

**ISOLATION AND EFFECTS OF CITRUS LIMONOIDS ON CYTOCHROME  
P450 INHIBITION, APOPTOTIC INDUCTION AND CYTOTOXICITY ON  
HUMAN CANCER CELLS**

A Dissertation

by

SHIBU M. POULOSE

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

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Horticulture

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## ABSTRACT

Isolation and Effects of Citrus Limonoids on Cytochrome P450 Inhibition,  
Apoptotic Induction and Cytotoxicity on Human Cancer Cells. (December 2005)

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This dissertation illustrates an efficient purification method for citrus limonoids and flavonoids, while examining their effects on cytochrome P450 inhibition and apoptotic induction on human neuroblastoma (SH-SY5Y) and colonic adenocarcinoma (Caco-2) cells. The first study developed a bulk purification method for limonoids, from seeds and molasses of citrus fruits, using a combination of chromatographic techniques. This also resulted in an efficient purification method for naringin and hesperidin from citrus byproducts.

The second study investigated the inhibitory effects of purified limonoids and flavonoids on the enzymatic activities of different isoforms of human cytochrome P450. *O*-Dealkylase and hydroxylase activities of CYP1A2, CYP1B1, CYP3A4 and CYP19, using specific substrates such as ethoxyresorufin (ethoxyresorufin *O*-dealkylase, EROD), methoxyresorufin (methoxyresorufin *O*-dealkylase, MROD), and dibenzylfluorescein (DBF), were

found to be significantly ( $P < 0.001$ ) reduced at micromolar levels. A kinetic analysis showed competitive and non-competitive modes of inhibition by limonoids, on CYP19 hydroxylase activity. The results corroborate the active role of limonoids in the redox cycling mechanisms.

The third study examined the antioxidant and apoptotic inducing ability of limonoid glucosides on human neuroblastoma cells. Four limonoid glucosides, LG (17 $\beta$ -D glucopyranoside limonin), OG (obacunone 17 $\beta$ -D glucopyranoside), NAG (nomilinic acid 17 $\beta$ -D glucopyranoside), and DNAG (deacetylnomilinic acid 17 $\beta$ -D glucopyranoside), have shown superoxide scavenging at millimolar levels. Micromolar amounts of LG and OG induced rapid necrosis of SH-SY5Y cells. Cytotoxicity was correlated ( $P = 0.046$ ) to a concentration and time-dependent increase in caspase 3/7 activity. Analyses of DNA content during the S phase of the cell cycle indicated reductions of 86.6% for LG and 82.3% for OG as compared to untreated. The results validate the antineoplastic distinctiveness of limonoid glucosides.

In the fourth study, cytotoxic effects of limonoid aglycones and glucosides were assessed on human SH-SY5Y neuroblastoma and colon carcinoma (CaCo-2) cell lines and compared with the non-cancerous Chinese hamster ovary (CHO) cells. Significant ( $P < 0.001$ ) cytotoxic effects were observed only on cancerous cells, over 24 to 36 h. The study revealed a marked increase in the DNA content of aneuploidic cells, which results in cell cycle arrest. The results confirm that glycosides are the most active apoptotic inducing form.

This dissertation is dedicated to my beloved wife, parents, siblings, mentors, friends and to those who are affected by cancer, for their faith and patience.

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## CHAPTER I

### INTRODUCTION

#### 1.1 Background

Citrus is an important fruit crop of tropical and subtropical areas with over 4000 years history of cultivation (1). The United States contributes nearly one sixth of the world citrus production, accounting for nearly 70 million metric tons (2). Consumed both as fresh fruits and processed products, the U.S. export value of citrus is estimated to be \$300 million in 2004-2005 season (3). Consumption of citrus has long been known to prevent many human diseases, from scurvy to several types of cancers. The health enhancing effects of citrus consumption is attributed to the wide assortment of bioactive compounds that makeup citrus phytochemicals (4). The major class of citrus phytochemicals includes flavonoids, carotinoids, limonoids, folic acid, pectin, coumarins, vitamin C, high quality soluble fiber and mineral potassium (5). Unique among these are the limonoids found predominantly in Rutaceae, Meliaceae, Cneoraceae and Simaroubaceae families, with citrus being the only edible fruits among these families. Limonoids are triterpenoids derived from the precursor 4,4,8-trimethyl-17-furanylsteroid, with furan and lactone moieties on their ring structures.

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This dissertation follows the style and format of the Journal of Nutrition.

Limonoids occur both as aglycones, otherwise known as citrus bitter principles and as tasteless water-soluble glycosides (6,7). Two enzymes, UDPG-limonoid glycosyl transferase and limonoid D-ring lactone hydrolase perform the inter-conversion of free to carbohydrate bearing moieties during fruit maturation (8). More than thirty-six limonoid aglycones and seventeen limonoid glucosides have thus far been isolated from citrus and its hybrids (9). Limonoid aglycones contribute to the “delayed bitterness” problem in citrus juices (10). The most common aglycones are limonin, nomilin, obacunone and deacetylnomilin which are present relatively large concentrations in citrus seeds and molasses (6,10).

A glucose moiety attached to C-17 position of aglycones by a  $\beta$ -glycosidic linkage makes the respective limonoid glucosides nonbitter and water-soluble. Most common among them are limonin 17 $\beta$ -D glucopyranoside (LG), nomilin17 $\beta$ -D glucopyranoside (NG), nomilinic acid 17 $\beta$ -D glucopyranoside (NAG), obacunone 17 $\beta$ -D glucopyranoside (OG), and deacetylnomilinic acid 17 $\beta$ -D glucopyranoside (DNAG). These are found in relatively higher concentrations in seeds, and molasses (6-8,11,12). Orange, grapefruit, and lemon juices contain an average of 320, 190 and 82 parts per million (ppm) of limonoid glucosides (13) and a glass of orange juice contains on average of 66 mg/L of limonoid glucosides (14).

Contemporary studies have shown that numerous health benefits are derived from limonoid ingestion such as reducing the effects of HIV infections

(15,16), serving as antimalarial and anti-inflammatory agents (17), and acting as cytotoxic agents against cancers of breast (18), colon (19,20), stomach (21), buccal pouch (22-24) and blood (25). Limonoids also have been found to lower blood cholesterol (26,27) and induce detoxifying enzymes such as glutathione S-transferase (GST) (28,29) and cytochrome P450 (30), which are among the main targets for antineoplastic strategies. Some, if not all of these properties appear to be associated with the ability of limonoids to affect cell growth (31). With the plethora of health promoting properties, citrus limonoids appear to be potential food supplementations and nutrient additives (11).

It is necessary to evaluate the biological activity of pure compounds to determine the chemopreventive agents in citrus fruits. However, the low concentration of limonoids in fruits and their byproducts makes their extraction and purification difficult (12). Several analytical methods using reversed-phase and normal-phase HPLC methods coupled with UV detection have been used to separate and detect limonoid aglycones and glucosides in small quantities (9,32). Additionally, the methods to isolate, identify and quantify limonoids lack specificity and sensitivity and, therefore, have not been very successful in the bulk purification of individual compounds required for large scale biological activity studies. Many of the carcinogenesis studies involving limonoids were performed only with topical applications in animals because of the dearth of purified compounds. Recently ion exchange and affinity based synthetic resins

have been used for the purification of naturally occurring compounds and could be applied for the bulk purification of limonoids from seeds and molasses.

Despite remarkable treatment advances, cancer remains the leading death causing disease among children up to age 14 years in the U.S. and the second leading cause of death among adults next to coronary heart disease (33). As many as one-third of all cancer deaths in the U.S. could be prevented with a healthy, balanced diet emphasizing vegetables, fruits, whole grains and beans which have a proven effect in maintaining a healthful weight (33). Generation of reactive oxygen species, activation of cytochrome P450 enzymes, alteration of cell cycles, and suppression of tumor suppressor genes are among the leading causes of the onset of several types of cancers (34,35). Consequently, strategies to counter the malignancies of these cancers are vitally important.

## **1.2 Cytochrome P450 isoenzymes**

Cytochrome P450 (CYP) enzymes are hemoprotein monooxygenases that catalyze a wide variety of endogenous and xenobiotic compounds (36,37). In humans, CYP enzymes have been classified into 18 gene families with over 50 enzymes in each. Individual isoenzymes are thought to be responsible for the metabolism of specific substrates. Some of the most important CYP isoenzymes are CYP3A4, CYP1A2, CYPB1 and CYP9, which have a specific role in the onset of several types of cancers (38-42). CYP3A4 isoenzyme, the most

abundant subfamily, has been implicated in the etiology of prostate cancer (38). A higher CYP1A2 level is known to influence risk of lung and colorectal cancers (39,40). Recent studies on high levels of E2-4 hydroxylation in estrogen responsive tissues have shown CYP1B1 to play an important role in estrogen related tumorigenesis (41). CYP19 aromatase catalyzes the conversion of androgens to estrogens in the last step of the estrogen biosynthesis pathway. It is believed that increased exposure to estrogens is a risk factor for breast cancer and, therefore, the human aromatase (CYP19) gene is a plausible candidate for low-penetrance breast cancer susceptibility (42). Since CYP enzymes are involved in both activation and detoxification in the first pass metabolism, their partial inhibition by naturally occurring compounds may represent a novel anticarcinogenesis strategy (42). Further, they could reduce the costs on drugs by increasing the bioavailability of orally administered drugs (43). Flavonoids of grapefruit have been shown to inhibit the activity of certain other human cytochrome P450 enzymes (44). Assessing the alkoxyresorufin *O*-dealkylase activity by CYP isoforms is reported to be a good assay system to test the inhibitory effects of naturally occurring compounds from citrus and to determine the kinetic properties of the inhibition (44). Since bioactive compounds in citrus juice have been shown to interact with phase I enzymes, it is imperative to determine the inhibitory effect of purified citrus limonoids on CYP isoforms.



### 1.3 Antioxidant activity

An array of bioactive compounds from fruits and vegetables has been shown to act against free radicals that have the potential to damage DNA and lead to cancer, coronary heart disease, and other chronic diseases (45-47). Activation of GST (28,29) and inhibition of CYP enzymes (30), both enzymes are known to work against redox cycling, by citrus limonoids has led to speculation that these compounds could be considered as antioxidants. However, no firm experimental data are available that support limonoids as having free radical quenching abilities. Pyrogallol (1,2,3-benzenetriol) undergoes auto-oxidation in the presence of superoxide anion radicals ( $O_2^{\cdot-}$ ) to form purpurogallin. Superoxide dismutase (SOD) inhibits this reaction (48) by quenching the superoxide radicals which can be measured spectrophotometrically. This *in vitro* assay serves as an excellent method to test the superoxide quenching ability of citrus limonoids.

### 1.4 Apoptotic induction

One of the molecular mechanisms underlying the chemoprevention properties of limonoids are thought to be through the induction of apoptosis. Although there is evidence that suggests limonoids restrain the proliferation of some cancerous cell lines, the underlying mechanism is unknown. The human SH SY5Y neuroblastoma cell line is an excellent experimental model for the study of apoptosis. Members of the Caspase 3/7 family of proteases have been

found to be crucial effectors of the downstream apoptosis signaling (49). So deciphering the cytological and biochemical evidence of limonoid induction of apoptosis is very revealing.

Neuroblastomas are embryonal tumors of the peripheral nervous system that arise from the neural crest; they account for about 10 percent of childhood cancer in U.S. (50). Chemotherapy has not made a significant impact on curing these pediatric malignancies (51). A few citrus flavonoids have been shown to protect against neuronal injury presumably through caspase pathway (52). Human SH-SY5Y neuroblastoma cell lines have been widely used to study anticancer drug therapy (53) and could be used to study the biochemical effects of citrus limonoids on the induction of apoptosis. Apoptosis, i.e., programmed cell death, plays a crucial role in development of several diseases. Biochemically, apoptosis is characterized by fragmentation of the genome and cleavage and/or degradation of several cellular proteins. The inhibitory effects of citrus limonoids on human cancer cell proliferation in certain types of cancer cell lines are postulated to be through the induction of apoptosis. High concentrations of limonoids have been shown to induce apoptosis in breast cancer (MCF-7) cells through the use of flow cytometry (31). Members of caspase 3/7 family of proteases have been found to be crucial mediators of the complex biochemical events associated with apoptosis. These enzymes have substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleave a number of different proteins including poly (ADP-ribose) polymerase

(PARP), DNA dependent protein kinase, and actins. Z-DEVD-R110 (N-CBC-Lasp-Lglu-Lval-Lasp-Rhodamine110) is a profluorescent substrate which is cleaved at the aspartic acid residue by caspase 3/7, releasing highly fluorescent rhodamine-110. This assay system may be used with either cell extracts or primary cell suspensions and enables one to identify the regulatory events in the apoptotic death process. Overall, studying the effects of limonoids on human neuroblastoma cells and their regulatory mechanisms could give fundamental evidence for the molecular mechanism of apoptotic induction. Furthermore, studying the effects of these compounds at different stages of the cell cycle provides critical evidences for the cytostatic effects of these bioactive compounds.

### **1.5 Cytotoxicity on colonic adenocarcinoma**

Colorectal cancer is the second most common cause of deaths related to cancer in U.S. Based on the American Cancer Society's *Facts and Figures*, about 145,000 new cases and about 57,000 deaths due to colorectal cancer will occur in 2005 in the U.S. alone (33). Epidemiological studies and animal experiments consistently proved the protective effects citrus fruits and their bioactive compounds against a wide range of cancers in humans (5). Grapefruit juice has been shown to suppress the carcinogen (PhIP) induced colon DNA damage (54). Furthermore, orange juice, which is rich in limonoids and flavonoids, significantly inhibited azoxymethane induced colon cancer in male

Fisher 344 rats (55). Similarly, limonin and obacunone inhibited aberrant crypt foci (ACF) formation, thereby, inhibiting the pathogenesis of colon cancer (20). Considering the advantages of limonoid glucoside over aglycones in water solubility and absorption, it is imperative to study the cytotoxic effects of limonoid glucosides and other aglycones on human colon cancer cells. It is also important to assess the effect of these bioactive compounds on non-cancerous mammalian cells, to understand the toxic effects. Hence Chinese hamster ovary cells provide an excellent non-cancerous cell model to assess the toxicity of these bioactive compounds.

## CHAPTER II

### ISOLATION OF LIMONOIDS AND FLAVONOIDS FROM SEEDS AND MOLASSES OF CITRUS FRUITS

#### 2.1 Synopsis

Limonooids with putative cancer preventive properties were purified from seeds and molasses of citrus fruits using a combination of ion exchange and size exclusion chromatographies. pH-dependent cold precipitation of a large quantity of naringin, prior to the separation of limonooids from flavonooids on a styrene/divenylbenzene anionic resin using an increasing salt gradient, was found to be critical for higher yields of semi-purified limonoid glucosides. Substantial differences were observed with use of different resins on the total recovery of limonoid glucosides. High purity limonoid glucosides were obtained via reverse phase preparative high performance liquid chromatography and high purity limonoid aglycones through direct crystallization. The purity and structures of individual compounds were confirmed using LC-MS and NMR. Limonilic acid was synthesized from limonin for the structure-bioactivity studies. Hesperidin, a major flavonoid of oranges was isolated from orange peels using a pH dependent potassium iodide precipitation method. A large scale purification method of limonooids and flavonooids from citrus byproducts is presented.

## 2.2 Introduction

Plant compounds are sources for the development of new drugs and model structures for synthetic formulations (56). Citrus fruits contain most of the important class of health promoting bioactive compounds including flavonoids, limonoids, carotenoids, vitamin C, coumarins, pectin, folic acid, glucaric acid, high quality soluble fiber, and minerals such as potassium (5). Although initial speculations on the biological activities of citrus compounds were associated with vitamin C, it was later proved that other components impart health promoting properties. Limonoids are abundant in citrus fruits, and occur both as water insoluble aglycones and soluble glucosides (6,7). The bitter limonoid aglycones are converted to nonbitter and tasteless glucosides by UDP-D-glucose-limonoid glucosyltransferase during fruit maturation (8). Evidence suggests that intestinal absorption of limonoids and flavonoids is greatly enhanced when ingested as glucosides (57,58).

Citrus limonoids have numerous health promoting properties in animals and human cell culture studies (15-31). Major flavonoids of citrus fruits, naringin, narirutin and hesperidin have also been shown to have numerous health promoting properties (59-64). Flavonoids are being evaluated as food additives by the FDA (65). Because of the growing evidence on the health promoting properties, these bioactive compounds could be developed as food nutrient additives. Limonilic acid, a modified limonin, has been shown to possess

significant antifeedant activity with Colorado potato beetle larvae (66), and thus could be used for insect control.

However, the activity studies of these bioactive compounds rely on the availability of pure compounds. Many of the carcinogenesis investigations with limonoids were performed either with mixtures or using topical applications in animals due to inadequate supplies of pure compounds. Citrus byproducts from processing plants are excellent sources for limonoids with more than 500 ppm in seeds and molasses (6-8,11,67).

Procedures to isolate pure limonoids (both aglycones and glucosides) have advanced steadily and it is now feasible to prepare and evaluate pure materials for anticancer effectiveness. Several methods such as normal-phase HPLC (32, 68, 69) reversed-phase HPLC (70,71), NMR (7,72,73), LC/ESI/MS (74 -76), have been developed to quantify and/or identify limonoids with different degrees of specificity and sensitivity. Adsorption and ion exchange resins have greatly facilitated the isolation and purification of many flavonoid and glucoside mixtures (11,77). Despite these recent advances, there is still room to improve the separation efficiency and increase yields. The current study establishes an efficient method for isolation of limonoids, hesperidin, and synthesis of limonilic acid from limonin. In addition, a bulk purification method for naringin that involves a combination of chromatographic techniques is described.

## 2.3 Materials and methods

***Limonoids and flavonoids source.*** Seeds of Mexican oranges (*varieties*; Valencia & Navel) were obtained in 100 Kg lots from Monterrey, Mexico, dried under shade, and finely powdered in a coffee blender, before solvent extraction. Orange and grapefruit molasses were obtained from the Texas Citrus Exchange (TCX), a commercial citrus juice plant in Mission, Texas. The molasses was stored at 4°C with 0.2% sodium azide added until used.

***Resins and other chemicals.*** Commercial macroporous, neutral resins, such as SP-70, XAD-16 and XAD-2, were purchased from Supelco. Weak anion exchangers, i.e., WA-30 and high performance Q-sepharose with exchange and binding capacities of 150-200  $\mu\text{eq/mL}$  and of 120 mg/L human serum albumin (HSA/mL) respectively, were obtained from Sigma-Aldrich Co. The strong cationic exchanger Dowex-50 was also purchased from Sigma Chemical Co. Dowex-50 had an ion exchange capacity of 1.7 meq/mL wet resin, of 100-200  $\mu$  mesh size, and 50-58% water retention capacity. Small columns (2.5 cm X 10 cm) were used for evaluation and selection of appropriate resins, e.g., XAD-16, XAD-2 and WA30. Bulk purification was accomplished using Dowex-50 (10 cm x 40 cm column), SP-70 (10 cm x 30 cm column), and Q-sepharose (10 cm x 30 cm column). The resins were presoaked in methanol, made into a slurry using deionized water and added to the column until about two thirds of it was filled. The top end of the first column was fitted to a low pressure peristaltic pump and the series of columns were interconnected (**Fig. 1**). The resins were washed

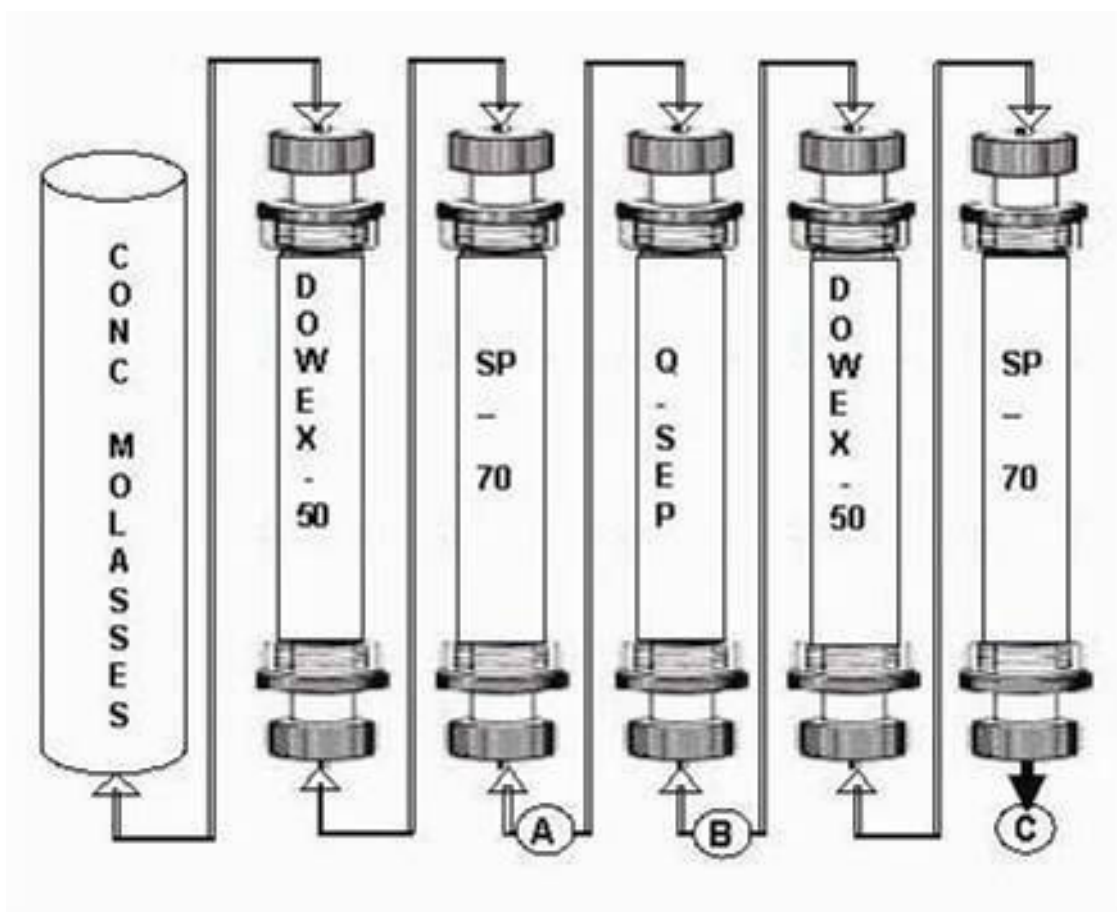


until the conductivity of the eluant decreased below 100  $\mu\Omega$  and backwashed with three column volumes (CV) of deionized water.

***Selection of resins for glycosidic extracts from seeds.*** Ethanolic extracts of Mexican orange seeds were concentrated by rotary-vacuum evaporation. A portion (100 g) of the dried extract was mixed 1:3 with water. The pH was adjusted from 4.0 to 6.5 to determine the optimum pH for purification and yield with the various resins. After loading, the columns were washed with four column volumes of water and then the compounds were eluted with 80% ethanol. The solvent was removed by rotary-evaporation and the product weights were determined after freeze-drying.

***Separation of limonoid glucosides from flavonoids.*** Orange and grapefruit molasses (1 kg; 56 and 53° brix of total soluble sugars respectively (TSS)) were placed in beakers and the TSS adjusted to 13° Brix with deionized water. The slurry was centrifuged at 10,000  $g_{max}$  for 45 min to remove solids and the supernatant was vacuum filtered through Whatman #5 (2.5  $\mu\text{mol/L}$ ) filter paper. The clarified solution (2400 ml) was loaded onto the Dowex-50 at a flow rate 46 mL/min using a peristaltic pump. The Dowex-50 column was connected directly to SP-70 adsorbent column. After loading, the columns were washed with 5 CV water. The loading capacity and washing efficiency was determined by sampling the discharge from SP-70 and analyzing by TLC (silica gel). The compounds adsorbed to SP-70 were eluted with 80% methanol at a flow rate of 50 mL/min. The eluant was concentrated and the solvent was recovered. The

method described by Schoch *et al.*(11) for efficient separation of limonoids from flavonoids was improved by removing naringin in large quantities. After passing through Dowex-50 and SP-70 resin columns, 1 Kg of molasses yielded 650 ml concentrate (from roto-evaporating the methanol eluant) of pH 2.68. Naringin was precipitated from this concentrate by adjusting the pH to 3.5, stirring overnight at 4° C. The precipitate was vacuum filtered using Whatman #5 filter paper. The precipitate was then washed with ice cold water to remove all the impurities. The precipitated naringin was subsequently dried at 60°C and analyzed for purity. The pH of the remaining filtered supernatant was adjusted to 6.5 and the supernatant loaded onto a Q-sepharose anion exchange column at 35 mL/min. A linear gradient of NaCl (0-0.6 mol/L) was used to elute the compounds and 200 fractions (25 mL each) were collected. The fractions were desalted by passing them through a SP-70 column (2.5 cm x 30 cm) and eluted with 70% methanol. Limonoid glucosides were detected by silica gel TLC analysis (mobile phase of 5:3:1:1 ethylacetate: ethylmethylketone: formic acid: water). Compounds were detected on the TLC plate using Elrich's reagent (1% *p*-dimethylaminobenzaldehyde in ethanol) in an HCl gas chamber. The flavonoids were detected on a UV spectrophotometer at 254 and 280 nm. The fractions were freeze-dried and then were weighed. The purification scheme through ion-exchange chromatography and the points where mass balances were taken is shown in **Figure 2.1**.



**FIGURE 2.1** Sequence of ion-exchange columns in the purification of limonoids glucosides and flavonoids from molasses of oranges and grapefruits. A, B, and C represents the points where the mass balances were taken.

**Preparative HPLC separation.** Semi-purified limonoid glucoside mixture (1 g) was dissolved in 10 ml deionized water and injected to a preparative HPLC. Previously described methods (75,78) were modified to optimize resolution of individual peaks by using a mobile phase of 10-25% acetonitrile gradient, with 0.003% phosphoric acid. A reverse phase C-18 column (10 cm x 40 cm, Novapak, Waters Inc.) at 950/ 1150 psi (radial/ back) pressure was used. Peaks were monitored using a Waters UV-Photo Diode Array (PDA) detector. Individual peaks were collected based on the retention time. Acetonitrile was removed using a rotary-evaporator, and the compounds were lyophilized. The purity of the compounds and their structures were determined through, HPLC, LC-MS and NMR.

**Extraction of limonoid aglycones.** The limonoid aglycones were extracted by modification of existing methods (79,80). Finely powdered grapefruit (*varieties*; Mexican red and Rio red) and orange seeds (*varieties*; Navel and Valencia) (10 kg/batch) were extracted with 15 L hexane in the Soxhlet apparatus for 24 h at 60°C. The hexane extract was recovered and filtered using Whatman # 5 filterpaper. The hexane extract was partitioned with methanol at 1:1 ratio and allowed to settle for 1 hour. The methanol fraction was separated and subject to concentrated under reduced pressure (rotary-evaporation). The resultant concentrate was dissolved in hexane and allowed to stand overnight at room temperature. The next day the precipitants were filtered using Whatman # 5 filterpaper. The silica gel TLC analysis of the filtered

precipitate indicated high concentrations of obacunone and nomilin. This mixture was vacuum dried, powdered, and used for open silica column chromatography. The hexane defatted seed powder (6.5 kg) was further extracted with acetone (15 L) at 60°C for 12 h in a Soxhlet apparatus to remove the aglycones. The acetone extract was concentrated under reduced pressure and a rotary-evaporator. The aglycone mixture dissolved in acetone was spotted on silica gel TLC plates (Sigma Aldrich Co.) using ethylacetate:hexane (3:2) mobile phase, sprayed with 10% sulfuric acid in methanol, and developed at 110°C for 1 min. The R<sub>f</sub> values were compared with those of the standards. The compounds were further quantified on an analytical HPLC (Waters, Novapack RP-18, 8 mm x 40 mm, UV 6000LP detector at 210 nm) using a 10-50% linear gradient of water:acetonitrile with 0.003% phosphoric acid. The major aglycone limonin was precipitated from the concentrate by dissolving it 1:2 in dichloromethane, and allowing it to stand for 1 hour and then vacuum filtering through a Buckner funnel using Whatman # 5 filterpaper. Limonin was further purified by washing repeatedly with isopropanol and recrystallizing in dichloromethane. Most of the limonin was removed by recrystallization (3–4 times). The remaining filtrate was rotoevaporated and redissolved in minimum acetone and left at 4°C for 72 h to crystallize nomilin. The nomilin crystals were washed with cold isopropanol. The leftover filtrate was concentrated and subjected to vacuum liquid chromatography to purify minor individual aglycones.

**Vacuum liquid chromatography (VLC).** VLC has been used as an alternative technique to conventional chromatographic methods (81) and for the purification of bioactive diterpenoids and other compounds (82). This technique was used to purify individual aglycones from bulk limonoid aglycone mixtures. The filtrate obtained after removal of limonin and nomilin contained concentrated amounts of deacetylnomilin, obacunone and traces of limonin and nomilin. The filtrate was mixed with TLC grade silica gel (EM Science) with gypsum binder (mesh size 2-2.5  $\mu\text{m}$ , pore size 0.75  $\text{cm}^2/\text{g}$ , pore diameter 60 $^{\circ}\text{A}$ , Bet surface area 500  $\text{m}^2/\text{g}$ , pH 6.8 at 10% aqueous suspension) and subjected to VLC to purify individual compounds. The mixture was finely powdered (until free flowing) and added to dry TLC grade silica gel of same properties already in a VLC column. A vacuum was applied for about 40 minutes for uniform packing and then washed with 100% hexane to check the uniform movement of solvent through the column. The mixture was firmly placed on the top of the silica packing, covered with a filter paper and vacuum was applied to compact the silica packing. A step gradient of 20-80% of ethylacetate:hexane at 5% increments (50 mL/step) was used to elute the aglycones from the column and individual fractions were analyzed with TLC and pooled based on the individual compounds content of the fraction.

**Open column chromatography.** Open silica column chromatography with a range of mobile phases is widely used for the efficient purification of individual bioactive compounds. This is a rapid technique for the separation of

minute quantities of compounds (83). Aglycone mixtures devoid of limonin and other crystallized aglycones were dissolved in acetone and mixed with equal quantities (w/w) of 63-200 mesh size silica gel. The mixture was packed into a 24/40 column 2.5 cm x 30 cm with 54 g of silica and eluted with 40-70% ethylacetate-hexane. The effluent was collected (20 mL fractions) until complete elution. The fractions were analyzed by TLC and then pooled depending on their content. The unresolved limonin-deacetylnomilin was further separated on 1:1 mixture of 230-400 mesh silica, eluted with 55% ethylacetate-hexane.

***Synthesis of limonilic acid from limonin.*** The method for synthesis of limonilic acid reported by Emerson (84) was modified. Limonin (8 g) was suspended in 250 ml of 0.2 N KOH in methanol contained in a round bottom flask equipped with a water cooled condenser. The solution was refluxed for 30 minutes. After cooling, the methanol was removed under vacuum. The residue was dissolved in 100 mL water and 3 g each of Iodine and potassium iodide were added. To this mixture 2N NaOH was added until the iodine color was changed to a light red/strong orange yellow. After 15 minutes, 5 mg/L of sodium sulfite was added and the solution was acidified with 5N HCl. At pH 1 the limonilic acid was precipitated. The precipitate was filtered and washed with isopropanol and then with hexane to remove Iodine. The purity was confirmed via analytical HPLC.

***Isolation of hesperidin from orange peels.*** Large quantities of hesperidin were isolated via modification of a previously described method (85). 2.5 Kg of freshly peeled mandarin and orange peels were finely ground with a coffee blender, and mixed with 4 L of deionized water. The pH of the slurry was raised to 11-12 by addition of calcium hydroxide granules. The elevated pH slurry was stirred for 90 minutes and filtered using cheese cloth (3 layers). The filtrate was collected and the solids were discarded. The pH of filtrate was adjusted to 4.7 with concentrated HCl. The solution heated to 60°C in a water bath for 4 h, stirred for 2 h and held at room temperature overnight. The precipitated slurry was vacuum filtered, washed with methanol, and then with hexane to remove the impurities. The filtrate was dried and analyzed using analytical HPLC.

## **2.4 Results**

***Selection of resins.*** The total glucoside yield results, using different resins at different pH are summarized in **Table 2.1**. The yields were relatively higher for SP-70 and XAD-16, compared to other resins tested. Total glucoside yield was higher at pH 4.5, compared to other pH ranges tested (**Table 2.1**).



**TABLE 2.1**

*Recovery of semi-purified limonoid glucosides from 100 mL of seed concentrates using different ion exchange columns at different pH. Values are mean (g)  $\pm$  SD, n= 3.*

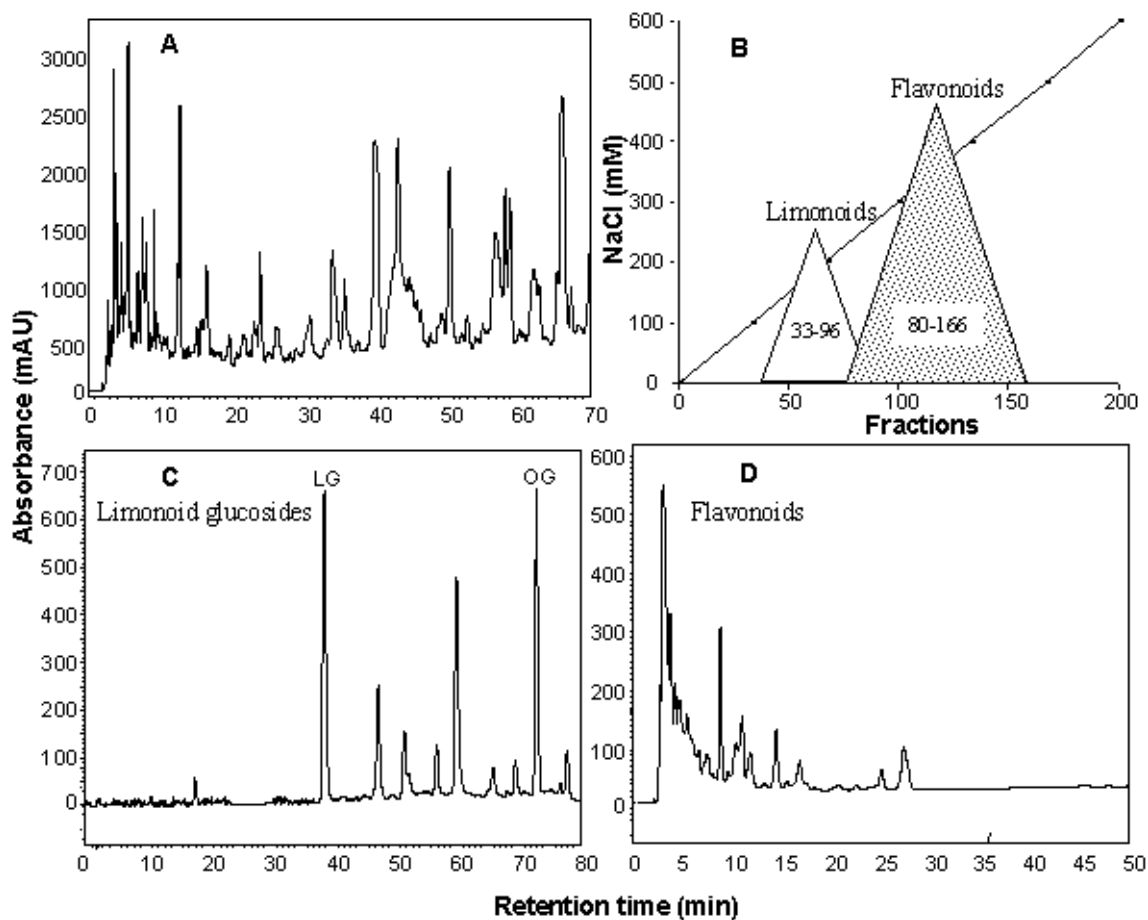
	pH				
	4.0	4.5	5.0	6.0	6.5
<b>SP-70</b>	2.69 $\pm$ 0.12	3.24 $\pm$ 0.16	3.07 $\pm$ 0.32	3.14 $\pm$ 0.31	2.98 $\pm$ 0.13
<b>XAD-2</b>	1.54 $\pm$ 0.17	1.56 $\pm$ 0.09	1.42 $\pm$ 0.21	1.32 $\pm$ 0.24	1.47 $\pm$ 0.14
<b>XAD-16</b>	2.98 $\pm$ 0.19	3.14 $\pm$ 0.11	2.97 $\pm$ 0.17	2.65 $\pm$ 0.12	2.64 $\pm$ 0.24
<b>SP70 &amp;WA-30</b>	1.38 $\pm$ 0.21	1.69 $\pm$ 0.17	1.17 $\pm$ 0.22	1.32 $\pm$ 0.17	1.12 $\pm$ 0.16

**TABLE 2.2**

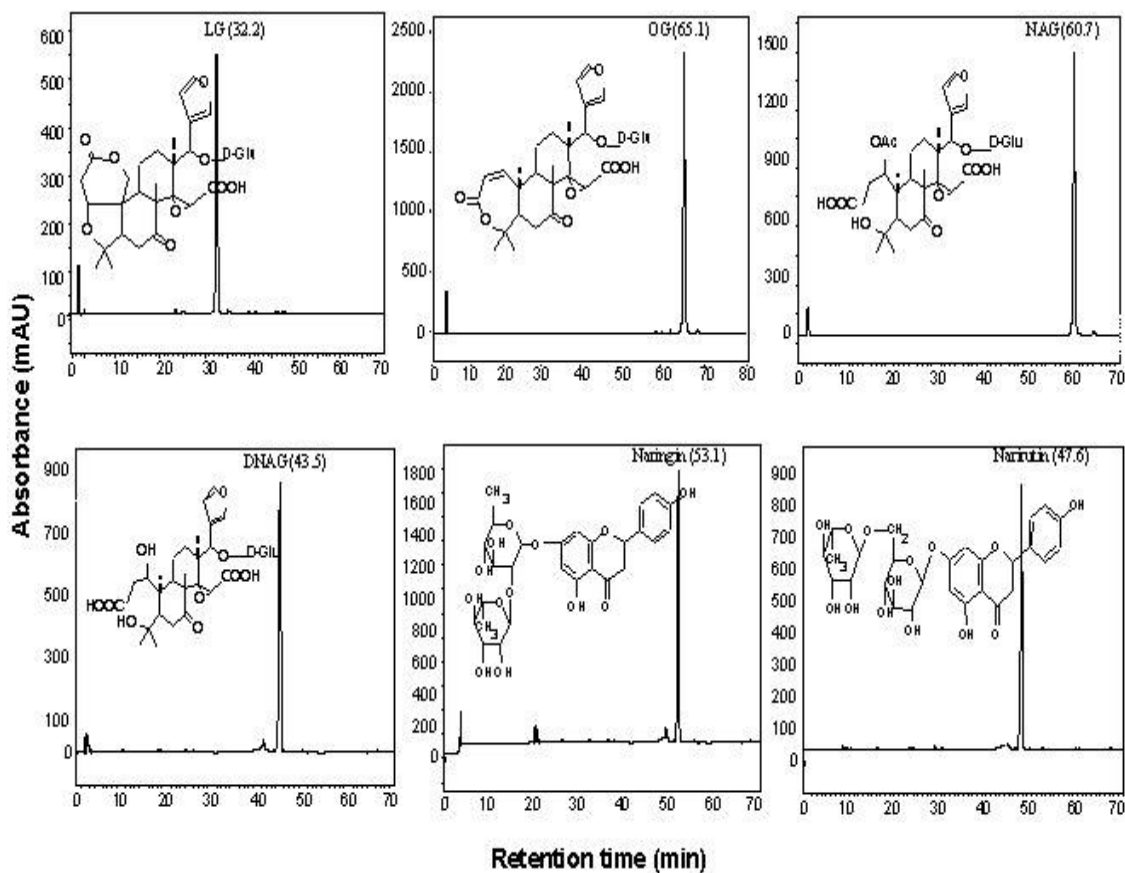
*Recovery of limonoid glucosides (g) from 1 Kg of clarified methanolic molasses concentrate after the removal of naringin. Values are mean (g)  $\pm$  SD, n= 4.*

	Mixture	LG	OG	NAG	DNAG
<b>Orange molasses</b>					
After SP-70	14.21 $\pm$ 1.28	1.92 $\pm$ 0.21	0.91 $\pm$ 0.18	0.88 $\pm$ 0.27	0.53 $\pm$ 0.12
After Q-sep	9.63 $\pm$ 1.01	1.17 $\pm$ 0.14	0.79 $\pm$ 0.15	0.73 $\pm$ 0.11	0.55 $\pm$ 0.16
Final mixture	6.32 $\pm$ 0.36	1.28 $\pm$ 0.12	0.77 $\pm$ 0.21	0.56 $\pm$ 0.09	0.43 $\pm$ 0.19
<b>Grapefruit molasses</b>					
After SP-70	12.45 $\pm$ 1.23	1.06 $\pm$ 0.26	0.98 $\pm$ 0.22	0.74 $\pm$ 0.32	0.51 $\pm$ 0.13
After Q-sep	7.71 $\pm$ 0.82	0.94 $\pm$ 0.29	0.82 $\pm$ 0.28	0.59 $\pm$ 0.19	0.47 $\pm$ 0.14
Final mixture	5.82 $\pm$ 0.81	0.97 $\pm$ 0.12	0.77 $\pm$ 0.19	0.47 $\pm$ 0.19	0.39 $\pm$ 0.16

**Separation of limonoids, flavonoids and naringin.** The removal of naringin was found to be critical for the efficient separation of limonoid glucosides from flavonoids. The array of compounds in molasses was fractionated on Q-sepharose anion exchanger into limonoids and flavonoids with an increasing salt gradient (**Fig. 2.2**). Methanolic concentrates of 1 Kg grapefruit and orange molasses from SP-70 yielded  $29.86 \pm 3.26$  and  $22 \pm 2.78$  g of naringin with a higher than 94% purity. Naringin alone comprised of 89% of the total flavonoids isolated followed by hesperidin and narirutin. Dowex-50 and SP-70 resins effectively reduced the impurities (**Table 2.2**). The HPLC and TLC profiles of the freeze-dried Q-sepharose fraction showed limonoid glucosides in the initial 33-96 fractions followed by flavonoids in the 80-166 fractions (**Fig. 2.2**) with traces of naringin in all the fractions. Limonoids were separated on the NaCl gradient of 0.16 to 0.21 mmol/L while flavonoids separated at 0.23 to 0.48 mmol/L. An average of 16 g of dried limonoid glucosides and 9.8 g flavonoid mixtures were obtained from 1 Kg of clarified citrus molasses concentrate, after removing the naringin. The weight of the individual glucosides was calculated based the dried isolates from 1 g of limonoid mixture on preparative HPLC (**Table 2.2**). The peaks collected from the preparative HPLC were confirmed as the listed compounds (**Fig. 2.3**) with more than 90 % purity, by LC-MS and NMR. Other limonoid glucosides, e.g., NG and DNG, were isolated in minor quantities through preparative HPLC, but the purity of these compounds was determined to be less than 75%.



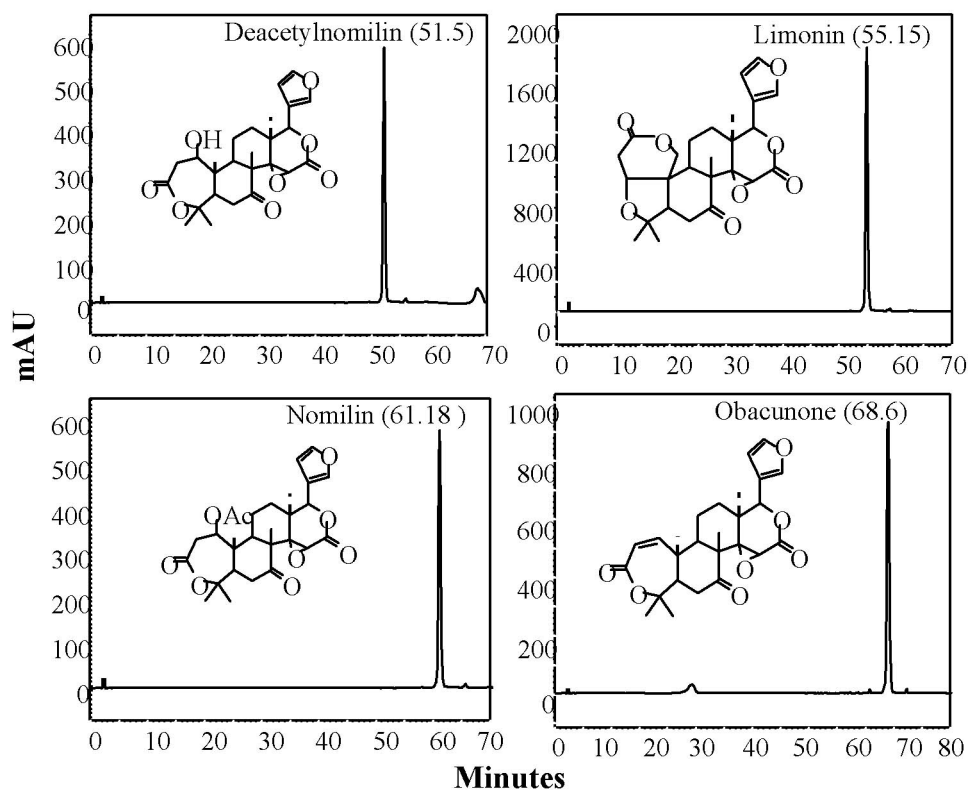
**FIGURE 2.2** Separation of limonoids from flavonoids using clarified molasses on an anion exchange column Q-sepharose, with increasing gradient of sodium chloride. Panel A represents the analytical HPLC chromatogram of the clarified molasses. Panels B, C represent, chromatograms of limonoid glucosides and flavonoids, separated on Q-sepharose.



**FIGURE 2.3** Structures and analytical HPLC chromatograms with retention time for the individual compounds purified by ion-exchange chromatography and preparative HPLC. LG = limonin 17 $\beta$ -D glucopyranoside, OG = obacunone 17 $\beta$ -D glucopyranoside, NAG = nomilinic acid 17 $\beta$ -D glucopyranoside, DNAG = deacetylnomilinic acid 17 $\beta$ -D glucopyranoside.

**Purification of limonoid aglycones.** For large scale purification of limonoid aglycones, soxhlet extraction, direct crystallization, and vacuum liquid chromatography, shown to be an efficient method. Crystallization yielded limonin with over 95% purity. Though nomilin and deacetylnomilin were directly crystallized, the purity of those compounds was observed to be 76% on HPLC; therefore, open silica column chromatography and VLC were effective in separating large quantities of aglycones (**Fig. 2.4**). One kilogram of dried orange, grapefruit seeds yielded 8.62 and 9.31 g of limonin, 0.62 and 0.47 g nomilin, 0.9 and 0.63 g of deacetylnomilin and 0.21 and 0.17 g of obacunone, respectively. Considering the abundance of aglycones in seeds, and glucosides in molasses, citrus byproducts provide excellent sources for bulk purification purposes.

**Limonilic acid, hesperidin and narirutin.** Synthesis from 8 g of limonin resulted in 6.3 g of pure limonilic acid of 87% purity. The procedure was found to be efficient in yield and purity as compared to the previously described method (84). Similarly the method reported here for the isolation of hesperidin from orange peels was found to be efficient yielding an average 28.7 g for 2.5 Kg peels. However, purity of the compound was similar to that the previously described. Preparative HPLC separation yielded a 93% pure narirutin product (**Fig. 2.3**), on analytical HPLC.



**FIGURE 2.4** Structures and analytical HPLC chromatograms with retention time for the individual limonoid aglycones. Resolved on a mobile phase of 10-50% acetonitrile in water with 0.003% phosphoric acid, using a reverse phase C-18 column at 1 mL/min flow rate.

## 2.5 Discussion

Adsorption and ion-exchange resin load capacity is a key parameter for limonoid purification. When compared to the other resins, SP-70 had an increased yield at pH 4.5. The ion-exchange mechanism involved in this purification process is well characterized for other compounds (86). The

adsorption-desorption properties of limonoids on adsorbent resins like SP-70 can be summarized in the following steps: (i) selective movement of limonoids in solution to the boundary of resin particles, (ii) attachment of limonoid glucosides to the surface of the resin, (iii) diffusion to the intra-particle active sites from the surface, (iv) attachment and desorption depending upon the molecular size, charge of the molecule and polarity of the mobile phase. The differences in yields at various pH's could be due to conformational changes that glucosides undergo as a function of pH.

The ion-exchange process depends on the ionic strength of the mobile phase and desorption increases with increase in the conductivity (87). A lower degree of cross-linking in a cationic exchanger like Dowex-50 eliminates polyelectrolytes and emulsifier anions (88) which aid in the elimination of colored impurities from molasses. The rate of the adsorption/desorption processes on an anionic resin is determined by the number of charged groups on the molecule. Structural properties of limonoid glucosides exhibit a negatively charged open D-ring with a glucose moiety attached to it. In the case of acidic glucosides like NAG and DNAG, both the A and D rings are open. From the fractionation pattern (**Fig. 2.2**) it is evident that the negative charges on limonoid glucosides and flavonoids molecules alter the conductivity and separation on anionic resins is affected by an increasing salt gradient. The increase in yield over the previously described method (11) can be ascribed to the separation of naringin at the initial stages of separation. Although more than 17 limonoid glucosides have been

identified from citrus (6), only the four most abundant glucosides have been characterized (**Table 2.2**) for use in further biological activity studies.

Extraction of aglycones using a Soxhlet apparatus remains to be proven an efficient method of extraction. Direct crystallization was successful in providing a large quantity of high purity limonin. The availability of seeds and molasses from the juice processing plants makes for an inexpensive source of raw materials. Large scale purification of desired products from seeds and molasses via the Soxhlet extraction, direct crystallization, VLC, and silica column chromatography proves to be inexpensive and efficient. These methods are applicable for industrial purification. Similarly, the synthesis of limonilic acid, precipitation of hesperidin, and separation of narirutin are also suitable for the bulk purification to meet commercial needs.

In conclusion, large amounts of pure limonoids and flavonoids are needed to elucidate their cancer preventive properties. The bulk purification methods reported in this chapter prove to be economical and efficient. The isolation and activity studies with pure compounds could provide critical information regarding the structure-activity relationship, molecular mechanisms, and biochemical interaction, thereby could aid as lead structures for new drug development.



## CHAPTER III

### INHIBITORY EFFECTS OF CITRUS LIMONOIDS AND FLAVONOIDS ON HUMAN CYTOCHROME P450 ISOENZYMES

#### 3.1 Synopsis

The effects of limonoids and flavonoids on *O*-dealkylase and hydroxylase activities of human cytochrome P450 (CYPs) isozymes such as CYP1A2, CYP1B1, CYP3A4 and CYP19 were measured using the substrates ethoxyresorufin (ethoxyresorufin *O*-dealkylase, EROD), methoxyresorufin (methoxyresorufin *O*-dealkylase, MROD), and dibenzylfluorescein (DBF). The kinetic studies resulted in variable inhibition of CYPs in concentration dependent assays. Significant ( $P < 0.001$ ) reductions in enzyme activities were observed, with purified compounds above 2  $\mu\text{mol/L}$ . Kinetic analysis indicated that limonin glucoside inhibited CYP19 hydroxylase competitively, while nomilinic acid glucoside in a non-competitive mode. IC<sub>50</sub> values were less for glucosides than for the aglycones. The results support prior suggestions for an active role of these compounds in the redox cycling. The variation in efficacy by different limonoids can be ascribed to the structural variation of the molecules. Limonoid inhibition of key CYPs involved in carcinogenesis supports growing evidences that citrus bioactive compounds can act as anticancer agents.

### 3.2 Introduction

Exposure to environmental chemicals is considered as a major risk factor for several types of cancers. These exposures can result in the generation of reactive oxygen (e.g., singlet oxygen, superoxide) and nitrogen (e.g., peroxyxynitrite, nitrogen dioxide) species (89) which have been implicated as causative agents for many diseases including inflammatory and degenerative diseases, arthritis, retinitis pigmentosa, coronary artery diseases, and many types of cancers (89,90). In humans, many of the chemicals are pre-carcinogens and are activated to carcinogenic and mutagenic substances by microsomal enzymes (89,91). The human microsomal enzyme system consists of scores of cytochrome P450 isoenzymes. *In vitro* studies with certain phytochemicals revealed a reduction in activities involved with generation of carcinogens through partial inhibition of these enzymes (44,92,93).

Cytochrome P450 enzymes are electron-transporting proteins that contain a heme-prosthetic group in which the iron alternates between reduced ferrous ( $\text{Fe}^{2+}$ ) and oxidized (ferric,  $\text{Fe}^{3+}$ ) state (**Fig. 3.1**) (94). The CYPs are known to be involved in the metabolic activation and detoxification of environmental carcinogens, orally administered drugs, steroids, bile acids, fatty acids, eicosanoids, and fat soluble vitamins (91,95). Activation involves the formation of reactive oxygen species (ROS) that may interact with DNA of target cells (91,96). In humans, CYP enzymes have been classified into 18 gene families with over 50 enzymes in each (97) and are mostly found on the

endoplasmic reticulum. Individual isoenzymes are thought to be responsible for the metabolism of specific substrates. Some of the most important cytochrome P450 isoenzymes are CYP3A4, CYP1A2, CYP1B1, and CYP19, which have specific roles in the onset of several types of cancers (38-41,91,96). Thus, inhibition of CYPs by naturally occurring compounds may represent a novel approach in the anticarcinogenesis strategy (42,95).

Citrus products contain several vitamins, minerals, and an array of bioactive compounds. The bioactive compounds in citrus fruits have been shown to possess numerous cancer preventive properties (2,5). Much of current research is being focused on limonoids and flavonoids. Limonoids are unique to citrus. Contemporary studies have shown that limonoids have numerous health benefits such as countering the effects of HIV infections, serving as antimalarials, anti-inflammatory agents, and as cytotoxic-cytostatic agents against several types of cancers (15-31). Limonoids are shown to be non-toxic to the normal cells (31). The effect of limonoids and flavonoids in inducing glutathione S-transferase (GST), a major phase II detoxifying enzyme (5,24,28) and specific role on certain CYP activity (30) lead us to postulate that one of the antineoplastic properties of these compounds could be through inhibition of cytochrome P450 enzymes, thereby reducing carcinogenesis. Here we report the effects of citrus limonoid aglycones, glucosides, and flavonoids on the activity of human cytochrome p450 isoenzymes (CYP1A2, CYP1B1, CYP3A4, CYP19) using alkoxyresorufins and dibenzylfluorescein (DBF) as substrates.



aglycone mixture used for the CYP activity assay consisted of 49% limonin, 17% deacetylnomilin, 14% nomilin, 8% obacunone, and 12% other aglycones in minor concentrations, based on the HPLC profiles. The stock solutions (1 mol/L) were prepared in DMSO for individual limonoid aglycones and in water for pure limonoid glucosides. The stock solutions for the mixtures were in ppm. The DMSO concentration was maintained below 0.1% in the CYP reaction mixtures.

***CYP isoforms and substrates.*** Alkoxyresorufins (e.g., methoxyresorufin, and ethoxyresorufin) were synthesized as described by Mayer *et al.*(98). DBF and ketoconazole were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Human liver expressed CYP isoenzymes CYP1A2, CYP1B1, CYP3A4, CYP19 and NADPH oxidoreductase were purchased from BD Gentest Inc. (Woburn, MA, USA). Activity of all these isoforms were expressed from human CYP1A2 cDNA using baculovirus infected (BTI-TN-5B1-4) expression system.

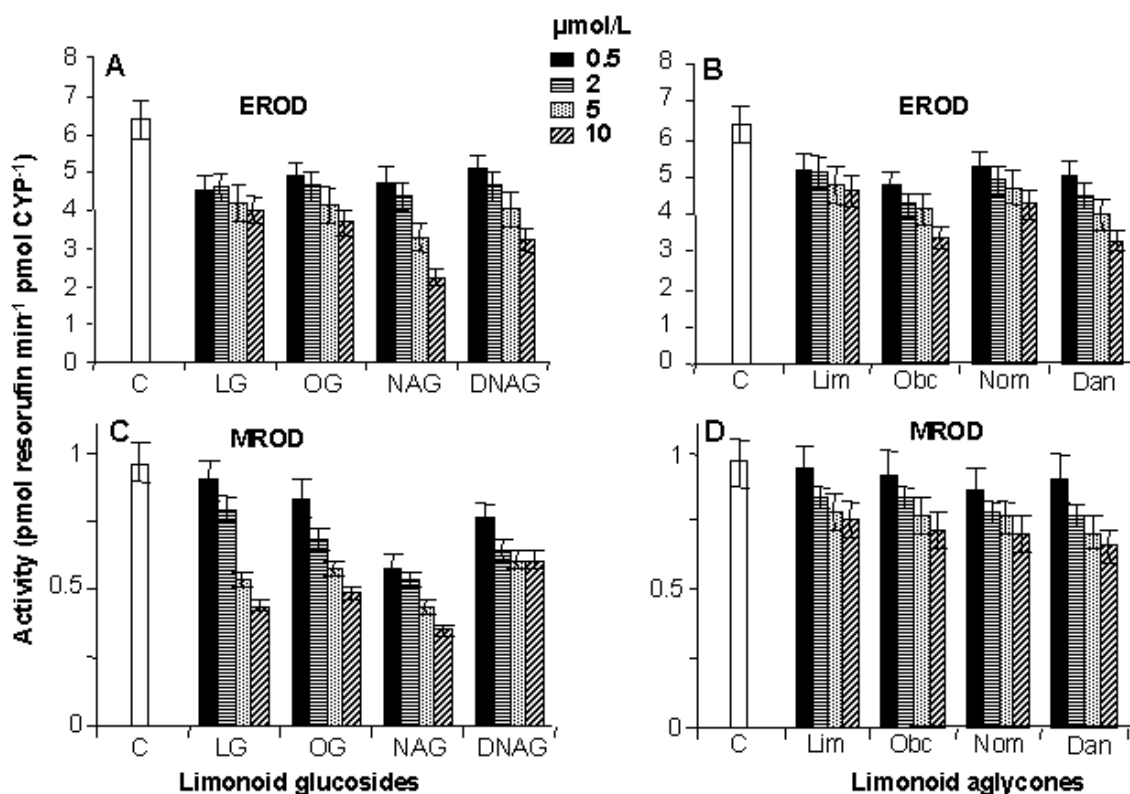
***Methoxy-, ethoxy-O-dealkylase and hydroxylase activities of CYPs.*** Methoxyresorufin and ethoxyresorufin were used as substrates for CYP1A2 and CYP1B1. DBF was the substrate for CYP3A4 and CYP19. Ketoconazole (0.25  $\mu\text{mol/L}$ ) was used as positive control. The MROD and EROD activities of CYP1B1 and CYP1A2 were determined fluorometrically as described by Burke *et al.* (99). The substrates (1 mol/L) were prepared in DMSO. The reaction mixture (500  $\mu\text{L}$ ) contained 0.5 to 10  $\mu\text{mol/L}$  citrus limonoids, 0.2 to 40  $\mu\text{mol/L}$  substrates, 2-10 pmol/L CYP. The volume was made up using proportional volumes of 1.3 mmol/L NADP<sup>+</sup>, 3.3 mmol/L glucose 6-phosphate. 0.4 units/mL

glucose 6-phosphate dehydrogenase, 3.3 mmol/L  $\text{MgCl}_2$  in 0.1M phosphate buffer, pH 7.4. Ice cold NADPH oxidoreductase was added just before the start of the reaction. A Synergy HT fluorescence plate reader (Bio-Tek Instruments, Inc., Winooski, VA), equipped with  $530 \pm 15$  nm excitation and  $590 \pm 15$  nm emission filters was used to determine the kinetic and endpoint fluorescence of resorufin. Hydroxylation of DBF was measured by following the formation of fluorescein ( $485 \pm 15$  nm excitation,  $538 \pm 15$  nm emission). The DBF reactions were terminated at 30 minutes duration with 2 mol/L NaOH, using the same plate reader. Reaction products were quantified using resorufin and fluorescein standards. Inhibitor concentrations that produce 50% inhibition ( $\text{IC}_{50}$ ) were calculated as previously described (100).

**Statistical analysis.** All the experiments were done in triplicate. The data were analyzed by one way ANOVA using SPSS statistical analysis software (Version 11.0). Comparisons were made within and between groups using Fisher's least significant difference test and Dunnet's paired comparisons.

### 3.4 Results

All of the limonoids significantly ( $P < 0.001$ ) inhibited EROD (ethoxyresorufin *O*-dealkylase) activity of CYP1B1 (**Fig. 3.2A,B**). However, there were no significant differences between 0.5 and 2.0  $\mu\text{mol/L}$  of LG ( $P = 0.350$ ), limonin ( $P = 0.120$ ), and 2 to 5  $\mu\text{mol/L}$  obacunone ( $P = 0.011$ ) and nomilin ( $P = 0.02$ ). Limonoid glucosides were better inhibitors than aglycones (**Fig. 3.2A,B**).



**FIGURE 3.2** Ethoxyresorufin and methoxyresorufin *O*-dealkylase (EROD, MROD) activities of CYP1B1 treated with limonoid glucosides (panels A,C) and limonoid aglycones (panels B,D). Values are mean  $\pm$  SEM,  $n = 3$ . C (control), Lim (limonin), Obc (obacunone), Nom (nomilin) and Dan (deacetylnomilin).

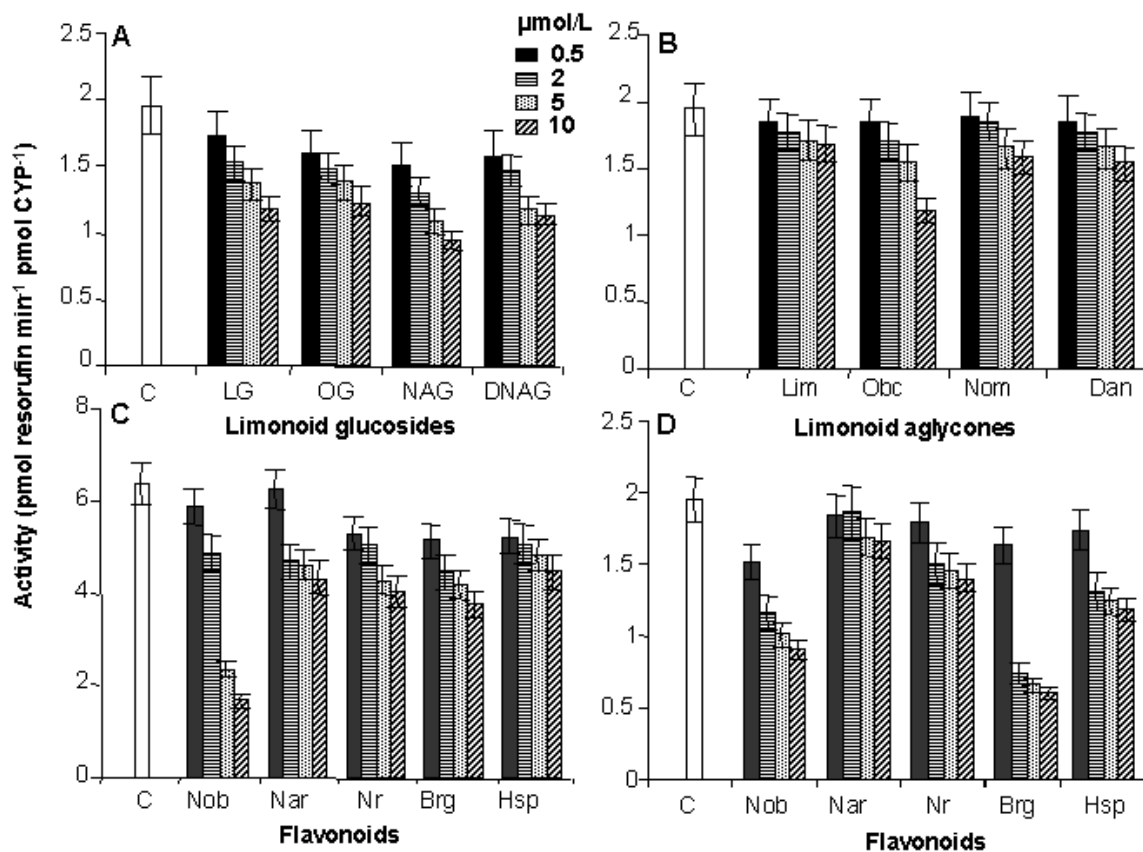
The acidic limonoid glucoside, NAG, had the highest inhibitory effect. All of the limonoid glucosides significantly ( $P < 0.001$ ) inhibited MROD activity of CYP1B1 (Fig. 3.2C,D). Fisher's least square differences on mean metabolite production (MROD) by CYP1A2 treated with different levels of limonoids indicated that all the compounds were effective ( $P < 0.005$ ) inhibitors when

compared to untreated human CYP (**Fig. 3.3A,B**). Likewise, all of the flavonoids significantly ( $P < 0.001$ ) inhibited CYP1A2 MROD activity.

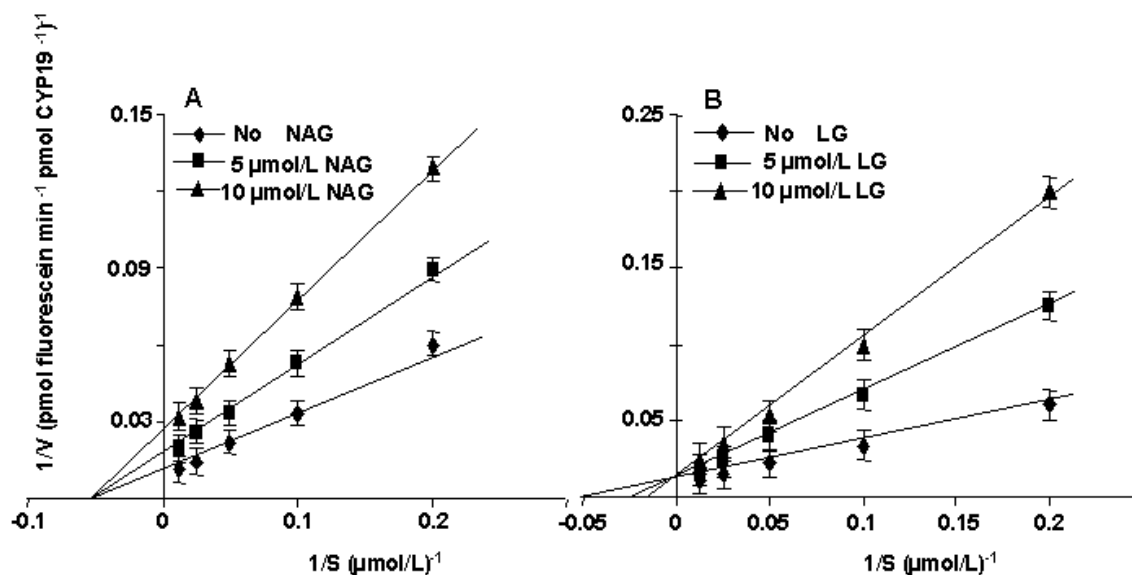
Human aromatase, CYP19, was inhibited by limonoid glucosides in two different modes as shown in **Figure 3.4**. Inhibitory effects of LG were reversible with increasing concentrations of DBF (**Fig. 3.4A**), whereas NAG, the acidic glucoside exhibited non-competitive inhibition (**Fig. 3.4B**), with the same enzyme. The limonoid glucosides and aglycones were effective inhibitors of CYP3A4 DBF hydroxylation (**Fig. 3.5**). NAG and nomilin were apparently the best inhibitors of CYP3A4 DBF hydroxylation (**Fig. 3.5**); this is similar to what was observed for CYP1B1 EROD activity.

The IC<sub>50</sub>'s of the limonoids for the various CYPs varied considerably (**Table 3.1**). Overall the IC<sub>50</sub> values were lower for limonoid glucosides, than those calculated for the aglycones. The nomilinic acid glucosides (NAG) were found to be the most effective limonoid inhibitors for most of the isoforms tested. The glucoside mixture used in this study was comprised of about 32% limonin glucoside, 34% obacunone glucoside, 12% nomilinic acid glucoside, 9% nomilin glucoside, 8% deacetylnomilinic acid glucoside, and 4% deacetylnomilin glucoside. The mixtures could not be compared against the pure compounds, instead used as a representative of the total glycosides in a juice or fruit. Similarly aglycone mixture used for the CYP activity assay consisted of 49% limonin, 17% deacetylnomilin, 14% nomilin, 8% obacunone, and other aglycones in minor concentration.

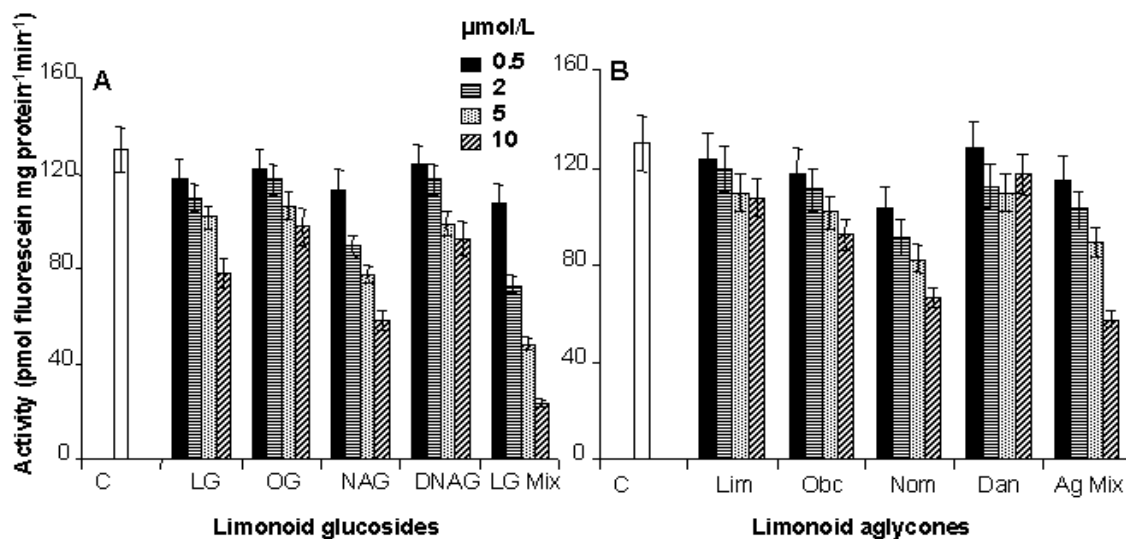




**FIGURE 3.3.** Effects of limonoid glucosides (A) and limonoid aglycones (B) on MROD activity of CYP1A2. Panels C,D indicate the effects of flavonoids on EROD and MROD activities of CYP1B1 and CYP1A2 respectively. Values are mean  $\pm$  SEM, n = 3.



**FIGURE 3.4** Lineweaver-Burk plot for the mode of inhibition of NAG and LG on CYP19 hydroxylase activity. Nomilinic acid glucoside (NAG) inhibited CYP19 in an non competitive mode (A), while limonin glucoside (LG) inhibited competitively (B). Values are mean  $\pm$  SEM,  $n = 3$ .



**FIGURE 3.5** Effect of limonoid glucosides and aglycones on the hydroxylation of DBF, a measure of CYP3A4 activity. Values are in mean  $\pm$  SEM,  $n = 4$ .

**TABLE 3.1**

*IC-50 values for citrus limonoids on different CYPs. Expressed as  $\mu\text{mol/L}$ . or as ppm (\*).*

	EROD1B1	MROD1B1	MROD1A2	CYP3A4	CYP19
LG	22.16	9.01	13.94	13.34	7.06
OG	13.68	10.09	17.16	23.56	13.71
NAG	8.25	4.36	9.41	8.77	9.43
DNAG	10.03	18.76	13.74	18.89	27.79
Glycoside Mix*	3.12	7.12	8.55	5.06	6.34
Limonin	31.97	26.5	54.86	36.63	29.92
Obacunone	11.36	21.36	13.24	20.9	27.97
Nomilin	20.62	24.06	31.01	10.43	16.43
Deacetylnomilin	10.74	17.06	28.24	63.21	51.24
Aglycone Mix*	6.77	13.76	12.08	8.7	14.64

### 3.5 Discussion

The variation in IC50 values for different limonoids can be ascribed to the affinity of substrates/limonoids towards various enzymes in the formation of enzyme-substrate/inhibitor complex. The better solubility and uptake of glycosides over aglycones cannot be considered a critical factor as the aglycones were dissolved in DMSO which may have influenced their solubility and uptake. The multiple open rings and two reactive carboxyl groups on the acidic limonoid glycoside, NAG could be a factor for better inhibitor efficacy. These results confirm a definite role of the acidic glucoside in the redox cycling

mechanism which involves many enzymes including CYPs. However, compared to the IC<sub>50</sub>'s of many citrus flavonoids and furocoumarins (37,42,101), limonoids are poorer inhibitors for the isoenzymes tested. This study confirms the previously reported non-significant effects of limonin and nomilin, on various isoforms of CYPs in liver and intestine of rats at lower doses (30).

In humans, cytochrome P450 monooxygenases catalyze a wide variety of both endogenous and xenobiotic compounds (37). Recent studies on high levels of E2-4 hydroxylation in estrogen responsive tissues have shown CYP1B1 to play an important role in estrogen related tumorigenesis (42). Limonoid glucosides are better inhibitors of CYP1B1 than the limonoid aglycones. Moreover, all of the compounds partially inhibited these isoenzymes at micromolar levels. Epidemiological studies have shown increased risk of colon cancer in individuals with high CYP1A2 activity based on caffeine metabolism (102). Higher CYP1A2 activity has also been shown to influence the risk of lung cancer (39). Tanaka *et al.* (19,20) had earlier reported that obacunone and limonin reduced the azoxymethane induced colon carcinogenesis in rats. This study confirms the effects of other limonoids as inhibitors of CYP1A2 which has a direct role in the etiology of colon cancer. This also corroborates earlier claims that bioactive compounds in orange and grapefruit juice can reduce the food carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo pyridine) and AOM (azoxymethane) induced colon cancer in rats (54,55). CYP19, the steroid aromatase, catalyzes the conversion of androgens to estrogens. An increased

exposure to estrogens is considered a risk factor for breast cancer. Therefore, CYP19 is a plausible candidate for low-penetrance breast cancer (41,95). The decreased expression of CYP19 is propitious for the reduction of estrogen dependent tumors (103). LG and NAG inhibited CYP19 aromatase activity in competitive and non-competitive modes, respectively. This supports earlier claims by Miller *et al.* (13), So *et al.* (18), and Tian *et al.* (31), that some citrus limonoids and other bioactive compounds in orange and grapefruit juice reduce mammary tumorigenesis in rats as well as in the human breast cancer cells. CYP3A4, the most abundant subfamily of CYPs, has been implicated in the etiology of prostate cancer (38). Therefore, partial inhibition of CYP3A4 by citrus bioactive compounds, could aid in the reduction of prostate cancer. Increased plasma concentrations of orally administered drugs can be caused by the inhibition of CYP3A4 enzymes which play a pivotal role in the oxidative biotransformation of many drugs (37,93,95,101). Consequently, partial inhibition of these enzymes would prolong the bioavailability of administered drugs by lowering their turnover; inhibition could reduce the medical costs by extending drug half-lives (43,95). Partial inhibition of CYP3A4 by limonoids, also could contribute to the increased bioavailability of orally administered drugs when co-administered with grapefruit juice. But the IC<sub>50</sub> values observed for limonoids are much higher compared to other compounds such as furocoumarins and flavonoids, present in the grapefruit juice. Further research on dose response relationships of drugs interacting with citrus bioactive compounds could reduce

the threat of potential toxicity problems resulting from elevated drug levels. In mammalian systems, the induction of CYPs is known to be mediated through the expression of specific receptors like aryl hydrocarbon receptor (AhR) (93). AhR receptors have been primarily implicated in the over-expression of CYP1A2, CYP1B1 and other CYP1 family of enzymes (93). Some flavonoids have been shown to bind the AhR receptors without activating the transcription factor, thereby acting as antagonists of CYPs (93). Thus, we can speculate that the limonoids bind to some of this specific receptor, triggering the inhibitory effect.

In conclusion, citrus limonoids and flavonoids have been shown to effect redox-cycling enzymes in a dose dependent and dose independent manner. Limonoids partially inhibited the dealkylase and hydroxylase activity of cytochrome P450 isoforms at the levels tested. Inhibition of CYP enzymes represents a unique mechanism in the anticarcinogenesis strategy, part of which includes reducing the generation of reactive oxygen species. The presence of citrus bioactive compounds increased the bioavailability of orally administered drugs, by inhibiting CYP3A4 and could reduce medical costs. Dose responses and interactions need to be determined with prescribed drugs.

## CHAPTER IV

### LIMONOID GLUCOSIDES INDUCE APOPTOSIS IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS AND HAVE ANTIOXIDANT PROPERTIES\*

#### 4.1 Synopsis

Limonoid glucosides have been postulated to have free radical-scavenging and apoptosis-inducing properties against certain types of cancers. In this study four highly purified limonoid glucosides, i.e, LG, OG, NAG, and DNAG, were tested for superoxide radical ( $O_2^-$ ) quenching activity and cytotoxicity against cultured undifferentiated human SH-SY5Y neuroblastoma cells. Four limonoid glucosides have shown superoxide scavenging abilities and NAG, in particular, emulated the quenching effect to an equivalent concentration of vitamin C. Micromolar amounts of LG and OG induced rapid necrosis of cultured SH-SY5Y cells. Viability studies showed significant reduction in live cells within 24 h of treatment with LG and OG. Cytotoxicity was correlated ( $P = 0.046$ ) to a concentration and time-dependent increase in caspase 3/7 activity. DNA content at the S phase of the cell cycle indicated a significant reduction with limonoid treatment. The results strongly suggest the antineoplastic properties of limonoid glucosides.

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## 4.2 Introduction

Limonoid glucosides are a class of furan-containing triterpenes that are highly water soluble, non-bitter in taste and that have been shown to be effective against several types of cancers (4,5). Thus far, more than 19 limonoid glucosides have been isolated and characterized from citrus and their related species. Two enzymes, UDPG-limonoid glycosyl transferase and limonoid D-ring lactone hydrolase perform the inter-conversion of bitter limonoid aglycones to carbohydrate-bearing limonoid glucosides (8). The transferase gene has been isolated and cloned (104). Glucoside content in citrus juice is much higher than that of aglycones (13) and the glucosides are absorbed better due to higher water solubility (11). However, bioavailability studies have shown that the once absorbed, glucosides lose the glucose moiety and appear as aglycones in the blood serum (105).

Recent studies have shown numerous health benefits from limonoid ingestion, including the prevention of several types of cancer (15-31). Some, if not all of these cancer preventive properties appear to be associated with limonoid glucosides effects on cell growth (31). This study focuses on the mechanism for blocking cancer cell growth.

Bioactive compounds are known to play pivotal roles in detoxifying reactive oxygen species (ROS) and limiting their severity or preventing the occurrence of many types of cancers. While there is one report on the



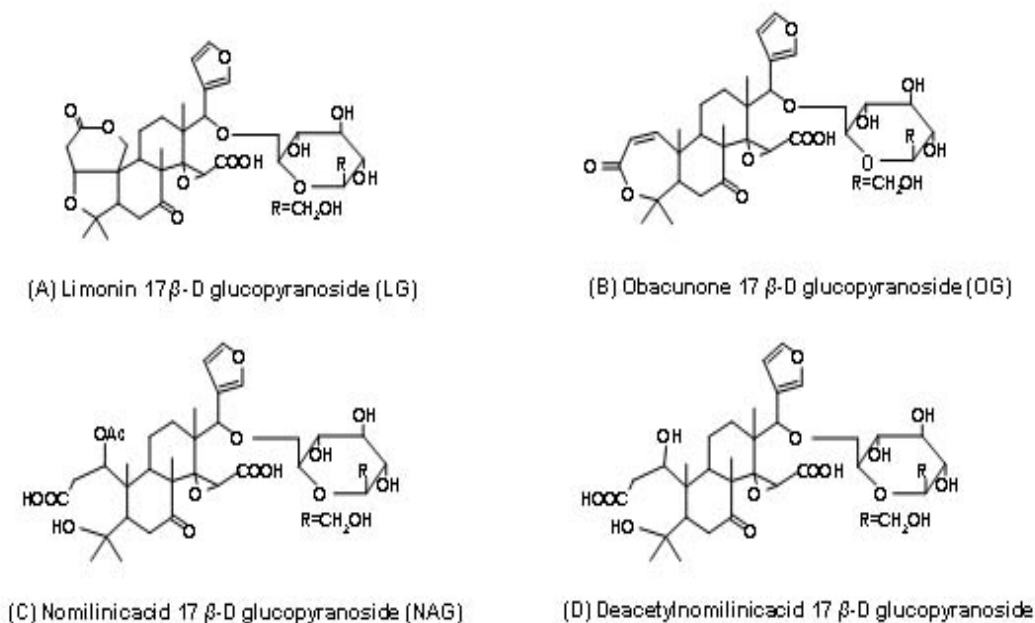
antioxidant capacity of a few limonoids (106), there is no concrete evidence on the type of reactive species involved in supporting such a role. Studies on carcinogen detoxification properties of limonoids, in mammalian system, have focused on the induction of glutathione S-transferase activity (12,16,17). We have shown the inhibitory effects of limonoids on several cytochrome P450 isoforms. GSTs and CYPs are known to work against redox cycling and assist in detoxification of many types of compounds. Here we provide biochemical evidence that specific limonoid glucosides have the capacity to quench superoxide radicals and intercede with the production of ROS.

Neuroblastoma, a common extra-cranial solid tumor in children, accounts for 10% of all cancers and up to 50% of malignancies among children (50,107). In this study reports a second facet of limonoid glucosides in inducing apoptosis, as measured by cytotoxic action against the neuroblastoma cell line SH-SY5Y. A surprising finding was an inequality in quenching and apoptotic-inducing properties of individual limonoid glucosides, suggesting that chemopreventative properties rely on the arrangement of specific functional groups within the structure of the molecule.

### **4.3 Material and methods**

***Limonoids and chemicals.*** Four limonoid glucosides (**Fig 4.1**), purified via preparative HPLC as described in the previous chapters were used for the activity studies described below. Pyrogallol, ascorbic acid, camptothecin, MTT

(bromure of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium), propidium iodide were purchased from Sigma Aldrich, Inc. Standard rhodamine, caspase specific substrate Z-DEVD-R110, and caspase inhibitor Z-DEVD-CHO were obtained from Molecular Probes.



**FIGURE 4.1** Purified citrus limonoid glucosides used for the study.

**Cell cultures.** SH-SY5Y human neuroblastoma cells (CRL-2266) were purchased from the American Type Culture Collection (Bethesda, MD) and cultured in 10% (v/v) fetal bovine serum in a 1:1 mixture of Eagle's minimum essential medium with non-essential amino acids, 0.001% antimycotic-antibiotic and Ham's F12 medium (108). Cells were grown on 25 cm<sup>2</sup> falcon culture flasks at 37°C under 5% CO<sub>2</sub>. When the cells reached 70-80% confluence they were

detached from the flasks with Pucks EDTA solution and quantified in a hemocytometer. Approximately  $15 \times 10^3$  cells were subcultured into 12, 24- and 96-well plates for experiments.

***Superoxide radical quenching.*** Assays for  $O_2^-$  radical quenching followed the procedure described by Marklund and Marklund (48) with the exception that the assays were performed at pH 7.6, which is closer to biological pH, and pyrogallol was present at a concentration of 0.2 mmol/L. Stock solutions of 1 mmol/L pyrogallol were prepared in 0.5 M HCl and diluted to the desired concentration and pH with 0.2 mol/L Tris-HCl. These adjustments allowed for a more sensitive measurement of limonoid quenching. Individual limonoid glucosides were added to a final concentration of 0.1-10 mmol/L and continuous absorbance readings were taken over 10 min in a Hitachi U2001 recording UV-visible spectrophotometer at wavelengths corresponding to purpurogallin (325 nm), purpurogalloquinone (600 nm) and the peroxidation species of purpurogalloquinone (440 nm). Decomposition rates were determined by the slope of the absorbance curve. Quenching was assessed by measuring decomposition rate against samples treated with 40 U of superoxide dismutase (Sigma-Aldrich).

***Measurements of cell viability.*** SH-SY5Y cells in triplicate were incubated in culture medium supplemented with limonoid glucosides (1-100  $\mu$ mol/L final concentration) and cell viability was monitored at 12 h intervals over the next 72 h. Viability determinations were based on trypan blue exclusion as

measured in a hemocytometer and the MTT reagent (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) as described (109).

**Assay of caspase 3/7 activity.** Upon reaching confluence, SH-SY5Y cells were harvested and co-cultured into 24-well culture plates. After 24 h individual cultures in triplicate were treated with limonoid glucosides. At 12, 24, 36 and 48 h after treatment, the cells were harvested, washed three times with PBS buffer and collected by centrifugation (5000 x *g*, 2 min, 4°C) and the cell pellet was taken up in 200 µL of lysis buffer (0.2 mol/L Tris, pH 7.5, 2 mol/L NaCl, 0.02 mol/L EDTA, 0.02% (v/v) Triton-X100). Aliquots were analyzed for protein using the BCA reagent (Pierce) and a lysate volume equivalent to 50 µg of protein was brought to 100 µL with buffer in a 96-well flat bottom black microtiter plate. An equal volume of caspase substrate (Z-DEVD-R110) was added with mixing and the plate was covered with aluminum foil and set aside at 37°C for 30 min. Fluorescence measurements were performed over a 2 h period in a Perkin-Elmer LS50B luminescence spectrometer, using 499 nm excitation and 521 nm emission wavelengths. Controls with no limonoid glucosides or with camptothecin, a strong apoptosis inducer, were run concurrently. Caspase activity was expressed as pmol/L rhodamine/min/µg protein. Other controls consisted of limonoid glucosides or camptothecin combined with a known caspase 3/7 inhibitor AC-DEVD-CHO.

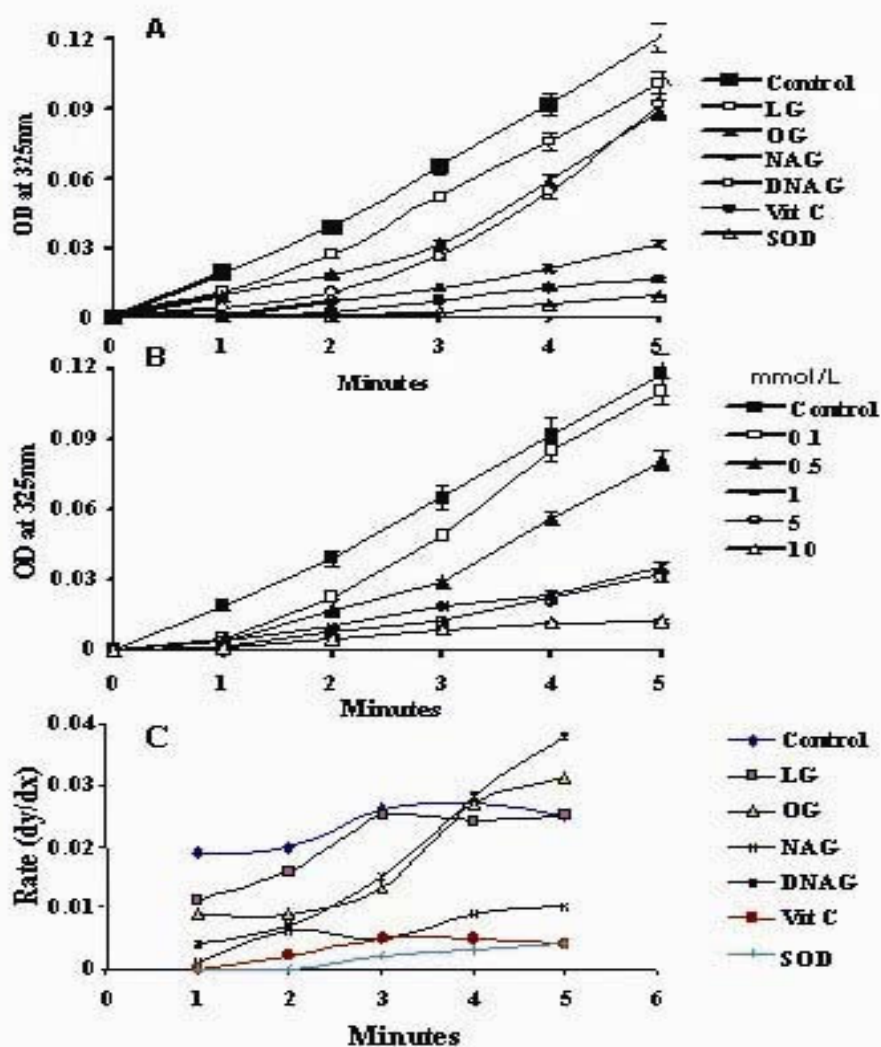
**Flow cytometry analysis.** The cells were treated with limonoid glucosides or camptothecin for 12, 24, 36, and 48 h and harvested as before.

Between  $10^6$  to  $10^7$  cells in PBS were centrifuged ( $200 \times g$ , 6 min,  $25^\circ\text{C}$ ), and the cell pellet was taken up in 50 mL of PBS, 450 mL of 70% ethanol, and stored at  $-20^\circ\text{C}$  until used. Cells were separated from the ethanol by centrifugation at  $200 \times g$  for 5 min, decanting the ethanol, and suspended in 1 mL of 0.1% (v/v) Triton X-100 in PBS, to which was added 200  $\mu\text{g}$  of DNase-free RNase A and 20  $\mu\text{L}$  of propidium iodide. After 15 min incubation at  $37^\circ\text{C}$  to destroy RNA, the suspension was filtered through nylon and collected in tubes that fit a Beckton Dickenson FACS Caliber flow cytometer. DNA fluorescence readings were taken with an excitation blue light set and 488 nm and a detector emission of red fluorescence through a 585 nm filter. Pulse width area signals were used to discriminate between  $G_2$  cells and cell doublets. The data were analyzed using ModFitLT software version 3.1 (PMac).

**Statistical analysis.** Experimental results represent triplicate experiments for each treatment group and are expressed as means  $\pm$  standard error. A two-way ANOVA was used with a  $P$ -value of less than 0.05 (Fisher's least significant difference test) considered to indicate significance and Dunnet's paired comparison. Analyses were done with SPSS statistical analysis software, (Version 11.0). The coefficient of variation in the DNA analysis was less than 6%. Reduced chi square goodness of fit was assumed to be 0.8 to 3.0. IC50 values for the inhibition concentration for individual compounds were calculated after Stahle *et al.* (100), using a linear regression equation.

#### 4.4 Results

***Superoxide quenching activity.*** The potential for the purified limonoid glucosides to quench free radicals was ascertained using pyrogallol in slightly alkaline (pH 7.6) solutions. Quenching superoxide anion ( $O_2^-$ ) effectively stops the spontaneous decomposition of the pyrogallol, which can be measured spectrophotometrically at wavelengths corresponding to the oxidized products of pyrogallol. Assays were run with limonoid glucoside concentrations varying between 0.1 and 10 mmol/L. Typically, a short lag phase at the start was used to measure quenching activity (**Fig. 4.2**). As shown in **Figure 4.2A**, at 5 mmol/L, clearly discernible differences in the quenching efficacies of the different limonoid glucosides were observed. NAG was superior to LG, OG, and DNAG over the 5 min assay time (**Fig. 4.2A**). At 5 mmol/L NAG had nearly the same quenching efficacy as L-ascorbic acid and superoxide dismutase. Testing NAG alone showed that strong and sustained inhibition, which was not seen until the concentration was above 1 mmol/L (**Fig. 4.2B**). Neither change in the pH nor pyrogallol concentration affected the profiles for the different limonoid glucosides. The rate of inhibition of purpurogallin formation by NAG was found to be diminishing after three minutes of the start of the reaction indicating a rapid turnover of the compound and unquenchable spontaneous auto-oxidation of pyrogallol (**Fig. 4.2C**). The acidic glucosides did not effect any noticeable change in pH on the buffer medium, at the levels tested.

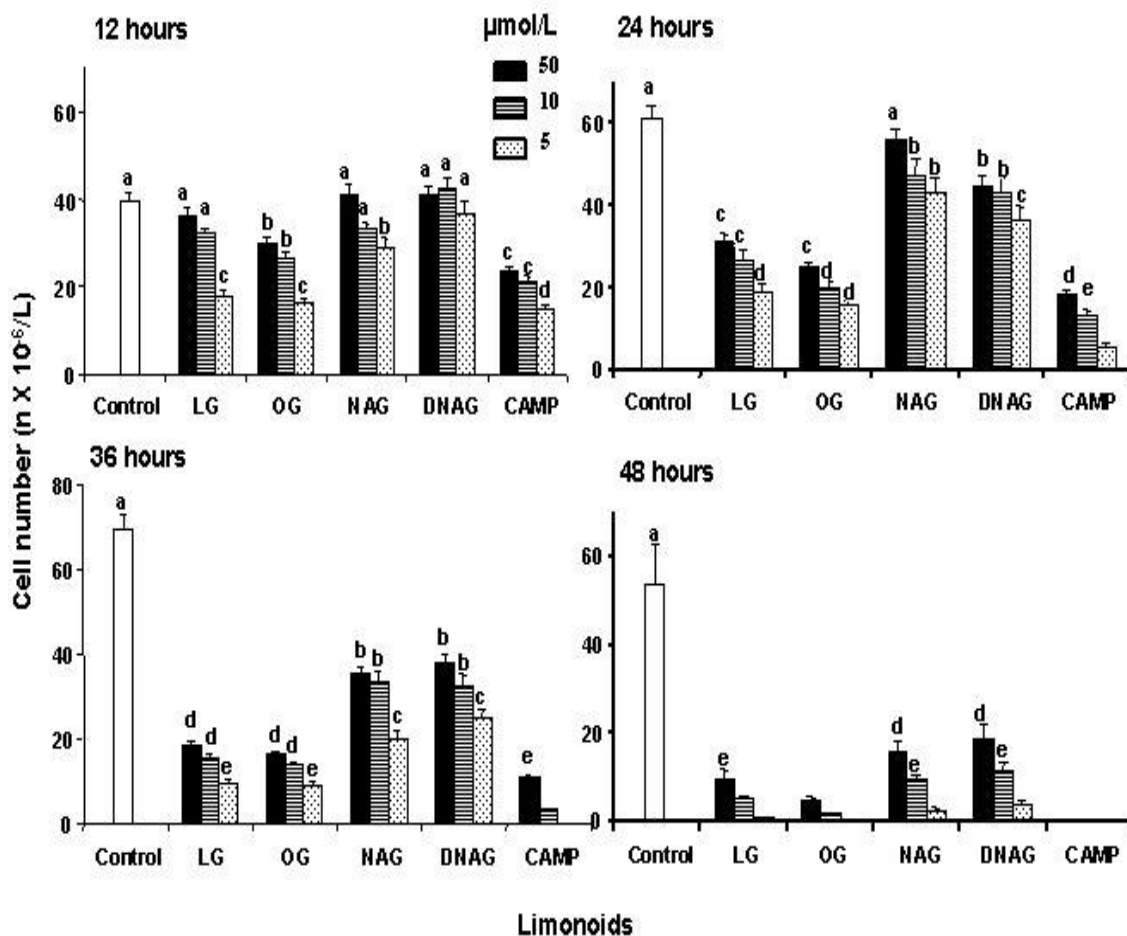


**FIGURE 4.2** Quenching of oxygen radicals by limonoid glucosides in vitro. (A) Limonoid glycosides (5 mmol/L) were tested against 0.2 mmol/L pyrogallol in 0.5 M Tris-HCl, pH 7.6. The kinetics of decomposition was followed by absorbance readings at 325 nm. Vitamin C (5 mmol/L) and superoxide dismutase (40 U) were used as positive controls. (B) Quenching of pyrogallol autooxidation by increasing concentrations of NAG (0.1-10 mmol/L). (C) Rate of pyrogallol oxidation based on the slopes for each compounds at 5 mmol/L level.

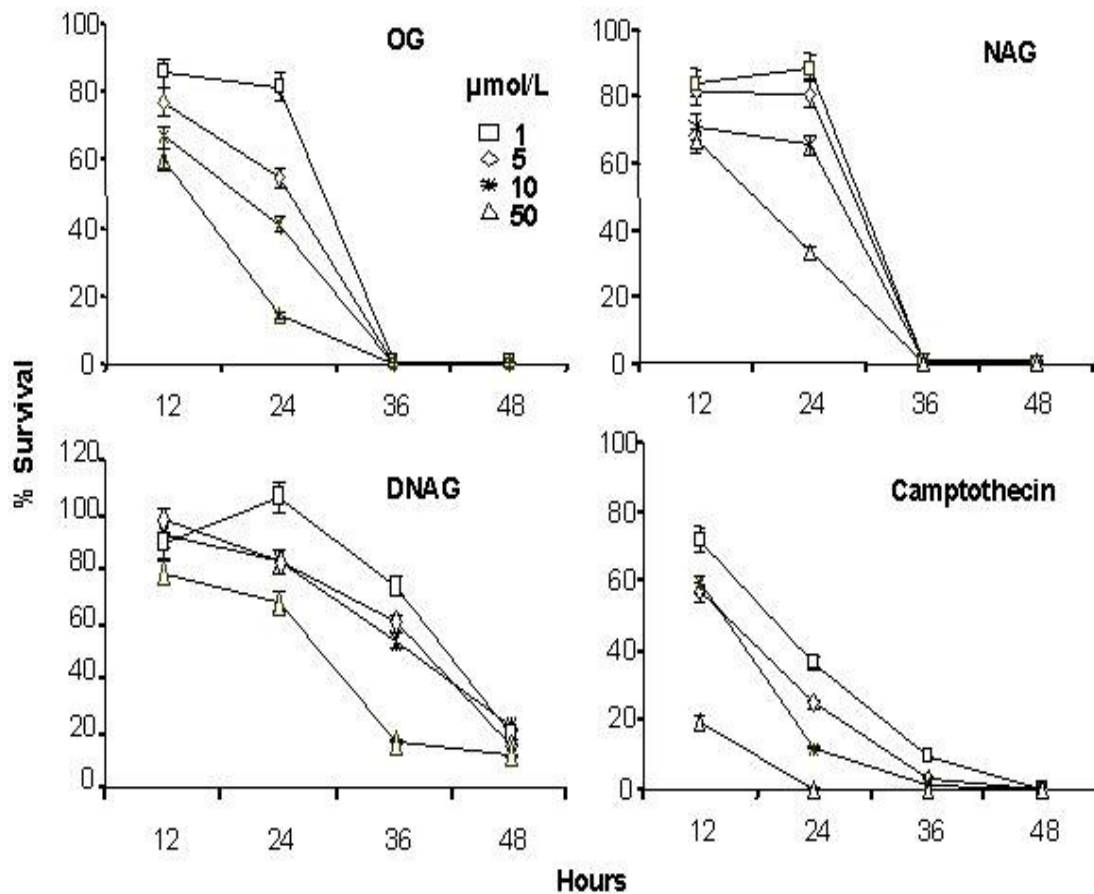
**Cell viability.** Human SH SY5Y neuroblastoma cells were treated with purified limonoid glucosides to test their cytotoxicity. After the cells had reached confluence, the growth medium was supplemented with the limonoid glucosides to final concentrations of 5, 10 or 50  $\mu\text{mol/L}$  and viability was measured at 12 h intervals. Depending on concentration, significant cytotoxicity was apparent as early as 12 h of exposure and continued downward for 48 h (**Fig. 4.3**). Cytotoxic potential was not uniform among the different limonoid glucosides, however. LG and OG, for example, were clearly more effective in killing cells than an equivalent concentration of NAG or DNAG. Indeed, the survival rate for the LG and OG showed no statistical difference when compared to cells that had been treated with similar concentrations of camptothecin. Untreated cells showed greater than 60% survival after 36 h and about 50% at 48 h, whereas survival for cells treated with 50  $\mu\text{mol/L}$  limonoid glucosides was less than 5% (**Fig. 4.3**).

A second experiment using MTT reduction also tested cell viability. By this analysis it was apparent that the cells survived a minimum of 36 h after exposure and that LG and OG had a stronger lethal potential than NAG or DNAG (**Fig. 4.4**). In fact, DNAG was least effective in the downward curve of survival rate. MTT reduction was undetectable after 36 h of exposure to LG, OG, or NAG. Depending on limonoid glucosides concentration, cell death occurred more rapidly between 24 and 36 h after exposure. Camptothecin, as expected showed the strongest lethal effects.





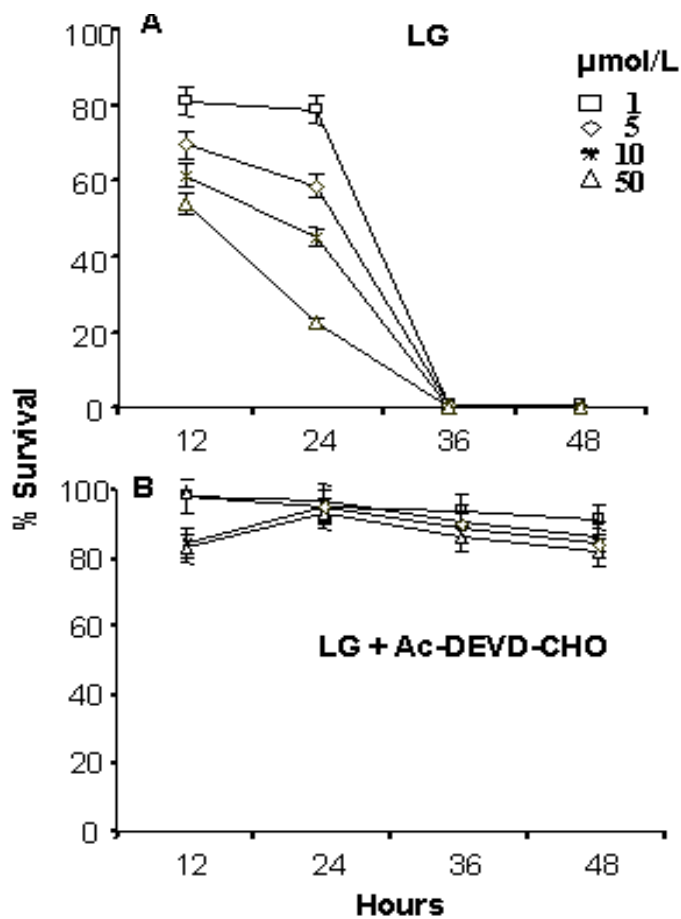
**FIGURE 4.3** Toxicity of limonoid glucosides against SH-SY5Y cells in culture. Assays were based on trypan blue exclusion. Cells were exposed to 5, 10 or 50 μM limonoid glucosides and tested for trypan blue-excluding cells at 12, 24, 36 and 48 h after exposure. Camptothecin was used as a positive control. Quantification was based on cell counts as performed in a hemocytometer and are expressed as 1000's/mL. Data shown represent triplicates readings for each treatment group and are expressed as the mean ± SE of the live cells.



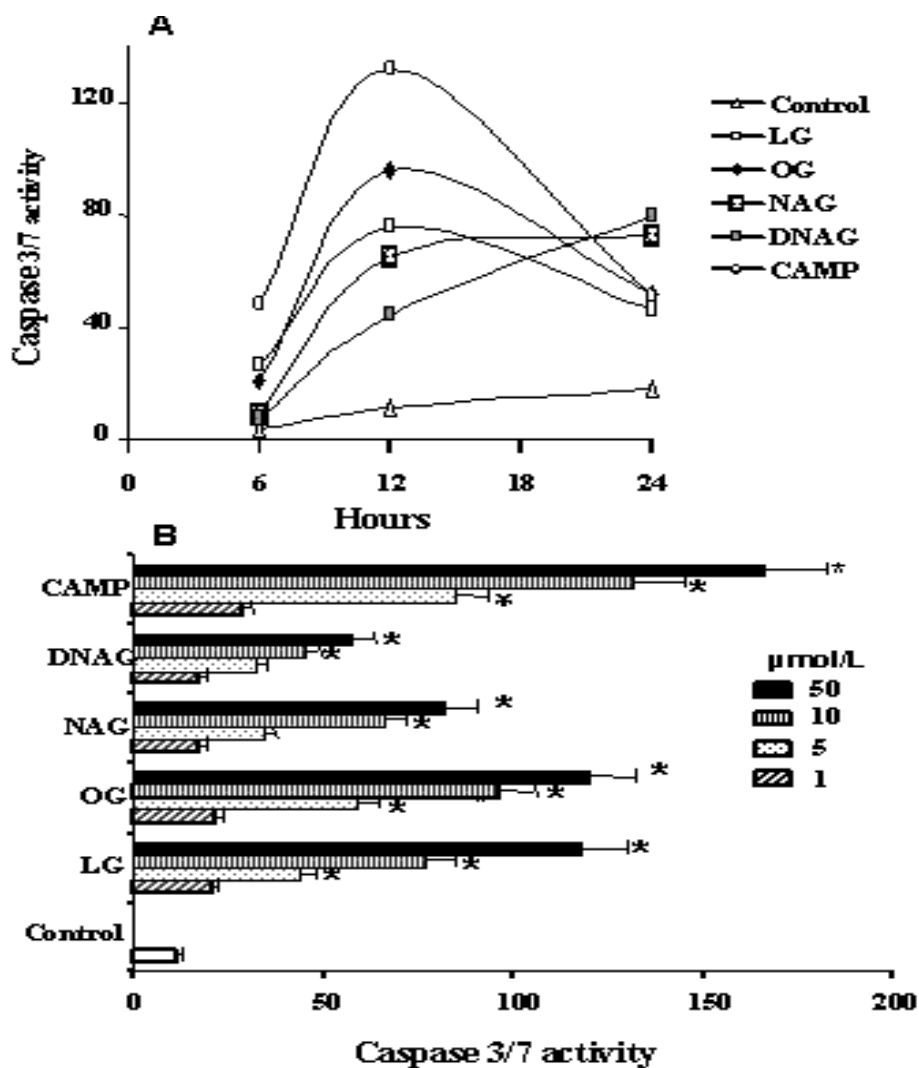
**FIGURE 4.4** Cell viability analysis based on MTT reduction, measured by the formation of formazon at 550 nm. The values shown represent mean  $\pm$  SE for  $n=3$ . Survival percentage was determined using control (untreated) cells as a norm. Camptothecin was used as a positive control.

A striking observation, however, was seen in cells that had been treated with LG and Ac-DVED-CHO in combination. Cell death was suppressed for at least 48 h, even with the concentration of LG, the most lethal limonoid glucoside, as high as 50  $\mu\text{mol/L}$  (**Fig. 4.5**). Since Ac-DVED-CHO is a potent inhibitor of caspase activity, there was a clear indication that toxic action of limonoid glucosides could be exerted through the induction of apoptosis.

***Test of apoptosis.*** It should be possible to observe apoptotic changes in the cells, if apoptosis induction is responsible for the decline in cell viability by limonoid glucosides. Therefore, we tested for caspase 3/7 induction and DNA fragmentation. In the caspase induction assay, the cells were treated with the limonoid glucosides for 12 h, harvested, washed with PBS and then lysed. Aliquots matched for their protein content were assayed for caspase 3/7 activity in the presence and absence of a caspase inhibitor. Camptothecin was used as a positive control. All of the limonoid glucosides at a set concentration (10  $\mu\text{mol/L}$ ) induced caspase 3/7 activity (**Fig. 4.6A**). Similar to the lethal potential on cell viability, there was a clear difference among the different limonoid glucosides. Though the basal caspase activity changed very little over the 24 h experimental period, the caspase activity appeared to peak at 12 h and declined thereafter. Although all limonoid glucosides were less effective than camptothecin, LG and OG were superior to NAG and DNAG in their caspase inducing efficacy.



**FIGURE 4.5** Survivability of human neuroblastoma cells treated with LG alone or LG + Ac-DEVD-CHO, a known caspase inhibitor. Values are based on MTT reduction, are means  $\pm$  SEM,  $n = 3$ .



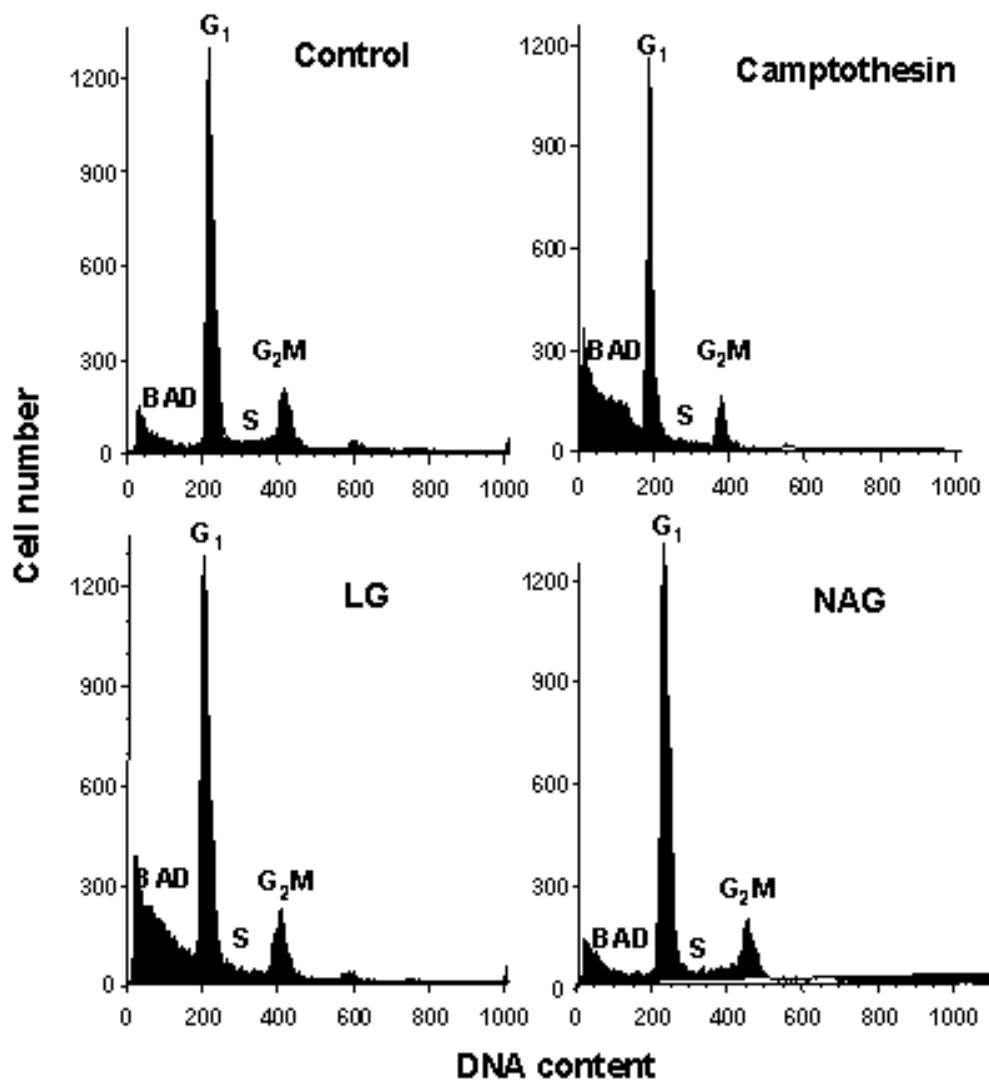
**FIGURE 4.6** Induction of caspase 3/7 activity by limonoid glucosides. The cells were exposed to 10  $\mu\text{mol/L}$  limonoid glucosides for 6, 12 or 24 h. (A) Kinetics of caspase induction for each of the different limonoid glucosides. Controls received no limonoids. (B) The induction of caspase 3/7 activity at 12 h as a function of different concentration of limonoids. Camptothecin (CAMP) was used as a positive control. Values are mean  $\pm$  SD,  $n = 3$ . \* Significantly different from control at  $P < 0.001$ . The activity is expressed as picomoles of rhodamine110 formed per  $\mu\text{g}$  of cell lysate protein per minute.

**Figure 4.6B** shows that caspase induction was a function of the concentration of limonoid glucosides in the medium. As little as 1  $\mu\text{mol/L}$  brought about a noticeable increment over basal (control) caspase activity; 5  $\mu\text{mol/L}$  brought about a significant difference ( $P < 0.05$ ). Importantly, those limonoid glucosides that were the most effective suppressors of cell growth, viz., OG and LG, were the strongest inducers of caspase 3/7 activity. The 12 h caspase activity had a strong correlation ( $r = -0.998$ ,  $P = 0.002$ ) to the cell survival. The data, therefore, confirmed the earlier study, which suggested that the cytotoxic effect of limonoid glucosides was manifested through apoptotic induction mechanism.

**Flow cytometry.** As a further confirmation of apoptosis induction by the limonoid glucosides, a flow cytometry analysis was performed. Cells were exposed to 10  $\mu\text{mol/L}$  limonoid glucosides for 24 h, harvested, fixed in 70% ethanol, and treated with propidium iodide before the analysis. Aggregates were removed by filtration through nylon. **Figure 4.7** shows that treated and control cells were mostly in the  $G_0$  and  $G_2M$  phases with less numbers of cells in the S phase subpopulation. The position marked A shows univariate DNA content, a marker of DNA degradation, was significantly higher for cells treated with LG, OG and camptothecin. Background aggregates and debris (BAD) between  $G_1$  and  $G_2$  phase, an indicator of apoptosis, was determined to be 35.4% for camptothecin, 31.3% for LG and 30.9% for OG, which was significantly higher ( $P < 0.05$ ) compared to control (8.6%), NAG (17.5%) and DNAG (11.1%). There

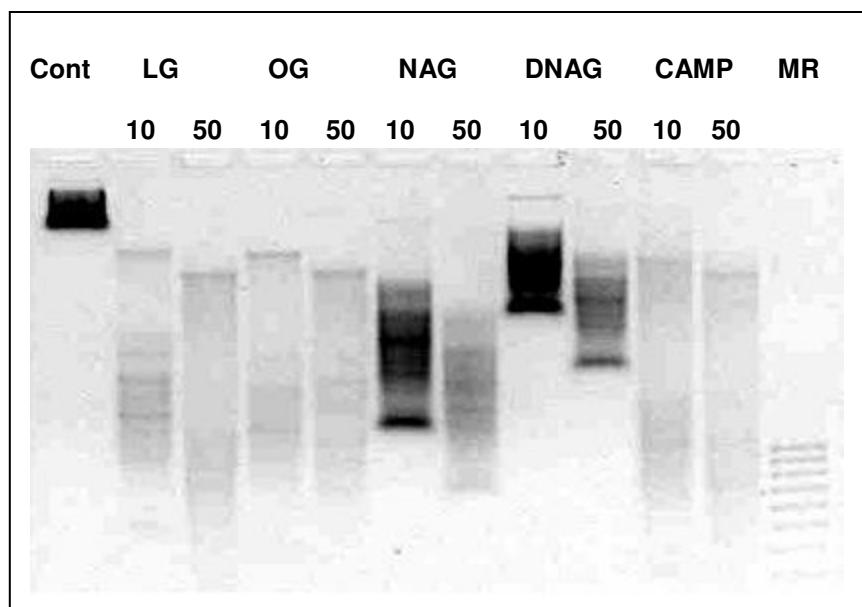
was no significant reduction in cell numbers at the G<sub>0</sub>/G<sub>1</sub> phase between all treatment groups, but OG, LG and camptothecin significantly reduced the number of S phase cells, which essentially blocks the crucial proliferation phase. DNA content at S phase of the cell cycle indicated a reduction of 86.67% with LG, 82.36% with OG, 57.46% with NAG and 38.35% with DNAG, as compared to the known inhibitor like camptothecin. OG also significantly suppressed the G<sub>2</sub>/M phase. Overall, the results provided evidence that limonoid glucosides act as antiproliferative agents through arresting critical growth stages of the cell cycle and inducing apoptosis.

***DNA fragmentation.*** The potential for limonoid glucosides to induce DNA breakage in sequel to apoptosis induction was determined through agarose gel electrophoresis. In this assay DNA was extracted from control and treated cells, precipitated with ethanol, and chromatographed on 1% (w/v) agarose. An equal amount of cellular DNA was applied to each lane of an electrophoresis gel. **Figure 4.8** shows that untreated cells had high molecular weight DNA that was virtually intact as shown by the heavy staining of slow moving components, whereas cells treated with LG, OG, or camptothecin showed a spectrum of fragments that were present in a familiar ladder pattern typical of endonuclease cleaved DNA. Fragmentation of DNA was less evident in cells treated with NAG and DNAG. These data support the flow cytometry results and provide direct evidence for DNA degradation of neuroblastoma cells as a consequence of limonoid treatment.



**FIGURE 4.7** Flow cytometry analysis of DNA in SH SY5Y cells as a function of limonoid glucoside treatment. Approximately  $10^6$  cells were treated with  $10 \mu\text{M}$  limonoids for 24 h. Measurements were taken with the excitation mode set at 488 nm and the emission at 585 nm. The results were quantified with ModFit V3 software.





**FIGURE 4.8** DNA fragmentation analysis of SH-SY5Y cells after limonoid treatment. Approximately 106 cells were treated with 10 or 50  $\mu\text{mol/L}$  limonoid glucosides for 24 h. An equal amount of DNA from each treatment group was applied to a 1% agarose gel. Lane marked MR shows 100 bp to 1000 bp molecular weight ladder.

#### 4.4 Discussion

Many epidemiological and controlled diet studies have reported that high intake of fruits and vegetables lower the risk of numerous degenerative diseases including cancer (110). Although many bioactive compounds from citrus fruits were implicated, the potential of limonoid glucosides is believed to dwell in antioxidant and apoptosis inducing properties.

Two important facets of limonoid glucosides action, such as, an ability to quench  $O_2^-$  radicals and to induce apoptotic changes in neuroblastoma cells were studied here. Quenching, as detected by an *in vitro* spectrophotometric assay, was evident only when relatively high (mmol/L) levels of the limonoid glucosides were present, which may limit chemopreventative significance. Furthermore, quenching appeared to occur only in the early stages of the reaction and, depending on concentration, did not endure beyond the first two or three minutes. The fact that quenching did not persist suggests the limonoid glucosides act stoichiometrically with  $O_2^-$  and, once modified, are unable to regenerate active forms. Because of the high concentrations needed and the failure to recycle to active forms weakens the suggestion that limonoid glucosides are free radical scavengers *in situ*.

The limonoid glucosides tested in this study caused rapid death to undifferentiated neuroblastoma cells in culture. Lethality occurred at micromolar concentrations, which suggests neuroblastoma cells are highly sensitive to their action. Tian *et al.* (31) showed that the obacunone glucoside was superior to

nomilinic acid glucoside in causing the death of MCF-7 breast cancer cells in culture. Moreover, the aglycones of NG and LG were as effective as the glucosides in killing cells, suggesting the glucoside appendage on the molecule is not important for toxic action. The previous study with some limonoid glucosides, however, ruled out apoptosis as a cause because of the relatively high amounts (100  $\mu\text{g/L}$ ) needed to induce an apoptotic response (31). In the present study, it has been demonstrated that limonoid glucosides as low as 1-5  $\mu\text{mol/L}$  (0.6-3.0  $\mu\text{g/L}$ ) induced caspase activity and fragmented DNA in SH-SY5Y cells. Why MCF-7 cells fail to show a similar heightened sensitivity against limonoid glucosides is not clear. Apoptosis can be induced in SH-SY5Y cells by a number of external factors and conditions such as tumor necrosis factor- $\alpha$  (111), amyloid- $\beta$ -peptides (112), UV radiation (113) and ceramide (114). In contrast, retinoic acid, PGE-2, leptin, guanosine, interleukin (IL)-1 $\alpha$  and IL-6 appear to protect SH-SY5Y cells against oxidants and neurotoxins (115-118), as do flavones such as luteolin, apigenin, and epigallocatechin (119-122). The data suggest that limonoid glucosides are more apt to fit the category of toxic agents as opposed to protective agents, and unlike the flavones offer little or no protection against oxidants and neurotoxins *in situ*. This hypothesis, however, is tempered by the observation that cancer cell lines HL-60, NCI-SNU-1, HeLa, SCOV-3, and HepG2 are unaffected by limonoid glucosides (31), which implies lethal action may be selective and dependent on cell type or differentiation state.

**TABLE 4.1**

*Summary of activity of limonoid glucosides on pyrogallol auto oxidation, caspase 3/7 induction and cell viability. Values expressed are mean  $\pm$  SE. DNA content is expressed as percent of untreated.*

	<b>LG</b>	<b>OG</b>	<b>NAG</b>	<b>DNAG</b>
Inhibition of superoxide radical (mmol/L) <sup>a</sup>	7.88 $\pm$ 0.8	4.62 $\pm$ 0.13	3.11 $\pm$ 0.09	3.53 $\pm$ 0.12
Induction of caspase 3/7 ( $\mu$ mol/L) <sup>b</sup>	8.36 $\pm$ 1.15	8.17 $\pm$ 0.88	14.89 $\pm$ 0.51	18.40 $\pm$ 0.57
Cell death at 24 h, trypan blue exclusion ( $\mu$ mol/L) <sup>c</sup>	5.14 $\pm$ 0.88	4.197 $\pm$ 0.66	28.59 $\pm$ 2.02	29.53 $\pm$ 0.57
Cell death at 24 h, MTT reduction ( $\mu$ mol/L) <sup>d</sup>	6.05 $\pm$ 0.73	5.54 $\pm$ 0.77	22.43 $\pm$ 1.57	25.53 $\pm$ 1.45
Percent reduction in DNA of S phase (% CV) <sup>e</sup>	86.67(4.7)	82.36(3.7)	57.46(4.7)	38.35(4.4)

<sup>a</sup> IC50 values calculated according to Stahle *et al.* (101) using a linear regression equation.

<sup>b</sup> Induction of 50% of caspase activity as compared to 10  $\mu$ mol/L camptothecin used as positive control.

<sup>c</sup> Cell survival (50%) after 24 h of treatment. Measured using hemocytometer and compared with that of control.

<sup>d</sup> Cell survival based on MTT reduction by live cells measures at 550 nm and compared with that of control.

<sup>e</sup> Reduction in DNA content at S-phase was measured using flow cytometer with propidium iodide staining and compared with that of control.

It is important to note the difference in the efficacy by different limonoid glucosides. It is clear that LG and OG induced cell death at one-fourth the concentrations of NAG and DNAG (**Table 4.1**). Caspase induction and DNA fragmentation was also achieved at lower concentrations of LG and OG than NAG and DNAG. Quenching, however, favored NAG. A careful inspection of the structures (**Fig. 4.1**) shows that NG and LG share the same structural features as NAG and DNAG with the exception of a sealed A ring in the OG and LG molecules. Miller *et al.* (23) earlier had speculated that the A ring as opposed to the D, is more decisive in its biological activity of limonoid glucosides. Our data strongly support that by showing greater apoptosis-inducing potential, that may also be associated with the A ring of the molecule.

Future research endeavors can now turn to the mechanism of apoptosis induction to explain the underlying mechanism of the limonoid glucosides on SH-SY5Y cells. Calcium, acting through the activation of two  $\text{Ca}^{2+}$ -dependent proteases, mu-calpain, and caspase 12, has been strongly implicated in induction of apoptosis and the regulation of the apoptotic signaling pathway (123). Mitochondria regulate apoptosis in vertebrates by releasing cytochrome *c*, an inducer of caspase 3, and Bcl-2 regulates this release (124). Activation of the JNK signaling pathway, an early event in the induction of apoptosis in neuronal cells (111), leads to the phosphorylation of c-Jun and Bcl-2, two factors that control apoptosis. What effect limonoid glucosides may have on these internal events is ripe for further investigation. One more consideration is the

synergistic action by which one limonoid promotes the action of another. Mertens-Talcott (125), showed that ellagic acid significantly potentiated the action of quercetin in stimulating caspase activity in cultured MOLT-4 human leukemia cells. A combination of the two fruit polyphenols gave robust anticarcinogenic effects than either one alone. This observation suggests limonoid glucosides could act in concert with flavones to promote an action. In nutrition synergistic health promoting effects achieved by whole fruit consumption, rather than individual molecules.

In conclusion, the data in this study strongly support limonoid glucosides at micromolar concentrations as being toxic to cultured neuroblastoma. Because limonoid glucosides are not lethal to all cancer cells (31), they may target specific cells types. Consequently, it is vital to recognize the vulnerable site(s) in cells that underlie their mechanisms of action.

## CHAPTER V

### EFFECT OF LIMONOIDS ON COLONIC ADENOCARCINOMA (Caco-2), NEUROBLASTOMA AND NON-CANCEROUS OVARY (CHO) CELLS

#### 5.1 Synopsis

Purified limonin, limonin glucoside, obacunone, obacunone glucoside, nomilin, and deacetylnomilin along with mixtures of limonoid aglycones and glucosides were tested for toxic effects on human SH-SY5Y neuroblastoma and colon carcinoma (Caco-2) cell lines and were compared with the mammalian non-cancerous Chinese hamster ovary (CHO) cells. Limonoid glucosides have been shown to have greater cytotoxic effects than aglycones and that the effects were specific to cancerous cells. The effects were significant ( $P < 0.001$ ) over 24 to 36 h when compared with the untreated cells. Flow cytometric analyses of limonoid treated neuroblastoma cells showed a marked increase in DNA content of aneuploidic cells within 24 h, which are considered to be a prognostic factor in neuroblastoma. The percentage of aneuploidic cells following treatment were 36% for LG, 26% for limonin and more than 42% for limonoid glucoside mixtures. The results confirm that both limonoid aglycones and glycosides are lethal to cancer cell lines; however, glycosides are the most active apoptotic inducing form, even though both forms arrest cell growth.

## 5.2 Introduction

Secondary metabolites have often been proven to be beneficial to human health. More than 120 important prescription drugs in the U.S. are derived from secondary plant metabolites which comprise about 25% of the total drugs in use (126). With innumerable chemopreventive properties, these phytochemicals have been divided into either cancer-blocking agents or cancer-suppressing agents (92). The blocking agents work through scavenging the carcinogens by CYP/GST mediated metabolism and detoxification, quenching ROS, and blocking the cellular targets. The suppressing agents restrain the proliferation and progression of the cancer by activating several transcription factors), inducing apoptosis, arresting the cell cycle (3-19, 92).

Statistics from American Cancer Society show an estimated 1,372,910 new cases and a total of 570,280 deaths by cancer in U.S. in 2005 (33). Of these, about 145,000 new cases and about 56,000 deaths will occur due to colorectal cancer. Neuroblastomas account for about 10% childhood cancer with an annual incidence of 6-8 per million (50,127). Furthermore, the overall costs of cancer for the year of 2005 is estimated to be \$189.8 billion with \$69.4 billion in direct medical expenditures and \$103.5 billion in indirect mortality costs, which include the costs of lost productivity due to premature death (33). An estimate by the National Cancer Institute indicates that one in four cancer deaths are diet related and that eight of ten cancer cases have a nutrition/diet component (33).



National Cancer Institute designer programs identified citrus fruits as especially nutrient rich foods containing more than a dozen classes of beneficial phytochemicals (4,5). Chemopreventive properties of citrus limonoids have been discussed in detail in the previous chapters. Epidemiological, cell culture and animal studies have shown that citrus limonoids can prevent several types of cancers (15-31). Though the antiproliferative effects of citrus limonoids are found to be mediated through inhibition of cytochrome P450, antioxidant activity and apoptotic induction in earlier experiments, their effects on normal cells and other cancer cell lines have yet to be ascertained. Strategies to offset the malignancies of colorectal cancer and neuroblastomas are vital. This study report the cytotoxic and apoptotic effects of the purified limonoids on human SH-SY5Y neuroblastoma (CRL-2266), colonic adenocarcinoma (Caco-2) and assessed the effects with a non-cancerous Chinese hamster ovary cells (CHO).

### **5.3 Materials and methods**

**Cell cultures.** Human SH-SY5Y neuroblastoma cells (CRL-2266), colonic adenocarcinoma cells (Caco-2), and Chinese hamster ovary cells (CHO) were purchased from the American Type Culture Collection (Bethesda, MD). SH-SY5Y cells were cultured in 10% (v/v) fetal bovine serum in a 1:1 mixture of Eagle's minimum essential medium with non-essential amino acids, 0.001% antimycotic-antibiotic and Ham's F12 medium as previously described (108). HepG2 and Caco-2 cells were grown in 10% (v/v) and 20% (v/v) fetal bovine

serum in Eagle's minimum essential medium with 2 mmol/L L-glutamine and Earle's balanced salt solutions (BSS) adjusted to contain 17.85 mmol/L sodium bicarbonate, 0.1 mmol/L non-essential amino acids, and 1 mmol/L sodium pyruvate. CHO cells were cultured in 10% (v/v) fetal bovine serum Ham's F12K medium with 2 mmol/L L- glutamine adjusted to contain 17.85 mmol/L sodium bicarbonate. Cells were grown on 25 cm<sup>3</sup> Falcon culture flasks at 37°C under 5% CO<sub>2</sub>. When the cells reached 70-80% confluence they were detached from the flasks using either Pucks EDTA (SH-SY5Y, CHO) or trypsin EDTA (Caco-2) and quantified in a hemocytometer. Approximately 10<sup>4</sup> cells/mL (1000/well) were sub-cultured into 96-well culture plates and incubated for 24 h (SH-SY5Y, CHO) and 48 h (Caco-2), to attain 70% confluence before the treatments. Fresh medium was added to each well at the beginning of the treatments. Stock solutions of aglycones were prepared in DMSO and glucosides in water.

**Cell viability.** The cells in triplicate were incubated in culture medium supplemented with limonoid glucosides (5-50 µmol/L final concentration) and cell viability was monitored at 12 h intervals over the next 48 h. Care was taken to keep the DMSO levels below 0.5% which had no apparent effect on cell growth. Viability determinations were based on the reduction of MTT reagent (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) by active mitochondria as described (109). The morphological differences between limonoid treated and untreated cancer cell lines were ascertained using a confocal microscopy using a green fluorescent filter.

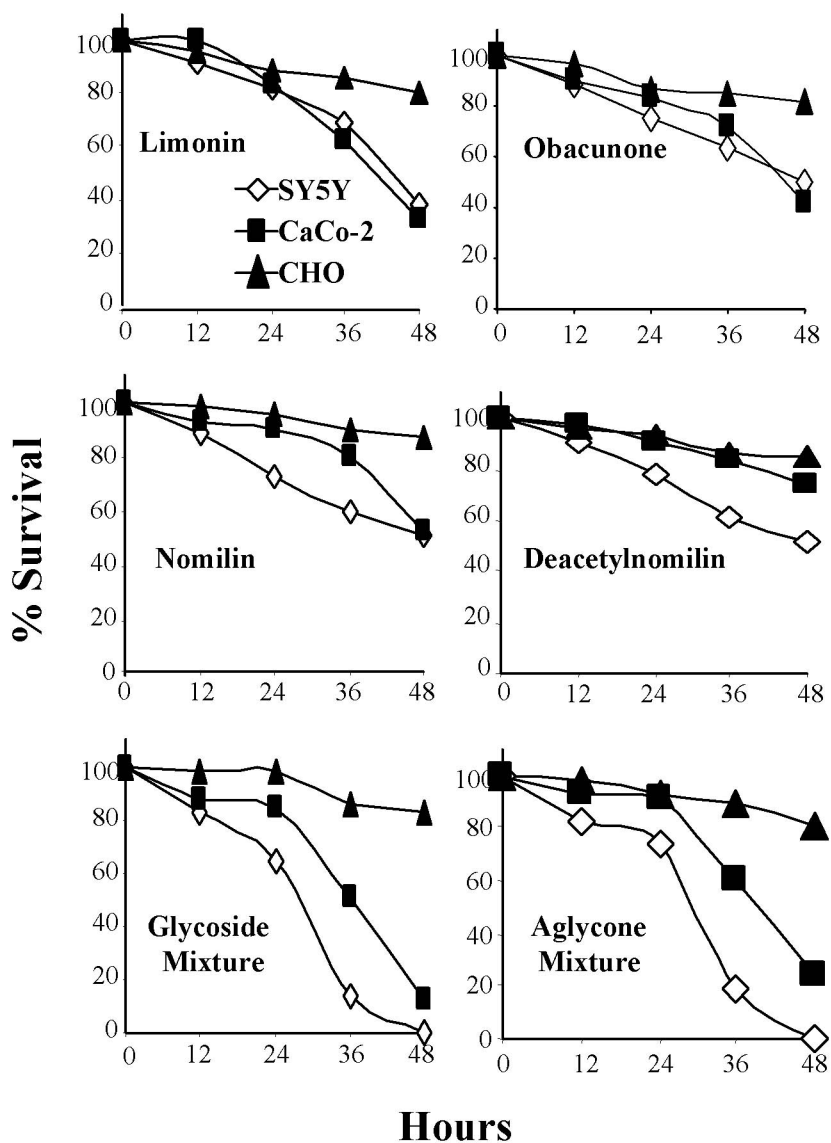
**Flow cytometry analysis.** Cells were treated with limonoids for 36 h and harvested as before. Between  $10^6$  to  $10^7$  cells in PBS were centrifuged ( $200 \times g$ , 6 min,  $25^\circ\text{C}$ ), washed 3 times with PBS and the cell pellet was resuspended in 500  $\mu\text{L}$  of PBS to which added 0.1% (v/v) Triton X-100 in PBS, 200  $\mu\text{g}/\text{mL}$  of DNase-free RNase A and 50  $\mu\text{L}/\text{mL}$  of propidium iodide in 4 mmol/L sodium citrate buffer. After a 15 min incubation at  $37^\circ\text{C}$  to destroy RNA, the suspension was filtered through nylon and collected in separate tubes that fit a FACS Caliber flow cytometer (Beckton Dickenson, San Jose, CA USA), equipped with a 15 mW argon ion laser. DNA fluorescence readings were taken with an excitation of 488 nm and emission with a 585 nm filter, with a doublet discrimination module to differentiate  $G_2$  cells from cell doublets using pulse width area. The existence of DNA aneuploidy was described as the existence of two  $G_0/G_1$  peaks in the DNA histogram as described by Abad *et al.* (128).

**Statistical analysis.** Experimental results for cell viability represent triplicate for each treatment group and are expressed as means  $\pm$  standard error (percent). A one-way ANOVA was used with a *P*-value of less than 0.001 with Fisher's least significant difference test for inter group comparisons. Analyses were done with SPSS statistical analysis software (Version 11.0). Distribution analysis of  $G_0/M$ , S and  $G_2/M$  phases of the cell cycle were performed on propidium iodide stained gated cells for the exclusion of doublets using ModFitLT software (Version 3.1, PMac, Beckton Dickenson).

## 5.4 Results

### ***Effect of limonoids on the viability of SH-SY5Y, Caco-2 and CHO cells.***

In accordance with the previous report on the effect of limonoid glucosides on the induction of apoptosis in human SH-SY5Y neuroblastoma cell lines (127), a slow but similar effect for the limonoid aglycones was observed. The morphological differences observed using confocal microscopy clearly indicated a rapid response for the limonin 17 $\beta$ -D glucopyranoside (LG) as opposed to that of limonin and obacunone aglycones (**Fig. 5.1**). The effects were consistent with both time and dose dependent assays (**Table 5.1**). While limonoid mixtures had definitive effects on the viability of both Caco-2 and SH-SY5Y cell lines, the response of both pure as well as mixtures was selective towards cancerous cell lines (**Fig. 5.2**). Neuroblastoma cell lines were found to be more sensitive than the colon carcinoma cells for nomilin (**Fig. 5.2**). Although all of the pure compounds seemed to have similar levels of cytotoxicity on SH-SY5Y cells the relative effects appeared to be strong for obacunone followed by limonin, nomilin, and deacetylnomilin (**Table 5.1**). There were no significant differences ( $P < 0.001$ ) between limonin and obacunone. Limonin, nomilin, and obacunone had same cytotoxicity towards Caco-2 and SH-SY5Y cell lines at 48 h.



**FIGURE 5.1** Time-dependent changes in percent viable cells following treatment with pure aglycones or limonoid mixtures. Limonoid concentration, 25 mmol/L. Values are mean  $\pm$  SEM,  $n = 3$ , based on untreated controls.

**TABLE 5.1**

*Percent viability of SH-SY5Y neuroblastoma cells treated with purified limonoid aglycones. Values are mean  $\pm$  SEM (%), n = 3.*

Time (h)	Conc. ( $\mu$ mol/L)	Limonin	Nomilin	Obacunone	Deacetylnomilin
12	5	96.85 $\pm$ 2.46	98.17 $\pm$ 3.98	98.23 $\pm$ 4.41	100.0 $\pm$ 3.88
	10	93.27 $\pm$ 3.54	95.56 $\pm$ 4.25	96.53 $\pm$ 5.62	97.32 $\pm$ 2.15
	25	91.18 $\pm$ 4.87	88.32 $\pm$ 1.13	88.91 $\pm$ 4.36	91.13 $\pm$ 4.11
	50	87.12 $\pm$ 6.13	81.17 $\pm$ 2.34	84.24 $\pm$ 6.67	85.34 $\pm$ 4.23
24	5	83.37 $\pm$ 4.60	87.89 $\pm$ 2.60	85.62 $\pm$ 5.32	88.74 $\pm$ 1.81
	10	87.20 $\pm$ 3.73	83.59 $\pm$ 2.41	84.25 $\pm$ 1.72	90.89 $\pm$ 1.71
	25	82.45 $\pm$ 5.23	72.94 $\pm$ 3.00	74.96 $\pm$ 1.93	77.97 $\pm$ 4.10
	50	65.98 $\pm$ 5.07	59.87 $\pm$ 1.36	57.18 $\pm$ 3.12	67.75 $\pm$ 1.44
36	5	79.85 $\pm$ 4.65	84.99 $\pm$ 4.46	80.96 $\pm$ 3.33	85.93 $\pm$ 3.78
	10	74.54 $\pm$ 6.35	71.03 $\pm$ 3.71	67.27 $\pm$ 2.43	76.25 $\pm$ 4.67
	25	69.67 $\pm$ 3.34	60.20 $\pm$ 2.76	62.68 $\pm$ 3.65	61.37 $\pm$ 2.76
	50	55.95 $\pm$ 3.12	58.19 $\pm$ 2.65	53.36 $\pm$ 1.65	61.99 $\pm$ 3.56
48	5	68.13 $\pm$ 2.35	74.35 $\pm$ 1.24	71.25 $\pm$ 4.09	75.34 $\pm$ 3.21
	10	62.65 $\pm$ 2.27	65.46 $\pm$ 4.65	58.54 $\pm$ 3.87	65.43 $\pm$ 2.13
	25	58.42 $\pm$ 5.54	62.46 $\pm$ 5.23	49.41 $\pm$ 3.91	51.25 $\pm$ 2.11
	50	45.87 $\pm$ 5.34	48.54 $\pm$ 3.67	41.32 $\pm$ 2.88	50.28 $\pm$ 2.28

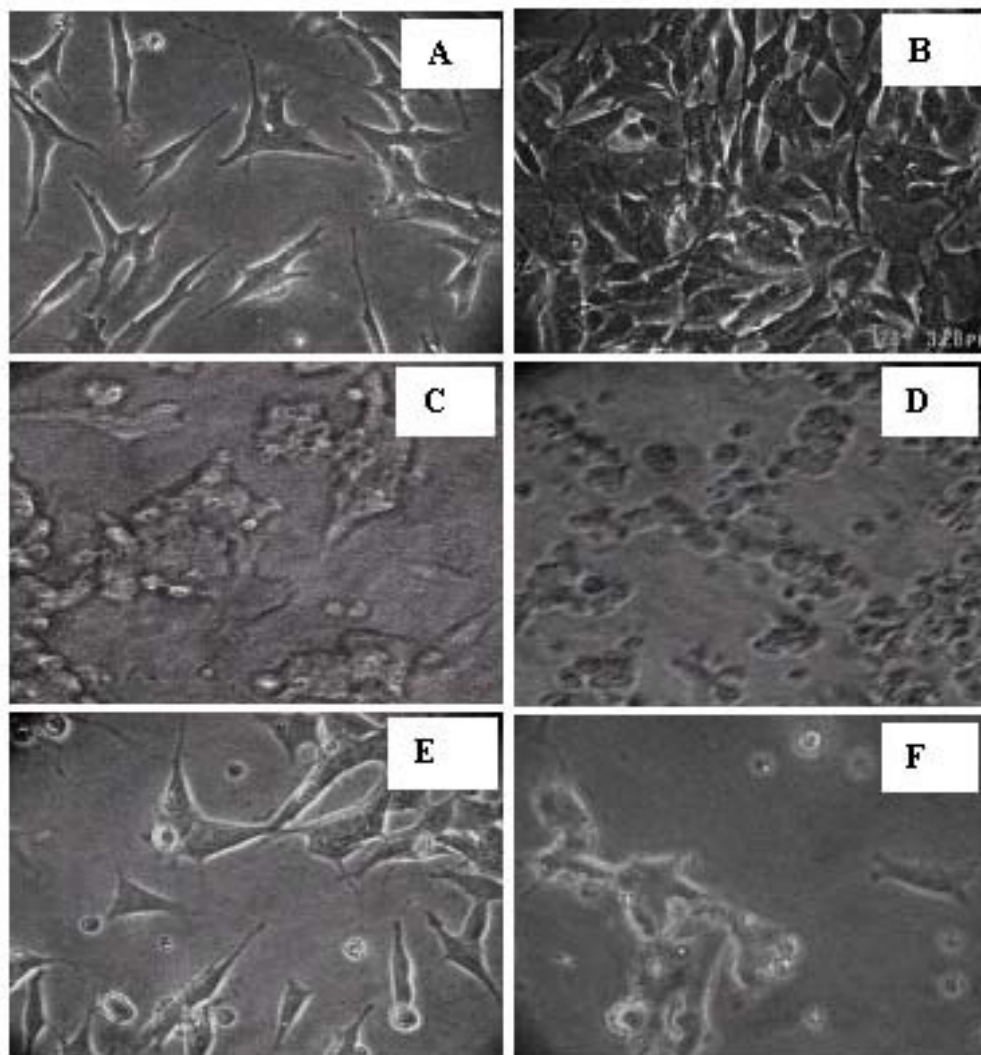
There was a significant difference ( $P < 0.001$ ) between glucoside and aglycone mixtures with the former being more effective within 24 h of treatment (**Table 5.2**). The results clearly indicated the minimal effect on the Chinese

hamster ovary cells (**Fig. 5.3**) and thus confirm non-toxicity on normal cells. Inter group comparisons showed no significant differences ( $P = 0.227$ ) between the aglycone and glucoside mixture from oranges at 25 ppm after 24 h.

**TABLE 5.2**

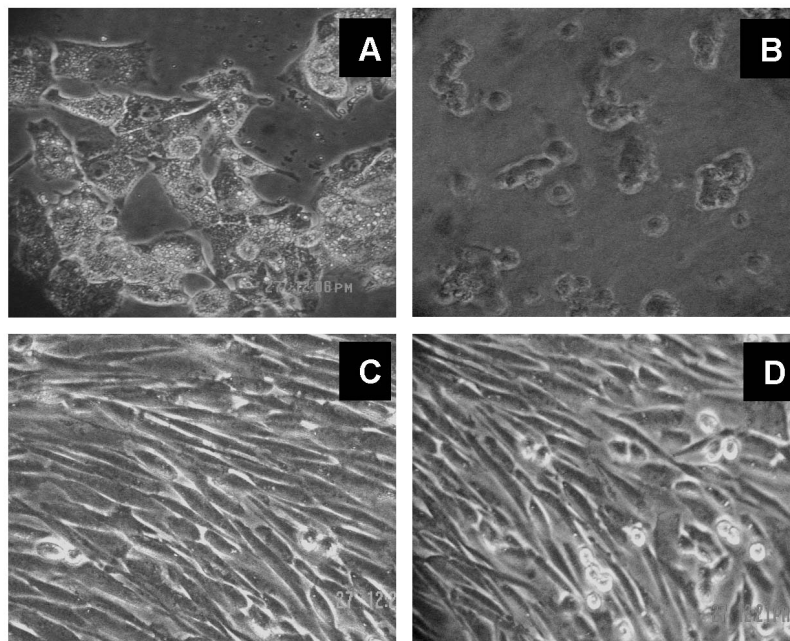
*Percent viability of SH-SY5Y neuroblastoma cells treated with mixtures of limonoid aglycones and glucosides from orange and grapefruits. Values are mean  $\pm$  SEM (%),  $n = 3$ .*

Time (hr)	Conc. (ppm)	Grapefruit aglycone mixture	Orange aglycone mixture	Orange glucoside mixture	Grapefruit glucoside mixture
12	5	91.12 $\pm$ 4.23	94.21 $\pm$ 1.94	95.78 $\pm$ 4.61	96.32 $\pm$ 2.31
	10	89.57 $\pm$ 3.56	87.16 $\pm$ 2.05	89.07 $\pm$ 3.21	91.18 $\pm$ 4.12
	25	88.12 $\pm$ 2.11	81.65 $\pm$ 2.76	82.34 $\pm$ 2.34	89.43 $\pm$ 3.33
	50	82.55 $\pm$ 3.57	80.23 $\pm$ 3.29	81.16 $\pm$ 1.65	83.54 $\pm$ 2.34
24	5	85.69 $\pm$ 2.69	88.91 $\pm$ 2.46	84.60 $\pm$ 6.93	94.61 $\pm$ 4.69
	10	90.61 $\pm$ 2.80	90.31 $\pm$ 3.55	83.82 $\pm$ 1.43	79.57 $\pm$ 2.06
	25	76.81 $\pm$ 3.35	73.95 $\pm$ 5.51	64.29 $\pm$ 3.04	60.53 $\pm$ 0.94
	50	58.91 $\pm$ 1.86	58.49 $\pm$ 1.05	46.80 $\pm$ 3.57	38.30 $\pm$ 1.96
36	5	25.10 $\pm$ 2.56	24.48 $\pm$ 1.07	21.61 $\pm$ 1.34	25.26 $\pm$ 1.86
	10	18.26 $\pm$ 1.43	25.64 $\pm$ 1.78	24.16 $\pm$ 1.17	26.33 $\pm$ 1.13
	25	19.39 $\pm$ 1.05	18.30 $\pm$ 1.63	13.31 $\pm$ 2.09	12.84 $\pm$ 2.34
	50	14.26 $\pm$ 1.96	12.98 $\pm$ 1.13	6.80 $\pm$ 1.76	6.59 $\pm$ 1.46
48	5	17.15 $\pm$ 1.46	19.12 $\pm$ 1.32	11.24 $\pm$ 1.06	13.15 $\pm$ 1.07
	10	11.10 $\pm$ 1.78	11.16 $\pm$ 0.65	6.31 $\pm$ 1.98	4.18 $\pm$ .97
	25	7.32 $\pm$ 1.71	0.32 $\pm$ 0.13	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
	50	2.31 $\pm$ 0.95	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00



**FIGURE 5.2** Confocal images of SH-SY5Y neuroblastoma cells treated with limonoid glucosides and aglycones, from **Figure 5.1**. Panels *A* and *B* untreated cells at 0 and 48 h. *C* and *D*, 25  $\mu\text{mol/L}$  LG for 24 and 48 h; *E* and *F*, 25  $\mu\text{mol/L}$  obacunone.

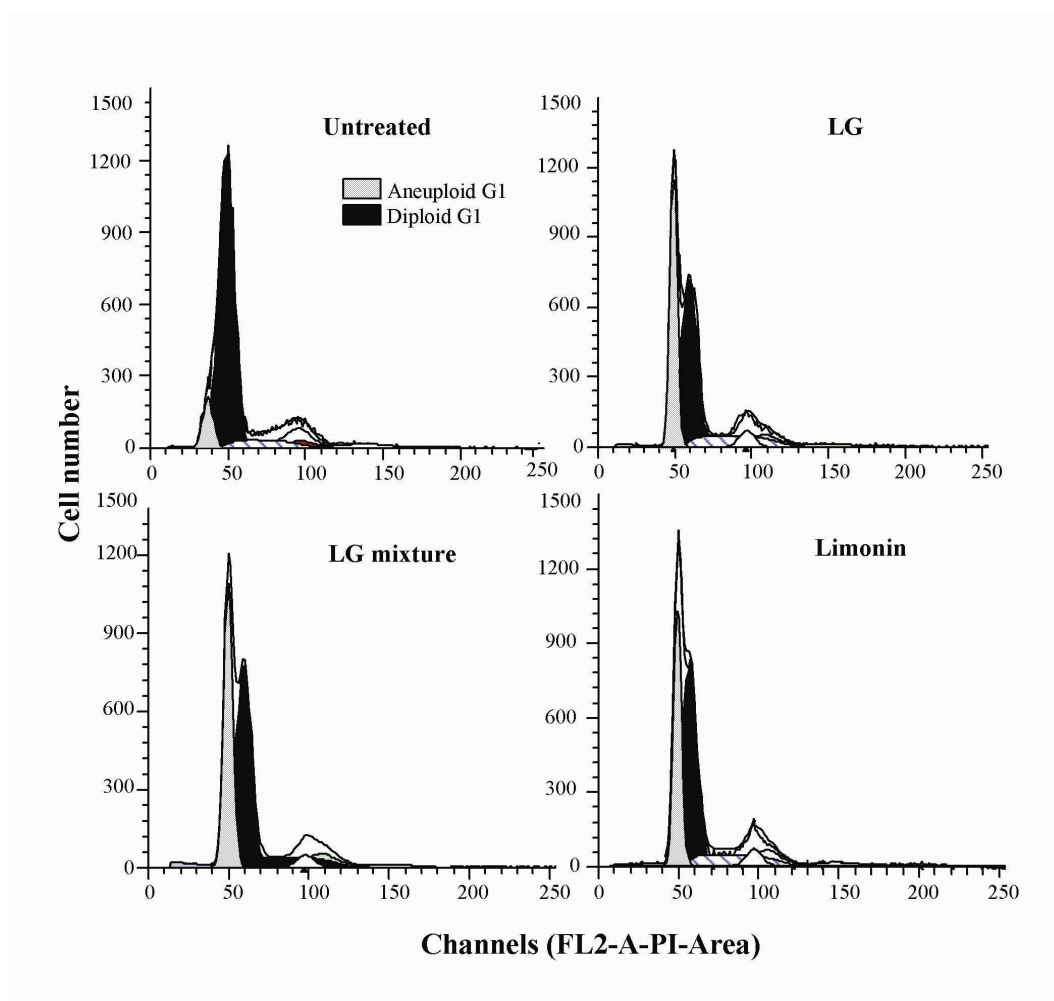




**FIGURE 5.3** Confocal images of Caco-2 and non-cancerous Chinese hamster ovary (CHO) cells treated with limonoids, from **Figure 5.1**. Panels *A,B*, Caco-2 cells treated with 25  $\mu\text{mol/L}$  of limonoid glycoside mixture for 24 and 48 h, respectively. Panels *C* and *D*, Chinese hamster ovary cells treated with the limonoid glycoside mixture.

***Limonoids induce aneuploidy.*** Limonoid treatment of neuroblastomas increased the percentage of aneuploidic cells to 2- to 3-fold over the untreated controls (**Fig 5.4 & Table 5.3**). The substantial increase of aneuploidy in the cells treated with limonoid mixtures. The mixtures consisted of 31% LG, 34% OG, 11% NAG, 8% DNAG, 7% nomilin glucoside, 4% DNG with several unknown compounds. The significant reduction in G2/M phase of the cell cycle can be ascribed to the inhibition of mitosis due to differing chromosome number,

which can lead to the non-division of aneuploidic cells, eventually leading to death of the cells. Thus, it can be discerned from the flow DNA frequency histogram data (**Table 5.3**) that both glycosides as well as aglycones inhibit cell proliferation.



**FIGURE 5.4** Flow cytometric DNA content analysis for cell cycle of SH-SY5Y neuroblastoma cells stained with propidium iodide after 36 h exposure. Panel A, untreated cells; panels B, C, and D, 25  $\mu\text{mol/L}$  limonin glucoside, limonoid glucoside mixture and limonin, respectively.

**TABLE 5.3**

*Percent DNA content of the SH-SY5Y neuroblastoma cells in the various phases of the cell cycle, treated with limonoids at 25  $\mu$ mol/L for 36 h. The values were obtained based on the DNA frequency histogram (ModFitLT software V3.1).*

	Diploid Total (%)	%G1	CV	Aneuploid Total (%)	%G1	%G2/M
Untreated	88.2	87.3	10.1	11.9	77.5	22.5
Limonin	73.3	81.5	6.1	26.8	83.0	17.0
Obacunone	63	77.6	8.1	37.0	85.7	14.4
Nomilin	56.1	78.1	8	44.0	88.9	11.1
LG	64	77.6	9	36.0	86.2	13.8
OG	61.2	79	8	38.8	88.7	11.3
NAG	72.4	83.4	5.3	27.6	84.7	15.3
Glu Mix(O)	52.3	73.2	5.9	47.7	93.3	6.7
Glu Mix (G)	57.1	80.7	7.9	42.9	92.1	7.9
Agl Mix (O)	58.4	77.2	5.1	41.6	91.6	8.4
Agl Mix (G)	60.3	81.6	5.1	41.6	89.2	10.8

## 5.5 Discussion

Previous studies have shown that apoptosis is the primary cause of neuroblastoma cell death by limonoid glycosides. In this study the cytotoxicity of limonoids was assessed in human SH-SY5Y neuroblastoma and colonic adenocarcinoma (Caco-2) cells and compared with two non-cancerous Chinese hamster ovary (CHO) cell lines. It was evident that neuroblastoma cells were more sensitive to limonoids than were colon carcinoma cells. Limonoid

cytotoxicity varied depending on the type of cancer cell and the type of limonoid applied. While both limonoid aglycones and glucosides at micromolar levels appeared to arrest cell growth, biochemical and morphological data showed that the glycosides were clearly more effective in inducing rapid cell death. Neither pure compounds nor mixtures showed toxicity against the viable mammalian CHO epithelial cells.

Corresponding to our previous report showing the apoptotic inducing ability of LG and OG, here the cell death on both SY5Y as well as Caco-2 cells by limonin and obacunone was observed, but at a significantly slower pace and never to the same extent. This confirms the killing potential of limonoid aglycones as reported by Miller *et al.* (22, 23). Considering the solubility factor that could hinder the killing potential, the aglycones were supplemented through non-toxic concentrations of DMSO, giving both compounds equal access to the cell's interior and sensitive site(s). Obacunone and other purified aglycones were as effective as glycosides in stopping cell division, but considerably inferior in rapid killing. The data, therefore, imply that stopping cell division and inducing cell death by apoptosis may be two separate and independent actions of limonoids on cancer cells. Aglycones are capable of arresting the cell proliferation, but weak in inducing the apoptosis. The propensity of aglycone and glycosidic mixtures speaks strongly for synergistic events beyond absorption, and from a nutritional perspective, whole fruit or whole juice consumption to maximize beneficial effects.

Chromosomal aberrations in cell DNA content (aneuploidy) measured by flow cytometry provide objective information and major prognostic factors for neuroblastomas (127). Aneuploidy is common in neuroblastoma cells and has been used to predict tumor aggressiveness and successful response to chemotherapy. In neuroblastoma tumor cells absence of aneuploidy (near diploid or near tetraploid DNA content) is confirmed to be the more aggressive pattern of neuroblastoma (127-130). Look *et al.* (130) reported that tumors with greater numbers of aneuploidic cells responded well to chemotherapy. Limonoids increased the number of aneuploidic SH-SY5Y cells. A previous report correlated cell cycle arrest with an increase in G1 phase cells, thus inferring a block in the transition to the S phase of the cell cycle. Enhancing aneuploidy raises the possibility that cell cycle arrest could also be occurring at a later stage of cell division, particularly the separation into two daughter cells which is shown as an arrest at the G2/M phase.

In conclusion, with proven anticancer properties, limonoids can now be considered as lead structures for anticancer drugs. Viability studies indicate that molecules patterned after limonoid glycosides may have greater commercial potential. As cellular permeability, water solubility, and metabolic change all play pivotal roles in the overall efficacy of a natural compound's performance (131), the glycosides appear to excel over aglycones. These studies advocate citrus fruit as a valuable functional food which may thwart cancer in human and could augment demand for citrus and citrus products.

## CHAPTER VI

### CONCLUSIONS

Combination of ion-exchange and crystallization techniques presented in this dissertation provides an efficient and economical method for the large scale purification of limonoids and flavonoids from citrus byproducts. The methods developed were found to be highly specific and with more than 80-fold increase in total yields, than present methods in practice for the isolation of limonoid glucosides. The removal of large amounts of naringin in particular, increased the purification efficiency. One kilogram of dried orange and grapefruit seeds yielded 8.62 and 9.31 g of limonin, 0.62 and 0.47 g nomilin, 0.9 and 0.63 g of deacetylnomilin and 0.21 and 0.17 g of obacunone respectively. An average of 16 g of dried limonoid glucosides and 9.8 g flavonoid mixtures were obtained from 1 Kg of clarified citrus molasses concentrate. Preparative HPLC separation of these semi-purified mixtures resulted in high yields of limonoid glucosides with more than 90% purity. Considering the abundance of aglycones in seeds, and glucosides in molasses, citrus byproducts provide an excellent source for bulk purification.

In our first biological activity study using the purified compounds, limonoids and flavonoids have shown to effect redox-cycling enzymes in a dose dependent and dose independent manner. Limonoids selectively and quantitatively inhibited the dealkylase and hydroxylase reactions performed by cytochrome P450

isoforms. All the limonoids significantly ( $P < 0.001$ ) inhibited EROD and MROD activity of CYP1B1 and CYP1A2. Limonoid glucosides were found to be better inhibitors than aglycones. The acidic limonoid glucoside, NAG, had the highest inhibitory effect on most of the isoforms tested. Correspondingly, the flavonoids tested also significantly ( $P < 0.001$ ) inhibited EROD and MROD activity of CYP1B1 and CYP1A2. Hydroxylation activity of human CYP aromatase, CYP19, was inhibited reversibly by LG and non-competitively by NAG. The limonoid glucosides and aglycones were also found to inhibit CYP 3A4 hydroxylation. NAG and nomilin were the best inhibitors of CYP3A4, drug metabolizing CYP isoenzyme. The IC<sub>50</sub> for limonoids varied considerably, depending upon the type of isoforms. The limonoid glucosides were found to be the better inhibitors when compared to the aglycones. The effects of citrus bioactive compounds on the partial inhibition of CYP enzymes represent a unique mechanism in the anticarcinogenesis strategy. One of the mechanisms is through the reduction in the generation of reactive oxygen species. This study also supports the role of citrus bioactive compounds on the increased bioavailability of orally administered drugs. Partial inhibition of CYP3A4 may represent an alternate strategy for the reduction in medical costs, but needs further studies on the mechanistic and dose response relations. Structural variations in different limonoid and flavonoid molecules can be ascribed to the differences in efficacy in inhibiting different CYP isoenzymes.

A complimentary means by which to address the question “Does limonoids acts as antioxidants?” relies on their ability to quench the reactive oxygen species. *In vitro* studies with four pure limonoid glucosides have shown effective superoxide scavenging and perceptible differences in the quenching efficacies, at 5 mmol/L. NAG emulated a quenching effect to an equivalent concentration of vitamin C, and was superior to LG, OG, and DNAG.

The study conducted to evaluate the cytotoxic effects of limonoid glucosides confirmed that micromolar amounts of LG and OG induce rapid necrosis of SH-SY5Y cells in culture. Viability studies showed significant ( $P < 0.001$ ) reduction in survivability by LG and OG within 24 h of treatment. The 12 h caspase 3/7 activity had a strong negative correlation ( $r = -0.998$ ,  $P = 0.002$ ) to the cell survival. The data confirmed that the cytotoxic effect of limonoid glucosides was manifested through apoptotic induction mechanism which was further endorsed through DNA fragmentation profile. Univariate DNA content was significantly higher for cells treated with LG, OG. Background aggregates and debris (BAD) between G1 and G2 phase, an indicator of apoptosis, was significantly higher ( $P < 0.05$ ) for LG and OG compared to control. Similarly DNA content at S phase of the cell cycle indicated a significant reduction by LG and OG as compared to NAG, DNAG (86.67% with LG, 82.36% with OG, 57.46% with NAG, and 38.35% with DNAG). Overall, these results confirm the antiproliferative property of limonoid glucosides are manifested through arresting critical growth stages of the cell cycle and inducing apoptosis.



The last study conducted to elucidate the cytotoxic effects of citrus limonoid aglycones and glucosides on human cancer cell lines such as SH-SY5Y neuroblastoma and colon carcinoma (Caco-2) cell lines showed a specific cytotoxic effects on cancerous cells and spared the non cancerous Chinese hamster ovary (CHO) cells. Limonoid glucosides have shown greater cytotoxic effects than aglycones and the effects were significant ( $P < 0.001$ ) over the period of 24 to 36 h when compared with the untreated cells. The morphological differences observed on the confocal microscopy clearly designate a rapid response for the LG as against limonin and obacunone aglycones. The effects were consistent with both time and dose dependent assay. Neuroblastoma cell lines were found to be more sensitive than the colon carcinoma cells in case of mixtures and nomilin during the initial period of exposure. The flow cytometric analysis of limonoid treated neuroblastoma cells showed a marked increase in DNA content of aneuploidic cells within 24 h. Percent aneuploidic cells were found to be 36% for LG, 26% for limonin and more than 42% for limonoid glucoside mixtures indicating a synergistic effect of different limonoid glucosides. The significant reduction in G2/M phase of the cell cycle confirms the results in chapter IV that limonoids inhibit S phase of the cell cycle. The inhibition of mitosis due to differing chromosome number in the aneuploidic cells, can lead to the non division of cells, eventually leading to death. These results confirm that both limonoid aglycones and glycosides are lethal to cancer cell lines; however

glycosides are the most active apoptotic inducing form, even though both forms arrest cell cycle.

In summary, with wide biological applications, anticancer properties and possible role in cell signaling mechanisms, limonoids can now be considered as a possible lead structure for a new class of anticancer drugs. The bulk purification methods could aid further research in the area of clinical trials, food additives, and nutritional supplements. As cellular permeability, water solubility, and metabolic change all play pivotal roles in the overall efficacy of a natural compound's performance, the glycosides appear to excel over aglycones. With citrus being a store house for many nutritionally important bioactive compounds, our studies strongly advocate citrus as a valuable functional food, particularly for the prevention of cancer in children.

## LITERATURE CITED

1. Calabrese, F. (2002) Citrus: Origin and history. In Citrus: The Genus Citrus (Dugo, G., & Giacomo, A. D., eds.) pp.1-15, Taylor & Francis Inc. New York.
2. USDA (2005) World Horticultural Trade & US Export Opportunities, Situation and outlook for citrus, [www.fas.usda.gov](http://www.fas.usda.gov), pp.1-8, Accessed April, 2005.
3. US Department of Commerce, (2005), US citrus exports, Florida Department of Citrus: Economic & market research report # EX-05-03, 1-2.
4. Miller, E.G., Taylor, S.E., Berry, C.W., Zimmerman, J.A. & Hasegawa, S. (2000) Citrus limonoids: Increasing importance as anticancer agents. In: Citrus Limonoids-Functional Chemicals in Agriculture and Foods (Berhow, M.A., Hasegawa, S., & Manners, G.D., eds.), pp.132-144. American Chemical Society, Washington D.C.
5. Montanari, A., Widmer, W. & Nagy, S. (1997) Health promoting phytochemicals in citrus fruit and juice products: Recent advances in phytochemistry. In: Functionality of Food Phytochemicals (Johns, T. A. & Romeo, J. T. eds.) pp.31 -52. Meeting of the Phytochemical Society of North America on Functionality, Plenum Press, New York.
6. Hasegawa, S. & Miyake, M. (1996) Biochemistry and biological functions of citrus limonoids. *Food Rev. Int.* 12: 413-435.
7. Hasegawa, S., Bennet, R. D., Herman, Z., Fong, C. H. & Ou, P. (1989) Limonoid glucosides in citrus. *Phytochemistry* 28: 1717-1724.
8. Hasegawa, S., Ou, P., Fong, C. H., Herman, Z., Coggins, C.W., Jr. & Atkin, D.R. (1991). Changes in the limonoate A-ring lactone and limonin 17 $\beta$ -D-glucopyranoside content of navel oranges during fruit growth and maturation. *J. Agric. Food Chem.* 39: 262-265.
9. Hasegawa, S., Berhow, M. A. & Fong, C. H. (1995) Analysis of bitter principles in citrus. In: Modern Methods of Plant Analysis. (Linskens, H. F., Jackson, J. F. & Allen, M. S., eds.) Vol.18, pp. 59-80. Springer-Verlag, Berlin, Germany.

10. Maier, V. P. & Beverly, G. D. (1968) Limonin monolactone, the nonbitter precursor responsible for delayed bitterness in certain citrus juices. *J. Food Sci.*, 33: 488-492.
11. Schoch, T. K., Manner, G. D. & Hasegawa, S. (2002) Recovery of limonoid glucosides from citrus molasses. *J. Food Sci.*, 67: 3159-3163.
12. Hasegawa, S., Fong, C. H., Herman, Z., & Miyake M. (1992) Glucosides of limonoids. Flavour precursors. *Am. Chem. Soc.* 86-97.
13. Miller, E. G., Porter, J. L., Binnie, W. H., Guo, I. Y. & Hasegawa, S. (2004) Further studies on the anticancer activity of citrus limonoids. *J. Agric. Food Chem.*, 52: 4908-4912.
14. Herman, Z., Fong, C. H., Ou, P. & Hasegawa, S. (1990) Limonoid glucosides in orange juices by HPLC. *J. Agric. Food Chem.* 38: 1860-1861.
15. Battinelli, L., Menqoni, F., Lichtner, M., Maqzzanti, G., Saija, A., Mastroianni, C. M. & Vullo, V. (2003) Effect of limonin and nomilin on HIV-1 replication on infected human mononuclear cells. *Plant Medica* 69: 910-913.
16. Sunthitikawinsakul, A., Kongkathip, N., Kongkathip, B., Phonnakhu, S., Daly, J.W., Spande, T.F., et al. (2003) Anti HIV-1 limonoid: first isolation from *Clausena excavata*. *Phytother. Res.* 17: 1101-1103.
17. Bray, D. H., Warhurst, C. C., Connolly, J. D., O'Neill, M. J. & Phillipson, J. D. (1990) Plants as sources of antimalarial drugs. Part 7. Activity of some species of Meliaceae plants and their constituent limonoids. *Phytother. Res.* 4: 29-35.
18. So, F. V., Guthrie, N., Chambers, A. F., Moussa, M. & Carroll, K. K. (1996) Inhibition of breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer* 25: 167-181.
19. Tanaka, T., Kohno, H., Tsukio, Y., Honjo, S., Tanino, M., Miyake, M. & Wada, K. (2000) Citrus limonoids obacunone and limonin inhibit azoxymethane-induced colon carcinogenesis in rats. *Biofactors* 13: 213-218.
20. Tanaka, T., Maeda, M., Kohno, H., Murakami, M., Kagami, S., Miyake, M. & Wada, K. (2001) Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by the citrus limonoids obacunone and limonin. *Carcinogenesis* 22: 193-198.

21. Lam, L. K. T. & Hasegawa, S. (1989) Inhibition of benzo[a]pyrene-induced forestomach neoplasia in mice by citrus limonoids. *Nutr. Cancer* 12: 43-47.
22. Miller, E. G., Gonzales, S. A. P., Couvillon, A. M., Wright, J. M. Hasegawa, S. & Lam, L. K. T. (1992) Inhibition of hamster buccal pouch carcinogenesis by limonin 17  $\beta$ -D-glucopyranoside. *Nutr. Cancer* 17: 1-7.
23. Miller, E. G., Fanous, R., Rivera, H. F., Binnie, W. H., Hasegawa, S. & Lam, L. K. T. (1989) The effect of citrus limonoids on hamster buccal pouch carcinogenesis. *Carcinogenesis* 10: 1535-1537.
24. Miller, E. G., Porter, J. L., Binnie, W. H., Guo I. Y. & Hasegawa, S. (2004) Further studies on the anticancer activity of citrus limonoids. *J. Agric. Food Chem.* 52: 4908-4912.
25. Pettit, G. R., Barton, D. H. R., Herald, C. L., Polonsky, J., Schmidt, J. M. & Connolly, J. D. (1983) Evaluation of limonoids against the murine P388 lymphocytic leukemia cell line. *J. Natur. Prod.* 46: 379-390.
26. Kurowska, E. M. & Manthey, J. A. (2004) Hydrolipidemic effects and absorption of citrus polymethoxylated flavones in hamsters with diet-induced hypercholesterolemia. *J. Agric. Food Chem.* 52: 2879-2886.
27. Kurowska, E. M. & Manthey, J. A. Casaschi, A. & Theriault, A. G. (2004) Modulation of HepG2 cell net apolipoprotein B secretion by the citrus polymethoxyflavone, tangeretin. *Lipids* 39:143-151.
28. Lam, L. K. T., Li, Y. & Hasegawa, S. (1989) Effects of citrus limonoids on glutathione S-transferase in mice. *J. Agric. Food Chem.* 37: 878-880.
29. Lam, L. K. T., Zhang, J., Hasegawa, S. & Schut, H. A. J. (1994) Inhibition of chemically induced carcinogenesis by citrus limonoids. In: *Food Phytochemicals for Cancer Prevention I.* (Huang, M. J., Osawa, T., Ho, C. T. & Rosen, R. T. eds.), pp. 209-219. American Chemical Society, Washington D.C.
30. Kelly, C., Jewell, C. & O'Brien, N. M. (2003) The effect of dietary supplementation with the citrus limonoids, limonin and nomilin on xenobiotic metabolizing enzymes in the liver and small intestine of the rat. *Nutr. Res.* 23: 681-690.

31. Tian, Q., Miller, E. G. Hassan, A., Tang, L. & Patil, B. S. (2001) Differential inhibition of human cancer cell proliferation by citrus limonoids. *Nutr. Canc.* 40: 180-184.
32. Manners, G. D. & Hasegawa, S. (1999) A new normal phase liquid chromatographic method for the analysis of limonoids in citrus. *Phytochem. Anal.* 10: 76-81.
33. American Cancer Society. (2005) *Cancer Facts & Figures 2005*. Atlanta, GA.
34. Halliwell, B. (1991) Reactive oxygen species in living systems: source biochemistry and role in human disease. *Am. J. Med.* 9: 14-22.
35. Guengerich, F. P., & Shimada, T. (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem. Res. Toxicol.* 4: 381-407.
36. Kadlubar, F. F., & Hammons G. J. (1987) The role of cytochrome P-450 in the metabolism of chemical carcinogens. In: *Mammalian cytochromes P-450* (Guengerich, F. P. ed.), pp. 81-130, CRC Press, Boca Raton, FL.
37. Guengerich F. P., & Shimada T. (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chem. Res. Toxicol.* 4: 381-407.
38. Zeigler, J. C. (2001) CYP3A4: a potential prostate cancer risk factor for high-risk groups. *Clin. J. Oncol. Nurs.* 5: 153-154.
39. Seow, A., Zhao, B., Lee, E. J., Poh, W. T., The, M. et al. (2001) Cytochrome P4501A2 activity and lung cancer risk: a preliminary study among Chinese women in Singapore. *Carcinogenesis.* 22: 673-677.
40. Marchand, L. L., Hankin J. H., Wilkens L. R., Pierce L. M., Franke A. et al. (2001) Combined effects of well-done red meat, smoking, and rapid N-acetyltransferase 2 and CYP1A2 phenotypes in increasing colorectal cancer risk. *Cancer Epidem. Biomarkers & Prev.* 10: 1259-1266.
41. Yager, J. D., & Liehr, J. G. (1996) Molecular mechanisms of estrogen carcinogenesis. *Ann. Rev. Pharmacol. Toxicol.* 36: 203-232.
42. Guengerich, F. P., Chun, Y. J., Kim, D., Gillam, E. M. J. & Shimada, T. (2003) Cytochrome P450 1B1: a target for inhibition in anticarcinogenesis strategies. *Mut. Res.* 523: 173-182.

43. Lilja, J. J., Kivisto, K. T. & Neuvonen, P. J. (1998) Grapefruit juice-simvastatin interaction: Effect on serum concentrations of simvastatin, simvastatin acid, and HMG/L-CoA reductase inhibitors. *Clinic. Pharmac. Therap.* 64: 477-483.
44. Doostdar, H., Burke, M. D. & Mayer, R. T. (2000) Bioflavonoids: Selective substrates and inhibitors of CYP 450 1A1 and CYP 1B1, *Toxicology* 144: 31-38.
45. Cao, G., & Sofic, E. (1996) Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* 44: 3426 -3431.
46. Steinmetz, K. A. & Potter, J. D. (1996) Vegetables, fruits and cancer prevention: A review. *J. Am. Diet. Assoc.* 96: 1027–1037.
47. Block, G., Patterson, B. & Subar, A. (1992) Fruit, vegetables and cancer prevention; a review of the epidemiological evidence. *Nutr. Cancer* 18: 1–29.
48. Marklund, S. & Marklund, G. (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47: 469-474.
49. Ikonen, M., Liu, B., Hashimoto, Y., Ma, L., Lee, K. W., Niikura, T., Nishimoto, I. & Cohen P. (2003) Interaction between the Alzheimer's survival peptide humanin and insulin-like growth factor binding protein regulates cell survival and apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 100: 13042-13047.
50. Jaffe, N. (1976) Neuroblastoma: Review of the literature and an examination of its enigmatic character. *Cancer Treat. Rev.* 3: 61–82.
51. Souhami, R. & Tobias, J. (1998) *Cancer and its Management* (3rd Ed), Blackwell Science, Oxford, U.K.
52. Wang, C. N., Chi, C. W., Lin, Y. L., Chen C. F., & Shiao, Y. J. (2001) The neuroprotective effects of phytoestrogens on amyloid  $\beta$  protein-induced toxicity are mediated by abrogating the activation of caspase cascade in rat cortical neurons. *J. Biol. Chem.* 276: 5287-5295.
53. Nicolini, G., Rigolio, R., Scuteri, A., Miloso, M., Saccomanno, D., Cavaletti, G. & Tredici, G. (2003) Effect of *trans*-resveratrol on signal transduction pathways involved in paclitaxel-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Neurochem. Internat.* 43: 419-429.

54. Miyataa, M., Takanoa, H., Takahashia, K., & Sasakib, Y. F. (2002) Suppression of 2-amino-1-methyl-6-phenylimidazo [4,5- $\square$ ] pyridine-induced DNA damage in rat colon after grapefruit juice intake. *Cancer Lett.* 183: 17-22.
55. Miyagi, Y., Om, A. S., Chee, K. M., & Bennik, M. R. (2000) Inhibition of azoxymethane-induced colon cancer by orange juice. *Nutr. Cancer* 32: 224-229.
56. Balandrin, N. F., Kinghorn, A. D., Farnsworth, N. R. (1993) In: *Human Medicinal Agents from Plants* (Kinghorn, A. D. & Balandrin, M. F., eds.), pp. 2-12. American Chemical Society (Symposium Series 534), Washington, D.C.
57. Manners, G. D., Jacob, R. A., Breksa, A. P., Schoch, T. K. & Hasegawa, S. (2003) Bioavailability of citrus limonoids in humans. *J. Agric. Food Chem.* 51: 4156-4161.
58. Hollmam, P., Hertog, M. & Katan, M. (1996) Role of dietary flavonoids in protection against cancer and coronary heart disease. *Biochem. Soc. Trans.* 24: 785-789.
59. Jagetia, G. C., Venkatesha, V. A. & Reddy, T. K. (2003) Naringin, a citrus flavonone, protects against radiation-induced chromosome damage in mouse bone marrow. *Mutagenesis* 18: 337-343.
60. Jeon, S. M., Bok, S. H., Jang, M. K., Lee, M. K., Nam, K. T., Park, Y. B., Rhee, S. J. & Choi, M. S. (2001) Antioxidative activity of naringin and lovastatin in high cholesterol-fed rabbits. *Life Sciences* 69: 2855-2866.
61. Gorinstein, S., Leontowicz, H., Leontowicz, M., Krzeminski, R., Gralak, M. et al. (2005) Changes in plasma lipid and antioxidant activity in rats as a result of naringin and red grapefruit supplementation. *J. Agric. Food Chem.* 53: 3223-3228.
62. Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. & Yano, M. (1999) Antiproliferative activity of flavonoids on several cancer cell lines. *Biosci. Biotech. Biochem.* 63: 896-899.
63. Tanaka, T., Kohno, H., Murakami, M., Shimada, R., Kagami, S., Sumida, T., Azuma, Y. & Ogawa, H. (2000) Suppression of azoxymethane-induced colon carcinogenesis in male F344 rats by mandarin juices rich in  $\square$ -cryptoxanthin and hesperidin. *Int. J. Cancer* 88: 146-150.



64. Scambia, G., Ranelletti, F. O., Benedetti P. P., Piantelli, M., Bonanno, G. et al. (1990) Inhibitory effect of quercetin on OVCA 433 cells and presence of type II estrogen binding sites in primary ovarian tumors and cultured cells. *Brit. J. Cancer* 62: 942-946.
65. Food and Drug Administration (1982) Evaluation of the health aspects of hesperidin, naringin, and citrus bioflavonoid extracts as food ingredients. SCOGS-II-3, FDA/BF-82/62; Order No. PB82-192931, Gov. Rep. Announce Index (U.S.A.) 82(15), 2962.
66. Bentley, M. D., Rajab, M. S., Alford, A. R., Mendel, M. J. & Hassanali, A. (1988) Structure-activity studies of modified citrus limonoids as antifeedants for Colorado potato beetle larvae, *Leptinotarsa decemlineata*. *Entomol. Exper. Applic.* 49: 189-193.
67. Ozaki, Y., Ayano, S., Inaba, N., Miyake, M., Berhow, M. A. & Hasegawa, S. (1995) Limonoid glucosides in fruit, juice, and processing by-products of Satsuma mandarin (*Citrus unshiu* Marcov.). *J. Food Sci.* 60: 186-189.
68. Rouseff, R. L. & Fisher, J. F. (1980) Determination of limonin and related limonoids in citrus juices by high performance liquid chromatography. *Anal. Chem.* 52: 1228-1233.
69. Sawabe, A., Hasegawa, S., Bennet, R. D. & Wong, R.Y. (2000) LC-MS and NMR technique for the analysis and characterization of citrus limonoids. In: *Citrus Limonoids; Functional Chemicals in Agriculture and Foods*. (Berhow, M. A., Hasegawa, S. & Manners, G. eds) pp. 40-58. American Chemical Society (Symposia series 758), Washington, D.C.
70. Shaw, P. E. & Wilson, C. W. (1984) A rapid method for the determination of limonin in citrus juice by reverse-phase high-performance liquid chromatography. *J. Chromatogr.* 464: 375-386.
71. Van Beek, T. A. & Blaakmeer, A. (1989) Determination of limonin in grapefruit juice and other citrus juices by high-performance liquid chromatography. *J. Chromatogr.* 161: 375-86.
72. Bennet, R. D., Hasegawa, S. & Herman, Z. (1989) Glucosides of acidic limonoids in citrus. *Phytochemistry* 28: 2777-2781.
73. Hasegawa, S. & Bennett, R. D. (1980) Isolimonic acid, a new citrus limonoid. *Phytochemistry* 19: 2417-2419.

74. Manners, G. D., Breska III, A. P., Schoch, T. K. & Hidalgo, M. B. (2003) Analysis of bitter limonoids in citrus juices by atmospheric pressure chemical ionization and electrospray ionization liquid chromatography-mass chromatography. *J. Agric. Food Chem.* 51: 3709-3714.
75. Tian, Q. & Ding, X. (2000) Screening for limonoid glucosides in *Citrus tangerina* (Tanaka) Tseng by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr.* 874: 13-19.
76. Schoch, T. K., Manner, G.D. & Hasegawa, S. (2001) Analysis of limonoid glucosides from citrus by electrospray ionization liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* 49: 1102-1108.
77. Manthey, J. A. (2004) Fractionation of orange peel phenols in ultrafiltered molasses and mass balance studies of their antioxidant levels. *J. Agric. Food Chem.* 52: 7586-7592.
78. Tian, Q., Liu, Y., Bhat, N. G. & Patil, B. S. (2000) Total limonoids in citrus. *Proc. Intl. Citricult. Cong. IX.* 1261-1264.
79. Emerson, O. H. (1948) Bitter principles in citrus fruit. I. Isolation of nomilin, a new bitter principle from the seeds of oranges and lemons. *J. Am. Chem. Soc.* 70: 545-549.
80. Dreyer, D. L. (1965) Citrus bitter principles. III. Isolation of deacetylnomilin and deoxylimonin. *J. Org. Chem.* 30: 749-51.
81. Targett, N. M., Kilcoyne, J. P. & Green, B. (1979) Vacuum liquid chromatography: an alternative to common chromatographic methods. *J. Org. Chem.* 44: 4962-4964.
82. Coll, J. C. & Bowden, B. F. (1986) The application of vacuum liquid chromatography to the separation of terpene mixtures. *J. Nat. Prod.* 49: 934-936.
83. Turcant, A., Premel, C. A., Cailleux, A. & Allain, P. (1988) Screening for neutral and basic drugs in blood by dual fused-silica column chromatography with nitrogen-phosphorus detection. *Clin. Chem.* 34: 1492-1497.
84. Emerson, O. H. (1952) Bitter principles in citrus. III. Some reactions of limonin. *J. Am. Chem. Soc.* 74: 688-693.

85. Hendrickson, R. & Kesterson, J. W. (1956) Crude hesperidin purification. *Citrus Industry* 37: 7-10.
86. Scordino, M., Di, M. A. Passerini, A. & Maccarone, E. (2004) Adsorption of flavonoids on resins: cyanidin 3-glucoside. *J. Agric. Food Chem.* 52: 1965-1972.
87. Levison, R. P. (1996) Ion exchange chromatography and secondary adsorption effects. In: *Downstream Processing of Natural Products, a practical handbook* (Michael S. V. ed). Wiley J & Sons, Inc. Chichester, U.K.
88. Vandenhul, H. J. & Wanderhoff, J. W. (1972) The characterization of latex particle surfaces by ion exchange and conductometric titration. *J. Electroanal. Chem.* 37: 161-168.
89. Halliwell, B. (1991) Reactive oxygen species in living systems: source biochemistry and role in human disease. *Am. J. Med.* 9: 14-22.
90. Loo, G. (2003) Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation. *J. Nutr. Biochem.* 14: 64-73.
91. Wrighton, S. A. & Stevens, J. C. (1992) The human hepatic cytochrome P450 involved in drug metabolism. *Crit. Rev. Toxicol.* 22: 1-21.
92. Russo, M., Tedesco, I., Iacomino, G., Palumbo, R., Galano, G. & Russo, G. L. (2005) Dietary phytochemicals in chemoprevention of cancer. *Curr. Med. Chem. Immunol. Endocr. Metab. Agents* 5: 61-72.
93. Hodek, P., Trefil, P. & Stiborova, M. (2002) Flavonoids-potent and versatile biologically active compounds interacting with cytochromes p450. *Chem. Biol. Interact.* 139: 1-21.
94. Ortiz de Montellano, P. R. (1986) Oxygen activation and transfer. In: *Cytochrome P-450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed) pp. 217-271. Plenum Press, New York.
95. Guengerich, F. P. (2003) Cytochrome P450, drugs and disease. *Mol. Interv.* 3: 194-204.
96. Miller, E. C. & Miller, J. A. (1976) The metabolism of chemical carcinogens to reactive electrophiles and their possible mechanisms of action in carcinogenesis, In: *Chemical Carcinogenesis Monograph*, pp. 737-762. American Chemical Society, Washington, D.C.

97. Bartsch, H., Nair, U., Risch, A., Rojas, M., Wikman, H. & Alexandrov, K. (2000) Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. *Cancer Epidemiol. Biomarkers Prev.* 9: 3-28.
98. Mayer R. T., Jermyn, J. W. & Burke, M. D. (1977) Methoxyresorufin as a substrate for the fluorometric assay of insect microsomal O-dealkylase. *Pestic. Biochem. Physiol.* 7: 349-354.
99. Burke, M. D., Thompson, S., Weaver, R. J., Wolf C. R. & Mayer, R. T. (1994) Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. *Biochem. Pharmacol.* 48: 923-936.
100. Stahle, E. L., Schloss, L., Sundqvist, V. A., Brytting, M., Hokeberg, I., Cox, S., Wahren, B. & Linde, A. (1998) Solid phase ELISA for determination of the virus dose dependent sensitivity of human cytomegalovirus to antiviral drugs in vitro. *Antiviral Res.* 40: 105-112.
101. Girenavar, B., Poulouse, S. M., Brodbelt, J. S., Jayaprakasha G. K., Bhat, N. G. & Patil, B. S. (2005) Whither grapefruit drug interaction?, American Chemical Society, (ACS symposium series) Washington, D.C. (in press).
102. Lang, N. P., Butler, M. A., Massengill, J., Lawson, M., et al. (1994) Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase the risk for colorectal cancer or polyps. *Cancer Epidemiol. Biomarkers Prev.* 3: 675–682.
103. Brodie, A. M. H. (1985), Aromatase inhibition and its pharmacological implications. *Biochem. Pharmacol.* 34: 3213-3219.
104. Kita, M., Hirata, Y., Moriguchi, T., Endo-Inagaki, T., Matsumoto, R., Hasegawa, S. Suhayda, C. G. & Omura, M. (2000) Molecular cloning and characterization of a novel gene encoding limonoid UDP-glucosyltransferase in citrus. *FEBS. Lett.* 469: 173-178.
105. Manners, G. D., Jacob, R. A., Breksa, A. P., Schoch, T. K. & Hasegawa, S. (2003) Bioavailability of citrus limonoids in humans. *J. Agric. Food Chem.* 51: 4156-4161.
106. Yu, J., Wang, L., Walzem, R. L., Miller, E. G., Pike, L. M. & Patil, B. S. (2005) Antioxidant activity of citrus limonoids, flavonoids, and coumarins. *J. Agric. Food Chem.* 53: 2009-2014.

107. Gale, G., D'Angio, G., Uri, A., Chatten, J. & Coop, C. E. (1982) Cancer in neonates: the experience at the children's hospital of Philadelphia. *Pediatrics* 70: 409–413.
108. Reddy, M. C. M., Majumdar, S. & Harris, E. D. (2000) Evidence for a Menkes-like protein with a nuclear targeting sequence. *Biochem. J.* 350: 855-863.
109. Hansen, M. B., Nielsen, S. E. & Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immun. Meth.* 119: 203-210.
110. Silalahi, J. (2002) Anticancer and health protective properties of citrus fruit components. *Asia Pac. J. Clin. Nutr.* 11: 79-84.
111. Deng, M., Zhao, J. Y., Ju, X. D., Tu, P. F. Jiang, Y. & Li, Z. B. (2004) Protective effect of tubuloside B on TNF-alpha-induced apoptosis in neuronal cells. *Acta Pharmacol. Sin.* 25: 1276-1284.
112. Ikonen, M., Liu, B, Hashimoto, Y., Ma, L., Lee, K. W., Niikura, T., Nishimoto, I. & Cohen, P. (2003) Interaction between the Alzheimer's survival peptide humanin and insulin-like growth factor binding protein regulates cell survival and apoptosis. *Proc. Natl. Acad. Sci.* 100: 13042-13047.
113. Berglund, C. M., Radesater, A. C., Persson, M. A., & Budd H. S. L. (2004) UV-induced apoptosis in SH-SY5Y cells: contribution to apoptosis by JNK signaling and cytochrome c. *J. Neurosci. Res.* 78: 580-589.
114. Lovat, P. E., Di Sano, F., Corazzari, M., Fazi, B., Donnorso, R. P. et al. (2004) Gangliosides link the acidic sphingomyelinase-mediated induction of ceramide to 12-lipoxygenase-dependent apoptosis of neuroblastoma in response to fenretinide. *J. Natl Canc Instit.* 96: 1288-1299.
115. Kweon, S. M., Lee, Z. W., Yi, S. J., Kim, Y. M., Han, J A., Paik, S. G. & Ha, S. S. (2004) Protective role of tissue transglutaminase in the cell death induced by TNF-alpha in SH-SY5Y neuroblastoma cells. *J. Biochem. Mol. Biol.* 37: 185-191.
116. Lee, E. O., Shin, Y. J., & Chong, Y.H. (2004) Mechanisms involved in prostaglandin E2-mediated neuroprotection against TNF-alpha: possible involvement of multiple signal transduction and beta-catenin/T-cell factor. *J. Neuroimmunol.* 155: 21-31.

117. Russo, V. C., Metaxas, S., Kobayashi, K., Harris, M. & Werther, G. A. (2004) Antiapoptotic effects of leptin in human neuroblastoma cells. *Endocrinology* 145: 4103-4112.
118. Pettifer, K. M., Kleywegt, S., Bau, C. J., Ramsbottom, J. D. Vertes, E., Ciccarelli, R., Caciagli, F., Werstiuk, E. S. & Rathbone M. P. (2004) Guanosine protects SH-SY5Y cells against beta-amyloid-induced apoptosis. *Neuroreport*. 15: 833-836.
119. Bissonnette, C. J., Klegeris, A., McGeer, P. L. & McGeer, E.G. (2004) Interleukin 1 alpha and interleukin 6 protect human neuronal SH-SY5Y cells from oxidative damage. *Neurosci Lett*. 361: 40-43.
120. Kang, S. S., Lee, J. Y., Choi, Y. K., Kim, G. S. & Han, B. H. (2004) Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblastoma cells. *Bioorg. Med. Chem. Lett*. 15: 2261-2264.
121. Jeong, J. H., Kim, H. J., Lee, T. J., Kim, M. K., Park, E. S. & Choi, B. S. (2004) Epigallocatechin 3-gallate attenuates neuronal damage induced by 3-hydroxykynurenine. *Toxicology* 195: 53-60.
122. Zhang, Y. & Zhao, B. (2003) Green tea polyphenols enhance sodium nitroprusside-induced neurotoxicity in human neuroblastoma SH-SY5Y cells. *J. Neurochem*. 86: 1189-1200.
123. Sergeev, I. N. (2004) Genistein induces Ca<sup>2+</sup>-mediated, calpain/caspase-12-dependent apoptosis in breast cancer cells. *Biochem. Biophys. Res. Common*. 321: 462-467.
124. Green, D. & Kroemer, G. (1998) The central executioners of apoptosis: caspases or mitochondria? *Trends Cell. Biol*. 8: 267-271.
125. Mertens-Talcott, S. U., Talcott, S. T. & Percival, S. S. (2003) Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J. Nutr*. 133: 2669-2674.
126. Setzer, W. N. & Setzer, M. C. (2003) Plant-derived triterpenoids as potential anticancer agents. *Mini Rev. Med. Chem*. 3: 540-556.
127. Taylor, C. P. F., Bown, N. P., McGuckin, A. G., Lunec, J., Malcolm, A. J., Pearson, A. D. J. & Sheer, D. (2000) Fluorescence in situ hybridization techniques for the rapid detection of genetic prognostic factors in neuroblastoma. *Br. J. Cancer* 83: 40-49.

128. Abad, M., Ciudad, J., Rincon, M. R., Silva, I., Paz-Bouza, J. I., Lopez, A., Alonso, A. G., Bullon, A. & Orfao, A. (1998) DNA aneuploidy by flow cytometry is an independent prognostic factor in gastric cancer. *Anal. Cell. Pathol.* 16: 223-231.
129. Look, A. T., Hayes F. A., Shuster J. J., Douglas E. C., Castleberry R. P., Bowman L. C., Smith E. I. & Brodeur G. M. (1991) Clinical relevance of tumour ploidy and N-myc gene amplification in childhood neuroblastoma: a pediatric oncology group study. *J. Clin. Oncol.* 9: 581-591.
130. Gansler T., Chatten J., Varello, M., Bunin G. R. & Atkinson B. (1986) Flow cytometric analysis of neuroblastoma: correlation with histology and clinical outcome. *Cancer* 58: 2453-2458.
131. Shoji, Y. & Nakashima, H. (2004) Nutraceuticals and delivery systems. *J Drug Targeting* 12: 385-391

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### EDUCATION

Ph.D. Horticulture	Texas A&M University	2005
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B.Sc. Agriculture	Univ. Agric. Sci., Bangalore, India	1995

### PROFESSIONAL EXPERIENCE

Postdoctoral Trainee, Vegetable and Fruit Improvement Center	2005
Graduate Research Assistant, Dept. of Hort. Sci. TAMU	2001-2005
Research Coordinator, GREEN Foundation, Bangalore, India	1998-2001

### PUBLICATIONS

- Poulouse, S. M., Harris, E. D. & Patil, B. S. 2005, Citrus limonoids induce apoptosis in human neuroblastoma cells and have radical scavenging activity. *J. Nutr.* 135: 870-877.
- Poulouse, S. M., Harris, E. D. & Patil, B. S. 2005, Proapoptotic and anticancer activity of citrus limonoids on human neuroblastoma cells. *The FASEB Journal*, 19 (5): 830.
- Poulouse, S. M., Harris, E. D. & Patil, B. S. Cytotoxic effects of citrus limonoids: glycosides are more lethal than aglycones against human neuroblastoma and colonic adenocarcinoma cells, in preparation.
- Poulouse, S. M., Mayer, R. T., Girenavar, B., Pike, L. M., & Patil, B. S., Purification and inhibitory effects of citrus limonoids on human CYP 450 isoenzymes, in preparation.

### HONORS AND AWARDS

- 2005 Outstanding Co-operative Ph.D. Student, 2004-2005, TAMUK
- 2005 Best Student Research Oral Presentation-Life Sciences. TAMU
- 2005 & 2004 Best Student Research Poster Award, Ag Program. TAMU
- 2004 Outstanding Graduate Student of the Year 2003-2004, TAMUK