CITRUS BIOACTIVE COMPOUNDS INFLUENCING PHASE II DETOXIFYING ENZYMES: POTENTIAL FOR CANCER CHEMOPREVENTION

A Thesis

by

JOSE LUIS PEREZ

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

MASTER OF SCIENCE

May 2007

Major Subject: Horticultural Sciences

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Approved by:

Chair of Committee,	Bhimanagouda S. Patil
Committee Members,	G. K. Jayaprakasha
	Kevin Crosby
	Ranjita Misra
	Mani Skaria
	Hassan Ahmad
Head of Department,	Tim Davis

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ABSTRACT

Citrus Bioactive Compounds Influencing Phase II Detoxifying Enzymes: Potential For Cancer Chemoprevention. (May 2007)

Jose Luis Perez, B.S., University of Texas Pan-American

Chair of Advisory Committee: Dr. Bhimanagouda S. Patil

Several cell culture and animal studies demonstrated that citrus limonoids have protective effects against certain types of cancer. These chemopreventive properties of citrus limonoids are attributed to the induction of phase II enzyme, glutathione S-transferase (GST). In the current study, six citrus limonoids and two modified limonoids were utilized for the evaluation of NAD(P)H: quinone reductase (QR) activity and glutathione S-tranferase (GST) activity against 1-chloro-2,4-dinitrobenzene (CDNB) and 4-nitroquinoline 1-oxide (4NQO) in A/J female mice.

In liver, limonoids that induced phase II enzyme activity were limonin-7-methoxime (32% CBNB), (270% 4NQO), (65% QR); and deacetylnomilin (180% QR). In stomach, limonin-7-methoxime (51% 4NQO); deacetyl nomilinic acid glucoside (55% 4NQO), nomilin (58% CDNB), (75% 4NQO); isoobacunoic acid (25% CDNB); deacetylnomilin (19% CDNB); limonoid mixture (45% 4NQO), (200% QR). Furthermore, in intestine, nomilin (280% 4NQO); deacetylnomilin (73% 4NQO), (22% QR); and the limonoid mixture (93% 4NQO) increase enzymatic activity. Finally in lung,

deacetyl nomilinic acid glucoside (67% CDNB); limonin-7-methoxime (32% QR); and defuran limonin (45% QR) diplayed induction properties.

Furthermore, D-glucaric acid (GA), a chemoprotective compound found in fruits and vegetables, was quantified using High Performance Liquid Chromatography (HPLC) grapefruit. Nine widely used grapefruit varieties were analyzed for the levels of D-glucaric acid. Seasonal levels of GA in each of the grapefruit varieties tested were found to be Thompson (58.36-126.8 mg/100ml), Henderson (29.6-49.7 mg/100ml), Rio Red (40.0-58.8mg/100ml), Star Ruby (25.5-46.7 mg/100ml), I-48 (26.6-58.3 mg/100ml), Ruby Red (49.3-63.0 mg/100ml), Ray's Ruby (58.2-72.1 mg/100ml), Marsh (53.7-65.8 mg/100ml) and Duncan (50.17 mg/100ml). The HPLC method developed for the quantification of D-glucaric acid was found to be simple, fast, and reproducible. Additionally, the labor intensity and cost of sample preparation were greatly reduced.

DEDICATION

This thesis is dedicated:

to my father, Jose Luis Perez, who taught me how to work and that a man's word is more valuable than all the money in the world. His teachings in the "school of life" have been and always will be helpful in confronting the challenges presented by life. He was not only my father, but also my best friend. I hope to one day be as great a man, father, son, brother, husband, and friend as he was.

to my mother, Mercedes Perez, who taught me the values of hard work. Her love, advice and encouragement were key factors in my fulfilling my career objectives. Her hard working nature inspired me to keep pushing forward despite all odds. I love you mom.

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to my fiancée, Mirna Gonzalez, who love, understanding and encouragement I had through the most difficult times in my life. Thanks to her support and faith in me, I was able to continue my education at time when I was about to give up. Thank you.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

History revealed that human illnesses were treated using traditional medicine, presently known as complementary or alternative medicines (Issa, Volate, & Wargovich, 2006). Written and archeological records dating back over 5000 years suggest the use of plant and plant products as treatments for ill physiological conditions (Raskin et al., 2002). Furthermore, in 480 BC, Hippocrates stated that "Positive health requires a knowledge of man's primary constitution and the powers of various foods, both those natural to him and those resulting from human skill", in other words genetics and diet influence health (Reddy, Odhav, & Bhoola, 2003). In recent nutritional research, the focus has shifted from the traditional study of vitamin and mineral deficiencies to the study of naturally occurring bioactive compounds having important effects on human health (Ejaz, Ejaz, Matsuda, & Lim, 2006). Knowledge of traditional medicine has paved the way for contemporary medicine. This is significant mainly in research involving cancer. From 1981-2002, a total of 1031 new chemical compounds were discovered for the treatment of various diseases, of which 57-67% were of natural origin. Among the medications used to treat or prevent cancers, approximately 75% of them are of natural origin (Newmann, Cragg, & Snader, 2003). In developed countries, the practice of contemporary medicine is mainly observed; however, 80% of the population of developing countries still rely on the use of traditional medicine (Kim et al., 2005).

This thesis follows the style of *Food Chemistry.*

The role of the consumption of fruits and vegetables in maintaining human health has been investigated by many researchers in the last two decades. Several recent reviews have evaluated benefits of consumption of fruits and vegetables and prevention of the onset of cardiovascular diseases and cancer (Steinmetz & Potter, 1996; van't Veer, Jansen, Klerk, & Kok, 2000; Surh, 2003; Mandel, Paker, Youdim, & Weinbreb, 2005). Studies show that limited consumption of fruits and vegetables increases the risk of lung, esophagus, bladder, prostate and stomach cancer by about two times compared to individuals with more frequent intake (Ejaz, Ejaz, Matsuda, & Lim, 2006).

During the last decade, health maintaining properties of citrus have been extensively studied using cell culture and animal models. Citrus fruits have been reported to induce hypocholesteroleic responses, enhance antioxidants in serum, prevent osteoporosis, and contain certain compounds that act as anticancer agent (Kurowska, et al., 2000b; Deyhim et al., 2006; Jayaprakasha, & Patil, 2007; Yu, Wang, Walzem, Miller, Pike, & Patil, 2005; Vanamala, Reddivari, Yoo, Pike, & Patil, 2006; Tanaka et al., 1997; Tanaka et al., 1998; Kim et al., 2005; Miyagi, Om, Chee, & Bennink, 2000).

Among different citrus species, grapefruit contains a range of bioactive compounds that have the potential to protect against carcinogenesis (Miyata, Takano, Takahashi, Sasaki, & Yamazoe, 2002). Among the compounds that have shown biological activity are flavonoids, pectin, vitamin C, folic acid, coumarin-related compounds, fibers, and limonoids (Patil, Brodbelt, Miller, & Turner, 2006).

The biological activities of citrus limonoids have been evaluated on several cell culture and animal model systems. Citrus limonoids have been shown to inhibit HIV-1

replication on infected human mononuclear cells (Battinelli et al. 2003). In two Japanese studies, it was reported that orange juice and citrus limonoids, obacunone and limonin, played an important role in the inhibition of azoxymethane-induced colon cancer (Miyagi, Om, Chee, & Bennink 2000; Tanaka et al., 2000). Furthermore, evidence shows that bioactive compounds found in grapefruit (one of them is limonin) protect against azoxymethane (AOM) induced abberant crypt foci (Vanamala, Reddivari, Yoo, Pike, & Patil, 2006). Inhibition of 7,12-dimethylbenz[a]anthracene-induced oral tumors by citrus limonoids has been the focus of in other laboratories (Miller, Porter, Binnie, Guo, & Hasegawa, 2004; Miller, Fanous, Rivera-Hidago, Hasegawa, & Lam, 1989). Strong evidence suggests that benzo[a]pyrene induced forestomach neoplasia in mice was inhibited by citrus limonoids (Lam & Hasegawa, 1989). Recently several cell culture studies have been conducted to observe the effects of citrus limonoids on various cell lines.

The chemopreventive properties of citrus limonoids have in some cases been attributed to their ability to induce phase II enzyme activity, mainly GST. Several studies have evaluated the ability of certain limonoids to induce GST activity in animal models (Lam, Li, & Hasegawa, 1989; Lam & Hasegawa 1989; Tanaka et al., 2000; Ahmad, Li, Polson, Mackie, Quiroga, & Patil, 2006; Kelly, Jewell, & O'Brien, 2003). The induction of GST activity has been correlated to the chemical structural components of citrus limonoids.

In addition to citrus limonoids, it has been observed that citrus also contains D-glucaric acid (Walaszek, Szemraj, Hanausek, Sherman, & Adams, 1996; Dwivedi, Heck,

Downie, Larroya, & Webb, 1990). Glucaric acid (GA), a compound whose presence has been reported in various fruits and vegetable, has also shown to be a potential anticancer agent. Accumulating evidence has positively correlated the consumption of dietary glucaric acid and cancer prevention (Walaszek, Hanausek-Walaszek, Minton, & Webb, 1986; Abou-Issa, Duruibe, Minton, Larroya, Dwivedi, & Webb, 1988; Abou-Issa, 1995; Walaszek, Flores, & Adams, 1988; Walaszek, 1990; Dwivedi, Downie, & Webb, 1989; Yoshimi, Walaszek, Mori, Hanasuek, Szeraj, & Slaga, 2000; Oredipe, Barth, Hanausek-Walaszek, Sautins, & Walaszek, 1987; Oredipe, Barth , Dwivedi, & Webb, 1992; Boone, 1992).

Many studies have demonstrated the effect of GA on different breast cancers at different stages of carcinogenesis. GA has been shown to inhibit 7,12 dimethylben[a]anthracene-induced mammary tumorogenesis (Walaszek, Hanausek-Walaszek, Minton, & Webb, 1986; Abou-Issa, Duruibe, Minton, Larroya, Dwivedi, & Webb, 1988). Furthermore, the effect of GA on the initiation and promotion stages of carcinogenesis was evaluated. Results from this study show that replication of breast tumors was reduced by 28% in the initiation stage and 42% at the promotion stage (Abou-Issa, 1995). In another study, it was shown that GA reduced estrogen receptors found on mammary carcinomas. The reduction of these receptors was correlated with a reduction of tumor growth (Walaszek, Flores, & Adams, 1988).

Anti-cancer properties of GA have also been investigated on other organs. In lung cancer induced by benzo(a)pyrene, GA proved to be an effective inhibitor in mice (Walaszek, 1990). In colon, GA was shown to inhibit the incidence and reduced the size

of colon and intestinal cancers (Dwivedi, Oredipe, Barth, Downie, & Webb, 1989), and azoxymethane–induced rat colon carcinogenesis (Yoshimi, Walaszek, Mori, Hanasuek, Szeraj, & Slaga, 2000). GA has demonstrated an inhibitory effect on the initiation and promotion of hapatocarcinogenesis (Oredipe, Barth, Hanausek-Walaszek, Sautins, & Walaszek, 1987; Oredipe, Barth, Dwivedi, & Webb, 1992). In skin cancer, GA and its precursor (1,4)-glucarolactone were shown to inhibit certain preliminary skin cancer biomarkers (Boone, 1992).

It is possible that chemopreventive properties of citrus may be attributed to the synergistic effects of two groups of anticancer agents, GA and limonoids. The possible mode of anticancer action may be through induction of phase II enzymes such as, glutathione S-transferase (GST), NAD(P)H: quinone reductase (QR), and UDP-glucuronosyltransferase (UDPGT). The objective of this investigation is to evaluate the potential of various structurally different citrus limonoids to induce phase II enzymes GST and QR in A/J female mice. Additionally, an analytical method for the quantification of D-glucaric acid in grapefruit varieties was also developed.

CHAPTER II

LITERATURE REVIEW

Chemoprevention potential of fruits and vegetables has been a topic that has captured the attention of researchers and consumers alike. The concept of chemoprevention is based on the potential biological activities of pharmaceuticals or dietary constituents which can inhibit or reverse the process of carcinogenesis (Walaszek, Hanausek, & Slaga, 2004). Carcinogenesis is a multi-step process consisting of three critical stages: initiation, promotion, and progression. The first step, initiation of carcinogenesis is a process, in which xenobiotics and some endogenous agents cause the damage of cellular DNA. The damaged DNA leads to the activation of oncogenes and inactivation of tumor suppressor genes, promotion. The promotion phase is a process that occurs over a long period of time and is usually reversible if detected early. The final stage of carcinogenesis is the progression phase. In this phase, damage experience on DNA in the earlier phases cause benign tumor to convert to malignant tumors which are capable of affecting surrounding tissues and metastasizing to distant sites (Walaszek, Hanausek, & Slaga, 2004). In order for bioactive compounds to be chemopreventive, one or more of the stages of carcinogenesis has to be blocked and or suppressed. Factors involved in these processes include, phase I enzymes, oxidative enzyme pathways, induction of phase II detoxifying enzymes, scavenging of reactive metabolites, altering DNA repair mechanism, inhibition of cell proliferation and inhibition of inflammation, and modulation of cell differentiation and apoptosis (Hanausek, Walaszek, & Slaga, 2003).

2.1. Citrus limonoids

Two decades ago, limonoids received more attention by the citrus industry due to their impact on bitterness in juice. During early investigations, limonin, was considered the more bitter compound, which caused a major concern in the juice industry and led to development of debittering techniques. In later studies, other limonoids, nomilin and obacunone, were also found to be responsible for the bitterness of citrus products. Currently, 37 limonoid aglycones and 17 limonoid glucosides have been identified (Roy, & Saraf, 2006). Limonoids are a group of highly oxygenated, terpenoids found in great proportion in Meliaceae and Rutaceae plants (Roy, & Saraf, 2006). Limonoids are present as secondary metabolites in plants which are reported to have significant antifeedant activity (Ruberto, Renda, Tringali, Napoli, & Simmonds, 2002). The typical limonoid structure contains mainly a 4,4,8-trimethyl-17-furanylsteroid skeleton (Fig 1). Furthermore, citrus limoniods are found in aglycone (bitter) and glucoside (tasteless) forms (Hasegawa, 2000)

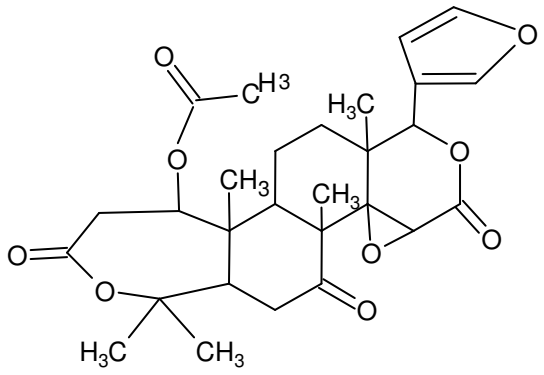

Fig.1. Structure of nomilin as an example of the typical limonoid structure

The biosynthesis of citrus limonoids is achieved in the phloem region via the terpenoid biosynthestic pathway (Ou, Hasegawa, Herman, & Fong, 1988). It is postulated that nomilin is the precursor of all of the limonoids isolated from *Citrus.* Nomilin, upon biosynthesis, is translocated to other parts of the plants, leaves, fruit, peels, and seed. Further, nomilin is converted to other limonoid aglycones by several pathways: limonin, calamine, ichangensin, and 7-acetate limonoid pathways (Hasegawa, Herman, Orme, & Ou, 1986). Each of these pathways yields a variety of different limonoids. In this study, we utilized limonoids produced by the limonin pathways. During maturation, these limonoid aglycones are converted to 17-β-D-glucopyranoside derivatives, mainly in fruit tissues and seeds (Hasegawa, 2000). The conversion of aglycones to glucosides is carried out by UDP-D-glucose:limonoid glucosyl transferase (Hasegawa, Suhayda, Hsu, & Robertson, 1997). Evidence suggests that the level of this enzyme is higher in sweet oranges as opposed to pummelo and its hybrids which are bitter even in late season (Ohta, & Hasegawa, 1995; Hsu, Berhow, Robertson, & Hasegawa, 1998). Commercially sold citrus juices contain low levels of limonoid aglycones, but high levels of limonoid glucosides. For example, glucoside levels in orange juice, grapefruit juice, and lemon juice are 320, 195 and 90 ppm, respectively (Lam, Li, & Hasegawa, 1989).

2.2. Citrus limonoids and potential cancer prevention

Accumulating results from decades of research provide conclusive evidence about the chemopreventive properties of citrus limonoids. Nomilin has been observed to reduce the number of mice with benzo[a]pyrene (BP)-induced tumors from 100-72%,

additionally the number of tumors per mouse was also decreased. Furthermore, limonin was less active as a chemopreventive agent compared to other limonoids. In another model of cancer protection, limonin and nomilin were also tested in the protection against 7,12-dimethylbenzy[a]anthracene (DMBA)-induced buccal carcinogenesis (Miller, Fanous, Rivera-Hidalgo, Hasegawa, & Lam, 1989). Interestingly, limonin, in the DMBA induced carcinogenesis model, was reported to reduce tumor burden by 60%, while nomilin was less inhibitory.

Early experiments involving cancer prevention in animal models have proposed that nomilin, in general, is a more potent inhibitor than limonin. Limonin on the other hand, was observed to be a potent inhibitor in the promotion phase of carcinogenesis (Lam, Hasegawa, Bergstrom, Lam, & Kenney, 2000). Nomilin was found to reduce the number of benzo[a]pyrene induced fore stomach tumors by 50%, to inhibit carcinogenesis induced by DMBA in a two stage model, and to inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced tumorigenesis by 36%. Furthermore, limonin in the two stage model was observed to inhibit the promotion of carcinogenesis by TPA.

As citrus limonoid research progressed, new limonoids were reported (Miller, Porter, Binnie, Guo, & Hasegawa, 2004). In this study, new limonoids, ichangensin, obacunone, and deoxylimonin, were examined to determine their effect on the inhibition of DMBA induced oral tumors. The activity of these limonoids was compared to the previously reported nomilin and limonin. Data from this study showed that, obacunone reduced tumor number (25%) and burden (40%). In addition, deoxylimonin, lacking the

epoxide group on the D-ring, also reduced tumor number and burden by 30% and 50 %, respectively. Data from initial studies showed that an intact A ring on the limonoid structure is essential for chemoprevention (Miller, Taylor, Berry, Zimmermann, & Hasegawa, 2000). Furthermore, it was observed that modifications to the D-ring did not affect biological activity (Miller, Taylor, Berry, Zimmermann, & Hasegawa, 2000, Miller, Porter, Binnie, Guo, & Hasegawa, 2004).

In addition to animal studies, citrus limonoids have also been tested on human cancer cell lines. Several citrus limonoids were inhibitory to the proliferation of estrogen receptor negative (MDA-MB-435) and receptor-positive (MCF-7) breast cancer cells (Guthrie, Morley, Hasegawa, Manners, & Vandenberg, 2000). Citrus limonoid glucosides were proven to be lethal at micromolar concentrations for neuroblastoma cells in culture, while normal cells were not affected (Poulose, Harris, & Patil, 2005). Furthermore, limonoid agylcones were shown to significantly shorten viability of cancer cell lines SH-SY5Y neuroblastoma and Caco-2 colonic adenocarcinoma, while non-cancerous Chinese hamster ovary cells did not showed any change in cell numbers or morphology (Poulose, Harris, & Patil, 2006). In a previous study, several human cancer cell lines were used to evaluate the effects of citrus limonoids. In this study, specific limonoids showed cytotoxicity on MCF-7 breast cancer cells (Tian, Miller, Ahmad, Tang, & Patil, 2001).

2.3. Glutathione S-transferase

Chemoprevention by citrus limonoids has been repeatedly attributed to the induction of GST activity. A study in the Netherlands evaluated the effect on habitual

consumption of fruits and vegetables on rectal GST. It seems that consumption of citrus fruit was positively correlated with human rectal GST activity (Wark et al., 2004). Several studies have attributed the induction of these enzymes to the structural components of limonoids (Kelly, Jewell, & O'Brien, 2003; Lam, Li, & Hasegawa, 1989; Lam, Sparnins, Wattenburg, 1982). In 1989 alone, three reports were published on chemoprevention by citrus limonoids. Lam and Hasegawa (1989) reported the use of citrus limonoids, nomilin and limonin, as chemopreventive agents in ICR/Ha mice. Nomilin was observed to induce GST activity 2.48 to 3.44 times in liver and 3.00 to 4.17 in intestine compared to the control. The limonin treatment group showed only slight induction of GST capabilities. Due to the fact that some induction of GST activity was observed by citrus limonoids, eight limonoids of varying structural variation were analyzed in mice. The most induction of GST activity was observed in the cytosol of the small intestine mucosa of the nomilin treated mice followed by obacunone, isoobacunoic acid, and ichangin (Lam, Li, & Hasegawa, 1989). Other limonoids tested in this study (limonin, limonol, and deoxylimonin), were mild or inactive GST inducers. Further studies demonstrate that dietary obacunone elevates GST and QR in liver significantly against azoxymethane (AOM)-induced aberrant crypt foci at 4 weeks, while limonin increase only QR Activity (Tanaka et al., 2000). GST induction activity was later tested using a nomilin, limonin and aglycone mixture (Ahmad, Li, Polson, Mackie, Quiroga, & Patil, 2006). In the stomach, all three limonoids increased GST activity, while in the liver only nomilin induced GST activity. Results from this study provided supporting evidence for Lam, Li, & Hasegawa's (1989) previous findings, that protection against

chemical carcinogenesis is prominent in the stomach. Recent animal studies reported that limonoids affect induction of GST activity in a dose-dependent manner (Kelly, Jewell, & O'Brien, 2003). Data showed that GST activity increased in a dose dependent fashion in the liver in limonin and nomilin fed animals, while only nomilin increased GST activity in the small intestine.

The furan moiety has been thought to be one of the components responsible for induction of GST activity (Lam, Li, & Hasegawa, 1989). Two furan containing diterpenes, kahweol and cafestol, isolated from green coffee beans were shown to induce GST activity in various tissues in mice (Lam, Sparnins, Wattenburg, 1982). In a later study, eight citrus limonoids were tested for induction of GST activity in mice. In this study, the authors looked at the structure-function relationship of different citrus limonoids and their effect on GST activity (Lam, Li, & Hasegawa, 1989).

GSTs are thought to play a curial physiological role in the initial stages of detoxification of potential alkylating agents (Booth, Boyland, & Sims, 1961). GST enzymes catalyze the adduct formation of glutathione (GSH), a tripeptide consisting of glycine, glutamic acid, and cysteine, with electrophilic xenobiotics (Beutler, Duron, & Kelly, 1963). GST catalyzes the reaction of xenobiotic and other environmental factors to react with the –SH of glutathione, neutralizing the toxin and making it more water soluble (Habig, Pabst, & Jacoby, 1974). GST enables the conjugation of GSH to electrophilic toxins. An increase in the activity of GST usually enhances the ability of an organism to detoxify carcinogens. Therefore, any substance that increases the activity of GST may be a potential anti-carcinogen with the ability to inhibit chemically induced

cancer formation (Lam, Li, & Hasegawa, 1989; Hahn-Obercyger, Stark, & Madar, 2005). In 2005, a study was completed on the effect of grapefruit and oroblanco on hepatic detoxification in rats. The data from this study indicated that citrus consumption has a positive impact on phase II enzymes glutathione S-transferase and NAD(P)H: quinone reductase (Hahn-Obercyger, Stark, & Madar, 2005).

2.4. NAD(P)H: quinone reductase

NAD(P)H: quinone reductase (QR) is important in the protection against toxic and neoplastic effects of xenobiotics. QR catalyses the two electron reduction of quinones to protect cells from free radicals and reactive oxygen species that arise from one electron reduction. Quinones are toxic products of oxidative metabolism of aromatic hydrocarbons (Horning, Thenot, & Helton, 1978). The reduction of quinones can lead to their detoxification or to the production of biologically reactive products depending upon the mechanism of reaction and subsequent deposition of hydroquinone products (Benson, Hunkeler, & Talalay, 1980). In a previous study, nimbolide, a limonoid isolated from neem flowers, showed induction of QR activity in Hepa1c1c7 cells (Sritanaudomchai et al., 2005). Furthermore, Tanaka et al. (2000) have reported the induction of QR by certain citrus limonoids.

2.5. UDP-glucuronosyltransferase

In the case of, UDP-glucuronosyltransferase, a different approach is taken. UDP-glucuronosyltransferase (UDPGT) catalyzes a biosynthetic reaction called glucuronidation. Glucuronidation, a major drug metabolizing reaction, increases the polarity of xenobiotic agents by conjugating the electrophile with glucuronic acid. The

product of this conjugation reaction is then excreted in bile or urine as glucuronides, thus potential carcinogenic agents from the body are removed (Miner & Mackenzie, 1991; Shipkova & Wieland, 2005). Glucuronidation leads to significant reduction in the toxic or pharmacological activity of electrophilic substances (Walaszek, 1990). Glucuronides are then excreted in urine or bile. As bile travels through the intestinal track, it can become a substrate for β-glucuronidase. Interestingly, β-glucuronidase counteracts the action of UDPGT by de-conjugating the xenobiotic back to its electrophilic state, allowing its re-absorption and enterohepatic circulation (Walaszek, 1990). In de-glucuronidation, the action of the UDPGT is reversed. β-glucuronidase deconjugates the compound bound to glucuronic acid making it available to interact or be transported to other areas in the biological system (Dwivedi, Downie, & Webb, 1987). Furthermore, β-glucuronidase is known to be highly inhibited by (1,4)-glucarolactone. At physiological pH, glucaric acid is in equilibrium with (1,4)-glucarolactone and (6,3)-glucacrolactone (Horton & Walaszek, 1982), but when glucaric acid is orally administered it is converted to (1,4)-glucarolactone (Walaszek, Hanausek-Walaszek, Minton, & Webb, 1986). (1,4)-Glucarolactone does not directly interact or modify the activity of UDPGA, but inhibits the deconjugation of xenobiotics, therefore increasing the net glucuronidation. *In vivo*, metabolism of glucaric acid to (1,4) glucolactone has been shown to increase the detoxification of carcinogens and inhibit chemical carcinogenesis by inhibiting β-glucuronidase (Lampe, Li, Potter, & King, 2002).

2.6. D-glucaric acid

Glucaric acid, or D-glucarate in its salt form, is a non-toxic natural compound produced in small amount in mammals and by a few plants (Walaszek, Szemaraj, Hanausek, Adams, & Sherman, 1996). Glucaric acid is an end metabolite of the D-glucuronic acid pathway metabolism in mammals (Marsh, 1963). The product of the oxidation of D-glucuronic acid yields glucaric acid (GA), D-glucaro-1,4-lactone (1,4-GL) and D-glucaro-6,3-lactone (6,3-GL). These compounds have been observed to be excreted normally in human urine (Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). The excretion was used as an indirect indicator of hepatic microsomal enzymes induction by exogenous chemicals or xenobiotics (Dutton, 1980). GA has also been measured in fruits and vegetables (Dwivedi, Heck, Downie, Larroya, & Webb, 1990; Walaszek, Szemaraj, Hanausek, Adams, & Sherman, 1996). Several studies have evaluated the health aspect of GA on various biological systems. Many of these studies observed that the consumption of dietary GA was an effective chemoprevention strategy. Researchers attributed this activity to the effect of 1,4-GL on glucuronidation (Dwivedi, Heck, Downie, Larroya, & Webb, 1990). Glucuronidation is a process in which phase II enzyme UDPGT conjugates xenobiotics making them more polar by addition of a glucuronide moiety in order to facilitate their expulsion from the body, therefore removing toxins from the body (Shimoi & Nakayama, 2005; Shipkova & Wieland, 2005). The conjugated products are then excreted in bile or urine. In some instances, the conjugated products are deconjugated by β-glucuronidase (Dwivedi, Downie, & Webb, 1987). In the deconjugation process, or deglucuronidation, β-glucuronidase hydrolyses

the glucuronide moiety, therefore reintroducing the toxin into circulation throughout the body (Speaker, Backman, & Kroemer, 1997). Several studies have shown that 1,4-GL is a potent inhibitor of β-glucuronidase (Levvy, 1952; Marsh, 1963; Kushinsky & Chen, 1967; Marselos, Dutton, & Hanninen, 1975; Narita, Nagai, Hagiwara, Aburada, Yokoi, & Kamataki, 1993). Since both GA and its precursor, 1,4-GL, are present in fruits and vegetables, dietary modulation of β-glucuronidase was investigated (Lampe, Li, Potter, & King, 2002). It seems that an inverse association of serum β-glucuronidase activity and plant-food intake in humans exists.

2.7. D-glucaric acid chemistry and biological activities

In 1949, the inhibitory properties of D-glucarate on animal β-glucuronidase were investigated. In this study, it was found that GA strongly inhibited the activity of β-glucuronidase (Karunairatnam & Levvy 1949). In the following year, several studies were published on the extent of inhibition of GA on β-glucuronidase (Karunairatnam, Kerr, & Levvy, 1949; Karunairatnam & Levvy 1951, Mills, Paul & Smith 1953; Campbell, 1949). In 1952, a report by G.A. Levvy stated that the compound actually responsible for the inhibition of β-glucuronidase was saccharo-1,4-lactone (1,4-GL). 1,4-GL was found to be a very powerful inhibitor of β-glucuronidase having an affinity 240 times greater than that of phenolphthalein glucuronide, a substrate with the highest know affinity for β-glucuronidase at the time.

Following these findings researchers began investigating how these interactions between enzymes and compounds could be used. In 1957, Boyland, Gasson, & Williams deduced that orally administering 1,4-GL to men exposed to carcinogenic aromatic

amine would reduce the amount of free carcinogens which were released by the action of β-glucuronidase against conjugation. A later study in Japan, reported that 1,4-GL, was absorbed through the intestinal tract and distributed throughout the body when administered orally (Kiyomoto, Harigaya, Ohshima, & Morita, 1963). The bioavailability of 1,4-GL was also reported, 10-20 % of the orally administered lactone was excreted in urine and more than half of the lactones remained unchanged after a few hours post administration. Studies carried out by Charles Marsh in the early to mid 1960's highlighted the importance of the inhibition of β-glucuronidase by 1,4-GL (Marsh, 1963; Marsh & Reid, 1963; Marsh & Carr, 1965). The results of Marsh's work demonstrated that GA and its precursors were normal constituents of urine. These constituents were present in small amounts in the liver and other tissues. The inhibition of β-glucuronidase also sparked the curiosity of other researchers on how different parameters might influence the activity of 1,4-GL on enzymatic activity. In 1967, a study was carried out analyzing the inhibition of β-glucuronidase in bovine liver. It was found that the interconversion between GA and the inhibitory 1,4-GL was dependent on pH, temperature and time. Furthermore, the pH optima of β-glucuronidase changed in the presence of 1,4-GL (Kushinsky & Chen, 1967).

Reports of the structure of GA and its precursors were initially produced in the late 1970's– early 1980's. In 1976, a report was published on the crystalline structure of 1,4-GL monohydrate (Gress & Jeffrey, 1976). The main focus of this study was to elucidate the structural information of 1,4-GL in a crystalline state as compared to in solution. In a later study, the conformation of both GA and its lactone were studied by

the use of nuclear magnetic resonance (NMR) spectrometry (Horton & Walaszek, 1982). It was demonstrated that GA and its lactones (1,4-GL and 6,3-GL) are in equilibrium in solution.

Now that the presence of GA in urine was proven and information on the chemical behavior of GA and its precursor was available, Charles Marsh reported the biosynthesis of GA in mammals. His earlier work showed that GA is excreted at a rate of 30-100 μmol per day (Marsh, 1985). In 1986, Marsh reported that in the presence of iron salts and hydrogen peroxide, D-Glucuronic acid was converted into GA. The reaction inhibited by free-radical scavengers, is pH dependent and occurs in the presence of some iron complexing agent. After oxidation, the first product observed was a lactone presumed responsible for the inhibition of β-glucuronidase (Marsh, 1986). Accumulative evidence on the inhibition of β-glucuronidase provided many researchers the knowledge to further investigate the role of GA in cancer prevention.

2.8. D-glucaric acid and potential cancer prevention

Several studies, reviewed in later sections, have termed dietary D-glucarate as a chemoprotective agent with strong supporting evidence. The chemopreventive property that has been linked to D-glucarate is due to the ability of this compound to affect the overall efficiency of phase II enzyme UGP-glucuronosyltranferase (UDPGT) by preventing de-glucuronidation. It has been hypothesized by many studies that by modulating the activity of β-glucuronidase the net glucuronidation is increased, leading to the efficient removal of toxins, carcinogens, and other carcinogenic substances. In 1952, Levvy published that 1,4-GL is a potent inhibitor of mammalian β-glucuronidase

(Levvy, 1952). Later studies showed that β-glucuronidase was higher in patients with bladder cancer (Boyland, Gasson, & Williams, 1957). These results plus the fact that 1,4-GL shortened the pharmacologic action of drugs (Marselos, Dutton, & Hanninen, 1975), lead to theory that GA or its derivatives could be used as chemopreventive agents. The inhibition of β-glucuronidase by 1,4-GL was also observed to repress marker oncofetal protein, which has been observed to be correlated with the appearance of pre-neoplastic foci (Walaszek, Hanausek-Walaszek, & Webb, 1988).

Fruits and vegetables have been reported to contain high levels of D-glucarate (Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996; Dwivedi, Heck, Downie, Larroya, & Webb, 1990). In both the of these studies, various fruits and vegetables were examined using several of the previously described methods for the evaluation of GA. Interestingly, in both studies citrus fruits reported the highest values. Since previous studies have evaluated the oral administration of GA in animals and the presence of GA in fruits and vegetables, the concept of GA acquisition by dietary means for cancer prevention was proposed. It was later, shown that the inhibitory 1,4-GL was formed in the acidic conditions of the stomach, when GA salt was orally administered (Walaszek et al., 1997). Furthermore, it was also shown that 1,4-GL was absorbed through the intestine, transported within the blood to different organs, and finally excreted in urine. Reports on the effect of GA on detoxification and its presence in fruits and vegetables, enticed researchers to investigate the correlation between plant-food intake and levels of serum β-glucuronidase (Lampe, Li, Potter, & King, 2002). This study reported an

inverse association with the consumption of plant-food and β-glucuronidase levels, suggesting the possibility of enhancing physiological well being by dietary means.

GA has proven to be effective in the prevention if various types of cancer, but overall, breast cancer has been the most studied. Initial studies showed, that 1,4-GL exhibited a 70% reduction in the number of rats with mammary tumors induced by 7,12-dimethylbenzanthracene (DMBA), 72% decrease in the number of mammary tumors per rat, and a reduction in the induction of oncofetal proteins (Walaszek, Hanausek-Walaszek, & Webb, 1984). Using the same model, further reports on the use of calcium D-glucarate for the prevention of mammary tumorigenesis were published (Walaszek, Hanausek-Walaszek, Minton, & Webb, 1986). In a later study, rats fed GA for 2-4 weeks showed inhibited β-glucuronidase activities leading to a marked antiproliferative effect in mammary epithelium tissue (Walaszek, Hanausek, Sherman, & Adams, 1990). Furthermore, When GA and *N*-(4-hydroxyphenyl) retinamide were administered independently a 50-65% inhibition of tumor incidence and tumor multiplicity was observed, similar observations were noted when a combination of GA and *N*-(4-hydroxyphenyl) retinamide was administered (Abou-Issa, Duruibe, Minton, Larroya, Dwivedi, & Webb, 1988). Additionally, It was later shown that *N*-(4-hydroxyphenyl) retinamide used in combination with GA suppressed the growth of MCF-7 human breast cancer cells *in vitro* (Bhatnagar, Abou-Issa, Curley, Koolemans-Beynan, Moeschberger, & Webb, 1991). It was later reported that the basis for the chemopreventive properties of these compounds used individually and/or in combination is that they raise cellular cAMP levels, repress protein kinase C and inhibit DNA synthesis (Abou-Issa et al.,

1993). Furthermore, it was later demonstrated that when GA or *N*-(4-hydroxyphenyl) retinamide were administered during the promotion phase of DMBA induced tumorigenesis, there was increased tumor latency while tumor multiplicity was decreased (Abou-Issa, 1995). In addition, it was also observed that when fed during initiation or promotion separately or throughout both phased continually, GA and *N*-(4-hydroxyphenyl) retinamide were observed to reduce tumor incidence compared to the control.

Besides breast cancer, the effects of GA on other types of cancer has also been previously studied. GA was initially tested for chemopreventive properties against bladder cancer (Boyland, Wallace, Avis, & Kinder, 1964; Boyland, Kinder, Manson, & Wallace, 1965). Additionally, the role of GA on colon cancer was also explored (Takada, Hirooka, Hiramatsu, & Yamamoto, 1982; Dwivedi, Oredipe, Barth, Downie, & Webb, 1989; Yoshimi, Walaszek, Mori, Hansusek, Szenraj, & Slaga, 2000). Chemopreventive properties of GA were also reported on skin, liver and lung cancer (Dwivedi, Downie, & Webb; 1989; Gupta & Singh, 2004; Walaszek, Hanausek, Narog, Zoltaszek, & Slaga 2005; Oredipe, Barth, Hanausek-Walaszek, Sautins, Walaszek, & Webb, 1987; Oredipe, Barth, Dwivedi, & Webb, 1992; Walaszek, Hanausek, Zoltaszek & Slaga, 2004; Hanausek, Walaszek, Szemraj, Zoltaszek, & Slaga, 2004). Interestingly, phase I clinical trials initiated by Walaszek, Hanausek, Narog, Raich, & Slaga (2004) found that increased doses of GA consistently suppressed β-glucuronidase levels and that at highest concentrations calcium D-glucarate was well tolerated with no signs of toxicity.

2.10. Analytical methods for D-glucaric acid

Early research demonstrated that GA was found in urine and that its precursor, 1,4-GL, was a potent β-glucuronidase inhibitor. These findings led to a need to quantitatively determine the amount of GA in bodily fluid, mainly urine and blood serum. A quantitative method for the determination of GA in urine was initially reported by Marsh & Reid in 1963. This method consisted of boiling of GA to yield the highly specific β-glucuronidase inhibitor 1,4-GL. A later study by a group of Japanese scientists reported that, even though 1,4-GL is highly specific, many other compounds present in urine also inhibit β-glucuronidase activity (Ishidate, Matsui, & Okada, 1965). In this study, quantification of GA using chemical determination was achieved which involved the treatment of urine with ion exchange chromatography, then the eluate was oxidized with periodic acid and the product was subjected to colorimetric determination. In later investigations, a method was developed using gas-liquid chromatography of the silylated derivatives of GA, but in this study GA was determined to the 0.1μg level (Gangolli, Longland, & Shilling, 1974). In a study analyzing lactone-forming acid in succulent plants, thin layered chromatography was employed to identify GA content of the plants. This method involved the isolation of the acid from the plant material, pretreatment of the TLC plate with a mixture of solvents, detection by either UV light or Hydroxylamine-ferric chloride and bromophenol blue, and finally heating for one hour at 105°C (Kringstad & Nordal, 1975). While new methods were emerging, Marsh's 1963 method was still the most widely used to evaluate the GA content in urine and serum. Several investigations were published from 1965-1977 citing this method (Aarts,

1965; Hunter, Maxwell, & Williams, 1971; Davidson, McIntosh, & Ford, 1974; Latham, 1974; Smith & Rawlins, 1974; Herzberg, Tenenbaum, Fishel, & Wiener, 1977; Simmons, Davis, Dordoni, & Williams, 1974; March, Turner, Shanley, & Field, 1974). Thereafter, a new method was proposed that permitted the determination of GA with a linear calibration curve over a wide range versus the logarithmic calibration curve proposed by Marsh (Jung, Scholz, & Schreiber, 1981). Unfortunately, the previously published methods were laborious and in many instances lacking reproducibility and accuracy. In 1983, a new low-pH method was proposed by a group of Italian scientists. Using the knowledge of the equilibrium of GA and its lactones in solution, Marsh's enzymatic method was evaluated at various pH and found that by boiling GA at pH 3.8 inhibition of β-glucuronidase was greater. This change in pH allows the GA/lactone equilibrium to remain constant during the enzymatic assay (Colombi, Maroni, Antonini, Cassina, Gambini, & Foa, 1983). Interestingly, during the same year, two other methods were published describing use of high performance liquid chromatography (HPLC). While one of these HPLC methods combined the use of ion exchange chromatography, radioactivity monitoring, and UV absorption in urine (Walters, Lake, Bayley, & Cottrell, 1983), the other method described the separation of GA and its lactones from an aqueous solution (Laakso, Tokola, & Hirvisalo, 1983). Unfortunately, separation of GA was not effective enough to quantitatively determine the amount of GA present in urine from the previous methods.

In the mid 1980's Marsh's enzymatic method was still the most widely used method for the determination of GA in urine. Over time, the method was continually

modified for higher repeatability and ease. In 1984, Marsh's method was again optimized into a micro-method. The optimization is on the amount of samples that can be processed by a person per day, from 50 to 60 determinations in one day (Jung & Pergrande, 1984). Both enzymatic and chemical methods had advantages and disadvantages. In 1985 Marsh developed an enzymatic method for the determination of GA by converting it to pyruvate (Marsh, 1985). The theory behind this method is that GA, as a substrate for a dehydratase present in several bacteria, produces a mixture of glucarate derivatives that are then converted to pyruvic acid and tartronic acid by an aldolase enzyme. Since the measurement of pyruvic acid is a common practice, it seemed to be adequate for the spectrophotometric determination of GA.

The most prevalent methods for the determination of GA seemed to be the enzymatic method and the glycoxylic acid method. While comparing the results of these methods, it was observed that the enzymatic method is preferable. The enzymatic method is less subject to interference and less labor intensive compared to the glyoxylic acid method (Steinberg & Needham, 1986). Furthermore, a new method was proposed by Paolo Mocaraelli in 1988, which averaged about 100 determinations per day and still retained the precision and accuracy of previous enzymatic methods, with the added bonus of being suitable for automated instruments (Mocaraelli et al., 1988). This new method made the process more adequate for use in clinical settings. In 1993, a simple HPLC method for the determination of GA in urine was proposed (Poon, Villenueve, Chu, & Kinach, 1993). This simple isocratic HPLC method consisted of the treatment of urine with a gel that has a high affinity for GA, separating it from other substances.

Interestingly, in 1998, a group of cancer researchers reinforced the use of the pyruvate assay for the analysis of blood serum of breast and prostate cancer patients. The authors stated that the uses of other methods are not as sensitive for the quantification of GA as the pyruvate method (Walaszek, Hanausek, Szemraj, & Adams, 1998).

Due to increase in cancer incidence and the fact that previous studies correlate the consumption of dietary GA and cancer prevention, two studies were published concerning the content of GA in food. The presence of GA in plants has previously been reported (Kringstad & Nordal, 1975; Risch, Herrmann, & Wray, 1988). In 1990, a study evaluated the effect of GA on β-glucuronidase activity and the GA content in fruits and vegetables (Dwivedi, Heck, Downie, Larroya, & Webb, 1990). The fruits and vegetables were analyzed by the enzyme inhibition method and confirmed by HPLC. The results of this study indicated that orange contained the highest levels of GA (4.64mg/100g), followed by spinach, apples, carrot, alfalfa sprouts potatoes and broccoli. Later, researchers form MD Anderson Cancer Center, reevaluated the GA content of various fruits and vegetables and analyzed the cholesterol lowering effects of GA in rats (Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). In this study, both the enzymatic and the pyruvate method were used and found that grapefruit and apple had the highest levels of glucarate, 3.60 and 3.45 g/kg respectively, while in vegetables edible cactus and alfalfa sprouts contain high GA levels 3.49 and 3.45 g/kg, respectively.

CHAPTER III

ORGAN SPECIFIC INDUCTION OF PHASE II ENZYMES BY CITRUS LIMONOIDS IN MOUSE

3.1. Synopsis

Several cell culture and animal studies demonstrated that citrus limonoids have protective effects against certain types of cancer. These chemopreventive properties of citrus limonoids are believed to be attributed to the induction of phase II enzymes, in particular, glutathione S-transferase (GST). In the current study, the inductive effects of purified limonoids and their mixture have been investigated for GST and NAD(P)H: quinone reductase (QR) activities. Female A/J mice were treated with different citrus limonoids. Among the limonoids tested, the highest induction of GST against 1-chloro-2,4-dinitrobenzene (CDNB) was observed in stomach, 58% by nomilin, followed by isoobacunoic acid (25%) and deacetylnomilin (19%). Deacetlylnomilin in intestine as well as liver and a mixture of limonoids in liver demonstrated a significant reduction of GST activity against CDNB. Furthermore, induction of GST against 4-nitroquinoline 1-oxide (4NQO) differs for each limonoid. Nomilin showed significant induction of GST in intestine (280%) and stomach (75%) while deacetylnomilin showed significant induction only in intestine (73%) compared to control. Furthermore, a limonoid mixture significantly induced GST activity in intestine (93%) and stomach (45%). Finally, significant induction of QR activity was observed by isoobacunoic acid in liver (230%) followed by a limonoid mixture in stomach (200%) and deacetylnomilin in intestine

(22%). While limonoids statistically induced both GST and QR activity ($p<0.05$) in specific organs, certain limonoids also showed a reduction of these phase II enzyme activities. Our findings indicate that the structural differences in the limonoids may provide specificity for induction or reduction of phase II enzyme activity. This kind of organ specificity related to the structural differences of limonoids indicates potential of these compounds in prevention of cancer. Dietary intake of citrus limonoids may provide a powerful tool against the onset of various cancers.

3.2 Introduction

Citrus is the most widely consumed fruits in the world and found to possess important beneficial effects on human health. Citrus fruits have long been recognized to contain secondary metabolites including flavonoids, vitamin C, furocoumarins, carotenoids, folate, pectin, and limonoids (Manners, Jacob, Breska, Schoch, & Hasegawa, 2003). Limonoids are a group of chemically related triterpene derivatives. Many studies conducted in our laboratory and elsewhere, using limonoids, provided evidence of their protective properties in cancer prevention and cardiovascular diseases (Miller, Porter, Binnie, Guo, & Hasegawa, 2004; Kurowska, Hasegawa, & Manners, 2000a; Battinelli et al., 2003; Miyagi, Om, Chee, & Bennink, 2000; Tanaka et al., 2000; Vanamala et al., 2006; Miller, Fanous, Rivera-Hidalgo, Binnie, Hasegawa, & Lam, 1989). Citrus limonoids have been reported to lower cholesterol by reducing the production of medium apo B as seen in cultured human liver cells HepG2 (Kurowska, Hasegawa, & Manners, 2000a). Two citrus limonoids (limonin and nomilin) have been evaluated for their ability of inhibiting HIV-1 replication (Battinelli et al., 2003). During

the last decade, the health maintaining properties of citrus limonoids has been focused in anti-cancer properties using cell and animal models. In two Japanese studies, it was reported that orange juice and citrus limonoids, obacunone and limonin, played an important role in the inhibition of azoxymethane-induced colon cancer (Miyagi, Om, Chee, & Bennink, 2000; Tanaka et al., 2000). A recent study provided evidence that limonin, naringin, and whole grapefruit protect against azoxymethane (AOM) induced aberrant crypt foci (Vanamala et al., 2006). Inhibition of 7,12-dimethylbenz[a]anthracene- induced oral tumors by citrus limonoids has been the focus in our collaboration efforts (Miller, Porter, Binnie, Guo, & Hasegawa, 2004; Miller, Fanous, Rivera-Hidalgo, Binnie, Hasegawa, & Lam, 1989). Additionally, benzo[a]pyrene induced forestomach neoplasia in mice was inhibited by citrus limonoids (Lam & Hasegawa, 1989).

Recently cell culture studies from our laboratory have investigated the effects of citrus limonoids (Poulose, Harris & Patil, 2005; Poulose, Harris, & Patil, 2006; Tian, Miller, Ahmad, Tang, & Patil, 2001). In one study, citrus limonoid glucosides were proven to be lethal at micromolar concentrations for neuroblastoma cells in culture (Poulose, Harris & Patil, 2005). Furthermore, limonoid aglycones were shown to significantly reduce viability of SH-SY5Y neuroblastoma and Caco-2 colonic adenocarcinoma. The non-cancerous Chinese hamster ovary cells hardly showed any change in cell numbers or morphology when treated with the limonoids (Poulose, Harris, & Patil, 2006). Additionally, reports from our laboratory showed that the cytotoxic

effect of the specific limonoids tested on MCF-7 cells may have an effect limited to breast cancer cells (Tian, Miller, Ahmad, Tang, & Patil, 2001).

Over the past two decades, several articles have reported isolation of citrus limonoids from seeds, peels, fruit, juice and other by-products (Rouseff & Nagy, 1982; Bennet, Hasegawa, & Herman, 1989; Miyake, Ozaki, Ayano, Bennett, Herman, & Hasegawa, 1992; Ohta, Berhow, Bennett, & Hasegawa, 1992; Berhow, Omura, Ohta, Ozaki, & Hasegawa, 1994; Ozaki, Ayan, Inaba, Miyake, Berhow, & Hasegawa, 1995). Recently, more efficient methods have been reported for isolating a variety of limonoids, aglycones and glucosides, from citrus (Jayaprakasha, Brodbelt, Bhat, & Patil, 2006a; Raman, Cho, Brodbelt, & Patil, 2005; Jayaprakasha, Patil, & Bhat, 2006b). Limonoid glucosides are tasteless water soluble compounds formed in fruits during maturation from their corresponding aglycones. On the other hand, certain aglycones are bitter with low water solubility and principally found in seeds (Hasegawa, 2000). Citrus limonoids contain a furan moiety attached to the D-ring lactone at the 3- position. Studies on furan-containing natural products have suggested that the furan moiety is responsible for the induction of the phase II detoxifying enzyme glutathione S- transferase (GST) (Tian, Miller, Ahmad, Tang, & Patil, 2001). In 2003, Kelly, Jewell, & O'Brien reported an induction of GST activity when rats were fed limonin and nomilin. Potential induction of phase II enzymes, such as GST, using naturally occurring compounds has sparked an interest in the scientific community.

Gluthathione S-transferases are thought to play a crucial physiological role in the initial stages of detoxification of potential alkylating agents (Booth, Boyland, & Sims,

1961). An increase in the activity of GST usually enhances the ability of an organism to detoxify carcinogens. It has been reported that substances that increases the activity of GST can be a potential anti-carcinogen with the ability to inhibit chemically induced cancer formation (Lam, Li, & Hasegawa, 1989) Another phase II enzyme, which is also important in the protection against toxic and neoplastic effects of xenobiotics, is NAD(P)H: quinone reductase (QR). QR protects against cytotoxicity by catalysing the two electron reduction of quinones that arise from a one electron reduction. An increase in its level is positively correlated with chemoprevention (Benson, Hunkeler, & Talalay, 1980). Previous cell culture studies demonstrated that freeze dried grapefruit extracts induce both GST and QR activities (Williamson et al., 1997). A recent study also demonstrated that the consumption of citrus fruits modulate both phase I and phase II metabolizing enzymes in rats (Hahn-Obercyger, Stark, & Madar, 2005).

Accumulating evidence suggest that certain citrus limonoids have the ability to induce detoxifying enzymes, mainly GST (Tanaka et al., 2000; Miller, Fanous, Rivera-Hidalgo, Binnie, Hasegawa, & Lam, 1989; Lam & Hasegawa, 1989, Kelly, Jewell, & O'Brien, 2003; Lam, Li, & Hasegawa, 1989; Ahmad, Li, Polson, Makie, Quiroga, & Patil, 2006). In majority of these studies nomilin has proven to be the strongest inducer of GST activity against CDNB. While evaluating the induction of GST activity, CDNB is used as a general substrate, without exhibiting any substrate preference in GST isozymes α, μ, π (Ahmad, Tijerina, & Tobola, 1997). A later study showed that the specific activity of the α, μ, π isozymes varied widely when CDNB was used as a substrate (Townsend, Fields, Doss, Clapper, Doehmer, & Morrow, 1998). One study

suggests that the GST μ isozyme is more efficient in the conjugation of 4NQO (Aceto, Llio, Lo Bello, Bucciarelli, Angelucci, & Federici, 1990). Most major mammalian GST isozymes are grouped into four major classes namely, α, μ, π and θ (Mannervik & Danielson, 1988; Meyer, Coles, Pemble, Glimore, Fraser, & Ketterer, 1991). Due to the significant variability of GST isozymes in different tissues, it is important to evaluate the induction capabilities of citrus limonoids using substrate models other than CDNB.

In order to understand the role of the A and A' rings in the limonoids on biological activity, we analyzed the induction potential of specific citrus limonoids (fig. 2) with a closed A ring (nomilin, deacetylnomilin) and open A ring (isoobacunoic acid). Previous research suggest that modifications to the A and A' rings have significant differences in the ability to induce GST activity (Lam, Li, & Hasegawa, 1989). For example, ichangin with a closed A ring and an open A'ring, showed induction of GST activity (Lam, Li, & Hasegawa, 1989). The understanding of the relationship between the various structural properties of citrus limonoids and induction of phase II detoxifying enzymes is vital for cancer prevention. In this study, we intend to further elucidate the function of various structurally dissimilar citrus limonoids in the induction of phase II enzymes, GST and QR.

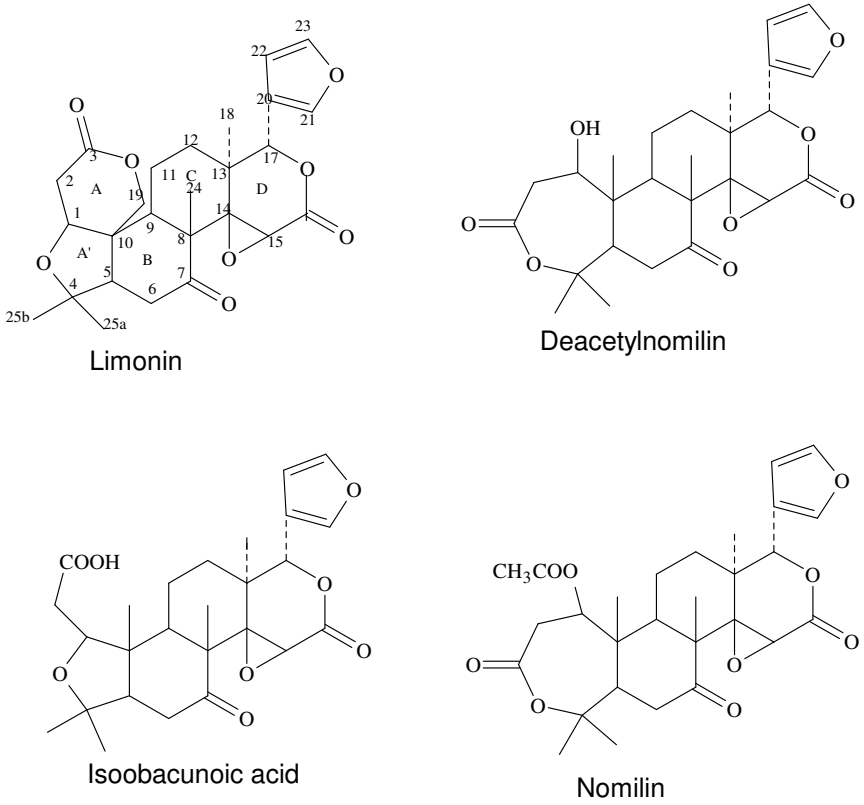

Fig. 2. Structures of citrus limonoids and mixture (limonin, nomilin, isoobacunoic acid) used in study

3.3. Materials and methods

3.3.1. Materials

All solvents / chemicals used in this study were obtained from Fisher Scientific (Houston, TX). β-NADPH reduced tetrasodium salt, 2,6-dichlorophenol-indophenol (DCPIP), 1-chloro-2,4-dinitrobenzene (CDNB), 4-nitroquinoline 1-oxide (4NQO), glutathione reduced (GSH), FAD disodium salt, and all solvents were purchased from Sigma-Aldrich company (St. Louis, MO). Bovine Serum Albumin (BSA) was obtained from Intergen (Purchase, NY). The citrus limonoids used in this investigation were isolated and purified in our laboratory.

3.3.2. Animal studies

Female A/JOlaHsd 8-9 weeks old mice were purchased from Harlan Sprague-Dawley Laboratory (Indianapolis, IN). The mice were kept on AIN-76 semi-purified custom diet without vitamin E obtained from MPBiomedical (Solon, OH) and tap water *ad libitum*. The mice were housed in plastic cages in an environmentally controlled room on a 12 h light / 12 h dark cycle.

The mice were divided (n=4) into four experimental groups and one control group. The treatments consisted of nomilin, deacetylnomilin, isoobacunoic acid and a limonoid mixture (limonin, nomilin, and isoobacunoic acid). Each sample (20 mg) was suspended in DMSO: corn oil (1:1) (v/v) and administered by oral gavage once every two days. The control group was given the corresponding DMSO: corn oil (1:1) treatment. A total of four treatments were administered. Forty eight hours after the last treatment the mice were sacrificed by cervical dislocation. Lung, intestine, stomach, and

liver were harvested immediately after sacrifice and washed with cold PBS. A portion of the tissue was stored for future use, while the remaining portion was weighed and homogenized using a Pro200 homogenizer with PBS (10mM), pH 7.0, containing β-mercaptoethanol (1.4mM) to obtain a 10% (w/v) homogenate. The cell extracts were centrifuged at 22,000 x g for 45 min in a refrigerated Beckman Avanti 30 centrifuge. Following centrifugation, the supernatant was carefully removed and stored in -20°C until further use.

3.3.3. Determination of enzyme activities and protein

GST activity against CDNB was determined by the slightly modified method developed by Habig, Pabst, & Jakoby (1974). This system involves the addition of GSH to CDNB, a nucleophilic aromatic substitution that occurs via an addition-elimination sequence involving a short lived σ-complex intermediate (Armstrong, 1997). The 1ml enzyme assay consisted of 100 mM phosphate assay buffer, pH 6.5; 20 mM CDNB and 10 mM GSH and 50 µl of tissue homogenate sample. The absorbance was measured at 340 nm in a Beckman DU 640 UV/Visible spectrophotometer against an appropriate blank.

GST activity against 4NQO was performed using a modified method developed by Stanley & Benson (1988). In this reaction the glutathione replaces the nitro group of 4NQO. The 1ml enzyme assay consisted of 100 mM phosphate assay buffer pH 6.5; 5 mM 4NQO and 10 mM GSH and 20 µl of tissue homogenate. The absorbance was measured at 350 nm in a Beckman DU 640 UV/ Visible spectrophotometer against an appropriate blank.

The QR activity was determined by slight modification of the method reported by Wang, Liu, Higuchi, & Chen (1998). The 1ml enzyme assay system consisted of 25 mM Tris/ HCl pH 7.5; 0.18 mg/ml BSA; 5 μM FAD; 0.2 mM NAD(P)H; 40 μM DCPIP and 20 μl of tissue homogenate. The blank contained all the above except the tissue homogenate and 0.2 mM NAD(P)H, while the control contained all except the enzyme sample. The absorbance was measured at 600 nm in a Beckman DU 640 UV/Visible spectrophotometer against the blank.

The spectrophotometer was equipped with enzyme kinetic software and programmed to calculate enzyme units. The amount of enzyme that used 1 μmole of substrate per minute at 25°C is equivalent to one unit of enzyme activity. Each organ homogenate represented one sample; all assays were performed in triplicates. Student's t-test was used to asses the significance of the data.

The protein content of the samples was quantified by Bradford's method (1976). The absorbance was read at 595 nm. Bovine serum albumin was used as a standard.

3. 4. Results

The effect of three limonoids and the mixture of limonoids was investigated on four organs of female A/J on the induction of GST activity (Table 1). Among the limonoids tested, the highest induction of GST was observed against CDNB by nomilin (58%), followed by isoobacunoic acid (25%) and deacetylnomilin (19%) in stomach. Interestingly, deacetlylnomilin in intestine as well as in liver and the mixture of limonoids in liver demonstrated significant inhibition of GST against CDNB. On the

contrary, no significant changes were observed in any of the treatment groups in lung homogenates.

Furthermore, GST activity was measured against 4NQO, in four organs mentioned earlier and the results are presented in Fig.3. The most noticeable induction of GST activity against 4NQO, was observed in intestine homogenates compared to control. Nomilin showed significant induction of GST in intestine (280%) and in stomach (75%) while the limonoid mixture (93%) and deacetylnomilin (73%) significantly induced GST activity in intestine homogenates. In stomach homogenates, nomilin (75%) and the limonoid mixture (45%) significantly ($p<0.05$) induced GST activity against 4NQO. Although not statistically significant, an increase in activity was also observed due to deacetylnomilin and isoobacunoic acid. In the other organs, lung and liver, no significant GST activity was observed. Interestingly, deacetlylnomilin in intestine as well as liver and the mixture of limonoids in liver demonstrated significant reduction of GST activity against CDNB.

Table 1
GST Activity against 1-chloro-2,4-dinitrobenzene, CDNB (Specific activity expressed as units/mg protein)

Samples	**Stomach**	**Intestine**	**Liver**	**Lung**
Control	0.728 ± 0.05	1.260 ± 0.13	2.520 ± 0.11	0.399 ± 0.07
Mixture	0.757 ± 0.02	1.191 ± 0.11	1.833 ± 0.26 [a]	0.414 ± 0.02
Nomilin	1.152 ± 0.16**	1.122 ± 0.24	2.223 ± 0.39	0.395 ± 0.01
Deacetylnomilin	0.872 ± 0.09**	0.715 ± 0.18 [a]	1.582 ± 0.24 [a]	0.449 ± 0.01
Isoobacunoic Acid	0.914 ± 0.13**	0.825 ± 0.84	1.390 ± 0.02	0.365 ± 0.01

Results are means ±SD (n=4)
[a] indicates significant reduction of activity at $p<0.05$
** indicates significant induction of activity at $p<0.05$

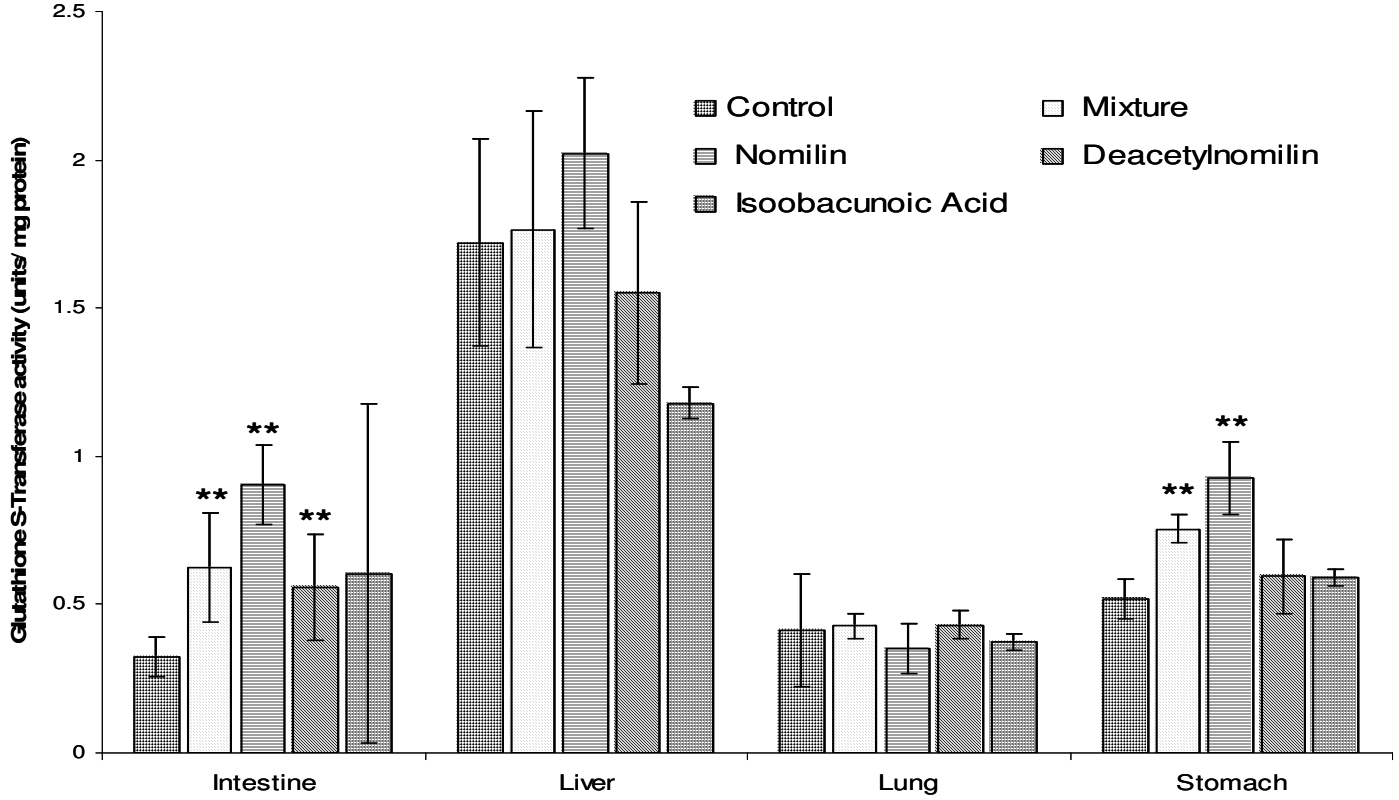

Fig. 3. GST activity in different organ homogenates against 4-NQO. Bars indicate mean ± S.D. (n=4).
** indicate significant induction ($p<0.05$).

Table 2 depicts QR activity in liver, intestine, lung and stomach homogenates. In stomach homogenates, significant induction of QR activity was observed in the limonoid mixture (200%) treatment group. Deacetylnomilin (22%) treatment group showed induction of QR activity in intestine homogenates and isoobacunoic acid (23%) treatment groups showed induction in liver. Similar to GST, none of the limonoids significantly induced QR activity in lung homogenates. Isoobacunoic acid, however, showed significant inhibition of QR activity compared to the control.

Table 2
Quinone reductase activity (Specific activity expressed as units/mg protein).
Results are means ± SD (n=4)

Samples	**Stomach**	**Intestine**	**Liver**	**Lung**
Control	6.592 ± 0.60	3.838 ± 0.54	0.618 ± 0.06	0.377 ± 0.12
Mixture	13.372 ± 4.17**	3.544 ± 0.92	0.623 ± 0.10	0.374 ± 0.05
Nomilin	6.226 ± 0. 71	2.892 ± 0.43	0.662 ± 0.17	0.354 ± 0.02
Deacetylnomilin	6.113 ± 2.12	4.702 ± 0.43**	1.137 ± 0.36**	0.356 ± 0.04
Isoobacunoic acid	4.982 ± 1.19 [a]	3.229 ± 2.57	1.477 ± 0.78**	0.349 ± 0.03

[a] indicate significant reduction of QR activity was observed at $p<0.05$
** indicate significant induction of QR activity was observed at $p<0.05$

3.5. Discussion

Recent research has been focused on citrus limonoids, from their isolation to the elucidation of their structures, and the effects of these compounds on different biological systems (Miller, Porter, Binnie, Guo, & Hasegawa, 2004; Kurowska, Hasegawa, & Manners, 2000a; Battinelli et al., 2003; Miyagi, Om, Chee, & Bennink, 2000; Tanaka et al., 2000; Vanamala et al., 2006; Miller, Fanous, Rivera-Hidalgo, Binnie, Hasegawa, & Lam, 1989; Lam & Hasegawa, 1989; Poulose, Harris & Patil, 2005; Poulose, Harris, & Patil, 2006; Tian, Miller, Ahmad, Tang, & Patil, 2001; Jayaprakasha, Brodbelt, Bhat, & Patil, 2006; Raman, Cho, Brodbelt, & Patil, 2005; Jayaprakasha, Patil, & Bhat, 2006b). A major focus in citrus limonoid research has been on their ability to induce the activity of phase II detoxification enzymes, mainly GST. A recent study in the Netherlands, evaluated the effect of habitual consumption of fruits and vegetables on rectal GST activity against CDNB. It seems that consumption of fruits and vegetables was positively correlated with human rectal GST activity (Wark et al., 2004). Several studies have attributed the induction of these enzymes to the structural components of limonoids. The furan moiety has been thought to be one of the components responsible for induction of GST activity (Lam, Li, & Hasegawa, 1989). Lam, Sparnins, & Wattenburg (1982) studied two furan containing diterpenes, kahweol and cafestol, which were shown to induce an increase in the activity GST in various tissues in mice. In a later study, eight citrus limonoids were tested for induction of GST activity in mice and the structural features of different citrus limonoids and their effect on GST activity has been reported. It seems glutathione S-transferase plays a crucial physiological role in the initial stages of

detoxification of potential alkylating agents (Booth, Boyland, & Sims, 1961). GST enzymes catalyze the adduct formation of glutathione (GSH), a tripeptide consisting of glycine, cysteine, and glutamic acid with electrophilic xenobiotics (Beutler, Duron, & Kelly, 1963). GST catalyzes the reaction of xenobiotic and other environmental factors to react with the –SH of glutathione, neutralizing the toxin and making it more water soluble (Armstrong, 1997). An increase in the activity of GST usually enhances the ability of an organism to detoxify carcinogens. Hence, it is possible that any substance that increases the activity of GST can be a potential anti-carcinogen with the ability to inhibit chemically induced cancer formation (Lam, Li, & Hasegawa, 1989). Other structural features that seem to play a role in the induction of GST activity are the A and A' rings, and modification to the B ring in limonoids. The present study was conducted under controlled dietary conditions. Furthermore, a mixture of limonoids was also used in order to observe any increase of GST activity, possibly due to synergistic effect.

Quinone reductase activity has also been observed along with other phase II enzymes (De Long, Proshaska, & Talalay, 1986). QR protects against cytotoxicity, and an increase in its levels is positively correlated with chemoprevention. QR levels increase along with the levels of other chemopreventive enzymes and it is induced by several chemically dissimilar substances (De Long, Proshaska, & Talalay, 1986). QR catalyses the two electron reduction of quinones to protect cells from free radicals and reactive oxygen species that arise from a one electron reduction. Quinones are one of the toxic products of oxidative metabolism of aromatic hydrocarbons (Horning, Thenot, & Helton, 1978). The reduction of quinones leads to their detoxification or to the production of

biologically reactive products depending upon the mechanism of reaction and subsequent deposition of hydroquinone products (Benson, Hunkeler, & Talalay, 1980). In a previous study, nimbolide, a limonoid isolated from neem flowers, showed induction of QR activity in Hepa1c1c7 cells (Sritanaudomchai et al., 2005).

In our study, nomilin, which is composed of intact A, B, C, and D rings showed the highest induction activity in the GST assays. It seems that, nomilin possesses most of the structural features that are reported to be essential in the induction of GST activity. Deacetylnomilin has a structural similarity to nomilin with the exception of the deacetylation of the A ring. Deacetylnomilin showed induction of GST in some organs, while also inducing QR activity in liver and intestine. These results reinforce our observations that the composition of the A ring is critical for the induction of phase II enzymes.

Isoobacunoic acid has an intact A' ring and an open A ring, while the rest of the limonoid structure is similar to nomilin. Induction of GST activity against CDNB was only observed in stomach homogenates. It is interesting that some induction of GST activity was observed even in the absence of an intact A ring. It is possible that other components of the limonoid structure may serve as a catalyst for enzyme induction. The limonoid mixture utilized showed induction of GST activity against 4NQO in stomach and liver homogenates and some induction of QR activity was observed in stomach homogenates. The limonoid mixture was composed of limonin, nomilin, and isoobacunoic acid. In a previous study (Lam, Li, & Hasegawa, 1989), it was concluded that limonin was ineffective as a GST inducer. This was attributed to the fact that the

structure of limonin is composed of intact A and A' rings. In the current study, the ability of the limonoid mixture to induce GST activity was probably due to the presence of deacetylnomilin. Deacetylnomilin showed induction in many of the assays performed and could be responsible for the induction of GST activity even in the presence of limonin.

In this study an interesting observation was made, the limonoids tested were not active inducers of GST activity in lung. This phenomenon could be due to the fact that the composition of GST isoenzymes vary in mammalian tissues. Previous reports suggest that variability of GST isoenzymes is influenced by tissue, species, and gender (Mitchell, Morin, Lakritz, & Jones, 1997; Hu & Singh, 1997; Singh et al., 1998). Furthermore, it was also demonstrated that one of the GST isoenzymes in stomach is efficient in the conjugation of glutathione but was not detected in lung homogenate from A/J female mouse (Hu & Singh, 1997). Further studies suggested that the gender of the organism also influenced isoenzyme composition, in addition to GST differences among tissues (Singh et al., 1998). It is possible that the absence of induction of GST activity in lung by citrus limonoids may be due to the fact that the substrates utilized were not specific for GST isoenzymes in the mice tested.

In addition to the inactivity of limonoid in lung homogenates, certain limonoids reduced GST activity in different organs. Previous studies have shown that GST activity can be inhibited, reversibly and irreversibly by various compounds (Ploemen, Can Ommen, Hann, Schefferlie, & van Balderen, 1993). It has been proposed that possible inhibition mechanisms are the covalent bonding of compounds to the GST enzyme, competitive inhibition against CDNB, and noncompetitive inhibition against GSH

(Yamada & Kaplowitz, 1980; Ploemen, van Ommen, van Bladeren, 1990; Clark & Sinclair, 1988). Inhibition of GST enzyme could also be beneficial for chemotherapeutic reasons. The inhibition GST could prevent the possible inactivation of chemotherapeutic drugs (Black & Wolf 1991).

3.6. Conclusion

The ability of citrus limonoids to induce GST and QR activity renders them as potent anti-carcinogens. Further studies need to be conducted in order to truly understand the structural-activity relationship between citrus limonoids and certain phase II enzymes. From our present study, it seems possible that an adequate intake of citrus fruit may lead to a better ability of detoxification by these phase II enzymes in the body, which may have potential benefits in prevention of cancer and xenobiotic related diseases. To the best of our knowledge, this is the first to report on the induction QR and GST against 4NQO by certain citrus limonoids.

CHAPTER IV

LIMONIN METHOXYLATION INFLUENCES INDUCTION OF GLUTATHIONE S-TRANSFERASE AND NAD(P)H: QUINONE REDUCTASE

4.1. Synopsis

Previous studies indicated that chemoprevention by citrus limonoids has been attributed to the induction of phase II detoxifying enzymes. In this study three purified and two modified limonoids were use to investigate the influence of Phase II enzymes NAD(P)H: quinone reductase (QR) and glutathione S-tranferase (GST) activities against CDNB and 4NQO in female A/J mice. Our results show that the highest induction (67%) of GST activity against CDNB was displayed by deacetyl nomilinic acid glucoside in lung homogenates followed by limonin-7-methoxime (32%) in treated liver homogenates. Interestingly, the limonin-7-methoxime showed the highest (270%) GST activity in liver against 4NQO, while the same compound in stomach induced GST by 51% compared to the control. The deacetyl nomilinic acid glucoside treatment group induced GST activity by 55% in stomach homogenates. Another Phase II enzyme, QR, was significantly induced by limonin-7-methoxime by 65 and 32% in liver and lung homogenates, respectively. Modified limonoid, defuran limonin, induced QR in lung homogenates by 45%. Our results indicate that modification to the functional groups of the limonin structure may differentially influence their ability to induce phase II enzyme activity. These findings are indicative of a possible mechanism for the prevention of cancer by aiding in detoxification of xenobiotics.

4.2. Introduction

Health maintaining properties citrus fruits have recently been promoted due to several cell culture and animal studies on cancer prevention. Recent research has transitioned from the study of classical vitamin deficiency related diseases such as scurvy to the study of thousands of bioactive compounds that may have important roles in prevention of several diseases such as cancer, heart and Alzheimer's (Ejaz, Ejaz, Matsuda, & Lim, 2006). Initial research on citrus limonoids was initiated to ameliorate the bitterness problem in citrus juice due to the bitter limonoid aglycones, limonin and nomilin (Miller, Porter, Binnie, Guo, & Hasegawa, 2004).

Limonoids are a group of structurally related triterpene derivatives found in plant families Rutaceae and Meliaceae (Hasegawa, Bennett, & Verdon, 1980). Citrus limonoids are composed of two main nucleus structures. The structure of limonin exemplifies the first general nucleus which consists of five rings designated A, A′, B, C, and D. The second limonoid structure, nomilin, consists of four rings designated as A, B, C, and D.

Several studies in our lab (Vanamala et al., 2006; Poulose, Harris & Patil, 2005; Poulose, Harris, & Patil, 2006; Tian, Miller, Ahmad, Tang, & Patil, 2001) and elsewhere (Kurowska, Hasegawa, & Manners, 2000a; Battinelli et al., 2003; Miyagi, Om, Chee, & Bennink, 2000; Tanaka et al., 2000; Miller, Fanous, Rivera-Hidalgo, Binnie, Hasegawa, & Lam, 1989) demonstrated the role of limonoids for potential health benefits. The biological activities have been reviewed in chapter IV.

Additionally, our laboratory has been isolating a variety of limonoids, aglycones and glucosides, from citrus (Jayaprakasha, Brodbelt, Bhat, & Patil, 2006 a; Raman, Cho, Brodbelt, & Patil, 2005; Jayaprakasha, Patil, & Bhat, 2006b). Limonoid glucosides are tasteless water soluble compounds formed in fruits during maturation from their corresponding aglycones. Certain aglycones, on the other hand, are bitter compounds with low water solubility and principally found in seeds (Hasegawa, 2000). Naturally occurring citrus limonoids contain a furan ring attached to the D ring at the 3-position. The presence of the furan moiety is thought to be responsible for the induction of the phase II detoxifying enzyme GST activity. In addition to GST, another important phase II enzyme which protects against toxic and neoplastic effects of xenobiotics is QR. QR protects against cytotoxicity and increases in its levels are positively correlated with chemoprevention (De Long, Prosjaska, & Talalay, 1986). Recent research suggest that the consumption of citrus fruits, specifically grapefruit and oroblanco, modulate both phase I and phase II metabolizing enzymes in rats (Hahn-Obercyger, Stark, & Madar, 2005).

In addition to chemoprevention, the phase II detoxification enzymes have also been reported to be involved in the aging process. According to the “free radical theory of aging”, the generation of free radicals play an important role in the aging process (Harman, 1956). Aerobic metabolism produces free radicals that accumulate over time. The accumulation of free radicals cause DNA, lipid and protein damage, which are continuously repaired by the body. Furthermore, the repair activity has been observed to decline with age (Valko, Leibfritz, Moncol, Cronin, Mazur, & Telser, 2007). It has been

observed that glutathione, glutathione related enzymes and other phase II enzymes protect cellular macromolecules from electrophilic attack by endogenous and exogenous substances. Previous research data suggest that the production of glutathione and glutathione utilizing enzyme activity decrease with age (Zhou, Gao, Cai, & Sparrow, 2006), which may be creating an imbalance in the redox status. Levels of glutathione and glutathione metabolizing enzymes have been observed to remain constant when calorie restriction was administered as opposed to *ad libitum* diet, which displayed a decrease of glutathione and its enzymes with age (Cho et al., 2003).

Previous findings have reported the induction of phase II enzymes by a few citrus limonoids (Tian, Miller, Ahmad, Tang, & Patil, 2001; Miyagi, Om, Chee, & Bennink, 2000; Tanaka et al., 2000; Lam, Li, & Hasegawa, 1989; Kelly, Jewell, & Brien, 2003; Ahmad, Li, Polson, Mackie, Quiroga, & Patil, 2006). In these studies, GST activity was tested against CDNB, which is a commonly used substrate for a variety of GST isozymes (Ahmad, Tijerina, & Tobola, 1997). A previous study suggests that the μ GST isozyme optimally conjugates 4NQO (Aceto, Llio, Lo Bello, Bucciarelli, Angelucci, & Federici, 1990). Most of the isozymes of GST found in mammalian tissues are grouped under four major classes namely α, μ, π and θ (Mannervik & Danielson, 1988; Meyer, Coles, Pemble, Gilmore, Fraser, & Ketterer, 1991). Due to the variability of these enzymes in different mammalian tissues, it is important to explore how citrus limonoids affect the activity of GST enzymes.

In the present study, we analyzed the induction of phase II detoxification enzymes, GST and QR using citrus limonoids purified from *Citrus aurantium* seed. In addition,

modified citrus limonoids were also used to further demonstrate the structural-activity relationship between the structurally different bioactive compounds and phase II enzymatic activity.

4.3. Materials and methods

4.3.1.Materials

β-NADPH reduced tetrasodium salt, 2,6-dichlorophenol-indophenol (DCPIP), 1-chloro-2,4-dinitrobenzene (CDNB), 4-nitroquinoline 1-oxide (4NQO), glutathione reduced (GSH), FAD disodium salt, and all solvents were purchased from Sigma-Aldrich company (St. Louis, MO). Bovine Serum Albumin (BSA) was obtained from Intergen (Purchase, NY). Citrus limonoids used in this study were isolated and purified in our laboratory. Two limonoids limonin-7-methoxime and defuran limonin were modified according to established methods. The structures of these compounds are presented in figure 4.

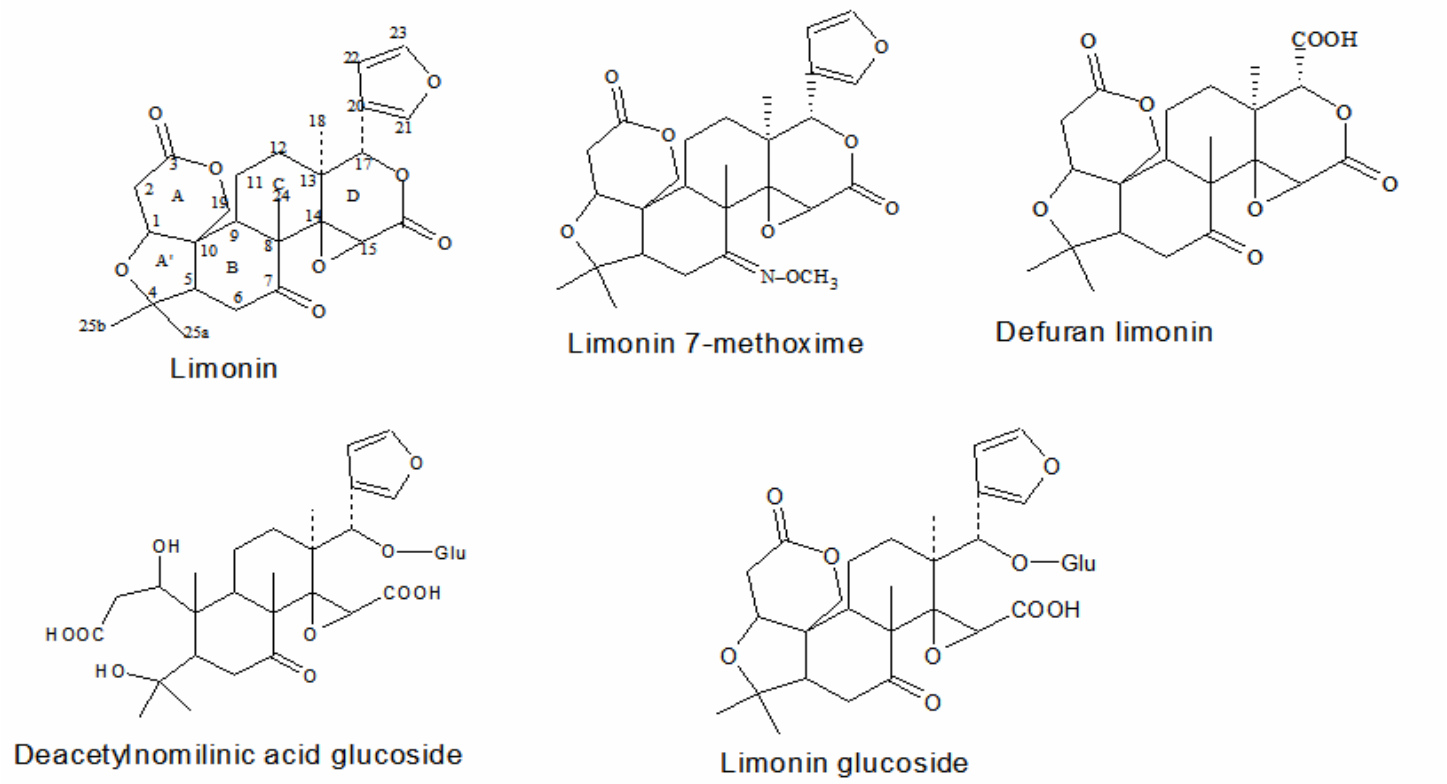

Fig.4. Structures of purified and modified limonoids

4.3.2. Animals

Female A/J 8-9 weeks old mice were purchased from Harlen Sprague-Dawley Laboratory (Indianapolis, IN). The mice were kept on a AIN-76 semi-purified custom diet without vitamin E obtained from MPBiomedical (Solon, OH) and tap water *ad libitum*. The mice were housed in plastic cages with "Aspen" Sani-chip bedding in an environmentally controlled room on a 12 h light / 12 h dark cycle.

4.3.3. Experimental design and treatment

The mice were divided (n=4) into five experimental groups and one control group. The treatments consisted of limonin, limonin glucoside, deacetyl nomilinic acid glucoside, limonin-7-methoxime, and defuran limonin. Each limonoid treatment (20 mg) was suspended in DMSO: corn oil (1:1) (v/v) and the control group was given the corresponding DMSO: corn oil (1:1) treatment. The treatments were administered by oral gavage once every 48h. and a total of four administrations were applied. Forty eight hours after the last treatment the mice were sacrificed by cervical dislocation.

4.3.4. Preparation of organ samples

Lung, intestine, stomach, and liver were harvested immediately after sacrifice and washed with cold PBS. A portion of the tissue was separated for storage while the remaining was weighed and homogenized by a Pro200 homogenizer in 10% (w/v) PBS (10mM), pH 7.0, containing β-mercaptoethanol (1.4mM). Homogenates were centrifuged at 22,000 x g in a Beckman Avanti 30 centrifuge for 45 min. After centrifugation, the supernatant was carefully removed and stored in -20°C until further use.

4.3.5. Protein assay

Cytosolic protein concentration was measured by Bradford's quantification method (1976). Bovine serum albumin was used as a standard.

4.3.6. GST assays

GST activities against 4NQO and CDNB were determined by a methods developed by Stanley et al. (1988) and Habig et al. (1974). The absorbance was monitored at 350 (4NQO) and 340 (CDNB) nm in a Beckman DU 640 UV/Visible spectrophotometer. The spectrophotometers were equipped with enzyme kinetic software and programmed to calculate enzyme units. The amount of enzyme that uses 1 μmole of substrate per minute at 25°C is equivalent to one unit of enzyme activity.

4.3.7. QR assay

The quinone oxidoreductase (QR) assay was performed by modifying a method reported by Wang et al. (1998), using DCPIP as substrate. The absorbance was measured at 600 nm in a Beckman DU 640 UV/Visible spectrophotometer. The spectrophotometers were equipped with enzyme kinetic software and programmed to calculate enzyme units. The amount of enzyme that used 1 μmole of substrate per minute at 25°C is equivalent to one unit of enzyme activity.

Each organ homogenate representing one sample and all assays were performed in triplicates. Student's t-test was used to asses the significance of the data obtained.

4.4. Results

Three purified citrus limonoids and two modified limonoids were tested for induction of phase II enzymatic activity. GST activities were assayed using CDNB and 4NQO as substrates. Induction of QR was also evaluated.

Mice were treated with the five limonoids in order to evaluate the induction of GST activity against CDNB. In lung, deacetyl nomilinic acid glucoside was the only limonoid that showed significant induction of GST activity, 67%, compared to the control. Interestingly, in liver, modified methoxylated limonin-7-methoxime showed significant induction of GST activity, while limonin glucoside and deacetyl nomilinic acid glucoside showed slight reduction of activity (Table 3).

GST activity was also measured against 4NQO. In stomach homogenates, deacetyl nomilinic acid glucoside (55%) showed the highest induction of GST activity, followed by limonin-7-methoxime (51%). In liver homogenates, limonin-7-methoxime induced GST activity was three times higher (270%) than that of the control. In intestine homogenates, defuran limonin and deacetyl nomilinic acid glucoside reduced GST activity, while no activity change was observed in lung homogenates (Fig 5).

Table 3
GST activity† against 1-chloro-2,4-dinitrobenzene

Sample	Stomach	Intestine	Liver	Lung
Control	0.887 ± 0.07	0.864 ±0.09	1.783 ± 0.02	0.365 ± 0.08
Limonin	0.837 ± 0.13	0.635 ± 0.01[a]	1.633 ± 0.27	0.342 ± 0.05
Limonin-7-methoxime	0.898 ± 0.18	1.346 ± 0.47	2.367 ± 0.05**	0.369 ± 0.03
Defuran limonin	0.866 ± 0.12	0.793 ± 0.09	1.458 ± 0.54	0.340 ± 0.08
Limonin glucoside	0.868 ± 0.13	0.756 ± 0.06	1.413 ± 0.08 [a]	0.447 ± 0.09
Deacetyl nomilinic acid glucoside	1.151 ± 0.38	0.707 ± 0.13	1.356 ± 0.20 [a]	0.609 ± 0.12**

Results are means ± (n=4)
† specific activity (units/mg protein)
[a] indicates significant reduction of activity at $p<0.05$
** indicates significant induction of activity at $p<0.05$

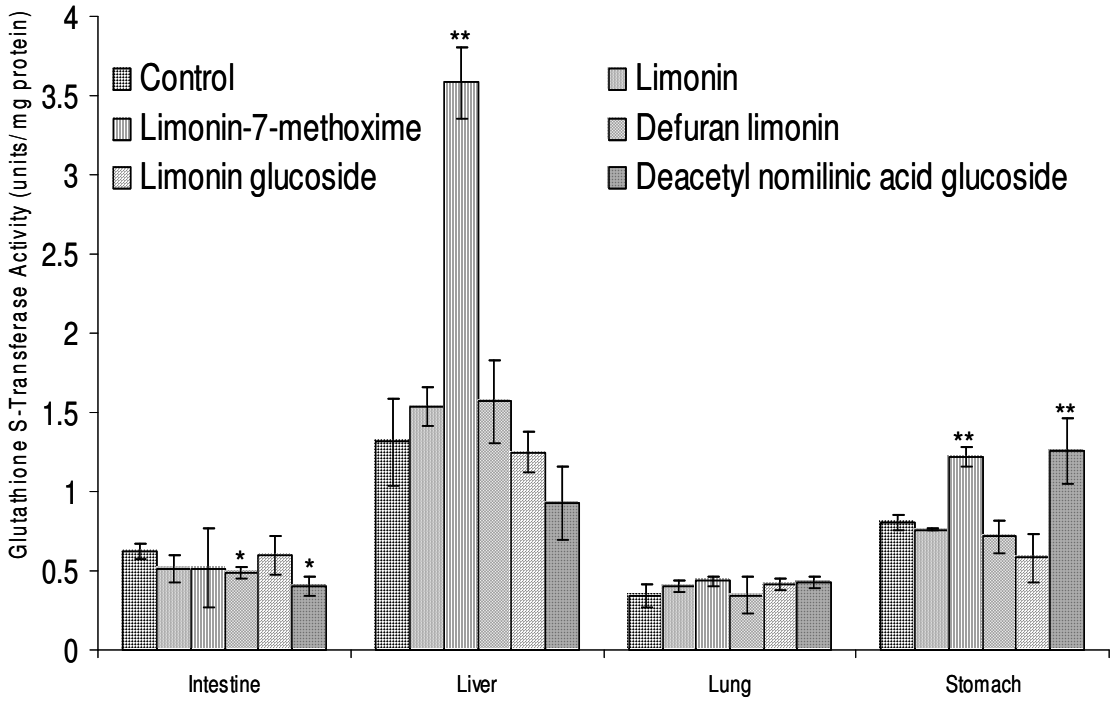

Fig. 5 GST activity in different organ homogenates against 4-Nitroquinoline 1-oxide, a potent xenobiotic tumorigenic to lung, esophagus, forestomach, glandular stomach, skin and other organs. Bars indicate mean ± S.D. (n=4). * indicate statistically significant inhibition ($p<0.05$). ** indicate significant induction ($p<0.05$).

Quinone reductase activity was measured in lung, intestine, liver and stomach homogenates. In liver and lung, limonin-7-methoxime showed significant induction of QR 65% and 32%, respectively compared to control. In intestine and stomach homogenates, limonin-7-methoxime increased QR activity, but induction was not statistically significant. In lung homogenates, defuran limonin showed the highest induction (45%) activity. Interestingly, deacetyl nomilinic acid glucoside reduced QR

activity in lung. No significant change in activity was observed in stomach homogenates (Table 4).

Table 4
Limonoid induction potential of quinone reductase activity†

Sample	Stomach	Intestine	Liver	Lung
Control	8.309 ± 1.73	2.336 ± 0.27	0.520 ± 0.04	0.263 ± 0.05
Limonin	8.569 ± 1.58	2.474 ± 0.72	0.389 ± 0.10	0.297 ± 0.04
Limonin-7-methoxime	10.455 ± 1.12	4.014 ± 1.15	0.858 ± 0.11**	0.349 ± 0.01**
Defuran limonin	8.985 ± 0.79	2.005 ± 0.24	0.414 ± 0.02	0.381 ± 0.05**
Limonin glucoside	8.125 ± 2.38	2.454 ± 0.14	0.479 ± 0.06	0.314 ± 0.05
Deacetyl nomilinic acid glucoside	9.581 ± 4.07	2.296 ± 0.50	0.238 ± 0.02 [a]	0.286 ± 0.01

Results are means ±SD (n=4)
† specific activity (units/mg protein)
[a] indicates significant reduction of activity at $p<0.05$
** indicates significant induction of activity at $p<0.05$

4.5. Discussion

A study conducted in the Netherlands, reported that the habitual consumption of fruits and vegetables was positively correlated with human rectal GST activity (Wark et al., 2004). Recent research on citrus limonoids shows that both limonin and nomilin could inhibit certain chemically induced carcinogenesis in different animal models (Lam & Hasegawa, 1989; Lam, Zhang, & Hasegawa, 1994; Lam, Zhang, Hasegawa, & Schut,

1994). In a two stage model for skin carcinogenesis, it was shown that nomilin was more effective as an inhibitor during the initiation stage of carcinogenesis, while limonin was more active during the promotional phase of carcinogenesis (Lam, Hasegawa, Bergstrom, Lam, Kenney, 2000). The main difference between these two limonoids, is that limonin has an A and A′ ring while nomilin has only a seven membered A ring. Furthermore, it was suggested that there is a possible induction of GST activity in mice by citrus limonoids (Lam & Hasegawa, 1989).

The differential induction potential of certain citrus limonoids to induce GST activity was later attributed to different structural components of the limonoid nucleus. It was suggested that an intact A ring is required for anti-neoplastic effects, such as present in nomilin (Miller, Taylor, Berry, Zimmermann, & Hasegawa, 2000). It has also been stipulated that modification to the B ring of the limonoid nucleus can also alter the induction of GST activity.

The D-ring of the limonoid nucleus has a furan ring attached to its third position. Several studies have been conducted on the importance of the furan ring in the induction of GST activity. Kahweol and cafestrol are two furan containing diterpenes, which are reported inducers of GST activity (Lam, Sparnins, & Wattenburg, 1982). All of the naturally occurring citrus limonoids contain this furan moiety. All the citrus limonoids tested in this study had the furan moiety present except for the modified, defuran limonin. Previous studies have shown that the furan moiety plays a role in the induction of GST activity (Lam & Hasegawa, 1989). In the current study, defuran limonin exhibited no induction in any of the GST assays. Interestingly, induction of QR activity

due to defuran limonin was observed in lung homogenates. It seems that the furan moiety may be important for induction of phase II enzymes but not essential to anti-neoplastic activity.

In order to understand the role of A and A' rings in the limonoids on biological activity, the induction potential of citrus limonoids with an open A ring (deacetyl nomilinic acid glucoside), and intact A and A′ rings (limonin, defuran limonin, limonin glucoside and limonin-7-methoxime) were analyzed. Previous work has indicated that modifications to the A and A′ rings produce significant differences in the ability to induce GST activity (Lam & Hasegawa 1989). Authors demonstrated that citrus limonoids with intact A and A′ rings (limonin, limonol, and deoxylimonin) are not active GST inducers, while ichangin, with an open A′ ring, showed induction of GST. It is clear from the previous study that citrus limonoids with only an intact A ring were responsible for a majority of the induction activity. Interestingly, on the contrary, our current results showed that limonin-7-methoxime, with intact A and A′ rings and with methoxylation in the B ring, had significant GST induction in liver against CDNB and in liver and stomach against 4NQO. In QR assays limonin-7-methoxime showed induction in lung and liver homogenates and defuran limonin showed induction in lung only. Deacetyl nomilinic acid glucoside, with an open A ring also showed induction of GST activity in lung against CDNB and in stomach against 4NQO.

Considering modifications to the B-ring, all of the tested limonoids, except for one, contained a ketone at the B ring. In limonin-7methoxime, the ketone was substituted by a methoxime functional group, the rest of the limonoid structure was identical to

limonin. While limonin did not show any induction activity in any of the enzymatic assays performed, limonin-7-methoxime showed induction of phase II enzymes in several organ homogenates assayed. Furthermore, limonin-7-methoxime showed induction of GST activity in liver homogenate against CDNB. In GST against 4NQO, induction was seen in liver and stomach homogenates. Additionally, limonin-7-methoxime showed QR induction in liver and lung homogenates. Our results strongly suggest that modification to the B ring with a methoxy group plays a very important role in the induction of phase II detoxifying enzymes, as compared to the inactive limonin.

Interestingly, limonin glucoside did not show any induction of Phase II enzyme activity in any of the assays performed. It seems that, addition of a glucose moiety attached to the D ring of the limonoid nucleus may not play a major role in biological activity in relation to Phase II enzymes.

The induction of phase II enzymes by citrus limonoids can potentially inhibit carcinogenesis by conjugating harmful substances into more water soluble form. The increase in polarity of these electrophilic substances facilitates their excretion form the body. Furthermore, it has been reported that plant-derived phase II enzyme inducers may be potentially important in the incidence of age-related macular degeneration (Zhou, Gao, Cai, & Sparrow, 2006). Phase II enzymes are important in the prevention of age-related degenerative conditions. Induction of phase II enzymes helps in the elimination of reactive oxygen species, which accumulate with age. Therefore, citrus limonoids could be considered as a potential anti-aging agent by inducing the activity GST and QR.

4.6. Conclusion

In this study, citrus limonoids with different structural characteristics were evaluated to understand their detoxification potential. To the best of our knowledge, this is the first study to report on the induction of GST using 4NQO as a substrate. Additionally, for the first time, two modified limonoids were also examined for induction of the phase II enzymes GST and QR. It is possible that an ample intake of citrus fruits may aid in the expulsion of xenobiotics by an increase in activity of phase II enzymes. The ability of these compounds to induce the activity of detoxifying phase II enzymes makes them valuable bioactive compounds in the quest of prevent cancer, anti-aging and oxidative related diseases, and deserves more in-depth research in order to improve human health.

CHAPTER V

THE PRESENCE OF PHASE II DETOXIFICATION ENHANCER, D-GLUCARIC ACID, IN GRAPEFRUIT (*Citrus paradisi* Macf.)VARIETIES

5.1. Synopsis

Fruits and vegetables have been reported to contain D-glucaric acid, especially in citrus. D-glucaric acid has been reported to possess anticancer properties. In this investigation, a method for the quantification of D-glucaric acid in grapefruit by High Performance Liquid Chromatography (HPLC) was developed. This HPLC method uses an isocratic mobile phase (0.1% phosphoric acid). Nine widely used grapefruit varieties were analyzed for the levels of D-glucaric acid. Seasonal levels of GA ranged as follows: Thompson (58.36-126.8 mg/100ml), Henderson (29.6-49.7 mg/100ml), Rio Red (40.0-58.8mg/100ml), Star Ruby (25.5-46.7 mg/100ml), I-48 (26.6-58.3 mg/100ml), Ruby Red (49.3-63.0 mg/100ml), Ray's Ruby (58.2-72.1 mg/100ml), Marsh White (53.7-65.8 mg/100ml) and Duncan (43.99-64.05 mg/100ml). Seasonal variation of D-glucaric acid within the individual varieties was also measured. The overall trend of GA level was increased from early to late season in the tested varieties. Our method has a sensitivity of detecting D-glucaric acid as low as 0.2 μg with accuracy and precision >95%. This method was found to be simple, fast, accurate and reproducible. Additionally, the labor intensity and cost of sample preparation were greatly reduced as compared to reported methods.

5.2. Introduction

Over the last decade the consumption of citrus products has been positively correlated with health benefits (Patil, Brodbelt, Miller, & Turner, 2006; Dwivedi, Heck, Downie, Larroya, & Webb, 1990; Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). Studies during this period have found citrus fruits to be rich in bioactive compounds, namely, carotenoids, limonoids, flavonoids, pectin, vitamin C, folate, furocoumarins, and D-glucaric acid (GA) (Patil, Brodbelt, Miller, & Turner, 2006). Previous studies have shown grapefruit and oranges contain high levels of GA (Dwivedi, Heck, Downie, Larroya, & Webb, 1990; Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). Data obtained from two decades of research suggests that consumption of dietary GA is helpful in the prevention of carcinogenesis (Walasek, Hanausek-Walaszek, Minton, & Webb, 1986; Oredipe, Barth, Hanausek-Walaszek, Sautins, Walaszek, & Webb; 1987; Walaszek, Flores, & Adams, 1988; Dwivedi, Oredipe, Barth, Downie, & Webb, 1989; Walaszek, 1990; Abou-Issa, Koolemans-beynen, & Meredith, 1992; Oredipe, Barth, Dwivedi, & Webb, 1992; Boone, 1992; Abou-Issa, 1995; Yoshimi, Walaszek, Mori, Hanausek, Szenraj, & Slaga, 2000).

During the last decade, few studies have attempted to measure the content of glucarate in fruits and vegetables. In 1990, GA in seven fruits and vegetables was measured using an indirect enzymatic inhibition method and HPLC for confirmation (Dwivedi, Heck, Downie, Larroya, & Webb, 1990). Sample preparation consisted of incubating the sample 0.1M sodium borate to convert the glucarolactone to GA, passing the sample through Dowex 1-X8 chromatography. GA was eluted with a 0.05 M sodium

borate and 0.02 M sodium sulfate mixture. The presence of GA was confirmed by HPLC using a Bio-Rad HPX- H organic acid column. The mobile phase consisted of an isocratic elution with 0.01N sulfuric acid containing 10% acetonitrile and detected at 210 nm. The enzymatic method has the disadvantage that other substances in the sample might compete with GA in the inhibition of the enzyme. Furthermore, HPLC was used to confirm the presence of GA in the sample. While HPLC procedures are more desirable, the use of buffers is tedious and could affect the function of the HPLC apparatus over time. In another study in 1996, the contents of 31 fruits and vegetables was evaluated by two methods; an enzymatic method and by a pyruvate assay (Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). The pyruvate assay employs the use of bacterial enzymes for the conversion of GA and its lactones to pyruvate. The disadvantage of this assay is that it is time consuming and the amount of purified enzyme might be limiting. Data from these studies suggest that grapefruit and oranges contain higher levels of GA (Dwivedi, Heck, Downie, Larroya, & Webb, 1990; Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). Other methods of determination of D-glucaric acid in urine using chromatographic have been published (Poon, Villenueve, Chu, & Kinach, 1993; Gangolli, Longland, & Shilling, 1974; Laakso, Tokola, Hirvisalo, 1983; Walters, Lake, Bayley, & Cottrell, 1983). These methods either employ the use of complex procedures for the volatilization of GA. In the present study, a method was established for the quantification of GA present in different grapefruit varieties. Additionally, the seasonal variation of D-glucaric acid content was studied in nine varieties of grapefruit.

5.3. Materials and methods

5.3.1. Materials

Nine varieties of grapefruits (*Citrus paradisi* Macf.)- such as Rio Red, Star Ruby, I-48, Henderson, Ray's Ruby, Thompson, Marsh White, and Duncan, were obtained from Texas A&M University-Kingsville Citrus Center (Weslaco, Texas). Dowex 50WX2 (mesh size 100), calcium D-saccharate tetrahydrate, and all HPLC grade solvents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Millipore water was used for sample preparation and HPLC analysis.

5.3.2. Resin activation

Activation of Dowex 50 (50 g) was carried out by washing of resin four times with 150 ml of H_2O: HCl (1:1) each wash was carried out for 15 min. The resin was filtered and rinsed with distilled water until neutral pH.

5.3.3. Sample collection and preparation

Ten fruits from each variety were harvested from the same tree, and the tree was labelled for future harvesting. The fruits were collected on: November 5 (early season), February 5 (mid- season), and May 5 (late season). Each variety of grapefruit was peeled and made into homogenous juice, separately. Samples were composed of two fruits, which yields 700-1000ml of juice. Aliquots (1.5ml) were centrifuged (Eppendorf 5417C, Westbury, NY, USA) at 10,000 rpm for 25 min., in order to remove pulp material. The supernatant was then filtered through a 0.45 μm syringe filter (Whatman, Florham Park, NJ, USA) and stored at -80°C until subjected to HPLC analysis of GA.

5.3.4. Preparation of free GA

At present, GA standard is not commercially available in market. Hence calcium D-saccharate tetrahydrate was deionized for the preparation of free GA as follows. Calcium D-saccharate tetrahydrate (300 mg) was suspended in 15 ml of water and was treated with 3g of Dowex 50 [H^+]. The mixture was stirred with a magnetic stirrer for 20 min and the supernatant was decanted into a 100 ml volumetric flask. Water wash was continued until neutral pH was achieved. Supernatants and washings were combined and the volume was made up to 100 ml.

5.3.5. HPLC analysis

Chromatographic separation of GA was achieved by means of a Perkin Elmer (Perkin Elmer, Boston USA) Series 200 pump coupled with a Perkin Elmer Series 200 autosampler and a Perkin Elmer Series 200 UV/VIS detector using a Hydro-RP column (250 mm x 4.6 I.D.) 4µm particle size (Phenomenex, Torrance, CA, USA). An isocratic mobile phase of 0.1% phosphoric acid (v/v) was used at a flow rate of 1 ml/min. GA was detected at 210nm. For quantification of GA, the Turbochrome Navigator software version 6.2.1. was used.

5.3.6. Calibration and linearity

The linearity of the method was analyzed by evaluating a series 1.2, 2.4, 4.9, 9.9, 19.8 µg of standard GA. Fifteen microliters of the five standard solutions of standard GA were injected into HPLC. A calibration curve was obtained by plotting the concentration of GA versus the average peak area from triplicate runs. A range of calibration

concentrations was chosen to reflect the normal D-glucaric acid concentration present in grapefruit samples.

5.3.7. Limit of quantification

The limit of quantification was defined as the lowest GA concentration which can be determined with an accuracy and precision of > 95%. The lower limit of quantification was determined to be to a level of 0.03 µg.

5.3.8. Quantification of GA in samples

Fifteen microliters of each sample were injected into HPLC for the analysis of GA in grapefruit samples. The concentration of GA present in each sample was calculated by application of the linear calibration function, incorporating a dilution factor. GA content of grapefruit was presented as mg /100 ml of grapefruit juice.

5.4. Results

We employed the use of a Synergy Hydro-RP column (Phenomenex, Torrance, CA, USA) of 4 micron particle size (250mm x 4.6 I.D.) for the separation of GA from grapefruit juice. D-Glucaric acid was separated with an isocratic mobile phase of 0.1% phosphoric acid (v/v), at a flow rate of 1 ml/min. The D-glucaric acid peak was eluted at 2.84 min retention time (Fig. 6). Figure 7 depicts a typical grapefruit sample and a grapefruit sample with a GA spike.

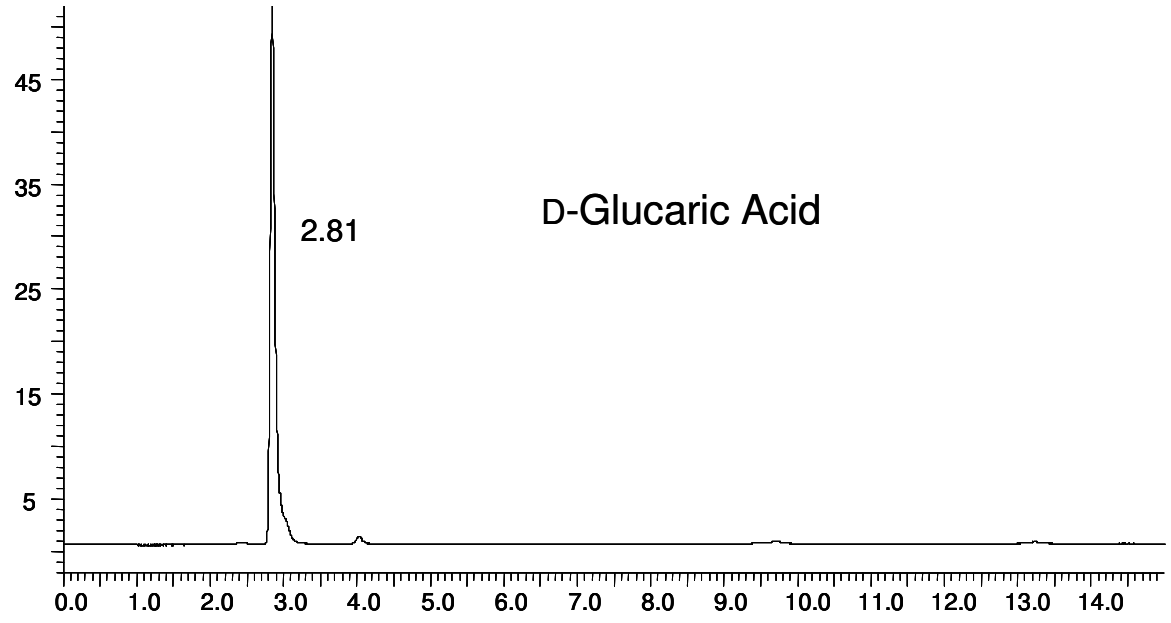

Fig.6. Chromatogram for standard D-glucaric acid

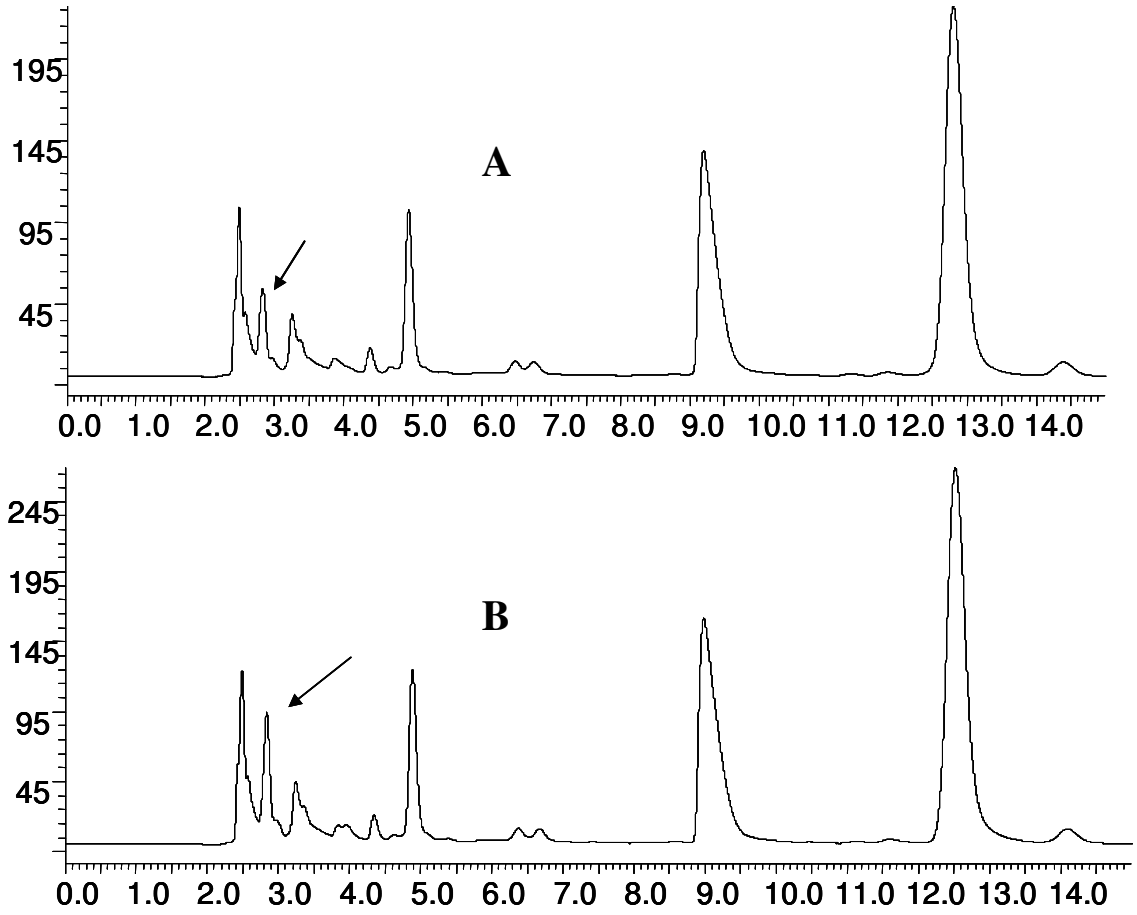

Fig.7. Chromatograms of grapefruit samples. (A) typical grapefruit sample, (B) grapefruit sample with a GA spike

Fig.8. depicts the D- glucaric acid concentration of fruit harvested in November. Thompson varieties had the highest content of D-glucaric acid, 126.8 mg/100ml. The Marsh (65.8 mg/100ml), Ruby Red (63.0 mg/100ml), Ray's Ruby (58.2mg/100ml), Duncan (50.17 mg/100ml), Henderson (44.3mg/100ml) and Rio Red (41.2mg/100ml) varieties all displayed similar midrange levels of GA, while the Star Ruby (25.5

mg/100ml) and the I-48 (26.7 mg/100ml) varieties had significantly lower levels of GA than Thompson variety.

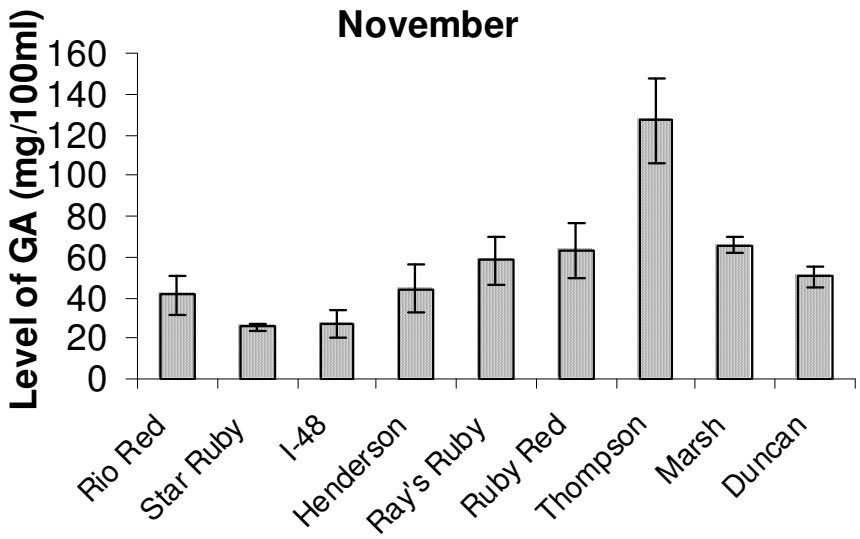

Fig.8. Level of D-glucaric acid (GA) in grapefruit varieties harvested in November

Concentrations of D-glucaric acid in grapefruit harvested in February followed a similar trend from that of the early season harvest. Thompson, a pink grapefruit, again had the highest concentration of D-glucaric acid, 85.9 mg/100ml. The midrange varieties were Duncan (64.05 mg/100ml), Ray's Ruby (63.4 mg/ml), Marsh (53.7 mg/100ml), Ruby Red (49.3 mg/100ml), Star Ruby (46.7 mg/100ml), I-48 (41.9), and Rio Red (40.1

mg/100ml). From the mid-season harvest, the Henderson yielded the lowest levels of D-glucaric acid, 29.6 mg/100ml (Fig.9).

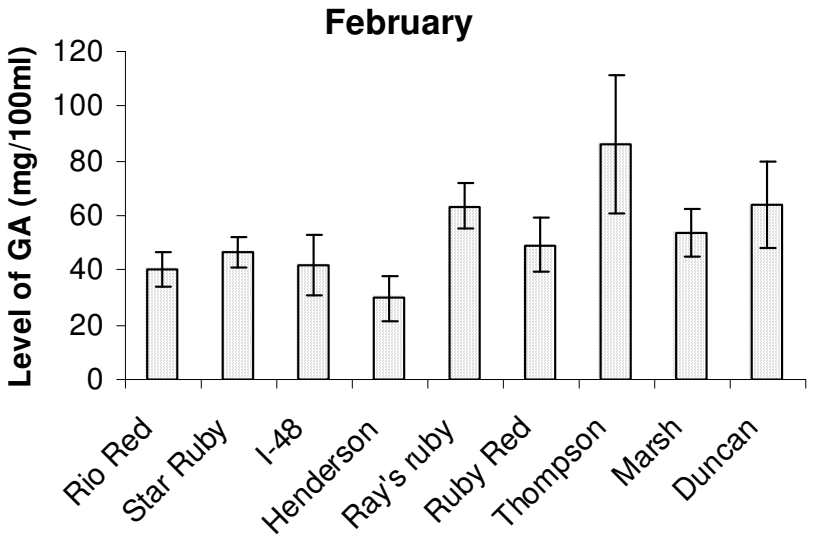

Fig.9. Level of D-glucaric acid (GA) in grapefruit varieties harvested in February

The results from the May harvest showed similar results. The Ray's Ruby variety displayed the highest content of D-glucaric acid, 72.2 mg/ml. The D-glucaric acid level of the Ray's Ruby variety was followed by the Rio Red (58.8 mg/100ml), Thompson (58.4 mg/100ml), I-48 (58.3 mg/100ml), Ruby Red (55.0 mg/100ml), and Marsh (54.0 mg/100ml) variety grapefruits. The Henderson (49.7 mg/100mg), Duncan (43.9 mg/100ml), and Star Ruby (43.5 mg/100ml) varieties were observed to contain the least

D-glucaric acid content in the fruit harvested in the month of May (Fig.10). The trend in seasonal changes of GA for the nine grapefruit varieties is illustrated in figure 11.

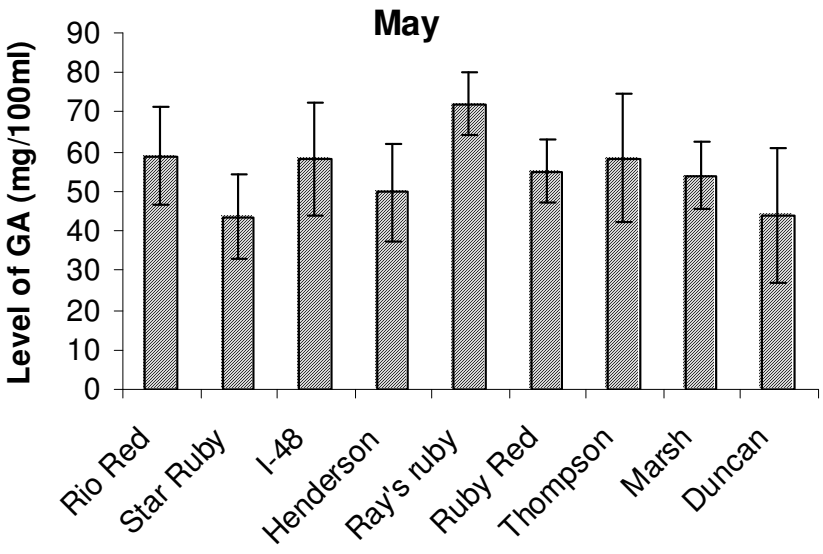

Fig.10. Level of D-glucaric acid (GA) in grapefruit varieties harvested in May

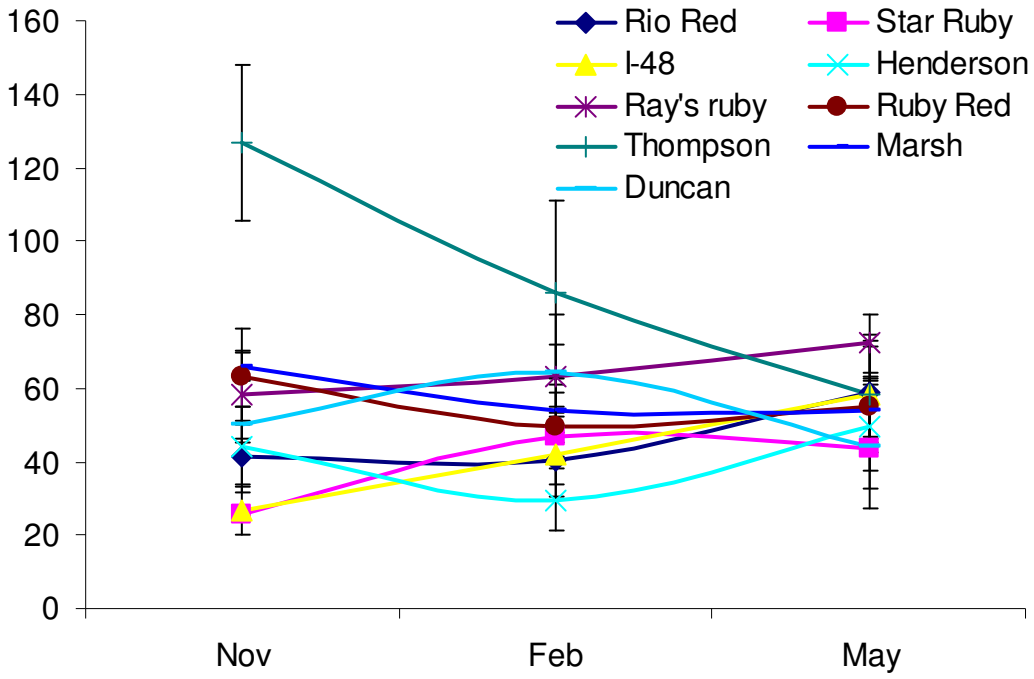

Fig.11. Seasonal variation of D-glucaric acid content in grapefruit

We have presented data obtained from a simple isocratic HPLC method for the determination of D-glucaric acid. This analytical procedure displayed optimal and reproducible results for the quantification of D-glucaric acid. The linear relationship between the D-glucaric acid concentration and the peak area using our reported method was determined using six concentrations, 1.2, 2.4, 4.9, 9.9, and 19.8µg injected in triplicate. The regression coefficient for the D-glucaric acid calibration curve was 0.996 (fig12). The lower limit for detection of D-glucaric acid was found to be 0.07 µg.

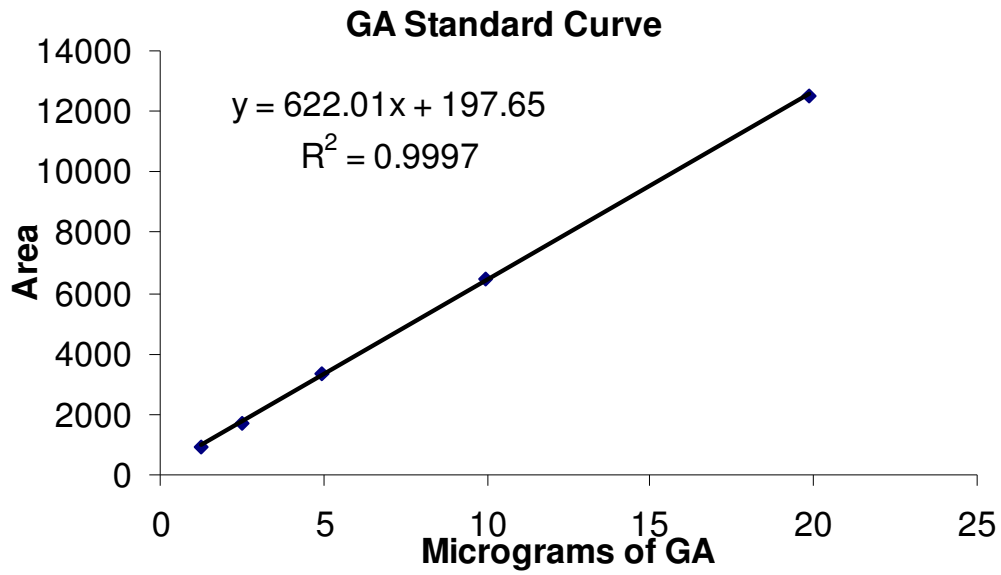

Fig.12. Calibration curve for D-glucaric acid (GA).

5.5. Discussion

Citrus fruit contain many bioactive compounds, including D-glucaric acid. Several studies have reported measurements of D-glucaric acid, but current methods are expensive, laborious, and in some cases only useful for qualitative analysis (Dwivedi, Heck, Downie, Larroya, & Webb, 1990; Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996; Poon, Villenueve, Chu, & Kinach, 1993; Gangolli, Longland, & Shilling, 1974; Laakso, Tokola, Hirvisalo, 1983; Walters, Lake, Bayley, & Cottrell, 1983). The work performed to date on the analysis of D-glucaric acid is summarized in Table 5. The purpose of this study is to develop a method that will be useful for the quantitative analysis of D-glucaric acid in fruit, namely grapefruit, while avoiding the previously stated disadvantages. Our reported method is a cost effective, fast and simple isocratic HPLC method for the quantification of D-glucaric acid in grapefruit.

Results from previous studies indicate a high content of D-glucarate in various fruits and vegetables (Dwivedi, Heck, Downie, Larroya, & Webb, 1990; Walaszek, Szemraj, Hanausek, Adams, & Sherman, 1996). Furthermore, these studies indicated that citrus fruits had the highest levels of D-glucaric acid. Based on the potential role of D-glucaric acid in human health, we developed a method for the quantification of D-glucaric acid in grapefruit varieties.

Table 5
Quantification of D-glucaric acid used by previous studies

Author	Year	Method	HPLC Conditions	Sample treat.	Origin
Walters *et al.*	1983	HPLC	Partisil-10 SAX (250x4.6) col., 0.06M KH2PO4 adjusted to 6pH w/NaOH @2ml/min, 200nm	Centrifugation	Urine
Laakso *et al.*	1983	HPLC	Spherisorb-NH2, 5um(228x5), 0.01M H2PO4-Acetonitrile (75:25) @2ml/min 220 nm	Made solution basic	Urine
Dwivedi *et al.*	1990	Enzyme/ HPLC	Bio-Rad HPX-87H organic acid col. Isocratic 0.01N Sulfuric w/10%acetonitrile 210nm	Cent., Boiling, Buffer	Fruits &Veg.
Poon *et al.*	1993	HPLC	Aminex HPX-87H (75x300), 0.004N sulfiric acid @0.5ml/min 210 nm	Affi-gel 601	Urine
Walaszek *et al.*	1996	Enzyme/ pyruvate			Fruit & Veg.
Lampe *et al.*	2002	Enzyme			Food
Jung *et al.*	1981	Enzyme			Urine
Mocarelli *et al.*	1988	Enzyme			Urine
Steinberg *et al.*	1986	Enzyme / Glyoxylic			Urine
Marsh	1985	Enzyme/ Pyruvate			Urine
Kringstad *et al.*	1975	TLC			Plants
Walaszek *et al.*	1997	HPLC/ Enzyme	Spherisorb-NH2, 0.01Mphosphoric acid -Acetonitrile @1.5ml/min 200nm		Urine/ organ extract.
Gangolli *et al.*	1974	GC		Volatilization	Urine
Colombi *et al.*	1983	Enzyme			Urine

Initially, difficulties faced to obtain standards. For our study, calcium D-saccharate tetrahydrate was subject to ion exchange chromatography to obtain free GA. Once free GA was obtained, the development and validation of the method was carried out. The analytical method consisted of a direct injection of sample to HPLC. The GA peak was eluted with an isocratic (0.1% phosphoric acid) mobile phase. The sample preparation was minimal, consisting mainly of homogenization and filtration. The analysis of grapefruit samples was completed in 15 min. Furthermore, the sensitivity of our method was able to detect level of GA as low as 0.03µg.

Differences were observed in the level of D-glucaric acid among the nine grapefruit varieties tested. Furthermore, seasonal changes of D-glucaric acid levels were also observed within individual varieties to varying degrees. In early season, grapefruit D-glucaric acid content varies from 25-126 mg/100ml. Interestingly, the range of D-glucaric acid levels in late season fruits is much narrower (43-72 mg/100ml). In some instances the D-glucaric aicd level increased in an individual grapefruit variety, while decreases in levels were observed in others. Levels of D-glucaric acid increased in the I-48, Star Ruby, Ray's Ruby, and Rio Red varieties from early to late season. Inversely, Duncan, Ruby Red, and Thompson varieties showed a decreasing trend in levels of D-glucaric acid as the season progressed. The Marsh variety showed no fluctuation in levels of D-glucaric acid, maintaining a mid range level, compared to other varieties through out the season (Table 6).

Table 6
Changes in D-glucaric acid levels from early to late season fruits.

Variety	Early Season (November) mg/100ml	Late Season (May) mg/100ml	Ratio Early/Late
Marsh	53.69 ± 8.6	54.03 ± 8.6	1.00
Star Ruby	25.59 ± 1.5	43.53 ± 10.6	1.70
Duncan	64.05 ± 16.0	43.99 ± 16.8	0.68
I-48	26.68 ± 6.6	58.36 ± 14.2	2.18
Ray's Ruby	58.19 ± 11.7	72.16 ± 7.7	1.24
Ruby Red	63.03 ± 13.2	55.01 ± 7.9	0.87
Henderson	44.29 ± 11.8	49.71 ± 12.4	1.12
Rio Red	41.20 ± 9.8	58.80 ± 12.4	1.42
Thompson	126.82 ± 21.1	58.43 ± 16.1	0.47

A general feature of the development of citrus fruits is the accumulation of organic acids at early fruit development (Erickson, 1968). This accumulation of GA reaches a peak and decreases at maturity. It has been postulated that the final organic acid content in mature fruit is determined by an equilibrium between synthesis, storage and mobilization (Laval-Martin, Farineau, & Diamond, 1977; Müller, Irkens-Kiesecker, Bubinstein, & Taiz, 1996; Ruffner, Possner, Brem, & Rast, 1984). In other instances, this decrease in organic acid content in Hamlin oranges, mainly citric and malic acid, has been attributed to their dilution due to fruit expansion (Ting & Vines, 1966). This decrease in levels of organic acids has been mainly observed in major organic acids, while less prominent acids, tartartic, succinic, and ascorbic acid, have been observed to remain relatively constant during fruit development (Albertini, Carcouet, Pailly, Gambotti, Luro, & Berti, 2006). Furthermore, it has also been noted that the organic acid content varies greatly within individual fruit species (Moing, Svanella, Gaudillere,

Gaudillere, & Monet, 1999). Recent studies have attributed to the changes in organic acid contents in fruit to the catabolic actions of various enzymes, mainly aconitase and phosphoenopyruvate carboxykinase (Sadka, Dahn, Cohen, & Marsh, 2000; Famiani et al., 2005). It is interesting to note that there have been very few studies measuring the D-glucaric acid levels in fruit. To the best of our knowledge, seasonal variations of D-glucaric acid have not been previously reported.

Several studies have developed HPLC methods for the quantification of D-glucaric acid, mainly from urine (Poon, Villenueve, Chu, & Kinach, 1993; Laakso, Tokola, & Hirvisalo, 1983; Walters, Lake, Bayley, & Cottrell, 1983). These methods involve tedious sample preparation and expensvive. Furthermore, the date obtained from some of these studies was mainly qualitative. In this investigation, we report a simple isocratic HPLC method for the quantification of D-glucaric acid in grapefruit samples which is quick, accurate, and reproducible.

5.6. Conclusion

A new method for the quantification of D-glucaric acid is developed which is accurate and reproducible compared to previously reported methods. D-glucaric acid has been extensively studied in several animal models for the prevention of chemical carcinogenesis. Furthermore, it has also been reported that D-glucaric acid is present in various fruits and vegetables, including citrus. We measured the D-glucaric acid content of nine grapefruit varieties and found that level of D-Glucaric acid ranged from 25-126 mg/100ml of grapefruit homogenate. Data from this investigation could be helpful in the fight against cancer by introducing a new, quick and reliable analytical method for food

analysis. By using this method and results from animal studies, nutritional scientists could recommend an appropriate intake of fruits and vegetable high in D-glucaric acid content for chemoprevention.

CHAPTER VI

CONCLUSION

Phase II enzyme activity is known to be one of the major mechanisms for chemoprevention. Several bioactive compounds have been reported to induce the activity of these enzymes. In this project, the inductive properties of citrus limonoids on GST and QR were investigated *in vivo*. It was found that variations in the limonoid structures influence the degree in induction of these enzymes. This was most notable in the methoxylation of limonin.

Furthermore, a method for the quantification of D-glucaric acid, a reported phase II detoxification enhancer, was developed. GA has previously been reported to be present in fruits and vegetables. Our method consisted of sample filtration and analysis by high performance liquid chromatography using an isocratic mobile phase. Compared to previous methods, this method is simple, quick and reproducible. An accurate determination of GA is essential for dietary recommendations of fruits and vegetables for possible chemoprevention.

The consumption of fruits and vegetables has been repeatedly correlated with positive health effects. A time has progressed and technology improved, these health promoting properties have been linked to specific bioactive compound present in the food we consume. Further studies are needed to elucidate the chemopreventive mechanisms of bioactive compounds found in citrus. Additionally, studies are also needed on the synergistic effects various bioactive compounds.

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APPENDIX

Abbreviations	Definition
(1,4)-GL	(1,4)-Glucarolactone
4NQO	4-nitroquinoline 1-oxide
(6,3)-GL	(6,3)-Glucarolactone
BSA	Bovine serum albumin
CDNB	1-chloro-2,4-dinitrobenzene
DCPIP	2,6-dichlorophenol-indophenol
GA	D-Glucaric acid
GST	Glutathione S-transferase
GSH	Glutathione (reduced)
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
PBS	Phosphate buffer solution
QR	NAD(P)H: quinone reductase
UDPGT	UDP-glucuronosyltransferase

VITA

Name: Jose Luis Perez

Address: Vegetable and Fruit Improvement Center, 1500 Research Parkway, Suite 120A, College Station, TX, 77843.

Email Address: jlperez@ag.tamu.edu

Education: B.S., Biology, The University of Texas Pan-American, 2004.
M.S., Horticulture, Texas A&M University, 2007

Professional Experience:

Texas A&M University, College Station ***September 2005- May 2007***
Description: Isolation and Purification of Limonoids found in citrus and evaluating their effects on human health; seasonal study on enzymes that convert bitter limonoids to tasteless limonoids; supercritical extraction of natural compound from citrus seeds; and evaluation of antioxidant activity of grapefruit varieties. HPLC determination of glucaric acid in grapefruit varieties.

University of Texas Pan American ***February 2006- March 2006***
Description: Carry out animal studies to evaluate the chemopreventive properties of limonoids isolated from citrus by enzymatic assays and induction of GST and QR activities and the effect of flavonoids on Hep G2 and Hepa 1c1c7 .

Texas A&M University Kingsville ***January 2005-August 2005***
Description: Carry out research involving the identification of plant pathogenic fungi(*Phytophthora spp. and Ganoderma spp.*) and method development for the examination soil for the nematode *Tylenchulus semipenetrans*, conduct field surveys, collect soil samples, produce field maps, DNA extraction, PCR, DNA purification, media preparation, transformation, cloning, and electrophoresis.

Texas A&M University, Citrus Center ***June 2003-December2004***
Description: Carry out several procedures in the lab: extraction, column separations, chromatography (HPLC, MPLC, Flash, size exclusion, ion affinity) anti-oxidant activity assays, keep a detail log book of all procedures, and research on subject.

University of Texas Pan American ***January 2004- December 2004***
Description: Isolation and Identification of mycological flora found on citrus leaves affected by Sooty Mold.

University of Texas Pan American ***June 2003- July 2003***
Description: Synthesis of organic compounds, NMR, and Column Chromatography.