

DIETARY APIGENIN AND NARINGENIN PROTECT AGAINST COLON
CARCINOGENESIS BY LOWERING HIGH MULTIPLICITY ABERRANT CRYPT
FOCI AND ENHANCING APOPTOSIS IN AZOXYMETHANE-TREATED RATS

A Thesis

by

TETY LEONARDI

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

MASTER OF SCIENCE

May 2005

Major Subject: Nutrition

DIETARY APIGENIN AND NARINGENIN PROTECT AGAINST COLON
CARCINOGENESIS BY LOWERING HIGH MULTIPLICITY ABERRANT CRYPT
FOCI AND ENHANCING APOPTOSIS IN AZOXYMETHANE-TREATED RATS

A Thesis
by
TETY LEONARDI

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

MASTER OF SCIENCE

Approved as to style and content by:

Nancy D. Turner
(Co-Chair of Committee)

Joanne R. Lupton
(Co-Chair of Committee)

Robert S. Chapkin
(Member)

Naisyin Wang
(Member)

Nancy D. Turner
(Chair of Faculty of Nutrition)

Gary R. Acuff
(Head of Department)

May 2005

Major Subject: Nutrition

ABSTRACT

Dietary Apigenin and Naringenin Protect Against Colon Carcinogenesis by Lowering High Multiplicity Aberrant Crypt Foci and Enhancing Apoptosis in Azoxymethane-Treated Rats. (May 2005)

Tety Leonardi, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Nancy D. Turner
Dr. Joanne R. Lupton

Colon cancer is the third most common cancer in the United States. However, evidence indicates that a proper diet abundant in fruits and vegetables may be protective against colon cancer development. Bioactive compounds in fruits and vegetables, such as flavonoids and limonoids, have been shown to possess anti-proliferative and anti-tumorigenic effects in various in vitro and in vivo models of cancer. Since there are few animal studies involving flavonoids and limonoids and colon cancer, this experiment investigated the potentially protective effects of four citrus flavonoids and one limonoid mixture against the promotion stage of chemically-induced colon cancer in rats. Male SD rats (n =60; 10 rats/group) were assigned to receive diets containing 0.1% apigenin, 0.02% naringenin, 0.1% hesperidin, 0.01% nobiletin, 0.035% limonin glucoside/obacunone glucoside mixture, or a control diet (0% flavonoid/limonoid). Rats received the diets for 10 wk and were injected with azoxymethane (15 mg/kg) at wk 3 and 4. The excised colons were evaluated for aberrant crypt foci (ACF) formation, cell proliferation (PCNA assay), apoptosis (TUNEL assay), and iNOS and COX-2 expression. When compared to the control diet, apigenin lowered the number of high multiplicity ACF (> 4 AC/focus) by 57% (P<0.05) and tended to lower the proliferative index (28%; P=0.07), while naringenin lowered both the number of high multiplicity ACF by 51% (P<0.05) and the proliferative index by 32% (P<0.05). Both apigenin and naringenin increased apoptosis of surface colon cells (78% and 97%, respectively; P<0.05) when compared to control diet. Hesperidin, nobiletin, and the limonin

glucoside/obacunone glucoside mixture did not have any effects on the above variables measured in this model of colon carcinogenesis. The colonic mucosal protein levels of iNOS or COX-2 were not different among the six diet groups. Evidence suggests that high multiplicity ACF are indicative of future tumor development in both humans and rats. Furthermore, dysregulated proliferation and apoptosis may also lead to tumorigenesis. Therefore, the ability of dietary apigenin and naringenin to reduce high multiplicity ACF, lower proliferation, and increase apoptosis may contribute toward colon cancer prevention. However, their protection is not due to their influence on iNOS and COX-2 protein levels.

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to all who helped me in my thesis research. Without your gracious assistance, I would not have gotten to where I am today. I would like to especially thank my mentor, major professor, and committee chair, Dr. Nancy Turner, whose support, encouragement, and intellectual input made the most daunting tasks seem doable. My gratitude also goes to co-chair Dr. Joanne Lupton, and committee members Dr. Robert Chapkin and Dr. Naisyin Wang for their valuable advice. A deep heart-felt thanks to Dr. Jairam Vanamala, who had greatly helped me all throughout this research project, and for being a great friend. I want to acknowledge Dr. Bhimu Patil and his laboratory for providing the experimental compounds used in this study. A big thank you to Mary Murphy for performing the statistical analysis in this study, Stella Taddeo, Chris and Heather van Velson, Kim Paulhill, and student workers for all their laboratory support, research scientist Dr. Mee Young Hong and my fellow graduate students Anne Newton, Dr. Lisa Sanders, Kristy Covert, and Cindy Warren for their assistance. I very much appreciate Dr. Laurie Davidson for her advice and for allowing me to use resources in her laboratory. Last but not least, I want to thank my husband, Kenny Kwong, who stood by my side and offered emotional support whenever I needed it.

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	viii
LIST OF TABLES	ix
CHAPTER	
I INTRODUCTION	1
II LITERATURE REVIEW	3
Multistage colon carcinogenesis	3
Proliferation and apoptosis	4
Aberrant crypt foci as a surrogate biomarker	5
Inflammation	6
Biological activities of citrus flavonoids and limonoids	8
Hypothesis	10
III MATERIALS AND METHODS	11
Animals and study design	11
Diets	12
Collection of tissue samples	14
Evaluation of aberrant crypt foci	16
Measurement of cell proliferation	16
Measurement of apoptosis	17
Western blot analysis of iNOS and COX-2	18
Statistical analysis	19

CHAPTER	Page
IV RESULTS	20
Diet analysis	20
Food intake and weight gain	20
Incidence of aberrant crypt foci	22
Colonocyte proliferation	22
Apoptosis	26
Expression of iNOS and COX-2	26
Correlation analysis	29
V DISCUSSION	31
VI CONCLUSIONS	37
LITERATURE CITED	38
APPENDIX A	50
APPENDIX B	54
APPENDIX C	62
APPENDIX D	63
VITA	64

LIST OF FIGURES

FIGURE	Page
1 Time line of the live animal portion of the experiment	11
2 Structures of the experimental compounds used in this study	13
3 Schematic diagram of rat colon sample collection	15
4 Cartoon illustrating a colonic crypt	17
5 Effects of experimental diets on the total number of aberrant crypts per colon (A), and number of aberrant crypts per centimeter of colon (B)	23
6 Effects of experimental diets on incidence of high multiplicity ACF per colon	24
7 Effects of experimental diets on cell proliferation in rat distal colon, measured by proliferative index	25
8 Effects of experimental diets on expansion of cell proliferation in rat distal colon, measured by proliferative zone	25
9 Effects of experimental diets on apoptosis in rat distal colon, measured by luminal surface apoptotic index	27
10 A representative blot of linearity test for iNOS and COX-2	28
11 Effects of experimental diets on the expression levels of iNOS enzyme	29
12 Effects of experimental diets on the expression levels of COX-2 enzyme	30

LIST OF TABLES

TABLE	Page
1 Composition of experimental diets	14
2 Food intake data for rats in each diet group	21
3 Weight data for rats in each diet group	21
4 Effect of experimental diets on number of proliferative cells, location of highest proliferative cell, and crypt height in colon of Sprague-Dawley rats	24
5 Effect of experimental diets on apoptosis within colonic crypt columns in male Sprague-Dawley rats	27

CHAPTER I

INTRODUCTION

It was estimated that by the end of the year 2004, approximately 1,500 Americans a day would have died of cancer, accounting for about one in four deaths (1). Out of the total number of deaths from cancer, the American Cancer Society projected that more than 100,000 new cases of colon cancer would be diagnosed and more than 56,000 deaths from colorectal cancer would occur in the United States (1). These statistics make colon cancer the third most common cancer in men and women individually, and the second leading cause in cancer mortality when numbers for men and women are combined (1). The 5-y survival rate following diagnosis of colon cancer is about 63% in the United States (2). In addition, the total economic burden of cancer imposed on society was estimated to be \$143.5 billion in 1996, the most recent year for which data were available (3). This figure included both the direct costs of cancer care and treatments and the indirect costs of productivity loss due to cancer illness and deaths. The costs associated with colorectal cancer alone accounted for about 13.1% of the total economic burden for all cancers (3).

Despite the unfavorable incidence of colon cancer, the 1999 Harvard Report on Cancer Prevention stated that colon cancer may be preventable when risk factors such as diet and lifestyle are modified (4). Furthermore, the implication of diet and lifestyle as probable risk factors for colon cancer was evidenced by studies of individuals migrating from low- to high-risk areas (5,6). Diet and its relationship with cancer have interested many scientists and is a topic of ongoing research. Where colon cancer is concerned, many scientists, backed by a large body of evidence, believe that a proper diet, especially one high in fruits and vegetables, may be protective against colon cancer development [reviewed in (7-9)]. This is an area of great contention, however, as others have found little or no relationship between consumption of fruits and vegetables and colon cancer (10-14).

This thesis follows the style and form of The Journal of Nutrition.

Nonetheless, more research in this area is warranted as new data emerge with promising associations between components in fruits and vegetables and the prevention of colon carcinogenesis. In a recent prospective cohort study of Swedish women, the risk of colon cancer was greatest for those with the lowest intake of fruits and vegetables, with fruit consumption having the greater association with lowered risk (6). A population study by Risch et al. (15) suggested that fruits, especially citrus fruits, contain biologically active compounds besides vitamin C that may play a role in preventing many chronic diseases including cancer. Although, to the best of our knowledge, there is no human studies specifically linking citrus fruits to lowered colon cancer risk, the use of citrus peel by subjects in Arizona was associated with a reduced risk of squamous cell carcinoma of the skin (16). However, some animal studies have reported some protection against developing colonic tumors from consuming citrus fruits. For example, feeding either orange juice (17) or 15% orange-pulp diet (18) was able to suppress chemically induced colon tumors in rats. Bioactive compounds such as certain flavonoids and limonoids have been suggested to be among the promising chemopreventive agents in citrus fruits (19). Recent experimental data also provide evidence that certain citrus flavonoids and limonoids may be protective against several cancers, including colon cancer (20-34).

CHAPTER II

LITERATURE REVIEW

Multistage colon carcinogenesis. The development of carcinogenesis is a multistep process involving the genetic mutation of normal cells (the initiation stage), transformation into and subsequent accretion of cells with abnormal phenotype through epigenetic mechanisms and alterations in cell dynamics (the promotion stage), and finally progressing into dysplastic, invasive, and metastatic cancer (the progression stage) (35). Very often, the process of carcinogenesis is the result of accumulated progressive changes in the expression of proto-oncogenes, tumor suppressor genes, apoptosis-regulating genes, and DNA repair genes – all causing the affected cells to acquire genetic instability (35).

Several major pathways to colon cancer development have been proposed [reviewed in (5,36,37)]. One major pathway involves an inherited or acquired mutation in the adenomatous polyposis coli (APC) gene, a tumor suppressor gene, which controls cell replication, adhesion, and migration via the wingless type (Wnt)/ β -catenin pathway. Activation of the Wnt/APC/ β -catenin pathway is said to enhance the expression of genes controlling cell growth and tumorigenesis. Also associated with this pathway are alterations in DNA methylation patterns and genetic changes such as the mutation of K-ras (a proto-oncogene) and mutation or loss of p53 (a tumor-suppressor gene). Another pathway involves microsatellite instability (36,37) of the affected cells caused by mutation, loss, or epigenetic hypermethylation in DNA mismatch repair (MMR) genes. Implicated in this pathway are transforming growth factor (TGF) β receptor II gene, which controls growth, and BAX gene, which controls apoptosis. One other pathway is via the inflammatory bowel disease (IBD) dysplasia-carcinoma sequence involving chronic inflammation and loss of epithelial integrity (5). Patients with ulcerative colitis and Crohn's disease, both categorized under IBD, have about a 20-fold excess risk of colon cancer than when IBD is not present (5). As will be discussed later, chronic inflammation creates a favorable environment for the promotion of cancer. Hypermethylation silencing of the estrogen receptor gene is also suggested as a separate

pathway to colon cancer (5,38). After the initial genetic modifications, however, the promotion of abnormal cells into subsequent colon adenocarcinoma takes many years to develop (36,37,39). This long latent period allows certain factors, including diet, a chance to modulate the disease (4,39).

Proliferation and apoptosis. Normal colonic epithelium maintains a tightly controlled equilibrium of cell growth and cell death (39,40). Typically, colonic cells proliferate in the lower part of the crypt and differentiate as they move up the crypt (39,40). Finally surface colonocytes undergo apoptosis or are sloughed off into the lumen and are lost into the fecal stream (39,40). In the promotion phase of carcinogenesis, including colon cancer, alterations of cell dynamics such as proliferation and apoptosis occur (35). For example, loss of the highly ordered process of colonic cell regeneration may cause the expansion of the proliferative zone towards the lumen or beyond the normal proliferative compartment within the crypt (37). In addition, it is widely accepted that disruptions of cell proliferation and apoptosis enable mutated cells to have a clonal growth advantage over normal cells, which can lead to tumor growth (41-43). Thus, measurements of proliferation and apoptosis are valuable biomarkers of tumorigenesis (44).

Certain components in the diet may modulate the promotion phase of carcinogenesis by influencing the rate of cell proliferation and apoptosis via various mechanisms (45). Some of the components reported in the literature are classic nutrients. For example, pectin, a highly fermentable dietary fiber, was shown to increase cell proliferation in the distal colon while decreasing apoptosis in the proximal colon (46). Another classic nutrient source, dietary corn oil, when provided to azoxymethane-treated Sprague-Dawley rats was found to suppress mitochondria-dependent apoptosis via inhibiting the tumor suppressor gene p53 (47). More recently, phytochemicals not usually considered as classic nutrients, have been the subject of much research. Apigenin, a flavonoid commonly found in fruits and vegetables such as oranges and onions, was able to inhibit proliferation of human colon carcinoma cell lines (SW480,

HT-29, and Caco-2) by inducing cell cycle arrest at G2/M phase (48). Genistein, an isoflavonoid found in soybeans, not only induced a G2/M phase cell cycle arrest in HT-29 cells but also enhanced apoptosis of the cells through regulation of p21^{WAF1} and Bax/Bcl-2 expression (49). A compound in green tea, (-)-epigallocatechin-3-gallate (EGCG), was found to exert a G1 phase cell cycle arrest in human ovarian cancer cell lines (SKOV-3 and OVCAR-3), and induce apoptosis with associated up-regulation in p21 and Bax (50). Quercetin, a flavonoid found in apples, onions, and tea, was shown to induce G2/M or G0/G1 cell cycle arrest in nasopharyngeal carcinoma cell lines, HK1 and CNE2, with up-regulation of retinoblastoma gene expression (51). The same study also showed that quercetin mediated apoptosis of HK1 and CNE2 by up-regulating proapoptotic protein Bad, and caspases-3 and -7 (51). Therefore, certain nutrients and non-nutritive bioactive compounds are capable of influencing proliferation and apoptosis by changing gene expression and affecting cell cycle checkpoints, as well as inflammation, which will be described later.

Aberrant crypt foci as a surrogate biomarker. In the mid-1980s, preneoplastic lesions termed as aberrant crypt foci (ACF) were proposed as distinct histological lesions that preceded polyp formation (52) in the polyp-cancer sequence of colon carcinogenesis (53). ACF were first detected by Bird (54) in the colons of carcinogen-injected rodents. Since then, ACF have been hypothesized as the earliest easily identifiable precursors to neoplastic lesions in animal models of colon carcinogenesis (55,56) and to lead towards colon tumorigenesis in humans (57,58). Under the microscope, ACF can be seen as single crypts or clusters of morphologically altered colonic crypts in methylene blue-stained colons of chemically treated rodents (55,56) and in humans (58,59). Compared to normal colon mucosa, ACF are described to have dilated and irregular luminal openings with thickened epithelial linings that appear to protrude into the lumen (55,56,58,59). Their occurrence in the colon can range from none to hundreds (57). Evidence supports the use of ACF as a surrogate biomarker predictive of future colonic tumor development (55-58). Firstly, ACF are present in

colons of patients with and before development of colon cancer (58). In addition, ACF occur more frequently in the distal part of the human colon, which is in accordance with the location of carcinomas observed in colon cancer (58). Genes frequently altered in colon cancers, such as APC, K-ras, and p53, had also been shown to be modified in ACF (57,58). Furthermore, changes in expression of β -catenin, iNOS, COX-2, and microsatellite instability due to defects in the DNA mismatch repair system commonly associated with colon cancers had also been demonstrated in ACF (57,58). Finally, the clonal and dysplastic nature of certain types of ACF, an important factor in tumorigenesis, has been reported (57,58). Cell proliferation was increased and the proliferative zone expanded towards the lumen in ACF of chemically treated rodents (58). Similarly, ACF with excessive proliferation or severe dysplasia were identified in colons of humans with colonic tumors (60). Although less defined in experimentally-induced ACF, one study reported that apoptosis was lower in ACF than in normal crypts of rats (58). Therefore, based on all the evidence discussed above, the ACF stage as an end-point is a suitable surrogate biomarker for colon cancer. In addition, the ACF system is also generally accepted and widely used to screen for colon chemopreventive agents (57,58).

Inflammation. The inflammatory process had been connected to the development of cancer (61,62). In fact, chronic inflammatory conditions such as ulcerative colitis have been proposed as a possible pathway to the development of colon cancer (5,62). Two pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) have both been implicated in colon carcinogenesis (63-66). Indeed, enhanced expression of iNOS and COX-2 have been shown in colorectal adenomas and adenocarcinomas in humans and chemically induced colonic tumors in rats (67-70).

During chronic inflammation, iNOS activity is greatly induced and excessive nitric oxide (NO) production occurs (61). NO has been suggested to modulate cell proliferation and apoptosis, induce DNA damage, and act as a reactive nitrogen species

(61,71). Using the ACF system, agents that inhibit iNOS also inhibit ACF formation, and thus may protect against colon tumorigenesis through this activity (72,73). Interestingly, NO is also implicated in the regulation of COX-2 activity (74,75). Salvemini et al. (74) demonstrated in both *E. coli* lipopolysaccharide (LPS)-activated macrophages and in LPS-treated male Sprague-Dawley rats that NO inhibition resulted in attenuated prostaglandin (PG) synthesis, a major product of the COX enzyme. A later study by Landino et al. (75) put forward a possible link between NO and PG synthesis by both the COX-1 and -2 enzymes. Peroxynitrite, a coupling product of NO and superoxide anion, was found to modify COX activity and suppress PG synthesis (75). The authors suggested that peroxynitrite, an inorganic hydroperoxide, may act as a substrate for the peroxidase portion of the COX enzyme, thus inducing its activity (75).

Cyclooxygenases, also known as prostaglandin endoperoxidase (PGH) synthases, are key enzymes involved in the production of prostaglandins using arachidonic acid as a precursor (76). As alluded to earlier, PGH synthases have both a peroxidase activity and a cyclooxygenase activity at two distinct sites (76). COX-2, the inducible isoform, is not usually detectable in normal cells, but its expression and activity are greatly induced in inflammation (76). Overexpression of COX-2 has been detected in human colorectal tumors (68,69). PGE₂, one of the products of COX-2, is implicated in abnormal cell proliferation, apoptosis inhibition, induction of angiogenesis, and also has been found at increased levels in colon cancer tissue (63,76). Thus, the inhibition of COX-2 levels or activity may protect against colon cancer development (76). In rodent models of colon carcinogenesis COX-2 inhibitors have been shown to reduce ACF and the incidence, multiplicity, and size of tumors (63,76,77).

There is evidence that both iNOS and COX-2 expression may be modulated by certain compounds in the diet (62,78-85). Several flavonoids, including naringenin and apigenin, were able to suppress in-vitro expression of iNOS and COX-2 (78,80-82,84). In those studies, the flavonoids were found not to inhibit iNOS activity but to attenuate its expression at the transcription level (78,81,84). One study by Raso et al. (82), however, demonstrated that both expression and activity of iNOS and COX-2 were

inhibited by some flavonoids. Although the exact mechanism through which the flavonoids were acting has not been elucidated, studies with other phytochemical compounds such as curcumin, EGCG, and resveratrol have shed some light on a possible mechanism (83). Nuclear factor-kappa B (NF- κ B), one the most ubiquitous eukaryotic transcription factors, is involved in regulating the transcription of genes promoting tumor growth, including the expression of iNOS and COX-2 enzymes (83). NF- κ B is normally prevented from binding to the nucleus by forming a complex with inhibitory protein I κ B (83). Curcumin, EGCG, and resveratrol were shown in several in vitro studies to possess the ability to inhibit nuclear translocation of NF- κ B by blocking the breakdown of I κ B, thus subsequently suppressing transcription of iNOS and COX-2 genes (83).

Biological activities of citrus flavonoids and limonoids. Citrus fruits contain a wide variety of phytochemicals, and many such substances are potentially anticarcinogenic (19). Flavonoids, a class of polyphenolic compounds, and limonoids, classified as triterpenoids, are naturally occurring substances in citrus fruits (86,87). Flavonoids are divided into six major classes, depending on variations in their structure, into flavones, flavonols, flavanones, catechins, anthocyanidins, and isoflavones (86). Flavonoids are observed to possess many biological activities in vitro, including antioxidant, antiproliferation, antibiotic, antiallergic, antidiarrheal, antiulcer, anti-inflammatory, antiestrogenic, and the ability to modify enzyme activity (86,88). The amount of data published on limonoids is much less when compared to those on flavonoids. Limonoids were discovered initially as bitter agents in citrus juices but recent work has demonstrated that limonoids may also contain health promoting properties such as being anticarcinogenic and having lipid-lowering effects (87). Indeed, certain flavonoids and limonoids present in citrus fruits have shown potential chemopreventive properties in both in vitro and in vivo animal models (20-34). In cell culture assays, the citrus methoxyflavonoid nobiletin inhibited proliferation of HTB43 human squamous cell carcinoma cells, A549 human lung carcinoma cells, CCRF-HSB-2

human T-cell leukemia, and TGBC11TKB human gastric cancer cells (20,21). Naringenin, another citrus flavonoid, inhibited proliferation of HT29 colon cancer cells and MDA-MB-435 human breast cancer cells (22,23). A citrus limonoid glucoside mixture (69.8% obacunone glucoside, 6.7% limonin glucoside) was shown to inhibit the proliferation of MCF-7 human breast cancer cells, with an IC₅₀ of 8.7 µg/ml (24). When added to the diets of F344 rats and CF-1 mice, the citrus flavonoids (hesperidin, nobiletin, apigenin) and limonoids (limonin, obacunone) lowered the number of chemically induced aberrant crypt foci and reduced tumorigenesis of the colon (25-28). To our knowledge, there is no study on naringenin and its effects on a rat model of colon cancer. However, when provided to Sprague-Dawley rats in an experimental model of breast cancer, naringenin reduced the weight of chemically induced mammary tumors by 52% (23). In addition, hesperidin has been shown to protect against oral tumors in F344 rats (29) and limonin, limonin glycoside, and obacunone protected against a hamster cheek pouch model of oral carcinogenesis (30-32). Limonin has also been shown to be protective against chemically induced forestomach, lung, and skin carcinogenesis in ICR/Ha and SENCAR mice (33,34).

Some studies have begun to investigate the mechanisms through which citrus flavonoids and limonoids could potentially exert their chemoprotective effects. As discussed previously, certain flavonoids were able to inhibit the expression of proinflammatory enzymes iNOS and COX-2. Another school of thought focused on the free radical scavenging/antioxidant activity of flavonoids as the basis of their chemopreventive effects (89,90). Since DNA damage caused by free radicals may be one of the pathways linked to carcinogenesis, the anticarcinogenic effects of certain flavonoids may be due to their ability to quench free radicals (89,90). Other possible chemoprotective mechanisms suggested are induction of phase II detoxifying enzymes and inhibition of protein kinases in signal transduction pathways (91). Certain limonoids like nomilin and obacunone were thought to exert their anticarcinogenic effects through induction of the phase II detoxifying enzyme, glutathione S-transferase (92).

Hypothesis. Based on evidence from published data discussed above, we hypothesized that certain citrus flavonoids and limonoids exert chemopreventive effects on the promotion stage of colon carcinogenesis when included in the diet. Specifically, our hypothesis was:

Citrus flavonoids (hesperidin, nobiletin, apigenin, naringenin) and limonoids (a limonin glucoside/obacunone glucoside mixture) would protect against development of aberrant crypt foci (ACF) by an associated decrease in colonic cell proliferation and increase in apoptosis via suppression of iNOS and COX-2 expression.

Specifically, our objectives were:

1. To determine the effect of dietary hesperidin, nobiletin, apigenin, naringenin, and a limonin glucoside/obacunone glucoside mixture on the number and multiplicity of aberrant crypt foci in rat colon.
2. To evaluate if there is a correlation between aberrant crypt foci formation and colonic cell proliferation and apoptosis in rats consuming these compounds.
3. To investigate if there is an association between colonic cell proliferation and apoptosis and the levels of iNOS and COX-2 expression in colonic tissues of rats consuming these compounds.

CHAPTER III

MATERIALS AND METHODS

Animals and study design. The animal use protocol in this study had been approved by the University Laboratory Animal Care Committee of Texas A&M University and conformed to the National Institute of Health guidelines. Sixty male weanling (21-d) Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX) were individually housed in polycarbonate cages with raised steel grid floors to diminish coprophagy and access to bedding. The rats were maintained in a temperature- and humidity-controlled animal facility with a 12-h light-dark photoperiod. Food and drinking water were freely available. The time line for this study is shown in Figure 1. Upon arrival, the rats were acclimated for 5-d and given regular pelleted rat food. On the fifth day, the rats were stratified according to their body weights into six groups (10 rats/group) and started on experimental diets for a total of 10 wk. All rats were injected twice with the colon carcinogen azoxymethane (AOM, Midwest Research Institute, Kansas City, MO; 15 mg/kg), spaced 1-wk apart. The first injection was given 3-wk after starting the experimental diets. Rats were terminated 6-wk after the last AOM injection and colon tissues collected. Body weights of the rats and diet intake data were recorded for 48-h at 7, 21, 56, and 77-d after starting experimental diets (93).

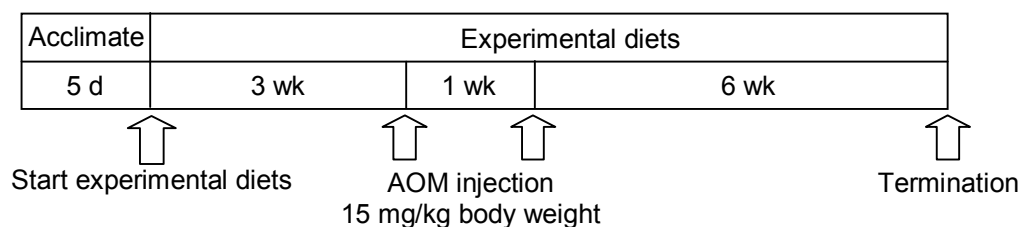
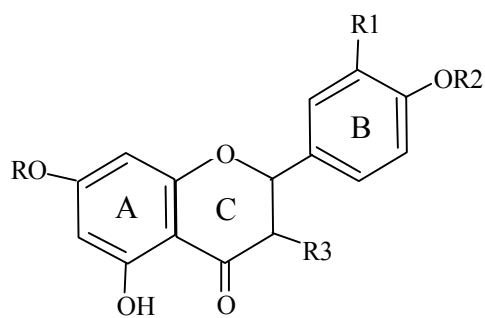


FIGURE 1 Time line of the live animal portion of the experiment. Abbreviation: AOM, azoxymethane.

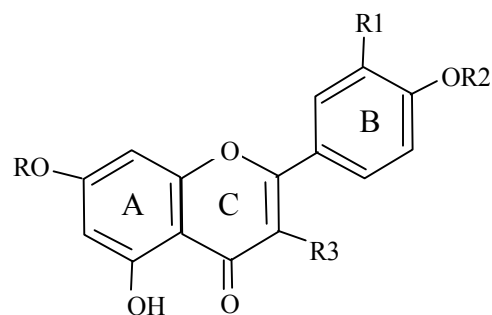
Diets. There were six experimental diets (basal, 0.1% hesperidin, 0.01% nobiletin, 0.02% naringenin, 0.1% apigenin, 0.035% limonin glucoside/obacunone glucoside mixture, Figure 2) that contained, with the exception of the basal diet, levels of experimental compounds chosen based on concentrations that were shown effective in the following studies. Tanaka and his colleagues showed that providing hesperidin at 0.1% and nobiletin at 0.01% of the diet had significantly suppressed the formation of aberrant crypt foci (ACF) in AOM-treated male F344 rats (25,26). In CF-1 mice, feeding apigenin at 0.1% of the diet was able to suppress ACF formation (27). Other studies found a significant reduction in DMBA-induced buccal pouch tumors by treating female Syrian hamsters with a 3.5% limonin glucoside solution (30-32). In another study done by our lab (94), naringin at 0.02% of the diet was fed to Sprague-Dawley rats. To compare the effects of naringenin, the aglycone of naringin, with naringin, the same concentration was chosen for this study.

The composition of the experimental diets is shown in Table 1. Except for the amount of dextrose and the experimental compounds, all other diet components were exactly the same among the six experimental diets. The experimental compounds were added at the expense of dextrose. Dextrose, casein, DL-methionine, and AIN-76 mineral mix were purchased from Bio-Serv, Frenchtown, NJ; AIN-76 vitamin mix and choline bitartrate were purchased from Harlan Teklad, Indianapolis, IN; pectin was purchased from Danisco, New Century, KS; corn oil was provided by Traco Labs, Champaign, IL. Hesperidin, nobiletin, naringenin, apigenin, and limonin glucoside/obacunone glucoside mixture were graciously provided by Texas A&M University-Kingsville Citrus Center. Briefly, hesperidin (97% pure), naringenin (96% pure), and apigenin (97% pure) were isolated from methanol extracts of powdered dried peel from citrus reticulata, citrus junos, and citrus aurantium L., respectively (95). Nobiletin (97% pure) was crystallized from hexane extracts of dried citrus peel (96) The limonin glucoside/obacunone glucoside mixture (76% pure) was purified from ethanol extracts of citrus seeds and molasses (97).

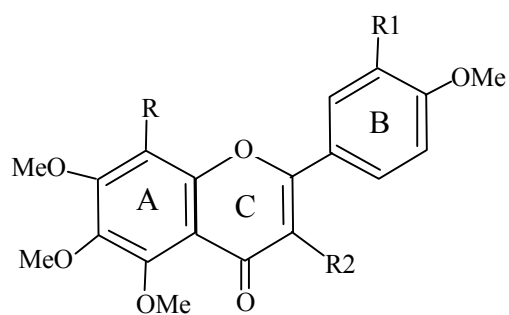
**Flavanone**

Hesperidin: R=Rutinose
 R1=OH
 R2=Me
 R3=H

Naringenin: R=R1=R2=R3=H

**Flavone**

Apigenin: R=R1=R2=R3=H

**Polymethoxyflavone**

Nobiletin: R=R1=OMe
 R2=H

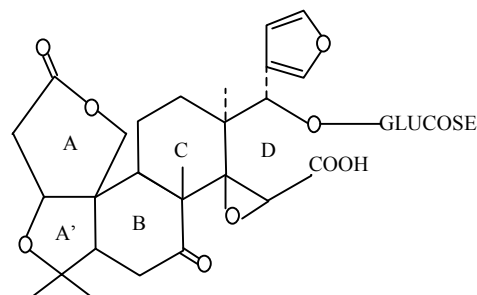
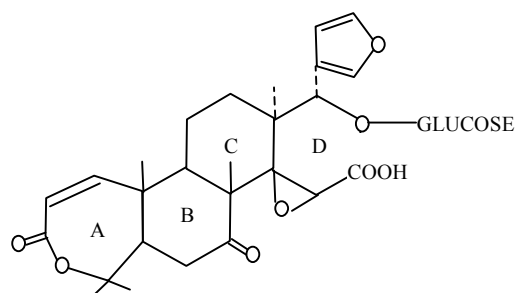
**Limonin glucoside****Obacunone glucoside**

FIGURE 2 Structures of the experimental compounds used in this study. Adapted from Kawaii et al. (98).

TABLE 1
Composition of experimental diets

Diet components	Basal (Control)	Hesperidin	Nobiletin	Naringenin	Apigenin	LG/OG mixture ¹
	g/100 g					
Dextrose ²	51.06	50.96	51.05	51.04	50.96	51.025
Casein ²	22.35	22.35	22.35	22.35	22.35	22.350
Pectin ³	6.00	6.00	6.00	6.00	6.00	6.000
Mineral mix ² (AIN-76)	3.91	3.91	3.91	3.91	3.91	3.910
Vitamin mix ⁴ (AIN-76)	1.12	1.12	1.12	1.12	1.12	1.120
DL-methionine ²	0.34	0.34	0.34	0.34	0.34	0.340
Choline bitartrate ⁴	0.22	0.22	0.22	0.22	0.22	0.220
Corn oil ⁵	15.00	15.00	15.00	15.00	15.00	15.000
Experimental compounds	0.00	0.10	0.01	0.02	0.10	0.035

¹ Limonin glucoside/obacunone glucoside mixture.

² Bio-Serv, Frenchtown, NJ.

³ Danisco, New Century, KS.

⁴ Harlan, Indianapolis, IN.

⁵ We acknowledge the generous donation of the dietary corn oil by Sid Tracy, Traco Labs, Champaign, IL.

Diets were mixed according to protocol (see Appendix A for diet mixing protocol) and stored in a -20°C freezer until use. To determine actual concentration of the experimental compounds (hesperidin, nobiletin, naringenin, apigenin, and limonin glucoside/obacunone glucoside mixture) in the diets, samples of the diets were analyzed by high performance liquid chromatography (HPLC) as described previously (93,99).

Collection of tissue samples. Six weeks after the second AOM injection, each rat was euthanized by CO₂ overdose and its entire colon excised. Figure 3 shows a schematic diagram of the sectioning of colon tissues. Two, 1-cm sections were cut from the distal end of each rat colon. One of the two sections from the distal end was fixed in

ethanol (70% EtOH) and the other was fixed in paraformaldehyde (4% PFA). After flushing with ice-cold PBS, the remaining colon was split open and cut in half longitudinally. One half of the colon was fixed in 70% EtOH for aberrant crypt foci assay and the other half was used for collecting protein samples. To collect protein samples, colonic mucosal cells were gently scraped off using a glass slide such that only the basement membrane was left. The mucosal scrapings were homogenized in a protein buffer (10% Tris-HCL, 5% Sucrose, 1% EDTA, 1% EGTA, 0.0125% NaF, 10% Triton X-100, 1% Sodium Orthovanadate, 4% Sigma Protease Inhibitor Cocktail, 0.07% Beta-mercaptoethanol) and the mucosal cells were lysed by passing the mucosal homogenate through a 27 gauge fine needle. The mucosal cell lysates were centrifuged at 15,000 g for 20 min at 4°C, and the supernatant, containing the released proteins, was aliquoted into 13-40 µL samples, and stored immediately in a -80°C freezer (93).

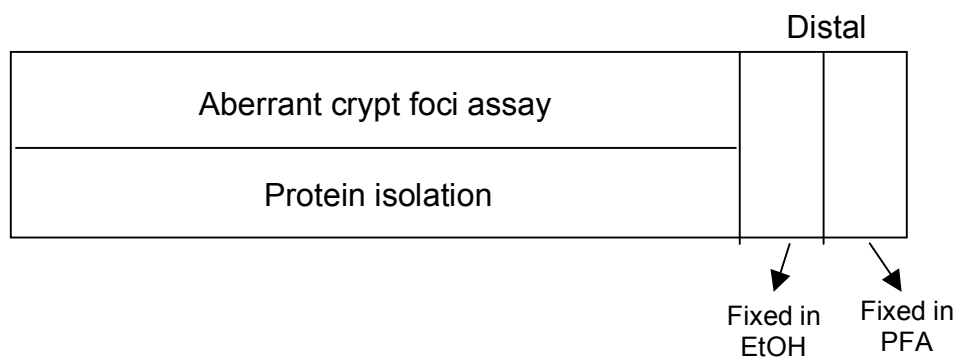


FIGURE 3 Schematic diagram of rat colon sample collection. Sections were used for aberrant crypt foci evaluation, western blot analysis, and immunohistochemistry assays. Abbreviation: EtOH, ethanol; PFA, 4% paraformaldehyde.

Evaluation of aberrant crypt foci. This assay was performed according to the procedure of Bird (54), with modifications (93). Each rat colon tissue prepared for this assay was aligned in a longitudinally folded piece of Whatman #1 filter paper with mucosal side facing up and fixed in 70% EtOH for 24 h. After 24 h of fixation, the colon tissue was taken off the filter paper and stained with 0.5% methylene blue. After staining, the entire colon was examined under a light microscope using 40X magnification for quantification of aberrant crypts. Two or more aberrant crypts clustered together were termed an aberrant crypt focus (ACF); a focus with more than four aberrant crypts was termed a high-multiplicity ACF. Dysplastic ACF, or ACF with a high number of aberrant crypts, are more likely to correlate with subsequent formation of adenomas and/or adenocarcinomas, thus more interest is placed on the evaluation of high-multiplicity ACF formation (57,58). The following were evaluated in each colon sample – high-multiplicity aberrant crypt foci, total aberrant crypts, and aberrant crypts per centimeter of colon.

Measurement of cell proliferation. This assay was performed according to the proliferating cell nuclear antigen (PCNA) staining procedure of Zheng et al. (100), with modifications (93). Colon tissue from the distal end of each rat colon was fixed in 70% EtOH, embedded in paraffin, cut into 4 μ m slices, and mounted onto glass slides. The deparaffinized and rehydrated tissue sections were incubated with a murine anti-PC-10 monoclonal antibody (dilution 1:50; Signet Lab, Dedham, MA). Subsequently, tissue sections were incubated with biotinylated anti-mouse IgG from the Vectastain ABC Elite kit (Vector Lab, Burlingame, CA). Negative control tissues were prepared by incubating with PBS instead of anti-PC-10 antibody. Tissue sections were then stained with diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical, St Louis, MO) and counterstained with hematoxylin (Sigma Chemical, St Louis, MO). PCNA-containing nuclei, indicative of proliferating cells, showed up as brown spots within colonic crypt columns. Twenty-five crypt columns per rat were selected for analysis. For each crypt

column, the proportion of proliferating cells and position of the highest proliferating cell were recorded (Figure 4).

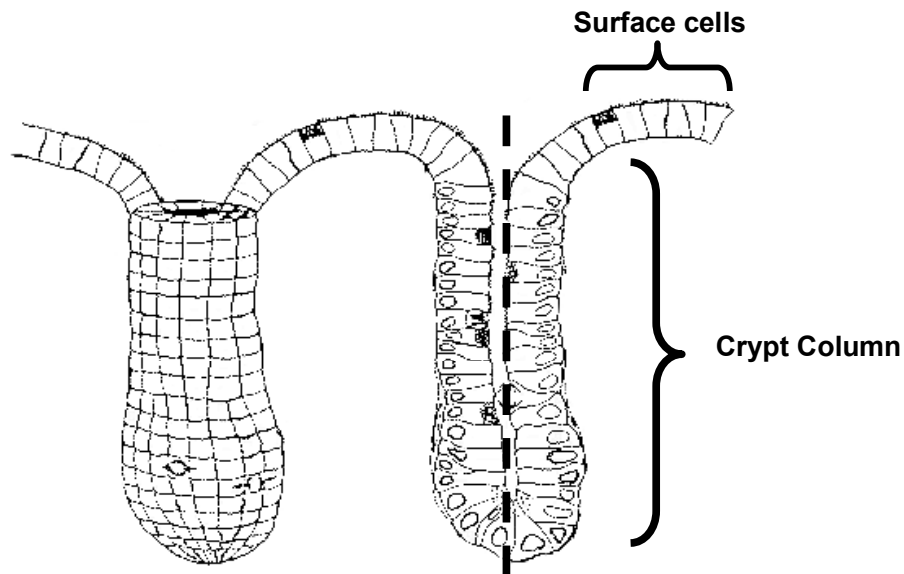


FIGURE 4 Cartoon illustrating a colonic crypt. A colonic crypt column is one side of the colonic crypt (shown above as crypt divided by dashed line). Five surface cells immediately after the highest cell of the crypt column were evaluated for incidence of apoptosis.

Measurement of apoptosis. The incidence of apoptosis in colonic tissues was measured by TUNEL assay according to the method of Hong et al. (101). Colon tissue (4% PFA-fixed and paraffin embedded) from the distal end of each rat colon was cut into 4 μm slices and mounted onto glass slides. The deparaffinized and rehydrated tissue sections were pretreated with proteinase K (Ambion, Austin, TX) to remove crosslinking caused by PFA. A working solution containing terminal deoxynucleotide transferase (TdT)-reaction buffer was incubated with tissue sections in a pre-warmed humidified chamber, with the subsequent addition of anti-digoxigenin peroxidase. This step labels

the free 3'OH termini of DNA fragments (a characteristic of apoptotic cells) with digoxigenin via TdT and forms an antibody-antigen complex. Visualization of the antibody-antigen complex was accomplished by staining with DAB and counterstaining with 1% methyl green. Positive control tissues were prepared by nicking DNA with deoxyribonuclease I (Ambion); negative control tissues were prepared by substituting PBS for TdT in the working solution. Apoptotic cells, seen as brown spots within colonic crypt columns and mucosal surface cells, were quantified for 50 crypt columns per rat. For each rat, the proportion of apoptotic cells within each crypt column and corresponding mucosal surface cells were recorded (Figure 4).

Western blot analysis of iNOS and COX-2. Protein concentrations in supernatant extracted from the centrifuged colonic mucosal homogenate of each rat were determined by a BCA Protein Assay kit according to manufacturer's instructions (Pierce, Rockford, IL). Thirty micrograms of protein (see Appendix B for western blot protocol) were separated by Novex® 4-12% Tris-Glycine gels for 3 h and 30 min at 15 mA and 150 v and electrophoretically transferred to Invitrolon PVDF membranes (Invitrogen, Carlsbad, CA) for 2 h at 300 mA and 25 v; both processes ran in a 4°C walk-in cooler. A color marker, TriChromRanger prestained protein molecular weight marker mix (Pierce, Rockford, IL), was loaded into one of the wells of the gel for identification of the relative positions of the desired protein bands. After the transfer step, the membranes were dipped in methanol and let air-dry. The membranes were then cut horizontally into three pieces, using the bands of the color marker as a guide, such that each piece of the membrane contained one protein band of interest. The three pieces of membranes were individually rehydrated with methanol and processed separately from this step onward. After blocking with 2% bovine serum albumin for 1 h at room temperature (Fisher, Pittsburg, PA), the membranes were incubated with a goat polyclonal anti- β -actin (dilution 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), a goat polyclonal anti-COX-2 (dilution 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit polyclonal anti-iNOS antibody (dilution 1:375; Cayman Chemicals, Ann Arbor, MI)

overnight at 4°C. The membranes were subsequently probed with a bovine anti-goat (dilution for β -actin 1:300,000; dilution for COX-2 1:60,000; Santa Cruz Biotechnology, Santa Cruz, CA) or a goat anti-rabbit IgG-HRP conjugate (dilution 1:60,000; Cayman Chemicals, Ann Arbor, MI) for 1 h at room temperature. The antibody-antigen complexes on the membranes were developed with a chemiluminescence kit (Pierce, Rockford, IL), intensities of bands were captured with a Bio-Rad Fluor-Imager (Bio-Rad Lab, Hercules, CA), and quantified using Quantity One software (Bio-Rad Lab, Hercules, CA). β -actin was used as a loading control.

Statistical analysis. Animal weight and diet intake data were analyzed by one-way ANOVA or a repeated measures model using the Proc GLM or Proc Mixed method in SAS (SAS Institute Inc., Cary, NC). Evaluation of ACF, proliferation and apoptotic indices, and protein expression levels of iNOS, COX-2, and β -actin were analyzed by one-way ANOVA using the Proc Mixed method in SAS (SAS Institute Inc., Cary, NC). Pearson's correlation analyses in SAS were used for evaluation between ACF formation and proliferation or apoptosis, and between proliferation or apoptosis and iNOS or COX-2 (SAS Institute Inc., Cary, NC).

CHAPTER IV

RESULTS

Diet analysis. The experimental compounds (hesperidin, nobiletin, naringenin, and apigenin) were identified by comparing their retention times with those of known standards. After analysis by HPLC, performed in the laboratory at the Fruit and Vegetable Improvement Center, Texas A&M University, the concentrations of experimental compounds in the diets were determined to be 0.096% for hesperidin, 0.007% for nobiletin, 0.037% for naringenin, and 0.115% for apigenin. Except for naringenin, the concentrations of the compounds in the diets were similar to the intended concentrations (0.1% for hesperidin, 0.01% for nobiletin, 0.02% for naringenin, and 0.1% for apigenin). The discrepancy could be due to the human error of measurement during the diet mixing step or diet analysis procedure. Since the diet samples that were used for HPLC analysis were obtained from the initial mixing of the diets and aliquots of samples taken periodically throughout the entire study period, the results of the HPLC analysis suggested that the compounds did not degrade extensively during storage at -20°C . Due to technical difficulties in analyzing the diet containing limonin glucoside/obacunone glucoside mixture and lack of a standard for the limonin glucoside/obacunone glucoside mixture, their concentration was not determined.

Food intake and weight gain. Rats appeared to tolerate the experimental diets well as there were no significant differences in food intake (Table 2) among any of the diet groups overall and at any of the time points measured. However, the average body weight of rats in the apigenin group was less than the body weight of rats from the hesperidin, nobiletin, naringenin, and limonin glucoside/obacunone glucoside mixture groups measured at day 56, and from all diet groups when measured at day 77 (Table 3). When weight gained over the experimental period was considered, there was only a difference in weight gained between rats in the apigenin group and the limonin glucoside/obacunone glucoside mixture group (Table 3).

TABLE 2
Food intake data for rats in each diet group¹

Diet Group	Amount of food consumed (g/d)				
	Day 7	Day 21	Day 56	Day 77	Overall Average
Basal (Control)	14.9 ^a	17.5 ^a	19.8 ^a	19.6 ^a	17.8 ^a
Hesperidin	15.3 ^a	18.1 ^a	20.0 ^a	18.9 ^a	17.9 ^a
Nobiletin	14.9 ^a	18.1 ^a	20.5 ^a	19.4 ^a	18.1 ^a
Naringenin	14.7 ^a	18.3 ^a	20.8 ^a	19.7 ^a	18.2 ^a
Apigenin	14.8 ^a	17.6 ^a	18.9 ^a	19.1 ^a	17.5 ^a
LG/OG mixture ²	15.2 ^a	18.2 ^a	20.4 ^a	20.2 ^a	18.4 ^a
SEM	0.6	0.6	0.8	0.8	0.4

¹ Values given are LSmeans \pm SEM, n = 10 rats/group. Means in a column without a common letter differ, $P < 0.05$.

² Limonin glucoside/obacunone glucoside mixture.

TABLE 3
Weight data for rats in each diet group¹

Diet Group	Body weight (g)					Weight Gain
	Day 0	Day 7	Day 21	Day 56	Day 77	
Basal (Control)	66.7 ^a	124.6 ^a	194.0 ^a	332.4 ^{a,b}	381.0 ^a	313.8 ^{a,b}
Hesperidin	66.9 ^a	126.5 ^a	199.4 ^a	336.0 ^a	381.3 ^a	313.9 ^{a,b}
Nobiletin	67.1 ^a	126.9 ^a	198.3 ^a	336.5 ^a	383.5 ^a	315.8 ^{a,b}
Naringenin	67.1 ^a	126.6 ^a	197.5 ^a	338.5 ^a	384.4 ^a	316.8 ^{a,b}
Apigenin	66.7 ^a	125.0 ^a	194.5 ^a	320.9 ^b	365.3 ^b	298.0 ^a
LG/OG mixture ²	67.4 ^a	123.8 ^a	196.5 ^a	335.7 ^a	386.5 ^a	318.6 ^b
SEM	4.3	7.8	7.8	7.8	7.8	9.5

¹ Values given are LSmeans \pm SEM, n = 10 rats/group. Means in a column without a common letter differ, $P < 0.05$.

² Limonin glucoside/obacunone glucoside mixture.

Incidence of aberrant crypt foci. In rats provided with the experimental compounds (hesperidin, nobiletin, naringenin, apigenin, and limonin glucoside/obacunone glucoside mixture), the total number of aberrant crypts (AC) per colon or the number of aberrant crypts per centimeter of colon were not lower than the number in rats provided with the basal diet (control group). These results differ from the results reported by Tanaka et al. (25,26) where they saw a significant reduction in total AC in F344 rats provided with 0.1% hesperidin or 0.01% nobiletin when compared with the control. The reason for the difference may be due to species differences between F344 and Sprague-Dawley rats (102,103). There was, however, significantly more total aberrant crypts (25%) and number of aberrant crypts per centimeter of colon (28%) in rats provided with nobiletin compared to the rats provided with apigenin ($P < 0.05$, Figure 5A and 5B, also see Appendix C). In addition, rats from the nobiletin group also had greater numbers of aberrant crypts per centimeter of colon than rats from the basal (control) and hesperidin group (21% and 20%, respectively, $P < 0.05$, Figure 5B, also see Appendix C).

In rats provided with the basal diet, AOM induced an average of 3.94 high multiplicity ACF per rat (Figure 6). Dietary naringenin resulted in a 51% lower number of high multiplicity ACF (average of 1.94 per rat, $P < 0.05$). Apigenin lowered the number of high multiplicity ACF by 57% to an average of 1.69 per rat ($P < 0.05$). The number of high multiplicity ACF in rats provided with hesperidin, nobiletin, and limonin glucoside/obacunone glucoside mixture were not significantly different from the rats provided with the basal diet.

Colonocyte proliferation. Dietary naringenin lowered both the number of proliferating colon cells and the proliferative index by 32% (Table 4, Figure 7, $P < 0.05$), compared to the basal diet. In addition, rats provided with naringenin had a smaller proliferative zone (18% lowered, $P < 0.05$) when compared with rats in the basal group (Figure 8). There were no significant differences in the proliferative index or proliferative zone in rats provided dietary hesperidin, nobiletin, apigenin, and limonin

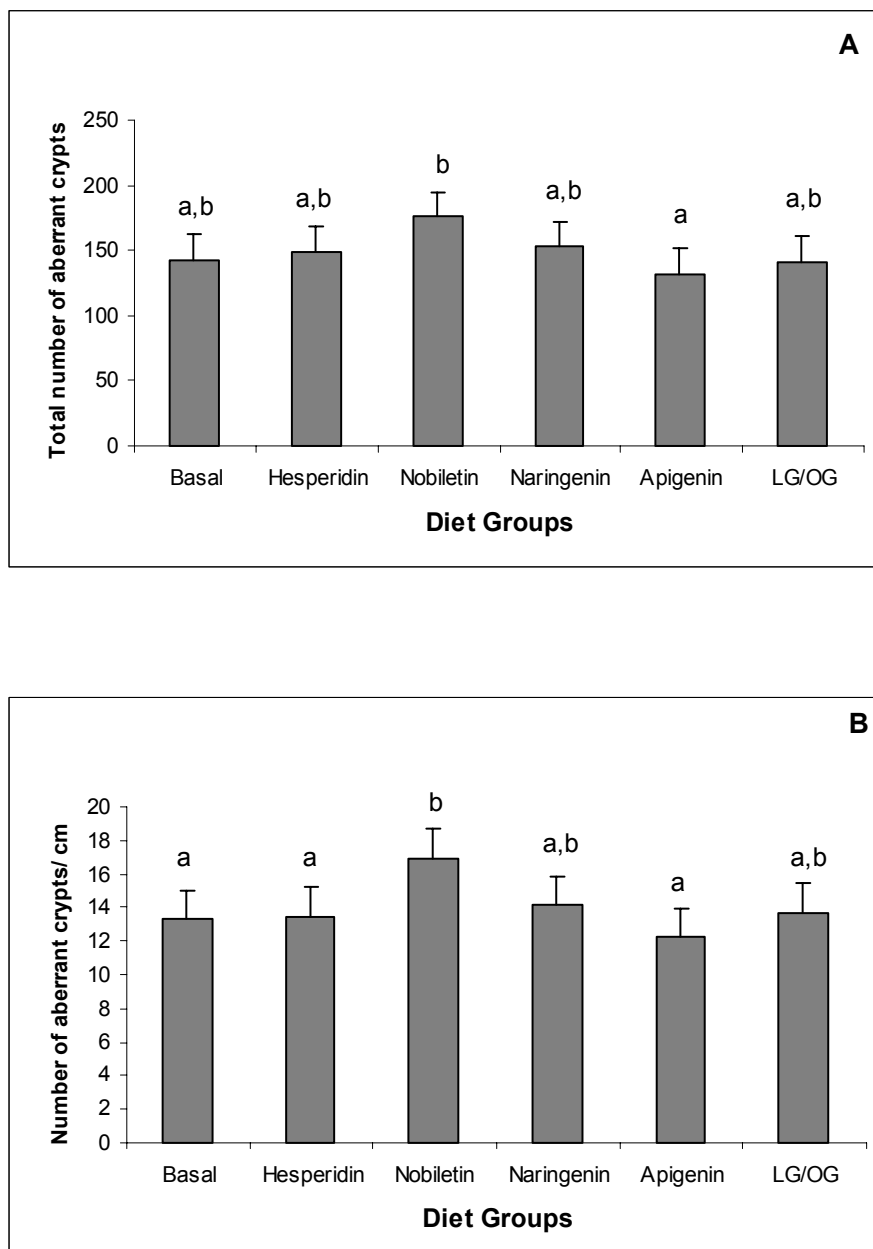


FIGURE 5 Effects of experimental diets on the total number of aberrant crypts per colon (A), and number of aberrant crypts per centimeter of colon (B). Values are LSmeans \pm SEM, $n = 10$ rats/group. Bars with different letters are significantly different, $P < 0.05$.

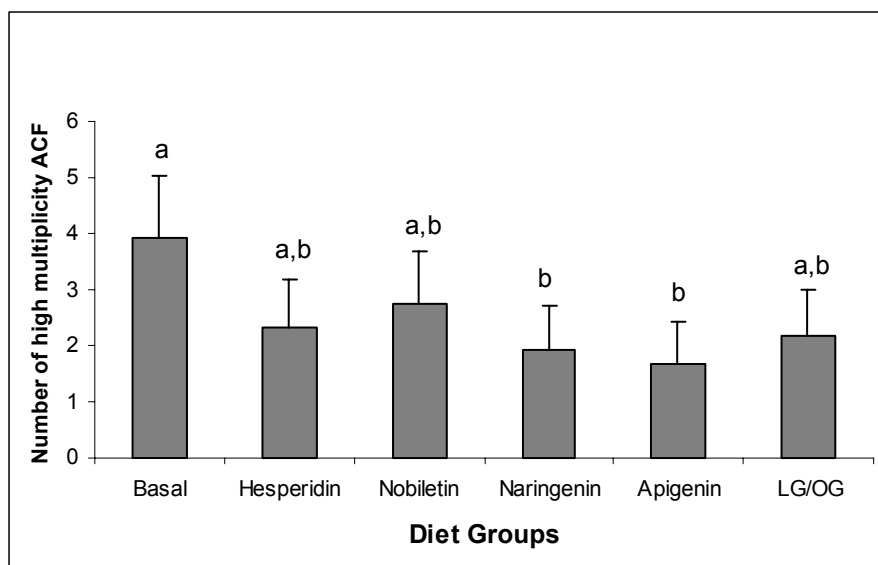


FIGURE 6 Effects of experimental diets on incidence of high multiplicity ACF per colon. High multiplicity ACF classified as > 4 aberrant crypts/focus. Values are LSmeans \pm SEM, n = 10 rats/group. Bars with different letters are significantly different, $P < 0.05$.

TABLE 4

Effect of experimental diets on number of proliferative cells, location of highest proliferative cell, and crypt height in colon of Sprague-Dawley rats¹

Diet Group	No. of proliferative cells	Location of highest proliferative cell	No. of cells in crypt column (crypt height)
Basal (Control)	6.64 ^a	14.98 ^a	32.05 ^a
Hesperidin	5.77 ^{a,b}	13.76 ^{a,b}	32.16 ^a
Nobiletin	4.94 ^{a,b}	12.58 ^{a,b}	31.21 ^a
Naringenin	4.47 ^b	12.12 ^b	31.98 ^a
Apigenin	4.77 ^{a,b}	12.78 ^{a,b}	32.11 ^a
LG/OG mixture ²	4.76 ^{a,b}	12.37 ^b	31.96 ^a
SEM	0.85	1.10	0.42

¹ Values are LSmeans \pm SEM, n = 10 rats/group. Means in a column without a common letter differ, $P < 0.05$.

² Limonin glucoside/obacunone glucoside mixture.

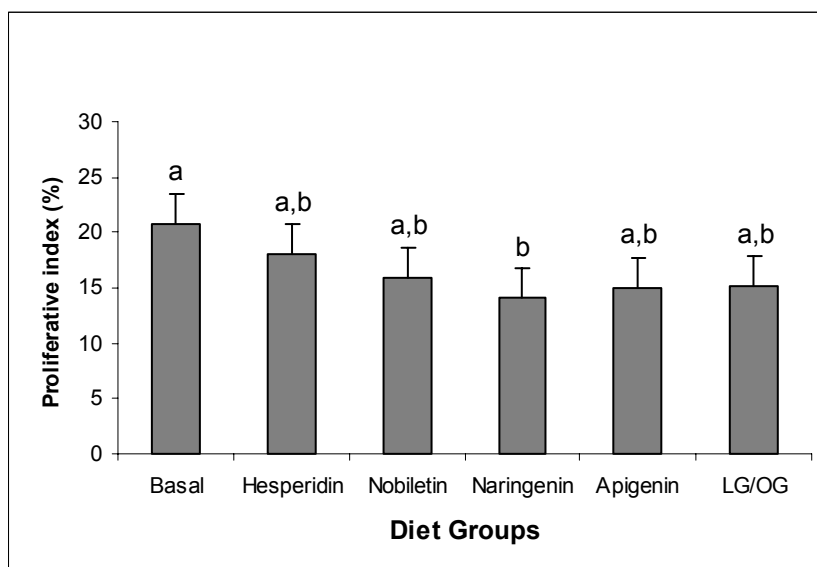


FIGURE 7 Effects of experimental diets on cell proliferation in rat distal colon, measured by proliferative index (number of proliferative cells/number of cells in crypt column). Values are LSmeans \pm SEM, $n = 10$ rats/group. Bars with different letters are significantly different, $P < 0.05$.

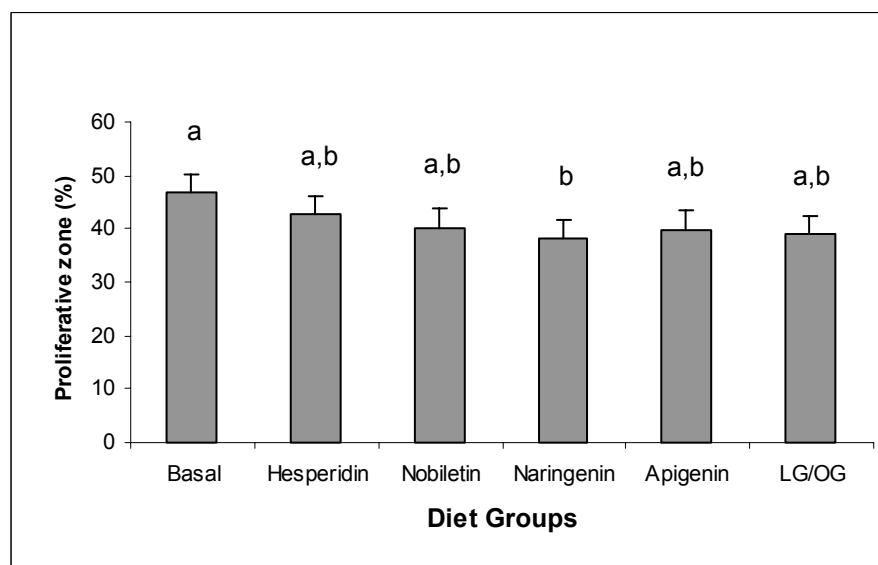


FIGURE 8 Effects of experimental diets on expansion of cell proliferation in rat distal colon, measured by proliferative zone (location of highest proliferative cell/number of cells in crypt column). Values are LSmeans \pm SEM, $n = 10$ rats/group. Bars with different letters are significantly different, $P < 0.05$.

glucoside/obacunone glucoside mixture compared to rats provided the basal diet. However, in addition to rats provided with naringenin, rats provided with the limonin glucoside/obacunone glucoside mixture did have a lowered position of the highest labeled cell from that of rats provided with the basal diet (Table 4). Colonic crypt heights were not found to be different among the diet groups (Table 4).

Apoptosis. To determine whether apoptosis of colonocytes is enhanced or suppressed, quantification of cells undergoing apoptosis is usually measured within colonic crypt columns. However, the extent of apoptosis within colonic crypts was limited in the present study. Apoptotic cells in the colonic crypts of all rats were not significantly different among the six diet groups, except between naringenin and limonin glucoside/obacunone glucoside mixture diet groups (Table 5). On the other hand, extensive staining of apoptotic cells was observed on the luminal surface. Thus, the luminal surface colon cells were evaluated for the extent of apoptosis as well. There was a significantly greater percentage of apoptotic cells on the luminal surface in rats fed naringenin and apigenin, when compared to control rats. The luminal surface apoptotic index for rats provided with dietary naringenin was 97% and apigenin 78% higher than rats provided with the basal diet ($P < 0.05$, Figure 9). Extent of apoptosis in the luminal surface cells in rats given dietary hesperidin, nobiletin, and limonin glucoside/obacunone glucoside mixture, however, were not significantly different from control rats (Figure 9).

Expression of *iNOS* and *COX-2*. Prior to performing western blot analysis on actual mucosal protein samples, *iNOS* and *COX-2* electrophoresis standards at varying concentrations (1000 – 62.5 pg/ μ l) were used to confirm the reproducibility and consistency of results of the protocol. A representative blot of the linearity test for *iNOS* and *COX-2* is shown in Figure 10. The linear regression of the three serial concentrations (1000, 500, 250 pg/ μ l) yielded a slope average of 0.97 for *iNOS* and 0.96 for *COX-2*.

TABLE 5

Effect of experimental diets on apoptosis within colonic crypt columns in male Sprague-Dawley rats¹

Diet Group	No. of apoptotic cells/crypt column
Basal (Control)	0.20 ± 0.05 ^{a,b}
Hesperidin	0.19 ± 0.05 ^{a,b}
Nobiletin	0.22 ± 0.06 ^{a,b}
Naringenin	0.32 ± 0.06 ^a
Apigenin	0.18 ± 0.05 ^{a,b}
LG/OG mixture ²	0.14 ± 0.04 ^b

¹ Values given are LSmeans ± SEM, n = 10 rats/group. Means in a column without a common letter differ, $P < 0.05$.

² Limonin glucoside/obacunone glucoside mixture.

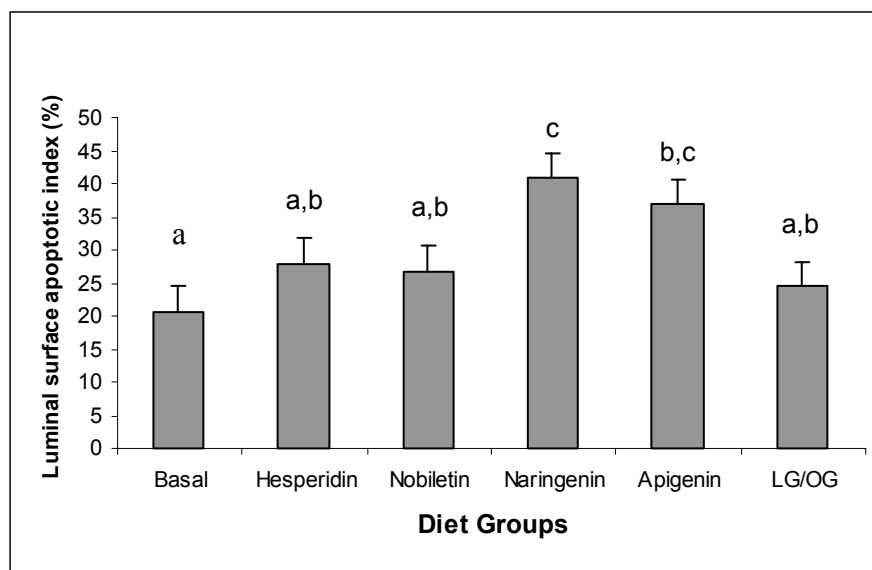


FIGURE 9 Effects of experimental diets on apoptosis in rat distal colon, measured by luminal surface apoptotic index (number of surface apoptotic cells/number of surface cells). Values are LSmeans ± SEM, n = 10 rats/group. Bars with different letters are significantly different, $P < 0.05$.

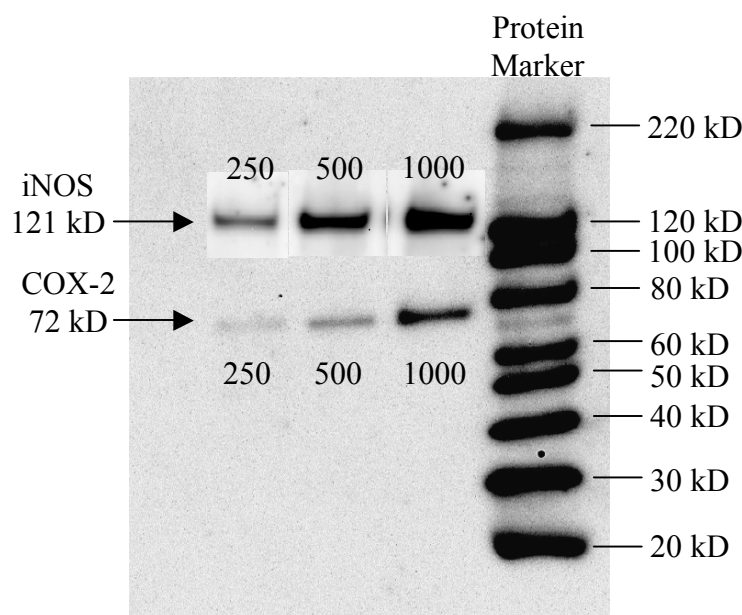


FIGURE 10 A representative blot of linearity test for iNOS and COX-2. Western blot analysis of iNOS and COX-2 electrophoresis standards using concentrations of 1000, 500, and 250 pg/ μ l.

Trial western blot runs on actual protein samples determined that loading 30 μ g of protein onto the 1.5 mm thick gels was optimal for band visualization. Since iNOS and COX-2 standards were loaded with mucosal protein samples for each gel, identification of iNOS and COX-2 protein bands in the mucosal protein samples were made by comparing bands that aligned with those from the standards. In addition, a protein marker was also loaded in each gel to confirm the identity of the bands according to their size (121 kD for iNOS and 72 kD for COX-2, Figure 10).

There were no differences in the expression of iNOS and COX-2 enzymes in colonic mucosa of rats in all the diet groups (Figures 11 and 12, also see Appendix D). Interestingly, when the data for rats in all diet groups were combined, there was a positive correlation between the levels of iNOS and COX-2 enzyme expression ($r = 0.504$, $P < 0.05$). The levels of β -actin in colonic mucosa of rats in all diet groups were

not different, indicating that the amounts of protein loaded were similar for all diet groups.

Correlation analysis. There was no correlation between colonocyte proliferation and high multiplicity ACF, or surface cell apoptosis and high multiplicity ACF for any of the six diet groups, or when data for all diet groups were combined. However, proliferative zone was negatively correlated with surface apoptotic index ($r=-0.279$, $P < 0.05$) when data for all diet groups were combined. In addition, there was a tendency for a negative relationship between proliferative index and surface cell apoptosis for the pooled data ($r=-0.237$, $P = 0.068$). It was also interesting to find that in rats provided with naringenin, COX-2 level was positively correlated with proliferative index ($r=0.896$, $P < 0.05$).

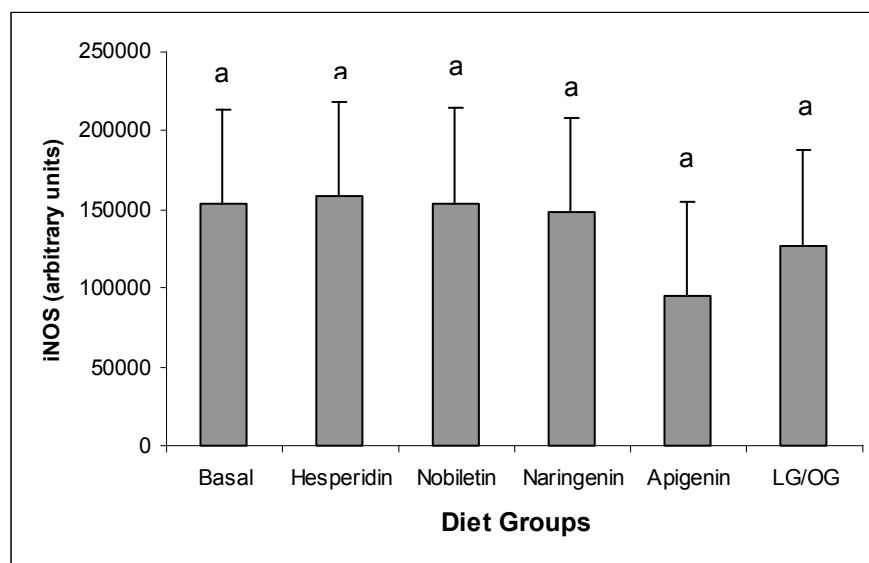


FIGURE 11 Effects of experimental diets on the expression levels of iNOS enzyme. Values are LSmeans \pm SEM, $n = 10$ rats/group. There were no significant treatment differences, $P > 0.05$.

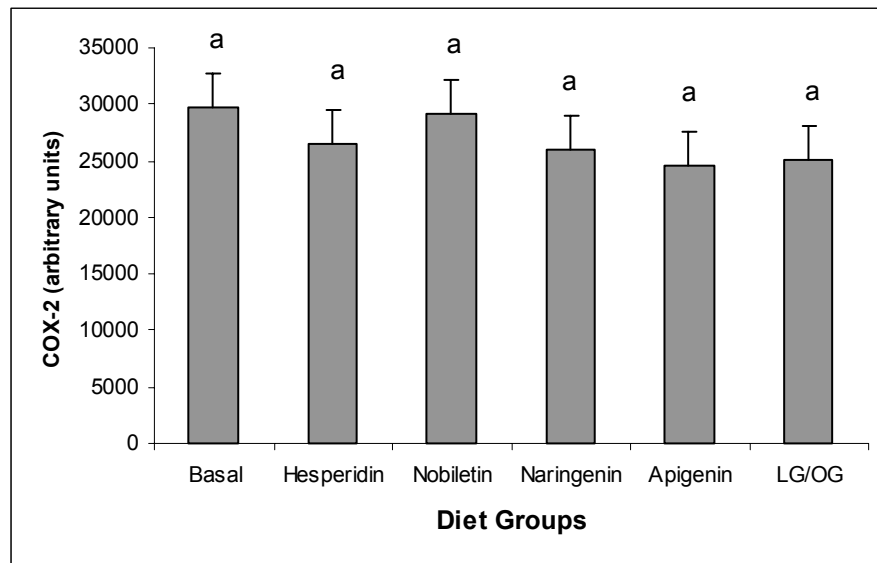


FIGURE 12 Effects of experimental diets on expression levels of COX-2 enzyme. Values are LSmeans \pm SEM, $n = 10$ rats/group. There were no significant treatment differences, $P > 0.05$.

CHAPTER V

DISCUSSION

The aim of this study was to investigate the chemopreventive ability of four citrus flavonoids (hesperidin, nobiletin, naringenin, and apigenin) and one citrus limonoid mixture (containing limonin glucoside and obacunone glucoside) to protect against the “promotion stage” of AOM-induced colon carcinogenesis in Sprague-Dawley rats. Using the formation of precancerous lesions, identified as ACF, as the end-point, it was determined that dietary naringenin at 0.037% of the diet and dietary apigenin at 0.115% of the diet (as determined by HPLC analysis) were able to reduce the incidence of dysplastic or high multiplicity ACF (> 4 aberrant crypts per focus) in the rat colon by 51% and 57%, respectively, from the control diet ($P < 0.05$, Figure 6). The protection was not a result of altered apparent nutrient intake or availability because body weight and amount of food consumed by the rats were similar among the diet groups and were not adversely affected by including the experimental compounds in their diet (Table 2 and 3). Although dietary apigenin at 0.1% of the diet has been previously shown to inhibit AOM-induced ACF in CF-1 mice (27), to our knowledge, this is the first study that showed the ability of naringenin, a major flavonoid found in grapefruit, to reduce AOM-induced ACF in Sprague-Dawley rats. Since high multiplicity ACF are more likely to correlate with future tumor formation (57,58), these results suggest that dietary naringenin and apigenin may protect against AOM-induced colon carcinogenesis. Naringenin and apigenin also showed promising chemoprotective effects in other in vivo cancer models. So et al. (23) provided naringenin at 0.24% of the diet to female Sprague-Dawley rats with 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors and observed a reduction in tumor weight from control without adverse effects in body weight. Subcutaneous injections of apigenin (0.75 or 1.5 mg/kg body weight) were able to significantly inhibit bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in male Wistar rats (104).

Hesperidin, nobiletin, and limonin glucoside/obacunone glucoside mixture (0.096%, 0.007%, as determined by HPLC analysis, and 0.035% of the diet,

respectively), on the other hand, were not observed to suppress ACF formation when compared to the control diet in this study. In fact, rats from the nobiletin group had higher numbers of aberrant crypts than rats from the apigenin group (25% higher, $P < 0.05$, Figure 5), and higher numbers of aberrant crypts per centimeter of colon than rats from basal (control), hesperidin, and apigenin groups (21%, 20%, and 28% higher, respectively, $P < 0.05$, Figure 5). Dietary hesperidin at 0.1% and nobiletin at 0.01% of the diet, however, was found to inhibit AOM-induced ACF in F344 rats in two studies done in Japan (25,26). The differences in the results may be due to strain differences between Sprague-Dawley and F344 rats (102,103), the time points at which the animals were sacrificed, or insufficient amounts of the flavonoids added to the diets in the current study. Taylor et al. (102) showed febrile differences between the two rat strains after being immunologically-challenged with intraperitoneal injections of lipopolysaccharide. Another research group found that Sprague-Dawley rats were more predisposed to certain types of tumors than F344 rats (103). One of the main pioneers who developed the ACF model had reported that the numbers of ACF, usually appearing within 2 wk after carcinogen injection, may peak at 8 wk and regress, and then reappear again at a later time point, usually around wk 20 (56,105). It is worthwhile to point out that the two studies in Japan terminated their rats 2 wk after the last carcinogen injection, thus there may not be sufficient time for the ACF to reach its peak developmental stage. Since our study sacrificed experimental rats 4 wk later than the two studies in Japan, the higher numbers of ACF seen in rat colons from the hesperidin and nobiletin diet groups may be due to the initial peak stage of aberrant crypt formation. Although hesperidin and nobiletin did not appear to be chemoprotective in our study, other studies had shown potential health benefits of those compounds in the laboratory setting (20,21,29,98,106). One of the most interesting health properties is the potential lipid-lowering effects of hesperidin and its aglycone, hesperetin (106,107).

Several studies reported the chemopreventive properties of citrus limonoids, in contrast to the non-chemoprotective results that was observed in our study with the limonin glucoside/obacunone glucoside mixture. A group of researchers from Japan fed

the aglycone form of limonin or obacunone at 0.02% of the diet and found significant inhibition of ACF in AOM-treated F344 rats for both diet groups (28). Thus, it may appear that the glycosidic form of limonin and obacunone was not effective in protecting against AOM-induced ACF. However, in addition to limonin and to a lesser extent obacunone, a 3.5% solution of limonin glycoside was shown to be a potent inhibitor of oral tumors in a hamster cheek pouch model for oral carcinogenesis (31,32). Therefore, it would be interesting to investigate the antineoplastic effects of a higher concentration of the limonin glucoside/obacunone glucoside mixture.

Loss of control in proliferating cells along with suppression of apoptosis are key cytokinetic modifications during tumorigenesis (41-43). In the current study, rats provided with naringenin had a reduced proportion of proliferating colon cells and smaller expansion of the proliferative zone than the control diet (32% and 38% respectively, $P < 0.05$, Figures 7 and 8). Naringenin's ability to suppress cell proliferation was observed in parallel with its ability to inhibit high multiplicity ACF, which may in part explain the chemoprotective effect. Furthermore, the antiproliferative effects of naringenin had been shown with HT29 colon cancer cells (22) and MDA-MB-435 human breast carcinoma cells (23). Hesperidin, nobiletin, apigenin, and the limonin glucoside/obacunone glucoside mixture were not observed to possess antiproliferative effects in this study. It is worthwhile to note, however, that several cell culture experiments had reported antiproliferative effects for hesperetin, the aglycone form of hesperidin, (23), nobiletin (20,21), apigenin (48), and a limonoid glucoside mixture (24).

In experimental models of colon cancer, apoptosis is traditionally measured within colonic crypt columns (see Figure 4). Luminal "surface" cells between crypts are not normally included in the analysis. However, tumors often develop when epithelial cells do not undergo apoptosis and instead accumulate on the luminal surface of the colon (46), thus it makes sense to measure apoptosis on surface cells. In the current study, the extent of apoptosis within the colonic crypt columns was limited, and there were no differences in the apoptotic staining index among the diet groups, except between diet groups naringenin and limonin glucoside/obacunone glucoside mixture.

Instead, a higher level of apoptotic staining was observed on surface cells of rat colons. When compared to the control diet, surface cell apoptosis was enhanced by 97% and 78% in rats provided with 0.02% naringenin and 0.1% apigenin, respectively ($P < 0.05$, Figure 9). This effect may further explain naringenin's ability to suppress high multiplicity ACF observed in this study. In a cell culture experiment, naringenin, applied at 40 and 80 μM for a 24-h period, induced caspase-3 mediated apoptosis in HL-60 leukemia cells (108). Since in colon carcinogenesis, changes in apoptosis may be more important in predicting tumorigenesis than cell proliferation alone (44), the up-regulation of apoptosis observed with apigenin may provide a possible explanation to apigenin's ability to inhibit high multiplicity ACF, even though there was no associated decrease in cell proliferation. Many natural compounds, including flavonoids, derived from the human diet had been shown to modulate the process of apoptosis in cell cultures (45,109). The ability of apigenin to induce apoptosis has been reported in a variety of cancer cell lines, including human prostate carcinoma DU145 cells (110), human cervical carcinoma HeLa cells (111) and in human leukemia HL-60 cells (112). Therefore, the results from this study suggest that naturally occurring flavonoids – naringenin and apigenin, may also modulate apoptosis in animal models. Pro-apoptotic effects were not seen in rat colons from hesperidin, nobiletin, and limonin glucoside/obacunone glucoside mixture diet groups.

While there were no statistical correlations between high multiplicity ACF and cell proliferation or apoptosis in this study, there was a slight negative correlation between proliferative zone within crypt column and surface cell apoptosis when data for all six diet groups were pooled ($r = -0.279$, $P < 0.05$). There was also a tendency for a negative relationship between proliferative index within crypt column and surface cell apoptosis for the pooled data ($r = -0.237$, $P = 0.068$). These results suggest that cell kinetics such as cell proliferation and apoptosis might be modulated in this study but the individual effects of each diet were not strong enough to achieve significance. It is possible that significance may be achieved if the sample size were larger. In any case, the negative relationship between cell proliferation and apoptosis is in agreement with

evidence from current literature that increased cell proliferation together with decreased apoptosis may serve as a permissive platform for cancer development (41-43).

Another reason for the development of cancer may be due to inflammatory processes (61,62). Increased expression of two pro-inflammatory enzymes, COX-2 and iNOS, has been reported in human colorectal adenomas and adenocarcinomas and in chemically induced colonic tumors in rats (67-70). In the current study, levels of COX-2 and iNOS proteins in rat colon mucosa were detected via western blot analysis. However, no differences in the levels of COX-2 or iNOS proteins among the six diet groups were observed. This may mean that dietary hesperidin, nobiletin, naringenin, apigenin, and limonin glucoside/obacunone glucoside mixture do not modulate the expression levels of COX-2 or iNOS proteins in this colon cancer model, and that the protective effects seen with naringenin and apigenin were not associated with regulation of COX-2 or iNOS enzyme expression. However, despite the results of this study, several investigations involving cell cultures showed that hesperidin, nobiletin, naringenin, and apigenin, with varying potencies, were able to inhibit induction of COX-2 or iNOS proteins (78,80-82,84).

One possible explanation for the discrepancy of results between in vitro and in vivo studies, may be that most cell culture assays use cancerous cell lines that are transformed, so the products of tumor promoting genes, such as iNOS or COX-2 in this case, are highly expressed or induced. In addition, the test media usually contain only one type of cell line. Consequently, any inhibitory effect by compounds of interest is likely to be obvious and significant. On the other hand, animal models present a challenge in that there is a diverse mixture of cell types in animal tissues. Also, not all cells in a tissue sample are “transformed” or cancerous, especially at early time points in carcinogenesis like the ACF stage. The expression of iNOS and COX-2 may be comparatively lower at the precancerous ACF stage than at the tumor or carcinoma stage. As a result, any protective effects in such scenarios would more likely be harder to detect. Thus, results produced with cell culture assays may not necessarily mean that the same results will be observed in higher biological systems such as laboratory rats. Due to

the promising *in vitro* inhibitory effects of hesperidin, nobiletin, naringenin, and apigenin on iNOS or COX-2 enzymes, it would be very interesting to find out if the inhibitory effects will be observed at the tumor stage as opposed to the ACF stage in an animal model. In addition, since the current study only measured the levels but not activities of iNOS and COX-2, it would be interesting to determine whether the levels of NO and PGE₂, the products of iNOS and COX-2 respectively, which are suggested to promote tumorigenesis, will be affected by the compounds in the current study. Raso et al. (82) reported that apigenin and, to a lesser extent, naringenin dose-dependently suppressed not only iNOS and COX-2 induction but also NO and PGE₂ levels in LPS-stimulated macrophages.

Interestingly, there was a positive correlation between COX-2 level and proliferative zone in rats provided with naringenin ($r=0.896$, $P < 0.05$). Evidence in the literature suggests that PGE₂, one of the products of COX-2 enzyme, promotes cell proliferation (76), thus may in part explain the positive correlation observed between COX-2 level and cell proliferation in the current study. There was also a positive correlation between the enzyme expression levels of iNOS and COX-2 ($r = 0.504$, $P < 0.05$) in the current study, when the data for rats in all diet groups were combined. This finding suggests that increased levels of iNOS correlate with increased levels of COX-2. Several cell culture studies and animal inflammatory models have shown the co-induction or co-regulation of iNOS and COX-2 enzymes (83). The positive correlation between iNOS and COX-2 may also indicate that these two enzymes work cooperatively to create an environment conducive to tumor promotion. In DMBA-induced hamster buccal pouch tumors, the levels of iNOS increased concomitantly with COX-2 when measured in untreated animals to dysplastic lesions to invasive squamous cell carcinoma stages (113).

CHAPTER VI

CONCLUSIONS

Dietary naringenin and apigenin showed promise as naturally-occurring chemopreventive agents against colon carcinogenesis. It would be interesting to investigate other possible mechanisms through which naringenin and apigenin exerted their protective effects *in vivo*. One cell culture study showed the ability of apigenin to modulate the mitogen-activated protein kinase signal transduction pathway (114). Another study reported on the suppression of protein kinase C in 12-*O*-tetradecanoyl-phorbol-13-acetate-(TPA)-mediated tumor promotion of mouse skin by apigenin (115). Although hesperidin, nobiletin, and the limonin glucoside/obacunone glucoside mixture did not show any protective effects in this model for colon cancer, they should not be discounted from future consideration since they have demonstrated effectiveness in other disease models. Furthermore, the differences in effect or lack of effect between different rat strains provided with the same compounds highlights the fact that higher biological systems, especially humans, are heterogeneous and diverse. A compound at a certain dosage may be beneficial for a group of people with a certain disease profile but not for another. Therefore, more research in the area of bioactive compounds and their interaction with disease is needed to determine the optimum nutrition profile for the prevention of disease for each individual person.

LITERATURE CITED

1. American Cancer Society (2004) Cancer facts & figures 2004. American Cancer Society, Atlanta, GA, <http://www.cancer.org/>. Accessed 1/12/05.
2. National Cancer Institute (2004) Cancer progress report - 2003 update. National Cancer Institute, NIH, DHHS, Bethesda, MD, <http://progressreport.cancer.gov/>. Accessed 1/12/05.
3. Ries, L. A. G., Eisner, M. P., Kosary, C. L., Hankey, B. F., Miller, B. A., Clegg, L., Mariotto, A., Feuer, E. J., Edwards, B. K. (eds) (2004) SEER cancer statistics review, 1975-2001. National Cancer Institute, Bethesda, MD, http://seer.cancer.gov/csr/1975_2001/. Accessed 1/12/05.
4. Tomeo, C. A., Colditz, G. A., Willett, W. C., Giovannucci, E., Platz, E., Rockhill, B., Dart, H. & Hunter, D. J. (1999) Harvard report on cancer prevention. volume 3: Prevention of colon cancer in the United States. *Cancer Causes Control* 10: 167-180.
5. Potter, J. D. (1999) Colorectal cancer: Molecules and populations. *J. Natl. Cancer Inst.* 91: 916-932.
6. Terry, P., Giovannucci, E., Michels, K. B., Bergkvist, L., Hansen, H., Holmberg, L. & Wolk, A. (2001) Fruit, vegetables, dietary fiber, and risk of colorectal cancer. *J. Natl. Cancer Inst.* 93: 525-533.
7. Steinmetz, K. A. & Potter, J. D. (1996) Vegetables, fruit, and cancer prevention: A review. *J. Am. Diet. Assoc.* 96: 1027-1039.
8. Block, G., Patterson, B. & Subar, A. (1992) Fruit and vegetables and cancer prevention: A review of epidemiological evidence. *Nutr. Cancer* 18: 1-29.
9. Steinmetz, K. A. & Potter, J. D. (1991) Vegetables, fruit, and cancer. I. *Epidemiology. Cancer Causes Control* 5: 325-357.
10. Hung, H.-C., Joshipura, K. J., Jiang, R., Hu, F. B., Hunter, D., Smith-Warner, S. A., Colditz, G. A., Rosner, B., Spiegelman, D. & Willett, W. C. (2004) Fruit and vegetable intake and risk of major chronic disease. *J. Natl. Cancer Inst.* 96: 1577-1584.

11. Deneo-Pellegrini, H., Boffetta, P., De Stefani, E., Ronco, A., Brennan, P. & Mendilaharsu, M. (2002) Plant foods and differences between colon and rectal cancers. *Eur. J. Cancer Prev.* 11: 369-375.

12. Willett, W. C. (2001) Diet and cancer: One view at the start of the millennium. *Cancer Epidemiol. Biomark. Prev.* 10: 3-8.

13. Voorrips, L. E., Goldbohm, R. A., van Poppel, G., Sturmans, F., Hermus, R. J. J. & van den Brandt, P. A. (2000) Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study. *Am. J. Epidemiol.* 152: 1081-1092.

14. Michels, K. B., Giovannucci, E., Joshipura, K. J., Rosner, B. A., Stampfer, M. J., Fuchs, C. S., Colditz, G. A., Speizer, F. E. & Willett, W. C. (2000) Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers. *J. Natl. Cancer Inst.* 92: 1740-1752.

15. Risch, H. A., Jain, M., Choi, N. W., Fodor, J. G., Pfeiffer, C. J., Howe, G. R., Harrison, L. W., Craib, K. J. P. & Miller, A. B. (1985) Dietary factors and the incidence of cancer of the stomach. *Am. J. Epidemiol.* 122: 947-959.

16. Hakim, I. A., Harris, R. B. & Ritenbaugh, C. (2000) Citrus peel use is associated with reduced risk of squamous cell carcinoma of the skin. *Nutr. Cancer* 37: 161-168.

17. Miyagi, Y., Om, A. S., Chee, K. M. & Bennink, M. R. (2000) Inhibition of azoxymethane-induced colon cancer by orange juice. *Nutr. Cancer* 36: 224-229.

18. Kossoy, G., Ben-Hur, H., Stark, A., Zusman, I. & Madar, Z. (2001) Effects of a 15% orange-pulp diet on tumorigenesis and immune response in rats with colon tumors. *Oncol. Rep.* 8: 1387-1391.

19. Silalahi, J. (2002) Anticancer and health protective properties of citrus fruit components. *Asia Pac. J. Clin. Nutr.* 11: 79-84.

20. Kandaswami, C., Perkins, E., Soloniuk, D. S., Drzewiecki, G. & Middleton, Jr. E. (1991) Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma in vitro. *Cancer Lett.* 56: 147-152.

21. Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. & Yano, M. (1999) Antiproliferative activity of flavonoids on several cancer lines. *Biosci. Biotech. Biochem.* 63: 896-899.
22. Frydoonfar, H. R., McGrath, D. R. & Spigelman, A. D. (2002) The variable effect on proliferation of a colon cancer cell line by the citrus fruit flavonoid naringenin. *Colorectal Dis.* 5: 149-152.
23. So, F., Guthrie, N., Chambers, A. F., Moussa, M. & Carroll, K. K. (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer* 26: 167-181.
24. Tian, Q., Miller, E. G., Ahmad, H., Tang, L. & Patil, B. S. (2001) Differential inhibition of human cancer cell proliferation by citrus limonoids. *Nutr. Cancer* 40: 180-184.
25. Tanaka, T., Makita, H., Kawabata, K., Mori, H., Kakumoto, M., Satoh, K., Hara, A., Sumida, T., Tanaka, T. & Ogawa, H. (1997) Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* 18: 957-965.
26. Kohno, H., Yoshitani, S., Tsukio, Y., Murakami, A., Koshimizu, K., Yano, M., Tokuda, H., Nishino, H., Ohgashi, H. & Tanaka, T. (2001) Dietary administration of citrus nobiletin inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Life Sci.* 69: 901-913.
27. Au, A., Li, B., Wang, W., Roy, H. & Birt, D. F. (1999) Apigenin inhibits colonic ornithine decarboxylase activity in cultured cells and mouse colon and aberrant crypt foci formation in CF-1 mice. *FASEB J.* 13: A582
28. Tanaka, T., Maeda, M., Kohno, H., Murakami, M., Kagami, S., Miyake, M. & Wada, K. (2000) Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by the citrus limonoids obacunone and limonin. *Carcinogenesis* 22: 193-198.
29. Tanaka, T., Makita, H., Ohnishi, M., Hirose, Y., Wang, A., Mori, H., Satoh, K., Hara, A. & Ogawa, H. (1994) Chemoprevention of 4-nitroquinoline 1-oxide-induced

oral carcinogenesis by dietary curcumin and hesperidin: Comparison with the protective effect of β -carotene. *Cancer Res.* 54: 4653-4659.

30. 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 food* (Berhow, M. A., Hasegawa, S., Manners, G. D., ed.), pp. 132-143. American Chemical Society, Washington, D. C.

31. Miller, E. G., Gonzales-Sanders, A. P., Couvillon, A. M., Wright, J. M., Hasegawa, S. & Lam, L. K. T. (1992) Inhibition of hamster buccal pouch carcinogenesis by limonin 17- β -D-glucopyranoside. *Nutr. Cancer* 17: 1-7.

32. Miller, E. G., Fanous, R., Rivera-Hidalgo, 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.

33. 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.

34. 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: Fruits and vegetables* (Huang, M. T., ed.), pp. 209-219. American Chemical Society, Washington, D. C.

35. Hursting, S. D., Slaga, T. J., Fischer, S. M., DiGiovanni, J. & Phang, J. M. (1999) Mechanism-based cancer prevention approaches: Targets, examples, and the use of transgenic mice. *J. Natl. Cancer Inst.* 91: 215-225.

36. Grady, W. M. & Markowitz, S. D. (2002) Genetic and epigenetic alterations in colon cancer. *Annu. Rev. Genomics Hum. Genet.* 3: 101-128.

37. Lynch, J. P. & Hoops, T. C. (2002) The genetic pathogenesis of colorectal cancer. *Hematol. Oncol. Clin. N. Am.* 16: 775-810.

38. Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E. & Baylin, S. B. (1994) Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nature Genet.* 7: 536-540.

39. Lipkin, M., Reddy, B., Newmark, H. & Lamprecht, S. A. (1999) Dietary factors in human colorectal cancer. *Annu. Rev. Nutr.* 19: 545-586.
40. Neutra, M. R. (1988) The gastrointestinal tract. In: *Cell and tissue biology: A textbook of histology*, 6th ed. (Weiss, L., ed.), pp. 642-683. Urban & Schwarzenberg, Baltimore.
41. Green, D. R. & Evan, G. I. (2002) A matter of life and death. *Cancer Cell* 1: 19-30.
42. Evan, G. I. & Vousden, K. H. (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature* 411: 342-348.
43. Pucci, B., Kasten, M. & Giordano, A. (2000) Cell cycle and apoptosis. *Neoplasia* 2: 291-299.
44. Chang, W. L., Chapkin, R. S. & Lupton, J. R. (1997) Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. *Carcinogenesis* 18: 721-730.
45. Watson, W. H., Cai, J. & Jones, D. P. (2000) Diet and apoptosis. *Annu. Rev. Nutr.* 20: 485-505.
46. Hong, M. Y., Chang, W. L., Chapkin, R. S. & Lupton, J. R. (1997) Relationship among colonocyte proliferation, differentiation, and apoptosis as a function of diet and carcinogen. *Nutr. Cancer* 28: 20-29.
47. Wu, B., Iwakiri, R., Ootani, A., Tsunada, S., Fujise, T., Sakata, Y., Sakata, H., Toda, S. & Fujimoto, K. (2004) Dietary corn oil promotes colon cancer by inhibiting mitochondria-dependent apoptosis in azoxymethane-treated rats. *Exp. Biol. Med.* 229: 1017-1025.
48. Wang, W., Heideman, L., Chung, C. S., Pelling, J. C., Koehler, K. J. & Birt, D. F. (2000) Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Mol. Carcinog.* 28: 102-110.
49. Yu, Z., Li, W. & Liu, F. (2004) Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. *Cancer Lett.* 215: 159-166.

50. Huh, S. W., Bae, S. M., Kim, Y.-W., Lee, J. M., Namkoong, S. E., Lee, I. P., Kim, S. H., Kim, C. K. & Ahn, W. S. (2004) Anticancer effects of (-)-epigallocatechin-3-gallate on ovarian carcinoma cell lines. *Gynecol. Oncol.* 94: 760-768.
51. Ong, C. S., Tran, E., Nguyen, T. T., Ong, C. K., Lee, S. K., Lee, J. J., Ng, C. P., Leong, C. & Huynh, H. (2004) Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expression. *Oncol. Rep.* 11: 727-733.
52. McLellan, E. A. & Bird, R. P. (1988) Aberrant crypts: Potential preneoplastic lesions in the murine colon. *Cancer Res.* 48: 6187-6192.
53. Muto, T., Bussey, H. J. R. & Morson, B. C. (1975) The evolution of cancer of the colon and rectum. *Cancer* 36: 2251-2270.
54. Bird, R. P. (1987) Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: Preliminary findings. *Cancer Lett.* 37: 147-151.
55. Bird, R. P. & Good, C. K. (2000) The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Toxicol. Lett.* 112-113: 395-402.
56. Bird, R. P. (1995) Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett.* 93: 55-71.
57. Cheng, L. & Lai, M.-D. (2003) Aberrant crypt foci as microscopic precursors of colorectal cancers. *World J. Gastroenterol.* 9: 2642-2649.
58. Roncucci, L., Pedroni, M., Vaccina, F., Benatti, P., Marzona, L. & Pol, A. D. (2003) Aberrant crypt foci in colorectal carcinogenesis. Cell and crypt dynamics. *Cell Prolif.* 33: 1-8.
59. Pretlow, T. P., O'Riordan, M. A., Pretlow, T. G. & Stellato, T. A. (1992) Aberrant crypts in human colonic mucosa: Putative preneoplastic lesions. *J. Cell Biochem.* 16G: 55-62.
60. Siu, I.-M., Pretlow, T. G., Amini, S. B. & Pretlow, T. P. (1997) Identification of dysplasia in human colonic aberrant crypt foci. *Am. J. Pathol.* 150: 1805-1813.

61. Ohshima, H. & Bartsch, H. (1994) Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mutat. Res.* 305: 253-264.
62. Philpott, M. & Ferguson, L. R. (2004) Immunonutrition and cancer. *Mutat. Res.* 551: 29-42.
63. Watanabe, K., Kawamori, T., Nakatsugi, S. & Wakabayashi, K. (2000) COX-2 and iNOS, good targets for chemoprevention of colon cancer. *Biofactors* 12: 129-133.
64. Crowell, J. A., Steele, V. E., Sigman, C. C. & Fay, J. R. (2003) Is inducible nitric oxide synthase a target for chemoprevention? *Mol. Cancer Therapeu.* 2: 815-823.
65. Sporn, M. B. & Suh, N. (2000) Chemoprevention of cancer. *Carcinogenesis* 21: 525-530.
66. Taketo, M. M. (1998) Cyclooxygenase-2 inhibitors in tumorigenesis (part II). *J. Natl. Cancer Inst.* 90: 1609-1620.
67. Ambs, S., Merriam, W. G., Bennett, W. P., Felley-Bosco, E., Ogunfusika, M. O., Oser, S. M., Klein, S., Shields, P. G., Billiar, T. R. & Harris, C. C. (1998) Frequent nitric oxide synthase-2 expression in human colon adenomas: Implication for tumor angiogenesis and colon cancer progression. *Cancer Res.* 58: 334-341.
68. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S. & Dubois, R. N. (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 107: 1183-1188.
69. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. & Hla, T. (1995) Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.* 55: 3785-3789.
70. Takahashi, M., Fukuda, K., Ohata, T., Sugimura, T. & Wakabayashi, K. (1997) Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. *Cancer Res.* 57: 1233-1237.

71. Lopez-Farre, A., Rodrigues-Feo, J. A., Sanchez de Miguel, L., Rico, L. & Casado, S. (1998) Role of nitric oxide in the control of apoptosis in the microvasculature. *Biochem. Cell Biol.* 30: 1095-1106.
72. Kawamori, T., Takahashi, M., Watanabe, K., Ohta, T., Nakatsugi, S., Sugimura, T. & Wakabayashi, K. (2000) Suppression of azoxymethane-induced colonic aberrant crypt foci by a nitric oxide synthase inhibitor. *Cancer Lett.* 148: 33-37.
73. Rao, C. V., Kawamori, T., Hamid, R. & Reddy, B. S. (1999) Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. *Carcinogenesis* 20: 641-644.
74. Salvemini, D., Settle, S. L., Masferrer, J. L., Seibert, K., Currie, M. G. & Needleman, P. (1995) Regulation of prostaglandin production by nitric oxide: An in vivo analysis. *Br. J. Pharmacol.* 114: 1171-1178.
75. Landino, L. M., Crews, B. C., Timmons, M. D. & Morrow, J. D. (1996) Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci.* 93: 15069-15074.
76. Wendum, D., Masliah, J., Trugnan, G. & Flejou, J.-F. (2004) Cyclooxygenase-2 and its role in colorectal cancer development. *Virchows Arch.* 445: 327-333.
77. Kawamori, T., Rao, C. V., Seibert, K. & Reddy, B. S. (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.* 58: 409-412.
78. Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y. & Kim, H. P. (1999) Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem. Pharmacol.* 58: 759-765.
79. Narayanan, B. A., Narayanan, N. K., Simi, B. & Reddy, B. S. (2003) Modulation of inducible nitric oxide synthase and related proinflammatory genes by the omega-3 fatty acid docosahexaenoic acid in human colon cancer cells. *Cancer Res.* 63: 972-979.

80. O'Leary, K. A., de Pascual-Teresa, S., Needs, P. W., Bao, Y.-P., O'Brien, N. M. & Williamson, G. (2004) Effect of flavonoids and vitamin E on cyclooxygenase-2 (COX-2) transcription. *Mutat. Res.* 551: 245-254.
81. Olszanecki, R., Gebaska, A., Kozlovski, V. I. & Gryglewski, R. J. (2002) Flavonoids and nitric oxide synthase. *J. Physio. Pharmacol.* 53: 571-584.
82. Raso, G. M., Meli, R., Di Carlo, G., Pacilio, M. & Di Carlo, R. (2001) Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. *Life Sci.* 68: 921-931.
83. Surh, Y.-J., Chun, K.-S., Cha, H.-H., Han, S. S., Keum, Y.-S., Park, K.-K. & Lee, S. S. (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: Down-regulation of COX-2 and iNOS through suppression of NF-kB activation. *Mutat. Res.* 480-481: 243-268.
84. Tsai, S., Lin-Shiau, S. & Lin, J. (1999) Suppression of nitric oxide synthase and the down-regulation of the activation of NFkB in macrophages by resveratrol. *Br. J. Pharmacol.* 126: 673-680.
85. Wu, G. & Meininger, C. J. (2002) Regulation of nitric oxide synthesis by dietary factors. *Annu. Rev. Nutr.* 22: 61-86.
86. Ross, J. A. & Kasum, C. M. (2002) Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* 22: 19-34.
87. 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.
88. Manthey, J. A., Guthrie, N. & Grohmann, K. (2001) Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Curr. Med. Chem.* 8: 135-153.
89. Pietta, P.-G. (2000) Flavonoids as antioxidants. *J. Nat. Prod.* 63: 1035-1042.
90. Rice-Evans, C. (2001) Flavonoid antioxidants. *Curr. Med. Chem.* 8: 797-807.
91. Lopez-Lazaro, M. (2002) Flavonoid as anticancer agents: Structure-activity relationship study. *Curr. Med. Chem.* 2: 691-714.

92. Lam, L. K. T., Li, Y. & Hasegawa, S. (1989) Effects of citrus limonoids on glutathione s-transferase activity in mice. *J. Agric. Food. Chem.* 37: 878-880.
93. Vanamala, J. (2004) Postharvest irradiation treatment effect on grapefruit functional components and their role in prevention of colon cancer. Dissertation, Texas A&M University, College Station.
94. Vanamala, J., Leonardi, T., Murphy, M. E., Taddeo, S. S., Patil, B. S., Pike, L. M., Chapkin, R. S., Lupton, J. R. & Turner, N. D. (2004) Natural and irradiated grapefruit pulp and their functional compounds suppress aberrant crypt foci and colonocyte proliferation. *FASEB J.* 18: A894
95. Raman, G., Jayaprakasha, G. K., Cho, M., Brodbelt, J. S. & Patil, B. S. (2004) Isolation of structurally similar citrus flavonoids by flash chromatography. *Analytical Lett.* 37: 3005-3016.
96. Raman, G., Jayaprakasha, G. K., Cho, M., Brodbelt, J. & Patil, B. S. (2004) Rapid Adsorptive Separation of Citrus Polymethoxylated Flavones in Non-aqueous Conditions. *Separation Purification Technol.* (in press).
97. 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 (in press).
98. Kawaii, S., Tomono, Y., Katase, E., Ogawa, K. & Yano, M. (1999) Effect of citrus flavonoids on HL-60 cell differentiation. *Anticancer Res.* 19: 1261-1270.
99. Patil, B. S., Vanamala, J. & Hallman, G. (2004) Irradiation and storage influence on bioactive components and quality of early and late season 'Rio Red' grapefruit (*Citrus paradisi* Macf). *Postharvest Biol. Technol.* 34: 53-64.
100. Zheng, Y., Kramer, P. M., Olson, G., Lubet, R. A., Steele, V. E., Kelloff, G. J. & Pereira, M. A. (1997) Prevention by retinoids of azoxymethane-induced tumors and aberrant crypt foci and their modulation of cell proliferation in the colon of rats. *Carcinogenesis* 18: 2119-2125.

101. Hong, M. Y., Chapkin, R. S., Wild, C. P., Morris, J. S., Wang, N., Carroll, R. J., Turner, N. D. & Lupton, J. R. (1999) Relationship between DNA adduct levels, repair enzyme, and apoptosis as a function of DNA methylation by azoxymethane. *Cell Growth Diff.* 10: 749-758.
102. Taylor, A. N., Tio, D. L. & Romeo, H. E. (2005) The febrile response to intraperitoneal lipopolysaccharide: Strain and gender differences in rats. *J. Neuroimmunol.* 158: 86-93.
103. Tennekes, H., Kaufmann, W., Dammann, M. & van Ravenzwaay, B. (2004) The stability of historical control data for common neoplasms in laboratory rats and the implications for carcinogenic risk assessment. *Reg. Toxicol. Pharmacol.* 40: 293-304.
104. Tatsuta, M., Iishi, H., Baba, M., Yano, H., Murata, K., Mukai, M. & Akedo, H. (2001) Suppression by apigenin of peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in Wistar rats. *Clinical Exp. Metast.* 18: 657-662.
105. McLellan, E. A., Medine, A. & Bird, R. P. (1991) Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: Putative preneoplastic lesions. *Cancer Res.* 51: 5270-5274.
106. Miwa, Y., Yamada, M., Sunayama, T., Mitsuzumi, H., Tsuzaki, Y., Chaen, H., Mishima, Y. & Kibata, M. (2004) Effects of glucosyl hesperidin on serum lipids in hyperlipidemic subjects: Preferential reduction in elevated serum triglyceride level. *J. Nutr. Sci. Vitaminol.* 50: 211-218.
107. Lee, S.-H., Jeong, T.-S., Park, Y. B., Kwon, Y.-K., Choi, M.-S. & Bok, S.-H. (1999) Hypocholesterolemic effect of hesperetin mediated by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and acyl coenzyme A: Cholesterol acyltransferase in rats fed high-cholesterol diet. *Nutr. Res.* 19: 1245-1258.
108. Chen, Y.-C., Shen, S.-C. & Lin, H.-Y. (2003) Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. *Biochem. Pharmacol.* 66: 1139-1150.

109. Taraphdar, A. K., Roy, M. & Bhattacharya, R. K. (2001) Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Curr. Sci.* 80: 1387-1396.
110. Shukla, S. & Gupta, S. (2004) Molecular mechanisms for apigenin-induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells. *Mol. Carcinog.* 39: 114-126.
111. Zheng, P.-W., Chiang, L.-C. & Lin, C.-C. (2005) Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. *Life Sci.* 76: 1367-1379.
112. Wang, I. K., Lin-Shiau, S. Y. & Lin, J. K. (1999) Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukemia HL-60 cells. *Eur. J. Cancer* 10: 1517-1525.
113. Kim, S.-A., Ahn, S., Kim, D. K., Kim, S. G., Lee, S. H., Kim, J. & Yoon, J. H. (2004) Sequential expression of inducible nitric oxide synthase and cyclooxygenase-2 during DMBA-induced hamster buccal pouch carcinogenesis. *In vivo* 18: 609-614.
114. Dross, R. V., Xue, Y., Knudson, A. & Pelling, L. C. (2003) The chemopreventive bioflavonoid apigenin modulates signal transduction pathways in keratinocyte and colon carcinoma cell lines. *J. Nutr.* 133: 3800S-3804S.
115. Lin, J.-K., Chen, Y.-C., Huang, Y.-T. & Lin-Shiau, S.-Y. (1997) Suppression of protein kinase C and nuclear oncogene expression as possible molecular mechanisms of cancer chemoprevention by apigenin and curcumin. *J. Cell. Biochem. Suppl.* 28/29: 39-48.

APPENDIX A
DIET MIXING PROTOCOL

Prior preparation checklist:

1. Make sure there are adequate diet components
2. Test compounds
3. Adequate clean and labeled tubs and ziplock bags for diet components
4. Arrange for manpower
5. Weighing scales (2): 50 g-5 kg and >50 g
6. Measuring tubs and scoops (& spatulas)
7. Stirring tool
8. Adequate labeled diet bags and outer bags
9. Colored Tapes
10. Sharpies
11. Aluminum foil
12. 5 L jugs for oil (2-3)
13. Cart, tub for ice and ice
14. Gloves, bench papers, napkins
15. Diet specification sheets
16. Mixing bowl and mixer (2)
17. Labeled vials and specimen cups for diet samples

Note: Each mixing bowl can only contain up to 20 kg of diet, thus the total weight of each diet need to be divided up into 2 to 3 portions

For each diet group, there will be 2 to 3 portions named Bowl 1, Bowl 2, and Bowl 3, if necessary.

Each Bowl group will consists of 3 tubs plus 1 ziplocked bag of diet components. See example below:

Diet	Bowl 1	Bowl 2	Bowl 3
Nobiletin –100	3 tubs + 1 ziplock	3 tubs + 1 ziplock	3 tubs + 1 ziplock
Tangeretin –200	3 tubs + 1 ziplock	3 tubs + 1 ziplock	3 tubs + 1 ziplock
Hesperidin –300	3 tubs + 1 ziplock	3 tubs + 1 ziplock	3 tubs + 1 ziplock
Obacunone –400	3 tubs + 1 ziplock	3 tubs + 1 ziplock	3 tubs + 1 ziplock
Basal –500	3 tubs + 1 ziplock	3 tubs + 1 ziplock	3 tubs + 1 ziplock
LG/OG – 600	3 tubs + 1 ziplock	3 tubs + 1 ziplock	3 tubs + 1 ziplock
Total	18 tubs + 6 ziplocks	18 tubs + 6 ziplocks	18 tubs + 6 ziplocks

Day 1 – Diet components weighing day

- GOALS:**
1. Measure diets into appropriate tubs and ziplock bags
 2. Arrange in order
 3. Seal and store in freezer, if necessary
 4. Record diet lot numbers
 5. Document process and take pictures
- SUPPLIES:** See checklist
- START TIME:** 8 AM
- LOCATION:** Kleberg basement (diet mixing room)
- MANPOWER:** At least 2; preferably at least one guy.
- PROCEDURE:**
1. Transport diet components from storage and/or freezer to mixing room.
 2. Bring supplies to mixing room.
 3. Weigh out diet components (according to specification sheet) in the following order:

1 st – Dextrose (tub)	
2 nd – Casein (tub)	
3 rd – Pectin (ziplock bag)	
4 th – Salt mix	
5 th – Vitamin mix	all in
6 th – Methione	one tub
7 th – Choline*	
 4. Arrange tubs that belong to a diet group together; within diet groups, arrange into bowl number.
 5. Store tubs containing salt/vitamin/met/choline mix in freezer.
 6. Record diet lot numbers.
 7. Seal, label and store any excess diet components.
 8. Tidy up.

*Note: Choline is kept in a dessicator because it absorbs moisture easily. It will only be taken out when it is time to measure choline. Once measured, the choline needs to be mixed in with the salt/vitamin mix to ensure dryness.

Day 2 and 3 – Diet mixing day

- GOALS:
1. Measure oil needed for the day
 2. Mix diet components into each diet groups
 3. Package and double-seal into ziplock bags
 4. Store diet bags
 5. Document process and take pictures
- SUPPLIES: See checklist
- START TIME: 8 AM
- LOCATION: Kleberg basement (diet mixing room)
- MANPOWER: At least 4; preferably at least one guy
- PROCEDURE:
1. Get cart, 2-3 5 L jugs, tub big enough to fit jugs and ice, aluminium foil, bench papers, scissors, sharpies, napkins, weigh scale, plastic beaker.
 2. Go to Dr. Chapkin's lab to measure out corn oil, where it is also stored. When done, fill remaining barrel of oil with nitrogen gas, seal, date and store back in freezer. Record lot number.
 3. Cover jugs with foil, label, and put on ice.
 4. Bring 2 big stainless steel mixing bowls from lab to basement.
 5. Measure out test compounds, add to salt mix and mix well.
 6. Start with Bowl 1 of a diet group, mix by hand diet components in the following sequence:
 1. Pectin
 2. Salt mix
 3. Casein
 4. Dextrose
 7. Fit bowl onto electric mixer, cover, and mix dry components for 5 min at #1 setting.
 8. Still at #1 setting, start mixer for 10 min. During this time, pour oil into mix in 3 separate quantities and times. To get all oil out from jug, use diet to mop it up.
 9. Scrape sides of bowl and blade, mix for 5 min more.
 10. Lower bowl, scrape sides of bowl and blade, put bowl back into position, and mix for 10 min at #2 setting.
 11. Scrape diet off blade, lower and remove bowl. Take a sample of diet mix from 4 locations in the bowl (send for analysis).
 12. Scoop diet into labeled bags and double-bag. Store in labeled plastic tubs in freezer.
 13. Repeat the above process for all diet groups. Clean up.

Notes: Before starting on a new diet group, remember to wash bowls and scoops, and change gloves to prevent cross-contamination.

Equipment like bowls and scoops need to be sprayed with 70% EtOH after washing and drying.

The dry diet components are in powder form so dust clouds form when pouring; pour slowly to reduce dust.

Salt mix, vitamin mix, and methionine are stored in freezer, so put it back after measuring out into tubs.

The salt/vitamin/methione/choline mix might be lumpy so it is best to use gloved hands to break up the lumps and mix uniformly.

Diet samples are taken after mixing for analysis of composition. The small vials are used to collect samples on mixing day and sent for analysis soon after. The big specimen cups are used for collecting diet samples throughout the feeding period and analyzed at the end.

APPENDIX B

WESTERN BLOT PROTOCOL

For iNOS and COX-2
Optimized September 2004

Day before:

1. Prepare Western template (attached).
2. Identify the samples for WB analysis and organize them in sequence for gel loading and for easy retrieval from -80°C freezer.
3. Label 0.6 mL epi-tubes for number of lanes loaded (1-10).
4. Make sure that 0.3 g and 0.6 g BSA powder are weighed out and buffers (running and transfer buffers; 10X, 2X, and 1X PBS/Tween) are available.
5. Store in walk-in cooler: deionized distilled water (2 L), running buffer (2 L), and transfer buffer.
6. Transfer buffer to be pre-mixed in bulk (10 L). Add MeOH and 10% SDS to pre-mixed 2X transfer buffer about 30 min before use.
7. To be kept cold before use: epi-tubes of ddH₂O, 2X sample buffer, 1X sample buffer (made just before use; 1 part 2X + 1 part ddH₂O).

On the day of Experiment:

1. Prepare standards first (refer to standard preparation protocol).
2. Take out and keep markers, standards, samples, 2X and 1X sample buffers, and ddH₂O on ice.

NOTE: Switch on the Heating block and set 98°C .

Make sure protein samples are inverted, flicked, and spun in table-top centrifuge prior to pipetting sample from original epi-tube to new epi-tube for reducing protein.

3. Pipette markers, standards, and samples, Sample Buffer, and ddH₂O into 0.6 mL epi-tubes, mix by flicking and vortexing tubes. Then quick spin (15 sec) in tabletop centrifuge.

Samples:	Vol. Protein samples + 2X Sample Buffer + dd H ₂ O
Standards:	iNOS and COX-2 (Cayman); refer to protocol for preparing standards
1 ⁰ ABs	iNOS Rabbit polyclonal IgG (Cayman 160862; stored in -20°C) COX-2 Goat polyclonal IgG (Santa Cruz 1745; stored in 4°C) β -Actin Goat polyclonal IgG (Santa Cruz 1616; stored in 4°C); house-keeping protein as loading control
2 ⁰ AB:	Bovine anti-goat IgG-HRP conjugate (Santa Cruz 2350) Goat anti-rabbit IgG-HRP conjugate (Cayman 10004301)

4. Heat samples at 98°C for 10 min (**Do Not Heat Marker**).
How to use the heating block: Set the time, temperature, shaking to 300 rpm and start. Do steps 1 – 3 of Gel Preparation.
5. Remove samples from heating block and keep on ice for 10 sec. Quick spin in the tabletop centrifuge; may need a paper towel to wipe the epitubes. Continue step 4 of Gel Preparation.

GEL PREPARATION (NOTE: Gels are in walk-in-cooler)

1. Gel comes in a hard plastic casing enclosed in an outer plastic wrap containing buffer. Cut open the outer plastic wrap, drain buffer into the sink, wipe the gel's inner plastic casing with paper towel, and mark in the center of the wells with permanent marker. Gently remove the comb from the gel (Novex® 4-12% Tris-Glycine Gel 1.5 mm, 10 well, Novex #EC6035), peel off the white tape on the casing, and place the gel keeping the notched glass plate face down in the electrophoresis unit which is open all the way (unscrew all the way up). Clam gel to unit from the sides and leave the bottom at half-open position.
2. Place the unit in the chamber and pour Tricine SDS Running Buffer (Novex #LC1675) until wells are covered. Fill Buffer Chamber with Tricine SDS Running Buffer (Novex #LC1675) to the middle of min and max mark (slightly below max. line).
3. Transfer unit into 4°C cold room. Do NOT move unit after loading gels.
4. Using 10 µl tip and pipette, load the whole amount of markers, standards and samples into designated wells (use marks as guides). Change tips every time you load the samples. Make sure there is no trapped air in the tip before loading; put the tip to the wall where the mark indicating the center of the well is and gently unload the tip.
5. Put lid on electrophoresis unit and connect electrodes to power supply (red to red and black to black).
6. Run at 15 mA and 75 V for about 210-240 min (3.5-4 h), until dye front nears bottom of gel (**DOUBLE POWER FOR 2 GEL UNITS**). Ensure that protein is moving to the bottom of the gel after 5 min. Add additional running time if dye front is not near bottom of gel. Turn off power supply and unplug unit when finished.

	Start time		beginning V		mA
	End time		ending V		mA

NOTE: Pour back the running buffer into the running buffer bottle. This could be reused until it is cloudy.

TRANSFER GEL

1. Pour some Tris Glycine Transfer Buffer into clean large staining tray.
2. Soak blotting pads and filter papers in Tris Glycine Transfer Buffer.
3. Place gel on bench, large side down; crack open gel case with an end of a spatula. Cut corner above first lane of gel with a scalpel (right side - top corner). Using a rocking motion with scalpel cut off thickened bottom of gel. Cut off the well projections.

Keep the gel moist with the transfer buffer all the time.

4. Pick the gel up from the bottom and transfer gel from case to staining tray containing transfer buffer - AS IS- DO NOT FLIP GEL OVER. Leave for 5 min to equilibrate gel with transfer buffer.
5. Wet membrane (Invitrolon PVDF #LC2005) with methanol to soak ~15 sec (keep methanol in a squirt bottle – spray the methanol in the staining tray and then place the membrane). Pour out the methanol in the sink and run water for 2-3 min or in the waste bottle in the hood.
6. Grab edge of membrane and place in transfer buffer in the staining tray for 2-5 min, swirling to saturate the membrane.
7. Put transfer cassette into large staining tray (black side down). Open cassette and prepare transfer stack by placing one wet sponge on the black cathode side.
8. Lay one piece of the wet filter paper on the sponge.
9. Place gel onto filter paper, **AS IS**, wetting the gel surface with a few drops of transfer buffer, to keep moist.
10. Lay prepared membrane carefully onto gel, avoid trapping any bubbles.

Do Not Repositon The Membrane Once It Contacts The Gel.

11. Use a glass rod/roller to roll out any bubbles.
12. Lay other piece of filter paper wetted with transfer buffer on the membrane. Roll out any bubbles.
13. Lay other two packing sponge, wetted with transfer buffer, on the filter paper.
14. Close cassette, put 300 – 350 ml transfer buffer, tap the blotting cup to dislodge any bubbles, put cassette into the tank, with black side facing “labeled” black cathode.
15. Set on the bench on the 4°C cold room. Add cold ddH₂O to tank.
16. Run unit at 300 mA and 25 V for 120 min (2 h) (DOUBLE POWER FOR 2 UNITS).

Beginning time _____ **V** _____ **mA** _____

Ending time _____ **V** _____ **mA** _____

To check if everything transferred off the gel onto the membrane, gently place the gel into a staining tray with COMASSIE STAIN. Gently rock for one hour. Destain with destainer overnight in cold room.

17. Prepare 2% BSA/PBS/Tween. (0.6 g BSA in 30 ml PBS/Tween).

18. Put transfer cassette (black side down) into large tray. Open cassette, remove two sponges and upper filter paper. **Cut the membrane at the first well i.e. right-hand side top corner.**
19. Using tweezers, **flip membrane over** and wash briefly in ddH₂O. Transfer to staining tray containing MeOH and let saturate for 15 sec Then let dry on filter paper for 15 min.
20. Cut membrane to size according to color marker. Re-wet with MeOH for 15 sec.
21. Using tweezers, put membrane into 2% BSA/PBS/Tween, shake gently for 3 h in 4°C cold room or 1 h at RT.

Ensure the cut corner of the membrane is always on your left side from this step onwards.

NOTE: Discard transfer buffer.

BLOTTING

1. Prepare 1% BSA/PBS/Tween (0.3 g BSA in 30 ml PBS/Tween).
2. Pour 1% BSA/PBS/Tween into tray. Immediately before adding transfer membrane, add 1° antibody, and mix gently.

iNOS 1° AB: 80 µl

COX-2 1° AB: 30 µl

B-actin 1° AB: 3 µl

3. Put transfer membrane into 1° antibody, shake gently overnight in 4°C cold room. Record time and keep time consistent (at least 8 h).
4. Transfer membrane into tray with 2X PBS/Tween for brief wash.
5. Pour off PBS/Tween, add new 2X PBS/Tween to tray. Shake strongly 15 min x 3.
6. Prepare 1% BSA/PBS/Tween (0.3 g BSA in 30 ml PBS/Tween).
7. Pour 1% BSA/PBS/Tween into tray. Immediately before adding transfer membrane, add 2° Antibody. Shake gently for 1 h at RT.

iNOS 2° AB: 0.5 µl

COX-2 2° AB: 0.5 µl

B-actin 2° AB: 0.1 µl

NOTE: Secondary antibody has cholesterol, so it does not freeze. However, make sure it is properly mixed, as it is very dense.

8. Transfer membrane into tray with 10X PBS/Tween for brief wash.
9. Pour off PBS/Tween, add new 10X PBS/Tween to tray. Shake strongly 15 min x 3. During this time, gather items for chemiluminescence and imaging. Bio-Rad imager is located in room 435.
10. Just before imaging, mix chemiluminescence substrates gently as below:
 - iNOS – Femto Substrate, 0.5 ml each of reagent A and B; incubate 5 min.
 - COX-2 – Dura Substrate, 0.25 ml each of reagent A and B; incubate 5 min.
 - β-Actin – Dura Substrate, 0.5 ml each of reagent A and B; incubate 1.5 min.
11. Place membrane between acetate sheets. Pipette reagent mixture onto top of membrane and gently lower down top acetate sheet such that membrane is completely covered with reagent mixture.

12. After incubation, place membrane between new acetate sheets (so that reagent mix doesn't spill onto Bio Rad reader tray) and **image immediately**.

In room 435,

Switch on the phosphorimager (Switch is on the left hand side, back; bottom).

Switch on the computer

Click on 'Quantity One' program icon

File --->Flour-S

Chemiluminescent

Blotting

High Resolution

Open camera door completely and imager door

Click on **Position** to check on placement of blot (adjust the blot position and make sure the cut end is at the top left hand side)

Make sure aperture of camera is greater than 1.4

Place the paper with writing on top of the blot and then focus by rotating the camera focus knob.

After focusing well, enter amount of time for exposure (generally 30-60 sec)

Then hit ACQUIRE

If everything is OK (position of the blot and quality), acquire again for 600 sec.

When done imaging – hold APPLE down and hit B

then invert display

then autoscale

adjust the bright and dark as needed

To quantify protein in bands

>Volume Properties

>#5 Volume rectangle

> Draw bands around bands of interest

>#10 Volume report

>click on name, volume and adjusted volume. Print report

Prepare a tub with the following items for Chemiluminescence step:

1. Waffle paper liners
2. 1000P pipette and pipette tips
3. Pre-cut acetate sheets or acetate sheets and scissors
4. 2-mL eppetubes and holder
5. Chemiluminescence substrates – pipette 2 mL of each substrate into separate eppetubes

6. Timer
7. Blots in PBS/Tween
8. Waste pipette tips container
9. Tweezers

Solutions to make/Supplies

Tricine SDS Running Buffer (1 L)

100 ml 10X Tricine SDS Running Buffer (Novex#LC1675 from Invitrogen)
900 ml ddH₂O
Store at 4C

Pre-mixed 2X Tris-Glycine Transfer Buffer (1 L)

80 ml of 25X TrisGly Transfer Buffer (Novex#LC3675 from Invitrogen)
780 ml ddH₂O
Add 1 ml of 10% SDS (Sigma L-4522) in 1 L of water
Store at 4C

NOTE: Prepare 10 L of the above and store in walk-in cooler. Only add 140 ml MeOH to 860 ml of pre-mix ½ h before use.

10 X PBS/ 1% Tween

1 bottle PBS (Gibco #21600-069) + 1 L ddH₂O. Swirl to mix.
OR use 10X ready prepared PBS
add 10 g (~10 mL) Tween 20 (Sigma # P-1379). Swirl to mix.
Store at RT

5X PBS/1% Tween

Equal parts 10X PBS/Tween and ddH₂O

2X PBS/1% Tween (1L)

200 ml 10X PBS/Tween
800 ml ddH₂O

1X PBS/1% Tween (1L)

1 packet PBS/Tween powder
1 L ddH₂O

Coomassie Blue Gel Stain

0.2 g Coomassie Brilliant Blue R-250 (BioRad #161-0400)
add 40 mL methanol and add 10 mL acetic acid
add 50 mL dH₂O

Gel Destain for Western

90 mL methanol
20 mL acetic acid
Add 90 mL dH₂O

SUPPLIES (not specified in protocol)

Hoefer miniVE Vertical Electrophoresis System #80-6421-05
 Amersham Electrophoresis Power Supply EPS 601
 Bio-Rad PowerPac HC
 Electrophoresis Sample Buffer 2X, Santa Cruz sc-24945
 Acetate sheets, Wilton's office supply, CIL-00010
 Bovine Serum Albumin, Fisher BP 1600-106
 SuperSignal West Dura Extended Duraton Substrate, Pierce #34075
 SuperSignal West Femto Maximum Sensitivity Substrate, Pierce #34095
 TriChromRanger Prestained Protein Molecular Weight Marker Mix, Pierce #26691
 iNOS Electrophoresis Standard, Cayman 360862
 COX-2 (Ovine) Electrophoresis Standard, Cayman 360120
 MagicMark XP Western Standard, Invitrogen LC5602

Preparation of COX-2 and iNOS Electrophoresis Standards

Electrophoresis standards come in 100 ng/ μ l concentration from supplier.
 Pipette standards into 5 μ l aliquots and store in -80°C freezer.
 On day of use, prepare and dilute standards into various concentrations according to chart below.

Add 5 μ l of 2X sample buffer to 5 μ l of standard = 10 μ l of 50 ng/ μ l std.
 Heat at 90°C for 5 min. at 300 rpm on heating block. Cool on ice for 30 sec.
 Spin on bench-top centrifuge for 15 sec.
 Add 40 μ l of 1X sample buffer to the 10 μ l of 50 ng/ μ l std.
 You will now have 50 μ l of 10 ng/ μ l std (dilution of 1:5).

For this conc. of std	Add this amount	Of this	To this amount Of 1X sample buffer	You will get this final amount
1 ng/ μ l or 1000 pg/ μ l	5 μ l	10 ng/ μ l std	45 μ l	50 μ l
500 pg/ μ l	5 μ l	1000 pg/ μ l	5 μ l	10 μ l
250 pg/ μ l	5 μ l	1000 pg/ μ l	15 μ l	20 μ l
125 pg/ μ l	5 μ l	1000 pg/ μ l	35 μ l	40 μ l
62.5 pg/ μ l	6 μ l	1000 pg/ μ l	74 μ l	80 μ l

NOTE: Make sure all eppetubes are finger-flicked and vortexed to properly mix the solutions, and spun in bench-top centrifuge before proceeding to the next step.

For loading into gel, you will only need 1 μ l of stds at each concentration.

WESTERN TEMPLATE

Date:

Type of Gel:

Purpose:

1° Antibody:

2° Antibody:

WELL	SAMPLE	Protein Conc. (ug)	Sample (ul)	Water (ul)	Dye (ul)
1.					
2.					
3.					
4.					
5.					
6.					
7.					
8.					
9.					
10.					

APPENDIX C

TABLE OF ACF FORMATION

Effect of experimental diets on AOM-induced ACF formation in male Sprague-Dawley rats¹

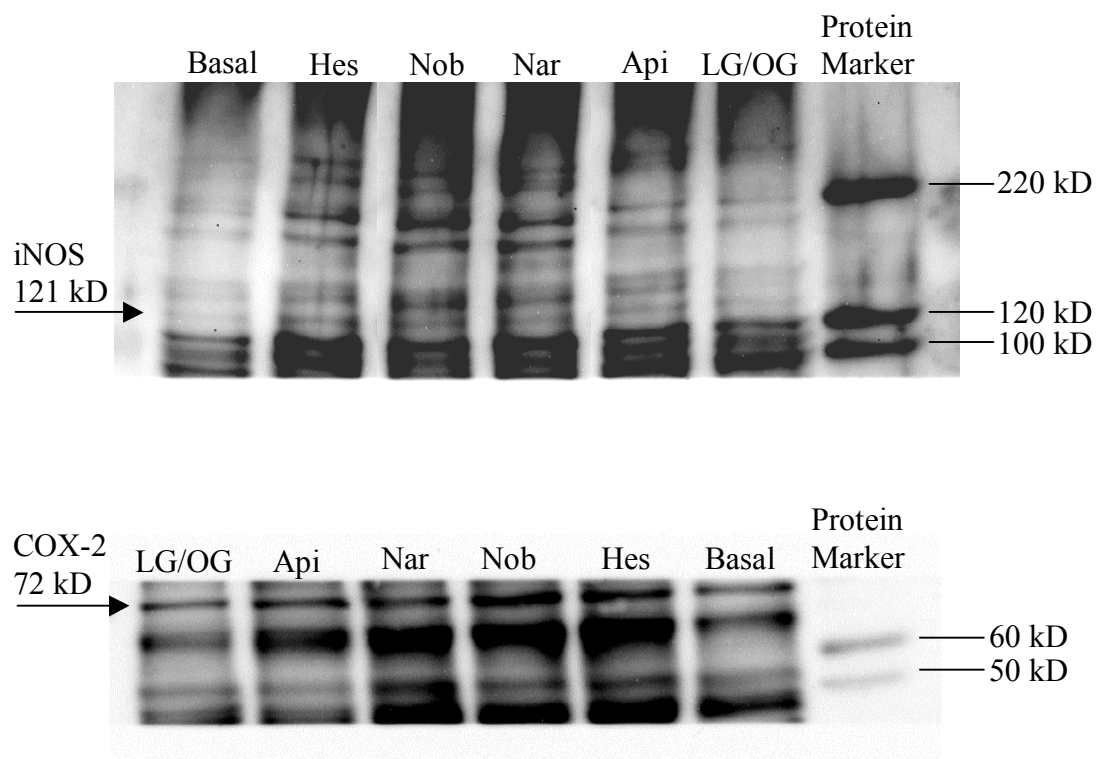
Diet Group	No. of high multiplicity ACF/colon	Total no. of aberrant crypts/colon	No. of aberrant crypts/cm colon
Basal (Control)	3.94 ± 1.11 ^a	142.80 ± 19.39 ^{a,b}	13.31 ± 1.73 ^a
Hesperidin	2.33 ± 0.85 ^{a,b}	149.50 ± 19.39 ^{a,b}	13.46 ± 1.73 ^a
Nobiletin	2.76 ± 0.93 ^{a,b}	176.10 ± 19.39 ^b	16.92 ± 1.73 ^b
Naringenin	1.94 ± 0.78 ^b	152.90 ± 19.39 ^{a,b}	14.16 ± 1.73 ^{a,b}
Apigenin	1.69 ± 0.73 ^b	132.00 ± 19.39 ^a	12.24 ± 1.73 ^a
LG/OG mixture ²	2.17 ± 0.86 ^{a,b}	140.57 ± 20.05 ^{a,b}	13.74 ± 1.78 ^{a,b}

¹ Values given are LSmeans ± SEM, n = 10 rats/group. Means in a column without a common letter differ, $P < 0.05$.

² Limonin glucoside/obacunone glucoside mixture.

APPENDIX D

FIGURE OF WESTERN BLOT



Representative blots of western blot analysis on actual mucosal protein samples for iNOS and COX-2 enzymes. Abbreviation: Hes, hesperidin, Nob, nobiletin, Nar, naringenin, Api, apigenin, LG/OG, limonin glucoside/obacunone glucoside mixture.

VITA

TETY LEONARDI

3320 Thornton Lane Apt. S-2, Temple, TX 76502

EDUCATION

MS, Nutrition, May 2005; Dietetic Internship, July 2004
Texas A&M University, College Station, TX

BS, Nutritional Sciences, August 2002
Texas A&M University, College Station, TX

CONFERENCES

The Center for Environmental and Rural Health Annual Scientific Symposium 2004 - Dietary Fiber and Colon Cancer, Texas A&M University, College Station, TX. Participated in poster competition (honorable mention).

Intercollegiate Faculty of Nutrition Research Symposium 2004, Texas A&M University, College Station, TX. Participated in oral competition (honorable mention).

Experimental Biology 2004, Federation of American Societies for Experimental Biology, Bethesda, MD. Presented talk in minisymposia. Participated in Diet and Cancer Research Interest Section poster competition (tied for 1st place).

Agriculture Program Conference 2004, Texas A&M University, College Station, TX. Participated in student research poster competition.

HONORS AND AWARDS

Regents Scholarship; Mendon B Krischer Memorial Scholarship;
TAMU Academic Achievement Award; Houston Rodeo Endowed Scholarship;
Karen Smith Scholarship; Dean's Honor Roll

PUBLICATIONS

Leonardi, T., Vanamala, J., Taddeo, S. S., Murphy, M. E., Patil, B. S., Wang, N., Chapkin, R. S., Lupton, J. R. & Turner, N. D. (2004) Apigenin and naringenin suppress high multiplicity aberrant crypt foci formation and cell proliferation in rat colon. *FASEB J.* 18: A477.

Vanamala, J., Leonardi, T., Murphy, M. E., Taddeo, S. S., Patil, B. S., Pike, L. M., Chapkin, R. S., Lupton, J. R. & Turner, N. D. (2004) Natural and irradiated grapefruit pulp and their functional compounds suppress aberrant crypt foci and colonocyte proliferation. *FASEB J.* 18: A894.