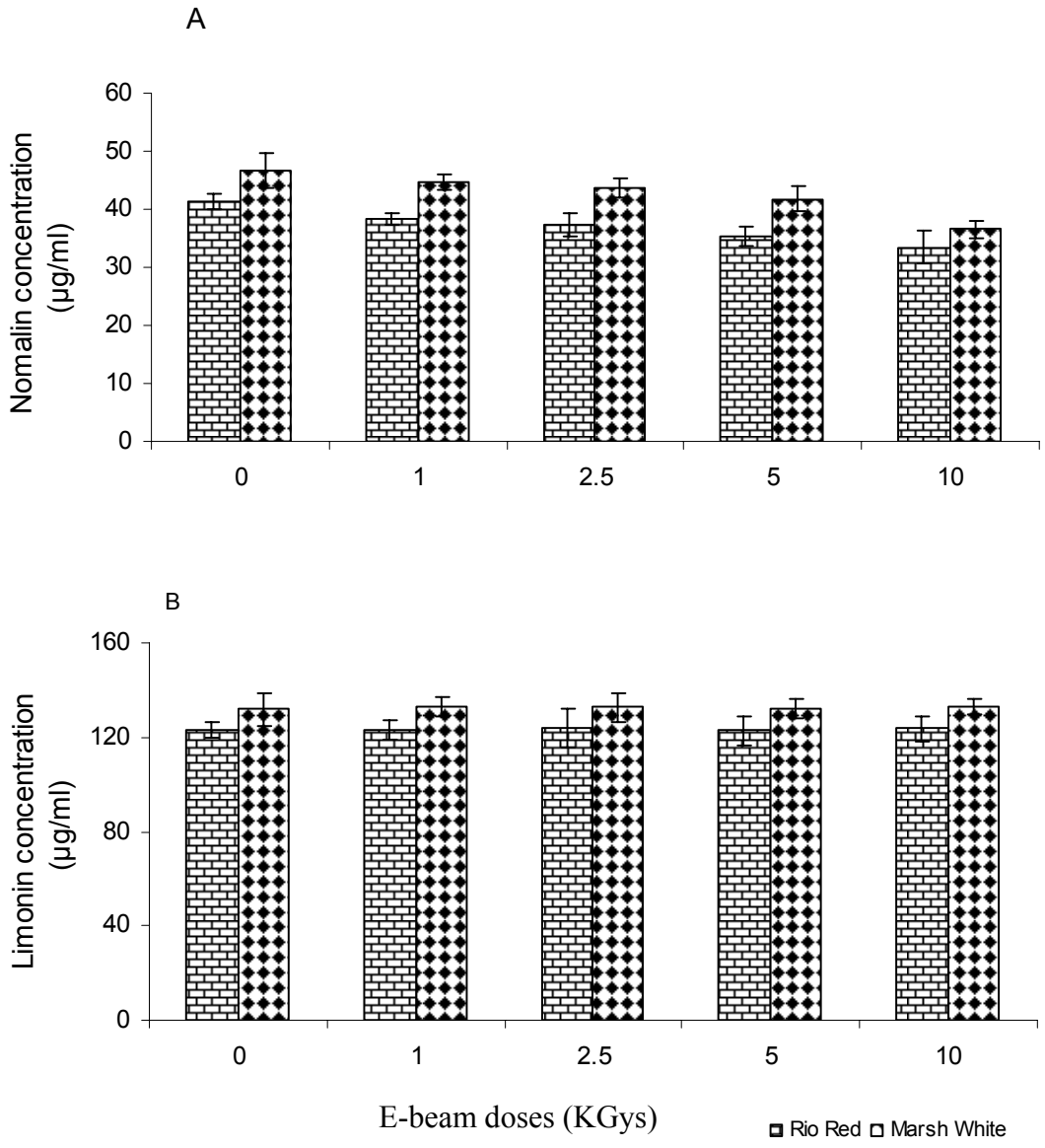


Figure 4.7. Effect of E-beam irradiation on A) nomilin, B) limonin levels of Rio Red and Marsh White grapefruit.

Influence of E-beam on Furocoumarins. Figure 4.5 shows the effect of E-beam irradiation on dihydroxybergamottin and bergamottin concentration. DHB levels show a decreasing trend from 1KGy to 10 KGys in both the cultivars. Rio Red fruits exposed to 1 KGys showed a 10.21% decrease over the control, while juice exposed to 10 KGys showed a 29.87% decrease. Marsh White showed 2.23% and 20.54% decrease at 1 and 10 KGys doses. However, bergamottin levels showed very little changes due to E-beam treatment.

Influence of E-beam on Flavonoids. Figure 4.6 shows the levels of naringin in Rio Red and Marsh White as influenced by e beam irradiation. Concentrations of naringin show an increasing trend in both the cultivars. There was a 4.96% and 4.93% increase over control at 1KGys and 10 KGys doses increased 18.90% and 15.37% increase in Rio Red and Marsh White, respectively. Naringin is the major flavonoid component responsible for grapefruit juice bitterness. Irradiation has been shown to increase the phenylalanine ammonia lyase (PAL) activity in citrus and other fruits *(110, 111)*. PAL enzyme catalyzes the deamination of L-phenylalanin to form *trans*- cinnamic acid, a precursor for flavonoids and tannins *(112)*. Irradiation induced de novo synthesis of naringin by PAL may be responsible for the increase in the content of naringin in treatments over the control.

Influence of E-beam on Limonoid Aglycones (Limonin and Nomilin). Figure 4.7 shows the effect of irradiation on the concentration of limonin and nomilin in Rio Red and Marsh White grapefruit juices. Nomilin showed decrease in concentration with increase in dose. At 1KGy treatment, nomilin decreased by 7.19% and 4.27%, where as 10KGys reduced the concentration by 19.02% and 21.56% in Rio Red and Marsh White

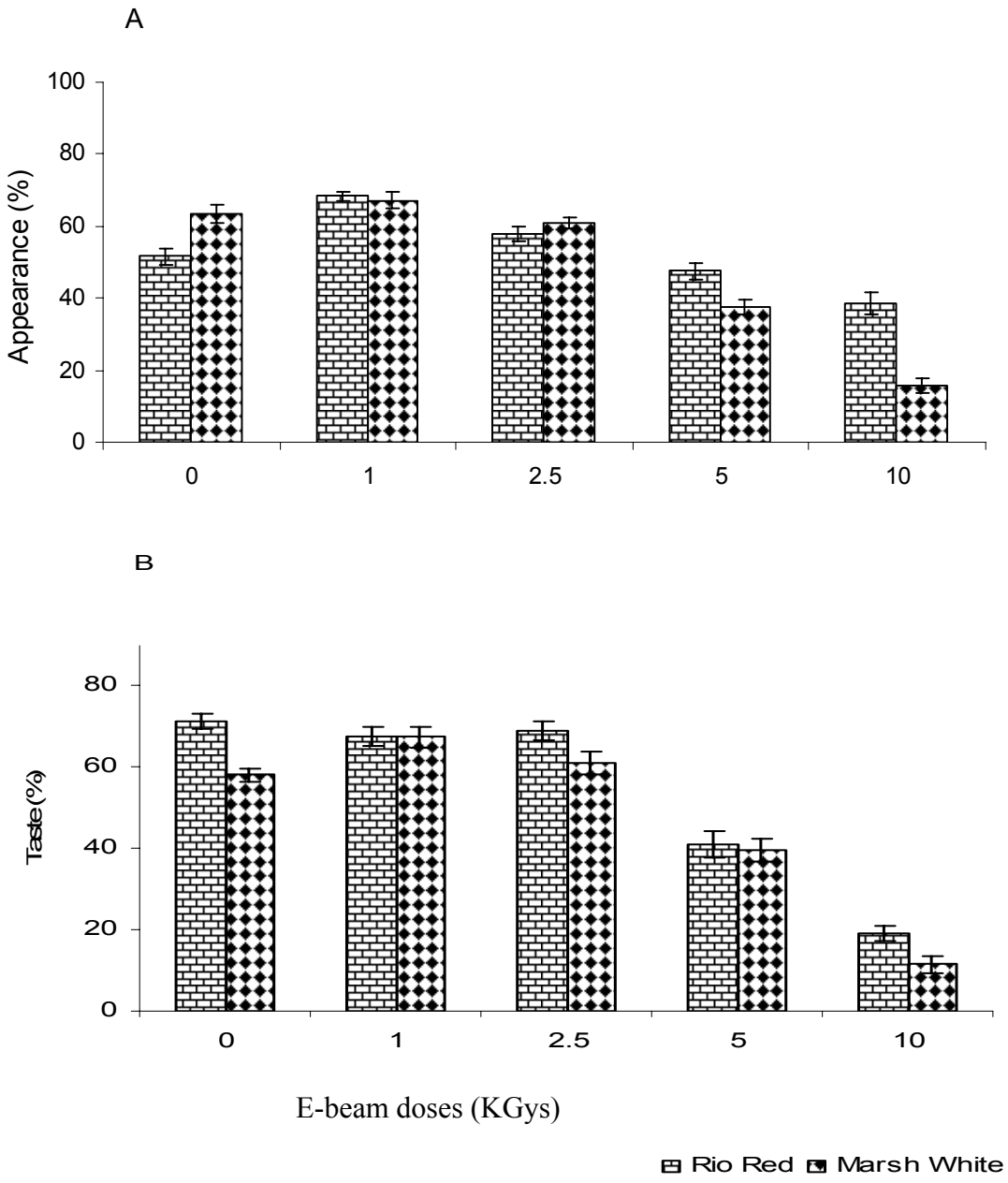


Figure 4.8. Effect of E-beam irradiation on A) appearance, B) taste of Rio Red and Marsh White grapefruit.

juices, respectively. In contrast limonin did not show significant change in the concentration with irradiation treatment. Vanamala et al., reported that the gamma-irradiation coupled with freeze drying is shown to influence limonoid aglycones; limonin and nomilin decreased by 15% and 47% respectively, however no significant difference was observed for obacunone concentration *(96)*.

Influence of Irradiation on Appearance and Taste. Figure 4.8 shows the effect of E-beam irradiation on appearance and taste of Rio Red and Marsh White grapefruits. Results from informal, untrained panel evaluation showed that 1KGys dose improved the appearance of fruits in both Rio Red and Marsh White grapefruits. There was 24.56% and 5.25% increase in appeal for 1KGys irradiated fruits over control for Rio Red and Marsh White respectively. However, at 10 KGys, a considerable decrease in appearance was noticed; the panel rated Rio Red and Marsh White 24.86% and 75.31% lower than control. In taste analysis of fruits exposed to 1KGy of irradiation, Rio Red was ranked 4.91% lower than control while Marsh White was ranked 16% higher. However, both grapefruit cultivars fruits were ranked lower in taste at 10 KGys. The taste rating of Rio Red decreased 73.03% and Marsh White decreased 80.20%, a significant drop compared to control.

Influence of γ-irradiation on Furocoumarins. Figure 4.9-4.11 shows the effect of γ-irradiation on dihydroxybergamottin, paradisin A and bergamottin concentration in Rio Red and Marsh White grapefruit. All the three furocoumarins showed decreasing levels in the juice as the dose of the irradiation increased. Paradisin A concentration change was prominent among the three, with 66.66% and 67.88% decrease in Rio Red and Marsh White cultivars.

Processing Effects on Furocoumarins Levels. Figure 4.12 depicts the levels of furocoumarins in hand squeezed and commercially processed grapefruit juices. Results indicate that DHB and bergamottin content were 1.98 and 3.03 times higher in hand squeezed juice than processed juice, respectively. The levels of paradisin A were not significantly ($P < 0.005$) different between hand squeeze and commercially processed juices.

Post-Harvest Storage Influence on Furocoumarins Levels. The levels of furocoumarins decreased quite remarkably in both cultivars stored at 24 ºC and 9 ºC **(Table 4.1)**. The DHB concentrations were decreased by 8.57% and 20.34% in Rio Red, while decrease was by 7.37% and 16.15% in Marsh White when the fruits were stored at 9 ºC and 24 ºC, respectively. The most pronounced decrease (30.43%) was noticed for paradisin A in Rio Red stored at 24 ºC. Bergamottin was relatively stable compound in both temperatures, with decrease of 4.14%, 12.16 % in Rio Red and 2.86% and 2.93% in Marsh White at 9 ºC and 24 ºC, respectively. In general, bergamottin was more stable during post -harvest storage followed by DHB and paradisin A.

Table 4.2 depicts the levels of three furocoumarins in three kinds of processed juice in containers such as cans, cardboard and cartoons. The levels of furocoumarins showed a decreasing trend in all three types of container as the storage time is extended. Levels of furocoumarins were higher in cartoon containers, compared to other containers. The levels of DHB, paradisin A and bergamottin were 2.065 ± 0.081, 0.099 ± 0.044 and 1.255 ± 0.028 µg/mL, respectively at the beginning of the study. After 90 days of storage levels of the furocoumarins decreased by 31.67%, 43.43% and 11.31% of DHB, paradisin A and bergamottin, respectively compared to the beginning of the season. The levels of

DHB, paradisin A and bergamottin in cardboard containers decreased by 33%, 57% and 32%, respectively over a period of 90 days. However, DHB, paradisin A and bergamottin were 24%, 32% and 35% lower over 60 days of storage in cartoons grapefruit juice. The juice in the cartoon container was made with pulp and stored under refrigerated conditions with maximum storage time of 60 days.

Wangensteen et al proposed that during commercial manufacturing of juice epoxybergamottin present in the grapefruit peel possibly distributed into the juice and by hydrolysis epoxybergamottin may convert to more potent CYP3A4 inhibitor, DHB *(86)*. However, we did not observe the increased levels of furocoumarins in the processed juice. Interestingly processed juice contains fewer amounts of DHB and bergamottin than hand squeezed juice. This fact is support by the study that, fresh squeezed grapefruit juice has been shown to increase the bioavailability of terfanadine by twofold compared to commercially processed juice *(17)*. Commercial grapefruit juice is manufactured in a multi-step process, which includes washing, puncturing of peel to get essential oils and squeezing of the whole fruit to juice. The juice is heated during pasteurization process and finally essential oil, an important constituent of grapefruit peel, is added as a flavor enhancer to the commercially produced grapefruit juice.

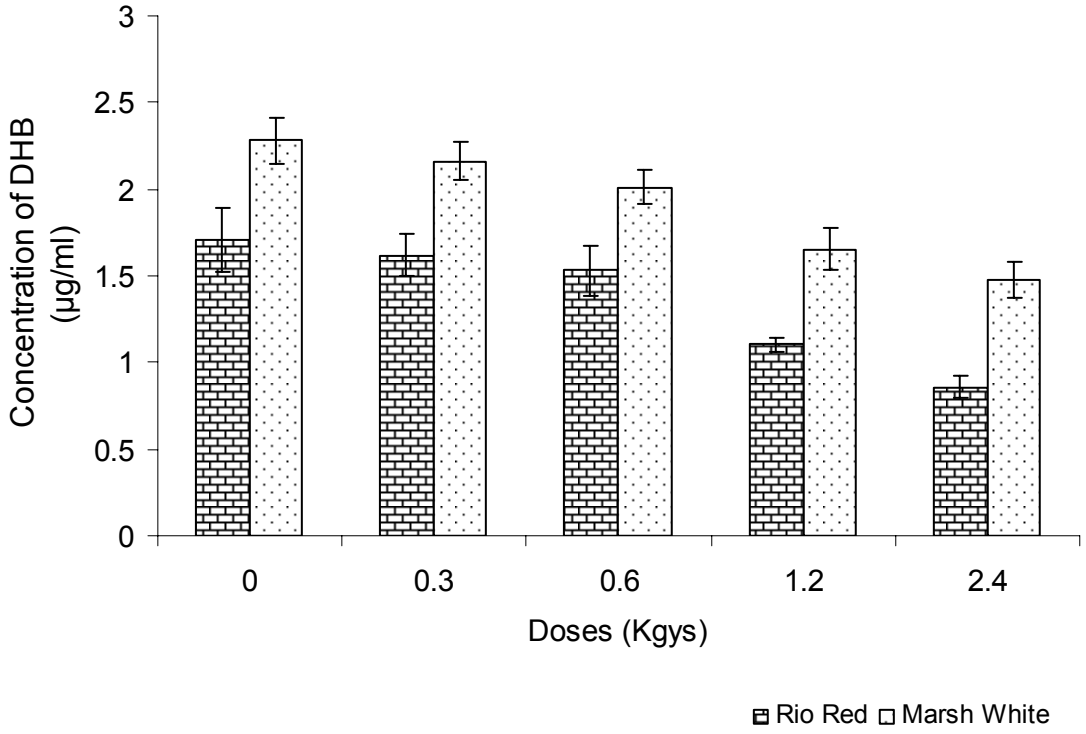


Figure 4.9. Effect of γ-irradiation on DHB levels of Rio Red and Marsh White grapefruit.

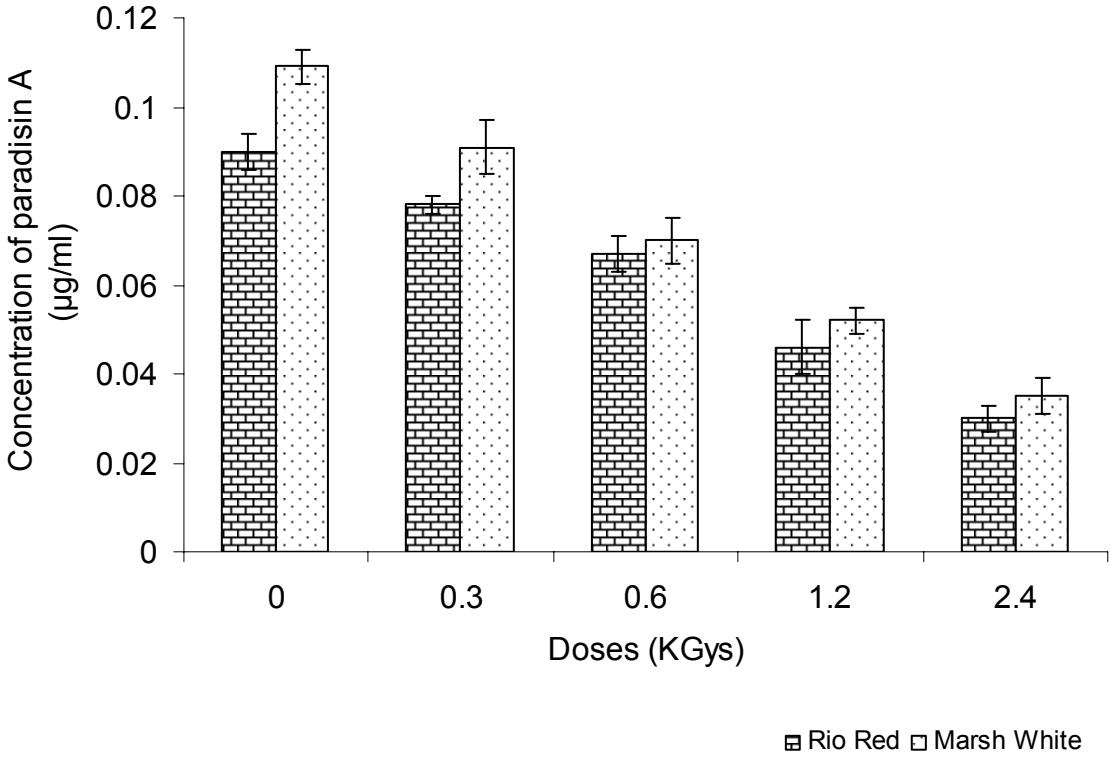


Figure 4.10. Effect of γ-irradiation on paradisin A levels of Rio Red and Marsh White grapefruit.

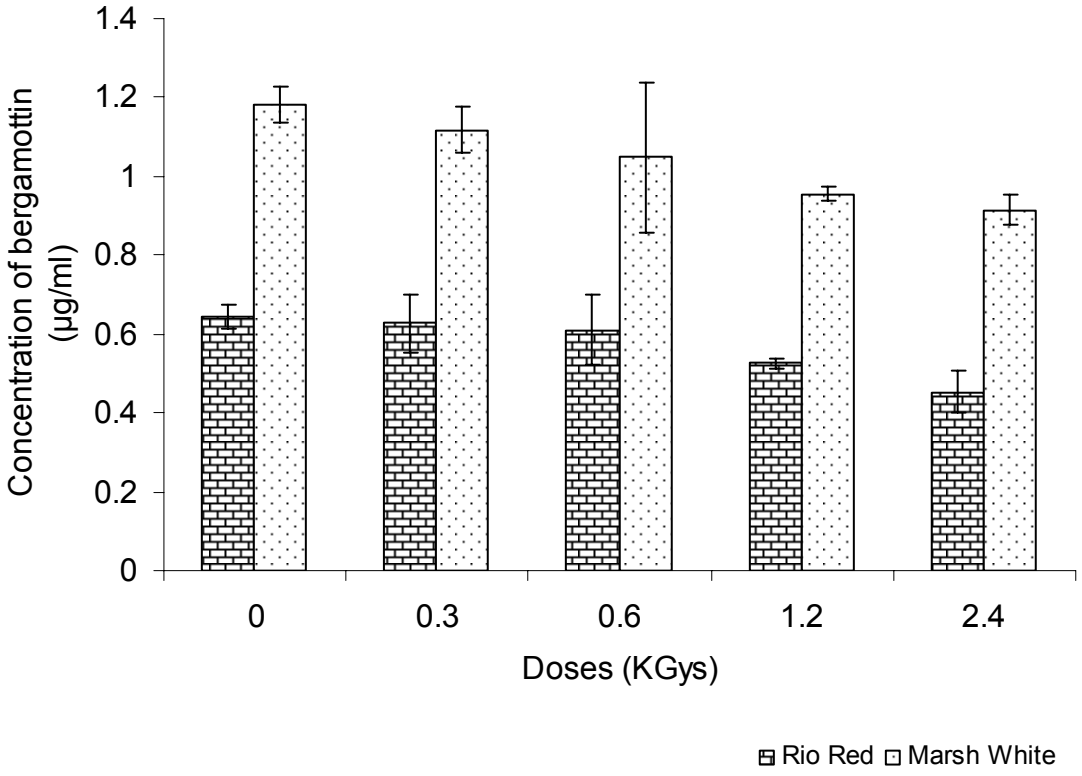


Figure 4.11. Effect of γ-irradiation on bergamottin levels of Rio Red and Marsh White grapefruit.

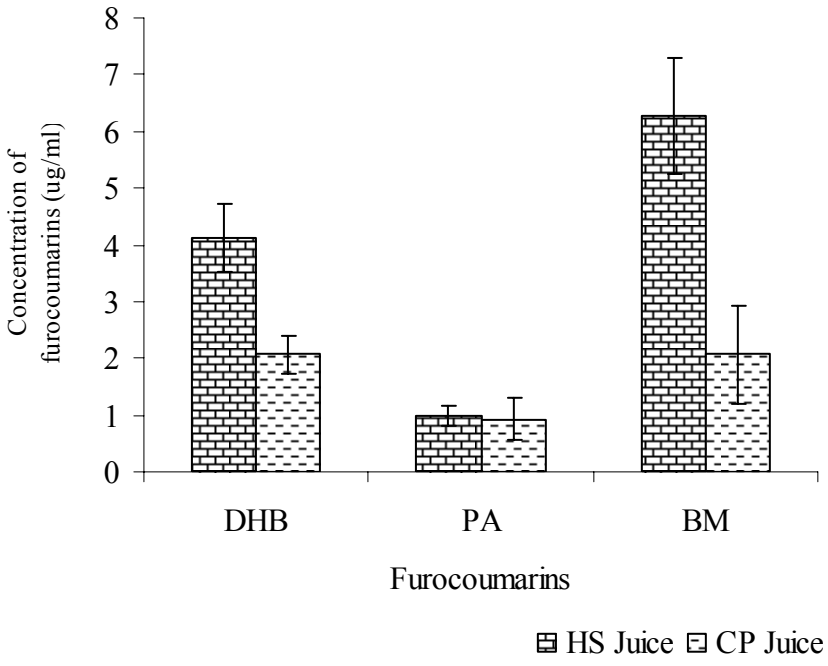


Figure 4.12. Levels of furocoumarins in hand squeezed (HS) juice and commercial processed (CP) juice. The results represent mean ± SD for 15 samples.

Table 4.1. Influence of temperatures during storage on the levels of furocoumarins in Rio Red and Marsh White grapefruits.

Grapefruit Cultivars	Storage Temperature	Days	Levels of Furocoumarins (µg/ml)		
			DHB	Paradisin A	Bergamottin
Rio Red	9 ºC	0	1.201 ± 0.061[a*]	0.09 ± 0.004 [a]	1.005 ± 0.049 [a]
		15	1.137 ± 0.078 [a]	0.088 ± 0.002 [a]	0.995 ± 0.062 [a]
		30	1.108 ± 0.069 [a]	0.087 ± 0.004 [a]	0.978 ± 0.028 [a]
		45	1.098 ± 0.068 [a]	0.076 ± 0.006 [b]	0.965 ± 0.37 [a]
	24 ºC	0	1.201 ± 0.061 [a]	0.09 ± 0.001[a]	1.005 ± 0.049 [a]
		15	1.091 ± 0.055 [a]	0.077 ± 0.004 [b]	0.909 ± 0.055 [b]
		30	0.998 ± 0.014 [b]	0.069 ± 0.007 [b]	0.896 ± 0.081 [b]
Marsh White	9 ºC	0	1.704 ± 0.043 [a]	0.092 ± 0.004 [a]	1.437 ± 0.038 [a]
		15	1.689 ± 0.076 [a]	0.089 ± 0.006 [a]	1.426 ± 0.04 [b]
		30	1.618 ± 0.091 [b]	0.086 ± 0.0052 [a]	1.408 ± 0.044 [c]
		45	1.587 ± 0.068 [c]	0.085 ± 0.003 [a]	1.397 ± 0.083 [d]
	24 ºC	0	1.704 ± 0.043 [a]	0.092 ± 0.005 [a]	1.437 ± 0.038 [a]
		15	1.593 ± 0.099 [d]	0.081 ± 0.003 [a]	1.406 ± 0.019 [e]
		30	1.467 ± 0.065 [e]	0.071 ± 0.002 [a]	1.396 ± 0.045 [f]

[*]Mean ± standard deviation, n=15.

[a]Means with a different letter, differ for the levels of the particular compound in the fruits significantly, $P < 0.05$.

Table 4.2. Influence of storage on the levels of furocoumarins in different grapefruit juice.

Type of Containers	Days	Levels of Furocoumarins (µg/ml)		
		DHB	Paradisin A	Bergamottin
Cans				
	0	2.065 ± 0.081[*a]	0.099 ± 0.004 [a]	1.255 ± 0.028 [a]
	15	1.985 ± 0.092 [b]	0.088 ± 0.003 [b]	1.223 ± 0.046 [b]
	30	1.885 ± 0.015 [c]	0.078 ± 0.003 [c]	1.213 ± 0.021 [c]
	45	1.772 ± 0.064 [d]	0.069 ± 0.004 [d]	1.193 ± 0.037 [d]
	60	1.598 ± 0.109 [e]	0.065 ± 0.015 [e]	1.166 ± 0.045 [e]
	75	1.499 ± 0.089 [f]	0.061± 0.028 [f]	1.142 ± 0.013 [f]
	90	1.411 ± 0.037 [g]	0.056 ± 0.007 [g]	1.1138 ± 0.049[g]
Cardboard				
	0	2.125 ± 0.073[a]	0.104 ± 0.008[a]	1.487 ± 0.071[a]
	15	1.985 ± 0.092[a]	0.097 ± 0.005[b]	1.354 ± 0.087[b]
	30	1.818 ± 0.108b	0.089 ± 0.001[c]	1.285 ± 0.051[c]
	45	1.789 ± 0.089[c]	0.076 ± 0.003[d]	1.116 ± 0.119 [d]
	60	1.5612 ± 0.089[d]	0.074 ± 0.006[e]	1.099 ± 0.19[e]
	75	1.4511 ± 0.061[e]	0.072 ± 0.002[f]	1.037 ± 0.017[f]
	90	1.4198 ± 0.183[f]	0.066 ± 0.004[g]	1.004 ± 0.105[g]
Cartons				
	0	2.3146 ± 0.091[a]	0.1009 ± 0.006[a]	1.8655 ± 0.091[a]
	15	2.1485 ± 0.092[b]	0.0981 ± 0.013[a]	1.6813 ± 0.086[b]
	30	1.9915 ± 0.105[c]	0.0883 ± 0.008[b]	1.4513 ± 0.107[c]
	45	1.8212 ± 0.064[d]	0.0769 ± 0.017[c]	1.3193 ± 0.045[d]
	60	1.7598 ± 0.109[e]	0.0685 ± 0.009[d]	1.2168 ± 0.021[e]

[*]Mean ± standard deviation, n=15.

[a]Means with a different letter, differ for the levels of the particular compound in the containers significantly, $P < 0.05$.

Finally, health benefits of bioactive compounds may not have any practical significance if the irradiation, storage and processing makes the fruits/juices unmarketable and unacceptable. Citrus fruits are an important source of Vitamin C in the human diet. Studies have shown that loss of vitamin C is minimal when citrus fruits were exposed to irradiation doses up to 1KGys *(113)*. Our study demonstrates that irradiation at 1KGys did not affect the Vitamin C content in Rio Red, but higher doses reduced it considerably. Vitamin C degradation can cause browning which leads to the problem of quality loss during high dose irradiation. Vitamin C degradation products react with amino acids leading to the formation of brown pigments; hydroxymethylfurfurol is one of the decomposition products of vitamin C and is a suggested precursor of brown pigments *(101)*. It is possible that, high dose of E-beam (10KGys) irradiated grapefruit received the lowest acceptability, due to decomposition of some of the bioactive compounds including vitamin C.

CHAPTER V

EVALUATION OF GRAPEFRUIT JUICE AND ITS FUROCOUMARINS ON CYTOCHROME P450 3A4, 2D6, AND 2C9 ISOENZYMES ACTIVITY AND P-GLYCOPROTEIN

Synopsis

Cytochrome P450 enzyme family is the most abundant and responsible for the metabolism of more than 60% of currently marketed drugs and is considered central in many clinically important drug interactions. Grapefruit juice increases the bioavailability of certain drugs mainly by inhibiting the cytochrome P450 enzymes and modulating the activity of transporter proteins. Seven different grapefruit juices and Pummelo juice as well as five furocoumarins isolated from grapefruit juice were evaluated at different concentrations on CYP3A4, CYP2C9 and CYP2D6 isoenzymes activity. Grapefruit and Pummelo juices were found to be potent inhibitors of cytochrome CYP3A4 and CYP2C9 isoenzymes at 5% concentration while CYP2D6 was scarcely affected. Among the five furocoumarins tested, the inhibitory potency was in the order of paradisin A>dihydroxybergamottin>bergamottin>bergaptol>geranylcoumarin at a range of 0.1 μM to 0.1 mM concentrations. The IC_{50} value was lowest for paradisin A for CYP3A4 with 0.11 μM followed by DHB for CYP2C9 with 1.58 μM.

Introduction

Cytochrome P450 enzymes are electron-transporting proteins that contain a heme-prosthetic group in which iron alternates between a reduced (ferrous, Fe^{2+}) and oxidized (ferric, Fe^{3+}) state *(114)*. In humans, cytochrome enzymes have been classified into 18

gene families with more than 50 enzymes in each and are mostly found on the endoplasmic reticulum. Individual isoenzymes are thought to be responsible for the metabolism of specific substrates. The cytochrome enzyme family is involved in the metabolism and detoxification of environmental carcinogens, drugs, steroids, bile acids, fatty acids, eicosanoids, and fat soluble vitamins *(115, 116)*. Certain cytochrome P450 isoenzymes, such as CYP3A4, CYP1A2, CYP1B1, CYP19, CYP32D6 and CYP32C9, have specific roles in the onset of several types of cancers *(117-120)*. Therefore, inhibition of cytochrome enzymes by naturally occurring compounds may represent a novel approach in the anti-carcinogenesis strategy *(116)*. Exposure to environmental chemicals is considered a major risk factor for several types of cancers. These exposures can result in the generation of reactive oxygen and nitrogen species such as singlet oxygen, super-oxide, nitrogen peroxynitrite and nitrogen dioxide *(121)*, which have been implicated as causative agents for many diseases including inflammatory and degenerative diseases, arthritis, retinitis pigmentosis, coronary artery diseases, and many types of cancers *(121)*. In humans, many of the chemicals are pro-carcinogens and are activated to carcinogenic and mutagenic substances by microsomal enzymes *(115, 121)*. The human microsomal enzyme system consists of scores of cytochrome P450 isoenzymes. In vitro studies with certain phytochemicals revealed a reduction in activities involved with generation of carcinogens through partial inhibition of these enzymes *(122-124)*.

Cytochrome P450 3A4, 2C9 and 2D6

Cytochrome P450 3A4 (CYP3A4) is the most abundant among all the cytochrome P450 enzymes. It is expressed in human liver and small intestine and also in some extra-hepatic tissues such as lungs, stomach, colon and adrenal. In fetal liver, P450 3A7 is the most abundant form of CYP3A4 *(125)*. Members of the CYP3A subfamily are the most abundant cytochrome enzymes in humans, accounting for 30% of total cytochrome in liver and 70% of those in the gut *(126)*. CYP3A family enzymes are inducible by barbiturates, rifampicin, dexamethasone, and others factors. A general correlation was shown between enzymes and mRNA levels in human livers; CYP3A4 is probably degraded by an ubiquitin linked pathway *(125)*. CYP3A4 contributes to the metabolism of approximately 50% of the drugs marketed or under development. Many of these are important drugs such as lovastatin, statins, prostate hypertrophy inhibitor, immunosuppressants, protease inhibitors and sildenafil *(125)*. This 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. CYP3A4 is also involved in the metabolism of cancer chemotherapeutic drugs and activation of carcinogens. Elevated enzyme levels reduce the bioavailability and variations in the CYP3A4 levels pose clinical challenge, when therapeutic window is narrow for a given drug *(125)*.

Cytochrome P450 2C9 is primarily a hepatic P450 but also expressed in small intestine and the levels of expression is next highest to CYP3A4. This is one of the major enzymes involved in the metabolism of drugs. This enzyme is induced by barbiturates and rifampicin and selectively inhibited by sulfaphenazole *(125)*. Cytochrome P450 2D6

is the first enzyme recognized in xenobiotics –metabolizing P450 enzyme. This enzyme expressed mainly in liver, lung and brain, accounts for approximately 5% of the total P450s, wide variation. However this enzyme accounts for oxidation of approximately 25% of the drugs metabolized by cytochrome P450 enzymes. Available information indicates that CYP2D6 is not inducible, rather expressed constitutively *(127)*. Wide variability in the activity of CYP2D6 is mainly due to the genetic variability.

Cytochrome P450 enzymes are the major drug metabolism enzymes and some of the main components of phase I metabolism. The cytochrome P450 enzyme system transforms lipophilic drugs to more polar compounds that can be excreted in urine *(126)*. The metabolites are generally less active than the parent compound, but in some cases the metabolites can be toxic, carcinogenic or teratogenic *(125)*. Induction and inhibition *(125)* are the most common causes of altered drug biotransformation during drug-drug and food-drug interaction. The effect of limonoids and flavonoids in inducing glutathione S-transferase, a major phase II detoxifying enzyme and inhibition of certain CYP enzymes activity is well documented *(128, 129)*. Unique bioactive compounds, which inhibit CYP enzyme, are furocoumarins from the grapefruit juice, which have been interfering with certain drugs by inhibiting CYP3A4 and interfering with transporter activity of membrane transporters. Bergamottin, a major component of grapefruit juice inhibited the activities of several cytochrome isoenzymes and inactivated CYP3A4 in a time- and concentration-dependent manner, via reactive furano-epoxide that covalently binds to CYP3A4 *(130)*.

P-glycoprotein

P-glycoprotein is a 170 KDa, membrane-localized efflux protein, which belongs to the ATP-binding cassette (ABC) super-family of transporters. This protein actively pumps out xenobiotics, including drugs from intercellular cytoplasm *(131)*, which may result in limited bioavailability of drugs. P-glycoprotein appears to be part of a mechanism to protect the body from harmful substances. It is the part of first-pass metabolism, acting as "gate keeper" for absorption of drugs into the systemic circulation. It has been implicated as a primary cause of multi-drug resistance in tumors *(132)*.

The objective of this study was to evaluate the effect of different grapefruit juices and isolated furocoumarins from grapefruit juice on the activity of CYP3A4, 2D6 and 2C9 isoenzymes and also on P-glycoprotein.

Materials and Methods

Juices and Furocoumarins. The compounds were isolated from grapefruit juice and grapefruit peel oil as described in the previous chapter. The pure compounds and grapefruit were used to evaluate the inhibitory effects on CYP3A4, CYP2C9, and CYP2D6 enzymes. Rio Red, Ruby Red, Ray Red, Star Ruby, Thompson Pink, Marsh White, Duncan and Pummelo grapefruits were collected from Texas A&M University Kingsville, Weslaco TX. Fruits were squeezed and juice was centrifuged at 4000 rpm, the supernatant was decanted and stored at -80 °C until used. Various concentrations of juices starting from 1% to 80% were prepared by diluting in millipore water. Stock solutions (50 mM) of DHB, paradisin A, bergamottin, bergaptol and geranylcoumarin were prepared in acetonitrile and kept at 4 °C. Working solutions of 0.01 to 1000 μM

solutions were prepared from the stock solution before starting the CYP3A4 inhibition assay experiment.

CYP3A4, CYP2C9, CYP2D6 and Substrates. Membrane preparation containing recombinant human CYP3A4, CYP2C9, and CYP2D6 (expressed from cDNA using a baculovirus expression system), specific cytochrome enzyme substrates luciferin 6' benzyl ether, 6' deoxyluciferin and ethyl glycol ester of luciferin 6' methyl ether, assay mixtures and luciferin were purchased from Promega (Promega Inc., Madison WI). Ketoconazol, sulfaphenazole and quinidine were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Luis, MO).

P-glycoprotein and Substrates. Membrane preparation containing recombinant human P-glycoprotein, verapamil and sodium orthovanadate, and assay mixtures were purchased from Promega (Promega Inc., Madison WI, USA).

CYP3A4, CYP2C9 and CYP2D6 Inhibition Assay. Inhibition assays were performed to evaluate the effect of grapefruit juices and furocoumarins on CYP3A4, CYP2C9, and CYP2D6 activity. Inhibition assays were performed as follows, to a 96-well microtiter plate 12.5 µl of luciferin free water (positive control) or ketoconazol/sulfaphenazole/quinidine (positive control for inhibition) and test compounds were added to the appropriate wells at 4X concentrations. Thawed, 12.5 µl of control reaction mixture and membrane preparations containing CYP3A4/2C9/2D6 were added at 4X concentration to the respective wells. The reaction mixture was mixed by shaking the plate and plate was pre-incubated at 37 ^{o}C for 10 minutes. The CYP3A4/2C9/2D6 assay reaction was started by adding 25 µl of 2X NADPH

regeneration systems to all the wells. The reaction mixture was mixed by shaking the plate and plate was incubated at 37 ^{o}C for 30 minutes. After 30 minutes 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 ^{o}C for 20 minutes. The luminescence was recorded using a plate-reading luminometer in terms of relative light units (RLU).

P-gp Activity Assay. To determine the effect of grapefruit juices and furocoumarins P-gp activity, assay was performed as follows. To a 96 well microtiter plate, 20 µl of P-gp-Glo assay buffer/20µl 0f 0.25mM Na_3VO_4 in assay buffer/20 µl of 0.5 mM verapamil in assay buffer/20µl of 2.5X of test compounds were added to control/sodium ortho-vanadate/ATP standard/verapamil/test compound wells, respectively. To each well, 20 µl of diluted P-gp membranes were added. The mixture was mixed by shaking the plate and plate was incubated at 37 ^{o}C for 5 minutes. The reaction was initiated by adding 10 µl of 25mM MgATP to all wells except those for ATP standards. Reaction mixture was mixed briefly on a plate shaker. The plate was incubated at 37 ^{o}C for 40 minutes. ATP standards of 10 µl were added to appropriate wells for ATP standard curve, at the end of 40 minutes incubation. After incubation, the reaction was stopped and luminescence was initiated by adding 50 µl of ATP detection reagent to all the wells. The reaction mixture was mixed briefly by shaking the plate, then the plate was incubated for 20 minutes at room temperature to develop luminescent signal. The luminescence was recorded using a plate-reading luminometer interms of relative light units (RLU).

Statistical Analysis. The inhibition was calculated on the basis of the relative activity of the positive control. The positive control was chosen as 100%. The percent inhibition was calculated from the formula 100 – [(relative AI activity/relative activity of positive control) X 100]. IC_{50} values were calculated using GraphPad Prism version 4.03 for Windows, (GraphePad Software, San Diego California, USA).

Results and Discussion

Inhibition of in vitro CYP3A4/2C9/2D6 Activity by Grapefruit and Pummelo Juices. Results of CYP3A4/2C9/2D6 isoenzymes inhibition by Rio Red, Ruby Red, Ray Red, Star Ruby, Thompson Pink, Marsh White, Duncan and Pummelo juices at 1%, 5% and 25% concentrations are summarized in **Figure 5.1 5.2** and **5.3** Differential Inhibition of cytochrome isoenzymes was demonstrated by grapefruit juices and Pummelo juice. Among the three enzymes, CYP2C9 was inhibited 48.12% by Ray Red and 73.88% by Pummelo juice at 1% concentration, while CYP2D6 was inhibited the least with maximum inhibition of 12.70% by Pummelo juice. However, maximum inhibition was at 10% and 25% concentration and observed in the range of 96.69% to 99.88% and 96.51% and 100% for CYP3A4 and CYP2C9, respectively. Inhibition of CYP2D6 at 10% was comparable with inhibition of CYP3A4 and CYP2C9 at 1%, while CYP2D6 inhibition was in the range of 73.20% to 89.99% at 25% of juice. The order of inhibition was found to be CYP2C9 > CYP3A4 > CYP2D6 at all the concentrations.

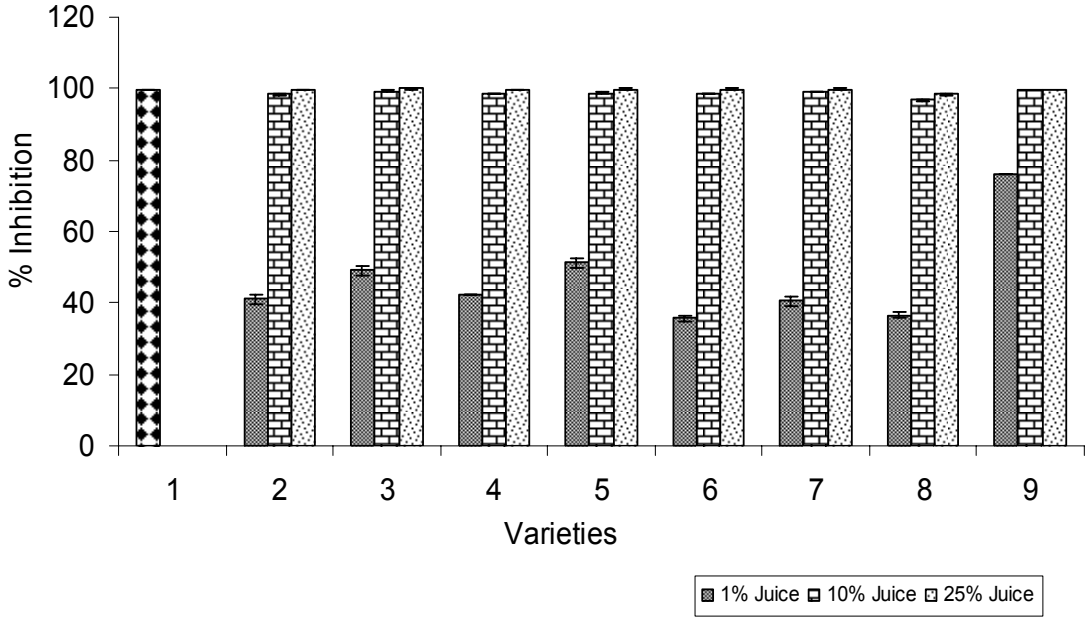


Figure 5.1 Inhibition of CYP3A4 activity by grapefruit and Pummelo juices. (1) ketoconazol (2) Rio Red grapefruit juice, (3) Ruby Red grapefruit juice, (4) Ray Red grapefruit juice, (5) Star Ruby grapefruit juice, (6) Thompson Pink grapefruit juice, (7) Marsh White grapefruit juice, (8) Duncan grapefruit juice and (9) Pummelo juices at 1%, 10% and 25% concentrations. Values are mean ± SD, n = 3.

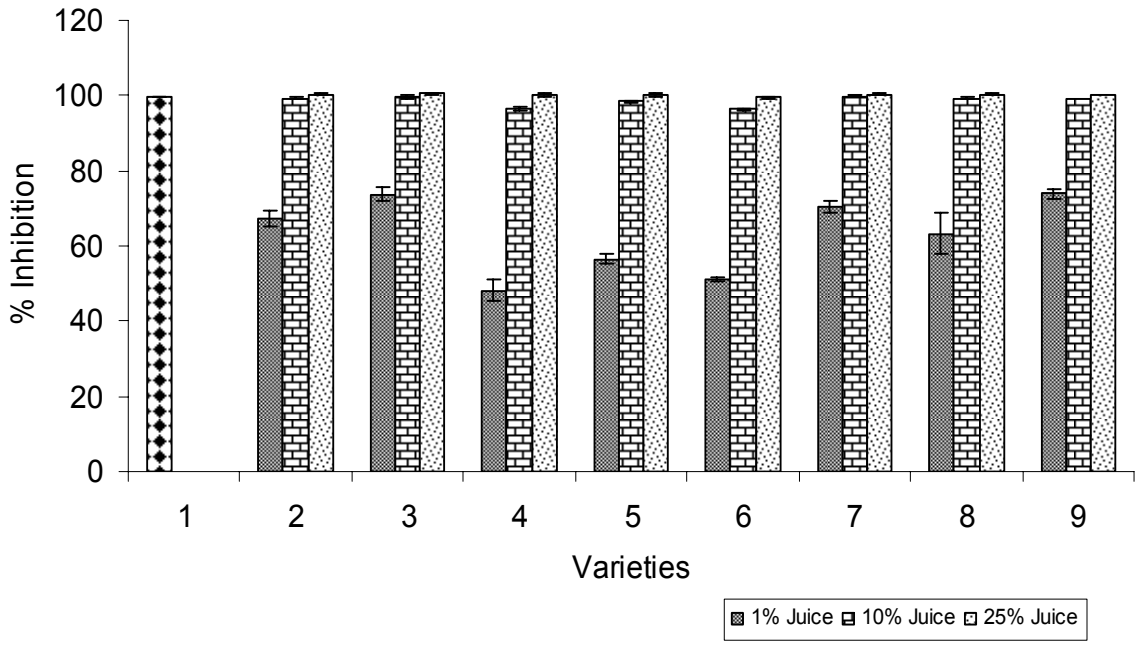


Figure 5.2 Inhibition of CYP2C9 activity by grapefruit and Pummelo juices. (1) sulfaphenazole, a known CYP2C9 inhibitor at 10 μM concentration, (2) Rio Red grapefruit juice, (3) Ruby Red grapefruit juice, (4) Ray Red grapefruit juice, (5) Star Ruby grapefruit juice, (6) Thompson Pink grapefruit juice, (7) Marsh White grapefruit juice, (8) Duncan grapefruit juice and (9) Pummelo juices at 1%, 10% and 25% concentrations. Values are mean ± SD, n = 3.

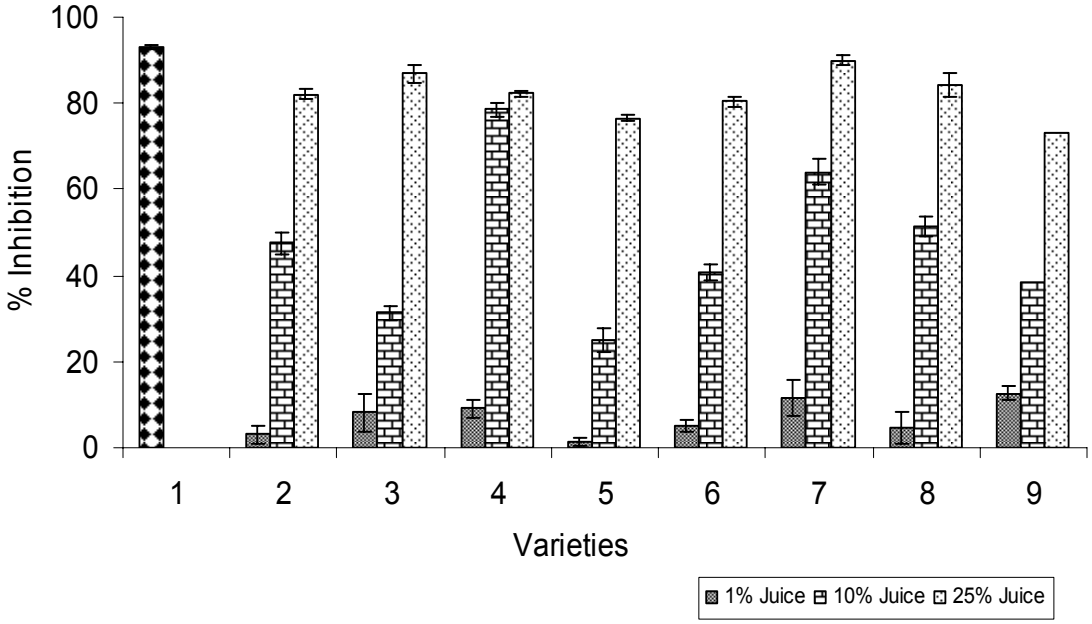


Figure 5.3 Inhibition of CYP2D6 activity by grapefruit and Pummelo juices. (1) quinidine, a known CYP2D6 inhibitor at 1 μM concentration, (2) Rio Red grapefruit juice, (3) Ruby Red grapefruit juice, (4) Ray Red grapefruit juice, (5) Star Ruby grapefruit juice, (6) Thompson Pink grapefruit juice, (7) Marsh White grapefruit juice, (8) Duncan grapefruit juice and (9) Pummelo juices at 1%, 10% and 25% concentrations. Values are mean ± SD, n = 3.

Inhibition of CYP3A4/2C9/2D6 Activities by the Isolated Furocoumarins. Various furocoumarins such as dihydroxybergamottin, paradisin A, bergamottin, bergaptol and geranylcoumarin were isolated from the grapefruit and results of CYP3A4/2C9/2D6 isoenzymes inhibition by at various concentrations are summarized in **Figure 5.4, 5.5** and **5.6.** Among the furocoumarins, paradisin A was the potent inhibitor of all the isoenzymes, followed by DHB, bergamottin, bergaptol and geranylcoumarin. CYP3A4 was inhibited almost completely by paradisin A at 10 μM and inhibition potential was more than the positive control ketoconazol, while DHB, bergamottin, bergaptol and geranylcoumarin inhibited >96% of activity at 100 μM. IC_{50} values for all three isoenzymes for dihydroxybergamottin, paradisin A, bergamottin, bergaptol and geranylcoumarin are presented in **Table 5.1.** Among all the furocoumarins the lowest IC_{50} was observed for paradisin A against CYP3A4 followed by CYP2C9 and CYP2D6. IC_{50} for bergamottin and geranylcoumarin against CYP2C9 was almost 10 times less than that of CYP3A4. The IC_{50} values for furocoumarins were in the order of geranylcoumarin > bergaptol > bergamottin > DHB > paradisin A.

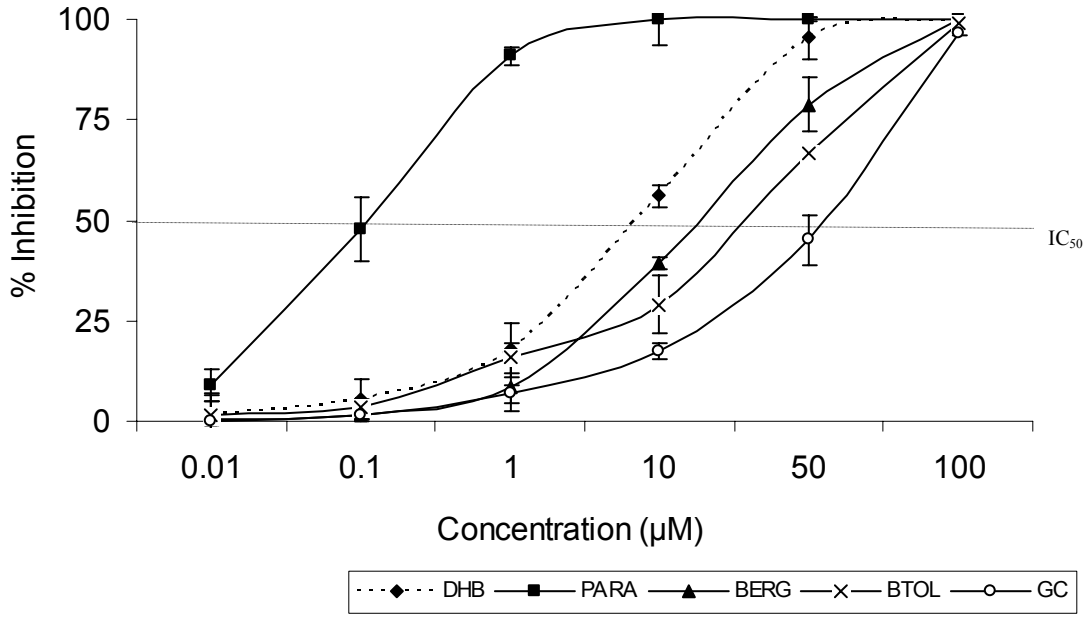


Figure 5.4 Inhibition of CYP3A4 activity by furocoumarins. Abbreviations for compounds are as follows DHB (Dihydroxybergamottin), PARA (Paradisin A), BERG (Bergamottin), BTOL (Bergaptol) and GC (Geranylcoumarin). Values are mean ± SD, n=3.

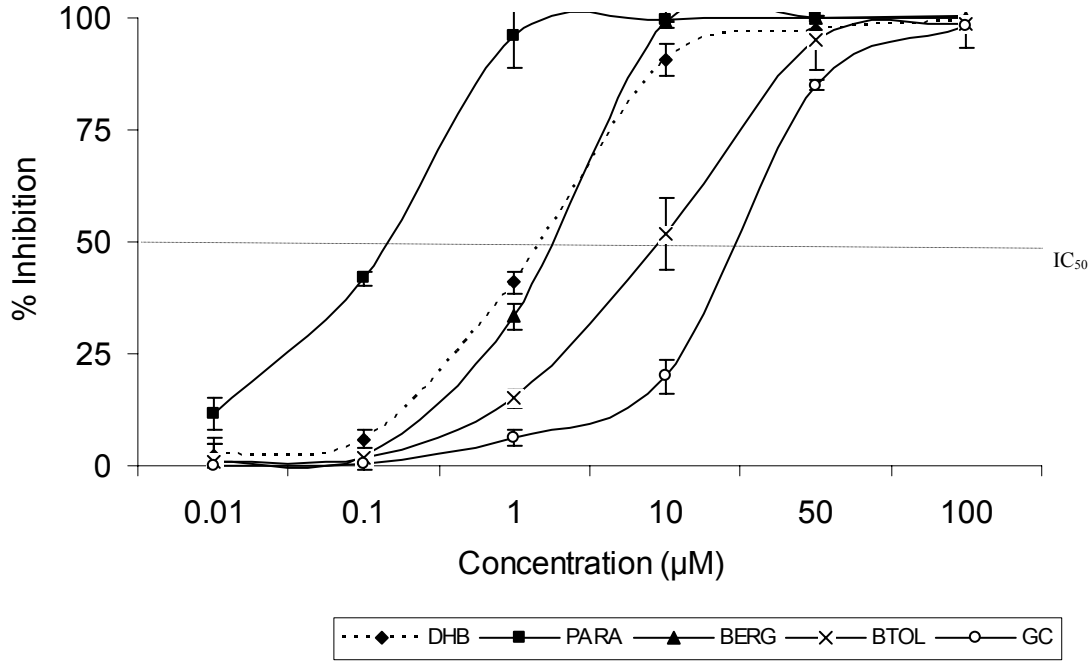


Figure 5.5 Inhibition of CYP2C9 activity by furocoumarins. Abbreviations for compounds are as follows DHB (Dihydroxybergamottin), PARA (Paradisin A), BERG (Bergamottin), BTOL (Bergaptol) and GC (Geranylcoumarin). Values are mean ± SD, n=3.

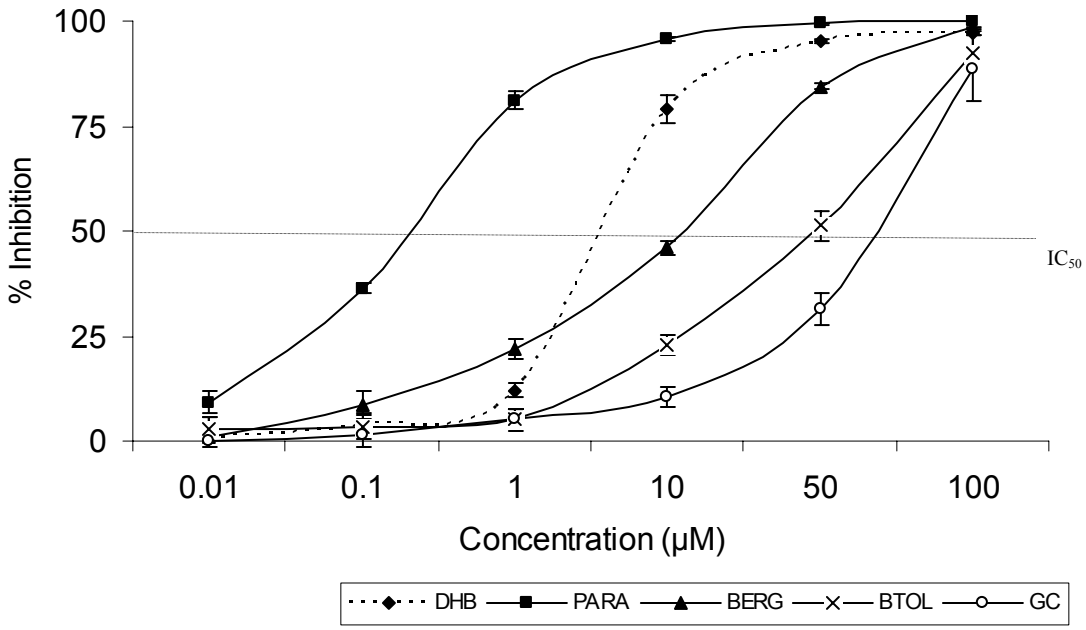


Figure 5.6 Inhibition of CYP2D6 activity by furocoumarins. Abbreviations for compounds are as follows DHB (Dihydroxybergamottin), PARA (Paradisin A), BERG (Bergamottin), BTOL (Bergaptol) and GC (Geranylcoumarin). Values are mean ± SD, n=3.

Table 5.1 IC_{50} values of DHB, paradisin A, bergamottin, bergaptol and geranylcoumarin for CYP3A4, CYP2C9 and CYP2D6 enzymes.

Compounds	**CYP3A4**	**CYP2C9**	**CYP2D6**
DHB	9.77	1.58	5.63
Paradisin	0.11	0.18	0.30
Bergamottin	22.91	4.50	11.74
Bergaptol	25.82	9.92	37.33
Geranylcoumarin	53.47	21.51	56.21

Effects of Grapefruit Juices and Furocoumarins on P-glycoprotein Activity. Effects of grapefruit juices and furocoumarins on P-glycoprotein activity are summarized in **Table 5.2.** Both Rio Red and commercial pink grapefruit juices increased the ATPase activity of P-gp in concentration dependent manner at 1% and 5% concentrations. Among the two juices, commercial pink grapefruit juice increased the activity of ATPase more than fresh Rio Red grapefruit juice. All five furocoumarins increased the ATPase activity of P-gp. The highest activity was observed with geranylcoumarin at 10 μM while lowest was observed with bergaptol at 1 μM concentration.

Cytochrome P450 is the single most important drug-metabolizing enzyme family. Among the isoenzymes, CYP3A, CYP2D and CYP2C are responsible for metabolism of 50%, 25% and 20% of drugs metabolized by cytochrome enzyme family, respectively *(59)*. Most lipophilic drugs are either metabolized by cytochrome P450 or pumped back into gut lumen by the P-glycoprotein transporter *(133)*. Thus, cytochrome P450 and P-glycoprotein act in tandem as a barrier to the oral delivery of many drugs *(134)*. Medications such as cyclosporine, ketoconazole erythromycin, itraconozole and diltizem inhibit both intestinal and hepatic CYP3A4 reducing pre-systemic drug metabolism, resulting in an increase in the oral bioavailability of the absorbed drug *(135-137)*.

Table 5.2 Effect of furocoumarins and grapefruit juice on P-glycoprotein ATPase activity.

Compounds	1 μM	10 μM
DHB	1.331 ± 0.262	1.509 ± 0.195
Bergamottin	1.242 ± 0.245	1.428 ± 0.134
Bergaptol	1.098 ± 0.171	1.890 ± 0.308
Geranylcoumarin	1.338 ± 0.347	2.599 ± 0.254
Juices	1%	5%
Rio Red	2.410 ± 0.081	3.628 ± 0.100
Commercial (Pink juice)	3.334 ± 0.105	4.074 ± 0.133
Controls/Standards		
Positive control	1.000 ± 0.152	
Varapamil (0.125 mM)	1.735 ± 0.082	
Na_3VO_4 (0.25 mM)	0 ± 0.190	

Co-administration of grapefruit juice with drugs including a number of dihydropyridine calcium channel blockers, cyclosporine, midazolam, triazolam and terfanadine drugs resulted in substantial increase in their oral bioavailability, with possible unintended reactions such as headaches, hypotension, facial flushing and lightheadedness *(138)*. Grapefruit juice increases the bioavailability of drugs mainly by inhibiting the first pass metabolism by cytochrome P450 isoenzymes. Grapefruit juice contains several bioactive compounds such as flavonoids, limonoids and coumarins. Some of the flavonoid aglycones such as naringenin, apigenin, hesperetin, quercetin and kaempferol are reported to inhibit microsomal CYP3A mediated oxidation of drugs in rat and human livers *(139)*. Initial investigations considered naringin and its aglycone naringenin as possible compounds responsible for drug interactions *(140)*. Nevertheless, when naringin was co-administered with felodipine in vivo, it did not show a clear influence on the pharmacokinetics of the drug *(140)*. Therefore, the activity of flavonoids on CYP3A4 in vivo remains unclear. Grapefruit juice contains several furocoumarins and its derivatives which are considered as possible candidates for drug interactions. Several furocoumarins have been reported from grapefruit juice with differential inhibitory effect on CYP P450 enzymes. Bergamottin and 6', 7' –dihydroxybergamottin are the major furocoumarins, whereas paradisin A and paradisin B are 100 times stronger inhibitors of microsomal CYP3A4 *(42)*. Interestingly, grapefruit juice has no effect on the bioavailability of drugs when the drugs are administered intravenously, suggesting the involvement of intestinal CYP3A inhibition, not the hepatic CYP 3A inhibition as the major cause for increased oral bioavailability *(49)*.

Variation in the IC_{50} values for different compounds and for different isoenzymes from this study may be ascribed to the affinity of furocoumarins towards formation of enzyme- inhibitor complex. Interestingly, IC_{50} values for furocoumarins are less than that of flavonoids and limonoids *(42)*. It is possible that furocoumarins are more non polar than flavonoids and may easily move towards the non-polar membrane environment where the majority of cytochrome P450 enzymes and transporter proteins are localized in the cells. Structural-functional variations in different furocoumarin molecules may be attributed to the differences in efficacy, in inhibiting the different cytochrome isoenzymes *(141)*. Bergamottin-induced inactivation led to the loss of up to 50% of CYP3A4 apoprotein, perhaps via apoprotein modification in the active site of the enzyme. Metabolites of bergamottin may undergo oxidation to form a reactive furanoepoxide that covalently binds to CYP3A4. Grapefruit juice seems to interact with P-glycoprotein as P-glycoprotein and CYP3A have overlapping substrate specificities. Soldener and co-workers discovered that furocoumarins shown to activate P-glycoprotein in intestinal cell monolayer in vitro resulting in an increased activity of P-glycoprotein *(142)*.

Inhibition of cytochrome P450 enzymes by grapefruit juices and its furocoumarins offers some clinical advantages in improving bioavailability of poorly absorbed drugs, such properties could help reduce the dose requirements and decrease the cost and harnessing the health benefits of consuming grapefruit juice. In addition, grapefruit juice bioactive components may act as potent inhibitor of cytochrome P450 enzymes which are involved in the activation pro-carcinogen to carcinogen. This represents a unique mechanism in the anti-carcinogenesis strategy, part of which includes reducing the generation of reactive oxygen species.

CHAPTER VI

EVALUATION OF GRAPEFRUIT JUICE AND ITS FUROCOUMARINS ON AUTO-INDUCER SIGNALING AND BIOFILM FORMATION IN PATHOGENIC BACTERIA

Synopsis

In the present study natural furocoumarins from grapefruit juice were evaluated for anti-quorum sensing activity and found the potent inhibition of AI-I and AI-2 activities at concentrations as low as 0.01%. Grapefruit juices at 5% concentration inhibited >99% of AI-1 and AI-2 activities. These results suggest that grapefruit juice can serve as a source of alternatives to the halogenated furanones to develop strategies targeted at microbial quorum sensing.

Introduction

The discovery of quorum sensing in bacteria opened another avenue to control microbial infections *(143)*. Quorum sensing appears to play a key role in the gene expression related to microbial infections and food spoilage *(144)*. In quorum sensing, small signaling molecules called auto-inducers (AI) mediate the ability to sense the size of a bacterial population *(145)*. Auto-inducers are constantly produced and received at a basal level by bacterial cells. Studies have shown that two auto-inducer systems, *N*-acylhomoserine lactone (AI-1) and a furanosyl borate diester molecule (AI-2) are involved in quorum sensing signaling in *Vibrio harveyi (146, 147)*. These molecules

interact with a transcriptional regulator, the LuxR homologues, to activate the expression of genes involved in the production of luminescence in *Vibrio* species *(148, 149)*. In other bacteria, these signaling molecules are also involved in regulation of virulence factors *(148, 150)*, antibiotic production and sporulation *(151)*.

Quorum sensing plays a major role in gene regulation in many environments and pathogens rely on these communication systems to promote infection *(144)*. Bacteria use auto-inducer molecules to communicate between each other and form structured, sessile communities or biofilms *(152)*. Bacterial biofilm formation is a trait closely related to pathogenicity *(144, 153)*. According to NIH report, biofilms associated with over 80% of all the microbial infections causing urinary tract infections, catheter infections, biliary tract infections, formation of dental plaque, gingivitis and lethal infections such as cystic fibrosis *(154)*. These communities develop structures that are morphologically and physiologically differentiated from free living bacteria and can survive antibiotic treatment and are often responsible for medical treatment failures *(155)*. Recently, there has been an increased research work aiming at prevention, control and eradication of biofilms *(156)* and one particular approach is the interruption or inhibition of the bacterial

quorum sensing. Several types of synthetic acylhomoserine lactones (AHL) analogues have been identified as quorum sensing inhibitors *(157)*. Synthetic halogenated furanones have been shown to inhibit bacterial quorum sensing in *P. aeruginosa* and exhibited favorable therapeutic effects on its infection *(157)*. However, halogenated furanone compounds are unstable and unsuitable for human use. Identification of naturally occurring compounds that interfere with intra- and inter-species cell-to-cell communication could provide new treatment strategies to prevent infections *(158)* and can be used as a safe alternative to the halogenated furanones. Furocoumarins and synthetic halogenated furanones share common structural "furan" moiety with AI signaling molecules *(159)*, which may interfere with the natural AI signaling system **(Figure 6.1)**. In the light of the above fact, in the present study we evaluated anti-quorum sensing properties of grapefruit juice and its bioactive furocoumarins, and also their effects on growth rate and biofilm formation in *Escherichia coli, Salmonella* Typhimurium and *Pseudomonas aeruginosa*.

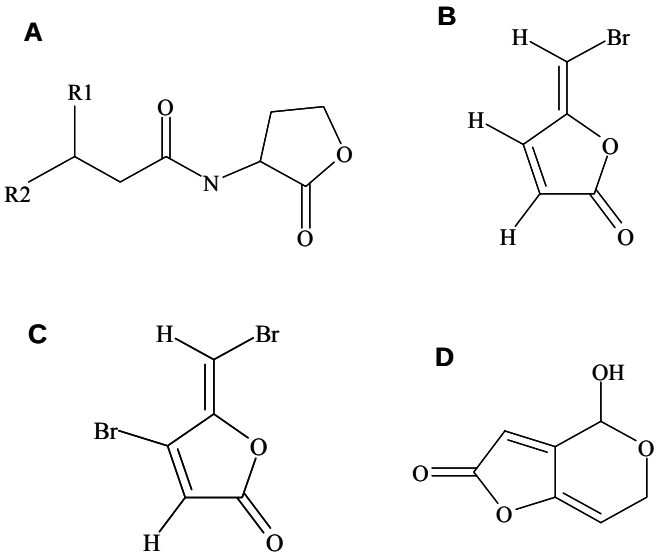


Figure 6.1. Structure of quorum sensing signal molecules. A) N-acyl homoserine lactones where R1 = H, OH, or O and R2 = C_1-C_{18} and synthetic furanone compounds B) furanone C30, C) furanone C56, and D) penicillic acid produced by fungi. Adopted from *(153, 157, 160).*

Materials and Methods

Preparation of Grapefruit Juices, Grapefruit Peel Oils and Furocoumarins. Rio Red and Marsh White grapefruits were harvested in the month of February 2006 from an orchard at the Texas A&M University-Kingsville Citrus Center Weslaco, Texas. Fruits were juiced in a home blender and juice was centrifuged at 4000 RPM to separate the pulp. The supernatant was stored at -80 ºC until they were used for study. Commercial grapefruit juice and grapefruit peel oils were obtained from Texas Citrus Exchange Mission, TX. Commercial grapefruit juice was pulp-free, made from concentrate grapefruit juice. Dihydroxybergamottin (DHB), paradisin A, bergamottin and bergaptol were isolated from grapefruit juice with modifications as described in chapter II. The purified furocoumarins were weighed and dissolved in DMSO to get 10 mg/ml stock solution. All the four furocoumarins were used at 0.01% concentration assays. However, juices and grapefruit peel oil were used at 1% and 5% concentrations.

Bacterial Strains and Growth Conditions. Strains of *Vibrio harveyi* BB120, BB886 and BB170 were used for bioluminescence assays. Wild type strain BB120 (AI-1+ and AI-2+) was used as a positive control for production of AI-1. Reporter strains BB886 (*luxP*::Tn5) and BB170 (*luxN*::Tn5) were used for detection of AI-1 and AI-2 respectively (Surette et al., 1999; Lu et al., 2004). All these strains were kindly provided by B. Bassler (Princeton University, Princeton, NJ) and were grown at 30 ºC in autoinducer bioassay (AB) medium prepared as described previously (Federle and Bassler, 2003). A solution consisting of NaCl (17.5 g/liter; Sigma, St. Luis, MO), $MgSO_4$ (12.3 g/liter; Fisher Chemicals, Fisher Scientific, Fair Lawn, NJ), and casamino acids (2 g/liter; Fisher Chemicals) was adjusted to pH 7.5 and sterilized by autoclaving.

When the solution had cooled, autoclave–sterilized 1.0 M potassium phosphate (pH 7.0, 10 ml/liter; Sigma), 50% glycerol (20 ml/liter; EM Science, Gibbstown, NJ) and filter sterilized 0.1 M L-arginine (10 ml/liter; Sigma) were added. The environmental isolate *E. coli* #5, used as positive control for production of AI-2 bioassay was grown at 37ºC on Luria-Bertani (LB) - Miller broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose *(161)*.

Preparation of Cell Free Supernatant (CFS) for AI-1 and AI-2 Assays. To prepare cell free supernatant containing high levels of AI-1, an aliquot (100 µl) of an overnight culture of *V. harveyi* BB120 was inoculated into 10 ml of fresh AB medium and incubated with shaking at 30ºC for 16 hours. To prepare cell free supernatant containing high levels of AI-2, an aliquot (100 µl) of an overnight culture of *E. coli* #5 (strain known to produce AI-2 molecules) *(161)* was inoculated into 10 ml of fresh LB supplemented with 0.5% glucose medium and incubated with shaking at 30ºC for 16 hours. The CFS was prepared by centrifuging (3300× g for 30 min at 4°C) the culture followed by filtration of the supernatant through 0.2 µm-pore size syringe filters (VWR, West Chester, PA). The CFS was stored at –20°C until AI-1 and AI-2 bioluminescence assays were performed.

Bioluminescence Assay for AI-1 and IA-2. To determine the effect of furocoumarins, grapefruit juices and grapefruit peel oil on AI-1 and AI-2 activity, bioluminescence assays were performed as described previously *(161)*. An overnight culture of *V. harveyi* BB886/BB170 was diluted with fresh AB medium (1:5,000) and 90 µl were added to each well of a 96 well microtiter plate (Perkin-Elmer –Wallac, Boston, MA). To test the inhibition of AI-1/AI-2 activity, 5 µl of *V. harveyi* BB120/*E. coli* #5

CFS was mixed with 4 µl of AB medium plus 1 µl of the different compounds dissolved in DMSO. This mixture was added to the wells containing 90 µl of the diluted (1:5,000) *V. harveyi* BB886/BB170 culture. The positive control consisted of 90 µl of diluted *V. harveyi* BB886/BB170 culture and 5 µl of *V. harveyi* BB120/*E. coli* #5 CFS mixed with 5 µl of AB media. To test if DMSO has any effect on the reporter strain, control wells were prepared with 90 µl of diluted *V. harveyi* culture, 5 µl of CFS (BB120/*E. coli* #5) and 4 µl of AB media with 1 µl DMSO. The negative control consisted of 90 µl of diluted *V. harveyi* BB886/BB170 culture and 9 µl of AB medium with 1 µl DMSO. The plate was shake-incubated (100 rpm) at 30 °C in a Lab-Line Orbital Shaker Incubator (Melrose Park, IL) for 4 hours; afterwards luminescence readings were recorded for every 20 minutes using Wallac Victor 2 luminomter (Perkin Elmer, Boston, MA). The readings were taken until the relative light units in the negative control wells reached approximately 100 light units.

Effect of Furocoumarins, Grapefruit Juice and Grapefruit Peel Oil on Biofilm Formation of *E. coli* O157:H7, *S. Typhimurium* and *P. aeruginosa.* Overnight cultures were diluted in CFA (colonization factor antigen) media at 1:25 proportion. Diluted culture of 190/198 µl were added to polystyrene 96-well plates, DHB, bergamottin, bergaptol and geranylcoumarin and Rio Red, Marsh White, commercial pink grapefruit juices and grapefruit peel oil were added to respective wells of plates, the plates were incubated at 37 °C for 24 hours. The appropriate amount of DMSO was added to all control wells to find effect of DMSO. To quantify the total biofilm mass, the suspension cultures were decanted, the plates were washed with phosphate buffer (pH 7.4), and the biofilms were stained with 0.3% crystal violet (Fisher, Hanover Park, IL) for 15 min. The

excess dye was removed by washing with phosphate buffer (pH 7.4). Dye associated with the attached biofilm was dissolved with 200 µl of 33% acetic acid. The OD was measured at 620 nm quantify the total biofilm mass. Each data point was averaged from six replicate wells and the standard deviations were calculated.

Data Analysis. The AI-1 and AI-2 activities were expressed as 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%. The percent inhibition was calculated from the formula 100 – [(relative AI activity/relative activity of positive control) X 100] *(161)*.

Results and Discussion

Inhibition of AI-1 and AI-2 Activity in *V. harvey* by Furocoumarins and Grapefruit Juice. The percent inhibition of AI-1 activity by various furocoumarins and grapefruit juices is shown in **Figure 6.2.** Among the furocoumarins, maximum inhibition was observed with bergaptol (99.15%) and minimum was seen with geranylcoumarin (80.88%), while the other two furocoumarins showed >96% of inhibition. Rio Red, Marsh White and commercial grapefruit juices showed 47%, 62% and 94% of inhibition, respectively at 1% concentration. However, all three juices showed >99% of inhibition activity at 5% concentration. It is evident from the results that DHB, bergamottin and bergaptol are potent inhibitors of AI-1 activity at 0.01% concentration, while grapefruit juice was most effective at 5% in inhibiting AI-1 activity. The percent inhibition of AI-2 activity by various furocoumarins and grapefruit juice is presented in Figure 3. The maximum and minimum inhibition activity was shown by bergaptol (98.85%) and

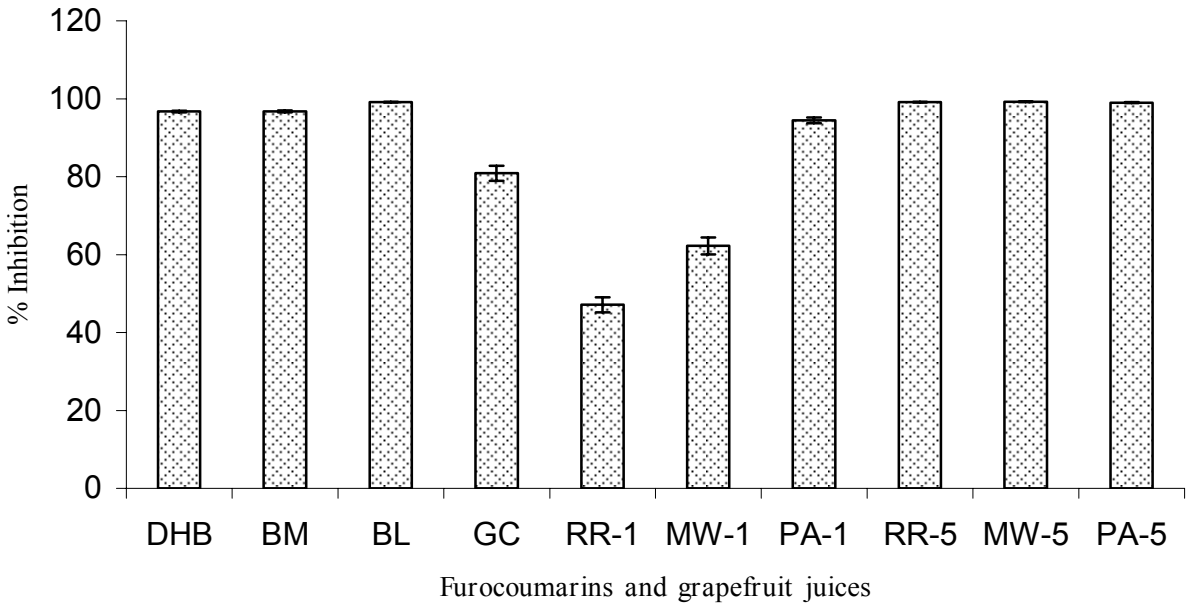


Figure 6.2. Inhibition of AI-1 activity by various furocoumarins, grapefruit juices and grapefruit peel oil. DHB, dihydroxybergamottin; BM, bergamottin; BL, bergaptol; GC, geranylcoumarin; RR-1, Rio Red grapefruit juice at 1%; MW-1, Marsh White grapefruit juice at 1%; PA-1, commercial pink grapefruit juice at 1%; RR-5, Rio Red grapefruit juice at 5%; MW-5, Marsh White grapefruit juice at 5%; PA-5, commercial pink grapefruit juice at 5% concentrations. Bars in the panel indicate ±SD, n=3.

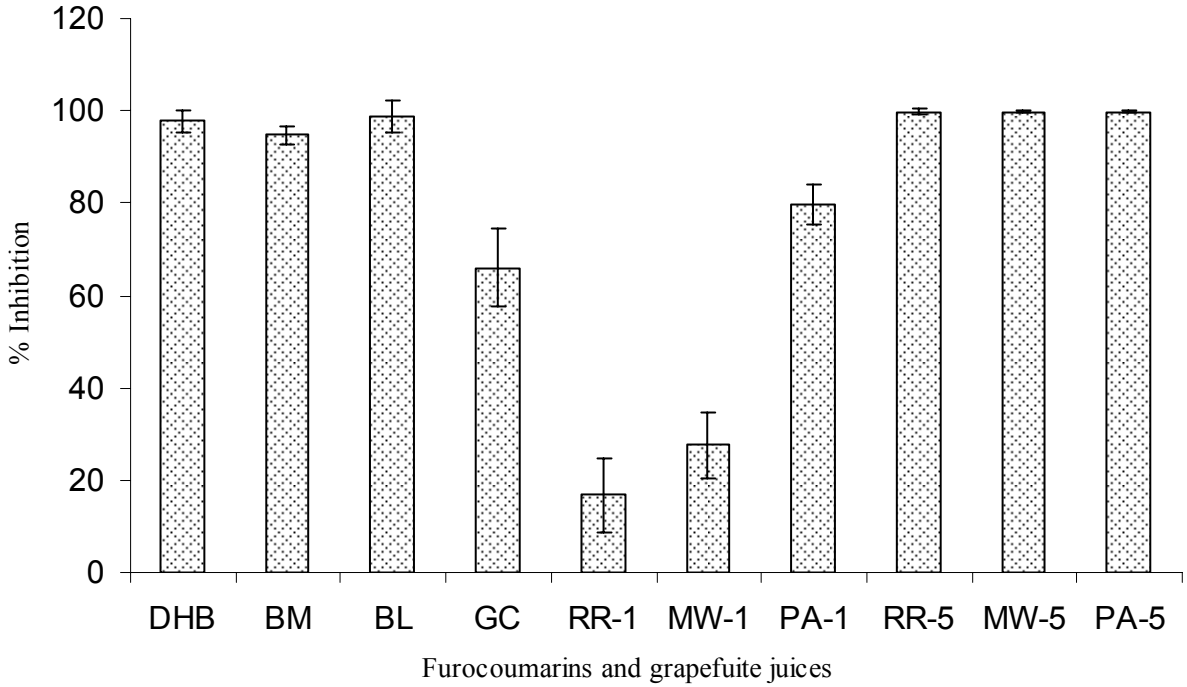


Figure 6.3. Inhibition of AI -2 activities by various furocoumarins, grapefruit juices and grapefruit peel oil. DHB, dihydroxybergamottin; BM, bergamottin; BL, bergaptol; GC, geranylcoumarin; RR-1, Rio Red grapefruit juice at 1%; MW-1, Marsh White grapefruit juice at 1%; PA-1, commercial pink grapefruit juice at 1%; RR-5, Rio Red grapefruit juice at 5%; MW-5, Marsh White grapefruit juice at 5%; PA-5, commercial pink grapefruit juice at 5% concentrations. Bars in the panel indicate ±SD, n=3.

geranylcoumarin (66.01%), respectively. Rio Red and Marsh White grapefruit juices showed 16.82% and 27.52% inhibition, respectively at 1% concentration. However, commercial grapefruit juice at 1% concentration showed 79.80% inhibition. All three grapefruit juices inhibited AI-2 activity by >99% at 5% concentration.

Effect of Furocoumarins, Grapefruit Juice and Citrus Oil on Biofilm Formation of *E. coli* O157:H7, *S.* Typhimurium and *P. aeruginosa*. Results of grapefruit juice and furocoumarins effect on biofilm formation are summarized in **Table 6.1.** Among the furocoumarins studied, DHB inhibits the biofilm formation by 27.32% and 25.75% in *P. aeruginosa* and *E. coli*, while geranylcoumarin showed 19.5% inhibition in *S.* Typhimurium. Bergaptol showed 32.65% of inhibition in *P. aeruginosa*, this is the highest among the furocoumarins at 0.01% concentration. All the grapefruit juices at 5% concentration were more effective than 1% in inhibiting the biofilm formation. Rio Red, Marsh White and pink commercial grapefruit juices showed 42.59%, 68.09% and 88.9% inhibition of biofilm formation in *E. coli*, *S.* Typhimurium and *P. aeruginosa* at 5% concentration.

Several studies involving plant extracts have demonstrated the anti-quorum sensing activity. Vanilla extract *(162)*, garlic extract *(163)* and several medicinal plants extracts from have shown potent anti-quorum sensing activity *(143)*. However, these studies lack the active agent of the plant extract responsible for the reported anti-quorum sensing activity. In our study, naturally occurring furocoumarins (DHB, bergamottin and bergaptol) extracted from grapefruit juice showed almost complete inhibition in both AI-1 and AI-2 activity at 0.01% concentration.

Table 6.1. Percent inhibition of biofilm formation in *E. coli* O157:H7, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* by furocoumarins and grapefruit juices.

Furocoumarins /Grapefruit juices	*E. coli*	*S.* Typhimurium	*P. aeroginosa*
DHB	25.75 ± 0.05[a]	4.90 ± 0.04	27.32± 0.07
BM	22.70 ± 0.04	6.01 ± 0.13	27.37 ± 0.09
BL	14.83 ± 0.02	8.04 ± 0.04	32.65 ± 0.08
GC	10.30 ± 0.03	19.52 ± 0.04	9.24 ± 0.05
RR-1%	30.94 ± 0.02	9.69 ± 0.06	11.97 ± 0.04
MW-1%	33.51 ± 0.02	8.91 ± 0.04	18.16 ± 0.02
PA-1%	32.85 ± 0.04	34.85 ± 0.05	26.44 ± 0.05
RR-5%	41.65 ± 0.07	43.99 ± 0.07	41.36 ± 0.08
MW-5%	42.70 ± 0.02	47.55 ± 0.05	44.90 ± 0.07
PA-5%	42.59 ± 0.06	88.99 ± 0.02	68.09 ± 0.03

DHB; dihydroxybergamottin, BM; bergamottin, BL; bergaptol, GC; geranylcoumarin, RR-1; Rio Red grapefruit juice at 1%, MW-1; Marsh White grapefruit juice at 1%, PA-1; commercial pink grapefruit juice at 1%, RR-5; Rio Red grapefruit juice at 5%, MW-5; Marsh White grapefruit juice at 5%, PA-5; commercial pink grapefruit juice at 5% concentrations. Data are presented as mean ± SD of absorbance at 620 nm.

The structural resemblance between these furocumarins, the auto-inducer molecules and all synthetic halogenated furanones is the furan moiety. Geranylcoumarin, which lacks the furan ring in its molecule, showed minimum inhibition of AI-1 and AI-2 activities. Natural and isolated furanones have shown to interference with quorum sensing systems in *V. fischeri*, *V. harveyi*, *Serratia liquefaciens, S. ficaria* and *P. aeruginosa (153)*.

It has been reported that halogenated furanones from the marine red alga *Delisea pulchra* are strong inhibitors of the bioluminescence labeled AHL-regulated system in *E. coli (164)*. Halogenated furanones have shown inhibitory activity in cultivar of assays intended to measure AHL-regulated gene expression and such inhibition was found to be partially relieved by increasing AHL concentrations in the bioassays *(165)*. This suggests the competition between the AHL-signaling molecules and furanones for a common binding site on LuxR and LuxR homologues *(164)*. AHLs are highly conserved and have the same homoserine lactone moiety with different cayl side chains and substitutions of carboxyl or hydroxyl group at the C3 position *(166)*. AHLs are co-regulatory ligands required for control of the expression of genes encoding virulence traits in many Gram-negative bacteria. The fact that bergaptol, bergamottin and DHB showed strong inhibition of quorum sensing without interfering with critical life process makes these quorum sensing inhibitors suitable candidates for treating microbial infections using a non-antibiotic based strategy, since the selective pressure for development of bacterial resistance could be avoided *(148, 157)*.

Food-borne illnesses resulting from food poisoning with pathogenic bacteria has been of vital concern to public. The recent outbreak of E. coli (August 2006) in spinach is vivid example. To reduce health hazards and economic losses due to food borne microbe,

the use of plant derived compounds as antimicrobial agents seem to be an interesting way to control the pathogenic microbial activity in the food system and extend the shelf life of food.

Plants have long been a source of drugs and continue to contribute to the process of drug discovery. Natural products such as alkaloids, phenols, polyphenols, saponins, tannins, terpenes, antraquinones and steroids are known to posses antimicrobial activity *(167)*. Essential oils are odorous, volatile products of an aromatic compounds produced during secondary metabolism. These are normally formed in special cells found in bark, stem, leaves, fruit and fruit peel as in case of citrus. Essential oils and their components are known to be active against a wide cultivar of bacteria *(167)*.

Biofilm formation has been shown to cause severe health problems. Biofilms constitute a protected mode of growth that allows survival of pathogens in hostile environments. Bacterial biofilms growing in natural and industrial environments are resistant to bacteriophages, amoeba and diverse antibiotics used against them. Medically, biofilms can withstand host immune responses, and they are much less susceptible to antibiotics than non-attached individual microbial counterparts. There is tremendous interest in naturally-occurring compounds as biofilm controlling and preventing agents. Recently ursolic acid was reported as potent inhibitor of biofilm formation in *E. coli* and natural furanones isolated from *D. pulchra* have been found to reduce biofilm formation in *E. coli* and in *B. subtilis (168, 169)*.

The results of this study have revealed that furocoumarins and grapefruit juice act as potent inhibitors of AI-1 and AI-2 activity. Furocoumarins from the grapefruit juice

offers safe alternative to the halogenated furanones to develop anti-quorum sensing strategies that might reduce bacterial infections.

CHAPTER VII

EVALUATION OF ANTI-PROLIFERATIVE ACTIVITIES OF FUROCOUMARIN MONOMERS AND DIMER ON CANCER AND NORMAL CELL LINES

Synopsis

Furocoumarins have shown spectrum of biological activities such as cytochrome P450 enzyme inhibition, modulation of P-glycoprotein activity, anti-quorum sensing and anti-oxidant activity. In this study, synthesized furocoumarin monomers and dimer were investigated for the anti-proliferative activities on normal and cancer cell lines. Compounds were found to be non-toxic to normal cells, as seen in case of NIH-3T3 cells. However, bergamottin showed a significant anti-proliferative activity in both HT-29 and MCF-7 adenocarcinoma cell lines.

Introduction

Furocoumarins are currently of great interest and research into these bioactive compounds is becoming increasingly intense due to their involvement in grapefruit-drug interaction. In humans, many of the chemicals are pro-carcinogens and are activated to carcinogenic and mutagenic substances by microsomal enzymes *(115, 121)*. Some of the important cytochrome P450 isoenzymes are CYP3A4, CYP1A2, CYP1B1, CYP19, 2D6 and 2C9, which have specific roles in the onset of several types of cancers *(115, 117-120)*. In vitro studies with certain phytochemicals revealed reduction in carcinogens through partial inhibition of these enzymes *(112, 124)*. Thus, inhibition of cytochrome

enzymes by naturally occurring compounds may represent a novel approach in the anti-carcinogenesis strategy *(116)*. The aim of the present work was to evaluate series of synthesized furocoumarin monomers and dimer on biological activity against normal and cancer cell lines in vitro.

Materials and Methods

Chemicals. All the chemicals were molecular biology and analytical grade were purchased from Sigma-Aldrich, VWR International (1310 Goshen Parkway, West Chester, PA).

Furocoumarin Monomers and Dimer. Compounds were synthesized from bergapten as described in chapter II. Stock solutions (10 mM) of bergapten, bergaptol, bergamottin, epoxybergamottin, DHB and dimer, paradisin A were prepared in DMSO and kept at 4 °C. Working solution of 0.625 to 100 μM solutions were prepared from the stock solution before starting the experiment.

Cell Culture. NIH 3T3, HT-29 and MCF-7 were purchased from the American Type Culture Collection (Bethesda, MD) and cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum from Hyclone (925 West 1800 South Logan, UT). Cells were grown on 25 cm^2 falcon culture flasks at 37 ^oC in 5% CO_2. When cells reached 70-80% confluence they were detached with Trypsin- EDTA solution and quantified using Z1 coulter counter (Beckman coulter Inc, 4300 N. Harbor Boulevard Fullerton, CA).

Measurement of Cell Viability

A. MTT Assay. Cell proliferation was evaluated using MTT reagent (3[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium) assay, which measured the mitochondrial dehydrogenase activity of viable cells spectrophotometrically. Cells were seeded at a density of $5x10^4$ cells/well and allowed to attach for 24 hrs, these attached cells were treated with furocoumarins dissolved in DMSO at 6.25, 12.5, 25, 50 and 100 μM in a 96 -well cell culture plates and were incubated at 37 ºC in 5% CO_2 . After 24, 48 and 72 hrs, MTT was added to each well. After incubation for 4 h at 37 ºC, medium was removed carefully and the intracellular formazan was dissolved by adding 200 μl of DMSO to each well. After jolted by the shaker for 5 minutes, the upper solution was transferred to another 96-well plate. The absorbance was measured spectrophotometrically at 515 and 550 nm in microplate reader (KC4, v3.3 microplate reader). Samples were treated in triplicates and results were expressed as mean ±SD.

B. Cell Proliferation Assay by Coulter Counter. MCF-7 Cells were seeded at a density of $5x10^4$/well in 12-well plates, and after 24 hours they were treated with media mixed with known concentration of samples. Treatment was done at 25, 50 and 100 μM

concentration, DMSO at 100μM was used as control treatment and Campothecin at 25 μM was used as positive control. Cells were counted at 24 and 48 Hrs using a Coulter Z1 cell counter after detaching using Trypsin-EDTA. Each experiment was completed in triplicate and results are expressed as mean ± SD for each determination.

Assessment of Cytotoxicity by LDH Assay. Cytotoxity was assessed as lactate dehydrogenase (LDH) released into the media relative to cultures lysed with 1% Triton X-100. This end point was chosen since it coincide with membrane disruption, a late stage irreversible step in cytolethality *(170)*. MCF-7 cells were treated with furocoumarins at the dose of 25, 50 and 100 μM and the medium was subjected for assay after centrifugation to remove cells. Cells were allowed to attach for 24 h and then treated in triplicate with varying concentrations of furocoumarins. LHD was estimated using Roche (Hoffmann-La Roche Nutley, NJ) cytotoxicity detection kit and calculations were done as per instruction in kit using positive and negative control.

Table 7.1. Effect of furocoumarin monomers and dimer on the activity on COS7 cell lines. Data are expressed as mean ± SD, % of control.

			24 hrs			
Concentration (µM)	**5**	**10**	**25**	**50**	**75**	**100**
Campothecin	97 ± 2.7	97 ± 0.2	95 ± 2.2	90 ± 2.8	86 ± 0.84	80 ± 1.8
Bergapten	98 ± 4.3	98 ± 2.2	96 ± 2.0	98 ± 3.1	87 ± 1.4	80 ± 0.5
Bergaptol	97 ± 3.3	99 ± 1.0	103 ± 5.8	103 ± 6.1	103 ± 1.4	107 ± 3.2
Bergamottin	102 ± 4.0	107 ± 1.7	115 ± 3.9	125 ± 0.7	125 ± 4.1	129 ± 5.4
Epoxy-bergamottin	100 ± 0.8	100 ± 2.8	104 ± 2.9	112 ± 3.0	126 ± 4.0	132 ± 2.5
DHB	101 ± 7.9	104 ± 6.7	101 ± 4.9	108 ± 5.0	118 ± 6.5	127 ± 15.1
Paradisin A	111 ± 3.3	106 ± 9.7	98 ± 3.8	103 ± 1.7	106 ± 6.7	104 ± 2.9
			48 hrs			
Campothecin	83 ± 0.9	78 ± 2.1	76 ± 6.0	77 ± 2.4	80 ± 3.3	73 ± 1.2
Bergapten	100 ± 1.9	95 ± 0.7	95 ± 4.1	96 ± 3.9	83 ± 2.1	76 ± 4.7
Bergaptol	95 ± 1.4	96 ± 4.8	95 ± 4.4	97 ± 6.1	101 ± 0.5	104 ± 5.9
Bergamottin	100 ± 4.5	96 ± 5.6	95 ± 5.0	89 ± 5.6	80 ± 1.7	73 ± 5.9
Epoxy-bergamottin	99 ± 0.8	99 ± 1.3	97 ± 2.3	98 ± 3.2	104 ± 2.2	98 ± 3.1
DHB	105 ± 2.1	103 ± 2.2	102 ± 3.8	104 ± 4.3	110 ± 4.3	117 ± 5.8
Paradisin A	106 ± 2.1	87 ± 3.2	81 ± 1.5	87 ± 0.9	82 ± 0.8	86 ± 1.0

Results and Discussion

Furocoumarin monomers such as bergapten, bergaptol, bergamottin, epoxybergamottin, DHB and dimer, paradisin A were evaluated for their effect on normal and cancer cell lines. Results indicate that normal COS-7 cells were not affected by any of the furocoumarins, as seen by MTT assay results at 24 hrs and 48 hrs **(Table 7.1)**. Anti-proliferative activity of all the compounds was less significant on colon adenocarcinoma cells (HT 29) at 24 hrs; however bergamottin exhibited activity of 35.74 % at 100 μM concentration at 48 hrs **(Table 7.2)**. Other compounds did not show significant activity. All furocoumarin monomers and dimer showed similar results in case of MCF-7 cell lines as measured by MTT assay **(Table 7.3)**. Bergamottin showed ~30% inhibition activity at 100 μM concentration followed by epoxybergamottin. However, activities of other compounds were not significant.

All the monomers and dimer when screened for antiproliferation ability on MCF-7 cells, bergamottin showed more than 50% inhibition activity at the dose of 25, 50 and 100 μM at 48 hours, followed by paradisin A and DHB However, other compounds exhibited moderate activity which was not significant in comparison with campothecin **(Table 7.4)**. Furthermore, antiproliferative results were supported by LDH based cytotoxicity assay **(Table 7.5)**. Compounds which showed significant antiproliferation activity had higher amount of LHD leakage into media, indicating cytotoxicity of the compounds in vitro.

Furocoumarin are widely dispersed in nature and are secondary metabolites in a cultivar of plant belonging to such as *Umbellifera and Rutacea* families *(38)*. Extracts of

plants containing furocoumarins such as *Psoralea corylifolia* and *Ammi majus*, were used in India and Egypt, respectively, as early as 2000 BC to treat hyperproliferative skin diseases *(171)*. Furocoumarins have been extensively used in phototherapy coupled with ultraviolet light. Psoralen is employed in the treatment of different hyperactive skin conditions including vitilago, psoriasis and atopic dermatitis *(171)*. The main mechanism of furocoumarin action is based on ability to form photo-adducts with genetic material, DNA in some instances RNA, proteins and membrane components *(171, 172)*. The ability of these compound to form adduct, thereby suppressing the replication of genetic material would be the main reason for furocoumarins therapeutic activity. Our results indicate that there are potential use of bergamottin and other derivatives as inhibitors of carcinoma cells. Modification of monomers/dimers by chemical means would enhance the activity of these compounds. Further research into these compounds towards understanding the mechanism of action and dose optimization may lead to better understanding of these molecules as anticancer agents.

Table 7.2. Effect of furocoumarin monomers and dimer on the activity on HT-29 cell lines. Data are expressed as mean ± SD, % of control.

			24 hrs			
Concentration (µM)	**5**	**10**	**25**	**50**	**75**	**100**
Campothecin	63 ± 2.5	60 ± 1.1	57 ± 3.2	56 ± 1.9	55 ± 1.4	45 ± 10.4
Bergapten	113 ± 10.0	120 ± 12.9	119 ± 12.4	122 ± 10.0	119 ± 11.1	105 ± 11.8
Bergaptol	104 ± 13.2	113 ± 19.0	115 ± 12.8	113 ± 14.2	119 ± 16.9	122 ± 18.6
Bergamottin	110 ± 9.2	111 ± 7.2	117 ± 6.7	109 ± 6.7	109 ± 4.4	106 ± 2.6
Epoxy-bergamottin	113 ± 1.2	105 ± 2.6	114 ± 7.9	123 ± 2.5	131 ± 0.9	132 ± 1.1
DHB	117 ± 8.6	120 ± 8.7	129 ± 10.8	130 ± 9.8	129 ± 6.8	116 ± 6.8
Paradisin A	121 ± 4.2	124 ± 7.3	123 ± 5.7	123 ± 2.4	122 ± 2.2	118 ± 5.4
			48 hrs			
Campothecin	45 ± 0.8	46 ± 2.8	46 ± 0.2	45 ± 0.7	45 ± 0.9	42 ± 1.6
Bergapten	117 ± 4.7	112 ± 3.0	104 ± 28.8	113 ± 4.5	104 ± 2.2	91 ± 2.6
Bergaptol	112 ± 3.0	110 ± 1.9	111 ± 3.5	111 ± 5.8	110 ± 2.0	102 ± 7.5
Bergamottin	115 ± 8.7	111 ± 6.9	102 ± 1.3	86 ± 4.6	71 ± 4.4	64 ± 1.2
Epoxy-bergamottin	114 ± 5.5	112 ± 3.8	108 ± 3.0	107 ± 1.7	104 ± 12.6	83 ± 0.2
DHB	128 ± 7.0	118 ± 3.7	126 ± 15.1	120 ± 4.4	127 ± 28.9	93.00
Paradisin A	116 ± 7.5	113 ± 4.3	107 ± 7.3	111 ± 5.6	97 ± 19.5	100 ± 3.3

Table 7.3. Effect of furocoumarins monomers and dimer on the activity on MCF-7 cell lines. Data are expressed as mean ± SD, % of control.

				24 hrs				
Concentration (µM)	**0.625**	**1.25**	**2.5**	**5**	**10**	**25**	**50**	**100**
Bergapten	97.95	98.82	96.22	96.62	95.77	97.77	97.05	77.35
SD	4.14	4.86	3.04	4.43	4.74	1.03	1.69	3.82
Bergaptol	95.04	87.24	92.19	87.86	89.12	89.47	85.60	87.62
SD	1.46	2.96	7.08	1.25	0.32	0.66	1.06	3.28
Bergamottin	97.41	95.42	97.55	106.84	105.37	116.85	109.46	108.38
SD	1.04	0.03	0.33	4.98	1.82	2.90	9.02	1.74
Epoxybergamottin	96.18	89.53	89.38	89.51	91.84	97.96	109.38	111.49
SD	1.39	0.37	3.47	4.02	0.84	3.05	5.41	5.89
DHB	101.55	98.74	98.13	99.43	98.61	97.97	100.81	101.24
SD	0.92	0.02	4.71	0.64	1.96	2.55	1.79	
Paradisin A	109.48	107.38	106.76	113.44	113.24	127.20	128.63	129.83
SD	3.91	4.71	4.33	7.87	4.00	7.53	10.20	15.18
				48 hrs				
Bergapten	96.99	96.29	93.27	93.22	93.02	94.97	95.22	78.44
SD	3.00	0.95	0.15	1.72	1.09	0.22	1.02	1.85
Bergaptol	96.18	91.76	88.48	88.09	86.66	88.51	89.81	93.08
SD	7.17	4.80	8.39	10.17	8.15	5.01	8.26	6.98
Bergamottin	86.62	86.55	82.68	86.22	85.94	79.25	75.01	71.96
SD	6.71	3.69	1.28	2.11	0.88	1.33	0.74	4.10
Epoxybergamottin	87.51	89.75	93.30	89.97	89.84	89.68	82.73	85.91
SD	5.13	2.10	1.63	6.04	4.81	6.98	12.41	6.63
DHB	96.61	93.31	94.97	97.78	100.18	98.25	103.63	100.92
SD	4.80	1.34	1.02	7.98	7.87	11.38	9.71	11.04
Paradisin A	114.98	102.42	92.03	98.38	96.37	100.84	96.47	106.64
SD	15.32	7.17	0.30	1.31	6.59	4.35	2.38	9.84

Table. 7.4. Effect of furocoumarin monomers and dimer on proliferation of MCF-7 cells.

Treatment	Conc. In µM	Cell number x 1000	
		24 hrs	48 hrs
DMSO	100	16.2 ± 4.96 [a]	38.7 ± 4.49
Campothecin	50	8.4 ± 3.05	11.3 ± 7.17
Bergapten	25	13.7 ± 3.81	26.5 ± 5.08
	50	12.6 ± 3.31	32.9 ± 5.69
	100	18.7 ± 1.94	8.8 ± 2.09
Bergaptol	25	16.65 ± 1.63	51.25 ± 12.64
	50	20.6 ± 7.84	49.6 ± 9.67
	100	12.3 ± 1.51	33.3 ± 3.80
Bergamottin	25	14.9 ± 1.21	13.00 ± 0.83
	50	13.3 ± 2.31	6.6 ± 2.18
	100	10.2 ± 2.92	7.00 ± 0.99
DHB	25	21.8 ± 1.80	22.0 ± 3.08
	50	10.45 ± 2.03	19.25 ± 4.13
	100	9.2 ± 1.57	16.1 ± 3.17
Epoxybergamottin	25	19.9 ± 2.13	25.85 ± 3.26
	50	13.4 ± 2.00	28.6 ± 5.59
	100	13.3 ± 5.49	27.5 ± 4.41
Paradisin A	25	11.3 ± 1.52	22.8 ± 1.02
	50	10.6 ± 2.5	18.62 ± 0.95
	100	10.0 ± 0.86	14.4 ± 1.25

[a]Values are mean of six readings ± SD

Table 7.5. Cytotoxicity of furocoumarin monomers and dimer by LDH assay.

Treatment	Conc. (μM)	% LDH leakage	
		24 hrs	48 hrs
DMSO	100	49.86 ± 0.08 [a]	98.19 ± 0.04
Campothecin	50	59.95 ± 0.12	57.36 ± 0.04
Bergapten	25	64.11 ± 0.09	55.71 ± 0.19
	50	80.29 ± 0.08	49.34 ± 0.01
	100	70.35 ± 0.05	61.34 ± 0.12
Bergaptol	25	70.35 ± 0.10	58.00 ± 0.12
	50	62.03 ± 0.07	47.84 ± 0.15
	100	58.03 ± 0.09	42.63 ± 0.03
Bergamottin	25	59.03 ± 0.07	57.18 ± 0.13
	50	73.69 ± 0.03	48.63 ± 0.03
	100	58.15 ± 0.5	45.81 ± 0.05
DHB	25	69.25 ± 0.09	49.58 ± 0.17
	50	61.82 ± 0.10	39.78 ± 0.04
	100	60.78 ± 0.03	50.47 ± 0.11
Epoxybergamottin	25	58.03 ± 0.09	51.33 ± 0.13
	50	50.93 ± 0.04	44.98 ± 0.05
	100	51.23 ± 0.11	37.74 ± 0.06
Paradisin A	25	61.10 ± 0.09	32.30 ± 0.15
	50	51.90 ± 0.11	33.8 ± 0.08
	100	80.55 ± 0.16	37.6 ± 0.17

[a]Values are mean of six readings

CHAPTER VIII

SUMMARY AND CONCLUSION

Grapefruit is considered a “store house” of health-promoting compounds, such as carotenoids, limonoids, flavonoids, pectin and vitamin C. Grapefruit juice carries the American Heart Association’s “heart check” food mark. However, consumption of grapefruit along with a certain medications is posing a risk of drug toxicity and side reactions. Furocoumarins are the main components of grapefruit juice, interacting with cytochrome P450 enzymes and P-glycoprotein.

An efficient and improved technique has been developed for isolation and purification of furocoumarins using a combination of chromatographic techniques. Five furocoumarins namely, Dihydroxybergamottin, paradisin A, bergamottin, bergaptol and geranylcoumarin were isolated from grapefruit and series of furocoumarin monomers and paradisin A were synthesized. These compounds were influenced by various pre- and post-harvest factors. Variation of three furocoumarins such as dihydroxybergamottin, paradisin A and bergamottin were monitored in seven cultivars of grapefruits and its parent Pummelo. Considerable differences were observed in the levels of these compounds in different grapefruit cultivars. 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 contains the highest amount of DHB (2.587 ± 0.432 μg/ml) and bergamottin (1.004 ± 0.068 μg/ml), where as the highest levels of paradisin A was observed in Star Ruby. The highest levels of DHB (2.266 μg/ml) and bergamottin (2.411 μg/ml) were found in flame grown in California. The changes in the

levels of these furocoumarins during the season in Rio Red and March White grapefruit cultivars were evaluated.

The grapefruit juice along with isolated compounds such as bergamottin, 6' 7'-dihydroxybergamottin, paradisin-A, bergaptol and geranylcoumarin were tested for their inhibitory effects on human CYP3A4, CYP2C9 and CYP2D6. Grapefruit and Pummelo juices were found to be potent inhibitors of cytochrome CYP3A4 and CYP2C9 isoenzymes at 5% concentration while CYP2D6 was less affected. Among the five furocoumarins tested, the inhibitory potency was in the order of paradisin A>dihydroxybergamottin>bergamottin>bergaptol>geranylcoumarin at 0.1 μM to 0.1 mM concentrations. The IC_{50} value was lowest for paradisin A for CYP3A4 with 0.11 μM followed by DHB for CYP2C9 with 1.58 μM. Studies involving the grapefruit juice and furocoumarins on bacterial auto-inducer (AI) signaling found that, furocoumarins are potent inhibitors of both AI-1 and AI-2 activities at 0.01% concentration and grapefruit juices inhibited >99% of AI-1 and AI-2 activities at 5% concentration. Grapefruit juice and furocoumarins also affected biofilm formation in *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Pseudomonas aeruginosa*. In another study, involving synthesized furocoumarin monomers and dimer on anti-proliferative activities on normal and cancer cell lines, furocoumarins found to be non-toxic to normal cells. However, bergamottin showed a significant anti-proliferative activity in both HT-29 and MCF-7 adenocarcinoma cell lines.

Inhibition of cytochrome P450 enzymes by grapefruit juices and its furocoumarins offers some clinical advantages in improving bioavailability of poorly absorbed drugs and reduce the dose requirements and ultimately reducing the cost and

harnessing the health benefits of consuming grapefruit juice. In addition grapefruit juice bioactive components may act as potent inhibitor of cytochrome P450 enzymes which are involved in activation pro-carcinogen to carcinogen. This represents a unique mechanism in the anti-carcinogenesis strategy, part of which includes reducing the generation of reactive oxygen species. When grapefruit juice increases bioavailability of drugs by 1.5 to 15 fold, is it feasible to explore the possibilities of using grapefruit juice as a "super-pill" supplement?, especially in the context of costly and comprehensively used drugs, such as anti-retroviral drugs. Based on the extent of bioavailability obtained by single/double strength juice originating from white or red grapefruit cultivar, one can consider revising the dose of drugs. Considering the HIV epidemics and prices of anti-retroviral drugs, grapefruit juice induced drug interaction offers challenge and opportunity to explore supplementing the drug dose with grapefruit juice or natural furocoumarins. Alternatively, one can isolate or synthesize the furocoumarins and use them as a supplement to reduce the drug doses required for effectiveness.

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APPENDIX I

ABBREVIATIONS USED

µg: micro gram

µl: micro liter

AI signal: Auto Inducer signal

CYP2C9: Cytochrome P450 2C9

CYP2D6: Cytochrome P450 2D6

CYP3A4: Cytochrome P450 3A4

DEM: Delbecco's Modifies Eagle's Medium

DHB: dihydroxybergamottin

DMSO: Dimethyl sulfoxide

DQFCOSY: Double Quantum Filter Correlated Spectra

E-beam: electronic beam

HPLC: High-performance liquid chromatography

HSQC: Hetero Single Quantum Correlated Spectra

mg: milli gram

MS: Mass spectrometry

NMR: nuclear magnetic resonance

P-gp: P-glycoprotein

APPENDIX II

DEFINITION OF TERMS

Analytical HPLC: is a form of column chromatography used to separate components of interest from a mixture of compounds for analytical purposes

Preparative HPLC: is a form of column chromatography used for separation and isolation of components from the mixture.

^{1}H NMR: is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen. ^{1}H NMR is characterized by chemical shifts in the range of +12 to -4 PPM and spin coupling between protons. The integration spectrum gives the proton properties in the molecule

^{13}C NMR: application of NMR with respect to carbon. This helps in identifying the carbon atom in an organic molecule. This is important tool in structure elucidation.

Cytochrome P450 enzymes: are heme containing proteins with absorption maxima at 450 nm. Cytochtome P450 enzymes are major xenobiotics metabolism enzymes in human and play a major role in drug metabolism and interactions.

COS7: Normal cell line of African green monkey mainly used for virus replication studies.

NIH-3T3: Norma cell lines of Swiss NIH mouse embryo used mainly for DNA transfection studies.

MCF-7: Human breast adenocarcinoma cell line

HT-29: cell line of human colon adenocarcinoma origin

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