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**EFFECT OF CHLOROGENIC ACID AND NEOCHLOROGENIC
ACID ON HUMAN COLON CANCER CELLS**

Effect of Chlorogenic Acid and Neochlorogenic Acid on Human Colon Cancer Cells

An Undergraduate Honors Thesis

Submitted in partial fulfillment of the requirements for the

Bumpers College Honors Program in the

Department of Food Science

By:

Taylor Thurow

April 2012

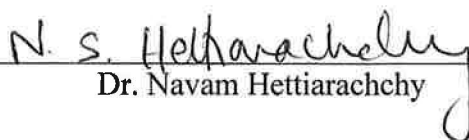
University of Arkansas



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ABSTRACT

Prune consumption has been associated with a decreased risk of colon cancer, yet there has been confusion as to which chemical component(s) of the prune are responsible for its anticarcinogenic properties. Previous studies have evaluated chlorogenic acid as a chemotherapeutic agent, however only a limited amount of studies have investigated neochlorogenic acid, the predominant phenolic compound found in the prune. The purpose of this study was to determine the effects that chlorogenic acid and neochlorogenic acid have as anticarcinogenics on the human adenocarcinoma, Caco-2 cell line. The anti-colon cancer effects of chlorogenic acid and neochlorogenic acid were analyzed by experiments that measured cell proliferation and morphology in culture of Caco-2 cells. Treatment of cells with chlorogenic acid and neochlorogenic acid significantly reduced cell proliferation at concentrations of 150-500 μ mol at 24, 48, and 72 hours by 63.7-96.0% and 69.7-94.2%, respectively ($p < 0.05$). At the majority of sample times and concentrations, chlorogenic acid and neochlorogenic acid did not significantly differ in percent reduction of viable cells ($p < 0.05$). The cell morphology of treated cells changed, as the surface of cells became more rough, uneven, and irregularly-shaped as the concentration of the treatment increased, compared to the untreated Caco-2 cell. These findings of significant cell proliferation inhibition suggest that both chlorogenic acid and neochlorogenic acid could be colon cancer suppressive components of the prune.

Chapter I. Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the United States. Based on the rates from 2005-2009, 54.0 men and 40.2 women out of 100,000 in the United States will be diagnosed with colorectal cancer each year. For future generations, this means 4.96% men and women born today will be diagnosed with cancer of the colon and rectum during their lifetimes. Although incidence and mortality rates have declined since 1998, colorectal cancer is still an imminent threat. Locally, trends for colorectal cancer are still unfavorable. Arkansas is well above the U.S. death rate for cancer of the colon and rectum in both men and women, having an annual death rate of 18.9 deaths per 100,000, compared to the national average of 17.1 per 100,000. Risk factors associated with colorectal cancer include increasing age, race, sex, heredity, preexisting conditions, tobacco usage and diet (1).

It is estimated that 30-60% of cancers may be diet-related. Thus, alterations of the diet to include whole-grain cereals, fruits and vegetables could provide protection from diet-related cancers. Consumption of these protective foods could prevent up to 66-75% of colon cancers (2).

The prune is linked to decreased risk of colon cancer and is rich in phenolic antioxidants that may be responsible for this anticarcinogenic behavior. Of all the phenolic compounds present in the prune, neochlorogenic acid is the most predominant, followed by chlorogenic acid (3). The goal of this study is to determine the effects of chlorogenic acid and neochlorogenic acid on human colon cancer cells. The hypothesis of this study is that one or more of the chlorogenic acid and neochlorogenic acid treatment concentrations tested will be able to effectively inhibit colon cancer cell

proliferation. The objectives of this study were to: 1) determine if chlorogenic acid and neochlorogenic acid can effectively inhibit colon cancer cell proliferation, 2) determine what concentrations of chlorogenic acid and neochlorogenic acid are effective at inhibiting cell growth, and 3) establish the time requirement to significantly reduce the number of viable cells. To achieve these objectives, cell viability was measured and cell morphology was observed.

Chapter II. Literature Review

Colon Cancer

Cancer is the leading cause of death worldwide, and was responsible for 7.6 million deaths in 2008. According to the World Health Organization (WHO), 608,000 of these cancer related deaths were due to colorectal cancer. Colorectal cancer is the second leading cause of cancer-related deaths in the United States in both men and women. In 2012, it is estimated that there will be more than 143,000 people diagnosed with CRC, and approximately 52,000 deaths due to CRC. This would make CRC the fourth most common cancer in men, after skin, prostate, and lung cancer, and the fourth most common cancer in women, after skin, breast, and lung cancer. Based on rates from 2006-2008, 1 in 20 men and women born today will be diagnosed with colorectal cancer at some point in their lifetime. The risk factors associated with CRC include increasing age, family history of CRC, genetic alterations, tobacco usage, and diet. The colon's function of absorbing water and nutrients from food support the strong correlation between diet and colorectal cancer. Diets high in fat and low in calcium, folate, and fiber are associated with a higher risk of CRC. A diet low in fruits and vegetables also suggest a higher risk of CRC. Due to the epidemiological studies that suggest a relationship between a diet high in fruits and vegetables and lower rates of cancers in the esophagus, prostate, colon, and stomach; the phenolic compounds found in fruits are of great interest because of their potential anticarcinogenic properties. It has been suggested that phenolic compounds, such as the hydroxycinnamic acids, chlorogenic acid and neochlorogenic acids in the prune, are responsible for the inverse association between fruit consumption

and cancer. This review will focus on two of the phenolic compounds found in the prune: neochlorogenic acid and chlorogenic acid.

Colorectal cancer (CRC) occurs in the tissue of the colon, the first four to five feet of the large intestine, and in the rectum, which is the passageway that connects the colon to the anus. Most cases of CRC are found in the form of an adenocarcinoma, meaning that the cancer begins in cells that make and release mucus and other fluids. Colorectal cancer affects both men and women and is seen in all racial and ethnic groups, but found most significantly in people 50 years of age and older. CRC is responsible for substantial medical and societal costs, with an estimated direct medical cost of \$14 billion in 2010 (1).

General Information of Prune/Plum

Plums are the fruits of *Prunus* in the Rosaceae family (4). The drying of plums will yield dried plums, otherwise known as prunes. The majority of prunes are the dried fruits of cultivars of *Prunus domestica* L., which originated from the Caucasus region in Eurasia (5). Prunes are the byproduct of crossbreeding a cherry plum and a tetraploid blackthorn (3). Currently, plums are produced to become whole canned plums, prunes, prune juice, pastes, sauces, juice concentrates, and prune bits (6). A recent interest has been taken in prunes due to having the best nutrient score among dried fruits, along with figs (7). For these reasons and more, dried plums have recently been promoted as a health food (8). However, since edible plants are not considered to be a type of medicine, prunes are now being thought of as a natural source of anticancer compounds (9). Although, a few studies have been done on the prune, more are needed to investigate the

health benefits of the prune, including the possibility of being chemopreventative and chemotherapeutic.

Of the prunes produced, half will be consumed while the other half will be further processed (10). An estimation of the daily consumption of the raw plums is 0.89g daily intake (6). The plum and the black plum account for 50% each of the total plum consumption (10).

California is the world's largest producer of dried plums, producing 67% or 178 million kilograms of prunes each year (3). According to the National Agricultural Statistics Service, the estimate of the 2010 California dried plum crop production was 150,000 tons. This was a 14% increase from the previous five years average (11). Dried plums or prunes are made by dehydrating plums at 85-90°C for 18 hours, so that the plums can be processed into prunes, prune juice, prune puree, or other prune products. This process allows dried plums to be stored up to one year due to decreased water activity of less than 25% and an average of 82mg of ascorbic acid to inhibit molds and yeasts. The effects of processing a new plum to a dried plum cause many differences in the chemical composition of the prune. The sugar concentration increases due to the dehydration and the disappearance of sucrose which has been hydrolyzed into glucose and fructose instead (12). Processing will degrade half of the phenolic compounds, flavonols, hydrocinnamic acids, and will completely degrade anthocyanins and flavan-3-ols in the dried plum (13). In addition, a decrease in ascorbic acid, vitamin C, chlorogenic acid, and neochlorogenic acid will occur after processing raw plums (14). It has been shown that processing plums into dried plums will cause a 90% decrease in β -carotene, 20% decrease in amino acids, 34% decrease in thiamin, 20% decrease in niacin,

and a 42% increase in riboflavin (3). However, the processing of prunes into prune juice will cause a reduction in fiber due to the lack of skin and pulp, but have no effect on the phenolic compounds (3,13).

Components of a Prune

Basic Nutritive Values and Components

The basic nutritive value for a 100g dried plum consists of 239 calories, 0.52g fat, 62.7g carbohydrates, 2.61g protein, and 7.1g of fiber (15). Prunes are found to be high in fiber, sorbitol, simple sugars, and phenolic compounds (3). Prunes provide energy due to their high sugar composition. Fresh prunes can have 12.8-29g of total sugar of which 31% is glucose, 18% fructose, 23% sucrose, and 28% sorbitol (3). Fresh prunes contain approximately 2.7 g of sorbitol while dried prunes and prune juice contain 15 and 17.8g of sorbitol per 100g of prune plum (12). Sugar proportions stay similar in fresh, dried, and juice except for the absence of sucrose in prune juice (12). Prunes contain 6-16g/100g of fiber. They also contain very little fat. The small amount of fat is due to the presence of lipids in the prune's pulp, plum seeds, and in the epicuticular wax that covers the fruit. Prunes are considered to be a good source of vitamin C, riboflavin, niacin, and pyridoxine, since they contain greater than 10% of the recommended daily intake. Fresh prunes contain 5.1-16.5mg vitamin C, while dried prunes contain between 4.0-8.7mg. They also contain 5% recommended daily intake of vitamin A, because fresh prunes contain between 0.22-0.57mg of β -carotene, the provitamin A. Total, between 15-30 carotenoids are present. The mineral content on the other hand, usually depends on the

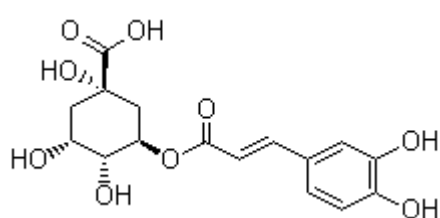
soil of the region that the fresh prune is grown in. Prunes are considered to be high in boron with 1.88mg/100g when the requirement for humans is only 1mg/day. Dried prunes also contain 50mg calcium, 50mg magnesium, and 80mg phosphorus. Prunes also contain a fair amount of acids, such as 6.9mg salicylic acid to protect from spoilage, 2g/100g organic acids (mostly citric acid), and very few free amino acids (majority are asparagines) (3).

Phenolic Compounds in Prunes: Neochlorogenic Acid and Chlorogenic Acid

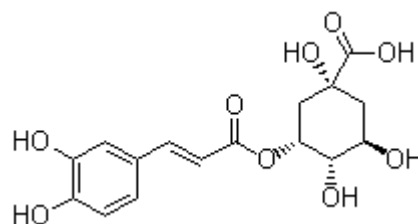
Phenolics are of high abundance in fresh and dried prunes, containing 1.84 mg/g phenolics in a dried plum (4). There are five times more phenolics in the skin of the prune than there are in the pulp (3). The total phenolics include caffeoylquinic acids such as chlorogenic acid and neochlorogenic acid, flavanols such as catechin and procyanidins, flavanols such as quercetin and its derivatives, and anthocyanins such as cyanidin, 3-B-glucoside, and cyanidine 3-B-rutinoside (8,9). The color of the prune is due to the presence of anthocyanins, which are water soluble plant pigments responsible for the blues, purples, and reds present in plants. Fresh plums contain approximately 12.5mg of anthocyanins (10). However, the odor and flavor of the fresh prune are from volatile compounds such as B-ionone and nonanal. Other components of the prune are B-carboline alkaloids, antioxidants, and cyanogenic glycosides (3). Polyphenolics can vary depending on the cultivar climate, storage conditions, and processing. With total phenolics ranging from 30.8-97.6%, flavanols will account for 1.6-19%, and anthocyanins will account for 12.6-50.3% (16). It has been shown that prunes are the highest in antioxidant activity with 204.9-567mg/100g, followed by raisin, blueberry,

blackberry, strawberry, and raspberry based on oxygen radical absorbance capacity (ORAC) (16, 5). Of these antioxidants, neochlorogenic acid is the most predominant in amount, followed by chlorogenic acid (16). For 100g, there are 81mg of neochlorogenic acid in a fresh prune, 131mg in a dried prune, and 22.5mg in prune juice. For chlorogenic acid in 100g, 14.4mg are present in fresh prunes, 44mg present in a dried prune, and 19.3mg present in prune juice (3).

Phenolics are natural protectants, whose main purpose is to provide an antifungal defense mechanism in fruit (17). However, phenolics, specifically chlorogenic acid and neochlorogenic acid, can have protective qualities for humans too. Chlorogenic acid has weak antimicrobial activity, even to the point of being active against the polio virus at low concentrations. Chlorogenic acid also causes dissipation of the sodium electrochemical gradient so that more glucose will pass through the bowel (3).



Chlorogenic Acid



Neochlorogenic Acid

Figure 1. Chemical structure of chlorogenic acid (3-O-Caffeoylquinic acid, $C_{16}H_{18}O_9$) and neochlorogenic acid (5-Caffeoylquinic acid, $C_{16}H_{18}O_9$) (18)

Health Effects of Neochlorogenic Acid and Chlorogenic Acid

Role as an Antioxidant

The prune has the highest antioxidant capacity of all dried fruits (8). In fact, the antioxidant capacity of chlorogenic acid itself is higher than those of vitamin C and

vitamin E (5). Phenolics like chlorogenic acid and neochlorogenic acid exhibit antioxidative properties by chelating metal ions, inhibiting lipid oxidation, inhibiting radical forming enzymes, and eliminating free radicals (16). With these properties, neochlorogenic acid and chlorogenic acid can protect low-density lipoprotein (LDL) from oxidation by 86-97% so that age-related diseases can be prevented (4).

Role as Chemopreventative/ Chemotherapeutic Agent

Neochlorogenic acid and chlorogenic acid fractions may demonstrate chemopreventative and chemotherapeutic activities. They decrease cancer risk by protecting scavenging reactive oxygen species (ROS), enhancing DNA repair and carcinogen detoxification, and modifying carcinogen uptake and metabolism. Phenols target molecular pathways of cancer cells while having a low toxicity in normal cells (9). From *in vivo* studies with ileostomy subjects, it has been found that when digested, 33% of chlorogenic acid is absorbed in the small intestine, and the rest is transported to the colon, where the bioavailability depends on the metabolism of certain microflora (9). It has been proven that chlorogenic acid in a person's diet can inhibit the formation of tumors in the bowel and liver (3). However, in *in-vitro* studies, it has also been found that chlorogenic acid and neochlorogenic acid at concentrations of 17mg/L and 10mg/L can suppress breast cancer cell growth, MD-MGA-435, while having no effect on normal breast epithelial cells, MCF-10A (9).

In-Vitro Studies

There have been numerous studies that have used chlorogenic acid and neochlorogenic acid at various concentrations, times, and on several carcinoma cell lines, which have displayed anticarcinogenic behavior. **Table 1** summarizes these studies.

Table 1. Summary of anticarcinogenic activities of chlorogenic acid and neochlorogenic acid

Phenolic Compound	Cell Line	Concentration	Effect	Time
Chlorogenic Acid; Neochlorogenic Acid [9]	MDA-MB-435estrogen negative breast cancer cells	17 mg/L; 10mg/L (IC ₅₀) (47.98µmol/L ; 28.22µmol/L)	Dose-dependent reduction in cell growth and viability in MDA-MB-435	Net growth measured at 3 days
Chlorogenic Acid [19]	AH109A, rat ascites hepatoma cells	0-40µmol/L	No effect on proliferation	44hr in presence of chlorogenic acid
Chlorogenic Acid [20]	Hep3B, human liver carcinoma	200-300 µg/ml (564.5-846.7 µmol/L)	Reduction in cell growth and viability	24 hours after incubated with chlorogenic acid
Chlorogenic Acid [21]	U-87, glioblastoma brain-tumor derived cells	100µmol/L	Chlorogenic acid inhibited growth of U-87 glioma cells	18 hours after incubated with chlorogenic acid
Type of Chlorogenic Acid: CDCQ [22]	HT-1080, fibrosarcoma	20µmol/L (inhibited invasion by 50%)	Suppressed invasion and migration	24 hours after incubated with CDCQ
Chlorogenic Acid [23]	HT29, human colon cancer	289.2 µmol/L (EC ₅₀)	Killed 50% of cells	After 72 hour treatment
Chlorogenic Acid Derivative: 1,3-dicaffeoyl-muco-quinic acid diacetal [24]	Dnmt3a	35 µmol/L (IC ₅₀); 0-500 µmol/L	Dose-dependent inhibition of Dnmt3a (causes cancer development)	10 minutes after treated with chlorogenic acid derivative

Phenolic Compound	Cell Line	Concentration	Effect	Time
Chlorogenic Acid [25]	HT29, colon cancer cells; HepG2 liver cancer cells	0-500 $\mu\text{mol/L}$ (EC ₅₀) HT29; 413 $\mu\text{mol/L}$ HepG2	Time and Dose-dependent reduction in cell viability and growth	72 hours after treatment with chlorogenic acid

Chapter III. Materials and Methods

Materials

Chlorogenic acid (CA) and neochlorogenic acid (NCA) were purchased from Quality Phytochemicals LLC (New Jersey, USA). These compounds were dissolved in dimethyl sulfoxide (DMSO, ATCC, Rockville, MD) and then added to media at treatment concentrations of 0, 150, 300, and 500 μmol . The control treatment consisted of only DMSO and media.

Methods

Cell Culture

Caco-2 cells, a human epithelial colorectal adenocarcinoma (cancer) cell line, were purchased from the American Type Culture Collection (ATCC, Rockville, MD) at passage number 18 and cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% Non-essential amino acids, and 2% antibiotic-antimycotic solution. Cell cultures were maintained under sterile conditions and incubated at 37°C in a humidified 5% CO₂ atmosphere (VWR® symphony™, VWR® International LLC, Radnor, PA). All media components and reagents were obtained from Gibco® through Life Technologies (Carlsbad, CA).

Cell Viability Assay

Cells at passage number 22 were used for the proliferation assays. Passage number refers to the number of times the cell line has been re-plated and allowed to grow back to confluency (maximum density). The effects of test compounds, chlorogenic acid

and neochlorogenic acid, on the cellular proliferation and viability of Caco-2 cells were measured using the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega Corp. Madison, WI). This assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter reagent contains a novel tetrazolium compound and an electron coupling reagent (phenazine ethosulfate; PES). The tetrazolium compound is reduced by living cells into a colored formazan product which is soluble in culture medium. The quantity of this product, measured at absorbance of 490nm by a plate reader, is directly proportional to the number of living cells in the culture.

To perform this assay, (3×10^3) Caco-2 cells in 100 μ l of Dulbecco's modified Eagles medium (DMEM) media containing 10% fetal bovine serum were seeded in the wells of a 96 well plate and incubated at 37°C and 5% CO₂. Cells were incubated for 24 hours to allow stable attachment before exposure to test compounds. The used media was removed and replaced with test media containing chlorogenic acid and neochlorogenic acid at treatment concentrations of 0, 150, 300, and 500 μ mol designated as time point 0.

Viability measurements were made at 0, 24, 48, and 72 hours after the addition of treatment. This was accomplished by adding 20 μ l of CellTiter reagent directly to the treatment wells containing cells and wells containing only the test media (sample control). A negative control was used to measure the viability of Caco-2 cells in the absence of test compounds. Experiments were conducted using quadruplicate determinations for each concentration for a test compound per plate. Microplates were incubated for 4 hours after the addition of the reagent, before measuring the absorbance

at 490nm with the plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT). After all absorbance readings were made, data was corrected for the background absorbance of the control media as well as the sample control media which contained chlorogenic acid or neochlorogenic acid but no cells. Absorbances were then converted into cell numbers using an equation from the Caco-2 cell standard curve completed earlier in which a serial dilution of cells was prepared in triplicate in a 96 well plate which ranged from 350 cells per ml to 70,000 cells per ml (Figure 1.). This was accomplished by seeding cells as in the actual experiment, adding CellTiter reagent directly to wells, and then measuring the absorbances of these cells. The results were plotted to compare the change in absorbance as the total cell number increased. From this standard curve, an equation of $y=29676.18x$ with an R^2 value of 0.99 was obtained. Calculated cell numbers were then used to plot cell growth curves.

Light Microscopy of Cells

Cell morphology was observed using a Nikon Eclipse E400 light microscope with Nikon Camera Head DS-Fi1. Cells were used to visualize and describe morphology changes. After 24 and 48 hours of treatment with chlorogenic acid and neochlorogenic acid, cells were harvested and an aliquot was taken from the cell suspension. A portion of the aliquot was then stained with trypan blue dye to provide contrast and determine viable from non-viable cells. 10 μ l of the aliquot was then pipetted onto a slide and covered with a cover-slip. The prepared slides were viewed under low power magnification until an acceptable viable cell was identified. All viable cells located on the slide were examined, after which the most representative cell with the clearest image was chosen. The cell of interest was then viewed under higher power (400x). Using the

Nikon camera software, contrast and color was adjusted to provide the best possible image, after which, a still shot was taken and saved for later viewing.

Statistical Analysis

Data were analyzed using Statistical Analysis System (Version 9.2; SAS Institute Inc., Cary, NC). Results were analyzed using one-way analysis of variance with an alpha level of 0.05, followed by Fisher's least significant difference (LSD) test. Data was presented as means \pm standard error of the mean (SEM).

Chapter IV. Results

Neochlorogenic Acid and Chlorogenic Acid Inhibit Proliferation of Caco-2 Cells

Treatment of Caco-2 cell line with both chlorogenic acid and neochlorogenic acid resulted in a significant reduction in cell proliferation in comparison to the untreated control (Table 2, Figure 3. (a) & (b)). At 24 hours, all treatments and concentrations (150, 300, 500 μ mol) significantly decreased the viable cell number compared to the control. At concentrations of 150, 300, and 500 μ mol, the number of living cells decreased by 63.7, 90.1, and 85.7% for chlorogenic acid and 69.7, 90.2, and 85.7% for neochlorogenic acid respectively compared to the control (Figure 3. (a) & (b)). At 48 hours, chlorogenic and neochlorogenic acid decreased viable cell numbers by 66.1, 94.7, and 92.3% for chlorogenic acid and 33.1, 91.9, and 90.7% for neochlorogenic acid (Figure 3. (a) & (b)). After the final time point of 72 hours, viable cell numbers were reduced by 56.0, 92.2, and 96.0% for chlorogenic acid and 63.8, 92.8, and 94.2 for neochlorogenic acid (Figure 3. (a) & (b)). The 300 and 500 μ mol concentrations for both chlorogenic acid and neochlorogenic acid were the most effective for suppressing cell growth and maintaining the inhibitory effect, being significantly different from the 150 μ mol concentration. The effectiveness of reduced cell proliferation was strongly correlated with the increasing concentration of the treatment.

Light Microscopy of Cells

Figure 3 shows the cell morphology observations of Caco-2 cells treated with chlorogenic acid (Figure 4. (b) & (d)) and neochlorogenic acid (Figure 4. (c) & (e)) at concentrations of 150, 300, and 500 μ mol. Figure 4. (b) & (c) represents Caco-2 cells at 24 hours and Figure 3. (d) & (e) represents Caco-2 cells at 48 hours. It can be seen in all

of the isolated cell images that the Caco-2 cell surfaces become more rough, uneven, and irregularly-shaped as the treatment time and concentration increased.

Chapter V. Discussion

The prune is an important source of polyphenols in the United States. Prunes have the highest antioxidant activity of all fruits, of which neochlorogenic acid is the most dominant followed by chlorogenic acid (16). There are many health benefits associated with the consumption of the prune, including chemopreventative and chemotherapeutic properties (3). The phenolic compounds in the prune have been suspected for these health-promoting activities, but there is little research to confirm this matter. There have been numerous *in-vitro* studies testing the antiproliferative behavior of chlorogenic acid, yet there is little consistency between studies (9, 19, 20, 21, 22, 23, 24, 25). Numerous cell lines, concentrations of treatment, length of study, and methods used have been explored, and therefore various results have been concluded. Also, there has been little to no research on neochlorogenic acid as a phenolic compound with anticarcinogenic properties

Of all the theories for the anticarcinogenic action by the prune, there are many studies that have investigated phenolic compounds for their inhibitory effect on cancer cells. The present study investigated this hypothesis by evaluating the effects of the main phenolic compounds found in the prune, neochlorogenic acid and chlorogenic acid, on cell proliferation and morphology of Caco-2 human colon cancer cells. It was found that neochlorogenic acid and chlorogenic acid significantly inhibited the growth and viability of Caco-2 cells in a concentration-dependent manner compared to the untreated control. Treatment with chlorogenic acid and neochlorogenic acid visibly altered the cell morphology of viable human colon cancer cells as well.

Results found corresponded most with studies by Veeriah *et al.* (23) and Glei *et al.* (25). Both studies utilized the HT29, human colon adenocarcinoma cell line with a doubling time of approximately 25 hours (26). In both studies, it was found that only the highest concentrations of 500 μ mol/L (25) and 289.2 μ mol/L (23) of chlorogenic acid were able to achieve a 50% cell survival number (EC_{50}) after 72 hours of treatment. Studies seeded cells in 96-well microtiter plates, allowed to attach for 24 and 48 hours (25, 23), incubated cells for 24, 48, and 72 hours, added 4'6'-diamino-2-pheynlindole dihydrochloride (DAPI) DNA dye and then measured the percentage of surviving cells by fluorimetric analysis at 360 and 465nm with a 96-well microtiter plate reader. The present study differed only in cell line and the assay used to measure the surviving cells. Despite the common type of cell line, chlorogenic acid appeared to be more effective in reducing the cell number in Caco-2 cells compared to HT29 cells. Even at the lowest concentration of 150 μ mol, both chlorogenic acid and neochlorogenic acid were able to reduce cell growth by 63.7% and 69.7% respectively in as short as 24 hours.

Other *in-vitro* studies that measured cell proliferation found dose-dependent reduction in cell growth at high enough concentrations (9, 19, 20, 21). However, it is important to recognize that these studies used different methods and cell lines that were not derived from the colon. There was only one study (9) found on the effects of neochlorogenic acid on cell proliferation compared to chlorogenic acid. This *in vitro* study tested the effects of neochlorogenic acid and chlorogenic acid on cell proliferation in an estrogen receptor negative MDA-MB-435 breast cancer cell line (9). Net growth of cells compared to control was reduced by 50% at concentrations of 17 and 10mg/L (47.98 and 28.22 μ mol/L) for chlorogenic acid and neochlorogenic acid at 72 hours (9).

Noratto *et al.* suggests that neochlorogenic acid may be a more potent bioactive compound against metastatic cancer than chlorogenic acid (9). Although this may have been the case for the MDA-MB-435 breast cancer cell line, there were no significant differences in percent reduction of cell number between chlorogenic acid and neochlorogenic acid in most concentrations and time points in the present study with Caco-2 human colon cancer cell line. Based on other *in vitro* studies, the concentration of chlorogenic acid needed to inhibit cell growth varied with the type of cell line used. Jin *et al.* (20) tested the effects of chlorogenic acid on Hep3B human liver carcinoma cell line and saw only a 12% reduction in cells at concentrations of 200µg/ml (564.5µmol/L) and above at 24 hours. Compared to the Caco-2 colon adenocarcinoma cell line, liver carcinoma was much less sensitive to chlorogenic acid and required a larger concentration for a lesser cell reduction effect. An *in vitro* study with chlorogenic acid on cell proliferation of U-87 human brain glioblastoma required only 100µmol/L for approximately a 70% reduction of cells in 18 hours (21). The brain glioblastoma cell line was similar to colon adenocarcinoma, with a larger reduction of cell growth with a smaller concentration of chlorogenic acid. A similar study required only 20µmol/L of chlorogenic to suppress HT-1080 human connective tissue fibrosarcoma by 50% in 24hours (22). Human connective tissue fibrosarcoma proved to be much more sensitive than all other cell lines, with the smallest concentration to provide a significant reduction in cell growth.

In the present study, there was significant inhibition of Caco-2 cell viability compared to the control cells for all concentrations of chlorogenic acid and neochlorogenic acid and time points. There was no significant difference in cell

reduction among the chlorogenic acid and neochlorogenic acid treatments at all concentrations and time points, with the exception of the 150 μ mol treatment at 48 hours. The 150 μ mol concentration was the least effective, having a significantly smaller reduction in cell growth compared to the higher concentration treatments and failing to maintain inhibition. Cell growth did rebound at 48 hours for chlorogenic acid and 24 hours for neochlorogenic acid at 150 μ mol. It can be hypothesized that this observation may be due to mitohormesis, the phenomenon of increased net cell growth in the presence of low doses of a compound that inhibits cell proliferation. A low dosage of the inhibitor can actually trigger repair mechanisms in the cell, which can neutralize the effect of this compound as well as repair other non-toxin related effects and therefore increase cell growth (9). In this study, mitohormesis would have occurred after 48 hours in the chlorogenic acid treatment and after 24 hours in the neochlorogenic acid treatment. The higher concentration treatments were significantly higher in cell reduction than the 150 μ mol concentration, yet were not significantly different from one another (Table 2). Although, the 300 μ mol treatment was observed to be more efficient at reducing the Caco-2 cell number through 48 hours compared to the 500 μ mol treatment, the difference was not significant. The 500 μ mol treatments were the most effective, with a time-dependent reduction in cell proliferation and the highest cell viability reduction of 96% for chlorogenic acid and 94.2% for neochlorogenic acid at 72 hours.

Chapter VI. Conclusion

In conclusion, it has been observed in this study that treatment of Caco-2 human adenocarcinoma cells with phenolic compounds from the prune strongly inhibited cell proliferation and changed the morphology of the cells. Only at the higher concentrations of 300 and 500 μ mol, was the inhibitory effect of cell proliferation able to be maintained in a time-dependent manner. The findings from this study suggest that chlorogenic acid and neochlorogenic acid may be colon-cancer suppressive components of the prune.

Appendix

Figure 2. CellTiter 96[®] AQueous One Solution Caco-2 cell standard curve

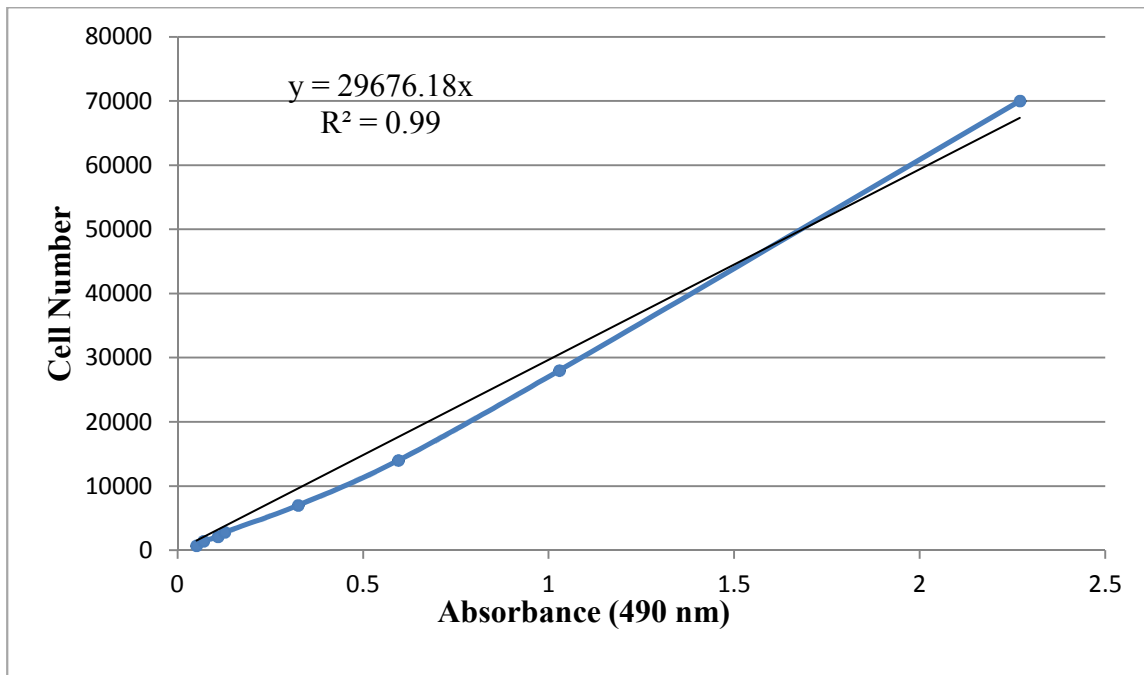
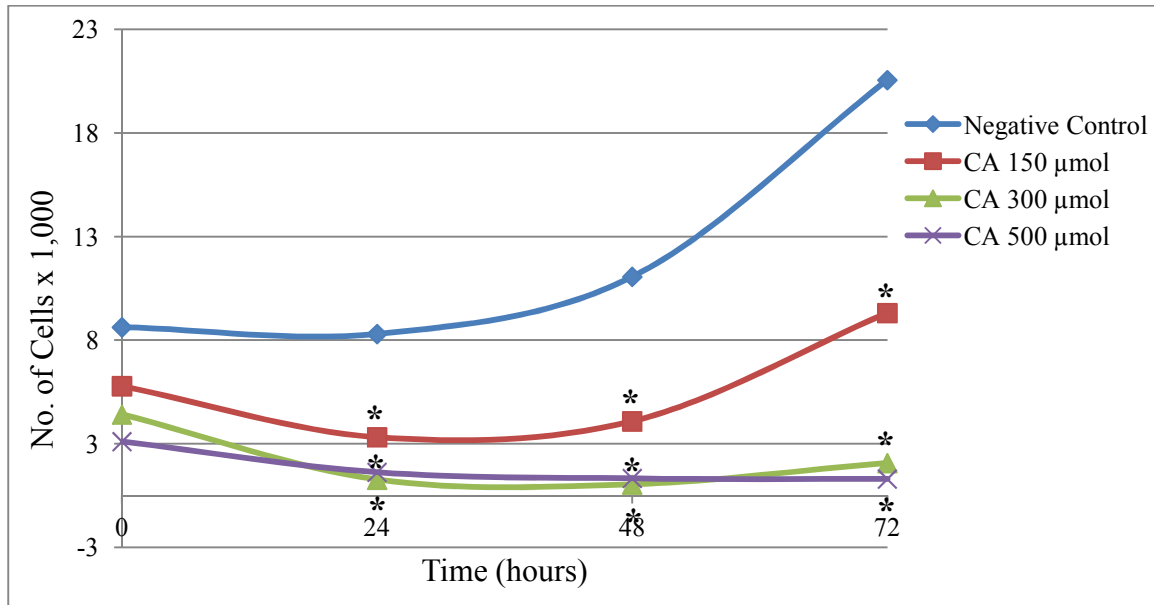
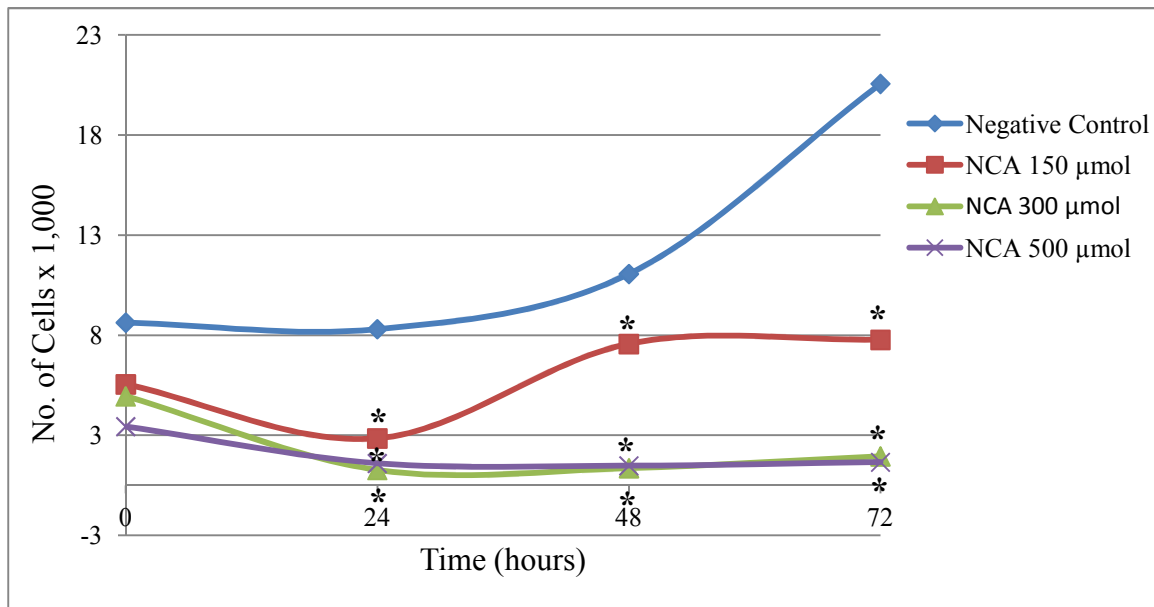


Figure 3. (a) Chlorogenic acid effects on CaCo-2 cell proliferation



Different concentrations of Chlorogenic Acid (CA) at each incubation time were compared against the negative control using Dunnett's test. Points marked with (*) represent significant differences from the control ($p < 0.05$).

(b) Neochlorogenic acid effects on Caco-2 cell proliferation



Different concentrations of Neochlorogenic Acid (NCA) at each incubation time were compared against the negative control using Dunnett's test. Points marked with (*) represent significant differences from the control ($p < 0.05$).

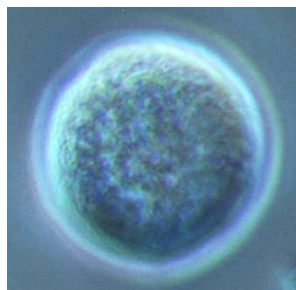
Table 2. Chlorogenic and neochlorogenic acid percent reduction in cell viability

Treatment	Conc. (μmol)	24 Hours		48 Hours		72 hours	
		Percent Reduction	SEM	Percent Reduction	SEM	Percent Reduction	SEM
Chlorogenic Acid	150	63.7 ^a	3.99	66.1 ^b	6.00	56.0 ^a	3.73
	300	90.1 ^b	0.68	94.7 ^c	0.72	92.2 ^b	0.20
	500	85.7 ^b	0.64	92.3 ^c	1.03	96.0 ^b	0.17
Neochlorogenic Acid	150	69.7 ^a	2.88	33.1 ^a	4.14	63.8 ^a	5.67
	300	90.2 ^b	1.46	91.9 ^c	0.19	92.8 ^b	0.84
	500	85.7 ^b	1.85	90.7 ^c	0.61	94.2 ^b	0.37

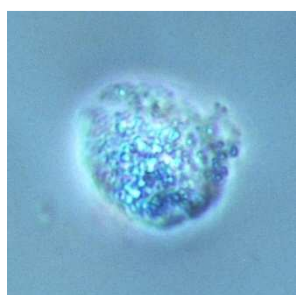
Data represents the percent reduction (%) compared with negative control and the standard error of the mean (SEM) (n = 3). Values in a column without common superscripts are significantly different ($p < 0.05$).

Figure 4. Morphology effects of treatments with different concentrations of chlorogenic acid and neochlorogenic acid

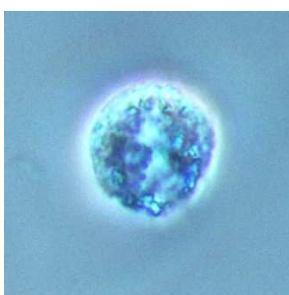
(a) Untreated Caco-2 cell



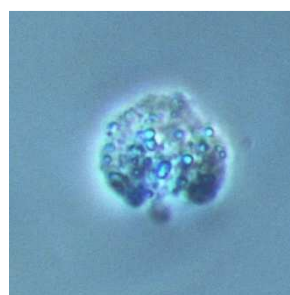
(b) Chlorogenic acid treatment for 24 hours



(i) 150

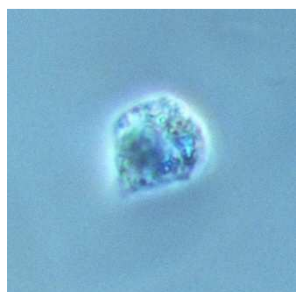


(ii) 300 μmol

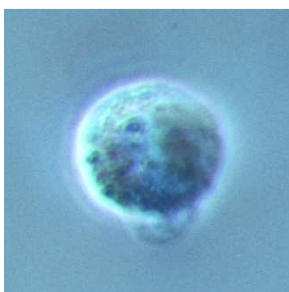


(iii) 500 μmol

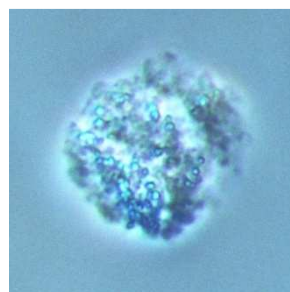
(c) Neochlorogenic acid treatment for 24 hours



(i) 150

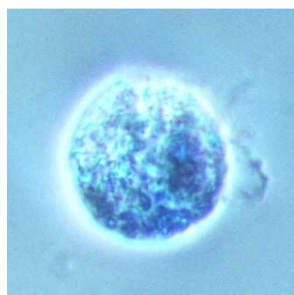


(ii) 300 μmol

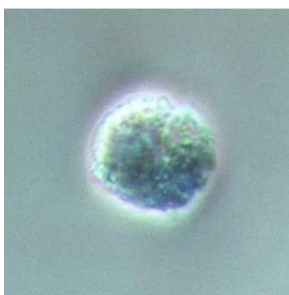


(iii) 500 μmol

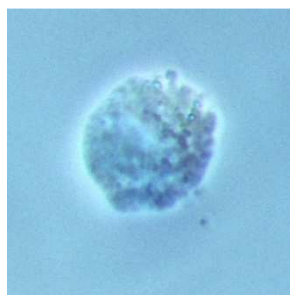
(d) Chlorogenic acid treatment for 48 hours



(i) 150

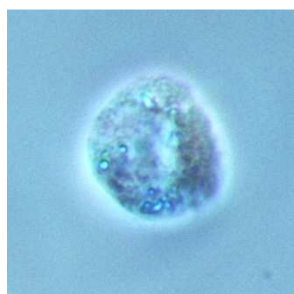


(ii) 300 μmol

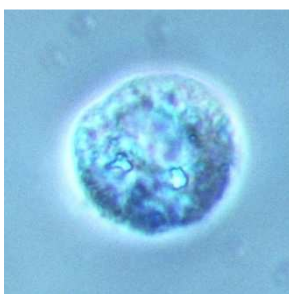


(iii) 500 μmol

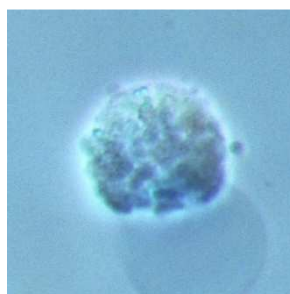
(e) Neochlorogenic acid treatment for 48 hours



(i) 150



(ii) 300 μmol



(iii) 500 μmol

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