ROLE OF SELECTED FRUITS AND PHYTOCHEMICALS IN CANCER PREVENTION: MECHANISMS OF ACTION

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ROLE OF SELECTED FRUITS AND PHYTOCHEMICALS IN CANCER PREVENTION: MECHANISMS OF ACTION

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Epidemiological studies have shown that high consumption of fruits and vegetables is associated with reduced risk of cancer. Phytochemicals, mainly phenolics and flavonoids, have been suggested to be responsible for these health benefits. However, the molecular mechanisms of the anticancer effects of fruits and vegetables are not fully understood.

Fourteen grape varieties were analyzed for the profiles of total phenolics, total flavonoids, and trans-resveratrol. Phytochemicals in those grapes have potent antioxidant and antiproliferative activities. In addition, thirteen grape varieties and fourteen common phytochemicals were evaluated for their ability to induce mammalian phase II detoxification enzyme – quinone reductase. Grape phytochemicals had strong activity in quinone reductase induction and potent antiproliferative activities toward Hepa1c1c7 cells. These results support the hypothesis that phytochemicals prevent cancer by acting as free radical scavengers and/or inducers of phase II detoxifying enzymes in the initiation stage, and/or as cell proliferation suppressors in the promotion/progression stages.

Combinations of selected fruits and phytochemicals were studied for their antioxidant activity, antiproliferative activity, and induction of quinone reductase. The combination of apple extracts and quercetin 3-β-D-glucoside (Q3G) exhibited more potent antiproliferative activity when compared to the apple extracts and Q3G alone. The combination of grape extracts and quercetin, genistein, and resveratrol showed...
higher induction activity of quinone reductase than grape extracts and phytochemicals alone.

In conclusion, we have demonstrated that the additive and synergistic effects of phytochemicals in fruits are responsible for their potent antioxidant activity, antiproliferative activity, and phase II detoxifying enzyme induction activity, and that the health benefit of a diet rich in fruits is attributed to the complex mixture of phytochemicals and their interactions present in those foods.
BIOGRAPHICAL SKETCH

Jun was born on October, 1969 in Wuhan, Hubei, China. In June of 1987 he graduated from Huazhong Agricultural University affiliated High School in Wuhan, China. The following September he enrolled in the Department of Soil and Agricultural Chemistry at Huazhong Agricultural University. After getting his Associate Degree in Brewing two years later, he accepted a job at Wuhan Seasonings and Food Company, and worked there for three years.

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Dedicated to:

My parents, Shidong Yang and Yuying Lei

My Wife, Yan Mei, our daughter, Jingke.

and my sister’s family
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CHAPTER ONE

LITERATURE REVIEW

1.1. Introduction

Cardiovascular diseases and cancer are the leading causes of death in the United States and the most industrialized countries. Both diseases are thought to be partially the result of oxidative stress, which can damage biomolecules. Based on the American Cancer Society (ACS) estimation, in 2007 a total of 1,444,920 new cancer cases are expected in the United States, and 559,650 Americans are expected to die of cancer, more than 1,500 people a day (American Cancer Society, 2007). Cancer is the second most common cause of death in the US, exceeded only by heart disease. In the US, cancer accounts for 1 of every 4 deaths. The three leading cancer types among new cancer cases are cancers of the genital system, with 33% prostate cancers in male and 31% breast cancers in female, followed by about 13% cancers of lung and bronchus, and about 11% cancers of colon and rectum. Diet ranks second only to smoking as a leading contributor to cancer incidence and mortality. Data suggest that as much as one third (range 20-42%) of all cancer deaths in the United States could be avoidable by dietary modification (Doll and Peto, 1981; Willett, 1995).

The federal government’s 2005 Dietary Guidelines for Americans provides advice on healthy food choices for the population over the age of two and about how a proper diet can promote health and reduce the risk of major chronic diseases (USDA, 2005). The key recommendation about the types and amounts of foods to eat is to make smart choices from every food group, and get the most nutrition out of the calories consumed. A healthy eating plan from 2005 Dietary Guidelines for Americans
emphasizes fruits, vegetables, whole grains, and fat-free or low-fat milk products. Fruits and vegetables contain many phytochemicals with positive biological effects, including phenolics, flavonoids, isoflavonoids, thiols, carotenoids, ascorbic acid, tocopherols, sulforaphane, indoles, isothiocyanates, and glucosinolates, which may protect against cancer through a variety of mechanisms. Therefore, the National Academy of Sciences of the United States in 1982 had guidelines in their report on diet and cancer, strengthening the importance of fruits and vegetables (NAS, 1982). The 5-a-Day program launched by National Academy of Sciences in 1989 was developed as a tool to increase public awareness of the health benefits of fruit and vegetable consumption and promote adequate intake of known vitamins (NAS, 1989). However, the median consumption of fruit and vegetables among US adults is 3.4 servings/day, and only approximately 23% of US adults consume the recommended 5 servings of fruit and vegetables every day (Subar et al., 1995). The majority of adults fall well short of meeting the guidelines. Thus, the Dietary Guidelines for Americans 2005 recommends 9-13 servings of fruits and vegetables daily.

1.2. Oxidative Stress and Cancer

1.2.1. Free radicals: Sources and Reactions

Normal cellular metabolism is well established as the source of endogenous reactive oxygen species (ROS: singlet oxygen, superoxide anion radical, peroxide anion, and hydroxyl radical) and reactive nitrogen species (RNS: nitric oxide and peroxynitrite), which account for the background levels of oxidative and nitrogen damage detected in normal tissue. On the other hand, exposure of normal cells to exogenous sources of carcinogens is also known to generate ROS and electrophilic stress and has a profound impact on the survival of living organisms. Endogenous oxidative events are produced by the Fenton reaction, mitochondrial electron transport,
respiratory bursts, oxygenating enzymes, reductive cleavage of peroxides, and xenobiotic metabolism. External sources of oxidants include UV radiation, natural radioactive gases, environmental pollutants such as automobile and cigarette smoke, as well as xenobiotics such as polycyclic aromatic hydrocarbons and phorbol esters. Oxidation is a multistage process, and its complexity forms the basis for multiple mechanisms of pathogenesis (Ames et al., 1993). Oxidative damage to macromolecules such as lipids, DNA, and proteins is an inevitable consequence of cellular metabolism, with increased levels following toxic insult.

1.2.2. Free Radical Defense Systems in Organisms

Changing the balance between oxidant damage and antioxidant protection alters the risks and benefits coming from free radicals. When the level of ROS exceeds the antioxidant capacity of the cell, intracellular redox homeostasis is altered and oxidative stress ensues (Halliwell, 1999). The crucial issue is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions in the body. To prevent or slow down the oxidative stress induced by free radicals, sufficient amounts of antioxidants need to be consumed (Ames et al., 1993). Generally, three defense systems exist in the organism to prevent oxidant-caused toxicity and damage, including antioxidant enzymes, metal-chelating proteins, and antioxidants (Bourgeois, 2003). Antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase (GSH-Px), which are involved in protecting the sites that are particularly preventive to oxidative stress. Metal-chelating proteins with antioxidant capacity such as transferrin are associated with the Fenton reaction. Endogenous antioxidants include vitamin C, vitamin E, and β-carotene. Normally, there exists a balance in vivo between the production of ROS and the capacity of the endogenous antioxidant defense system. However, endogenous antioxidant defense
are usually inadequate to scavenge ROS completely. Disturbance of this balance will cause oxidative stress, resulting in cellular damage to macromolecules, which is implicated in degenerative diseases (Ames et al., 1993). Dietary phytochemicals have been recognized as potentially being involving in the oxidant-antioxidant balance. Fruit and vegetables contain a wide variety of antioxidants such as phenolics and carotenoids that may help protect cellular systems from oxidative damage, and thus lower the risk of chronic diseases.

1.2.3. Oxidative Stress and Disease

Overwhelming evidence indicates that oxidative stress can lead to cell, tissue, and organ injury, resulting in oxidants- or free radicals-caused diseases such as CVD, atherosclerosis, cancer, cataracts, type 2 diabetes, and aging (Ames et al., 1993). Free radicals are constantly generated and quenched in our body. In normal metabolism, the level of oxidants and antioxidants in humans are maintained in balance, which is important for sustaining optimal physiological conditions (Temple, 2000). However, in a highly oxidative environment, accumulated long-term damage by free radicals is observed. It has been estimated that there are 10,000 damaging hits to DNA per cell per day in humans (Ames et al., 1993). It appears that certain phytochemicals such as phenolics may chemically retard the pathological processes of chronic diseases caused by oxidative stress such as cancer, CVD, autoimmunity, postischemia injury, trauma, and aging, which has led to the hypothesis that the consumption of phytochemicals may prevent or ameliorate these disease processes.

Much epidemiological data, animal work, and cell culture evidence supports the health benefits of plant-based foods such as fruit, vegetables, whole grains, and nuts in the prevention of cancer and cardiovascular disease (CVD) (Block et al., 1992;
Joshipura et al., 2001; Hu and Willett, 2002). Updated studies have demonstrated that the mechanisms of action of phytochemicals in the prevention of cancer go beyond antioxidant activity of scavenging free radicals (Liu 2003). Phytochemicals, especially for phenolics, in fruits, vegetables, whole grains, and other plant-based foods can have complementary and overlapping mechanisms of action through antioxidant activity and scavenging free radicals, chelating metal ions (Liu 2004); inhibition or reduction of different enzymes such as telomerase (Naasani et al., 2003), cyclooxygenases (Laughton et al., 1991), lipoxygenases (Sadik et al., 2003), xanthine oxidase (Van Hoorn et al., 2002), and protein kinases (Agullo et al., 1997); induction of enzyme activities in detoxification, oxidation, and reduction; modification of platelet activity (Dutta-Roy, 2002; Birt et al., 2001), homocysteine concentration (Brattstrom et al., 1988), and blood pressure (Appel et al., 1997); interaction with signal transduction pathways (Wiseman et al., 2001; Spencer et al., 2003), cell receptors (Mueller et al., 2004), and caspase-dependent pathways (Way et al., 2005); regulation of gene expression in cell proliferation and apoptosis and hormone metabolism; interfering with cyclin-dependent regulation of the cell cycle (Fischer and Lane, 2000); inhibition of nitrosamine formation; provision of substrate for formation of antineoplastic agents; dilution and binding of carcinogens in the digestive tract (Steinmetz and Potter, 1991a); and antibacterial and antiviral effects (Dragsted et al., 1993). Convincing evidence suggests that a change in dietary behavior such as increasing consumption of fruit, vegetables, and whole grains is a practical strategy for significantly reducing the incidence of some chronic diseases (Willett 1994). On the other hand, the identification of dietary patterns in populations with different disease incidence will be useful in understanding the relationship between diet and associated diseases.
1.3. *Health Benefits of Fruits and Vegetables*

Epidemiological evidence suggests that dietary phytochemicals present in fruits and vegetables can reduce cancer risk. In the first comprehensive review of the relation between consumption of fruits and vegetables and cancer, (Steinmetz and Potter, 1991a; Steinmetz and Potter, 1991b) concluded that diets rich in fruits and vegetables are related consistently to reduced risk for a variety of tumors, especially epithelial cancers of the respiratory and gastrointestinal tract (e.g. lung, esophagus, stomach, and colon). After investigating approximately 200 studies, (Block et al., 1992) examined the relationship between fruits and vegetables intake and cancers of the lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas, and ovary. The consumption of fruit and vegetable was found to have a significant protective effect against different cancers in 128 of 156 dietary studies. The risk of cancer for most cancer sites was twice as high in persons whose intake of fruit and vegetables was low compared with those with high intake. Fruit was significantly protective in cancers of the esophagus, oral cavity, and larynx. A protective effect from fruits and vegetables was found to be strong for breast cancer. A prospective study involving 9959 men and women (age 15–99 y) in Finland had an inverse association between the intake of flavonoids and the incidence of all sites of cancer (Knekt et al., 1997). Based on the international surveys, World Cancer Research Fund (WCRF, 1997) reported that convincing evidence supported that high intake of vegetables decreases the risk of cancers of the mouth and pharynx, esophagus, lung, stomach, colon, and rectum; that it probably decreases the risk of cancers of the larynx, pancreas, breast, and bladder; and that it possibly decreases the risk of cancers of the liver, ovary, endometrium, cervix, prostate, thyroid, and kidney. In a cohort study of atomic-bomb survivors in Japan, the positive relationship between high consumption of vegetables and fruit and lowered risk of bladder cancer was found (Nagano et al.,
A prospective study performed by (Feskanich et al., 2000) found that higher fruit and vegetable intakes were associated with lower risks of lung cancer in women but not in men. A population-based case-control study examined the relationship between intake of fruits and vegetables and a reduced risk in breast cancer in women in Shanghai, China (Malin et al., 2003). The pre-menopausal women who ate more dark yellow-orange vegetables and more citrus fruits tended to have lower breast cancer risk. Increased plasma levels of quercetin following a meal of onions were accompanied by increased resistance to strand breakage by lymphocyte DNA and decreased levels of some oxidative metabolites in the urine (Boyle et al., 2000). Intake of quercetin from onions and apples was found to be negatively correlated with lung cancer risk in Hawaii (Le Marchand et al., 2000). Fruit and vegetable consumption also appears to have a protective effect against coronary heart disease (CHD) (Joshipura et al., 2001). About 84,000 women were followed for 14 years and 42,000 men were followed for 8 years. It was found that people who ate the highest amount of fruits and vegetables had a 20% lower risk for CHD, and the lowest risks were observed in people who consumed more green leafy vegetables, and fruits rich in vitamin C. In the Zutphen Elderly Study, an approximately 30 mg/day intake of flavonoids was associated with around a 50% decreased risk in CHD mortality rate compared with individuals who had a less than 19 mg flavonoid/day intake (Hertog et al., 1993) In a cohort study of 5,133 men and women aged between 30 and 69 years conducted in Finland, onions and apples rich in dietary flavonoids, were related to a reduced risk of coronary mortality. People in the highest quartile for apple or onion intake showed an approximately 50% reduction in coronary mortality (Knekt et al., 1996). In a prospective study of 34,492 postmenopausal women in Iowa, flavonoid intake was associated with a lowered risk of cardiovascular disease in the group with the highest flavonoid intake (Yochum et al., 1999).
Compelling support for the involvement of free radicals in chronic disease development comes from epidemiological, animal, and cell culture studies showing an enhanced antioxidant status is associated with lowered risk for certain diseases. Many constituents from fruits and vegetables, including phenolics, flavonoids, vitamins C and E, carotenoids, folic acid, and mineral micronutrients, have antioxidant activity, contributing to their health promotion properties. The presence of phenolics in fruit and vegetables is now being recognized as playing an important role in disease prevention, possibly through their effect on oxidative damage beyond antioxidants (Heinonen et al., 1998; Record et al., 2001; Liu, 2003). Also, consumption of flavonoids in the diet was shown to be inversely associated with morbidity and mortality resulted from CHD (Hertog et al., 1993; Knekt et al., 1996). In response to oxidative/electrophilic stress, cells initially activate defense mechanisms that lead to coordinated activation of a battery of defensive genes that protect cells against oxidative/electrophilic stress. For instance, Induction of phase II enzymes in general leads to protection of cells/tissues against exogenous and/or endogenous carcinogenic intermediates. Phenolics found in fruits and vegetables are potential phase II enzymes inducers.

A diet high in fruits and vegetables may not only help prevent cancer and heart disease, but it may also help protect against a variety of other diseases. For instance, a diet high in fruits and vegetables may help protect against Alzheimer disease, cataracts, diabetes, and even asthma (Willett, 2002; Woods et al., 2003). It is believed that the effect of whole fruits and vegetables tends to be more pronounced than that of the individual dietary constituents they provide. This is due mainly to the combination of phytochemicals and other dietary constituents in fruits and vegetables.
1.4. Health Benefits of Grapes and Wines

Grapes, the largest single fruit crop grown in the world, are rich in phenolic acids, flavonoids, and resveratrol, which have been suggested to be responsible for the health benefits of grapes. At the beginning of the nineties, the ‘French paradox’ signified paradigmatically the health benefits due to regular and moderate consumption of red wine (Frankel et al., 1993a). That a diet high in saturated fat accompanied by regular consumption of red wine is associated with a low risk of CHD is called the French paradox (Renaud and Lorgeril, 1992). Lately, it was extensively demonstrated that the observed epidemiological relationship between wine intake and health benefits was attributable to the red wine phenolics (Kinsella et al., 1993; Frankel et al., 1993a). Grapes and wines, especially the skin, seeds, and red wine, are a reliable and rich source of phytochemicals, particularly of phenolics, whose individual and summated actions are suggested to be responsible for health benefits.

1.4.1. Epidemiological Evidence

Epidemiological studies have indicated that red wine protects against many diseases including cancer (Kimura et al., 1983; Jang et al., 1997; Clement et al., 1998; Das et al., 1999; Pendurthi et al., 1999; Hsieh et al., 1999a; Banerjee et al., 2002). Grapes and wines are rich in dietary flavonoids. Consumption of flavonoids was shown to be inversely associated with morbidity and mortality resulted from cancers and CHD (Hertog et al., 1993; Knekt et al., 1996; Knekt et al., 1997). A case-control study showed that increased consumption of grapes is associated with reduced risk of cancer (Zheng et al., 1993). In another case-control study, (Freudenheim et al., 1995) found that a lowered risk of breast cancer in western New York was associated with wine intake.
Epidemiological evidence from various populations around the world supports the ideas that moderate wine consumption is associated with increased longevity and reduced atherosclerotic mortality (St. Leger et al., 1979; Langer et al., 1992; Gronbeak and Sorensen, 1996; Rimm, 1996; Gronbeak, 1997; Criqui, 1998; Wannamethee and Shaper, 1999). A strong negative association between wine consumption and CHD mortality in eighteen developed countries was observed (St. Leger et al., 1979). The Danish study examined more than 13,000 men and women with age from 30 to 70 to look at the health benefits of wine and other types of alcoholic beverages (Gronbaek et al., 1995). It showed that people who drank only wine as an alcohol source had 50% of the risk of dying of people who never consumed wine after a 12-year heart study, and drinking other types of alcoholic beverages lacked benefits.

1.4.2. In Vitro and In Vivo Studies

1.4.2.1. Antioxidant Activity

Grapes and wines, especially grape seeds and red wines, have been found to have potent antioxidant activity. The antioxidant effects of grape phenolics were determined in a number of different systems (Kanner et al., 1994). It was concluded that effective antioxidants were found in all grape varieties, which corresponded to the concentration of phenolics in the system. The antioxidant activity of red grapes has been correlated with the phenolics and/or flavonoid content (Teissedre et al., 1996; Meyer et al., 1997; Mayer et al., 2001). In vitro oxidation positively correlated with the content of total phenolics, anthocyanins, and flavonols after investigation of phenolic extracts from fourteen different types of fresh grapes. Red wine is a concentrated source of dietary phenolic acids and flavonoids. Paganga et al. (1999) calculated that the antioxidant activity in 1 glass of red wine (150 mL) was equivalent
to that found in 12 glasses of white wine, 2 cups of tea, 5 apples, 5 (100-g) portions of onion, 500 mL of beer, or 7 glasses of orange juice.

A positive correlation has been observed between the antioxidant activity of wine and RSV level (Alonso et al., 2002). RSV has been shown to possess a stronger 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and hydroxyl radical-scavenging capacity than propyl gallate, vitamin E, and vitamin C (Soares et al., 2003).

Anthocyanins possess antioxidant capacity, and this is an important physiological function (Rice-Evans et al., 1995; Van Acker et al., 1996; Satué-Gracia et al., 1997; Wang et al., 1999; Degenhardt et al., 2000; Espin et al., 2000; Ehlenfeldt and Prior, 2001; Kalt et al., 2001; Connor et al., 2002; Moyer et al., 2002). There was a positive correlation between anthocyanin content and antioxidant activities of red grape extracts (Meyer et al., 1997), grape juices (Frankel et al., 1998), and red wines (Burns et al., 2000). Consumption of an anthocyanin-repleted diet in rats significantly improved ($p < 0.01$) plasma antioxidant capacity and decreased ($p < 0.001$) vitamin E deficiency-enhanced hydroperoxides and 8-oxo-deoxyguanosine concentrations in liver (Ramirez-Tortosa et al., 2001). Cyanidin 3-O-β-D-glucoside (C3G) functions as a potent antioxidant under oxidative stress in rats (Tsuda et al., 2000). Cyanidin-DNA copigmentation might be a possible defense mechanism against oxidative damage of DNA and may have in vivo physiological function attributable to the antioxidant ability of anthocyanins (Sarma and Sharma, 1999). It was found that antioxidant capacity of proanthocyanidins from grape seed is 20 times greater than vitamin E and 50 times greater than vitamin C (Uchida, 1980).
1.4.2.2. Antiproliferative Activity

Grapes and wines are rich source of RSV, being found to inhibit cellular events related to initiation, promotion, and progression of carcinogenesis both in vitro and in vivo models, including breast cancer (Gehm et al., 1997; Clement et al., 1998; Mgbonyebi et al., 1998; Subbaramaiah et al., 1998; Schneider et al., 2000; Ahmad et al., 2001; Soleas et al., 2001; Waffo-Teguo et al., 2001; Pozo-Guisado et al., 2002); prostate cancer (Hsieh and Wu, 1999; Mitchell et al., 1999; Hsieh and Wu, 2000; Kampa et al., 2000; Narayanan et al., 2002); liver cancer (Ciolin et al., 1998; Carbó et al., 1999; De Ledinghen et al., 2001; Sun et al., 2002b); Colorectal and intestinal cancers (Schneider et al., 2000; Wolter et al., 2001; Delmas et al., 2002; Wolter et al., 2002); skin cancer (Jang and Pezzuto, 1999; Soleas et al., 2002; Adhami et al., 2003); lung cancer (Hecht et al., 1999; Kimura and Okuda, 2001); blood cancer (Surh et al., 1999; Gautam et al., 2000; Dörrie et al., 2001; Gao et al., 2002; Tsan et al., 2002); and thyroid cancer (Shih et al., 2002). RSV functions as antiproliferative activities in a dose-dependent manner (Jang et al., 1997; Lu and Serrero, 1999). The antiproliferative activity of RSV was observed in a number of cancer cell lines and may be due partly to the induction of apoptosis (Surh et al., 1999; Ding and Adrian, 2002; Joe et al., 2002), and to the arrest of the cell cycle (Wolter et al., 2002; Larrosa et al., 2004; Castello and Tessitore, 2005). The molecular mechanisms associated with the anti-proliferative effects in cancer cells involve the activation of p53 (Huang et al., 1999), suppression of nuclear factor-κB (NF-κB) (Banerjee et al., 2002), and activator protein-1 (AP-1) (Kundu et al., 2004). The antiproliferative activities of RSV may also be explained by the direct inhibition of ribonucleotide reductase through efficiently scavenging the tyrosyl radical of the small protein, which supplies proliferating cells with deoxyribonucleotides required for DNA synthesis (Reichard, 1987; Fontecave et al., 1998; Schneider et al., 2000). RSV has also been found to inhibit DNA polymerase
(Sun et al., 1998), and ornithine decarboxylase, a key enzyme of polyamine biosynthesis that is enhanced in cancer growth (Schneider et al., 2000). The effects of RSV in breast cancer cell lines are inconsistent. For instance, it was found that RSV enhances MCF-7 cell growth (Basly et al., 2000), and induced growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in HL-60, SW-480, HCE-7, Seg-1, Bic-1, and MCF-7 human cancer cell lines (Joe et al., 2002). On the other hand, RSV was reported to slow down the proliferation of several human malignant cell lines (Della Ragione et al., 1998; Hsieh et al., 1999a). RSV induced significant dose-dependent inhibition in human oral squamous carcinoma cell (SCC-25) growth and DNA synthesis, and decreased viability and DNA synthesis capability of human promyelocytic leukemia (HL-60) cells through an induction of apoptosis by the Bcl-2 pathway (Surh et al., 1999). After investigation of the effect of RSV on growth, induction of apoptosis, and modulation of prostate-specific gene expression in DU-145, JCA-1 and PC-3 human CaP cells, Hsieh and Wu’s study (1999) suggested that RSV negatively modulates CaP cell growth by affecting mitogenesis and inducing apoptosis. RSV might be responsible for the growth inhibition of LNCaP cells by modulation of multiple signaling pathways (Narayanan et al., 2002). Potter et al. (2002) have suggested that the antiproliferative activities of RSV on cancer cells is the consequence of its conversion to piceatannol by CYP1B1. After inoculation into mice, the trans-RSV-3-O-D-glucoside (piceid) also inhibited the proliferation of Lewis lung carcinoma (LLC) cells, but only at a concentration of 1000 μM, while 2,3,5,4'-tetrahydroxystilbene-2-O-D-glucoside was more effective with an IC₅₀ of 81 μM. They concluded that the antitumor and antimetastatic activity of the stilbene glucosides, piceid and 2,3,5,4'-tetrahydroxystilbene-2-O-D-glucoside might be due to inhibition of DNA synthesis in LLC cells and angiogenesis of human umbilical vein endothelial cells (Kimura and Okuda, 2001). It was also reported that pterostilbene, a natural
methoxylated analog of RSV, suppressed the development of mammary lesions with an ED_{50} of 4.8 \mu M in a mouse mammary gland organ culture (Rimando et al., 2002).

Cyanidin, pelargonidin, and delphinidin showed antiproliferative activity in two estrogen-dependent human breast cancer cell lines (MCF-7 and BG-1), but not in the estrogen receptor-negative MDA-MB-231 cell line (Schmitt and Stopper, 2001). Cyanidin and delphinidin contribute to the potent inhibitors of the epidermal growth-factor receptor and were found to inhibit the growth of human vulva carcinoma cell line A431 in vitro (Meiers et al., 2001).

1.4.2.3. Inhibition of Lipid Oxidation

The oxidation of low-density lipoproteins (LDL) is an important event in the development of atherosclerosis. Several studies have indicated that grape, wine, and grape seed extracts inhibit the LDL oxidation (Mangiapane et al., 1992; Frankel et al., 1993a; Frankel et al., 1995; Teissedre et al., 1996). Frankel et al. (1993b) were first to demonstrate that trans-RSV reduced the copper-catalyzed oxidation of human LDL. In vivo, RSV blocked copper-catalyzed LDL oxidation in healthy human subjects by 70\% and 81\%, respectively. RSV was demonstrated to inhibit ROS production and lower lipid peroxidation, though not to the extent achieved by vitamin C, in blood platelets (Olas and Wachowicz, 2002). RSV has been also found to inhibit copper-initiated and, to a lesser extent, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-initiated oxidation of porcine LDL (Belguendouz et al., 1997; Frémont et al., 1999). LDL peroxidation was blocked better by RSV than by a phenolic extract from red wine. The effect of wine extracts, after 1,000 times dilution, in inhibiting the oxidation of isolated human LDL far exceeded that of the vitamin C and E (Frankel et al., 1993b). The effects of pretreatment with the anthocyanins (delphinidin, cyanidin,
and their glycoside and rutinoside derivatives) on induction of DNA damage were evaluated in rat smooth muscle and in rat hepatoma cell lines using the Comet test (Lazzé et al., 2003). The results showed that anthocyanins were effective against cytotoxicity, DNA single strand breaks formation and lipid peroxidation induced by tert-butyl-hydroperoxide.

1.4.2.4. Inhibition of Platelet Aggregation

The accepted mechanism of cardioprotection by phenolics from grapes and wines is inhibition of platelet aggregation. Quercetin, the predominant flavonoid in grape, suppressed platelet aggregation in vitro (Chung et al., 1993), and lowered thromboxane synthesis in vivo (Tzeng et al., 1991). RSV was also reported to be an inhibitor of platelet aggregation (Bhat et al., 2001a). RSV blocks adenosine diphosphate (ADP), collagen, and thrombin-stimulated human platelet aggregation in vitro (Wang et al., 2002). Pretreatment of platelets with RSV was found to inhibit lipopolysaccharide (LPS) and LPS plus thrombin-stimulated platelet adhesion to collagen and fibrinogen in a nondose-dependent pattern (Olas and Wachowicz, 2002). RSV supplementation decreased rate of platelet aggregation in rabbits when fed with a high cholesterol diet (Dobrydneva et al., 1999). The prevention of calcium influx through the storage operated calcium channels has been suggested as a target for RSV inhibiting thrombin-induced platelet aggregation. RSV was also observed to have vasorelaxation properties. RSV might mediate vasorelaxation in endothelium-intact and endothelium-independent aortic rings via both nitric oxide-dependent and -independent mechanisms (Chen and Pace-Asciak, 1996). The release of NO has been suggested as a mechanism for the reduction of ischemia-reperfusion injury in rat hearts after RSV treatments (Bradamante et al., 2003). The effects of extracts of grape seed (GSD) and grape skin (GSK) on collagen-induced whole blood platelet
aggregation (PA) were examined in vitro (human platelets) and ex vivo (dog platelets) (Shanmuganayagam et al., 2002). The results suggest that the components of GSD and GSK, when present in combination as in red wine, grape juice or in a commercial preparation containing both extracts, have a greater antiplatelet effect than when present individually.

The inhibition of platelet aggregation by grapes and wines was also demonstrated in animal and human studies. A study (Bagchi et al., 1998) indicated that grape seed proanthocyanidin extract showed higher protective effects than vitamin E, C, vitamin E plus C, and β-carotene against 12-O-tetradecanoylphorbol-13-acetate-induced lipid peroxidation and DNA fragmentation in liver and brain tissues, as well as against the production of free radicals in peritoneal macrophages of mice. Studies (Osman et al., 1998; Folts, 2002) showed that 5 ml/kg of red wine or 5-10 ml/kg of purple grape juice but not orange or grapefruit juice inhibited platelet activity, and protected against epinephrine activation of platelets in the dog, monkey, and humans. In a study of feeding subjects with 7mL/kg body weight/day for 14 days, whole juice from purple grapes decreased platelet aggregation, increased platelet-derived nitric oxide release, and suppressed superoxide production (Freedman et al., 2001). After Concord grape juice was given orally (10 mL/kg/day) to subjects, serum antioxidant capacity was increased, and LDL oxidation deceased similarly to that from 400 IU α-tocopherol per day (O'Byrne et al., 2002).

1.4.2.5. Anti-inflammatory Activity

The anti-inflammatory activity may be partially responsible for the chemopreventive and cardioprotective effects of phenolics present in grapes and wines. RSV non-competitively inhibited the activity of cyclooxygenase-1 (COX-1) in
a dose-dependent manner (Shin et al., 1998). In comparison to non-steroidal anti-inflammatory drugs such as aspirin and piroxicam, RSV also suppressed COX-1 hydroperoxidase activity and, to a lesser extent, cyclooxygenase-2 (COX-2) hydroperoxidase activity (Jang et al., 1997). RSV decreased the expression of COX-2 both in \textit{in vitro} and \textit{in vivo} models. It significantly decreased elevated levels of PGD$_2$ in rats (Martín et al., 2004), while it dose-dependently inhibits induced production of PGE$_2$ in human peripheral blood leukocytes \textit{in vitro} (Richard et al., 2005). It was observed that RSV lowered induced-COX-2 activity by inhibiting expression of the enzyme via signal transduction pathways (Kundu et al., 2004), and blocked the inflammatory actions of cytokines, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1\(\beta\) (IL-1\(\beta\)) (Culpitt et al., 2003). RSV also inhibited induced COX-2 activity via targeting the protein kinase C (PKC) signal transduction pathway in human breast epithelial cells, blocking the translocation of PKC into the membrane (Subbaramaiah et al., 1998). Pterostilbene exhibited moderate inhibition of COX-1 with an IC$_{50}$ of 19.8 \(\mu\)M, and was weakly active against COX-2 with an IC$_{50}$ of 83.9 \(\mu\)M, whereas RSV strongly inhibited both isoforms of the enzyme with IC$_{50}$ values of approximately 1 \(\mu\)M (Rimando et al., 2002). Anthocyanins present in grapes have been also reported to be anti-inflammatory (Wang et al., 1999).

\textbf{1.4.2.6. Phytoestrogenic Activity}

Some phenolics from grapes have a phytoestrogen effect. Typically, RSV has been categorized as a phytoestrogen: it is a partial estrogen receptor agonist at low levels; it antagonizes the growth-stimulatory effect of E$_2$ at higher doses in the presence of 17-\(\beta\)-estradiol (E$_2$) (Lu and Serrero, 1999). The estrogenic properties of RSV appear to vary in cell lines. RSV can stimulate estrogen-regulated progesterone receptor (PR) expression in MCF-7 cells (Gehm et al., 1997; Lu and Serrero, 1999;
Bhat et al., 2001b). It showed a superagonist activity by inducing gene activity 2- to 3-fold more than estradiol, while it exhibited superagonist activity in MVLN cells, an estrogen-dependent MCF-7 cell line (Basly et al., 2000). Conversely, RSV has been observed to have no superagonist activity (Bhat et al., 2001b), and demonstrated anti-estrogenic activity, through estradiol-induced gene expression inhibition, in the MCF-7 cell line (Lu and Serrero, 1999). In MCF-7, T47D, LY2, and S30 mammary cancer cell lines, RSV functioned as an agonist in the MCF-7 and the S30 cell lines, while antagonizing estrogen activity in T47D and LY2 cells (Bhat et al., 2001b). Bhat and Pezzuto (2001) also reported the anti-estrogenic activity of RSV in human endometrial adenocarcinoma (Ishikawa) through the suppression of PR expression, estradiol-induced alkaline phosphatase activity, and an estrogen receptor-mediated reporter gene.

1.4.2.7. **Effects on the Cell Cycle and Apoptosis**

Inhibition of cell cycle progression is a possible target for chemopreventive agents like phenolics. The effect of RSV on the cell cycle in tumor cells occurs at the S phase (Bellofernandez et al., 1993; Hsieh et al., 1999a; Schneider et al., 2000; Ahmad et al., 2001; Park et al., 2001; Wolter et al., 2001; Joe et al., 2002; Kuwajerwala et al., 2002; Estrov et al., 2003). However, a G1 phase arrest could also be observed in HepG2 cells (Kuo et al., 2002). RSV arrests stellate cells in G1-phase by selectively reducing the level of cyclin D1 (Kawada et al., 1998), and induces apoptotic cell death in HL-60 cells, particularly in cells arrested in the G0/G1 phase (Surh et al., 1999), which was linked to a decrease in the expression of the anti-apoptotic oncoprotein, Bcl-2. However, a RSV-induced arrest of HL-60 cells at the S/G2 phase transition and a subsequent increase in the number of cells in the G1/S phases have also been observed (Ragione et al., 1998), which was attributed to an
increase in cyclins A and E and inactive cdc2, without any modification of $p21^{WAF/CYPI}$ expression. Similar to RSV, piceatannol is also a cell cycle inhibitor that functions in the S phase. The suppression effect of RSV on normal cell growth is accompanied by the accumulation of cells in the S and G2 phases (Hsieh et al., 1999b).

Apoptosis, programmed cell death, is necessary for the maintenance of normal tissue homeostasis. Phenolics in grapes and wines are able to induce cell death both in vitro and in vivo. RSV has been reported to induce apoptosis in a number of cell lines (Carbó et al., 1999; Hsieh et al., 1999a; Adhami et al., 2001; Wolter et al., 2001; Joe et al., 2002; Kim et al., 2003; Liontas and Yeger, 2004; Scifo et al., 2004). On the other hand, RSV supressed oxidative-induced apoptosis in a variety of cell lines, including Swiss 3T3 mouse fibroblasts (Kutuk et al., 2004), rat pheochromocytoma (PC12) (Jang and Surh, 2001), human peripheral blood mononuclear (PBM) (Losa, 2003), and human retinal pigment epithelium (RPE) cells (King et al., 2005). The apoptosis induced by RSV is accompanied by induction of p53, activation of caspase 9, upregulation of Bax, and a decrease in Bcl-2 levels (Kim et al., 2004). RSV-induced apoptosis coincides with the enhancement of CD95L (Fas L) expression and preferentially targets CD95 (Fas, APO-1) high-expressing cells (Clement et al., 1998). RSV-induced cell death is consequently tumour-specific and involves the CD95–CD95L system as the apoptotic trigger, suggesting that this system could activate a series of intracellular events culminating in the death cascade. RSV shows anti-leukaemic activity against some mouse and human cell lines by irreversibly inhibiting cell proliferation and inducing apoptosis (Gautam et al., 2000). In acute lymphoblastic leukemia cell lines, depolarization of mitochondrial membranes and activation of caspase 9 could be observed after treatment with RSV (Dörrie et al., 2001). In a human colon cancer cell line (SW480), Delmas et al. (2003) found that the apoptosis
induced by RSV was not mediated directly through modulation of Fas/FasL interaction, but was attributable to caspase activation and increased accumulation of Bax and Bak. In human prostate carcinoma cells (DU145), RSV upregulated Bax protein and mRNA expression in a dose-dependent manner, whereas Bcl-2 and Bcl-xL levels were not significantly affected (Kim et al., 2003). The tumor suppressor p53-dependent pathway is involved in the induction of apoptosis in HepG2 cells (Kuo et al., 2002) and JB6 mouse epidermal cells (Huang et al., 1999) after treatment with RSV. In thyroid cancer cells, RSV-induced cell death was blocked by addition of the p53 inhibitor pifithrin-α or by transfection of a p53 antisense oligonucleotide (Shih et al., 2002). Piceatannol has been also exhibited to be a potent inducer of apoptosis in human SK-Mel-28 melanoma cells (Larrosa et al., 2004).

1.4.2.8. Effects on the Signal Transduction

Phenolics in grapes and wines are able to target signal transduction pathways, and further act with different biological effects. Mitogen-activated protein kinase (MAPK) pathways, including p38, c-Jun N-terminal protein kinase (JNK), and extracellular signal-regulated kinase (ERK), are well-characterized mammalian signal transduction pathways (El-Mowafy and White, 1999). MAPKs convert extracellular signals into intracellular events. Activator protein-1 (AP-1) is a dimeric transcription factor and is responsible for modulating the expression of several tumor-promoting genes (Kundu et al., 2004). The interaction between RSV and signal transduction pathways may explain many of the beneficial effects of RSV. TNF-α-induced JNK, AP-1, and MEK (MAPK kinase) activation were inhibited by pretreatment with RSV in U937 lymphoma cells (Manna et al., 2000). The modulation of ERK signaling by RSV is suggested to play an important role in angiotensin II (Ang II)–induced proliferation and ET-1 gene expression in rat aortic smooth muscle cells (Chao et al.,
2005). In porcine coronary arteries RSV was found to inhibit the induced activation of p38, JNK1, and ERK1/2 by endothelin-1 (ET-1) (El-Mowafy and White, 1999). RSV has been reported to target MEK, and ERK activation in the inhibition of cardiac fibroblast mitogenic signaling, proliferation, and differentiation into myofibroblasts (Olson et al., 2005).

The anticancer properties of RSV are due partly to activation of p53 and inhibition of NF-κB and AP-1 through suppressing signaling cascades. RSV could induce apoptosis in wild-type p53 mouse fibroblast cells, but was not able to induce apoptosis in p53-deficient fibroblast cells (Huang et al., 1999). It was observed that RSV-induced apoptosis and p53 activation (via phosphorylation) are mediated by the ERK and p38 pathways (She et al., 2001; Lin et al., 2002).

AP-1 is also provided as a potential target for RSV. Induced AP-1 activity in human cervical squamous carcinoma (HeLa) cells using 12-myristate 13-acetate (PMA), and ultraviolet light-C (UV-C) was reported (Yu et al., 2001). PMA was a strong activator of the ERK1/2 pathway, while UV-C activated the MAPK pathways. This study showed that RSV suppressed both PMA-induced and UV-C-induced AP-1 activity. Through the inhibition of AP-1 activity and ERK phosphorylation, topical application of RSV to mouse skin was found to be able to inhibit TPA-induced COX-2 expression (Kundu et al., 2004).

Piceatannol acts as an inhibitor of protein-tyrosine kinase activity of p72^{Syk} and p56^{Lck} in lymphoid cells (Geahlen and Mclaughlin, 1989), and inhibits the focal adhesion kinase and Src in thrombocytes (Law et al., 1999), and the tyrosine kinase activity of human placenta (Palmieri et al., 1999). Banerjee et al. (2002) observed that
RSV decreased mammary tumor incidence, tumor number, and extended cancer latency in female Sprague-Dawley rats by inhibiting the expression of NF-κB, COX-2, and matrix metalloprotease 9 enzyme. Although the mechanisms by which RSV suppresses NF-κB activation remains uncertain, it was found that RSV inhibited TNF-induced NF-κB activation by blocking phosphorylation and nuclear translocation of the NF-κB subunit p65 (Manna et al., 2000), and by preventing NF-κB DNA binding and inhibiting IκB kinase (IKK) activity via an upstream signaling component (Holmes-McNary and Baldwin, 2000).

Recently, it was reported that anthocyanidins directly cause human promyelocytic leukemia cells (HL-60) to generate intracellular hydrogen peroxide, and trigger apoptosis, possibly through an oxidative stress-involved JNK signaling pathway. It was observed that delphinidin stimulates JNK pathway activation including JNK phosphorylation and c-jun gene expression, and activates caspase-3 (Hou et al., 2003).

1.4.2.9. Other Health Effects

Using Tg2576 mice to model Alzheimer's disease (AD)-type amyloid beta-protein (Abeta) neuropathology, it was tested if moderate consumption of the red wine, Cabernet Sauvignon, modulates AD-type neuropathology and cognitive deterioration (Wang et al., 2006). The results showed that Cabernet Sauvignon significantly attenuated AD-type deterioration of spatial memory function and Abeta neuropathology in Tg2576 mice relative to control, supporting epidemiological evidence that moderate wine consumption may help reduce the relative risk of AD clinical dementia. RSV administrated to male Wistar rats resulted in a significant decrease in tumor cell content after inoculated with Yoshida AH-130 ascites hepatoma
tumors, which was linked to a G2/M phase arrest and apoptosis (Carbó et al., 1999). Recently, it was observed that daily oral administration of trans-RSV to adult male rats enhanced sperm production by stimulating the hypothalamic-pituitary-gonadal axis (Juan et al., 2005). The estrogenic activity of RSV may help prevent bone loss in post-menopausal women. RSV was shown to increase the proliferation of osteoblastic MC3T3-E1 cells and induce alkaline phosphatase activity, an enzyme believed to be involved in bone mineralization (Mizutani et al., 1998). Ovariectomized rats treated with RSV have been observed to exhibit significantly greater femur bone length, epiphysis bone mineral density, and bone calcium content than ovariectomized rats without treatment (Liu et al., 2005).

1.5. **Phytochemicals in Grapes and Wines**

Phytochemicals, an array of bioactive nonnutrient compounds in fruits, vegetables, whole grains, wine, tea, extra virgin olive oil, chocolate and other cocoa products, as well as other plant-based foods, have been related to reductions in the risk of major chronic diseases. It is estimated that over 5,000 phytochemicals have been identified (Shahidi and Naczk, 1995), but a large proportion still remain unknown and need to be characterized before their health benefits can be completely understood. Generally, phytochemicals can be classified as alkaloids, carotenoids, phenolics, nitrogen-containing compounds, and organosulfur compounds (Figure 1.1) (Liu 2004). Phenolics constitute one of the largest and most ubiquitous groups of phytochemicals. They can be divided into more than ten types based on their chemical structure (Strack, 1997). There are thousands of identified phenolic structures that vary from simple compounds such as phenolic acids with a C6 ring structure to highly polymerized molecules such as tannins. Phenolics share a common chemical structure
and differ in their linkages to other compounds. All phenolics possess an aromatic ring bearing one or more hydroxyl groups. The majority of phenolics have a sugar residue such as a monosaccharide, disaccharide, or oligosaccharide linked to the carbon skeleton. Other residues include amines, organic acids, carboxylic acids, and lipids.

Grapes contain a large array of bioactive phytochemicals. The main phytochemicals in grape (Vitis vinifera) arise from acetyl-CoA and shikimic acids. Phenolic acids, flavonoids, stilbenes, and proanthocyanidins are biosynthesized through the phenylpropanoid pathway (Kurkin, 2003). Grapes are rich in phenolics; most of them are present in the peel. Phenolics in grape are generally classified into two groups: the flavonoids and nonflavonoids. The flavonoids include flavan-3-ols, flavonols, and anthocyanins. The nonflavonoids encompass phenolic acids and stilbenes. In the grape berry, the flavonoids are mainly localized in the skins, such as the anthocyanins, while the flavan-3-ols are present both in the skins and seeds. However, the composition and concentration of phenolics in grapes vary with variety, species, season, and environmental and management factors such as soil conditions, climate, and crop load (Jackson and Lombard, 1993).

1.5.1. Phenolic Acids

The most common phenolic acids occurring in Vitis are usually divided in two main groups (Figure 1.2): benzoic acid derivatives, comprising 7 carbon atoms (C6-C1) and cinnamic acid derivatives, containing 9 carbon atoms (C6-C3). These compounds are present predominantly in the hydroxylated form, therefore being generally named hydroxybenzoic and hydroxycinnamic acids, respectively. Natural phenolic acids in grapes, either occurring in the free or conjugated forms, generally appear as esters or amides (Fiuza et al., 2004). Benzoic acid derivatives are a minor
component in new wines. Hydroxycinnamic acid derivatives are the major phenolics in grape juice and white wine. Three common hydroxycinnamic acid derivatives in grapes and wine are caftaric acid (caffeic acid), coutaric acid (coumaric acid), and fertaric acid (ferulic acid).

1.5.2. **Flavonoids**

Flavonoids are present in plants and have been studied extensively in fruits, vegetables, whole grains, legumes, nuts, olive oil, tea, and wine. This group contains over 8,000 known compounds, and this number is constantly growing due to the great structural diversity arising from various hydroxylation, glycosylation, methoxylation, and acylation (Rice-Evans and Packer, 2003). The generic structure of flavonoids consists of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring called C ring (Figure 1.3). Based on differences in the heterocycle C ring, flavonoids (Figure 1.4) are categorized as flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechins, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein, daidzein, dihydrodaiadzein, and equol) (Liu 2004). For naturally occurring flavonoids, they are mostly conjugated in glycosylated or esterified forms but can occur as aglycones, especially as a result of the effects of food processing (Hollman and Arts, 2000).

Anthocyanins, widely distributed throughout the plant kingdom, are natural, nontoxic, and water-soluble flavonoid pigments, being particularly common in fruits and vegetables where they are responsible for the red, orange, blue, and purple colors. The anthocyanins themselves are subdivided into the sugar-free anthocyanidine aglycons and and the anthocyanin glycosides. The aglycone is referred to as an anthocyanidin. There are 6 commonly occurring anthocyanidin structures (Figure 1.5). However,
anthocyanidins are rarely found in plants - rather they are almost always found as the more stable glycosylated derivatives, referred to as anthocyanins. These pigments act as powerful antioxidants helping to protect the plant from radicals formed by UV light and during metabolic processes. Absorption of anthocyanins appears to be much less than that of the flavonol quercetin, perhaps as little as one out of ten (Prior, 2003). Catechins are ubiquitous in plant-based foods, being particularly important in a large number of fruits, vegetables, and legumes, and occur in beverages such as red wine and tea (Arts et al., 2000). It is estimated that flavonoids account for nearly 60% of the phenolics in our diet and the remaining 30% are from phenolic acids.
Figure 1.1. Classification of dietary phytochemicals (modified from Liu, 2004)
1) Benzoic Acid

<table>
<thead>
<tr>
<th>Benzoic acid Derivatives</th>
<th>Substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Hydroxybenzoic acid</td>
<td>H OH H</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>H OH OH</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>OCH$_3$ OH H</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>OCH$_3$ OH OCH$_3$</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>OH OH OH</td>
</tr>
</tbody>
</table>

2) Cinnamic Acid

<table>
<thead>
<tr>
<th>Cinnamic acid Derivatives</th>
<th>Substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Coumaric acid</td>
<td>H OH H</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>OH OH H</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>OCH$_3$ OH H</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>OCH$_3$ OH OCH$_3$</td>
</tr>
</tbody>
</table>

Figure 1.2. Structures of common phenolic acids: 1) benzoic acid derivatives; 2) cinnamic acid derivatives.
Flavonoids in grapes and wines are primarily categorized into flavonols, flavones, flavanols, flavanones, and anthocyanidins. Flavonols are the most abundant class of flavonoids in grapes and wine, and they are found in the seed and skin in the grape. These are often specifically called the flavan-3-ols to identify the location of the hydroxy group on the C ring. Colorless flavanols contain catechin and epicatechin, the monomeric units of proanthocyanidins, whereas colored flavanones include quercetin, and anthocyanins are pigments responsible for grape and wine color (Iwashina, 2000; Shi et al., 2003). Proanthocyanidins, also known as condensed tannins, are characterized by a polymerization degree (PD) ranging mainly between 3 and 11, up to 17 and more (Iriti and Faoro, 2006).

Catechin belongs to flavan-3-ols, which are the most reduced form of the flavonoids. Trans and cis forms are found in grapes. The trans form is (2R,3S) (+)-catechin and the cis form is (2R,3R) (−)-epicatechin. Catechin and epicatechin are empimers, with (+)-catechin and (-)-epicatechin being the most common optical isomers found in nature (Figure 1.6). Epigallocatechin (EGC) and gallocatechin (GC) contain an additional phenolic hydroxyl group when compared to epicatechin and catechin, respectively. Catechin gallates are gallic acid esters of the catechins; such as epigallocatechin gallate (EGCG), which is commonly the most abundant catechin in tea. EGC is found in grape skin, but GC is not found in significant amounts. The monomeric catechins are bitter and astringent.

Quercetin is a flavonol, which are always found in a glycoside form in plants including in grape berries where it is found in grape skin. There are three forms of the simple flavonoid aglycones in grapes: quercetin, myricetin (3′4′5′ trihydroxy), and kaempferol (4′ hydroxy) (Figure 1.6).
Anthocyanins provide the red and blue colors found in the skins of red or black grapes, and also provide the color in red wine. The five basic anthocyanidins in wine are cyanidin, peonidin, delphinidin, petunidin and malvidin (Wrolstad, 2000). It is estimated that approximately 10,000 tons of anthocyanins from black grapes are consumed annually (Walford, 1980). The anthocyanins are a part of the flavonoid group of compounds that are characterized by the flavylium nucleus (Figure 1.5). The anthocyanin molecule consists of two or three portions: the aglycone base on the flavylium nucleus, a group of sugars, and a group of acyl acids. The common aglycones are listed in Figure 1.5. *V. vinifera* may contain up to 17 pigments (Riberau-Gayon, 1982). They are the 3-monoglucosides of cyanidin, peonidin, malvidin, petunidin, and delphinidin, and the same compounds acylated with acetic, coumaric, or caffeic acid. The anthocyanins in *V. Labrusca* contain the monoglucosides and diglucosides acylated with the above acids, in different proportions depending on the cultivar. The anthocyanins in grape skins are predominately the 3-O-glucosides of malvidin, cyanidin, delphinidin, peonidin, and petunidin (Wrolstad, 2000). Malvidin, the reddest of all anthocyanins, is the major one in dark red *vinifera* grape with higher proportions of cyanidin in red grape (Jackson, 1994). Concord grapes may contain up to 20 pigments with the major aglycones being cyanidin and delphinidin. Cyanidin 3-monoglucoside and delphinidin 3-monoglucoside are major anthocyanins in Concord grapes. Grape anthocyanins play a crucial role in the color quality of red wines, and have been increasingly used as food colorants and nutraceuticals (Mazza 1995).

Proanthocyanidins, also named condensed tannins, are the second most abundant phenolics after lignin (Santos-Buelga and Scalbert, 2000; Gu et al., 2003). They are oligomers or polymers of flavan-3-ols subunits. The most common types of
proanthocyanidins are shown in Figure 1.7. An ester bond between C2→C7 resulting in linkage of the flavan-3-ol units is named an A-type linkage. Units are linked mainly through C4→C8 bond, but the C4→C6 linkage also exists. These linkages are both called B-type linkages. The flavan-3-ol subunits may contain acyl or glycosyl substituents. The most common acyl substituent is gallic acid, which is bound as an ester with the hydroxyl in the C3 position in wine or tea (Santos-Buelga and Scalbert, 2000). The predominant phenolics in red wine are from the condensation of flavan-3-ol units to yield the oligomers (proanthocyanidins) and polymers (condensed tannins). Epicatechin is the major unit in condensed tannins from grapes and wine, catechin is the next most abundant. Grape seeds are waste products of the winery and grape juice industry, but they contain 5~8% phenolics depending on the variety (Shi et al., 2003). Major phenolics in grape seeds are proanthocyanidins and flavonoids, including gallic acid, the monomeric flavan-3-ols catechin, epicatechin, gallocatechin, epigallocatechin, and epicatechin 3-O-gallate, procyanidin dimers, trimers, and more highly polymerized procyanidins. Among them, the most abundant phenolics isolated from grape seed are catechins, epicatechin, procyanidin, and some dimers and trimers. In the USA, the daily intake of proanthocyanidins is approximately 57.7 mg/day including the monomers (Gu et al., 2004). Apples (32%), chocolate (17.9%), and grapes (17.8%) are the major contributors to proanthocyanidin consumption in the USA. The other sources of proanthocyanidins in daily diet are pears and wines.

The phenolics in grape seeds are primarily all flavonoids. The presence of flavan-3-ol monomers, dimers, and trimers has been extensively reported (Ricardo da Silva et al., 1991; Prieur et al., 1994). Monomers include catechin, epicatechin, and epicatech-3-O-gallate. The dimeric procyanidins are referred to as the B-series, while the trimeric procyanidins are referred to as the C-series (Figure 1.7). Five different
dimers (procyanidin B1, B2, B3, B4, and B5) and two trimers (C1 and C2) were identified from grape skin and seeds. Red wine contributes a significant amount of proanthocyanidins compared to other beverages. The common types of proanthocyanidins in grapes and wines are procyanidins, propelargonidin (epiafzelechin), and prodelphinidin (epigallocatechin). Due to being specifically extracted from the grape seeds and skin during the mash fermentation of red wine, proanthocyanidins have been suggested as a potential candidate to cause the superior effect of red wine as compared to white wine and other alcoholic beverages.

1.5.3. Stilbenes

More than 30 stilbenes and stilbene glycosides have been identified in plants (Soleas et al., 1997). Stilbenes (Figure 1.8) are phenolic compounds exhibiting two aromatic rings linked by an ethane bridge. Stilbenes usually include resveratrol (RSV: 3,5,4'-trihydroxystilbene), piceid (a resveratrol glucoside), pterostilbene (a dimethylated derivative of resveratrol), and viniferins (resveratrol oligomers) (Langcake and Pryce, 1976; Langcake, 1981; Jeandet et al., 1991). RSV is the parent molecule of viniferins, a family of phytoalexin that inhibit the progression of fungal infection. Another derivative, pterostilbene, has much greater antifungal activity than RSV, despite being present in a lower level (Langcake et al., 1979). Stilbenes are responsible for the bright blue fluorescence observed under long wavelength UV-light on grape leaf surfaces or grape berries following their accumulation within plant tissues.

RSV, one of phytoalexins, is synthesized by a wide variety of plant species, including grapes, peanuts, and mulberries, in response to pathogenic attack and environmental stress such as injury, UV irradiation and fungal infection. RSV was identified as the bioactive constituent of the dried roots of Polygonum cuspidatum in
1963. It has been used in traditional Chinese medicine to fight against favus, suppurative dermatitis, gonorrhea, and hyperlipemia. It was first detected in *V. vinifera* grape in 1976 (Langcake and Pryce, 1976), and found in wine in 1992 (Siemann and Creasy, 1992). RSV is synthesized in the leaf epidermis and the skin of grape berries, but not in the flesh. RSV exists in two isomers, the *cis* and *trans*, as shown in Figure 1.8. Both forms are found in wine, although it appears that only the *trans* isomer is found in grapes. Direct light (UV-light-induced) can cause its isomerization from *trans* to *cis* (Trela and Waterhouse, 1996). Grape variety affects RSV concentration, with higher concentrations found in red grape varieties compared to white grape varieties. In addition, wilting conditions, climate, and amount of fungal infection (*Botrytis cinerea*) also influence the level of RSV in grape skin. Due to its presence in grapes, RSV is also found in wines. Other compounds considered as oligomers of RSV and termed viniferins have also been found in grapevines as a result of infection or stress. The major components of these appear to be ε-viniferin (Figure 1.8), a cyclic resveratrol dehydrodimer (Langcake and Pryce, 1977; Jeandet et al., 1997), and α-viniferin, a cyclic resveratrol dehydrotrimer (Pryce and Langcake, 1977).

**1.5.4. Others**

Carotenoids as representatives of isoprenoid tetraterpenes (C₄₀) are produced in ripening grapes. Oxidation of carotenoids forms volatile and odoriferous compounds such as β-ionone, damascenone, and β-ionol (Baumes et al., 2002). Monoterpenes are major components of essential oils, and represent C₁₀ isoprenoids. The presence of isoprenoid monoterpenes in grape is the primary source of wine aroma (Pisarnitskii, 2001). Melatonin has been recently found in grape (Iriti and Faoro, 2006).
Figure 1.3. The generic structure of flavonoid.

Figure 1.4. Structures of main classes of dietary flavonoids.
<table>
<thead>
<tr>
<th>Anthocyanidin Derivatives</th>
<th>Substitutions</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin</td>
<td>OH H</td>
<td>Orange red</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH OH</td>
<td>Bluish red</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH$_3$ OCH$_3$</td>
<td>Bluish red</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>H H</td>
<td>Orange</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH$_3$ OH</td>
<td>Red</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OCH$_3$ OH</td>
<td>Bluish red</td>
</tr>
</tbody>
</table>

Figure 1.5. Structures of anthocyanidins.
Figure 1.6. Chemical structures of common dietary flavonoids.
Figure 1.7. Chemical structures of proanthocyanidins in grapes.
### Stilbene Substitutions

<table>
<thead>
<tr>
<th>Stilbene</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₁’</th>
<th>R₂’</th>
<th>R₃’</th>
<th>R₄’</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>trans</em>-resveratrol</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>trans</em>-resveratroloside</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>GlcO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>trans</em>-astringin</td>
<td>GlcO</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>trans</em>-pterostilbene</td>
<td>CH₃O</td>
<td>CH₃O</td>
<td>H</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>trans</em>-piceid</td>
<td>GlcO</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>trans</em>-piceatannol</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cis</em>-resveratrol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
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<tr>
<td><em>cis</em>-resveratroloside</td>
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<td>OH</td>
<td>O</td>
<td>GlcO</td>
</tr>
<tr>
<td><em>cis</em>-astringin</td>
<td>GlcO</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cis</em>-piceid</td>
<td>GlcO</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td></td>
<td></td>
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</tbody>
</table>

Glc: glucosyl (C₆H₁₁O₅).

Figure 1.8. Structures of the main stilbenes found in grape and wine.
In grapes and wines, phenolics are the third most abundant constituent after carbohydrates and acids (Singleton, 1980). The total extractable phenolics in grapes are 10% or less in pulp, 28-35% in the skin, and 60-70% in the seeds (Shi et al., 2003). It is well known that both genetic and agronomic or environmental factors play important roles in phenolic composition, concentration, and thus nutritional quality of crops. The average number of phenolic compounds for seeded \textit{V. vinifera} varieties from California is about 4,000 and 5,500 mg gallic acid equivalents (GAE)/kg for white and red grapes, respectively (Singleton and Esau, 1969). In 2002, Mattivi \textit{et al} studied 25 high-quality red grape cultivars \textit{(V. vinifera)}, and found that the Schiava variety contained the lowest phenolic content with 1358 mg/kg, and the Sagrantino variety had the highest phenolic content with 4628 mg/kg, resulting in a variability factor of 2.7 in the richness of phenolics between the values of the varieties with the average concentrations. Kanner \textit{et al.} (1994) also found a variation in phenolic content among Thomson seedless, Flame seedless, and Black seedless grapes, ranging from 260 to 930 mg/kg of fresh weight. It was estimated that the total phenolics in juice, pulp, skins, and seeds are approximately 5%, 1%, 30%, and 64%, respectively (Singleton and Esau, 1969; Singleton, 1982). Generally, the total phenolics of red grape skins is greatly higher than that of white grapes due to the loss of the ability to produce anthocyanins in the skins of white grapes. It was observed that the total phenolic content in winemaking grapes is significantly higher than that of table grapes. Caftaric acid is the predominant cinnamate in grapes, averaging roughly 170 mg/kg in \textit{V. vinifera} grapes, while the \textit{p}-coutaric and fertaric acids have around 20 and 5 mg/kg, respectively (Singleton \textit{et al.}, 1986). The variability of phenolics is influenced largely by genetic factors, environmental conditions, and the stage of development of the plant organ.
The level of phenolics in wine varies throughout their concentration in the grapes, the maturity state of berries, the vinification techniques used, stabilization and storage, as well as condensation, polymerization, oxidation and precipitation of different molecules. Polymeric condensed tannins and pigmented tannins comprise the majority of wine phenolics. Red wine contains phenolics in high concentration up to 4 g/L, but relatively low levels are present in white and rose wines, approximately a tenth of those of red wines (Waterhouse and Teissedre, 1997; Waterhouse, 2002). Total hydroxycinnamates’ concentrations are about 130 mg/L in white wines and 60 mg/L in reds (Waterhouse, 2002). The levels of benzoic acids in white wines are around 10 mg/L, while red wines are about 70 mg/L. In red wines, flavonoids usually comprise more than 85% of the total phenolic content, whereas in white wines, flavonoids typically constitute less than 20% of the total phenolic content (Soleas et al., 1997). Total monomeric flavan-3-ols levels in red wine ranges from 40 to 120 mg/L with the majority frequently being catechin (Ritchey and Waterhouse, 1999). The concentrations of total flavonols are about 53 mg/L for Cabernet Sauvignon. The amounts of proanthocyanidins and condensed tannins are in the range of 500 – 1500 mg/L or even higher in some red wines, while in white wine, amounts are between 10 mg/L and 50 mg/L (Waterhouse, 2002). The levels of total phenolics are 209.5 mg/L for young white wine, and 285.5 mg/L for aged white wine; while the amounts of total phenolics are 1,732 mg/L for young red wine, and 1,742 for aged red wine.

Grapes are the major dietary sources of anthocyanins, being responsible for the coloring of black, red, and purple grapes; however, anthocyanins are lacking in white grapes. In particular, anthocyanins mostly accumulate in the skins, whereas procyandins are located in the seeds. The study of anthocyanins is important for differentiating the quality and health benefits of grapes and red wines derived from
grapes. As a characteristic associated with the variety, the level of anthocyanins in grapes can be served as a direct estimate of the red pigments, and be useful for the classification of grape varieties and of relevant wines (Mattivi and Nicolini, 1997). Hence, anthocyanins might be proposed as chemical markers to differentiate grape varieties and red wines. The types of anthocyanins occurring in grapes, wines, juices, and their distribution were extensively reviewed (Mazza, 1995). It was reported that Lomanto and Colobel hybrid grape cultivar had the highest anthocyanin content with 603 mg/100g; Midsouth cultivar contained the lowest content with 5.5 mg/100g. The total anthocyanin content of red grapes is from about 30 to 750 mg/100g of fresh weight of ripe berries. The content of anthocyanins in the skin of grapes was estimated from 200 ~ 5000 mg/kg of fresh whole grape (Riberau-Gayon, 1982). The average content of anthocyanins in red wines is estimated at 26 mg/L (Frankel et al., 1995). The content of anthocyanins in skin of three *V. vinifera* L. grapes was 753-803 mg/kg for Pinot Noir, 826-1048 mg/kg for Cabernet Franc, and 981-1043 mg/kg for Merlot (Mazza et al., 1999). Muñoz-Espada et al. (2004) reported the total anthocyanin contents in skin of three non-*Vitis vinifera* grapes, which were 258 ± 37 mg/100 g of wet weight for Marechal Foch, 888 ± 78 mg/100 g for Norton, and 326 ± 5.9 mg/100 g for Concord. As a standard of malvidin 3-monoglucoside chloride, the lowest anthocyanin content in Primitivo variety was found to be 250 mg/kg whereas the highest amount was found in a Teroldego grape with 2323 mg/kg (Mattivi et al., 2002). The concentration and composition of the anthocyanins in red grape varies greatly with the variety, species, maturity, production area, and climate. In assessing red grape varieties for total anthocyanin content, broad variability exists. This is not surprising since these secondary metabolites are synthesized under genetic control in different varieties.
RSV is synthesized particularly in the leaf epidermis and the skin of grape berries and only trace amounts are present in the fruit flesh. The RSV levels in grape skins and red wines are 50-100 μg/g, and 1.5-3 mg/L, respectively (Joe et al., 2002). The RSV concentration in wine is greatly variable, which is affected by grape varieties, fungal pressure, skin contact, growing conditions, and vinification. RSV is more concentrated in red than in white wine due to longer time skin extraction in the red wine fermentation process than white wine. It was observed that the concentrations of the piceid isomers decreased as fermentation continued, while the RSV isomer increased, with trans-RSV having the highest final concentration (Mattivi et al., 1995). There was 1.33-7.17 mg RSV/L in Cabernet Sauvignon, 3.14-6.03 mg/L in Merlot, and 3.22-5.93 mg/L in Pinot Noir (Mattivi, 1993). Pinot Noir wines were found to have the highest concentrations of RSV, while white Bordeaux and Chardonnay wines contained the lowest levels in comparison of wines from the north coast of California (Lamuela-Raventós and Waterhouse, 1993). Jeandet et al. (1993) exclusively examined red wines (Pinot Noir) and white wines (Chardonnay and Aligote) from Burgundy, and quantified the trans- and cis-RSV independently in these samples. It was reported that the trans-RSV concentrations were in the range of values previously reported for Californian Pinot Noir wines (Lamuela-Raventós and Waterhouse, 1993), ranging from 10-15% of the total RSV concentrations (0.34-1.83 mg/L). In a comparison of wines made from grapes grown in Japan, results were similar (Sato et al. 1997). Siemann and Creasy (1992) reported that RSV level in most white wines was less than 0.03 mg/L although some Chardonnays from New York State had up to 0.1 mg/L. Among the 40 Tuscan wines (mainly Sangiovese grapes), it was found that the concentration of trans-RSV ranged from 0.3 to 2.1 mg/mL, while the concentration of cis-RSV was between 0.5 and 1.9 mg/mL (Mozzon et al., 1996). Generally, the total levels of all RSV derivatives are around 7 mg/L for red wines.
(Lamuela-Raventós, 1995), 2 mg/L for rosés and 0.5 mg/L for white wines (Ribeiro de Lima et al., 1999; Wallerath et al., 2002).

The phenolics in grape seed are also affected by variety, climatic condition, site of production, and degree of maturity. Shi et al. (2003) summarized the phenolic composition and concentration of grape seeds from the *viniferia*, *hybrid*, and *labrusca* type of red and white grape cultivars commonly grown in the Niagara region of Ontario, Canada. It was found that Pinot Noir contained the highest phenolic content with 797 mg/100g, followed by Sauvignon with 778 mg/100g, and Vincent with 759 mg/100g. Among 5 white grapes, Chardonnay had the highest phenolic content with 293 mg/100g, followed by Niagara with 231 mg/100g, Elvira with 152 mg/100g, Riesling with 114 mg/100g, and Seyval with 60 mg/100g. There were trace amount of dimer gallates and trimers present in Seyval.

1.7. Grape Varieties

The U.S. grape and grape product industries are primarily concentrated in California, accounting for virtually all table grapes and raisins, and approximately 90% of the nation’s wine production, with New York State and Washington State each at about 3% and the rest of the States at 4% combined. Grape juice production is mainly focused in Washington State, New York, Pennsylvania and Michigan. Bases on a comprehensive study (MKF Research, 2007) unveiled on Capitol Hill by the Congressional Wine Caucus on January 17, 2007, the United States wine, grape and grape product industries make a contribution more than $162 billion annually to the American economy. There are 23,856 grape growers, 934,750 grape bearing acres, 27.3 million wine-related tourist visits, and $3.5 billion in farm gate grape sales in
2005. Grapes rank the highest value fruit crop produced in the US, and the sixth highest values among US crops. There were 4,929 US bonded wineries in 2005, with a 70% increase of wineries compared to that in 2000. Wineries are in all 50 States, making $11.4 billion in winery sales revenues. In addition, the economic impact of other grapes products include $1.669 billion retail value of grape juice and grape product sales, $3 billion retail value of table grape sales, and $560 million retail value of raisin sales. This study indicates that all kinds of grape production have increased by 5% since 2003, with the total value of the grape increasing by over 15%, mainly through rising values for wine grapes and raisins.

1.7.1. **Grape Varieties and Distribution**

The grape is one of the popular fruits and most widely cultivated throughout the world. Nearly all *Vitis* species originate in the Northern Hemisphere. Among them, over 70% of *Vitis* species are native to North America. Early cultivars of Alexander, Cape Grape, Catawba, Concord, and Isabella were from native American species (Booth, 1911). Hedrick (1907) listed over 1,400 varieties of cultivated American grape species. There are about 60 species of *Vitis*, which are mainly found in the temperate zones of the Northern Hemisphere, and almost equally distributed between America and Asia (Mullins et al., 1992). Among them, *V. aestivalis, V. cinerea, V. labrusca,* and *V. riparia* have been used in grape breeding programs. Cythiana or Norton selected from *V. aestivalis* is the most widely grown cultivar in Missouri. *V. rotundifolia* such as muscadine grape has a long history in southern US (Lane, 1997), and are exemplified by the Scuppernong variety which is disease resistant, long lived, and vigorous. *V. labrusca* has been applied in interspecific hybrids with various cultivated *V. vinifera* for grape juice, jelly, fresh fruit and wine. As the most widely distributed species, *V. riparia* is very adaptable (Remaily, 1987). Roughly 80% of the
total grape is used in winemaking annually, and 13% is consumed as table grapes. The main wine-making grape varieties include Cabernet Sauvignon, Chardonnay, Pinot Noir, Merlot, and Zinfandel. The major table grape varieties are Perlete, black Ribier, Tokay, and Emperor (Mazza, 1995). The *V. vinifera* grapes are commonly used for wine production around the world, principally distributed in Europe. Most studies in phenolic composition and related antioxidant activity have been done in grapes and wines using *V. vinifera* species. In the United States, species such as *V. labrusca*, *V. riparia*, *V. aestivalis*, *V. rupestris*, and *V. rotundifolia* are also used in wine-making. Two main grape species in North America are *V. labrusca* and *V. rotundifolia*. The *labrusca* grapes are grown mostly in the lower Great Lakes region of the U.S. and Canada. The representative grape variety is the large purple berry - Concord. Due to its abundance in the United States, this grape is popular in making of juices and jellies. The *V. rotundifolia* or Muscadine grapes are cultivated throughout the southeastern United States, from North Carolina to eastern Texas.

### 1.7.2. Main Grape Varieties in New York

In New York State, the grape and wine industry contributes $6 billion annually and supports many other businesses, becoming a powerful economic engine (NYGF, 2007). As the second largest grape juice producer, the third largest total grape acreage, and the third wine producer in America, New York State has 962 family-owned vineyards covering 33,000 acres, employs about 18,000 people, produces 175,000 tons annual harvest with 70% of grape juice, 28% of wine, and 2% of table grapes, totally making $45 million annual harvest value (NYGF, 2007).

Back in the mid-1600’s, Dutch settlers made Manhattan the first place in New York State to plant grapes for winemaking. Compared to Europe’s wine regions, New
York Wine country is huge, spanning some 500 miles (800 km) from eastern Long Island to the State’s western border with Pennsylvania. Like the vineyard of Burgundy, Champagne, and Germany in Europe, as well as Washington State, New York State is considered a cool climate wine area, although New York’s wine country is further south than those regions, and on similar latitude to Oregon and parts of northern California. The receding glaciers of the Ice Age made New York a unique combination of geography and climate. Bodies of water act as natural air conditioning, warming in the winter, cooling in the summer, and sheltering the vines from high temperature, which result in several major wine regions with temperature gradient from 38°C to 43°C, each with specific microclimates. Each with its own unique climate, character, scenery, and wine varieties, there are four distinct wine regions in New York State, including the Long Island area, the Hudson River area between New York City and Albany, the Finger lakes area with its thin parallel lakes flanked by steep hillsides, and grape juice counties of Niagara, Erie and Cattaraugus. The Finger Lakes region is known for its Cabernet Franc and Chardonnay, and specializes in Riesling, Pinot Noir, Sparkling, and Ice wines (NYGF, 2007).

Generally, varieties in New York can be categorized as Native American (V. labrusca), French-American, and European varieties (V. vinifera). Horticulturists at Cornell University have been conducting viticultural research since the 1880s. They have released 53 varieties of juice, table and wine grapes since 1906. Native American varieties were the only grapes grown in New York until the 1930’s. Like Concord and Niagara varieties, these hardy, productive varieties bring in wines with a pronounced grapey aroma and taste. The Native American varieties include Catawba, Concord, Delaware, Diamond, and Niagara. Catawba can be made into pink (rose or blush) or white wines depending on skin contact during fermentation, typically with both high
sugar and acid, lending a nice balance of sweetness and tartness. Concord is used to make grape juice, and is also used in many kosher wines. Delaware, a white wine, can be produced from dry to semi-sweet with a pleasant flowery aroma. Niagara is another grape juice variety with unique fruity aroma. Baco Noir and Seyval Blanc are examples of French-American varieties, mostly developed by French scientists about 100 years ago, combining the flavor characteristics of European grapes and the hardiness of American grapes together. The Cornell University's Agricultural Experiment Station in Geneva, N.Y. has crossbred several popular varieties such as Cayuga White and Traminette. Four other varieties in this group are Chancellor, Marechal Foch, Vidal Blanc, and Vignoles. Baco Noir, from medium to full-bodied, has deep color. When young, it has strong flavors like Cabernet Sauvignon. Cayuga White is a highly reliable and productive variety in terms of crop size and quality. From Gewürztraminer, Traminette with spicy aroma is a reliable grape to grow and yields the same basic aroma and taste characteristics as Gewürztraminer. Vignoles, like Riesling, is made from dry to very sweet with a great balance of acidity. European varieties such as Riesling and Merlot are also cultivated in New York. Other varieties include Cabernet Franc, Cabernet Sauvignon, Chardonnay, Gewürztraminer, Pinot Noir, Riesling, and Sauvignon Blanc. Cabernet Sauvignon is a dominant grape in most Bordeaux wines, requiring a long growing season. Most New York plantings of Cabernet Sauvignon are in the Long Island area. As a very popular varietal wine in U.S., New York Chardonnays are generally more like French Chablis (crisp, light, and acidic) than California ones (big, round, and oaky). As a signature grape and wine of Long Island, Merlot with a rich, full aroma is a blending grape in Bordeaux to add softness and velvety texture. As the holy grail of grape and wine, Pinot Noir, most commonly associated with France’s Burgundy area, is difficult to grow and vinify. Riesling, the signature grape and wine of the Finger Lakes, is versatile in its range of
styles. The white wine of Bordeaux, Sauvignon Blanc is mostly grown on Long Island, and is typically aromatic, light, and crisp. Taken together, more than 35 grape varieties are grown in New York, and make some of the finest wines in the world (NYGF, 2007).

1.8. Mechanisms of Phytochemicals in the Prevention of Cancer

Accumulation of ROS and electrophiles under adverse conditions are known to cause cellular membrane and biomacromolecule damage, mutagenicity, degeneration of tissues, apoptotic cell death, premature aging, cellular transformation, and being further implicated in chronic diseases such as cancer and CVD (Ames et al., 1993; Ward, 1994; Breen and Murphy, 1995). Free radicals react with biological molecules, and there is no single defense against all targets of oxidative damage. Therefore, organisms have evolved a spectrum of mechanisms to prevent oxidative damage and free radicals at multiple steps of oxidation. Exogenous defenses such as dietary phytochemicals are demonstrated for the preventive and/or therapeutic intervention of ROS disorders. The chemopreventive agents such as dietary phenolics are a very promising group of compounds, on account of their safety, low-toxicity, and general acceptance. Phenolics may offer an indirect protection by activating endogenous defense systems. One of strategies for protecting against ROS or electrophiles injury may be through chemically mediated upregulation of endogenous antioxidants and phase II enzymes in cells. A connection between exogenous and endogenous antioxidants that appear to act in a coordinated fashion are observed (Masella et al., 2005; Talalay, 2005), which is achieved partly through antioxidant responsive elements (AREs) present in the promoter regions of many of the genes inducible by oxidative and chemical stress, suggesting dietary phenolics can stimulate antioxidant
transcription and detoxification defense systems through ARE. The ARE contains AP1/AP1-like elements arranged either as inverse or direct repeats followed by a GC box (Dhakshinamoorthy and Jaiswal, 2000). NF-E2 related factors (Nrf1, Nrf2, and Nrf3) do not bind to the ARE as homodimers or heterodimers and require other leucine zipper proteins to form heterodimers that bind to the ARE (Chan et al., 1993; Moi et al., 1994; Venugopal and Jaiswal, 1996). Nrf2 is a critical transcription factor that binds to the ARE in the promoter region of a number of genes, encoding for antioxidative and phase II enzymes in animals and human cells and tissues (Kwak et al., 2004; Lee and Johnson, 2004; Kobayashi and Yamamoto, 2005; Zhu et al., 2005). Nrf2 is more potent than Nrf1 in inducing gene expression. It was believed that Nrf1 and Nrf2 positively and c-Fos, Fra1, small Maf proteins, Nrf3, and Bach 1 (Dhakshinamoorthy et al., 2005) negatively regulate ARE-mediated NQO1 and other detoxifying enzyme gene expression, as well as antioxidant induction (Nguyen et al., 2000; Dhakshinamoorthy and Jaiswal, 2001). The NF-E2-related factors are in the family of basic leucine zipper proteins. The upstream of the leucine zipper in the basic region is responsible for DNA binding. The acidic region is required for transcriptional activation. Taken together, ARE, Nrf expression, and coordinated induction of genes encoding chemopreventive proteins provide critical mechanisms for anticancer activity.

It is has become increasingly clear that intracellular signaling pathways are activated via changes in intracellular metabolic oxidation/reduction (redox) reactions associated with ROS in cancer biology (Oberley and Buettner, 1979; Abate et al., 1990; Sun and Oberley, 1996; Arnold et al., 2001; Nioi and Hayes, 2004; Spitz et al., 2004; Gius and Spitz, 2006). The mechanism of signal transduction from antioxidants to Nrf2 is the current subject of extensive research. Generally, redox signaling is
involved in activation of protein kinases and inhibition of protein phosphatases through exposure to oxidants in normal cells (Guyton et al., 1996; Claiborne et al., 1999; Tonks, 2005). Nrf2 protein binds strongly to the ARE sequence and positively regulates its activity. The interaction between Nrf2 and ARE is regulated by several inhibiting or activating cofactors. For instance, Kelch-like ECH-associated protein 1 (Keap1) cofactor which is bound to actin protein and localized in the perinuclear space, sequesters Nrf2 in the cytoplasm by forming heterodimers, and inhibits its translocation to the nucleus, thus making it unable to activate the ARE sequences (Itoh et al., 2003). The modulation of Keap1–Nrf2 binding seems to play a crucial role in the cellular response to oxidative stress, although the exact mechanism of dissociation of Nrf2 from its inhibitor and the signal transduction pathway from oxidants to Nrf2–Keap1, remains poorly understood. It was assumed that Nrf2 heterodimerizes with c-Jun or Small Maf partners, inducing ARE activation and the consequent transcription of detoxifying enzyme genes. Phosphorylation, a principal mechanism in Nrf2 stabilization, is involved in several signal transcription pathways such as the MAPK, PKC, and PI3K pathways (Figure 1.9) (Zipper and Mulcahy, 2000; Huang et al., 2002; Kang et al., 2002; Nakaso et al., 2003). Generally, Nrf2 is retained in the cytosol by a cytosolic inhibitor Keap1. The signals from phenolic antioxidants lead to phosphorylation of Nrf2 and/or redox modulation of Nrf2/Keap1, leading to separation of Nrf2 from Nrf2/Keap1. As a result of kinase activation, Nrf2 dissociates from Keap1 and translocates in the nucleus. It is therefore possible that the transcriptional activity of Nrf2 is regulated by multiple converging signaling pathways. The Nrf2 accumulates in the nucleus to cause its binding to ARE and activation of ARE-mediated gene expression. A hypothesis could be that, phenolics, through modifying the capability of Keap1 in sequestering Nrf2, can react with active sulfhydryl groups, therefore modulating several sensor proteins including Keap1 (Levonen et al., 2004).
Another possibility could be that phenolics affect ARE-dependent gene expression through the activation of MAPK proteins (ERK, JNK and p38), probably involved in Nrf2 stabilization through its phosphorylation (Yu et al., 1997).

Dietary phenolics can stimulate the transcription of antioxidant and detoxification defense systems through ARE elements by influencing the pathways that regulate ARE activation. ARE and nuclear NF-E2 related factors mediate the basal expression and induction of a battery of defensive genes in response to dietary antioxidants. For instance, intake of cruciferous vegetables, such as cabbage, broccoli, and brussels sprouts, has been demonstrated to induce detoxification enzymes and increase clearance of xenobiotics in animals (Wattenberg, 1985; Prochaska et al., 1992; Zhang et al., 1992), and has been associated with lowered incidence of cancers in humans (Graham et al., 1978; Pantuck et al., 1979). Transcriptional regulation of phase II enzymes by Isothiocyanates (ITCs) is via Keap1–Nrf2–ARE mediated pathways (Dinkova-Kostova et al., 2002; McMahon et al., 2003). ITCs modulate carcinogen metabolism by inhibition of phase I carcinogen-activating enzymes (Talalay and Fahey, 2001), induction of phase II detoxification enzymes (Maheo et al., 1997; Shapiro et al., 1998), and inhibition of cell growth by cell cycle arrest and activation of apoptosis, linked to the activation of caspase-8, JNK1 and tyrosine phosphorylation (Gamet-Payrastre et al., 2000; Kong et al., 2001; Chiao et al., 2002). RSV was found to activate PKB/Akt in MCF-7 cells (Pozo-Guisado et al., 2004), and MAPKs in human melanoma (Niles et al., 2003) and mouse epidermal JB6 cells (She et al., 2002). RSV was reported to increase the activity of phase II enzymes (Lang et al., 1997), suggesting that it may up-regulate phase II antioxidant/detoxifying enzymes through Nrf2 activation, probably mediated by PKB/Akt or MAPKs.
Figure 1.9. Activation of Nrf2 signaling and induction of phase II detoxifying and antioxidant genes (modified from Talalay, 2005)

The transcriptional activation of phase II detoxifying and antioxidant enzymes, including NQO1, glutathione peroxidase, glutathione reductase, glutathione S-transferase (GST), γ-glutamylcysteine synthetase, sulfotransferases, epoxide hydrolases (EH), and UDP-glucuronosyl transferase (UDPGT), has been related to cis-acting elements, detected in the promoter region of those genes. NQO1 is a family of two-electron reducing enzymes that decrease the oxidative damage resulting from one-electron reduction of quinines, thus bypassing the semiquinone intermediate. NQO1 plays a vital role in protecting cells against oxidative cycling and GSH depletion by quinones (Prochaska et al., 1987) and has provided an index for phase II gene status of cells (Dhakshinamoorthy et al., 2000). Through a cis-acting ARE located within the
regulatory region of the mouse, rat, and human genes, the redox sensitivity of NQO1 transcription occurs. Expression of NQO1 is induced upon oxidative stress, leading to increase in oxidoreductase protein that offers the cell multiple means of protection from environmental insults (Nioi and Hayes, 2004), including directly catalyzing the reduction and detoxification of highly reactive quinones; decreasing one electron reductions and associated redox cycling; acting as an antioxidant enzyme through reduction of the oxidized form of vitamin E to a product with antioxidant activity; regulating the stability of p53 and apoptosis in mouse and human cells. NQO1 is inducible in human cell lines derived from a range of tissues (Nioi and Hayes, 2004), including adult retinal pigment epithelial cells, microvascular endothelial cells, the MCF-7 and T47D mammary cell lines, the IMR-32 neuroblastoma cell line, the AGS and RF-1 stomach cell lines, the NCI-H209 and NCI-H661 lung cells, the HL-60 and THP-1 leukemia lines, the HT-29 and LS-174 colon cell lines, and the LNCaP and PC3 prostate cell lines.

1.9. Additive and Synergistic Effects of Whole Foods and Phytochemicals

While fruits and vegetables are recommended for prevention of cancer, the effect of their bioactive components at the molecular level and their mechanisms of action are less well understood. Extensive research has identified and characterized various molecular targets that can potentially be used not only for the prevention of cancer but also for treatment. However, lack of success with targeted mono-therapy resulting from bypass mechanisms has forced researchers to employ combination agents that interfere with multiple cell signaling transduction pathways. In general, the effect of whole foods is more pronounced than that of the individual micronutrients or other bioactive phytochemicals they supply. Dietary phenolics can exert their effects
on multiple pathways separately or sequentially; in addition, the occurrence of interactions between/among phenolics in different pathways plays an important role.

Does the single compound and phytochemical mixture have the same health benefits in the prevention of cancer? It was observed in some clinical trials that dietary supplements or individual antioxidants do not appear to possess the consistent preventive effects with a diet rich in fruits, vegetables, whole-grains, and other plant-based foods. Epidemiological evidence for dietary supplements such as vitamin E and β-carotene for chronic disease prevention has found no effect on CVD in cardiovascular mortality and cancer (Hennekens et al., 1996; Omenn et al., 1996; Rapola et al., 1997). The incidence of nonmelanoma skin cancer was unchanged in patients receiving a β-carotene supplement over a five-year period of treatment (Greenberg et al., 1990). Vitamin C supplementation also has been shown not to lower the incidence of cancer (Blot et al., 1993) and heart disease (Salonen et al., 2000). Since dietary supplements simply cannot offer the balanced natural combination of phytochemicals present in fruit and vegetables, suggesting the health benefits can’t be replaced by dietary supplements, we hypothesize that the additive and/or synergistic effects of phytochemicals in fruit and vegetables are responsible for their potential biological effects such as antioxidant and anticancer activities, and that the benefit of a diet rich in fruit and vegetables is ascribed to the complex mixture of phytochemicals present in whole foods (Liu 2004).

1.9.1. Evidence

Much evidence suggests that foods and diets act synergistically to reduce the risk of chronic diseases. There is considerable evidence in support of food synergy such as whole-grain, Mediterranean diet, DASH diet, and high intake of fruits and vegetables diets.
1.9.1.1. DASH Diet

Hypertension is one of the leading causes of heart attack, stroke, and kidney disease, and affects about 25% of the population in the United States (Champagne, 2006). Dietary factors may influence blood pressure since vegetarians tend to have lower blood pressure than nonvegetarians (Sacks et al., 1974). The Dietary Approaches to Stop Hypertension (DASH) trial is a multicenter, randomized feeding study that tested the effects of dietary patterns on blood pressure. A diet rich in fruits, vegetables, and low-fat dairy foods and with reduced saturated and total fat can substantially lower blood pressure, which was shown by a large, randomized feeding study of blood pressure reduction, the 8-wk DASH study in 459 people (Appel et al., 1997). This diet offered an additional nutritional approach to prevent and treat hypertension. It was concluded that the DASH diet with low-sodium intake lowered blood pressure in all subgroups studied, including non-hypertensive individuals. The combination of the DASH diet and the lowest sodium level tested was superior in lowering blood pressure than either dietary intervention alone.

Blood pressure can be lowered by following the DASH eating plan, which focuses on increasing intake of foods rich in nutrients that are expected to lower blood pressure, mainly minerals (like potassium, calcium, and magnesium), protein, and fiber. While each step alone lowers blood pressure, the combination of the eating plan and a reduced sodium intake gives the maximum benefit and may help prevent the development of high blood pressure. The DASH diet adequately shows that changing dietary pattern can alter physiological status. As a trial of dietary patterns rather than individual nutrients, DASH, which tested the combined effects of nutrients that occur together in food, has contributed to a lower risk of hypertension.
1.9.1.2. Prudent and Western Diets

Many epidemiological studies have examined dietary patterns and those are associated with lowered risk of chronic diseases (Hu et al., 2000; Fung et al., 2001; Terry et al., 2001; Van Dam et al., 2002). Consumption of a diet high in fruit and vegetables, whole grains, fish and poultry, and low-fat dairy products presented a significant inverse association with the risk of CVD. Conversely, intake of a diet high in red and processed meats, refined grains, high-fat dairy products, sweets, and desserts showed a significant positive association with colon cancer and CVD (Fung et al., 2001; Fung et al., 2003). The concept of prudent and western diets was proposed. The prudent dietary pattern was characterized by higher intake of fruits, vegetables, whole grains, fish/sea foods, legumes, poultry, lean meat, and nuts/vegetable oils, for example, the Asian and Mediterranean diets, whereas the Western dietary pattern involved higher consumption of red meat, processed meat, butter, potatoes, refined grains, sweets and dessert, high-fat dairy products, and foods with high saturated fat, cholesterol, and trans fats.

Diets in the Asian areas are commonly plant-based. Despite containing different amounts of total fat, these diets include ample amounts of fruit, vegetables, legumes, whole grains, and nuts and smaller amounts of red meat and refined grains. It is believed that these traditional plant-based diets make a greater contribution to longevity and a reduced risk of CVD in Asian countries than in Western countries (Willett, 1994). The common Mediterranean dietary pattern is characterized by high consumption of fruits, vegetables, bread and other cereals, beans, nuts and seeds, olive oil (major monounsaturated fat source), and wine (low to moderate amounts) (De Lorgeril et al., 1999). Despite being higher in fat (40%) than the 30% recommended by the American Heart Association, the Mediterranean diet is gaining in popularity as
a tasty, heart-healthy alternative to low-fat eating. Compared with most Western populations, CVD mortality rate is low in the Mediterranean countries where olive oil rich in oleic acid is the primary source of fat, suggesting potential beneficial effects of monounsaturated fat on CVD. The association between greater longevity and reduced mortality and morbidity for CVD in Mediterranean diet has also been observed for certain cancers. The people living in the Greek island of Crete and other Greeks are 20% less likely to die of coronary artery disease than Americans. They also have approximately 1/3 less cancer than in the U.S (Visioli et al., 2004). The French population consumes similar amounts of saturated fats and has similar risk factors and comparable plasma cholesterol to the population in the United States. Renaud and de Lorgeril (1992) reported that coronary mortality in the United States is 182/10,000 population; the overall mortality of the French is 102. This discrepancy is referred as the French Paradox mentioned previously, which was believed to be due to the consumption of red wine together with the Mediterranean diet rich in vegetables oils. Consumption by the French of a diet containing phytochemical antioxidants may decrease the peroxidative tendencies and retard processes involved in atherogenesis and thrombosis. In general, consumption of nutrient-dense plant-based foods, including whole-grain foods, fruit and vegetables, is considered to be beneficial to health.

Cumulative evidence indicates the great potential of diets that are primarily based on minimally processed plant foods to reduce the risks of chronic diseases. The health benefits are due mainly to the large amounts of essential fatty acids, amino acids, fiber, minerals, vitamins, and phytochemicals in these diets. The combination of these nutrients from various diets has contributed to greater health benefits than those from a single diet.
1.9.1.3. Pure Compounds and Whole Foods

Substantial epidemiological evidence concerning the potential role of antioxidant nutrients in the prevention of cancers has accumulated over the past few decades. However, some studies from intervention and clinic trials do not support a preventive effect against cancer for antioxidant supplementation. Performed in 29,133 male heavy smokers between 50-59 years of age in Finland, the α-Tocopherol and β-Carotene Lung Cancer Prevention Study (ATBC, 1994) included daily supplementation with 50 mg α-tocopherol and 20 mg β-carotene using a 2×2 factorial design. There was no benefit found in the β-carotene group; conversely, the incidence of lung cancers increased by 16%. In a randomized, double-blind, placebo-controlled trial of β-carotene (50 mg on alternate days), 22,071 male physicians, 40 to 84 years of age enrolled in the United States. 12 years of supplementation with β-carotene produced neither benefit nor harm in terms of the incidence of malignant neoplasms, CVD, or death from all causes (Hennekens et al., 1996). A multicenter, randomized, double-blind, placebo-controlled primary prevention trial - the β-Carotene and Retinol Efficacy Trial, involving a total of 18,314 smokers, former smokers, and workers exposed to asbestos, was conducted (Omenn et al., 1996). It was found that the combination of β-carotene and vitamin A had no benefit and might have an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer, CVD, and any cause in smokers and workers after an average of 4 years of supplementation. Rapola et al. (1997) found that, in a randomized, double-blind, placebo-controlled study, in which 1862 men received dietary supplements of α-tocopherol (50 mg/day), β-carotene (20 mg/day), both, or placebo, the percentage of major coronary events in men with a previous myocardial infarction who smoke was not decreased with either α-tocopherol or β-carotene supplements; the risk of fatal CHD increased in the groups that received either β-carotene or the combination of α-tocopherol and β-carotene.
The reasons why the above clinical trials showed the nonsignificant or negative outcomes between the intake of dietary (antioxidant) supplements and certain chronic diseases may be due partially to the antioxidant supplements either losing their bioactivity or not behaving the same way as the compound in a phytochemical mixture because these compounds differ in molecular size, polarity, and solubility, which may affect their bioavailability and distribution in different subcellular organelles, cells, and tissues and organs. Another possible explanation of the contradictory results between observational studies and randomised trials could be the fact that doses used in clinical trials were much higher than the maximum levels offered by the usual dietary consumption which were found to be associated with a lower risk of cancer in observational epidemiological studies.

Whole-grain consumption and the risk of chronic disease is an example of food synergy (Chatenoud et al., 1998; Jacobs et al., 1998). Risk of certain chronic diseases appears to be lower with consumption of whole grain than of refined grain, suggesting that health benefit increases when all edible parts of the grain (bran, germ, and endosperm) are included. A refined-grain food contains only the endosperm; therefore, most of the grain’s nutritional value is lost during milling. The protective effect of whole-grain intake on reduced risk of chronic diseases was observed in epidemiological studies such as the Iowa Women’s Health Study with 10 years of follow-up (Jacobs et al., 1999) and the Norwegian Study (Jacobs et al., 2001), and in animal studies (Ripsin et al., 1992; Pereira et al., 2002). Frequent Iowa and Norwegian whole-grain bread consumers showed a healthier lifestyle than did those who ate little whole-grain bread.
Work previously performed in our lab found that phytochemical extracts from fruits and vegetables have strong antioxidant and antiproliferative activities both in cell culture (Eberhardt et al., 2000; Yang et al., 2004) and animal studies (Liu et al., 2005); thus we proposed that the combination of phytochemicals in fruits and vegetables is critical to their powerful antioxidant and anticancer effects. For example, the vitamin C in apples with skin accounts for only 0.4% of the total antioxidant activity, suggesting that most of the antioxidant activity of fruit and vegetables may be from phenolics and flavonoids (Eberhardt et al., 2000). Phytochemical extracts from apple skin at a dose of 50 mg/mL (on a wet basis) inhibits HepG2 human liver cancer cell proliferation by 90%; however, phytochemical extracts in 50 mg/mL apple without skin suppress tumor cell proliferation only by 21% (Wolfe et al., 2003). The apple extracts with skin significantly reduced the tumor cell proliferation when compared with the apple extracts without skin, indicating phenolic composition and concentration from whole apple make a greater contribution than those from a part of an apple.

1.9.2. Additive and Synergistic Research

Synergism by two or more compounds is defined as therapeutic effects that are greater than those expected from addition of the effects of the individual compounds. It is believed that chemotherapeutic combination approaches have been used to reduce drug toxicity, and delay the development of cancer cells, and to reach a greater effect than with one active compound alone. Fruits and vegetables are rich in phenolics and other bioactive compounds, the additive and/or synergistic effects have been suggested to be responsible for their health benefits (Liu 2003, 2004).
1.9.2.1. Antioxidant Activity

Antioxidant synergism was observed in different experiments such as vitamin E and C (Scarpa et al., 1984), vitamin E and β-carotene (Palozza and Krinsky, 1992), catechin and malvidin 3-glucoside (Rossetto et al., 2002), flavonoids and urate (Filipe et al., 2001), and tea polyphenols and vitamin E (Zhou et al., 2000). Interaction between/among various antioxidants would be expected to occur based on the order of reactivity of their oxidation-reduction potentials (Buettner, 1993).

Vitamin E reacts with lipid peroxyl radicals to form a relatively stable lipid hydroperoxide and vitamin E radical intermediate, which, in turn, may be recycled by other reductants. The interaction between the water-soluble antioxidant ascorbic acid and the lipid-soluble antioxidant tocopherol has been recognized, and ascorbic acid may be involved in the regeneration of the antioxidant capacity of vitamin E (Chen and Chang, 1979; Bendich et al., 1986; Chow, 1991). Vitamin C protected against loss of vitamin E in cultured rat hepatocytes (Halpner et al., 1998). It was reported that lipid-soluble free radical generator (AMVN) reduced by 35% the accumulation of α-tocopherolquinone, suggesting a recycling rather than an exclusive sparing action of vitamin C.

α-Tocopherol can protect β-carotene by the recycling of a α-tocopherol radical. A synergistically protective relationship between α-Tocopherol and β-carotene was observed (Leibovitz et al., 1990; Palozza and Krinsky, 1992). The authors hypothesized that a chain reaction initiating β-carotene peroxyl radical can be formed upon oxygen attack. The addition of α-tocopherol and β-carotene substantially facilitate the antioxidant activity of the β-carotene by limiting the production or reactivity of β-carotene peroxyl radical. The added α-tocopherol is consumed as it protects β-carotene
from autoxidation. Palozza and Krinsky (1991) observed that \( \alpha \)-Tocopherol and \( \beta \)-carotene have an additive effect in inhibiting radical-initiated lipid oxidation of lipid extracts from rat liver microsomes using hexane solutions. It seems that the cooperative interaction between ascorbic acid and \( \beta \)-carotene is unlikely at the lipid bilayer of membrane or at the core of lipoproteins due to the localization of \( \beta \)-carotene (Niki et al., 1995). The inhibiting action of ascorbic acid, \( \alpha \)-tocopherol, and \( \beta \)-carotene, alone or together, was studied by Böhm et al. (1998). Interestingly, they found a synergistic effect of the three antioxidants compared with the individual antioxidants, explaining such a synergism by assuming an electron transfer reaction in which the \( \beta \)-carotene radical is repaired by vitamin C.

1.9.2.2. Antiproliferative and other Biological Effects

In addition to antioxidant activities, phenolics have demonstrated other specific biological activities interfering with cellular mechanisms. Conte et al. (2003) reported synergistic protection of PC12 cells from \( \beta \)-amyloid toxicity by reservatrol and catechin. Compounds can offer additive or synergistic interaction against different biochemical targets. It was found that quercetin could enhance the action of carboxyamidotriazole (CAD) in human breast carcinoma MDA-MB-435 cells (Yeh et al., 1995). When quercetin and CAD were added to the MDA-MB-435 cells, synergism was observed in isobolograms in growth inhibition and clonogenic assays. The most effective combination was 20 \( \mu \)M quercetin with 4 \( \mu \)M CAD in growth inhibition assay; 30 \( \mu \)M quercetin with 1.2 \( \mu \)M CAD in clonogenic assay. RSV and quercetin additively activate caspase 3 in human pancreatic carcinoma cells (Mouria et al., 2002), and synergistically induce apoptosis in human leukemia cells (Mertens-Talcott and Percival, 2005). The effect of a combination of quercetin (25 \( \mu \)M) and \textit{trans}-RSV (25 \( \mu \)M) on mitochondrial cytochrome c release and caspase-3 activity was
greater than the expected additive response. The synergistic action with those two agents support the concept that the flavonoids (quercetin) and nonflavonoids (trans-RSV) act on the membrane permeability transition by distinct pathways, suggesting that the pathways are interactive (Mouria et al., 2002). Studies in cell cycle kinetics, proliferation, and apoptosis (caspase-3 activity) in human leukemia cells (MOLT-4) were conducted after incubation with ellagic acid, quercetin, and RSV as single compounds and in combination. A synergistic interaction with a CI of 0.64 for the combination of ellagic acid and resveratrol, and a CI of 0.68 for quercetin and RSV were observed after isobolographic analysis, indicating that the anticarcinogenic potential of foods containing phenolics may not be based on the effects of individual compounds, but may involve a synergistic enhancement of the anticancer effects (Mertens-Talcott and Percival, 2005). Genistein acted in synergism with eicosapentaenoic acid (EPA) on MCF-7 cells (genistein > 93.2 μM and EPA > 210.9 μM) and on MDA-MB-231 cells (genistein > 176.1 μM and EPA > 609.3 μM) (Nakagawa et al., 2000). The effects of EPA and an angiogenesis inhibitor (TNP-470) on the suppression of breast cancer cell growth were examined in five human breast cancer cell lines (MDA-MB-231, T-47D, MCF-7, KPL-1, and MKL-F). A combination of EPA and TNP-70 exerts synergistic activity against human breast cancer cells by accelerating apoptosis (Yamamoto et al., 1999). In MDA-MB-231 cells, diallyl disulfide (DADS) antagonized the effect of linoleic acid (LA), and synergized the effect of EPA (Nakagawa et al., 2001).

Anticancer activities of grape seed extract (GSE) and doxorubicin (Dox), either alone or in combination, in estrogen receptor-positive MCF-7 and receptor-negative MDA-MB468 human breast carcinoma cells were examined in order to enhance the efficacy of chemotherapy agents against breast cancer treatment (Sharma et al., 2004).
It was found that 100 µg/ml GSE in combination with 25–75 nM Dox treatment for 48 h had a strong synergistic effect (CI < 0.5) in cell growth inhibition, but mostly an additive effect (CI ~ 1) in cell death in both MCF-7 and MDA-MB468 cells.

Antioxidant vitamins of β-carotene, ascorbic acid and α-tocopherol, either combined or alone, were administered to rabbits with pacing-induced cardiomyopathy to assess the progression of congestive heart failure (Shite et al., 2001). It was observed that antioxidant vitamins combination decreased the myocardial oxidative stress, attenuated cardiac dysfunction, and prevented myocardial β-receptor downregulation. Administration of α-tocopherol alone produced similar effects, but the effects were less marked than those produced by the three vitamins together. A study of supplementing the diet with vitamin E, ginkgo biloba, pycnogenol, and ascorbyl palmitate in ApoE-deficient mice resulted in a significant increase in life span and a marked reduction of inclusion body histopathology in the hippocampus (Veurink et al., 2003). It is suggested that the combined actions of selected antioxidants may be therapeutically effective against neurodegenerative diseases such as Alzheimer’s disease. Using a balanced combination of several antioxidant nutrients, the SU.VI.MAX study (Hercberg et al., 2006) reported that a combination of antioxidants at nutritional doses lowered the incidence of cancers in healthy men by 31 %, without any evident increased cancer risk, where the doses used is similar to a healthy diet with high consumption of fruits and vegetables. Carotenoids are involved in quenching singlet oxygen and in scavenging reactive oxygen radicals. Experiments with zeaxanthin alone and in combination with vitamin E or C were carried out to examine their ability to protect cultured human retinal pigment epithelium ARPE-19 cells against oxidative stress (Wrona et al., 2004). It was concluded that cells with added antioxidants showed increased viability and accumulated less lipid
hydroperoxides than cells without the antioxidant, suggesting a synergistic action of zeaxanthin and vitamin E or C in efficient protection of cell membranes against oxidative damage induced by photosensitized reactions. Enhanced oxidative stress leads to a situation that favors atherothrombotic processes, thrombogenesis, and endothelial dysfunction in diabetes mellitus (Aydin et al., 2001). A recent study found that, in diabetic rats, the combination of α-tocopherol and aspirin exhibited a greater inhibitory effect on platelet aggregation than in untreated control animals with diabetes (Gonzalez-Correa et al., 2006). Also, the combination of the two compounds improved the thromboxane/prostacyclin balance in comparison to untreated diabetic animals and untreated healthy animals. This combination increased tissue concentrations of reduced glutathione; however, it did not potentiate the antioxidant effect of either compound alone.

In a recent antioxidant combination study of NNK-induced lung carcinogenesis in smoke-exposed ferrets, combined β-carotene, ascorbic acid and α-tocopherol was found to maintain normal tissue levels of retinoic acid, and inhibit the activation of mitogen-activated protein kinase pathways, cell proliferation and phosphorylation of p53 (Kim et al., 2006), suggesting that the combination of nutrients, rather than individual agents, could be an effective chemopreventive strategy against lung cancer in smokers.

1.9.2.3. **Induction of Phase II Detoxifying Enzymes**

Phenolics present in fruits and vegetables have numerous other biological effects, such as inducing phase II enzymes, that may be totally separate or act in concert with antioxidant activities. The interactions between sulforaphane and apigenin in the regulation of UGT and GST expression were examined in
undifferentiated CaCo-2 cells (Svehlíková et al., 2004). Sulforaphane in combination with apigenin led to a synergism, approximately 12-fold induction of UGT1A1 mRNA, although this interaction was not observed for GSTA1, proposing that UGT1A1 and GSTA1 are regulated by sulforaphane through different signal transduction pathways. The combinations of phytochemicals in the whole vegetable may provide additional protection through the synergistic induction of chemoprotective enzymes, suggesting that the health benefits of whole vegetables may be greater than those of the isolated compounds (Jeffery and Stewart, 2004). The synergistic upregulation of phase II enzymes by glucosinolate breakdown products in cruciferous vegetables has been reported in animal models. Four glucosinolate derivatives were examined individually and as a mixture for their effects on NQO1, hepatic P4501A (CYP1A), GST, and glutathione reductase (G-Rd) levels (Staack et al., 1998). Indole-3-carbinol (I3C, 56 mg/kg) and crambene (50 mg/kg) had a 1.9- and 2.5-fold increase in NQO1, respectively, in adult male F344 rats. It was observed that the I3C and crambene combination produced a synergistic effect on induction of NQO1 and GST. Furthermore, this lab tested whether the synergism is at the level of transcription. It was found that, after evaluating three subunits, GST Ya2 mRNA had a synergistic upregulation by crambene and I3C, while Yc1 and Yc2 showed only an additive response in adult male rats (Nho and Jeffery, 2001). Finally, it was concluded that a nitrile product of glucosinolate hydrolysis induced NQO1 mRNA levels and triggered the ARE, suggesting that synergistic upregulation of NQO1 is due to co-activation of the ARE and the xenobiotic response element (XRE) by I3C acid condensates and crambene (Nho and Jeffery, 2004).

The challenge for our research is to design and implement experimental studies to target the development of an understanding of many phytochemical interactions.
CHAPTER TWO

PHYTOCHEMICAL PROFILES AND ANTIOXIDANT ACTIVITIES
OF WINE GRAPES

2.1. Introduction

A large number of epidemiological studies have revealed that the consumption of fruits and vegetables is associated with a lowered risk for developing chronic diseases, such as coronary heart disease (CHD), cancer, diabetes, and Alzheimer’s disease (Block et al., 1992; Ames et al., 1993; Temple, 2000; Joshipura et al., 2001; Willett, 2002). The Caerphilly Study, which looked at 2,112 Welsh men, has shown that the consumption of vegetables and fruits could considerably reduce the risk of mortality from cancer (Hertog et al., 1996). A cohort study in Finland among 9,959 men and women aged 15-99 years reported an inverse association between the intake of flavonoids and incidence of lung cancer (Knekt et al., 1997). The positive relationship between the consumption of fruits and vegetables and a reduced risk for CHD was reported by other prospective cohort studies, for examples, the Zutphen Elderly Study (Hertog et al., 1993), the Iowa Women’s Health Study (Yochum et al., 1999), the US Health Professionals Study (Rimm et al., 1996), and a study of 5,000 men and women in Finland (Knekt et al., 1996). Fruits and vegetables possess many bioactive compounds, including phenolics, vitamins, minerals, thiols, carotenoids, and glucosinolates. However, phytochemicals in fruits and vegetables, mainly phenolics and flavonoids, are suggested to be the major bioactive compounds responsible for their health benefits. Therefore, the National Cancer Institute recommends consuming five or more servings of fruits and vegetables per day.
The disturbance of \textit{in vivo} ROS production and of the capacity of the endogenous antioxidant defense system will cause oxidative stress, resulting in cellular damage to DNA, proteins, or lipids, which is implicated in degenerative diseases (Ames et al., 1993). The presence of phytochemical compounds in fruits and vegetables play an important role in disease prevention, possibly through their effect on relieving oxidative damage by acting as antioxidants (Heinonen et al., 1998; Record et al., 2001; Liu, 2003; Liu, 2004). The traditional Western diet offers roughly 1g/day of mixed flavonoids (Kühnau, 1976). Consumption of flavonoids was shown to be inversely associated with morbidity and mortality resulted from CHD (Hertog et al., 1993; Knekt et al., 1996). Besides antioxidant activity, flavonoids have many biological activities such as the inhibition of plasma platelet aggregation and cyclooxygenase (COX) activity, the suppression of histamine release and SRS-A biosynthesis \textit{in vitro} (Hope et al., 1983); potent nitric oxide (NO) radical scavenging activity (Van Acker et al., 1995), and antibacterial, antiviral, anti-inflammatory, and antiallergenic effects (Cook and Samman, 1996). Flavonoids’ antiaging, anticarcinogenicity, and antimutagenicity properties originate from these activities, thus they potentially play a role in preventing a wide range of degenerative physiological processes. Phenolics and other plant antioxidants can scavenge free radicals, quench singlet oxygen, and act as chelators, and thus protect biological macromolecules from ROS attack. Hence, diet-derived antioxidants may be especially important in protecting against chronic diseases. There is an increasing interest in the health benefits of naturally occurring antioxidants contained in diets.

Grapes are one of the popular fruits and are widely cultivated throughout the world. There are about 60 species of \textit{Vitis}, which are mainly found in the temperate zones of the Northern Hemisphere, and almost equally distributed between America
and Asia (Mullins et al., 1992). Approximately 80% of the total grapes are used in winemaking, and 13% is consumed as table grapes (Mazza, 1995). The main wine-making grape varieties include Cabernet Sauvignon, Chardonnay, Pinot Noir, Merlot, and Zinfandel. The major table grape varieties are Perlete, black Ribier, Tokay, and Emperor. *V. vinifera* grapes are commonly used for wine production around the world, principally distributed in Europe. Most studies in phenolic composition and related antioxidant activity have been done on grapes and wines using *V. vinifera* species. In the United States, species such as *V. labrusca*, *V. riparia*, *V. aestivalis*, *V. rupestris*, and *V. rotundifolia* are also used in wine-making. There are two main grape species in North America: *V. labrusca* and *V. rotundifolia*. The *labrusca* grapes are grown mostly in the lower Great Lakes region of the U.S. and Canada. The representative grape variety is the large purple berry - Concord. Due to its abundance in the United States, this grape is popular in making of juices and jellies. Grapes are rich in phenolics; most of them are present in the peel (Singleton, 1982). Phenolics in grape are classified into two groups: the flavonoids and nonflavonoids. The flavonoids include flavan-3-ols, flavonols, and anthocyanins. The nonflavonoids encompass hydroxybenzoates, hydroxycinnamates, and stilbenes. In the grape berry, the flavonoids are mainly localized in the skins, such as the anthocyanins and resveratrol, while the flavan-3-ols are presented both in the skins and seeds. However, the composition and concentration of phenolics in grapes vary with variety, species, season, and environmental and management factors such as soil conditions, climate, and crop load (Jackson and Lombard, 1993).

Anthocyanins, widely distributed throughout the plant kingdom, are natural, nontoxic, and water-soluble flavonoid pigments, being particularly present in fruits and vegetables where they are responsible for the red, orange, blue, and purples colors.
Anthocyanins contain in daily diet after consuming certain fruits and vegetables. Grapes and berries are the major dietary sources of anthocyanins (Rechkemmer and Pool-Zobel, 1996). Anthocyanins are responsible for the coloring of black, red, and purple grapes, however, they are lacking in white grapes. In particular, anthocyanins mostly accumulate in the skins, whereas procyanidins are located in the seeds. It was reported that Lomanto and Colobel hybrid grape cultivar had the highest anthocyanin content with 603 mg/100g; Midsouth cultivar contained the lowest content with 5.5 mg/100g (Mazza, 1995). The content of anthocyanins in the skin of grapes was estimated from 200 ~ 5000 mg/kg of fresh whole grape (Riberau-Gayon, 1982). The average content of anthocyanins in red wines is estimated at 26 mg/L (Frankel et al., 1995). The anthocyanins in grape skins are predominately the 3-O-glucosides of malvidin, cyanidin, delphinidin, peonidin, and petunidin (Wrolstad, 2000). Malvidin, the reddest of all anthocyanins (Jackson, 1994), is the major one in dark red *vinifera* grape with higher proportions of cyanidin in red grape. Cyanidin 3-monoglucoside and delphinidin 3-monoglucoside are major anthocyanins in Concord grapes. Grape anthocyanins play a crucial role in the color quality of red wines, and have been increasingly used as food colorants and nutraceuticals. Anthocyanins possess antioxidant activity, being considered to be an important physiological function (Wang et al., 1997; Ehlenfeldt and Prior, 2001; Kalt et al., 2001; Connor et al., 2002; Moyer et al., 2002). Additively, anthocyanins have been reported to have anti-inflammatory (Wang et al., 1999), anticancer activity (Kamei et al., 1995; Bomser et al., 1996; Hou, 2003), apoptotic induction effect (Katsube et al., 2003), α-glucosidase inhibition activity (Matsui et al., 2001), vision improvement (Matsumoto et al., 2003), and effects on collagen, blood platelet aggregation, and capillary permeability and fragility (Kalt and Dufour, 1997). Dietary consumption of anthocyanins has been demonstrated to improve overall antioxidant defense status of human plasma (Cao and Prior, 1999).
In LPS/IFN-γ-activated RAW 264.7 macrophages, anthocyanins have been demonstrated to have significant inhibitory effect on NO production (Wang and Mazza, 2002). In 2003, (Katsube et al., 2003) have reported that anthocyanins inhibit the growth of HL60 cells through the induction of apoptosis. The growth inhibitory effects of anthocyanins in K562 leukemia and HCT-15 carcinoma cells are greater than those of other flavonoids, such as flavanons and flavonols (Kamei et al., 1995). Thus, anthocyanins as naturally occurring bioactive compounds and pigments present in foods have attracted interest due to their safety and health benefits.

Resveratrol (3, 4’, 5-trihydroxystilbene, RSV), synthesized by some plants in response to adverse conditions such as pathogenic attack and environmental stress, is found in various food products. It is particularly high in grape skins, seeds, and in red wine (Langcake and Pryce, 1976). RSV is present in cis and trans isoforms and the major trans isomer is the biologically active one (Bhat et al., 2001b). The ‘French Paradox’ has been suggested that RSV might be the main active component in red wine (Frankel et al., 1993a; Kopp, 1998). Thus, RSV has attracted considerable attention due to its cardioprotective and cancer chemopreventive activities (Jang et al., 1997), which provide great interest in grapes, wines, and dietary products containing RSV. The proposed mechanisms related to RSV’s health effects can be summarized as scavenging intracellular ROS (Manna et al., 2000), inhibiting the oxidation of LDL (Frankel et al., 1993b), preventing platelet aggregation (Pace-Asciak et al., 1995), suppressing cell proliferation via steps in the signal transduction pathways (Pozo-Guisado et al., 2002), inducing apoptotic cell death through activation of mitochondria-dependent pathways (Huang et al., 1999), exhibiting anti-inflammatory activity via down-regulation of proinflammatory cytokines (Wadsworth and Koop, 1999), promoting cellular differentiation (Mizutani et al., 1998), exhibiting
antiestrogenic activity (Lu and Serrero, 1999), and inhibiting of CYP1 enzymes (Chang et al., 2001).

The beneficial health-related effects of certain phenolics and flavonoids in grapes are of importance to consumers, breeders, and the grape industry. There is limited knowledge about the phytochemical profiles, antioxidant and antiproliferative activities in both Vitis vinifera and non-Vitis vinifera wine grapes grown in Finger Lake area in New York State. The objectives for this study were: (1) to determine the profiles of total phenolics, total flavonoids, total anthocyanins, and resveratrol in selected grapes grown in Finger Lake areas; (2) to measure the total antioxidant activity; (3) to determine the antiproliferative activity of grape extracts against human colon, liver, and breast cancer cells in vitro.

2.2. Materials and Methods

2.2.1. Chemicals

Sodium nitrite, (+)-catechin, Folin-Ciocalteu reagent (FCR), hydrochloric acid, glucagon, hydrocortisone, insulin, α-keto-γ-methiolbutyric acid (KMBA), and tran-RSV were purchased from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride, sodium hydroxide, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA). Gallic acid was purchased from ICN Biomedical Inc. (Costa Mesa, CA). 2,2’-Azobis(amidinopropane) (ABAP) was purchased from Wako Chemicals (Richmond, VA).
2.2.2. Grapes

Fourteen wine grape varieties (Baco Noir, Cabernet Franc, Catawba, Cayuga White, Chancellor, Chardonnay, Concord, DeChaunac, Marechal Foch, Niagara, Pinot Noir, Riesling, Sheridan, and Vidal Blanc) were provided by New York State Wine and Grape Foundation. General descriptions of the grape varieties are given in Table 2.1. Wine grapes were harvested upon ripening in 2003 and 2004. Grapes were selected that were free from visible blemish or disease. For quantitative analysis, 50–70 grape berries randomly selected from each grape variety were collected for extraction. All data collected for each grape variety were reported as means ± SD for at least three replications.

2.2.3. Extraction of Total Phenolic Compounds

Total phenolics were extracted from fresh grapes by the modified method reported previously in our laboratory (Sun et al., 2002a; Yang et al., 2004). Briefly, 100 g of grapes were blended for 1 min in 100 g of 80% acetone using a Waring blender with medium speed to remove seeds. After removal of seeds and adding additional 100 g of 80% acetone, the grapes were blended for 3 min using a Waring blender with high speed. The mixture was then homogenized in a Virtis High Speed Homogenizer for 3 min and filtered with vacuum under ice bath. The acetone in the filtrate was evaporated using a rotary evaporator at 45°C until the weight of the evaporated filtrate was less than 10% of the weight of the original filtrate. All extracts were stored at –40°C until use. All extractions were performed in triplicate.
Table 2.1. Description of grape varieties

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Color</th>
<th>Storage</th>
<th>Vintage</th>
<th>Growing Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vinifera</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabernet Franc</td>
<td>Dark purple/blue</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>Green</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Pinot Noir</td>
<td>Dark purple/blue</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Riesling</td>
<td>Green</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td><em>Hybrid</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baco Noir</td>
<td>Dark purple</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Catawba</td>
<td>Pink</td>
<td>- 40°C</td>
<td>2003</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Cayuga White</td>
<td>Green</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Chancellor</td>
<td>Dark purple/blue</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Concord</td>
<td>Red, purple</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>DeChaunac</td>
<td>Dark blue</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Marechal Foch</td>
<td>Dark purple</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Niagara</td>
<td>Green</td>
<td>- 40°C</td>
<td>2004</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Sheridan</td>
<td>Red, purple</td>
<td>- 40°C</td>
<td>2003</td>
<td>Finger Lakes area, NY</td>
</tr>
<tr>
<td>Vidal Blanc</td>
<td>Green</td>
<td>- 40°C</td>
<td>2003</td>
<td>Finger Lakes area, NY</td>
</tr>
</tbody>
</table>
2.2.4. Determination of Total Phenolic Content

The total phenolic content in grapes was determined using the Folin-Ciocalteu colorimetric method (Singleton et al., 1999), which was modified by our laboratory (Yang et al., 2004). Briefly, all extracts were diluted 1:10 with distilled water to obtain readings within the standard curve ranges of 0.0 - 600.0 µg of gallic acid/mL. The grape extracts were oxidized by Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance was measured at 760 nm after 90 min at room temperature by a MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). The absorbance values were then compared with those of standards with known gallic acid concentrations. All values were expressed as the mean (milligrams of gallic acid equivalents per 100 g of fresh sample) ± SD for three replications.

2.2.5. Determination of Total Flavonoid Content

The total flavonoid content of the grape extract was determined using a modified colorimetric method (Jia et al., 1999; Yang et al., 2004). Briefly, 0.25 mL of 1:10 diluted grape extract was mixed with 1.25 mL of distilled water, and subsequently with 0.075mL of 5% sodium nitrite solution, and allowed to react for 5 min. Then 0.15 mL of 10% aluminum chloride was added and allowed to further react for 6 min before 0.5 mL of 1 M sodium hydroxide was added. Distilled water was added to bring the final volume of mixture to 3 mL. The absorbance of the mixture was immediately measured at 510 nm wavelength against a prepared blank using a MRX II DYNEX spectrophotometer. The flavonoid content was determined by a catechin standard curve and expressed as mean (milligrams of catechin equivalents per 100 g of fresh sample) ± SD for the triplicate extracts.
2.2.6. Determination of Anthocyanin Content

Monomeric anthocyanin content of the grape extract was measured using a modified pH differential method (Boyles and Wrolstad, 1993; Wolfe et al., 2003). The grape extracts were mixed thoroughly with 0.025 M potassium chloride pH 1 buffer in 1:2 ratio of extract to buffer. The grape extracts were then mixed similarly with sodium acetate buffer pH 4.5. A Beckman DU640B spectrophotometer was used to measure absorbance at 510 and 700 nm against a buffer blank at pH 1.0 and 4.5. Absorbance readings were converted to total milligrams of cyanidin 3-glucoside (C3G). The anthocyanin content was calculated as follows:

\[
\text{Total monomeric anthocyanins (mg/100g)} = \Delta A \times MW \times 1000 / (\varepsilon \times C)
\]

\[
\Delta A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}
\]

where \( A \) is absorbance; MW (449.2) is molecular weight for C3G; \( \varepsilon \) (26,900) is the molar absorptivity of C3G; and \( C \) is the concentration of the grape extract in milligrams per milliliter. Anthocyanin content was expressed as milligrams of C3G equivalents per 100 g of fresh grape for the triplicate extracts.

2.2.7. Reverse-Phase HPLC Analysis of Resveratrol

A 3-mL grape sample was extracted in a test-tube with 5 mL of ethyl ether, and then the mixture was put into shaker with 200 rpm for 15 mins. Organic phase was transferred into a new test-tube. The residues were extracted with 5 mL of ethyl acetate twice using the same condition. The organic solvent in a new test-tube was evaporated through flushing with \( \text{N}_2 \). The dry residue was dissolved in 1 mL methanol, and the aliquots were than analyzed by RP-HPLC.

Stock solution containing 14.4 mg/mL of resveratrol in methanol was prepared. Solution was stored at -4°C in the darkness after elimination of oxygen with
a N₂ to avoid the oxidation or decomposition of phenolic compounds. Resveratrol in grape extracts was quantified using a RP-HPLC procedure employing a Supelcosil LC-18-DB, 150 mm × 4.6 mm, 3 μm column. Samples of 20 μL standard or grape methanolic extracts were directly injected into the column. Elution was carried out with a mobile phase delivered using a Waters 515 HPLC pump (Waters Corp., Milford, MA) at a flow rate of 1.2 mL/min according to the following gradient: the initial mixture was acetonitrile – water (9:91) adjusted to pH 2 with triflouroacetic acid for 10 mins; linear gradient to (25:75) in 10 mins, hold for 10 mins; linear gradient to (70:30) in 1 min, hold for 12 mins. A Waters 2487 wavelength absorbance detector (Waters Corp. Milford, MA) was used for UV detection of analytes at 307 nm. Data signals were acquired and processed on a PC running the Waters Millennium software, version 3.2 (1999) (Waters Corp. Milford, MA). Three HPLC injections were performed for each extract. Peak heights were used for all calculations. The recovery for RSV analyses was 104.78 ± 4.87% (n = 4).

2.2.8. Determination of Total Antioxidant Capacity

The total antioxidant capacity of grape extracts was measured using a total oxyradical scavenging capacity (TOSC) assay (Winston et al., 1998) as modified in our laboratory (Yang et al., 2004). In this assay, peroxy radicals produced from 2,2'-azobis-amidinopropane (ABAP) oxidize α-keto-γ-methiolbutyric acid (KMBA) to form ethylene gas, which was measured by gas chromatographic headspace analysis. Briefly, antioxidant activity was quantified after 15, 30, 45, and 60 min for four different grape extract concentrations and a control. The amount of ethylene generated by the reaction was expressed as peak area. The TOSC value corresponding to each extract concentration was calculated by integrating the area under the kinetic curve, and assessed as the following equation: \[ TOSC = 100 - \left( \frac{\int SA}{\int CA} \right) \times 100 \], where, \( \int SA \)
is the integrated area from the sample reaction, and \( \int CA \) is the integrated areas from the control reaction. The median effective dose (EC\(_{50}\)) was determined for each grape variety from the dose-response curve of grape concentration versus TOSC value. The TOSC value is expressed as \( \mu \text{mol of vitamin C equivalents per gram of sample.} \) All values were presented as the mean ± SD at least three replicates.

2.2.9. Measurement of Inhibition of Caco-2, HepG\(_2\), and MCF-7 Cell Proliferation

The antiproliferative activity of different grape extracts was assessed by measurement of the inhibition of Caco-2, HepG\(_2\), and MCF-7 (The American Type Culture Collection, ATCC, Rockville, MD) human cancer cell proliferation. Antiproliferative activities were determined by the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) (Promega, Madison, WI) reported previously (Yang et al., 2004). HepG\(_2\) - human liver cancer cells were cultured in Williams medium E (WME), containing 10 mM Hepes, 5 \( \mu \text{g/mL insulin, 0.05} \mu \text{g/mL hydrocortisone, 2} \mu \text{g/mL glucagon, and 5} \% \text{ fetal bovine serum (Gibco, Life Technologies, Grand Island, NY), 50 units/mL penicillin, 50} \mu \text{g/mL streptomycin, and 100} \mu \text{g/mL gentamicin. Caco-2 - human colon cancer cells were maintained in DMEM, containing 10 mM Hepes, 5} \% \text{ FBS, 50 units/mL penicillin, 50} \mu \text{g/mL streptomycin, and 100} \mu \text{g/mL gentamicin. MCF-7 human breast cancer cells were maintained in Alpha Minimum Essential Medium (MEM-a), containing 10 mM Hepes, 0.01 mg/mL insulin, 50 units/mL penicillin, 50} \mu \text{g/mL streptomycin, 100} \mu \text{g/mL gentamicin, and 10} \% \text{ fetal bovine serum (Gibco, Life Technologies, Grand Island, NY).} \) HepG\(_2\), Caco-2, and MCF-7 cells were maintained in a 5\% CO\(_2\)/37°C incubator. A total of 2.5 \times 10^4 \text{ HepG}_2 \text{ or Caco-2 or MCF-7 cells in growth media were placed in each well of a 96-well flat-bottom plate. Cell proliferation was measured by the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-}
carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium (MTS) to formazan. After 4 h of incubation, the growth medium was removed and media containing various concentrations (2, 5, 10, 20, 30, 40, 50, 75, and 100 mg/mL) of grape extracts were added to the cells. Control cultures received the extraction solution minus the grape extracts, and blank wells contained 100 µL of growth medium without cells. Cell proliferation (percent) was determined at 96 h from the MTS absorbance (490 nm) reading for each concentration compared to the control, using at least three replications for each sample. The effective median dose (EC₅₀) was determined and expressed as milligrams of grape extracts per milliliter ± SD.

2.2.10. Statistical Analysis

Statistical analysis was performed using Minitab Student Release 12 (Minitab Inc., State College, PA) and SigmaStat Version 8.0 (Jandel Corp., San Raphael, CA). Results were subjected to ANOVA, and differences between means were located using Tukey’s multiple comparison test. Correlations between various parameters were also investigated. Significance was determined at \( p<0.05 \). All data were reported as the mean ± SD of three replications.

2.3. Results

2.3.1. Total Phenolic Content

Total phenolic content of fourteen wine grapes are presented in Figure 2.1. Among all the grape varieties analyzed, Cabernet Franc and Pinot Noir had the highest total phenolic content (424.6 ± 3.8 and 396.8 ± 12.4 mg of gallic acid equivalents/100g of grape, respectively), followed by Concord (334.0 ± 13.6), Sheridan (331.39 ± 8.21), Chancellor (325.8 ± 21.7), Marechal Foch (312.5 ± 10.9),
Catawba (311.7 ± 9.1), DeChaunac (293.5 ± 21.6), Riesling (255.8 ± 8.8), Niagara (229.6 ± 3.9), Vidal Blanc (230.0 ± 5.5), Baco Noir (217.0 ± 14.1), Cayuga White (206.3 ± 8.2), and Chardonnay (201.1 ± 4.9). Significant differences were found in total phenolic content in comparisons between Cabernet Franc and Concord, Pinot Noir and Sheridan, Chancellor and Riesling, and DeChaunac and Niagara (p<0.05); however, significant differences in total phenolic content were not found between Cabernet Franc and Pinot Noir, between Riesling and Niagara, among Concord, Sheridan, Chancellor, Marechal Foch, and Catawba, and among Vidal Blanc, Baco Noir, Cayuga White, and Chardonnay (p>0.05). The results show that the red grape varieties except Baco Noir contain high concentrations of total phenolics. In contrast, the green grapes have lower phenolic content. There was a 2.1-fold difference in total phenolic content between the highest and lowest ranked varieties, Cabernet Franc and Chardonnay (p < 0.05).

Total flavonoids of the fourteen grape extracts were measured (Figure 2.2). The Pinot Noir showed the highest flavonoid content (301.8 ± 6.2 mg of catechin equivalents/100 g of fresh grapes, p<0.05), followed by Catawba (181.0 ± 5.4), Cabernet Franc (181.0 ± 15.3), Cayuga White (176.1 ± 10.7), Niagara (173.1 ± 11.3), Concord (168.2 ± 6.0), Sheridan (166.8 ± 1.4), Chardonnay (166.4 ± 20.4), Chancellor (140.0 ± 18.8), Riesling (133.5 ± 13.7), Marechal Foch (127.0 ± 14.2), DeChaunac (114.0 ± 12.0), Vidal Blanc (101.0 ± 9.4), and Baco Noir (98.0 ± 10.0). The flavonoid content of Pinot Noir, Catawba, Chancellor, and Baco Noir was significantly different from each other (p<0.05). However, significant differences in the total flavonoid content were not found in comparisons among Catawba, Cabernet Franc, Cayuga White, Niagara, Concord, Sheridan, and Chardonnay, and among Chancellor, Riesling, Marechal Foch, DeChaunac, Vidal Blanc, and Baco Noir (p>0.05). An about
3.1-fold difference in total flavonoid content was found between the highest and lowest ranked varieties, Pinot Noir and Baco Noir ($p < 0.05$).

Total anthocyanin contents of nine red grape extracts were determined (Figure 2.3). DeChaunac had the highest total anthocyanin content ($239.6 \pm 25.4$ mg of cyanidin 3-glucoside equivalents/100g of grapes, $p < 0.05$), followed by Chancellor, Marechal Foch, Baco Noir, Concord, Cabernet Franc, Sheridan, Pinot Noir, and Catawba. There was significant difference ($p < 0.05$) in anthocyanin content between DeChaunac, Chancellor, Baco Noir, Cabernet Franc, and Catawba. However, significant differences in the anthocyanin content were not observed between Chancellor and Marechal Foch, between Baco Noir and Concord, among Cabernet Franc, Sheridan, and Pinot Noir ($p > 0.05$). In addition, anthocyanins were not detected for all five green grape varieties.

Resveratrol content of fourteen grape extracts was quantified (Figure 2.4). Baco Noir variety contained the highest RSV content ($571 \pm 30$ μg/100g of fresh sample), followed by Pinot Noir ($421 \pm 54$), Vidal Blanc ($263 \pm 16$), Marechal Foch ($130 \pm 8$), Cabernet Franc ($119 \pm 8$), Chancellor ($117 \pm 7$), Sheridan ($112 \pm 10$), Riesling ($80 \pm 7$), DeChaunac ($75 \pm 6$), Chardonnay ($73 \pm 9$), Catawba ($72 \pm 5$), Concord ($65 \pm 8$), Niagara ($53 \pm 4$), and Cayuga White ($38 \pm 2$). There were significant differences ($p < 0.05$) in RSV content among Baco Noir, Pinot Noir, Vidal Blanc, Marechal Foch, and Chardonnay. However, no significant differences in RSV content were found among Marechal Foch, Cabernet Franc, Chancellor, Sheridan, DeChaunac, and Riesling, among Chardonnay, Catawba, Concord, Niagara, and Cayuga White ($p > 0.05$).
Figure 2.1. Total phenolic content of fourteen grape varieties (mean ± SD, n = 3). Bars with no letters in common are significantly different ($p < 0.05$).
Figure 2.2. Total flavonoid content of fourteen grape varieties (mean ± SD, n = 3). Bars with no letters in common are significantly different (p < 0.05).
Figure 2.3. Total anthocyanin content of nine red grape varieties (mean ± SD, n = 3). Bars with no letters in common are significantly different (p < 0.05).
Figure 2.4. Resveratrol content of fourteen grape varieties (mean ± SD, n = 3). Bars with no letters in common are significantly different (p < 0.05).
2.3.2. **Total Antioxidant Activity**

Total antioxidant activities of the fourteen grape varieties, expressed as micromoles (μmol) of vitamin C equivalents per gram of fresh grape, are shown in **Figure 2.5**. Phytochemical extracts of Cabernet Franc had the highest antioxidant activity (149.0 ± 10.0 μmol/g, \(p < 0.05\)), followed by Pinot Noir (122.4 ± 5.7), Concord (106.0 ± 6.0), Sheridan (106.6 ± 3.6), Chancellor (102.8 ± 6.0), Marechal Foch (100.2 ± 6.0), Catawba (98.0 ± 4.6), DeChaunac (96.3 ± 6.1), Riesling (79.8 ± 4.3), Niagara (65.3 ± 3.8), Vidal Blanc (64.7 ± 2.5), Baco Noir (63.4 ± 4.6), Cayuga White (63.3 ± 4.6), and Chardonnay (61.9 ± 6.1). A statistically significant difference \((p<0.05)\) was found among Cabernet Franc, Pinot Noir, Chancellor, Riesling, and Chardonnay. The total antioxidant activities of Pinot Noir and Concord were similar \((p > 0.05)\) but lower \((p < 0.05)\) than that of Cabernet Franc. No significant difference \((p>0.05)\) was found among Concord, Sheridan, Chancellor, Marechal Foch, Catawba, and DeChaunac, among Riesling, Niagara, and Vidal Blanc, and among Vidal Blanc, Baco Noir, Cayuga White, and Chardonnay grapes. The varieties containing high total phenolic content had higher antioxidant activity.

2.3.3. **Inhibition of Human Cancer Cell proliferation**

The effect of 14 wine grape varieties on the growth of Caco-2 human colon cancer cells, HepG\(_2\) human liver cancer cells, and MCF-7 human breast cancer cells *in vitro* are summarized, respectively, in **Figure 2.6**. Grape extracts had potent antiproliferative activity against human colon, liver, and breast cancer cells in a dose-dependent manner. Among the 14 grape varieties tested, Cabernet Franc, Pinot Noir, Chardonnay, Catawba, Concord, Sheridan, Niagara, and Riesling show relatively high antiproliferative activities toward both Caco-2 and HepG\(_2\) cells. Cabernet Franc and Catawba exhibit relatively strong antiproliferative activities toward MCF-7 cells.
Figure 2.7 presents the EC$_{50}$ of the antiproliferative activity of different grape varieties. Lower EC$_{50}$ values represent higher antiproliferative activities. The phytocemical extracts of Pinot Noir and Cabernet Franc possess the greatest antiproliferative activity toward Caco-2 cells with the lowest EC$_{50}$ of 9.5 ± 1.4 and 10.0 ± 1.0 mg/mL ($p$<0.05), respectively, followed by Chardonnay, Catawba, Concord, Sheridan, Niagara, Riesling, DeChaunac, Cayuga White, Baco Noir, Chancellor, Vidal Blanc, and Marechal Foch. The EC$_{50}$ doses were significantly different among Pinot Noir, Concord, Niagara, Baco Noir, and Marechal Foch ($p < 0.05$). The phytochemical extract of Marechal Foch exhibited a weak antiproliferative activity toward Caco-2 cells at a higher dose with an EC$_{50}$ of 37.2 ± 1.5 mg/mL.

The antiproliferative activities of grape extracts toward HepG$_2$ human liver cancer cells were somewhat different from those toward Caco-2 cells. The phytochemical extract of Pinot Noir and Chardonnay varieties exhibit the strongest antiproliferative effect toward HepG$_2$ cells with the lowest EC$_{50}$ of 17.0 ± 0.8 mg/mL and 18.1 ± 0.1 mg/mL ($p < 0.05$), respectively, followed by Cabernet Franc, Sheridan, Catawba, Riesling, Niagara, Concord, Cayuga White, Vidal Blanc, and DeChaunac. Cabernet Franc and Sheridan had similar EC$_{50}$ values ($p > 0.05$). The phytochemical extracts of Vidal Blanc and DeChaunac showed a weak antiproliferative activity at higher doses with the EC$_{50}$ of 52.1 ± 2.1 and 52.2 ± 3.0 mg/mL, respectively. Although demonstrated HepG$_2$ cell proliferative, the EC$_{50}$ values of the Baco Noir, Chancellor, and Marechal Foch varieties could not be calculated at the maximum doses used in this experiment.

The antiproliferative activities of grape extracts on MCF-7 human breast cancer cells were different from both HepG$_2$ and Caco-2 cells. The phytochemical
extract of Cabernet Franc contained the lowest EC$_{50}$ at 64.0 ± 3.9 mg/mL, indicating the most antiproliferative effect toward MCF-7 cells of the varieties examined ($p < 0.05$). There were statistical differences among the EC$_{50}$ values of Catawba, Chardonnay, and Riesling varieties ($p<0.05$). Although they exhibited inhibition of MCF-7 cell proliferation, the EC$_{50}$ values of the Pinot Noir, Concord, DeChaunac, Cayuga White, Baco Noir, Chancellor, Vidal Blanc, and Marechal Foch varieties could not be calculated from the maximum doses used in this experiment.

2.3.4. Relationship Between Total Phenolic Content and Antioxidant Activity

Correlations between total phenolics, total flavonoids, total antioxidant activity, and cell proliferation EC$_{50}$ values were analyzed for the 14 grape varieties. A significant linear relationship was found between total phenolic and total antioxidant activity in the phytochemical extracts from different grape varieties ($R^2 = 0.9775$, $p < 0.05$, Figure 2.8). The positive correlation indicates that the higher total phenolic content resulted in a higher total antioxidant activity. There were no other significant relationships observed.
Figure 2.5. Total antioxidant activity of phytochemical extracts of fourteen grape varieties (mean ± SD, n = 3). Bars with no letters in common are significantly different (p < 0.05).
Figure 2.6. Percent inhibition of Caco-2 (a), HepG2 (b), and MCF-7 (c) cancer cell proliferation by fourteen grape extracts.
Figure 2.6 (Continued)
Figure 2.7. $EC_{50}$ of antiproliferative activity of phytochemical extracts of fourteen grape varieties (mean ± SD, $n = 3$).
Total Phenolic Content (mg gallic acid equivalents/100g grapes)

Total Antioxidant Activity (μmol vitamin C equivalents/g grape)

$R^2 = 0.9775$

Figure 2.8. Relationship between total phenolic content and total antioxidant activity of fourteen grape varieties.
2.4. Discussion

Oxidative stress arising from an imbalance in human antioxidant status contributes to the pathology of chronic diseases (Ames et al., 1993; Temple, 2000; Record et al., 2001). Besides endogenous defenses, the consumption of dietary antioxidants contained in fruits and vegetables plays an important role in protecting against those pathological events. Previously, much attention has been paid to the antioxidant properties of ascorbic acid, tocopherol, and β-carotene. In recent years, phytochemicals, especially phenolics, have attracted increasing attention for their antioxidant activities. A study from our group has shown that the vitamin C content in whole apples contributes only 0.4% of the total antioxidant activity; suggesting that most antioxidant activity is derived largely from phytochemicals such as phenolic compounds, flavonoids, and anthocyanins (Eberhardt et al., 2000). This work has clearly shown that the phytochemicals present in grapes have potent antioxidant and antiproliferative activities, and that antioxidant activity in grapes is positively correlated with total phenolic content.

It is well known that both genetic and agronomic or environmental factors play important roles in phenolic composition and thus the nutritional quality of crops (Tomas-Barberan and Espin, 2001). In our study, the total phenolic contents of Cabernet Franc and Pinot Noir were the highest \((p < 0.05)\) at \(424.6 \pm 3.8\) and \(396.8 \pm 12.4\) mg/100g of fresh sample, respectively. Chardonnay had the lowest \((p < 0.05)\) total phenolic content at \(201.1 \pm 4.9\) mg/100g of fresh grape. There exists a 2.1-fold difference in total phenolic content between the highest and the lowest ranked varieties, Cabernet Franc and Chardonnay \((p < 0.05)\). The total flavonoid content of Pinot Noir was around 3.1-fold higher than that of Baco Noir, which had the lowest content. The average number of phenolic compounds for seeded *Vitis vinifera* varieties
from California is about 4,000 for white grape and 5,500 mg gallic acid equivalents (GAE)/kg for red ones (Singleton and Esau, 1969). In 2002, Mattivi et al have studied 25 high-quality red grape cultivars (V. vinifera), and found that the Schiava variety contained the lowest phenolic content with 1358 mg/kg, and the Sagrantino variety had the highest phenolic content with 4628 mg/kg, resulting in a variability factor of 2.7 in the richness of phenolics between the values of the varieties with the average concentrations. (Kanner et al., 1994) also found a variation in phenolic content among Thomson seedless, Flame seedless, and Black seedless grapes, ranging from 260 to 930 mg/kg of fresh weight. It was estimated that the total phenolics in juice, pulp, skins, and seeds are approximately 5%, 1%, 30%, and 64%, respectively (Singleton and Esau, 1969; Singleton, 1982). Generally, the total phenolics of red grape skins is greatly higher than that of white grapes due to the loss of the ability to produce anthocyanins in the skins of white grapes. However, our result showed that the phenolic content of different grapes depends mainly on the varietal differences, not on grape skin color. For example, Baco Noir variety had lower contents of total phenolics and flavonoids than Riesling, Niagara, and Vidal Blanc.

The study of anthocyanins is important for differentiating the quality and health benefits of grapes and red wines derived from grapes. As a characteristic associated with the variety, the level of anthocyanins in grape can serve as a direct estimate of the red pigments, and be useful for the classification of grape varieties and of relevant wines (Mattivi and Nicolini, 1997). Hence, anthocyanins have been proposed as chemical markers to differentiate grape varieties and red wines (Santos et al., 1991; Mattivi and Nicolini, 1997). The concentration and composition of the anthocyanins in red grapes varies greatly with the variety, species, maturity, production area, and climate. In assessing red grape varieties for total anthocyanin content, we
found that broad variability exists. This is not surprising since these secondary
metabolites are synthesized under genetic control in different varieties. In our study,
DeChaunac variety contained the highest total anthocyanin content at 239.6 ± 25.4
mg/100 g of sample, whereas Catawba had the lowest one at 8.1 ± 0.8 mg/100g of
sample, indicating almost 30-fold difference. The total anthocyanin content of red
grapes is from about 30 to 750 mg/g of fresh weight of ripe berries (Mazza, 1995).
The content of anthocyanins in skin of three *Vitis vinifera* L. grapes was 753-803
mg/kg for Pinot Noir, 826-1048 mg/kg for Cabernet Franc, and 981-1043 mg/kg for
Merlot (Mazza et al., 1999). (Muñoz-Espada et al., 2004) reported the total
anthocyanin contents in skin of three non-*Vitis vinifera* grapes, which were 258 ± 37
mg/100 g of wet weight for Marechal Foch, 888 ± 78 mg/100 g for Norton, and 326 ± 5.9 mg/100 g for Concord. With a standard of malvidin 3-monoglucoside chloride, the
lowest anthocyanin content in Primitivo variety was found to be 250mg/kg whereas
the maximum amount was found in a Teroldego grape with 2323 mg/kg (Mattivi et al.,
2002). RSV was first found in grapevines (*Vinis vitifera*) in 1976 (Langcake and
Pryce, 1976), and then reported in wine in 1992 (Siemann and Creasy, 1992). It is
synthesized particularly in the leaf epidermis and the skin of grape berries and only
trace amounts are present in the fruit flesh. Grape skins and red wines contain 50-100
µg RSV/g, and 1.5-3 mg/L, respectively (Joe et al., 2002). Our study showed that
Baco Noir grape extract contained the highest RSV content (571 ± 30 µg/100g). The
reservatrol content in Cayuga White variety was lowest at 38 ± 2 µg/100g.

A positive correlation between total phenolics and total antioxidant activity in
a number of different fruits has been reported both in our lab and others (Heinonen et
al., 1998; Prior et al., 1998; Velioglu et al., 1998; Karakaya et al., 2001; Sun et al.,
2002a; Yang et al., 2004). The present study reveals a strong correlation between total
antioxidant activity and total phenolics ($R^2 = 0.9775, p<0.05$). The antioxidant effects of grape phenolics were determined by Kanner et al (1994) in a number of different systems. It was concluded that effective antioxidants were found in all grape varieties, which corresponded to the concentration of phenolics in the system. The antioxidant activity of red grapes has been correlated with the phenolics and/or flavonoid content (Teissedre et al., 1996; Meyer et al., 1997; Mayer et al., 2001). The antioxidant activity was measured by the inhibition of human low-density lipoprotein (LDL) oxidation. In vitro oxidation positively correlated with the content of total phenolics, anthocyanins, and flavonols after investigation of phenolic extracts from fourteen different types of fresh grapes (Meyer et al., 1997). After Concord grape juice was given orally (10 mL/kg/day) to subjects, serum antioxidant capacity was increased, and LDL oxidation deceased similarly to that obtained with 400 IU α-tocopherol/d (O'Byrne et al., 2002). Anthocyanins have been proven to be very efficient antioxidants (Rice-Evans et al., 1995; Van Acker et al., 1996; Satué-Gracia et al., 1997; Wang et al., 1997; Wang et al., 1999; Degenhardt et al., 2000; Espin et al., 2000). Anthocyanins are potent antioxidants, similar to quercetin because of similar structures (Pietta, 2000). There was a positive correlation between anthocyanin content and antioxidant activities of red grape extracts (Meyer et al., 1997); grape juices (Frankel et al., 1998), and red wines (Burns et al., 2000). Vascular endothelial cells enriched with elderberry anthocyanins (1 mg/mL) could incorporate anthocyanins into the membrane and cytosol, conferring significant protective effects against oxidative damage (Youdim et al., 2000). Consumption of an anthocyanin-repleted diet in rats significantly improved ($p < 0.01$) plasma antioxidant capacity and decreased ($p < 0.001$) vitamin E deficiency-enhanced hydroperoxides and 8-Oxo-deoxyguanosine concentrations in liver (Ramirez-Tortosa et al., 2001). Cyanidin 3-O-β-D-glucoside (C3G) functions as a potent antioxidant under oxidative stress in rats
(Tsuda et al., 2000). (Frankel et al., 1993b) were first to demonstrate that trans-RSV reduced the copper-catalyzed oxidation of human LDL. LDL peroxidation was inhibited more by RSV than by a phenolic extract from red wine. However, neither anthocyanin nor RSV content correlated with total antioxidant activity of grapes in our experiment. This could be because individual compounds may act additively or synergistically with other compounds and the total expressed antioxidant activity may be dependent on the relative proportions of each compound in the system.

The phytochemicals present in all grape variety extracts showed a potent inhibitory effect on Caco-2, HepG2, and MCF-7 human cancer cell proliferation. The inhibition of cell proliferation was observed in a dose-dependent manner after exposure to extracts of Cabernet Franc, Pinot Noir, Chardonnay, Catawba, Concord, Sheridan, Niagara, and Riesling; demonstrating that these varieties contained greater antiproliferative activity than DeChaunac, Cayuga White, Baco Noir, Chancellor, Vidal Blanc, and Marechal Foch grapes. However, there was a significant difference among the varieties with respect to antiproliferative activity toward different cell lines. Pinot Noir and Cabernet Franc grapes contained the highest level of inhibitory action, and Marechal Foch possessed the lowest level toward Caco-2 human colon cancer cells. The phytochemical extract of Pinot Noir and Chardonnay varieties exhibited the highest antiproliferative effect toward HepG2 human liver cancer cells with the lowest EC50, and DeChaunac and Vidal Blanc grapes had the highest EC50 values. The antiproliferative activities of grape varieties differed for the Caco-2, HepG2, and MCF-7 cell lines. Overall, the 14 grape varieties possessed more inhibitory activity for Caco-2 colon cancer cell proliferation than for HepG2, and MCF-7 human cancer cell proliferation. Also, most grape extracts exhibited higher antiproliferative activity against HepG2 than MCF-7. The possible explanation may be that phytochemicals in
grape target different organs showing different effects. Since antiproliferative activity in cells involves uptake, metabolism, and transcriptional events, it is not surprising that different cell types exhibit different degrees of inhibition when presented with the same array of phytochemicals. It was observed that there was direct intestinal uptake of red fruit anthocyanins (cyanin 3-glucoside and cyanin 3,5-diglucoside) in human and rats, demonstrating that anthocyanins are taken up as structurally intact glycoside forms from the digestive tract into the blood circulation system in mammals (Miyazawa et al., 1999). The effects of pretreatment with the anthocyanins (delphinidin, cyanidin, and their glycoside and rutinoside derivatives) against induction of DNA damage were evaluated in rat smooth muscle and in rat hepatoma cell lines using the Comet test. The results showed that anthocyanins were effective against cytotoxicity, DNA SSB formation and lipid peroxidation induced by tert-butyl-hydroperoxide (TBHP) (Lazzé et al., 2003). Cyanidin-DNA copigmentation might be a possible defense mechanism against oxidative damage of DNA and may have an in vivo physiological function attributable to the antioxidant ability of anthocyanins (Sarma and Sharma, 1999). Cyanidin, pelargonidin, and delphinidin showed an estrogen-inducible cell proliferation effect in two human breast cancer cell lines (MCF-7 and BG-1), but not in the receptor-negative line MDA-MB-231 (Schmitt and Stopper, 2001). Cyanidin and delphinidin contribute to potent inhibition of the epidermal growth-factor receptor and were found to inhibit the growth of a human vulva carcinoma cell line A431 in vitro (Meiers et al., 2001). Recently, it was reported that anthocyanidins directly cause human promyelocytic leukemia cells (HL-60) to generate intracellular hydrogen peroxide, and trigger apoptosis, possibly through an oxidative stress JNK signaling pathway. Also, it was shown that delphinidin stimulates JNK pathway activation including JNK phosphorylation, c-jun gene expression, and activates caspase-3 (Hou et al., 2003). RSV has been reported to have
antiproliferative activity in different human cancer cell lines, such as MCF-7 (Mgbonyebi et al., 1998), HL60 promyelocytic leukemia cells (Clement et al., 1998), A431 epidermoid carcinoma cells (Ahmad et al., 2001), and Caco-2 colorectal carcinoma (Schneider et al., 2000). The effects of RSV in breast cancer cell lines are inconsistent. Some researchers found that RSV enhances MCF-7 (Basly et al., 2000) cell growth. It was demonstrated that RSV induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in MCF-7, SW480, HCE7, Seg-1, Bic-1, and HL60 human cancer cell lines (Joe et al., 2002). On the other hand, RSV was reported to slow down the proliferation of several human malignant cell lines (Della Ragione et al., 1998; Hsieh et al., 1999a). The suppression effect of RSV in normal cell growth is accompanied by the accumulation of cells in S and G2 phases (Hsieh et al., 1999b). The antiproliferative activities of RSV may be explained by the direct inhibition of ribonucleotide reductase, which supplies proliferating cells with deoxyribonucleotides required for DNA synthesis (Reichard, 1987; Fontecave et al., 1998). However, a correlation between RSV content and the EC50 value of antiproliferative activity for the three human cancer cell lines examined was not found in our study. A possible reason may be the low dose of RSV in the grape extracts.

No relationships were found between total antioxidant activity and antiproliferative activity for Caco-2, HepG2, and MCF-7 cell lines (p > 0.05). Additionally, the total phenolic and flavonoid contents of grapes did not correlate with antiproliferative activity for all three cell lines (data not shown). Similar results were also found in studies of raspberries and strawberries by our group (Liu et al., 2002; Meyers et al., 2003), which suggests that phytochemicals other than those tested in this experiment are responsible for inhibiting cell proliferation, or that the combination
of different phytochemicals functions additively and synergistically or antagonistically accounting for the antiproliferative activity of grapes.

Our results have shown that significant differences in phytochemical content exist among grape varieties. Phytochemicals in grape extracts have potent antioxidant and antiproliferative activities, and that antioxidant activity is well correlated with total phenolic content. However, information on the bioavailability and metabolism of phenolics in humans is still scarce; therefore, further studies need to be carried out.
CHAPTER THREE

INDUCTION OF ANTICARCINOGENIC MARKER ENZYME, QUINONE REDUCTASE, IN MURINE HEPATOMA CELLS IN VITRO BY GRAPE EXTRACTS AND SELECTED PHYTOCHEMICALS

3.1. Introduction

Extensive epidemiological evidence has shown that diets rich in fruits and vegetables are associated with a reduced risk of developing chronic diseases, such as cardiovascular disease, cancer, diabetes, and Alzheimers’s disease (Block et al., 1992; Ames et al., 1993; Temple, 2000; Joshipura et al., 2001; Willett, 2002). Fruits and vegetables contain many antioxidant and anticarcinogenic compounds, including phenolics, carotenoids, thiols, tocopherols, and glucosinolates, which may protect against cardiovascular diseases and cancer through a variety of mechanisms. These findings lead to the USDA Food Guide Pyramid recommendations of 5-9 servings of fruits and vegetables every day, and to the guideline of the National 5 a Day for Better Health Program which also aims to increase the consumption of fruits and vegetables from 5 to 9 servings per day in the United States.

The main carcinogenic agents are exogenous or metabolically generated reactive oxygen species (ROS) and electrophiles arising from in vivo normal oxidative processes and from the environment. For example, insufficient oxygen consumption from lipid peroxidation in mitochondria may result in the overproduction of ROS. Enhanced ROS levels can initiate DNA damage, are involved in tumor initiation and promotion, and may ultimately lead to carcinogenesis (Halliwell et al., 2000). Dietary phytochemicals are able to inhibit phase I enzymes and/or induce phase II enzymes,
including quinone reductase (QR, DT-diaphorase, NAD[P]H:quinone-acceptor oxidoreductase, nicotinamide quinone oxidoreductase 1 (NQO1), EC 1.6.99.2), glutathione S-transferase (GST, EC 2.5.1.18), epoxide hydrolase (EH, EC 3.3.2.3), and UDP-glucuronosyl transferase (UDPGT, EC 2.4.1.17). The isolated bioactive compounds from fruits and vegetables that have shown anticarcinogenic capacity are β-carotene (Peto et al., 1981), monoterpenes D-limonene and D-carvone (Wattenberg et al., 1989), sulforaphane (Zhang et al., 1992), and pinostrobin and pinocembrin (Fahey and Stephenson, 2002). Phytochemicals in fruits and vegetables may inhibit carcinogen activation by keeping the balance between carcinogen-activating phase I enzymes and phase II detoxifying enzymes, which are likely to play a protective role against xenobiotic cellular damage (Prochaska et al., 1992). A number of fruits and vegetables contain substantial quantities of compounds that modulate mammalian enzymes of xenobiotic metabolism. For example, edible plants belonging to the family Cruciferae such as broccoli, cauliflower, and Brussels sprouts contain glucosinolate, isothiocyanates, and sulforaphane, some of which are very potent inducers of phase II enzymes (Fahey et al., 1997). (Benson et al., 1978) have reported that phenolic antioxidants such as 2(3)-tert-Butyl-4-hydroxyanisole (BHA), which blocked tumor formation, induced several Phase II enzymes in a variety of animal tissues. The elevation of phase II detoxification and antioxidant enzymes by isothiocyanates, sulforaphane, carotenoids, flavonoids, and other phytochemicals is documented and recognized as one of the mechanisms by which fruits and vegetables exert their chemoprotective effects (Talalay and Fahey, 2001). Currently, upregulation of QR is thought to be a useful biomarker for anticarcinogenesis (Jaiswal, 2000).

QR is a flavoprotein that catalyzes the two-electron reduction and detoxification of quinones and its derivatives, leading to the protection of cells against
redox cycling, oxidative stress, and neoplasia. QR acts as an important regulator of a widely varied biological functions and may play a particularly significant role in cancer. The major functions of QR are to lower formation of ROS by decreasing one electron reductions and the associated redox cycling (Ernster, 1987), to maintain the overall antioxidant functions of the cell by keeping \( \alpha \)-tocopherolquinone and ubiquinones in their reduced and active states (Siegel et al., 1997), and to act as a phase II detoxifying enzyme involved in cancer prevention (Prochaska et al., 1987; Ross et al., 1993). Additionally, QR plays an important role in activating some anticancer drugs (Workman, 1994) and regulating the stability of p53 and apoptosis in mouse cells and human (Asher et al., 2001; De Long et al., 2002).

There exists convincing evidence that induction of phase II enzymes is an important mechanism responsible for cancer chemoprevention. The microtiter plate assay utilized to determine QR induction has been used extensively to characterize the inducer potency of a variety of natural and synthetic compounds as well as crude plant extracts (Prochaska and Santamaria, 1988; Dinkova-Kostova and Talalay, 2000; Talalay and Fahey, 2001). This bioassay has provided a rapid and quantitative method for detecting inducer activity and determining inducer potency. Induction of QR detoxication enzyme provides a strategy for achieving protection against ROS and carcinogenesis. It is therefore of great interest to see if grapes contain inducing compounds. The objective of this study was to determine potential induction of QR and antiproliferative activity to Hepa1c1c7 cells by wine grapes widely grown in the Finger Lakes region in New York State. The information obtained here suggests a novel mechanism of action of wine grapes to provide health benefits.
3.2. Materials and Methods

3.2.1. Chemicals

Alpha minimal essential medium (α-MEM), fetal calf serum (FBS), hepes, and antibiotic-antimycotic solution were purchased from GIBCO (Life Technologies, Grand Island, NY). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium (MTS) and phenazinemethosulfate (PMS) were from Promega (Madison, WI). Ascorbic acid, caffeic acid (>99% HPLC grade), (+)-catechin (hydrate, >98%), chlorogenic acid (>95%), ferulic acid, folic acid (~98%), genistein (<98% HPLC grade), quercetin (dihydrate), rutin, resveratrol (trans-3,4’,5-trihydroxystilbene, ~99%), β-naphthoflavone (BNF, 90-95%), digitonin, glucose 6-phosphate, menadione, nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), albumin from human serum (≥96%), yeast glucose 6-phosphate dehydrogenase, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS, ~99%), ethylenediamine tetraacetic acid (EDTA, anhydrous, ~99%), and dicoumarol were purchased from Sigma (St. Louis, MO). Gallic acid was from ICN Biomedicals, Inc. (Aurora, OH). Delphinidin chloride and malvidin chloride were from Indofine Chemical Company, Inc. (Hillsborough, NJ). L-ergothionenine was obtained from OXIS International, Inc. (Portland, OR). Dimethyl sulfoxide (DMSO) and Tween 20 were from Fisher Scientific (Fair Lawn, NJ). Tris-base and crystal violet were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All the other materials used were also obtained from Sigma (St. Louis, MO).

3.2.2. Grapes

Thirteen wine grape varieties (Baco Noir, Cabernet Franc, Catawba, Cayuga White, Chancellor, Chardonnay, Concord, DeChaunac, Marechal Foch, Niagara, Pinot
Noir, Riesling, and Vidal) were provided by a Grapeyard located in Branchport, N.Y. Wine grapes were harvested upon ripening in 2003 and 2004. Grapes free from visible blemishes or disease were selected. For quantitative analysis, 50–70 grape berries randomly chosen from all samples were collected for each grape variety. All data collected for each grape variety were reported as means ± SD for at least three replications.

3.2.3. Grape Pytochemical Extraction

Phytochemicals were extracted from fresh grape by a modified method reported previously in our laboratory (Yang et al., 2004). Briefly, 100 g of grape were blended for 1 min in 100 g 80% acetone using Waring blender with medium speed in order to remove seeds. To get grape samples reflecting the actual phytochemical compounds present in grape skin and pulp, the seeds were not crushed. After removal of seed, adding another 100 g 80% acetone, the grapes were blended for 3 min using Waring blender with high speed. The mixture was then homogenized in a Virtis High Speed Homogenizer for 3 min and filtered with vacuum under ice bath. Water in the filtrate was evaporated using a rotary evaporator at 45°C until the weight of the evaporated filtrate was less than 10% of the weight of the original filtrate. All extracts were stored at –40°C until use. All extractions were performed in triplicate.

3.2.4. Determination of Quinone Reductase Activity in Hepa1c1c7 Cell Culture

This bioassay was modified from a previously described method (Prochaska and Santamaria, 1988; Zhang et al., 1992). Hepa1c1c7 murine hepatoma cells (The American Type Culture Collection, ATCC, Rockville, MD) were grown in α-MEM without nucleosides or deoxyribonucleosides, supplemented with 10% FBS, at 37°C in an atmosphere of 5% CO2 in a humidified incubator. Cultured Hepa1c1c7 cells were
plated at a density of $2 \times 10^4$ cells/mL in 96-well plates (Costar 3595, Corning Inc. Corning, NY), and decanted after a 24 h incubation. The fresh medium and test samples dissolved in 10% DMSO were introduced and serially diluted to a concentration range of 5 – 200 μM for compounds, and 0.5 – 10 mg/mL for grape extracts. The final DMSO concentration in the medium was less than 0.5%. The cells were incubated for an additional 48 h. Growth medium and 1 μM BNF were used as negative and positive controls, respectively. The medium was removed and the cells were lysed with 50 μL 0.8% (w/v) digitonin in 2 mM EDTA at pH 7.6, and incubated for 10 min at 37°C. The plates were then agitated on a shaker (120 rpm) for 10 min at room temperature. A 200-μL aliquot of reaction mixture (7.5 mL of 0.5 M pH 7.4 Tris-HCl buffer; 100 mg of albumin from human serum; 1 mL of 1.5% Tween-20 solution; 0.1 mL of 7.5 mM FAD; 1 mL of 150 mM glucose-6-phosphate; 90 μL of 50 mM NADP; 300 units of yeast glucose-6-phosphate dehydrogenase; 45 mg of MTT; 150 μL of 50 mM menadione in acetonitrile; and 140.16 mL distilled water added to total 150 mL volume) was added to lysed cells. Menadione solution was added just before the mixture was dispensed into the microtiter plates. QR activity was measured as the reduction of menadione to menadiol, this being coupled to the non-enzymatic reduction of MTT to a blue formazan. The reaction generated a blue color, which was stopped after 5 min by the addition of 50 μL of a solution containing a 0.3 mM dicoumarol in 0.5% DMSO and 5 mM pH 7.4 potassium phosphate. Readings were made with triplet for each sample at 590 nm. Total protein concentrations were determined in a duplicate set of plates using crystal violet staining, and subsequently scanned at 490 nm.

The specific activity of QR is defined as nmol MTT blue formazan reduced per min and per mg protein. Induction was expressed as the ratio of the specific activity of
QR in the presence and absence of the test sample. The concentration required to double specific activity (CD) is determined via a curve of the ratio of QR specific activities of sample-treated cells to solvent-treated control cells as a function of inducer concentration. The EC\textsubscript{50} values of both pure compounds and grape extracts (half-maximal inhibitory concentration of cell viability) were examined. Chemopreventive Index (CI) values were determined by dividing EC\textsubscript{50} values by CD values (Gerhäuser et al., 1997).

3.2.5. Measurement of Inhibition of Hepa1c1c7 Cell Proliferation

The antiproliferative activity of pure compounds (antioxidants) and grape extracts was assessed by measurement of the inhibition of Hepa1c1c7 murine hepatoma cell proliferation. Antiproliferative activities were determined by the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) (Promega, Madison, WI) reported previously (Yang et al., 2004). Hepa1c1c7 cells were cultured in α-MEM without nucleosides or deoxyribonucleosides, containing 10% FBS, 50 units/mL penicillin, 50 µg/mL streptomycin, and 50 µg/mL amphotericin B. Hepa1c1c7 cells were maintained in a 5% CO\textsubscript{2}/37°C incubator. A total of 2.5 × 10\textsuperscript{4} Hepa1c1c7 cells in growth media were placed in each well of a 96-well flat-bottom plate (Costar 3595, Corning Inc. Corning, NY). Cell proliferation was measured by the ability of viable cells to reduce MTS to formazan. After 4 h of incubation, the growth medium was removed, and media containing various concentrations of pure compounds or grape extracts were added to the cells. Control cultures received the extraction solution minus the grape extracts, and blank wells contained 100 µL of growth medium without cells. Cell proliferation (percent) was determined at 72 h from the MTS absorbance (490 nm) reading for each concentration compared to the control, using at least three replications for each sample. The effective
median dose (EC\textsubscript{50}) was determined and expressed as milligrams of grape extract per milliliter ± SD or as micromole of pure compounds ± SD.

### 3.2.6. Statistical Analysis

Statistical analysis was performed using Minitab Student Release 12 (Minitab Inc., State College, PA) and SigmaStat Version 8.0 (Jandel Corp., San Raphael, CA). Correlations between various parameters were also investigated. Significance was determined at \( p < 0.05 \). All data were reported as the mean ± SD of three replications.

### 3.3. Results

13 wine grapes and 14 common antioxidants were examined \textit{in vitro} for their potential to induce QR, a representative phase II chemoprotective enzyme. The BNF with an induction of 7.5 ± 0.5-fold over solvent control at a concentration of 1 \( \mu \)M and with a CD value of 0.027 ± 0.003 \( \mu \)M was used as a positive control. Grape extracts which induced QR in Hepa1c1c7 cells are shown in Figure 3.1. Table 3.1 summarizes the concentrations of these extracts needed to double the induction. Compared to the solvent-treated control cells, at 2 mg/mL concentration, the induction of Cabernet Franc and Baco Noir were 3.1 ± 0.3 and 2.3 ± 0.2, respectively. The calculated CD values of Cabernet Franc and Baco Noir were 0.6 ± 0.1 and 1.4 ± 0.3 mg/mL, respectively. The induction of Pinot Noir and Niagara were 2.5 ± 0.3 and 3.1 ± 0.1 at concentration of 3 mg/mL, respectively. The calculated CD values of Pinot Noir and Niagara were 2.2 ± 0.2, and 2.4 ± 0.1 mg/mL, respectively. At concentration of 5 mg/mL, the induction of Concord, Chardonnary, and Vidal Blanc were 4.0 ± 0.4, 2.6 ± 0.4, and 2.2 ± 0.6, respectively. The generated CD values of Concord, Chardonnary, and Vidal Blanc varieties were 3.2 ± 0.1, 4.0 ± 0.4, and 4.6 ± 0.7 mg/mL, respectively.
Chancellor, Cayuga White, DeChaunac, and Marechal Foch varieties, at a concentration of 8 mg/mL, cause a maximum of 2.0 ± 0.1, 2.6 ± 0.2, 2.2 ± 0.3, and 2.1 ± 0.1-fold induction over control, respectively, in inducing QR activity in Hepa1c1c7 cells. The calculated CD values of Chancellor, Cayuga White, DeChaunac, and Marechal Foch varieties were 8.0 ± 0.1, 6.4 ± 0.3, 7.4 ± 1.1, and 7.7 ± 0.3 mg/mL, respectively. However, compared with other grapes, the Catawba and Riesling varieties showed very weak induction at concentration ranging from 0.1 to 10 mg/mL. The maximum induction of Catawba and Riesling was 1.2 ± 0.1, and 1.6 ± 0.1-fold at concentration of 3 mg/mL, respectively. In general, Cabernet Franc and Baco Noir were more potent inducers than other grape varieties evaluated. Pinot Noir and Niagara also showed strong induction effect. Chancellor, Cayuga White, DeChaunac, and Marechal Foch varieties exhibited weak induction activity.

In addition to investigating the QR induction of grape extracts, the induction potency of some common antioxidants in fruits and vegetables were also evaluated. Among 14 antioxidants examined in this study, only quercetin, genistein, and resveratrol exhibited higher induction of QR than other compounds ([Figure 3.2](#) and [Table 3.2](#)). Quercetin at a concentration of 30 μM resulted in a maximum of 3.5 ± 0.2-fold induction over control in inducing QR activity in Hepa1c1c7 cells. At a concentration of 50 μM, genistein caused a maximum of 2.8 ± 0.2-fold induction over control. Resveratrol led to a maximum of 2.2 ± 0.1-fold induction over control at a concentration of 50 μM. The generated CD values of quercetin, genistein, and resveratrol were 2.5 ± 0.5, 15.0 ± 3.0, and 28.7 ± 2.3 μM, respectively. Although these inducers can not double QR activity in the tested concentrations, chlorogenic acid, ferulic acid, folic acid, caffeic acid, ascorbic acid need lower concentration to reach
Table 3.1. Effects of Grape Extracts on Quinone Reductase Induction

<table>
<thead>
<tr>
<th>Grape Variety</th>
<th>CD (mg/mL)(^a)</th>
<th>EC(_{50}) (mg/mL)(^b)</th>
<th>CI(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baco Noir</td>
<td>1.4 ± 0.3</td>
<td>73.3 ± 5.4</td>
<td>52</td>
</tr>
<tr>
<td>Cabernet Franc</td>
<td>0.6 ± 0.1</td>
<td>8.7 ± 2.4</td>
<td>15</td>
</tr>
<tr>
<td>Cayuga White</td>
<td>6.4 ± 0.3</td>
<td>54.7 ± 3.9</td>
<td>9</td>
</tr>
<tr>
<td>Catawba</td>
<td>NI(^d)</td>
<td>40.3 ± 1.6</td>
<td>ND(^d)</td>
</tr>
<tr>
<td>Chancellor</td>
<td>8.0 ± 0.1</td>
<td>67.4 ± 2.7</td>
<td>8</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>4.0 ± 0.4</td>
<td>49.5 ± 3.7</td>
<td>12</td>
</tr>
<tr>
<td>Concord</td>
<td>3.2 ± 0.1</td>
<td>40.5 ± 2.9</td>
<td>13</td>
</tr>
<tr>
<td>DeChaunac</td>
<td>7.4 ± 1.1</td>
<td>69.3 ± 2.1</td>
<td>9</td>
</tr>
<tr>
<td>Niagara</td>
<td>2.4 ± 0.1</td>
<td>36.4 ± 0.9</td>
<td>15</td>
</tr>
<tr>
<td>Marechal Foch</td>
<td>7.7 ± 0.3</td>
<td>76.9 ± 3.2</td>
<td>10</td>
</tr>
<tr>
<td>Pinot Noir</td>
<td>2.2 ± 0.2</td>
<td>27.0 ± 1.7</td>
<td>12</td>
</tr>
<tr>
<td>Riesling</td>
<td>NI(^d)</td>
<td>39.3 ± 2.9</td>
<td>ND(^d)</td>
</tr>
<tr>
<td>Vidal Blanc</td>
<td>4.6 ± 0.7</td>
<td>43.0 ± 2.3</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\)CD: Concentration to double QR activity in Hepa1c1c7 cells.
\(^b\)EC\(_{50}\): Concentration to inhibit Hepa1c1c7 cell growth by 50%.
\(^c\)CI: Chemopreventive Index (EC\(_{50}\)/CD).
\(^d\)NI = no induction; ND = not determined.

Table 3.2. Effects of Selected Phytochemicals on Quinone Reductase Induction

<table>
<thead>
<tr>
<th>Compound</th>
<th>CD (µM)(^a)</th>
<th>EC(_{50}) (µM)(^b)</th>
<th>CI(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>NI(^d)</td>
<td>&gt; 200</td>
<td>ND(^d)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Delphinidin chloride</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Folic acid</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Genistein</td>
<td>15.0 ± 3.0</td>
<td>&gt; 125</td>
<td>ND</td>
</tr>
<tr>
<td>L-ergothionine</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Malvidin chloride</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.5 ± 0.5</td>
<td>123.2 ± 2.7</td>
<td>50</td>
</tr>
<tr>
<td>Rutin</td>
<td>NI</td>
<td>&gt; 200</td>
<td>ND</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>28.7 ± 2.3</td>
<td>56.3 ± 3.4</td>
<td>2</td>
</tr>
<tr>
<td>(\beta)-Naphthoflavone(^e)</td>
<td>0.027 ± 0.003</td>
<td>&gt; 20</td>
<td>&gt; 740</td>
</tr>
</tbody>
</table>

\(^a\)CD: Concentration to double QR activity in Hepa1c1c7 cells.
\(^b\)EC\(_{50}\): Concentration to inhibit Hepa1c1c7 cell growth by 50%.
\(^c\)CI: Chemopreventive Index (EC\(_{50}\)/CD).
\(^d\)NI = no induction; ND = not determined.
\(^e\)QR assay positive control.
the maximum induction effect than that of (+)-catechin, delphinidin chloride, gallic acid, L-ergothionenine, malvidin chloride, and rutin (Table 3.2).

Figure 3.3 and Figure 3.4 also present the EC$_{50}$ of the antiproliferative activity of both grape varieties and antioxidants. Lower EC$_{50}$ values represent higher antiproliferative activities. The phytochemical extracts of Cabernet Franc possessed the greatest antiproliferative activity towards Hepa1c1c7 cells with the lowest EC$_{50}$ of 8.7 ± 2.4 mg/mL ($p < 0.05$), followed by Pinot Noir, Niagara, Riesling, Catawba, Concord, Vidal Blanc, Chardonnay, Cayuga White, Chancellor, DeChaunac, Baco Noir, and Marechal Foch (Table 3.1). The EC$_{50}$ doses were significantly different among Cabernet Franc, Pinot Noir, Niagara, Chardonnary, and Marechal Foch ($p < 0.05$). The phytochemical extract of DeChaunac, Baco Noir, and Marechal Foch exhibited a weak antiproliferative activity towards Hepa1c1c7 cells at a higher dose. The antiproliferativity was significantly different among resveratrol, quercetin, and genistein ($p < 0.05$). However, the EC$_{50}$ values of other antioxidants could not be determined since they did not exhibit antiproliferative activity towards Hepa1c1c7 cells at doses ranging from 0 to 200 μM used in this experiment (Table 3.2).

The induction effect of QR activity observed with 13 grape extracts and 14 common antioxidants are summarized in Table 3.1 and Table 3.2, respectively. Among the grape extracts examined in this study (Table 3.1), Baco Noir, Cabernet Franc, Niagara, Concord, Pinot Noir, Chardonnary, Marechal Foch, DeChaunac, Cayuga White, Vidal Blanc, and Chancellor varieties were promising inducers of QR activity with CI values of 52, 15, 15, 13, 12, 12, 10, 9, 9, 9, and 8, respectively. Catawba and Riesling varieties exhibited weak induction of QR. Of the compounds tested in this study (Table 3.2), quercetin and resveratrol were potential inducers of
QR activity with CI values of 50, and 2, respectively. Caffeic acid, chlorogenic acid, ferulic acid, and folic acid exhibited weak induction of QR at concentrations from 0 to 100 μM. Ascorbic acid, (+)-catechin, delphinidin chloride, gallic acid, L-ergothionenine, malvidin chloride, and rutin showed very low induction of QR at concentrations from 0 to 100 μM.

3.4. Discussion

The potential of grape extracts and some common antioxidants to induce QR activity in comparison with the activity of BNF was evaluated. This bioassay measures the specific activity of QR in Hepa1c1c7 murine hepatoma cells grown in 96-well microtiter plates and exposed to serial concentrations of the tested extracts and compounds. Induction of QR activity was calculated from the ratio of specific enzyme activities of extract- or compound-treated cells in comparison with solvent-treated control cells. In order to investigate the induction of QR and to avoid cytotoxic effects, the half-maximal inhibitory concentration of cell viability (EC\textsubscript{50}) was also determined. Ultimately, Chemopreventive indices (CI) were calculated from the ratio between the EC\textsubscript{50} and CD value, which provided a reasonable index of potency of anticarcinogenic activity and cytotoxicity. Our work has clearly shown that the phytochemicals present in grapes have potent QR induction and antiproliferative activities towards Hepa1c1c7 cells.

Carcinogens are metabolized \textit{in vivo} by phase I enzymes that activate these compounds to highly reactive electrophilic metabolites and reactive species capable of damaging DNA, and by phase II enzymes that transform these reactive electrophiles to
Concentration of grape extracts (mg/mL)

Induction Ratio of Quinone Reductase (treated/control)

Concentration of β-naphthoflavone (µM)

Cabernet Franc
Baco Noir
Pinot Noir
Niagara
Concord
Chardonnay
Vidal Blanc
Cayuga White
DeChaunac
Marechal Foch
Chancellor
Catawba
Riesling
β-naphthoflavone

Figure 3.1. Effect of β-naphthoflavone (positive control) and grape extracts (means ± SD) on induction of quinone reductase (QR) in Hepa1c1c7 cells.
Figure 3.2. Effect of β-naphthoflavone (positive control) and selected phytochemicals (means ± SD) on induction of quinone reductase (QR) in Hepa1c1c7 cells.
Figure 3.3. Percent inhibition of Hepa1c1c7 cell proliferation by selected grape extracts.
Figure 3.4. Percent inhibition of Hepa1c1c7 cell proliferation by selected phytochemicals.
less toxic and more easily excretable products (Talalay et al., 1988; Prestera et al., 1993). Individuals genetically deficient in QR are more susceptible to hematological toxicity and carcinogenicity of benzene exposure, and may be more prone to developing a number of malignant tumors (Dinkova-Kostova and Talalay, 2000). In addition, QR gene expression is coordinately induced with other detoxifying enzyme genes in response to xenobiotics, antioxidants, oxidants, heavy metals, and radiation. Deletion mutagenesis in the QR gene promoter have identified several cis-elements including antioxidant response element (ARE), a basal element, and AP-2 element (Begleiter and Fourie, 2004). The ARE element is essential for basal expression and antioxidant-induced QR gene induction. Therefore, inducers of QR such as some antioxidants in fruits and vegetables are major protectors of cells against oxidative and electrophile stress. QR also plays a role in preventing cells from the toxic and neoplastic effects of quinones resulting from their participation in redox cycling.

Theoretically, extracts or compounds with low CD value and high EC\textsubscript{50} may be promising inducers because at low concentrations they can double QR while having little toxic effect on Hepa1c1c7 cells. A maximum of 4.5 ± 0.2- and 4.0 ± 0.4-fold induction of QR activity was observed for both Cabernet Franc and Concord, although they showed a certain adverse effect upon cell growth at high concentrations. In our study, among grape extracts, Cabernet Franc variety required the lowest concentration to double QR activity; however, it also showed the maximum antiproliferative activity towards Hepa1c1c7 cells. Baco Noir could be considered the most promising inducer in terms of inducing potency and low toxicity. Our previous study showed that Baco Noir variety possessed the highest reservatrol content among grapes tested, which could partially explain its high induction capacity. Pinot Noir with a high content of reservatrol also demonstrated lower concentration needed to double QR activity. Both
Cabernet Franc and Pinot Noir grape varieties exhibited high induction activity, but also high cytotoxicity, resulting in low CI values, which is similar to resveratrol (Table 3.2).

The studies conducted by (Fahey and Stephenson, 2002) reported that there was a positive correlation between antioxidant capacity and QR induction in honey. These authors further reported that dark honey had higher antioxidant capacity with better inducers of mammalian phase II enzymes than light colored honey. However, (Williamson et al., 1996) have found that the ability to induce QR among quercetin, quercetin-4'-glucoside, rutin (quercetin-3-rutinoside), quercetin-3,4'-diglucoside, and isoquercitrin (quercetin-3-glucoside) did not correlate with antioxidant capacity. Similarly, no positive correlation between induction activity and antioxidant capacity was found in grape varieties examined in this study. Further, some white grape varieties such as Niagara and Chardonnay showed higher induction potency than red varieties, and the anthocyanins contents in red grape varieties did not correlate with induction activity, which provides evidence that wine grapes have a different mechanism of induction that is not associated with their antioxidant activities and color. There might exist different mechanisms of action in antioxidant activity and induction ability in grapes, although ARE is essentially required for expression and coordinated induction of QR and other detoxifying enzyme genes.

Flavonoids are the most common and widely distributed phenolics in plants. Based on different structure, flavonoids can be classified into several classes, including flavonols, flavonones, flavones, anthocyanins and isoflavones (Harbone, 1993). Flavonoids are omnipresent in human diet, including fruits, vegetables, tea, and wine. Quercetin is a flavonol, and is the predominant flavonoid in the human diet.
Based on epidemiological surveys, estimates of human consumption are from 4 to 68 mg per day (Skibola and Smith, 2000). Quercetin has been shown to be antioxidant, anti-inflammatory, antiproliferative, antiviral, and antimicrobial, to enhance phase I enzyme transcription activation (Cioloño et al., 1999), and to induce phase II enzymes (Williamson et al., 1996; Uda et al., 1997; Yannai et al., 1998). It was demonstrated that quercetin was a good inducer of QR, and the concentrations for doubling QR activity was around 2.6 μM (De Long et al., 1986; Gerhäuser et al., 2003), which is similar to the concentration (2.5 ± 0.5 μM) found to double QR in this study. Also in the Hepa1c1c7 cell model, quercetin aglycone was the most effective inducer of QR (CD = ~ 13 μM) among quercetin-4'-glucoside, rutin, quercetin-3,4'- diglucoside, and isoquercitrin. Rutin, isoquercitrin, and quercetin-3,4'- diglucoside showed a very weak induction activity of QR (Williamson et al., 1996). Flavans such as catechin and epicatechin have been reported to have no induction of QR at concentrations from 0 to 100 μM in the Hepa1c1c7 model; while flavonols, such as apigenin, myricetine, quercetin, kaempferol, and galangin, were effective QR inducers with a maximal induction level from 1.6 to 3.6 (Uda et al., 1997). The structural features in flavonoids and related compounds have a decisive influence in promoting QR activity. It has been postulated that a 2,3 double bond in the C ring in flavonoid structure is essential for QR induction, which was supported by taxifolin, catechin, and epicatechin. In normal human Chang liver cells, green tea aqueous extract, green tea polyphenols, and some catechins could induce QR activity; however, the studies also showed that EGCG is a poor inducer for QR (Steele et al., 2000). Other flavonoids and related compounds, such as caffeic acid, gallic acid, and catechin, exhibited much less potent induction of QR (Fahey and Stephenson, 2002), which is consistent with our results.
Resveratrol was found to act as an antioxidant, antimutagen, inducer of apoptotic cell death through activation of mitochondria-dependent pathways (Huang et al., 1999), anti-inflammatory activity via down-regulation of proinflammatory cytokines (Wadsworth and Koop, 1999), as well as inhibitor of phase I enzyme (Chang et al., 2001) and inducer of phase II enzymes (Jang et al., 1997). The resveratrol concentration in fresh grape skin is about 50 ~ 100 µg/g, and the concentration of resveratrol from grape skins required to double QR activity in Hepa1c1c7 cells was reported to be 21µM (Jang et al., 1997). Several phytoestrogens which induce QR activity in human cancer cells were reported (Wang et al., 1998; Bianco et al., 2005). Genistein with phytoestrogenic activity is an isoflavone found principally in soybeans. The CD value of genistein is broad from 0.14 to 16.2 µM (Wang et al., 1998; Gerhäuser et al., 2003). The CD values of resveratrol and genistein in our study are 28.7 ± 2.3 and 15.0 ± 3.0 µM, respectively. Both genistein and resveratrol up-regulate QR expression in MCF-7 and MDA-MB-231 breast cancer cells, and this kind of regulation occurs at the transcriptional level through ERβ (oestrogen receptor β) transactivation at the electrophile response element (EpRE) of the QR gene promoter (Bianco et al., 2005).

Only quercetin, genistein, and reservatrol produced strong induction of QR among 14 antioxidants examined in this study. Other antioxidant compounds showed weak induction activity at different concentrations. Probably, the concentrations of other antioxidants used in this study might be too low to cause significant induction compared with the solvent control; however, concentration was limited by the apparent cytotoxicity on the Hepa1c1c7 cell line, which was observed through EC50 values.
In grapes there might exist other unknown compounds, which have potential QR induction ability, further studies needed to be performed to identify those compounds. Besides antioxidant activity of wine grapes, our study has shown that wine grape varieties can induce QR activity, and have antiproliferative activity toward Hepa1c1c7 cells, which would shed new light on the mechanism of action of wine grapes to produce health benefits.
CHAPTER FOUR

COMBINATION EFFECTS OF COMMON FRUITS ON ANTIOXIDANT Activity AND ANTIPROLIFERATIVE ACTIVITY

4.1. Introduction

Chronic disease such as cancers and cardiovascular diseases (CVD) are the mainly leading causes of death in the United States. MyPyramid, which incorporates recommendations from the 2005 Dietary Guidelines for Americans released in January by the USDA and U.S. Dept. of Health and Human Services, delivers the main message: steps to a healthier you. It advises what to eat to promote health and reduce the risk of chronic diet-related disease. A healthy eating plan from this guideline emphasizes fruits, vegetables, and whole grains. Besides providing the essential nutrients for life, their diet also contains bioactive compounds good for disease prevention and health maintenance. There is overwhelming evidence indicating that increased uptake of fruits and vegetables in the diet reduce the risk of CVD, cancer, as well as brain and immune dysfunction (Doll and Peto, 1981; Block et al., 1992; Willett, 1994; Temple, 2000; Joshipura et al., 2001). Reactive oxygen species (ROS) such as free radicals are byproducts generated during oxidative metabolism in organisms. Usually, there exists a balance levels between the oxidants and endogenous antioxidants in normal cell metabolism. However, an imbalance could be caused by the overproduction of oxidants, leading to oxidative damage to nucleic acids, proteins, and lipids. Antioxidants are believed to play a very important role in the body defense system against ROS. Fruits and vegetables are important sources of antioxidants. Phenolics and other plant antioxidants can scavenge free radicals, and then inhibit
their oxidative reactions with macromolecules in the body. The antioxidant activity of fruits and vegetables is assumed to be of greatest importance in combating a number of chronic diseases.

It is documented that over 5,000 individual phytochemicals have been identified in fruits, vegetables, and grains, but a large proportion still remain unknown and need to be identified. Phytochemicals can be classified as alkaloids, carotenoids, phenolics, nitrogen-containing compounds, and organosulfur compounds. Phenolics and carotenoids among phytochemicals are mostly studied nowadays. It is estimated that flavonoids account for nearly two out of three of the phenolics in our diet and the remaining one out of three are from phenolic acids. Flavonoids, being phenolic compounds with a wide variety of biological activities that have been identified in fruits, vegetables, and other plant-based foods, have been associated with lowered risk of some chronic diseases. The generic structure of flavonoids consists of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring so-called C ring. Based on differences in the heterocycle the C ring, flavonoids are categorized as flavonols, flavones, flavanols, flavanones, anthocyanidins, and isoflavonoids. For naturally occurring flavonoids, they are mostly conjugated in glycosylated or esterified forms but can occur as aglycones, especially as a result of the effects of food processing (Hollman and Arts, 2000). Anthocyanins, widely distributed throughout the plant kingdom, are natural, nontoxic, and water-soluble flavonoid pigments, being particularly present in fruits and vegetables where they are responsible for the red, orange, blue, and purple colors. Anthocyanins contains in daily diet after consuming certain fruits and vegetables. Catechins are ubiquitous in plant-based foods, being particularly important in a large number of
fruits, vegetables, and legumes, and occur in beverages such as red wine and tea (Arts et al., 2000).

Many constituents from fruits and vegetables, including phenolics, flavonoids, vitamins C and E, carotenoids, folic acid, and mineral micronutrients, have antioxidant activity, contributing to their health promotion properties. Much attention was paid to the dietary antioxidant properties of vitamins C, E, and carotenoids. However, studies have shown that some common fruits and vegetables possess high antioxidant activity, which cannot be accounted for by their vitamin C content (Wang et al., 1996; Eberhardt et al., 2000). Furthermore, some studies have demonstrated that dietary phenolics derived from plant are more effective antioxidants in vitro than vitamins C or E (Liu, 2004). On the other hand, epidemiological studies of Vitamin E, and β-carotene supplements for chronic disease prevention have found either no effect on CVD or a slight increase in cardiovascular mortality and cancer (Hennekens et al., 1996; Omenn et al., 1996; Rapola et al., 1997). Phenolics which possess two ortho-positioned hydroxyl groups are potential antioxidants. In recent years, phenolics and flavonoids with powerful antioxidant capacity has been drawing much attention. Studies have reported that phytochemicals in fruit and vegetables possess complementary and overlapping mechanisms of action, including scavenging free radicals, chelating metal ions, stimulation of the immune system, modulation of detoxification enzymes, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects (Dragsted et al., 1993; Waladkhani and Clemens, 1998).

The synergistic effect of antioxidants such as vitamin E and C (Scarpa et al., 1984), vitamin E and β-carotene (Palozza and Krinsky, 1992), catechin and malvidin
3-glucoside (Rossetto et al., 2002), flavonoids and urate (Filipe et al., 2001), and tea polyphenols and vitamin E (Zhou et al., 2000) were observed. Vitamin C can regenerate vitamin E from the vitamin E radical, recycling vitamin E. Vitamin E becomes a radical in the process, but it can be recycled by interacting with other antioxidant systems. It has been postulated that, compared with individual activity, combinations have greater antioxidant capacities in different model systems (Teissedre et al., 1996; Vivas et al., 1997; Meyer et al., 1998; Filipe et al., 2001; Filipe et al., 2001; Rossetto et al., 2002). Antioxidant synergism of tea polyphenols and α-tocopherol against free radical induced peroxidation of linoleic acid in homogeneous solutions and in micelles was reported, proposing a mechanism involving the recycling of α-tocopherol by the tea polyphenol (Zhou et al., 2000). The mixture of phenolic antioxidants found in wine and plant foods may interact to yield synergistic protection against LDL oxidation (Kinsella et al., 1993). The combinations of phenolics characterized by different free radical scavenging efficiency may show an antioxidant activity different from that expected on the basis of the sum of their individual activities (Saucier and Waterhouse, 1999). It was previously reported from our lab that apple extracts exhibit strong antioxidant and antiproliferative activities and that the major part of total antioxidant activity is from the combination of phytochemicals (Liu, 2004). Beside antioxidant activities, phenolics have demonstrated other specific biological activities interfering with cellular mechanisms. For example, synergistic protection of PC12 cells from β-amyloid toxicity by reservatrol and catechin was observed (Conte et al., 2003); quercetin and kaempferol were shown to be active on signal transduction pathways in cells via inhibiting PI 3-kinase, protein kinase C, and phospholipase A2, and by reducing neuronal apoptosis (Gschwendt et al., 1983; Wang et al., 2001).
It has been postulated that combinations of fruits have greater antioxidant activities than would be expected on the basis of their individual effects. The phenolics in fruits and vegetables may act independently or in combination as anti-tumor or cardioprotective agents. The synergistic effect of pure compound combinations has been reported. Only limited knowledge is available about any interaction between/among phenolic compounds in inhibiting peroxyl free radical production and cell proliferation. Furthermore, there is no literature report related to synergistic, additional, and antagonism effects on inhibition of peroxyl radical production and cell proliferation in whole foods. Information on antioxidant and antiproliferative activities of different phenolics would give us a better understanding of the activities of phytochemicals in complex mixtures such as juice cocktails. The goal of this study is to seek possible synergies between/among different fruits combinations. The objectives for this study were to: (1) determine the phenolic and flavonoid contents, the total antioxidant and antiproliferative activity of cranberry, apple, and grape extracts on human liver and colon cancer cells in vitro; (2) measure the total antioxidant activity with different fruit extract combinations; (3) examine the antiproliferative activity of combination of different fruit extracts on human liver and colon cancer cells in vitro.

4.2. Materials and Methods

4.2.1. Chemicals

Sodium nitrite, (+)-catechin, Folin-Ciocalteu reagent (FCR), hydrochloric acid, glucagon, hydrocortisone, insulin, and α-keto-γ-methiolbutyric acid (KMBA) were purchased from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride, sodium hydroxide, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh,
PA). Gallic acid was purchased from ICN Biomedical Inc. (Costa Mesa, CA). 2,2’-azobis(amidinopropane) (ABAP) was purchased from Wako Chemicals (Richmond, VA).

4.2.2. Sample Preparation

Cranberry (Early Black variety), Apple (Red Declious variety), and Grape (Crimson variety) were purchased from local supermarkets. Samples were cleaned and dried before extraction. All data collected for each fruit were reported as means ± SD for at least three replications.

4.2.3. Sample Extraction

Phenolics were extracted from fresh cranberry, apple, or grape by the method reported previously in our laboratory (Yang et al., 2004). Briefly, 100 g of fruits were blended for 3 min in 200 g 80% acetone using Waring blender. The mixture was then homogenized in a Virtis High Speed Homogenizer for 3 min and filtered with vacuum. Water in the filtrate was evaporated using a rotary evaporator at 45°C until the weight of the evaporated filtrate was less than 10% of the weight of the original filtrate. All extracts were stored at –40°C until use. All extractions were performed in triplicate.

4.2.4. Measurement of Total Phenolic Content

The total phenolic content in fruits was determined using the Folin-Ciocalteu colorimetric method (Singleton et al., 1999), which was modified by our laboratory (Yang et al., 2004). Briefly, all sample extracts were diluted 1:5 with distilled water to obtain readings within the standard curve ranges of 0.0 - 600.0 µg of gallic acid/mL. The fruit extracts were oxidized with Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance was measured at 760 nm after 90
min at room temperature by a MRX II Dynex plate reader (Dynex Technologies, Inc., Chantilly, VA). The absorbance values were then compared with those of standards with known gallic acid concentrations. All values were stated as the mean (milligrams of gallic acid equivalents per 100 g of fresh sample) ± SD for three replications.

4.2.5. Measurement of Total Flavonoid Content

The total flavonoid content of the fruit samples was determined using a modified colorimetric method (Jia et al., 1999; Yang et al., 2004). Briefly, 0.25 mL of diluted fruit extract was mixed with 1.25 mL of distilled water, and subsequently with 0.075 mL of 5% sodium nitrite solution, and allowed to react for 5 min. Then a 0.15 mL of 10% aluminum chloride was added and allowed to further react for 6 min before 0.5 mL of 1 M sodium hydroxide was added. Distilled water was added to bring the final volume of mixture to 3 mL. The absorbance of the mixture was immediately measured at 510 nm wavelength against a prepared blank using a MRX II DYNEX spectrophotometer. The flavonoid content was determined by a catechin standard curve, and expressed as mean (milligrams of catechin equivalents per 100 g of fresh sample) ± SD for the triplicate extracts.

4.2.6. Determination of Total Antioxidant Capacity

The total antioxidant capacity of fruit extracts was measured using a total oxyradical scavenging capacity (TOSC) assay (Winston et al., 1998) as modified in our laboratory (Jia et al., 1999; Yang et al., 2004). Briefly, antioxidant activity was quantified after 15, 30, 45, and 60 min for four different fruit extract concentrations and a control. The amount of ethylene generated by the reaction was expressed as peak area. The TOSC value corresponding to each extract concentration was calculated by integrating the area under the kinetic curve, and assessed as the following equation:
\[ \text{TOSC} = 100 - \left( \frac{\int SA}{\int CA} \right) \times 100 \], where, \( \int SA \) is the integrated area from the sample reaction, and \( \int CA \) is the integrated areas from the control reaction. The median effective dose (EC₅₀) was determined for each fruit variety from the dose-response curve of fruit concentration versus TOSC value. The TOSC value is expressed as \( \mu \text{mol} \) of vitamin C equivalents per gram of sample. All values were presented as the mean ± SD at least three replicates.

4.2.7. Measurement of Inhibition of HepG₂ and Caco-2 Cell Proliferation

The antiproliferative activity of different fruit extracts was assessed by measurement of the inhibition of HepG₂ and Caco-2 human cancer cell proliferation. Antiproliferative activities were determined by the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) (Promega, Madison, WI) reported previously (Yang et al., 2004). HepG₂ cells were cultured in Williams’ medium E (WME), containing 10 mM Hepes, 5 \( \mu \text{g/mL} \) insulin, 0.05 \( \mu \text{g/mL} \) hydrocortisone, 2 \( \mu \text{g/mL} \) glucagon, and 5% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY), 50 units/mL penicillin, 50 \( \mu \text{g/mL} \) streptomycin, and 100 \( \mu \text{g/mL} \) gentamicin. Caco-2 human colon cancer cells were maintained in DMEM, containing 10 mM Hepes, 5% FBS, 50 units/mL penicillin, 50 \( \mu \text{g/mL} \) streptomycin, and 100 \( \mu \text{g/mL} \) gentamicin. Both HepG₂ and Caco-2 cells were maintained in a 5% CO₂/37°C incubator. A total of \( 2.5 \times 10^4 \) HepG₂ or Caco-2 cells in growth media were placed in each well of a 96-well flat-bottom plate. Cell proliferation was measured by the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium (MTS) to formazan. After 4 h of incubation, the growth medium was removed and media containing various concentrations (5, 10, 20, 30, 40, 50, and 60\( \mu \text{g/mL} \)) of fruit extracts were added to the cells. In the combination test, the concentration were \( 0.125 \times \text{EC}_{50} \), \( 0.25 \times \text{EC}_{50} \),
0.5×EC<sub>50</sub>, 0.75×EC<sub>50</sub>, 1.0×EC<sub>50</sub>, 1.25×EC<sub>50</sub>, and 1.5×EC<sub>50</sub>. Control cultures received the extraction solution minus the fruit extracts, and blank wells contained 100 µL of growth medium without cells. Cell proliferation (percent) was determined at 96 h from the MTS absorbance (490 nm) reading for each concentration compared to the control, using at least three replications for each sample. The effective median dose (EC<sub>50</sub>) was determined and expressed as milligrams of fruit component per milliliter ± SD.

4.2.8. Median-Effect Principle for Dose-Effect Analysis

The dose-effect analysis was modified from Chou and Talalay (Chou and Talalay, 1977; Chou, 1991). The median-effect principle was used to calculate single and combined fruit extract effects. Dose-effect curves for each fruit extract and their combinations with series diluted concentrations were plotted by using the median-effect equation as following:

\[
 f_a = \frac{1}{1 + (D_m / D^m)}
\]

Where D is the dose, Dm is the dose required for 50% effect, which is equivalent to median effect dose (EC<sub>50</sub>), f<sub>a</sub> is the fraction affected by dose D, and m is a coefficient of the sigmoidicity of the dose-effect curve.

4.2.9. Combination Index for Determining Addition, Synergism and Antagonism

The combination index (CI) (Chou, 1991; Chou et al., 1994) has been used for data analysis of two-way combinations as follow:

\[
 CI = \frac{(D)_{1} + (D)_{2}}{(D)_{1} \times (D)_{2}}
\]
For three-way combinations, a third term, \((D_3)/(D_x)_3\), is added. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. \((D_1)\) and \((D_2)\) are the doses of fruit extracts in the combination system; \((D_x)_1\) and \((D_x)_2\) are the doses of fruit 1 and fruit 2 alone, respectively.

4.2.10. Experiment Design

The experiment of different fruit combinations for total antioxidant activity and cell proliferation was designed in Table 4.1. Firstly, the EC\(_{50}\) value of each fruit extract was determined. Based on each EC\(_{50}\) value, a series of concentrations were designed as 0.125×EC\(_{50}\), 0.25×EC\(_{50}\), 0.5×EC\(_{50}\), 0.75×EC\(_{50}\), 1.0×EC\(_{50}\), 1.25×EC\(_{50}\), and 1.5×EC\(_{50}\). Finally, different dose from fruit extract alone would be combined together to inhibit peroxyl free radicals. The design of combinations in antiproliferative activity of three fruit extracts was similar to that of antioxidant activity.

4.2.11. Statistical Analysis

Statistical analysis was conducted using Minitab Student Release 12 (Minitab Inc., State College, PA) and SigmaStat Version 8.0 (Jandel Corp., San Raphael, CA). Results were subjected to ANOVA, and differences between means were located using Turkey’s multiple comparison test. Significance was determined at \(p < 0.05\).
Table 4.1. Experiment design of fruit combination for antioxidant activity and antiproliferative activity.

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<th>0</th>
<th>0.125×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>0.25×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>0.5×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>0.75×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>1.0×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>1.25×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
<th>1.5×(EC&lt;sub&gt;50&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(fa)&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
</tbody>
</table>
4.3. Results

4.3.1. Total Phenolic and Flavonoid Content

The total phenolic and flavonoid contents of the three fresh fruits is shown in Figure 4.1. Cranberry was found to have the highest phenolic content \((p < 0.05)\) at 428.0 ± 23.1 mg of gallic acid equivalents/100g sample, followed by apple (221.6 ± 12.8), and grape (154.5 ± 13.9). Significant differences were found in total phenolic content in comparisons among cranberry, apple, and grape \((p<0.05)\). Cranberry had the highest flavonoid content (292.2 ± 10.3 mg of catechin equivalents/100g sample, \(p<0.05\)), followed by apple (171.3 ± 24.5), and grape (93.2 ± 7.1). The flavonoid content of cranberry, apple, and grape were significantly different from each other \((p<0.05)\).

4.3.2. Total Antioxidant Activity for Single Fruit

The total antioxidant activities of the three fresh fruits, expressed as µg/mL sample, are summarized in Figure 4.2. Cranberry possessed the greatest antioxidant activity (228.2 ± 2.8 µg/mL, \(p<0.05\)), followed by apple (429.5 ± 6.6), and grape (524.1 ± 1.7). A statistically significant difference \((p<0.05)\) was found among cranberry, apple, and grape. The dose-effect relationships of three fruit extracts were subject to the median-effect plot to determine their potency \((EC_{50})\), shape \((m)\), and conformity \((r)\) in inhibition of peroxyl free radical production. The pooled results are summarized in Table 4.2. The \(EC_{50}\) and \(m\) values for single fruit extract and for their combination mixtures were used for calculating synergism, additive, or antagonism based on the CI equation.
4.3.3. Antiproliferative Activity for Single Fruit

The antiproliferative activities of fresh fruits are expressed as the median effective dose (EC$_{50}$), with a lower EC$_{50}$ value signifying a higher antiproliferative activity (Figure 4.3). The extract of cranberry had the highest antiproliferative activity toward HepG$_2$ cells with the lowest EC$_{50}$ of 16.4 ± 3.5 mg/mL ($p<0.05$), followed by apple (32.3 ± 6.4), and grape (54.9 ± 6.4). The antiproliferative activities of three fresh fruit extracts on Caco-2 human colon cancer cells were similar to those on HepG$_2$ cells. The phytochemical extract of cranberry exhibited the highest antiproliferative effect toward Caco-2 cells with the lowest EC$_{50}$ of 14.2 ± 2.2 mg/mL ($p<0.05$), followed by apple (23.1 ± 2.9), and grape (36.7 ± 5.6). Compared with cranberry extract, the phytochemical extract of grape exhibited a weak antiproliferative activity toward both HepG$_2$ and Caco-2 cells at higher doses. The dose-effect relationships of three fruit extracts were subject to the median-effect plot to determine their potency (EC$_{50}$), shape ($m$), and conformity ($r$) in inhibition of proliferations of HepG$_2$ and Caco-2 human cancer cells *in vitro*. The pooled results are summarized in Table 4.3. The $EC_{50}$ and $m$ values for single fruit extract and for their combination mixtures were used for calculating synergism, additive, or antagonism based on the CI equation.
Table 4.2. Dose-effect relationship parameters of fruit extracts in inhibiting peroxyl free radicals*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>$EC_{50}$ (μg/mL)</th>
<th>$m$</th>
<th>$r$</th>
<th>$N$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>429.5 ± 6.6</td>
<td>1.995 ± 0.307</td>
<td>0.960 ± 0.010</td>
<td>3</td>
</tr>
<tr>
<td>Cranberry</td>
<td>228.2 ± 2.8</td>
<td>1.793 ± 0.252</td>
<td>0.948 ± 0.031</td>
<td>3</td>
</tr>
<tr>
<td>Grape</td>
<td>524.1 ± 1.7</td>
<td>1.562 ± 0.183</td>
<td>0.956 ± 0.023</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4.3. Dose-effect relationship parameters of fruit extracts combination in inhibiting Caco-2 and HepG2 cell growth *in vitro*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Caco-2 $EC_{50}$ (mg/mL)</th>
<th>m</th>
<th>r</th>
<th>n</th>
<th>HepG2 $EC_{50}$ (mg/mL)</th>
<th>M</th>
<th>r</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>25.13±1.1</td>
<td>1.856±0.001</td>
<td>0.994±0.002</td>
<td>6</td>
<td>Apple</td>
<td>37.70±0.6</td>
<td>1.980±0.213</td>
<td>0.977±0.013</td>
</tr>
<tr>
<td>Cranberry</td>
<td>12.68±0.5</td>
<td>2.430±0.944</td>
<td>0.975±0.001</td>
<td>6</td>
<td>Cranberry</td>
<td>17.46±0.3</td>
<td>2.010±0.196</td>
<td>0.994±0.002</td>
</tr>
<tr>
<td>Grape</td>
<td>40.79±0.9</td>
<td>1.966±0.188</td>
<td>0.996±0.004</td>
<td>6</td>
<td>Grape</td>
<td>56.68±1.8</td>
<td>1.816±0.525</td>
<td>0.973±0.001</td>
</tr>
</tbody>
</table>

*The parameters $EC_{50}$, m, and r are antilog of x-intercept, slope, and the linear correlation coefficient of median-effect plot, which signifies the potency ($EC_{50}$), the shape of the dose-effect curve, and conformity of the data to the mass-action law, respectively; n is the number of sets of dose-effect relationship experiments tested (Chou et al., 1994).
Figure 4.1. Total phenolic and flavonoid content of fresh fruit extracts (mean ± SD, \( n = 3 \)). Bars with no letters in common are significantly different (\( p < 0.05 \)).
Figure 4.2. Total antioxidant activity of phytochemicals from fruit extracts (mean ± SD, n = 3). Bars with no letters in common are significantly different (p < 0.05).
Figure 4.3. Antiproliferative activity of phytochemicals from fruit extracts (mean ± SD, n = 3).
4.3.4. Antioxidant Activities of Different Fruit Combinations

Among three fruits, three pairs of two-way combinations and one pair of three-way combination were investigated. The average median effect dose (EC$_{50}$) and CI values at 50% induction rate are presented in Table 4.4. The dose-effect curves of different combination are plotted out in Figure 4.4. The dose-effect curve of antioxidant activity is shifted to the left after the combination. Compared with single compound, the EC$_{50}$ concentration on inhibition of peroxyl free radicals dramatically decreases among four pairs of combinations with different magnitude. For example, The EC$_{50}$ of apple and cranberry after combination is 2.3- and 2.4-fold, respectively, lower than the EC$_{50}$ of each fruit alone. The EC$_{50}$ of apple, cranberry, and grape after combination is 3.0-, 3.2-, and 3.1-fold, respectively, lower than the EC$_{50}$ of each fruit alone, suggesting synergistic effect after the combination of the three compounds. Apple and grape two-way combinations also resulted in fold reductions of 1.42, and 1.49, respectively. Both two-way and three-way fruit combinations were examined at concentrations ranged from 0 to 1.5 $\times$ EC$_{50}$. The CI at 50% inhibition rate in apple and cranberry, in cranberry and grape, and among apple, cranberry, and grape combinations are 0.853 $\pm$ 0.09, 0.681 $\pm$ 0.12, and 0.977 $\pm$ 0.02, respectively, indicating a synergistic effect in those combinations. However, the two-way combination of apple and grape demonstrated an antagonistic effect since the CI value was 1.374 $\pm$ 0.05, which is greater than 1.

4.3.5. Antiproliferative Activities of Various Fruit Combinations

The median effect dose (EC$_{50}$) and combination index (CI) values for two-way and three-way fruit combinations in antiproliferative activity for human liver and colon cancer cells are summarized in Table 4.5, and 4.6, respectively. The dose-effect curves of different combination are plotted in Figure 4.5, and 4.6, respectively. For
three pairs of two-way and one pair of three-way combination there was a decrease in the EC$_{50}$ dose for all fruits tested both in HepG$_2$ and in Caco-2 cell proliferation.

In HepG$_2$ cell line, compared fruit alone and combination, the fold reduction in EC$_{50}$ of apple and cranberry is 2.42 and 2.62, respectively, in the apple and cranberry combination. The fold reduction in EC$_{50}$ of cranberry and grape is 3.52 and 3.62, respectively, in the cranberry and grape combination. The fold reduction in EC$_{50}$ of apple, cranberry, and grape is 3.73, 4.03, and 4.36, respectively, in the apple, cranberry and grape combination. However, the fold reduction in EC$_{50}$ of apple and grape is 1.74 and 2.03, respectively, in the apple and grape combination. The CI at 50% inhibition rate in apple and cranberry, in cranberry and grape, and among apple, cranberry, and grape combinations are 0.862 ± 0.11, 0.774 ± 0.12, and 0.899 ± 0.165, respectively, indicating a synergistic effect in those combinations. However, the two-way combination of apple and grape demonstrated an antagonistic effect since the CI value was 1.128 ± 0.05, which is greater than 1.

For Caco-2 cell proliferation, in the apple and cranberry combination, the fold reduction in EC$_{50}$ of apple and cranberry is 1.18, and 1.39, respectively; the fold reduction in EC$_{50}$ of apple and grape is 1.20, and 2.07, respectively. Cranberry and grape combinations lower the EC$_{50}$ of cranberry and grape to 2.29-, and 4.38-fold, respectively. The 3-way combination also causes the fold reduction in EC$_{50}$ of apple, cranberry, and grape with 2.86, 3.36, and 3.61, respectively. The CI values at 50% inhibition of Caco-2 cell proliferation are 1.426 ± 0.04, and 1.295 ± 0.15, respectively, in apple and cranberry, and in apple and grape combinations, suggesting an antagonism. 0.770 ± 0.03 and 0.927 ± 0.124 of CI values are generated in cranberry and grape, and in apple, cranberry, and grape combinations, indicating a synergism.
Table 4.4. Summary of medium effect dose based on antioxidant activity from fruit combinations.

<table>
<thead>
<tr>
<th>Fruit</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µmol vitamin C equ./g sample)</th>
<th>CI at 50% Inhibition rate</th>
<th>Combination Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>Combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td>429.5 ± 6.6</td>
<td>76.8 ± 1.2</td>
<td>188.1 ± 20.8</td>
<td>175.4 ± 20.5</td>
</tr>
<tr>
<td>Cranberry</td>
<td>228.2 ± 2.8</td>
<td>144.6 ± 1.8</td>
<td>94.05 ± 10.4</td>
<td>350.8 ± 41.1</td>
</tr>
<tr>
<td>Apple</td>
<td>429.5 ± 6.6</td>
<td>76.8 ± 1.2</td>
<td>303.3 ± 11.5</td>
<td>109.1 ± 4.2</td>
</tr>
<tr>
<td>Grape</td>
<td>524.1 ± 1.7</td>
<td>63.0 ± 0.2</td>
<td>352.7 ± 13.4</td>
<td>93.5 ± 3.6</td>
</tr>
<tr>
<td>Cranberry</td>
<td>228.2 ± 2.8</td>
<td>144.6 ± 1.8</td>
<td>77.0 ± 13.2</td>
<td>428.6 ± 68.9</td>
</tr>
<tr>
<td>Grape</td>
<td>524.1 ± 1.7</td>
<td>63.0 ± 0.2</td>
<td>179.6 ± 30.8</td>
<td>183.7 ± 29.5</td>
</tr>
<tr>
<td>Apple</td>
<td>429.5 ± 6.6</td>
<td>76.8 ± 1.2</td>
<td>144.7 ± 2.8</td>
<td>228.0 ± 4.4</td>
</tr>
<tr>
<td>Cranberry</td>
<td>228.2 ± 2.8</td>
<td>144.6 ± 1.8</td>
<td>72.3 ± 1.4</td>
<td>456.1 ± 8.9</td>
</tr>
<tr>
<td>Grape</td>
<td>524.1 ± 1.7</td>
<td>63.0 ± 0.2</td>
<td>168.8 ± 3.3</td>
<td>195.5 ± 3.8</td>
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</tbody>
</table>

Table 4.5. Summary of medium effect dose based on HepG<sub>2</sub> cell proliferation from fruit combinations.

<table>
<thead>
<tr>
<th>Fruit</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/mL)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/mL)</th>
<th>CI at 50% Inhibition rate</th>
<th>Combination Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>Combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td>37.70 ± 0.6</td>
<td>15.57 ± 0.81</td>
<td>0.862 ± 0.11</td>
<td>Synergism</td>
</tr>
<tr>
<td>Cranberry</td>
<td>17.46 ± 0.3</td>
<td>6.67 ± 0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td>37.70 ± 0.6</td>
<td>21.68 ± 0.96</td>
<td>1.128 ± 0.05</td>
<td>Antagonism</td>
</tr>
<tr>
<td>Grape</td>
<td>56.68 ± 1.8</td>
<td>27.87 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cranberry</td>
<td>17.46 ± 0.3</td>
<td>5.37 ± 1.4</td>
<td>0.774 ± 0.12</td>
<td>Synergism</td>
</tr>
<tr>
<td>Grape</td>
<td>56.68 ± 1.8</td>
<td>15.66 ± 4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple</td>
<td>37.70 ± 0.6</td>
<td>10.11 ± 0.71</td>
<td>0.899 ± 0.165</td>
<td>Synergism</td>
</tr>
<tr>
<td>Cranberry</td>
<td>17.46 ± 0.3</td>
<td>4.33 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grape</td>
<td>56.68 ± 1.8</td>
<td>13.0 ± 0.91</td>
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<td></td>
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</table>
Table 4.6. Summary of medium effect dose based on Caco-2 cell proliferation from fruit combinations.

<table>
<thead>
<tr>
<th>Fruit</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/mL)</th>
<th>CI at 50% Inhibition rate</th>
<th>Combination Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Single</strong></td>
<td><strong>Combination</strong></td>
<td></td>
</tr>
<tr>
<td>Apple, Cranberry</td>
<td>25.13 ± 1.1, 12.68 ± 0.5</td>
<td>21.25 ± 0.63, 9.11 ± 0.27</td>
<td>1.426 ± 0.04, Antagonism</td>
</tr>
<tr>
<td>Apple, Grape</td>
<td>25.13 ± 1.1, 40.79 ± 0.9</td>
<td>20.98 ± 0.98, 19.73 ± 5.4</td>
<td>1.295 ± 0.15, Antagonism</td>
</tr>
<tr>
<td>Cranberry, Grape</td>
<td>12.68 ± 0.5, 56.68 ± 1.8</td>
<td>5.54 ± 0.19, 12.93 ± 0.45</td>
<td>0.770 ± 0.03, Synergism</td>
</tr>
<tr>
<td>Apple, Cranberry, Grape</td>
<td>25.13 ± 1.1, 12.68 ± 0.5, 40.79 ± 0.9</td>
<td>8.79 ± 1.2, 3.77 ± 0.5, 11.30 ± 0.5</td>
<td>0.927 ± 0.124, Synergism</td>
</tr>
</tbody>
</table>
Figure 4.4. The dose-effect curves for fruit combinations in inhibiting peroxyl free radicals: (a) apple + cranberry; (b) apple + grape; (c) cranberry + grape; and (d) apple + cranberry + grape.
Figure 4.5. The dose-effect curves for fruit combinations in inhibiting HepG2 cell proliferation: (a) apple + cranberry; (b) apple + grape; (c) cranberry + grape; and (d) apple + cranberry + grape.
Figure 4.6. The dose-effect curves for fruit combinations in inhibiting Caco-2 cell proliferation: (a) apple + cranberry; (b) apple + grape; (c) cranberry + grape; and (d) apple + cranberry + grape.
4.4. Discussion

Compelling evidence indicates that multiple servings of fruits and vegetables in the daily diet provide health benefits. Studies conducted by our group and others have shown that phytochemical extracts in fruits and vegetables exhibit strong antioxidant and antiproliferative activities and that the major part of total antioxidant activity is from the combination of phytochemicals. Based on antioxidant and antiproliferative activities of the individual fruits, these activities of common fruits with different combinations were examined. Our work has clearly shown that the phytochemicals from single fruit have potent antioxidant and antiproliferative activities, and that antioxidant activity is positively correlated with total phenolic and flavonoid contents. Furthermore, the phytochemicals in the two-way and three-way combinations of fruits have demonstrated more potent antioxidant and antiproliferative activities due to the EC\textsubscript{50} decrease. The combinations in apple and cranberry, in cranberry and grape, and in cranberry, apple, and grape showed a synergistic effect on inhibition of radical production; however, the apple and grape combinations had an antagonistic effect on radical production blocking. For the HepG\textsubscript{2} and Caco-2 cell proliferation, the phytochemicals in apple and cranberry, in apple, cranberry, and grape had synergistic effect on antiproliferative activity. The additive and synergistic effects of phytochemicals in fruits are responsible for their potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruit is attributed to the complex mixture of phytochemicals present in whole foods. The evidence suggests that antioxidant and antiproliferative activities are best acquired through whole-food consumption.

The possible reason for protection against chronic disease owing to consumption of fruits is the presence of antioxidant vitamins such as vitamin C,
vitamin E, and β-carotene. However, some studies doubt on this hypothesis (Vinson et al., 1995; Cao et al., 1996; Rapola et al., 1997; Ascherio et al., 1999; Eberhardt et al., 2000; Leppala et al., 2000; Yusuf et al., 2000). (Vinson et al., 1995) showed that many phenolics are stronger antioxidants than the vitamin antioxidants with a model of the oxidation of the lower density lipoproteins. There were no benefits of vitamin C or E for reducing the risk of stroke for an 8-year prospective study of more than 44,000 healthy men in the Health Professionals Follow-up Study (Ascherio et al., 1999). (Yusuf et al., 2000) reported that a 4-year vitamin E supplementation to over 9000 patients with high risk for cardiovascular events led to no significant benefit. Studies from (Leppala et al., 2000) showed that a 6-year daily vitamin E or β-carotene supplementation to cigarette smokers had a decreased risk of cerebral infarction but an increased risk of hemorrhage. Our group found that the vitamin C in apples with skin accounts for only 0.4% of the total antioxidant activity. It has been proposed that the majority of antioxidant activity of fruits may come from phytochemicals such as phenolics and flavonoids. Phenolic compounds widely distributed in fruits scavenge ROS and free radicals as antioxidant. In addition, phenolics can chelate transition metal ions, influence specifically the cell at a transcriptional level, affect signal transduction pathways in the cells, down-regulate pathways leading to cell death, and affect cell redox systems (Spencer et al., 2001; Wang et al., 2001). More than 5,000 individual phytochemicals have been identified in fruits, vegetables, and grains, and each phytochemical has been proven to have unique biological activities. For example, quercetin blocks phospholipase A2, PI 3-kinase, and protein kinase C, and kaempferol inhibits the activation of caspase-2, -3, -8, and -9 and reduces neuronal apoptosis (Gschwendt et al., 1983; Wang et al., 2001). The studies from Kuroda and Hara (1999) and Chung et al. (2001) have shown that catechins inhibit tumors and induce apoptosis. Resveratrol arrests the cell cycle at S/G2 phase. Resveratrol may contribute
to inhibit cell proliferation by the suppressing the activities of cyclooxygenase-2 (COX-2), nuclear transition factors NF-κB, AP-1, c-fos, and inducing the activities of CD95L, p21, and p53 (Manna et al., 2000).

Based on the principle of the mass-action law via mathematical induction and deduction in enzyme kinetic models, the median-effect equation and the CI method have been extensively applied in drug combinations (Chou and Talalay, 1977; Chou, 1991; Chou et al., 1994). Our study was carried out by quantitative analysis of synergism or antagonism at different fruit doses and different effect levels. Here three methods are used to express additive, synergistic, and antagonistic effects. One way is to compare the fold EC$_{50}$ reduction between single fruit and combined fruit system. Another way is to compare generated CI value with 1. The last way is to examine inhibition fraction in each fruit system on the basis of the sum of their individual activity by using the dose (EC$_{50}$) produced in combined fruit system. If the results from above 3 methods are consistent, conclusions could be drawn. For example, in apple, cranberry, and grape combinations, the reduction fold of EC$_{50}$ of apple, cranberry, and grape after combination is 3.0, 3.2, and 3.1, respectively, suggesting synergistic effect after the combination of the three compounds. Secondly, the CI at 50% inhibition rate among apple, cranberry, and grape combinations is 0.977 ± 0.02, which is less than 1, indicating a synergistic effect.

From single fruit effect, it was found that cranberry showed the highest antioxidant and antiproliferative activities ($p < 0.05$), followed by apple, and grape. Theoretically, it is extrapolated that cranberry would make the maximal contribution of synergism among apple, cranberry, and grape combination. However, it was not consistent with the observed results. For example, cranberry made a minimal
contribution of 3-way combination in antioxidant model (Figure 4.5 (d)). We postulated that the activity of certain bioactive compounds from cranberry has changed in the combined matrix. Particular bioactive compounds may act additively and antagonistically with some compounds in the combined matrix and the overall expressed activity may be dependent upon relative proportions of each and/or presence/absence of particular compounds. In this study, 2-way combinations of apple plus cranberry, cranberry plus grape, and 3-way combination of apple plus grape plus cranberry exhibited the synergistic action in inhibiting peroxyl free radicals. However, the 2-way combination of apple plus grape showed antagonistic action in suppressing peroxyl free radicals. The observed antagonism between catechin and quercetin (cyanidin, caffeic acid, and ellagic acid) was also found in inhibiting LDL oxidation (Meyer et al., 1998).

Many of the phytochemicals in whole foods have been found to provide a much stronger antioxidant activity than single supplement such as vitamin C, E, and β-carotene. This may be explained by the combination of different phytochemicals functioning synergistically or additively. (Bentsath et al., 1936) have found that Hungarian red pepper extracts possessed an ascorbate-protective factor, identified as a mixture of flavonoids, and demonstrated that the interaction between flavonoids and other antioxidants. The synergy between flavonoids and ascorbate has been also reported by other experiments (Harper et al., 1969; Kandaswami et al., 1993). Urate is an important plasma antioxidant. 12 different flavonoids based on the number and position of hydroxylations in the A and B rings, availability, and prevalence in the human diet were analyzed for their antioxidant effects on copper-induced lipid peroxidation in diluted human whole plasma (Filipe et al., 2001). It has been suggested that catechin, quercetin, luteolin, and rutin with concentrations range from
5.0 to 7.5 μM showed a synergistic antiperoxidant effect with urate. In terms of mechanism, the reduction potential of the urate radical/urate pair is higher than that of the ascorbyl radical/ascorbate, which permits the reaction of the latter with the urate radical, recycling urate (Maples and Mason, 1988). Due to redox potential similar to ascorbate (Hendrickson et al., 1994), it was postulated that some flavonoids could recycle urate from its radical. Generally, combination of phytochemicals such as phenolics and flavonoid from fruits and vegetables contain more potential antioxidant activity and anticancer activity. The antioxidant synergy between (+)-catechin and other antioxidants (SO₂, Trolox, ascorbate, and uric acid) was evaluated in vitro using the Folin-Ciocalteu and metmyoglobin assays (Saucier and Waterhouse, 1999). The results showed that the mixture of (+)-catechin and SO₂ resulted in a synergistic effect of their antioxidant activities, the interaction between (+)-catechin and Trolox, ascorbate and uric acid led to an additive effect of their antioxidant activities. Catechin is relatively inefficient at inhibiting linoleic acid oxidation in micellar systems mimicking LDL (Rossetto et al., 2002). However, a strong synergistic effect between catechin and malvidin 3-glucoside was observed, indicating a recycling of malvidin 3-glucoside by catechin. The chemical structure and antioxidant activity of phytochemicals was evaluated by measuring inhibition of copper-catalyzed human LDL oxidation in vitro (Meyer et al., 1998). 20 different combinations of two/three of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid were examined to investigate the potential synergistic or antagonistic effects on measuring the antioxidant activities on LDL. It was found that antioxidant effects of the hydroxyphenols were additive in both the two- and three-compound combinations except combination of ellagic acid with catechin, where ellagic acid had a significant antagonistic effect on the antioxidant activity of catechin, it was proposed that the effect is due to hydrogen bonding between carbonyls in ellagic acid and o-dihydroxyl
groups in catechin. Neither of which showed effect on platelet function when used alone, the combination of 25 µmol catechin/L and 5 µmol quercetin/L significantly inhibited collagen-induced platelet aggregation and platelet adhesion to collagen, suggesting a synergistic effect (Pignatelli et al., 2000). With 50 µM catechin and 10 µM resveratrol or 25 µM resveratrol and 10 µM catechin, the toxicity determined by 10^{-7} M β-Amyloid peptide (1–41) in PC12 cells is almost completely abolished, suggesting a synergistic protective effect (Conte et al., 2003).

The observed additive and synergistic effects may be important in human health since oxidative processes are involved in many diseases. Here, the greatest challenge is how to decide the concentration range of individual fruits in the combination. If the designed concentrations were too low, the combination would not function. On the other hand, if the doses were too high, the combination would cause cytotoxicity toward cell lines. Here, based on individual fruit’s EC_{50}, series concentrations designed were lower or higher than EC_{50} both in antioxidant and antiproliferative activities models. The above method was proven to work well in our study. Our current study clearly demonstrated that there existed synergistic and antagonistic effects in fruits in inhibition of peroxyl free radical production and HepG2 and Caco-2 cell proliferation. However, the studies strengthen the quantitative analysis of fruit combinations rather than the mechanism of synergistic and antagonistic interactions. It is worthwhile to investigate synergistic and antagonistic effects of the main bioactive compounds from fruits, and conduct research on the mechanism of actions related to these interactions.
CHAPTER FIVE

COMBINATION EFFECTS OF SELECTED PHYTOCHEMICALS AND FRUITS ON QUINONE REDUCTASE ACTIVITY

5.1. Introduction

Numerous epidemiological studies have consistently shown that a high dietary consumption of fruits and vegetables is strongly associated with lowered risk for developing chronic diseases, such as cardiovascular disease (CVD) and cancer, which are the top two leading killers in the United States. From Heart Disease and Stroke Statistics—2006 Update (Thom et al., 2006), preliminary mortality data show that CVD is the underlying cause of death accounting for 37.3% of all 2,440,000 deaths or 1 out of every 2.7 deaths, while cancers accounted for 554,643 in 2003 in the United States. Scientific evidence suggests that about one-third of the 564,830 cancer deaths expected to occur in 2006 will be related to nutrition, physical activity, and being overweight, and thus could also be prevented (American Cancer Society, 2006). The National Academy of Sciences of the United States in 1982 issued guidelines on diet and cancer, strengthening the importance of fruits and vegetables. The Food Pyramid developed by the US Department of Agriculture in 2005 recommends fruits and vegetables consumption of 5 to 9 servings per day.

Besides providing the essential nutrients for life, diet also supplies bioactive compounds which are important for disease prevention and health maintenance. There is overwhelming evidence indicating that increased uptake of fruits and vegetables in the diet reduce the risk of heart disease, cancer, stroke, as well as brain and immune dysfunction (Doll and Peto, 1981; Block et al., 1992; Willett, 1994; Temple, 2000;
Joshipura et al., 2001). A number of epidemiological studies have examined the relation between consumption of fruits and vegetables and chronic diseases, and most of these studies found protective effects for fruits and vegetables. Study from (Knekt et al., 1994) indicated an inverse relationship between intake of vegetables and the risk of coronary artery disease (CAD) in 5,133 Finnish men and women. A significant inverse association between flavonoid intake from apple, berries, and onions and CAD death has been documented (Knekt et al., 1996). In pooled analyses of the Nurses’ Health Study and the Health Professionals’ Follow-up Study, including 2190 cases of CAD and 570 cases of ischemic stroke, (Joshipura et al., 2001) have found that there existed an inverse association between intake of fruits and vegetables and CAD in a dose-dependent manner, and that consumption of at least 8 servings/day is associated with the lowest risk. Diets rich in fruit and vegetables have been suggested for preventing cancer. In 1997, World Cancer Research Fund and American Institute for Cancer Research made a conclusion that there was convincing evidence that high consumption of vegetables lowers the risk of the colon, lung, stomach, mouth and pharynx, esophagus, and rectum cancers; that it probably decreases the risk of the larynx, pancreas, breast, and bladder cancers; and that it possibly lowers the risk of the liver, ovary, endometrium, cervix, prostate, thyroid, and kidney cancers. High intake of fruits has been associated with a decreased risk of most of the cancers mentioned above (WCRF, 1997). After examining the epidemiological evidence from case-control and cohort studies on fruit and vegetable consumption at different cancer sites by summarizing it quantitatively with a meta-analytic approach, (Riboli and Norat, 2003) concluded that case-control studies overall show a significant reduction in the risks of esophagus, lung, stomach, and colorectum cancers related to consumption of fruits and vegetables; prospective studies provide weaker evidence than do case-
control studies of the association of fruit and vegetable intake with lowered cancer risk.

The protective effects of fruits and vegetables are probably mediated by multiple beneficial nutrients and/or non-nutrients present in fruits and vegetables, including phenolics, folate, antioxidant vitamins, carotenoids, thiols, and glucosinolates. Reactive oxygen species (ROS) such as free radicals are the byproducts generated during oxidative metabolism in organisms. Antioxidants are believed to play a very important role in the body defense system against ROS. Endogenous antioxidants with dietary antioxidants may be particularly important in diminishing the cumulative effects of oxidatively damaged molecules. Fruits and vegetables are the important sources of antioxidants. Phenolics and other plant antioxidants can scavenge the free radicals, and then inhibit their oxidative reactions with macromolecules in the body. Previously much attention was paid to the dietary antioxidant properties of vitamins C, E, and carotenoids. However, studies have shown that some common fruits and vegetables possess high antioxidant activity, which cannot be accounted for by their vitamin C content (Wang et al., 1996; Eberhardt et al., 2000). Furthermore, some studies have demonstrated that dietary phenolics derived from plant are more effective antioxidants in vitro than vitamins C or E (Liu, 2003; Liu, 2004). On the other hand, epidemiological studies of Vitamin E, and β-carotene supplements for chronic disease prevention have found either no effect on CVD or a slight increase in cardiovascular mortality and cancer (Hennekens et al., 1996; Omenn et al., 1996; Rapola et al., 1997). Phenolics and flavonoids with the powerful antioxidant capacity have been drawing much attention recently. Fruits and vegetables consumed by human diets contain thousands of phytochemicals. Flavonoids are the largest class of phenolic compounds, and more than 5,000
compounds have been identified. In particularly, anthocyanins, hydroxybenzoic and hydroxycinnamic acid derivatives, flavones, flavanols (catechins), flavonols, flavanones, isoflavones, and tannins are frequently present. Phenolics which possess two ortho-positioned hydroxyl groups are good antioxidants. The possible effects of synergism and antagonism in complex mixtures of these phytochemicals need to be considered. It is believed that phytochemicals in fruits and vegetables possess complementary and overlapping mechanisms of action, including scavenging free radicals, chelating metal ions, modulation of detoxification enzymes, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects (Dragsted et al., 1993; Waladkhani and Clemens, 1998).

Quercetin (3,3',4',5,7-pentahydroxyflavone) belongs to an extensive class of polyphenolic flavonoid compounds almost ubiquitous in plant food sources. Quercetin is the major bioflavonoid in the human diet. The estimated average daily dietary intake of quercetin by an individual in the United States is about 25mg (Davis et al., 2000; Davis et al., 2005). The biological functions of quercetin can be summarized as free radical scavenger and inhibitor of ROS production (Pietta, 2000); binding in the ATP-binding pocket of the kinase as protein kinase inhibitor (Walker et al., 2000); inhibiting the religation of the DNA double strands as topoisomerase inhibitor (Constantinou et al., 1995); and regulating NF-κB transcription factors and AP-1 which regulate gene expression (Muraoka et al., 2002; Moon et al., 2003).

Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen with a wide variety of pharmacological effects in animal cells, and with a range of potential health beneficial effects from epidemiological and animal model studies, including
chemoprevention of breast and prostate cancers, cardiovascular disease, and post-menopausal ailments (Adlercreutz et al., 1995; Dixon and Daneel, 2002). In several clinical studies, genistein showed chemoprotective and chemotherapeutic potential against tumors, including colon, prostate, and breast cancers via different mechanisms of action such as modulation of cell cycle activity by arresting cell cycle at the G2-M stage (Matsukawa et al., 1993); apoptosis induction; competitive inhibition of ATP binding to the catalytic domain of tyrosine kinase; inhibition of DNA topoisomerase-II and tyrosine protein kinase (Akiyama et al., 1987); stimulating the production of sex hormone-binding globulin, lowering the risk of hormone related cancers by decreasing the amount of free and active hormones in the blood (Messina et al., 1994); and inhibition of cell growth by modulating transforming growth factor (TGF) β1 signaling pathways (Kim et al., 1998).

Resveratrol (3, 4’, 5-trihydroxystilbene, RSV) is found in various food products, with particularly high abundance in grape skin, seeds, and red wine (Langcake and Pryce, 1976). RSV is an active ingredient of the oriental folk medicine kojokon which have many therapeutic uses (Kimura et al., 1985). The presence of RSV in wine has been suggested as a possible explanation for the French Paradox (Siemann and Creasy, 1992; Frankel et al., 1993a). Thus, RSV has attracted considerable attention due to its cardioprotective and cancer chemopreventive activities (Jang et al., 1997), which provide great interest in grapes, wines, and dietary products containing RSV. The proposed mechanisms related to RSV’s health effects can be summarized as scavenging intracellular ROS (Manna et al., 2000), inhibiting the oxidation of LDL (Frankel et al., 1993b), preventing platelet aggregation (Pace-Asciak et al., 1995), suppressing cell proliferation via steps in the signal transduction pathways (Pozo-Guisado et al., 2002), inducing apoptotic cell death through activation
of mitochondria-dependent pathways (Huang et al., 1999), exhibiting anti-inflammatory activity via down-regulation of proinflammatory cytokines (Wadsworth and Koop, 1999), promoting cellular differentiation (Mizutani et al., 1998), exhibiting antiestrogenic activity (Lu and Serrero, 1999), and inhibiting CYP1 enzymes (Chang et al., 2001).

Induction of detoxification enzymes by fruits and vegetables is one of proposed mechanisms to account for chemoprotective effect through an enhancement in excretion of chemical carcinogens (Wattenberg, 1985). For instance, intake of cruciferous vegetables, such as cabbage, broccoli, and Brussels sprouts, has been demonstrated to induce detoxification enzymes and increase clearance of xenobiotics in animals (Prochaska et al., 1992; Zhang et al., 1992), and has been associated with lowered incidence of cancers in humans (Graham et al., 1978; Pantuck et al., 1979). Quinone reductase (QR) is a family of two-electron reducing enzymes that decrease the oxidative damage resulting from one-electron reduction of quinone (Lind et al., 1982). It is induced coordinately with other phase II detoxifying enzymes, such as the glutathione S-transferases (GST), and plays an important role in protecting cells against oxidative cycling, detoxifying carcinogens, and providing an index for Phase II gene status of cells (Dinkova-Kostova and Talalay, 2000). A wide variety of chemical compounds, including quinones, Michael reaction acceptors, isothiocyanates, oxathiolene oxides, hydroperoxides, trivalent arsenicals, some heavy metals, vicinal dimercaptans, carotenoids, and flavanoids has been reported to induce QR in cells (Talalay et al., 1988; Prestera et al., 1993; Albena et al., 2004). Compared with the corresponding normal tissues, enhanced levels of expression of QR have been reported in colon, breast, liver, and lung cancers (Riley and Workman, 1992; Belinsky and Jaiswal, 1993).
QR is a flavoprotein that catalyzes two-electron reduction and detoxification of quinones and its derivatives, leading to the protection of cells against redox cycling, oxidative stress, and neoplasia. QR acts as an important regulator of a wide variety of biological functions and may play a particularly significant role in cancer. The major functions of QR are to catalyze the obligatory single-step two-electron reduction, bypassing reactive and toxic semiquinone intermediates (Talalay and Dinkova-Kostova, 2004); to lower formation of ROS by decreasing one electron reductions and the associated redox cycling (Ernster, 1987); to help maintain certain endogenous antioxidants in their reduced and active forms such as ubiquinone (Landi et al., 1997) and α-tocopherolquinone (Siegel et al., 1997); to negatively regulating Mdm-2/ubiquitin-independent degradation pathway for stabilization of p53 protein (Asher et al., 2001); to play an important role in activating some anticancer drugs (Ross et al., 1993; Workman, 1994); and to act as a phase II detoxifying enzyme involved in cancer prevention (Ross et al., 1993; Workman, 1994).

A complex interplay among the ROS defense systems exists, with various antioxidant cycles acting to prevent cell damage and chronic diseases. Synergistic effect of antioxidants such as vitamin E and vitamin C (Scarpa et al., 1984), vitamin E and β-carotene (Palozza and Krinsky, 1991), catechin and malvidin 3-glucoside (Rossetto et al., 2002), flavonoids and urate (Filipe et al., 2001), and tea polyphenols and vitamin E (Zhou et al., 2000) were observed. Vitamin C can regenerate vitamin E from the vitamin E radical, recycling vitamin E (Buettner, 1993). Vitamin E becomes a radical in the process, but it can be recycled by interacting with other antioxidant systems. Fruits and vegetables contain an array of phytochemicals and thus provide a mixture of unique compounds and their derivatives in the diet. Some phytochemicals from fruits and vegetables are linked to induction of both Phase I and Phase II
detoxification enzymes, which have been defined as bifunctional inducers, while others only induce Phase II enzymes, and they have been designated as monofunctional inducers (Prestera et al., 1993). It has been postulated that combinations of fruit have greater antioxidant activities than would be expected on the basis of their individual effects. The phytochemcials in fruits and vegetables may act independently or in combination as anti-tumor or cardioprotective agents. Information on inducer interactions of phytochemicals would provide better understanding of the QR induction effects in complex mixtures. However, little is known about the relative potencies of interaction between/among different compounds present in a single fruit/vegetable to induce Phase II enzymes. Furthermore, there is no literature on the effect of combinations of fruit extracts on QR induction. Based on our previous studies, therefore, it is of great interest to examine combinations of common phytochemicals to look at the additive, synergistic, and antagonistic effects in induction of Phase II enzymes. The goal of this study therefore was to seek possible synergy between/among different phytochemicals and fruit combinations. The objectives for this study were to: (1) determine the phytochemical combination effect of quercetin, genistein, and resveratrol on QR induction; (2) examine the phytochemicals and grape variety combination effect on QR induction; (3) investigate the fruit combinations effect on QR induction.

5.2. Materials and Methods

5.2.1. Chemicals

Alpha minimal essential medium (α-MEM), fetal calf serum (FBS), hepes, and antibiotic-antimycotic solution were purchased from GIBCO (Life Technologies, Grand Island, NY). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
sulfenyl)-2H-tetrazolium (MTS) and phenazinemethosulfate (PMS) were from Promega (Madison, WI). Genistein (<98% HPLC grade), quercetin (dihydrate), resveratrol (3,4',5-trihydroxystilbene, ~99%), β-naphthoflavone (BNF, 90-95%), digitonin, glucose 6-phosphate, menadione, nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), albumin from human serum (≥96%), yeast glucose 6-phosphate dehydrogenase, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS, ~99%), ethylenediamine tetraacetic acid (EDTA, anhydrous, ~99%), and dicoumarol were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) and Tween 20 were from Fisher Scientific (Fair Lawn, NJ). Tris-base and crystal violet were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All the other materials used were also obtained from Sigma (St. Louis, MO).

5.2.2. Sample Preparation

Fresh Cranberry (Early Black variety), Apple (Red Declious variety), and Grape (Cabernet Franc variety) were purchased from local supermarkets. Samples were cleaned and dried before extraction. All data collected for each fruit were reported as means ± SD for at least three replications.

5.2.3. Sample Extraction

Phytochemicals were extracted from fresh cranberry, apple, or grape by the method reported previously in our laboratory (Yang et al., 2004). Briefly, 100 g of fruits were blended for 3 min in 200 g 80% acetone using Waring blender. The mixture was then homogenized in a Virtis High Speed Homogenizer for 3 min and filtered with vacuum. Water in the filtrate was evaporated using a rotary evaporator at 45°C until the weight of the evaporated filtrate was less than 10% of the weight of the
original filtrate. All extracts were stored at –40°C until use. All extractions were performed in triplicate.

5.2.4. Determination of Quinone Reductase Activity in Hepa1c1c7 Cell Culture

This bioassay was modified from a previously described method (Prochaska and Santamaria, 1988; Zhang et al., 1992). Hepa1c1c7 murine hepatoma cells (The American Type Culture Collection, ATCC, Rockville, MD) were grown in α-MEM without nucleosides or deoxyribonucleosides, supplemented with 10% FBS, at 37°C in an atmosphere of 5% CO₂ in a humidified incubator. Cultured Hepa1c1c7 cells were plated at a density of 2 × 10⁴ cells/mL in 96-well plates (Costar 3595, Corning Inc. Corning, NY), and decanted after a 24 h incubation. Fresh medium and test samples dissolved in 10% DMSO were introduced and serially diluted to a concentration range of 5 - 200 μM for compounds, and 0.5 - 10 mg/mL for grape extracts. The final DMSO concentration in the medium was less than 0.5%. The cells were incubated for an additional 48 h. Growth medium and 1 μM BNF were used as negative and positive controls, respectively. The medium was removed and the cells were lysed with 50 μL 0.8% (w/v) digitonin in 2 mM EDTA at pH 7.6, and incubated for 10 min at 37°C. The plates were then agitated on a shaker (120 rpm) for 10 min at room temperature. A 200-μL aliquot of reaction mixture (7.5 mL of 0.5 M pH 7.4Tris-HCl buffer; 100 mg of albumin from human serum; 1 mL of 1.5% Tween-20 solution; 0.1 mL of 7.5 mM FAD; 1 mL of 150 mM glucose-6-phosphate; 90 μL of 50 mM NADP; 300 units of yeast glucose-6-phosphate dehydrogenase; 45 mg of MTT; 150 μL of 50 mM menadione in acetonitrile; and 140.16 mL distilled water added to total 150 mL volume) was added to lysed cells. Menadione solution was added just before the mixture was dispensed into the microtiter plates. QR activity was measured as the reduction of menadione to menadiol, this being coupled to the non-enzymatic
reduction of MTT to a blue formazan. The reaction generated a blue color, which was arrested after 5 min by the addition of 50 μL of a solution containing a 0.3 mM dicoumarol in 0.5% DMSO and 5 mM pH 7.4 potassium phosphate. Readings were made in triplet for each sample at 590 nm. Total protein concentrations were determined in a duplicate set of plates using crystal violet staining, and subsequently scanned at 490 nm (Prochaska et al., 1992).

The specific activity of QR is defined as nmol MTT blue formazan reduced per min and per mg protein. Induction was expressed as the ratio of the specific activity of QR in the presence and absence of the test sample. The concentration required to double specific activity (CD) is determined via a curve of the ratio of QR specific activities of sample-treated cells to solvent-treated control cells as a function of inducer concentration (Kang and Pezzuto, 2004).

\[
\text{Specific Activity} = \frac{\text{Absorbance change of MTT/min} \times 3247 \text{ nmol/mg of protein}}{\text{Absorbance of crystal violet}}
\]

\[
\text{Fold of Induction (ratio)} = \frac{\text{Specific Activity of Treated Group}}{\text{Specific Activity of DMSO Control Group}}
\]

5.2.5. Median-Effect Principle for Dose-Effect Analysis

The dose-effect analysis was modified from Chou and Talalay (Chou and Talalay, 1977; Chou, 1991). The median-effect principle was used to calculate single and combined fruit extract effects. Dose-effect curves for each phytochemical and fruit extract and their combinations with series diluted concentrations were plotted by using the median-effect equation as follows:
\[ f_a = \frac{1}{1 + (D_a/D)^m} \]  \hspace{1cm} (1)

Where D is the dose, Dm is the dose required for 50% effect, which is equivalent to median effect dose (EC\(_{50}\)), \( f_a \) is the fraction affected by dose D, and m is a coefficient of the sigmoidicity of the dose-effect curve.

5.2.6. Combination Index for Determining Addition, Synergism and Antagonism

The combination index (CI) (Chou, 1991; Chou et al., 1994) has been used for data analysis of two-way combinations as following:

\[ CI = \frac{(D_1)}{(D_x)_1} + \frac{(D_2)}{(D_x)_2} \]  \hspace{1cm} (2)

For three-way combinations, a third term, \((D_3)/(D_x)_3\), is added. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. \((D_1)\) and \((D_2)\) are the doses of phytochemical/fruit extracts in the combination system; \((D_x)_1\) and \((D_x)_2\) are the doses of compound 1 and compound 2 (fruit 1 and fruit 2) alone, respectively.

5.2.7. Experiment Design

The experiment of different phytochemical/fruit combinations for QR induction was designed in Table 4.1. Firstly, the CD value of each phytochemical/fruit extract was determined. Based on each CD value, a series of concentrations were designed as 0.125×CD, 0.25×CD, 0.5×CD, 0.75×CD, 1.0×CD, 1.25×CD, and 1.5×CD. Finally, different dose from phytochemical/fruit extract alone would be combined together to induce QR activity. The generated CD value was used as EC\(_{50}\) to design series of combined concentrations.
5.2.8. Statistical Analysis

Statistical analysis was conducted using Minitab Student Release 12 (Minitab Inc., State College, PA) and SigmaStat Version 8.0 (Jandel Corp., San Raphael, CA). Results were subjected to ANOVA, and differences between means were located using Turkey’s multiple comparison test. Significance was determined at $p < 0.05$.

5.3. Results

5.3.1. QR Induction Capacity for Single Phytochemical and Fruit Extract

Three phytochemicals commonly present in fruits and vegetables and three fruit extracts were in vitro examined for their potential to induce QR, a representative phase II chemoprotective enzyme. BNF with an induction of $7.5 \pm 0.5$-fold over solvent control at a concentration of 1 $\mu$M and with a CD value of $0.027 \pm 0.003$ $\mu$M was used as a positive control. Three phytochemicals and three fresh fruit extracts induced QR in Hepa1c1c7 cells are investigated in Figure 5.1, and Figure 5.2, respectively. Quercetin at a concentration of 30 $\mu$M resulted in a maximum of $3.5 \pm 0.2$-fold induction over control of QR activity in Hepa1c1c7 cells. At a concentration of 50 $\mu$M, genistein caused a maximum of $2.8 \pm 0.2$-fold induction over control. Resveratrol led to a maximum of $2.2 \pm 0.1$-fold induction over control at a concentration of 50 $\mu$M. The CD values for quercetin, genistein, and resveratrol were $2.5 \pm 0.5$, $15.0 \pm 3.0$, and $28.7 \pm 2.3$ $\mu$M, respectively. The calculated CD values of apple, cranberry, and grape were $1.84 \pm 0.2$, $1.95 \pm 0.2$, and $1.72 \pm 0.1$ mg/mL, respectively. Compared to the solvent-treated control cells, at 2 mg/mL concentration, the induction of apple, cranberry, and grape were $2.3 \pm 0.2$, $2.0 \pm 0.4$, and $2.44 \pm 0.3$, respectively.
5.3.2. Single Phytochemical and Fruit Extract Parameters

The dose-effect relationships of three phytochemicals and three fruit extracts were subject to the median-effect plot to determine their potency ($EC_{50}$), shape ($m$), and conformity ($r$) in induction of Hepa1c1c7 cells in vitro. The pooled results are summarized in Table 5.1. The $EC_{50}$ and $m$ values for single phytochemical/fruit extract and for their combination mixtures were used for calculating synergism, additive, or antagonism based on the CI equation.

5.3.3. Two- and Three-Way Combinations Among Phytochemicals

Among three compounds, three pairs of two-way combinations and one pair of three-way combination were investigated. The average median effect dose ($EC_{50}$) and CI values at 50% induction rate are presented in Table 5.2. The dose-effect curves of different combination are plotted in Figure 5.3. The dose-effect curve of QR induction activity is shifted to the left after the combination. Compared with the single compound, the $EC_{50}$ concentration in induction of QR dramatically decreases among four pairs of combinations. For example, the $EC_{50}$ of quercetin and genistein on combination is 2.07- and 2.86-fold, respectively, lower than the $EC_{50}$ of each compound alone. The $EC_{50}$ of quercetin, genistein, and resveratrol after combination is 3.05-, 4.20-, and 4.77-fold, respectively, lower than the $EC_{50}$ of each compound alone, suggesting synergistic effect for the combination of the three compounds. It has been demonstrated that all combinations among three phytochemicals showed moderate synergistic effect on induction of QR at 50% induction rate, being indicated by a CI value less than 1.
Table 5.1. Dose-effect relationship parameters of phytochemicals and fruit extracts in induction of Hepa1c1c7 cells *in vitro*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hepa1c1c7</th>
<th>Extract</th>
<th>Hepa1c1c7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$EC_{50}$ (μM)</td>
<td>m</td>
<td>r</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.468±0.368</td>
<td>0.381±0.055</td>
<td>0.957±0.023</td>
</tr>
<tr>
<td>Genistein</td>
<td>20.473±3.109</td>
<td>0.572±0.054</td>
<td>0.886±0.030</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>44.886±2.851</td>
<td>0.541±0.031</td>
<td>0.835±0.029</td>
</tr>
</tbody>
</table>

*The parameters $EC_{50}$, m, and r are antilog of x-intercept, slope, and the linear correlation coefficient of median-effect plot, which signifies the potency ($EC_{50}$), the shape of the dose-effect curve, and conformity of the data to the mass-action law, respectively; n is the number of sets of dose-effect relationship experiments tested (Chou et al., 1994).

Table 5.2. Summary of medium effect dose based on induction of quinone reductase by phytochemical combination in Hepa1c1c7 cells *in vitro*.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>$EC_{50}$ (μmol)</th>
<th>CI at 50% Induction Rate</th>
<th>Combination Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>2.47 ± 0.37</td>
<td>1.19 ± 0.01</td>
<td>Synergism</td>
</tr>
<tr>
<td>(Single)</td>
<td>20.47 ± 3.11</td>
<td>7.15 ± 0.55</td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>2.47 ± 0.37</td>
<td>1.21 ± 0.05</td>
<td>Synergism</td>
</tr>
<tr>
<td>(Resveratrol)</td>
<td>44.89 ± 2.85</td>
<td>14.50 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>Quercetin (Combination)</td>
<td>1.19 ± 0.01</td>
<td>0.850 ± 0.07</td>
<td>Synergism</td>
</tr>
<tr>
<td>Genistein (Combination)</td>
<td>7.15 ± 0.55</td>
<td>0.879 ± 0.04</td>
<td>Synergism</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>6.39 ± 0.90</td>
<td>0.651 ± 0.10</td>
<td>Synergism</td>
</tr>
<tr>
<td>(Single)</td>
<td>12.78 ± 1.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin (Combination)</td>
<td>0.81 ± 0.07</td>
<td>0.812 ± 0.10</td>
<td>Synergism</td>
</tr>
<tr>
<td>Genistein (Combination)</td>
<td>4.89 ± 0.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resveratrol</td>
<td>9.40 ± 0.28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1. Effect of β-naphthoflavone (positive control) and selected phytochemicals (mean ± SD) in induction of quinone reductase in Hepa1c1c7 cells.
Figure 5.2. Effect of β-naphthoflavone (positive control) and fruit extracts (mean ± SD) in induction of quinone reductase in Hepa1c1c7 cells.
Figure 5.3. The dose-effect curves for phytochemical combinations: (a) quercetin + genistein; (b) quercetin + resveratrol; (c) genistein + resveratrol; (d) quercetin + genistein + resveratrol.
Figure 5.3 (Continued)

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- **Figure c**: Graph showing the effect of different doses on Genistein/RSV. The graph includes data points and trend lines for Genistein and RSV.

- **Figure d**: Graph showing the effect of different doses on Quercetin/Genistein/RSV. The graph includes data points and trend lines for Quercetin, RSV, and Genistein.
5.3.4. Two-, Three-, and Four-Way Combinations of Phytochemicals and Grape Extract

The results of combinations between Cabernet Franc grape variety and three phytochemicals are presented in Table 5.3 and Figure 5.4. The combination of Cabernet Franc and resveratrol showed superior synergistic effects with the lowest CI value (0.310 ± 0.04) among all combinations. Also, Cabernet Franc and quercetin combination yielded a synergy at 50% induction rate for QR. However, the combination of grape and genistein yielded antagonistic effect with CI value of 1.97 ± 0.23. The EC$_{50}$ of Cabernet Franc and genistein after combination is 0.954- and 1.15-fold, respectively, similar to the EC$_{50}$ of Cabernet Franc and quercetin alone, indicating an antagonistic effect. The three- and four-way combinations had a slight synergistic effect with CI values of 0.948 ± 0.03, and 0.933 ± 0.11, respectively. The EC$_{50}$ of Cabernet Franc and quercetin after combination is 3.67- and 3.23-fold, respectively, lower than the EC$_{50}$ of each one alone, suggesting synergistic effect after the combination.

5.3.5. Two- and Three-Way Combinations Among Fruit Extracts

The combinations among three fruit extracts are summarized in Table 5.4 and Figure 5.5. With different extent, all combinations of three fruit extracts showed synergistic effect at 50% induction rate on QR activity. The EC$_{50}$ of apple and cranberry after combination is 1.48- and 3.44-fold, respectively, lower than the EC$_{50}$ of each fruit alone. The EC$_{50}$ of apple, cranberry, and grape after combination is 2.92-, 6.70-, and 3.51-fold, respectively, lower than the EC$_{50}$ of each fruit alone, suggesting synergistic effect after the combination of the three fruit extracts. Based on CI value,
cranberry and grape combination yielded a synergistic effect. A moderate synergy was observed between apple and grape, among apple, cranberry, and grape. Apple and cranberry combination showed a slight synergistic effect.

5.4. Discussion

Compelling evidence has indicated that frequent consumption of fruits and vegetables in the daily diet provide health benefits. Previous work from our lab has demonstrated that phytochemicals from single fruit have potent antioxidant and antiproliferative activities, and that antioxidant activity is positively correlated with total phenolic and flavonoid contents (Yang et al., 2004). Research performed by our group and others has shown that phytochemical extracts from fruits and vegetable exhibit strong antioxidant and antiproliferative activities and that the major part of total antioxidant activity is from the combination of phytochemicals (Liu, 2004). Induction of detoxification enzymes via phytochemicals is another mechanism proposed for anticancer activity in fruits and vegetables. Data in our study clearly showed that three compounds and three fruit extracts could induce QR activity. Our results further demonstrated that all combinations among three phytochemicals showed synergistic effect on induction of QR at 50% induction rate, being indicated by CI of less than 1. Among combinations of three phytochemicals and Cabernet Franc grape, except the 2-way combination of Cabernet Franc and genistein, all other combinations demonstrated synergism. It was proven that all combinations of apple, cranberry, and grape fruit extracts had synergistic effect at 50% induction rate on QR activity. The additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent QR induction activity.
Table 5.3. Summary of medium effect dose based on induction of quinone reductase by grape extract and phytochemical combination in Hepa1c1c7 cells *in vitro*.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/mL)</th>
<th>CI at 50% Induction Rate</th>
<th>Combination Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single</td>
<td>Combination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-way</td>
<td>Cabernet Franc Quercetin</td>
<td>2.28 ± 0.40 0.62 ± 0.07</td>
<td>0.630 ± 0.08</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>4.26 ± 0.70     1.32 ± 0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cabernet Franc Genistein</td>
<td>2.28 ± 0.40 2.39 ± 0.26</td>
<td>1.97 ± 0.23</td>
<td>Antagonism</td>
</tr>
<tr>
<td></td>
<td>27.67 ± 4.20    24.15 ± 2.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cabernet Franc Resveratrol</td>
<td>2.28 ± 0.40 0.35 ± 0.05</td>
<td>0.310 ± 0.04</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>51.22 ± 3.30    5.98 ± 0.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-way</td>
<td>Cabernet Franc Quercetin</td>
<td>2.28 ± 0.40 0.67 ± 0.02</td>
<td>0.948 ± 0.03</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>4.26 ± 0.70 1.45 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51.22 ± 3.30    11.53 ± 0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-way</td>
<td>Cabernet Franc Quercetin</td>
<td>2.28 ± 0.40 0.53 ± 0.06</td>
<td>0.933 ± 0.11</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>Genistein</td>
<td>4.26 ± 0.70 1.13 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Resveratrol</td>
<td>27.67 ± 4.20 5.30 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>51.22 ± 3.30 8.98 ± 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.4. Summary of medium effect dose based on induction of quinone reductase by apple, cranberry, and grape combinations in Hepa1c1c7 cells *in vitro*.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>EC₅₀</th>
<th>EC₅₀ (mg/mL)</th>
<th>CI at 50% Induction Rate</th>
<th>Combination Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀</td>
<td>Single</td>
<td>Combination</td>
<td></td>
</tr>
<tr>
<td>2-way Apple Cranberry</td>
<td>2.37 ± 0.21</td>
<td>1.60 ± 0.43</td>
<td>0.930 ± 0.25</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>2.48 ± 0.41</td>
<td>0.72 ± 0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apple Grape</td>
<td>2.37 ± 0.21</td>
<td>0.74 ± 0.08</td>
<td>0.775 ± 0.09</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>2.28 ± 0.40</td>
<td>0.93 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cranberry Grape</td>
<td>2.48 ± 0.41</td>
<td>0.46 ± 0.04</td>
<td>0.535 ± 0.06</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>2.28 ± 0.40</td>
<td>0.88 ± 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-way Apple Cranberry Grape</td>
<td>2.37 ± 0.21</td>
<td>0.81 ± 0.05</td>
<td>0.786 ± 0.05</td>
<td>Synergism</td>
</tr>
<tr>
<td></td>
<td>2.48 ± 0.41</td>
<td>0.37 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.28 ± 0.40</td>
<td>0.65 ± 0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.4. The dose-effect curves for phytochemical and grape combinations: (a) quercetin + grape; (b) genistein + grape; (c) resveratrol + grape; (d) quercetin + resveratrol + grape; (e) quercetin + genistein + resveratrol + grape.
Figure 5.4 (Continued)
Figure 5.4 (Continued)
Figure 5.5. The dose-effect curves for fruit combinations: (a) apple + cranberry; (b) apple + grape; (c) cranberry + grape; (d) apple + cranberry + grape.
Accumulation of ROS and electrophiles under adverse conditions are known to cause cellular membrane, proteins, lipids, and DNA damage (Ames et al., 1993), mutagenicity, degeneration of tissues, apoptotic cell death, premature aging, cellular transformation, and being further implicated in chronic diseases such as cancer and CHD (Ward, 1994; Breen and Murphy, 1995). Exogenous antioxidants from diet have been used for the preventive intervention of ROS disorders (Willett, 1994; Temple, 2000). Another strategy for protecting against ROS injury may be via chemically mediated upregulation of endogenous antioxidants and phase II enzymes in cells. The latter depends on a understanding of the chemical inducibility of antioxidants and phase II enzymes, as well as the underlying signaling transduction mechanisms. The initial response of cell to ROS and electrophiles are the activation of endogenous defense mechanisms that lead to coordinated activation of a battery of defensive genes that protect cells against oxidative/electrophilic stress (Jaiswal, 2000), which leads to neutralizing the adverse effects and cell survival. Nrf2 has been demonstrated to be a critical transcription factor that binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase II enzymes in animals and human cells and tissues (Kwak et al., 2004; Lee and Johnson, 2004; Kobayashi and Yamamoto, 2005; Zhu et al., 2005). Therefore, it is of great interest in studying both exogenous antioxidants from fruits and vegetables and endogenous antioxidants and phase II enzymes induced by phytochemicals.

Studies from our lab and others have proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruit and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (Liu, 2003; Liu, 2004). For instance, the vitamin C in apples with skin accounts
for only 0.4% of the total antioxidant activity, suggesting that most of the antioxidant activity of fruit and vegetables may come from phenolics and flavonoids in apples (Eberhardt et al., 2000). Over 5,000 phytochemicals have been identified, but a large portion still remain unknown (Shahidi and Naczk, 1995) and need to be identified before their health benefits are fully understood. The combinations of phytochemicals in the whole vegetable may provide additional protection through the synergistic induction of chemoprotective enzymes, suggesting that the health benefits of whole vegetables may be greater than those of the isolated compounds (Jeffery and Stewart, 2004). The synergistic upregulation of phase II enzymes by glucosinolate breakdown products in cruciferous vegetables has been reported in animal models. Four glucosinolate derivatives were examined individually and as a mixture for their effects on QR, hepatic P4501A (CYP1A), GST, and glutathione reductase (G-Rd) levels (Staack et al., 1998). Indole-3-carbinol (I3C, 56 mg/kg) and crambene (50 mg/kg) had a 1.9- and 2.5-fold increase in QR, respectively, in adult male F344 rats. It was observed that I3C and crambene combination produced a synergistic effect on induction of QR and GST. Furthermore, this lab tested whether the synergism is at the level of transcription. It was found that, after evaluating three subunits, GST Ya2 mRNA had a synergistic upregulation by crambene and I3C, while Yc1 and Yc2 showed only an additive response in adult male rats (Nho and Jeffery, 2001). Lastly, it was found that a nitrile product of glucosinolate hydrolysis induced QR mRNA levels and triggered the ARE, suggesting synergistic upregulation of QR is due to co-activation of the ARE and the xenobiotic response element (XRE) by I3C acid condensates and crambene (Nho and Jeffery, 2004). It was proposed that crambene and sulforaphane, considered as a monofunctional inducer to increase phase II enzyme activity, transcriptionally upregulate phase II enzymes through triggering the ARE-mediated mechanism. The two compounds disrupt the interaction between
transcription factor Nrf2 and the sequestering protein Keap1; leading to Nrf2 to
translocate to the nucleus and interact with the ARE, resulting in elevated transcription
of target genes (Itoh et al., 2003).

Based on the principle of the mass-action law via mathematical induction and
deduction in enzyme kinetic models, the median-effect equation and the CI method
have been extensively applied in drug combinations (Chou and Talalay, 1977; Chou,
1991; Chou et al., 1994). Our study was carried out by quantitative analysis of
synergism or antagonism at different phytochemical/fruit doses and different effect
levels. From our previous study, quercetin, genistein, and resveratrol exhibited strong
induction activity of QR among eighteen tested phytochemicals. Among all thirteen
grape varieties analyzed, Cabernet Franc contained the highest induction activity of
QR. The present study proved that Red Delicious apple and Early Black cranberry had
QR induction capacity. Apple and cranberry had similar CD values (1.84 ± 0.2, 1.95 ±
0.2 mg/mL, respectively), and grape showed a lower CD value (1.72 ± 0.1 mg/mL)
than apple and cranberry, suggesting more potential QR induction capacity. One
question which remains is how to determine a series of combined doses in the
experiment. Theoretically, the combined concentration of each phytochemical/fruit
was based on the generated CD value through dose-response curve. After obtaining
the CD value, a series of doses were designed from 0.125 × CD to 1.5 × CD; then
these doses were used to treat cells to produce responses. From the new dose-response
curve the CD value (EC$_{50}$) was then determined again. However, there were some
differences in CD values. For example, the former CD values of apple, cranberry, and
grape were 1.84 ± 0.2, 1.95 ± 0.2, 1.72 ± 0.1 mg/mL, respectively; the latter CD
values of apple, cranberry, and grape were 2.37 ± 0.21, 2.48 ± 0.41, and 2.28 ± 0.40
mg/mL, respectively. These differences in potency between treatments in vitro could
be due to differences in bioavailability and/or cell variation. Compared with EC\textsubscript{50} values in induction of QR before and after combinations, it was found that there was a 4.77-fold difference in EC\textsubscript{50} value in the quercetin, genistein, and resveratrol combination. A 5.70-fold difference in EC\textsubscript{50} of resveratrol was found between combination and single compound in Cabernet Franc, quercetin, genistein, and resveratrol combination. The EC\textsubscript{50} of cranberry lowers 6.70-fold, compared with single fruit, in apple, cranberry, and grape combination. However, an antagonistic effect was observed in Cabernet Franc and genistein combination since the change of EC\textsubscript{50} values is small before and after combination.

Our study demonstrated that there was a synergistic effect in phytochemicals and fruits in the induction of QR. However, the current studies strengthen the quantitative results of phytochemical/fruit combinations rather than the mechanism of synergistic and antagonistic interactions. Future research should be focused on understanding the mechanism of actions related to these interactions.
CHAPTER SIX

COMBINATION EFFECT OF APPLE EXTRACTS AND QUERCETIN 3-β-D-GLUCOSIDE ON ANTIPROLIFERATIVE ACTIVITY IN MCF-7 HUMAN BREAST CANCER CELLS IN VITRO

6.1. Introduction

Breast cancer is the most frequently diagnosed cancer in women. Approximately 1 million women are estimated to be newly diagnosed with breast cancer each year worldwide. The updated data in 2007 report that an expected 178,480 new cases of invasive breast cancer are expected to occur among women in the US. In addition to invasive breast cancer, 62,030 new cases in situ breast cancer is expected to occur among women in 2007. The estimated women deaths are 40,460 (American Cancer Society, 2007). Although a great deal of work has been done in prevention and treatment in breast cancer, the results are not satisfied. For example, one drug, Tamoxifen, has been shown to be effective in only one-third of the breast cancer patient (Forbes, 1997). Therefore, exploring for new approach for the prevention and treatment of breast cancer is of great interest.

One alternative strategy to reduce the risk of cancer is through dietary modification. It has been estimated that a healthy diet could prevent approximately 30% of all cancers (Doll and Peto, 1981; Willett, 1995). Numerous epidemiological and animal studies revealed what appeared to be a strong link between intake of fruits and vegetables and protection against cancer (Block et al., 1992). The Nurses’ Health Study reported a significantly reduced risk of premenopausal women with breast cancer in their families who consumed high quantities of β- carotene-rich fruit and
vegetables (Zhang et al., 1999). The case-control study from Sweden showed a significantly lowered breast cancer risk ratio at the highest consumption of brassica vegetables (Terry et al., 2001). In another case-control study of women in Shanghai, pre-menopausal women who ate more dark yellow-orange vegetables and more citrus fruits tended to be associated with lowered risk of breast cancer (Malin et al., 2003). Five of the eight cohort studies showed an inverse relationship between intake of fruits and vegetables and survival of breast cancer patient, with a 20–90% reduction in death risk (Rock and Demark-Wahnefried, 2002).

Apples are widely and commonly consumed and are the main contributors of phytochemicals including phenolics and flavonoids in human diet both in the US and in Europe (Boyer and Liu, 2004). In the United States, apples are attributed to 22% of the total phenolics consumed from fruits, making them the largest source of phenolics (Vinson et al., 2001). In Finland, apples and onions are major sources of dietary flavonoids, while in the Netherlands apples rank third behind tea and onions as top sources of flavonoids (Hertog et al., 1993; Knekt et al., 1997). Apples are rich in hydroxycinnamic acids, dihydrochalcones, flavan-3-ols/procyanidins, anthocyanins, and flavonols (Oleszek et al., 1988; Pefes-Ilzarbe et al., 1991). The sugar moieties involved in glycosylation are galactose, glucose, rhamnose, xylose, arabinose, and rutinose (McRae et al., 1990). Quercetin 3-glycosides, chlorogenic acid, catechin, epicatechin and their dimers, phloridzin, and cyanidin 3-glycosides are the main individual phenolics in apple (Oleszek et al., 1988; McRae et al., 1990; Awad et al., 2000). However, the amount of quercetin 3-glucodise was reported to be low in apple (Tsao et al., 2003; Kahle et al., 2005).
The frequent consumption of fruits, including apple, is associated with lowered risk of chronic diseases. It appears that apples were linked to lowered risk of cancer (Le Marchand et al., 2000), coronary heart disease (Arts et al., 2000), asthma and pulmonary function (Tabak et al., 2001), and type II diabetes (Knekt et al., 2002) when compared to other fruits and vegetables and other sources of flavonoids. Recently, a study focuses on the investigation of apple intake and the risk of different cancers in Italy (Gallus et al., 2005). It was found that there is a consistent inverse relationship between apples and risk of various cancers such as breast and prostate cancers. Animal and \textit{in vitro} studies have also exhibited that phytochemicals present in apples have strong antioxidant activity (Eberhardt et al., 2000), antiproliferative activity (Wolfe et al., 2003), inhibition of lipid oxidation both in humans and rats (Mayer et al., 2001), and Cholesterol-lowering effects (Aprikian et al., 2001). Our group reported that, corresponding to doses in human consumption of one, three, and six apples a day, the whole apple extracts prevent mammary cancer in a dose-dependent manner in rat model (Liu et al., 2005).

Synergism by two or more compounds is defined as therapeutic effects that are greater than those expected from addition of the effects of the individual compounds. It is believed that chemotherapeutic combination approaches have been used to reduce drug toxicity, and delay the development of cancer cells, and to reach a greater effect than with one active drug alone. Fruits and vegetables are rich in phenolics and other bioactive compounds, which have been suggested to be responsible for their health benefits. The antioxidant synergism was observed in different experiments such as vitamin E and C (Scarpa et al., 1984), vitamin E and β-carotene (Palozza and Krinsky, 1992), catechin and malvidin 3-glucoside (Rossetto et al., 2002), flavonoids and urate (Filipe et al., 2001), and tea polyphenols and vitamin E (Zhou et al., 2000). In addition
to antioxidant activities, phenolics have demonstrated other specific biological activities interfering with cellular mechanisms. For example, (Conte et al., 2003) reported synergistic protection of PC12 cells from β-amyloid toxicity by reservatrol and catechin.

The phenolics in fruits and vegetables may act independently or in combination as anti-cancer agents. It has been postulated that combinations of fruits have greater antioxidant activities than would be expected on the basis of their individual effects. We have proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities and that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (Liu, 2003; Liu, 2004). This hypothesis partially explains why single antioxidant cannot replace the combination of natural phytochemicals in fruits and vegetables in achieving health benefits. Only limited knowledge is available about any interaction between/among phenolics in suppressing MCF-7 cell proliferation. There is no direct evidence linked to synergistic, additional, and antagonistic effects on inhibition of cell proliferation in apples. In order to elucidate the hypothesis that the combination of phytochemicals is responsible for the health benefit in apples, two-way combination of apple extracts plus Q3G was designed. The objective for this study was to determine whether the apple extracts in combination with Q3G has additive and/or synergistic effect on MCF-7 human breast cancer cell proliferation.
6.2. Materials and Methods

6.2.1. Chemicals and Reagents

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium (MTS), Quercetin 3-β-D-glucoside (Q3G), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals used in this study were of analytical grade.

6.2.2. Apple Preparation

Apples of Red Delicious variety were obtained from Cornell Orchard (Cornell University, Ithaca, NY). Fruits were cleaned and dried before extraction of phytochemicals. All data collected were reported as means ± SD for at least three replications.

6.2.3. Apple Extraction

Apple phenolics were extracted using a method reported previously in our laboratory (Sun et al., 2002; Yang et al., 2004). Briefly, 100 g of apples were blended for 3 min in 80% acetone (1:2 w/v) using a chilled Waring blender. The sample was then homogenized in a Virtis High Speed Homogenizer for 3 min and filtered with vacuum through a No. 2 Whatman filter paper. The filtrate was evaporated using a rotary evaporator at 45°C until approximately 90% of the filtrate had been evaporated. The sample was then recovered with water to a final volume of 50 mL, and stored at –40°C until use.
6.2.4. Measurement of Cytotoxicity

Cell cytotoxicity was assessed by methylene blue assay. Briefly, MCF-7 human breast cancer cells (The American Type Culture Collection, ATCC, Rockville, MD) were plated at a density of $4 \times 10^4$ cells/well into 96-well plate for 24 hours. Then cells were treated with different concentrations of tested apple extracts / phytochemicals for 24 hours. The growth media was removed, and the wells were rinsed with PBS. 50 μL of Hanks Balanced Salt Solution (HBSS: 1.25% glutaraldehyde and 0.6% methylene blue) was added into each well to fix and stain the cells for one hour. The 96-well plate was rinsed several times by immersion in Milli-Q water after removing HBSS. At the end of treatment, 100 μL of elution solution (PBS plus 50% of ethanol and 1% of acetic acid) was added, and cells were incubated for a further half hour with gentle rotation at room temperature. Cytotoxicity was obtained by scanning at 570 nm using a MRX Microplate Reader (Dynex Technologies, Chantilly, VA).

6.2.5. Measurement of Inhibition of MCF-7 Cell Proliferation

The antiproliferative activity of apple extracts/phytochemicals was assessed by measurement of the inhibition of MCF-7 cell proliferation. Antiproliferative activities were determined by the colorimetric MTS assay (MTS-based cell titer 96 nonradioactivity cell proliferation assay) (Promega, Madison, WI) reported previously (Yang et al., 2004). MCF-7 cells were maintained in Alpha Minimum Essentail Medium (MEM-a), containing 10 mM Hepes, 0.01 mg/mL insulin, 50 units/mL penicillin, 50μg/mL streptomycin, 100 μg/mL gentamicin, and 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, NY). MCF-7 cells were maintained in a 5% CO$_2$/37°C incubator. A total of $2.5 \times 10^4$ MCF-7 cells in growth media were placed in each well of a 96-well flat-bottom plate. Cell proliferation was measured by
the ability of viable cells to reduce MTS to formazan. After 4 h of incubation, the
growth medium was removed and media containing various concentrations of apple
extracts (10, 30, 50, 75, 100, and 125 mg/mL) or Q3G (10, 20, 30, 40, 50, 60, and 70
μM) were added to the cells. Control cultures received the extraction solution minus
the apple extracts, and blank wells contained 100 μL of growth medium without cells.
Cell proliferation (percent) was determined at 96 h from the MTS absorbance (490
nm) reading for each concentration compared to the control, using at least three
replications for each sample. The effective median dose (EC\text{50}) was determined and
expressed as milligrams of apple per milliliter ± SD or micromole of phytochemical ±
SD.

6.2.6. Experiment Design

The two-way combination of apple extracts and Q3G for cell proliferation was
designed. The EC\text{50} value of apple extracts and phytochemical were determined firstly.
The EC\text{50} value of apple extracts was 70.69 ± 5.73 mg/mL. The EC\text{50} value of Q3G
was 46.43 ± 1.28 μM, respectively. Based on each EC\text{50} value, a series of
concentrations were designed. For apple extracts, the combined concentrations are
0.125×EC\text{50}, 0.25×EC\text{50}, 0.5×EC\text{50}, 0.75×EC\text{50}, 1.0×EC\text{50}, and 1.25×EC\text{50}. For Q3G,
the combined concentrations are 0.125×(EC\text{50})/2, 0.25×(EC\text{50})/2, 0.5×(EC\text{50})/2,
0.75×(EC\text{50})/2, 1.0×(EC\text{50})/2, and 1.25×(EC\text{50})/2. The different apple and
phytochemical concentrations were combined together to generate the dose-response
curve in MCF-7 cell proliferation model.

6.2.7. Median-Effect Principle for Dose-Effect Analysis

The dose-effect analysis was modified from Chou and Talalay (Chou, 1976;
Chou and Talalay, 1984; Chou, 1991). The median-effect principle was used to
calculate individual and combined apple extracts/phytochemicals effects. Dose-effect curves for apple extracts/phytochemicals and their combinations with series diluted concentrations were plotted by using the median-effect equation as follow:

$$f_a = \frac{1}{1+(D_m/D)^m}$$  \hspace{1cm} (1)

Where D is the dose, Dm is the dose required for 50% inhibition effect, which is equivalent to median effect dose (EC50), fa is the fraction affected by dose D, and m is a coefficient of the sigmoidicity of the dose-effect curve.

**The Medium-Effect Plot.** The medium-effect plot is based on the logarithmic form of Chou’s median-effect equation (Chou, 1976; Chou, 1980):

$$\log(f_u / f_a) = m \log(D) - m \log(D_m)$$  \hspace{1cm} (2)

Where fu is the fraction unaffected, \( f_a = 1 - f_u \).

**6.2.8. Combination Index for Determining Addition, Synergism and Antagonism**

The combination index (CI) (Chou, 1991; Chou et al., 1994) has been used for data analysis of two-way combinations as follow:

$$CI = \frac{(D_1)}{(D_x)_1} + \frac{(D_2)}{(D_x)_2}$$  \hspace{1cm} (3)

Where (D1) and (D2) are the doses of apple extracts and Q3G, respectively, in the combination system; (Dx)1 and (Dx)2 are the doses of apple and Q3G alone, respectively. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.
6.2.9. Statistical Analysis

Statistical analysis was performed using Minitab Student Release 12 (Minitab Inc., State College, PA) and SigmaStat Version 8.0 (Jandel Corp., San Raphael, CA). Results were subjected to ANOVA, and differences between means were located using Tukey’s multiple comparison test. Significance was determined at $p<0.05$. All data were reported as the mean ± SD of three replications.

6.3. Results

The antiproliferative activities of apple extracts, Q3G, and two-way combination of those two agents (apple extracts + Q3G) toward the growth of MCF-7 human breast cancer cells in vitro are shown in Figures 6.1a. Apple extracts inhibited the MCF-7 cell proliferation at the doses of 30-125 mg/mL ($p<0.05$) in a dose-dependent manner. No cytotoxicity was found in apple extracts at the concentration equal to or lower than 125 mg/mL (Figure 6.1b). The EC$_{50}$ value of apple extracts inhibiting MCF-7 cell proliferation was 70.7 ± 5.7 mg/mL (Table 6.1). Q3G exhibited significant antiproliferative activity against MCF-7 cells at the doses 20-60 μM ($p<0.05$) in a dose-dependent manner (Figure 6.1a). The EC$_{50}$ value of Q3G in inhibiting MCF-7 cell growth was 46.4 ± 1.3 μM. No cytotoxicity was found at the doses tested above in MCF-7 cells (Figure 6.1b).

Two-way combination of apple extracts and Q3G significantly increased antiproliferative activity toward the growth of MCF-7 human breast cancer cells in vitro when compared to the apple extracts and Q3G alone (Figure 6.1a). The EC$_{50}$ values of apple extracts and Q3G in the two-way combination were reduced to 33.8 ± 2.9 mg/mL and 10.8 ± 2.1 μM, respectively (Table 6.1), which were 2-fold and 4-fold
lower than that of apple extracts and Q3G alone. No cytotoxicity was observed in the two-way combination of apple extracts and Q3G at all concentrations tested in MCF-7 cells (Figure 6.1b).

In order to quantify the combination effects of apple extracts and Q3G, dose-reduction index (DRI) and combination index (CI) were calculated based on the method reported previously (Chou et al., 1994). The DRI values at the inhibition of 50, 75, 90, and 95% of MCF-7 cell growth are presented in Table 6.2. The DRI value of apple extracts and Q3G at the 50% of inhibition of MCF-7 cell growth were 2.03 ± 0.55 and 4.28 ± 0.39 - fold when compared to the values of apple extracts and Q3G alone. The DRI value of apple extracts and Q3G at the 95% of inhibition of MCF-7 cell growth were 4.32 ± 1.48 and 6.55 ± 0.66 - fold when compared to the values of apple extracts and Q3G alone. The CI values at the inhibition of 50, 75, 90, and 95% of MCF-7 cell growth were calculated based on the median effect plot (Figure 2; (Chou, 1991; Chou et al., 1994), and presented in Table 6.2. CI<1, CI=1, and CI>1 indicate synergism, additive effect, and antagonism, respectively, as reported previously (Chou et al., 1994). The CI values of the two-way combination of apple extracts and Q3G at 50, 75, 90, and 95% inhibition of MCF-7 cell growth were 0.76 ± 0.16, 0.60 ± 0.12, 0.47 ± 0.10, and 0.42 ± 0.10, respectively, indicating strong synergistic effect at all concentrations tested.
Table 6.1. EC\textsubscript{50} values of apple extracts, Q3G, and apple extracts in combination with Q3G in inhibiting MCF-7 cell growth.

<table>
<thead>
<tr>
<th>Component</th>
<th>EC\textsubscript{50} value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single</td>
</tr>
<tr>
<td>Apple Extracts</td>
<td>70.69 ± 5.73 mg/mL</td>
</tr>
<tr>
<td>Q3G</td>
<td>46.43 ± 1.28 μM</td>
</tr>
</tbody>
</table>

Table 6.2. Computer-stimulated CI and DRI values for apple extracts and Q3G combination at 50%, 75%, 90%, and 95% inhibition of MCF-7 cell growth.

<table>
<thead>
<tr>
<th>Component</th>
<th>DRI* values at inhibition of</th>
<th>CI values at inhibition of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td>Apple Extracts</td>
<td>2.03 ± 0.55</td>
<td>2.69 ± 0.79</td>
</tr>
<tr>
<td>Q3G</td>
<td>4.28 ± 0.39</td>
<td>5.00 ± 0.22</td>
</tr>
</tbody>
</table>

*DRI (dose-reduction index) represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each component alone. All DRI values are calculated on the basis if the classic isobologram equation and assumptions (Chou et al., 1994).
Figure 6.1. Cell proliferation (a) and cytotoxicity (b) of apple extracts, Q3G, and apple extracts plus Q3G combination toward MCF-7 cells. “*” indicates a significant difference from the control ($p < 0.05$).
Figure 6.2. Medium-effect plot for apple extracts and Q3G combination.
6.4. Discussion

Epidemiological studies have consistently shown that the consumption of fruits and vegetables is associated with a lowered risk for developing chronic diseases, such as coronary heart disease (CHD), cancer, diabetes, and Alzheimer’s disease (Block et al., 1992; Ames et al., 1993; Joshipura et al., 2001; Willett, 2002). Breast cancer is the most commonly diagnosed invasive cancer in women in the US and is one of the leading causes of death due to cancer. Compelling evidence indicates that multiple servings of fruits and vegetables in the daily diet provide health benefits. Phytochemicals present in fruits and vegetables, including phenolics and flavonoids, are suggested to be responsible for those health benefits. Studies conducted by our group and others have shown that phytochemical extracts in fruits and vegetables exhibit strong antioxidant and antiproliferative activities and that the major part of total antioxidant activity is from the combination of phytochemicals (Eberhardt et al., 2000; Chu et al., 2002; Sun et al., 2002; Yang et al., 2004). Although there are many therapeutic strategies including chemotherapy to treat clinical breast cancer, the result is not satisfied. There is an urgent need to develop alternative strategy to prevent and treat breast cancer. Dietary modification is a practical approach, applying a combination of non-toxic effective phytochemicals from fruit extracts, which could enhance the efficacy of chemotherapy and lower toxicity to normal cells. Our data here clearly showed that apple extracts in combination with Q3G had a potent synergistic effect on MCF-7 cell proliferation in vitro.

Substantial epidemiological evidence concerning the potential role of antioxidant nutrients in the prevention of cancers has accumulated over the past few decades. However, in some clinical trials, it is observed that antioxidant nutrients taken alone do not explain the observed health benefits of diets rich in fruits and
vegetables (Liu, 2004). In a randomized, double-blind, and placebo-controlled trial of β-carotene in 22,071 male physicians, 12 years of supplementation with β-carotene (50 mg on alternate days) produced neither benefit nor harm in terms of the incidence of malignant neoplasms, CVD, or death from all causes (Hennekens et al., 1996). A multicenter, randomized, double-blind, placebo-controlled primary prevention trial - the β-Carotene and Retinol Efficacy Trial, involving a total of 18,314 smokers, former smokers, and workers exposed to asbestos, was conducted (Omenn et al., 1996). It was found that the combination of β-carotene and vitamin A had no benefit and may have had an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer and CVD in smokers and workers after an average of 4 years of supplementation. Why the single antioxidant approach doesn’t work in clinical trials? The crucial point is whether a purified phytochemical has the same health benefit as does the whole mixture of foods in which the phytochemicals are present. It is hypothesized that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (Liu, 2003; Liu, 2004).

Here we showed that the EC50 values of apple extracts and Q3G in the two-way combination were dramatically reduced, being 2-fold and 4-fold lower than that of apple extracts and Q3G alone, indicating a strong synergistic action. The CI values were smaller than 1 at the doses of 50, 75, 90, and 95% inhibition of MCF-7 cell growth. The DRI is a measure of how much the dose of each component in a synergistic combination may be reduced at a given effect level compared with the doses for each component alone. The DRI is important in clinical situations, where dose-reduction leads to lowered toxicity toward the host while retaining the
therapeutic efficacy. Data here exhibited that the DRI values of apple extracts and Q3G at the 50% of inhibition of MCF-7 cell growth were 2.03 ± 0.55 and 4.28 ± 0.39 -fold when compared to the values of apple extracts and Q3G alone. The DRI value of apple extracts and Q3G at the 95% of inhibition were 4.32 ± 1.48 and 6.55 ± 0.66 -fold when compared to the values of apple extracts and Q3G alone.

The identified key bioactive components and mechanisms that contributed to the health benefits of fruits and vegetables have been drawn much attentions. However, increasing evidence suggested that these components are much more complex in scope, interaction, and magnitude. It is estimated that approximately 8,000 phytochemicals present in whole foods. Those compounds may act on different targets with different mechanisms of action (Liu, 2004). It is believed that phenolics can exert their effects on the different signaling pathways such as mitogen-activated protein kinases (MAPK), activator protein-1 (AP-1), or nuclear factor-κB (NF-κB) either separately or sequentially, as well as possible interaction between/among these pathways, which can offer complementary and overlapping mechanisms of action. Apples are rich in phenolics and Flavonoids. Some of the extensively studied bioactive compounds in apples include catechin, epicatechin, procyanidin, cyanidin-3-galactoside, coumaric acid, chlorogenic acid, gallic acid, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, and phloridzin. (Tsao et al., 2003) have examined the concentrations of the major phenolic compounds in eight apple cultivars. The total phenolics ranged from 101.7 to 235.0 mg/100g of fresh weight in the peel. Procyanidins were present in both the peel (59.7%) and the flesh (55.7%). Ranging from 22.03 to 34.99 mg/100 g in the peels of the eight varieties, Quercetin glycosides accounted for 17.9% of the total phenolics; most are quercetin 3-galactoside, 3-arabinoside, and 3-rhamnoside. The amount of Q3G present in apple is low (Tsao et
al., 2003; Kahle et al., 2005), which may be not enough to cause antiproliferative activity against MCF-7 cells. Our hypothesis is that combination of apple extracts and Q3G will have synergistic effect in inhibiting the growth of MCF-7 cells in vitro. The results reported here clearly supported our hypothesis and showed that the two-way combination of apple extracts and Q3G had strong synergistic effect against MCF-7 cell growth. The synergistic action of apple extracts and Q3G may be of interest in clinical trials for breast carcinoma.

Both synergistic and antagonistic effects against breast cancer cells were reported. Compounds can offer additive or synergistic interaction through against different biochemical targets. It was found that quercetin could enhance the action of carboxamidotriazole (CAD) in human breast carcinoma MDA-MB-435 cells (Yeh et al., 1995). When quercetin and CAD were added to the MDA-MB-435 cells, synergism was observed in isobolograms in growth inhibition and clonogenic assays. The most effective combination was 20 μM quercetin with 4 μM CAD in growth inhibition assay; 30 μM quercetin with 1.2 μM CAD in clonogenic assay. Resveratrol and quercetin additively activate the caspase 3 in human pancreatic carcinoma cells (Mouria et al., 2002), and synergistically induce apoptosis in human leukemia cells (Mertens-Talcott and Percival, 2005). The effect of a combination of quercetin (25 μM) and trans-resveratrol (25 μM) on mitochondrial cytochrome c release and caspase-3 activity was greater than the expected additive response. The synergistic action with those two agents support the concept that the flavonoids (quercetin) and nonflavonoids (trans-resveratrol) act on the membrane permeability transition by distinct pathways, suggesting that the pathways are interactive (Mouria et al., 2002). Studies in cell cycle kinetics, proliferation, and apoptosis (caspase-3 activity) in human leukemia cells (MOLT-4) were conducted after incubation with ellagic acid,
quercetin, and resveratrol as single compounds and in combination. A synergistic interaction with a CI of 0.64 for the combination of ellagic acid and resveratrol, and a CI of 0.68 for quercetin and resveratrol were observed after an isobolographic analysis, indicating that the anticarcinogenic potential of foods containing phenolics may not be based on the effects of individual compounds, but may involve a synergistic enhancement of the anticancer effects (Mertens-Talcott and Percival, 2005).

Here two methods were applied to evaluate additive, synergistic, and antagonistic effects. One way is to examine the shifted pattern of combination in dose-response curve, and then to compare the EC$_{50}$ reduction between individual component and combined components. Another way is to compare generated CI value with 1. For example, in Figure 6.1, the combined concentration was almost saturated in dose-effect curve, which didn’t show cytotoxicity in the maximum combined concentration. The dose-response curve of antiproliferative activity was shifted to the left after combination of apple extracts and Q3G. The EC$_{50}$ of apple extracts and Q3G after combination is $2.03 \pm 0.55$, and $4.28 \pm 0.39$ - fold, respectively, lower than the EC$_{50}$ of apple or Q3G alone, suggesting synergistic effect. Secondly, the CI at 50% inhibition rate in apple extracts and Q3G combination is $0.755 \pm 0.16$, which is less than 1, indicating a synergistic effect (Chou, 1991). The mechanism of action in apple plus Q3G combination might be explained as bioactive compounds in apple extracts tend to increase the antiproliferative activity in MCF-7 cells by suppressing one or more targets of the signal transduction pathway, by stabilizing the Q3G in the system or, by increasing the bioavailability of the Q3G (Hemalswarya and Doble, 2006). In this study, based on individual apple extracts or Q3G’s EC$_{25}$ and/or EC$_{50}$, series concentrations were designed in combination in antiproliferative activities model. This method was proven to work well in our study.
The health benefits of fruits and vegetables are due to the additive and synergistic effects of an array of phytochemicals, rather than to a single compound alone. The balanced natural combination of phytochemicals present in fruits and vegetables cannot simply be mimicked by dietary supplements. The reasons may be due partially to that the purified compounds either loses their bioactivity or may not behave the same way as the compound in whole food. In addition, these compounds differ in molecular size, polarity, and solubility, which may affect their bioavailability and distribution in different subcellular organelles, cells, and tissues and organs. Another possible explanation of the contradictory results between observational studies and randomised trials could be the fact that the doses used in clinical trials were much higher than the maximum levels in whole foods through the dietary consumption which were found to be associated with the lower risk of cancer in observational epidemiological studies. Consumers may gain more significant health benefits from whole foods in their balanced diet than from dietary supplements, which do not contain the same array of balanced, and complex compounds (Liu, 2003; Liu, 2004). Our findings have important implications for combinations of phenolics for cancer prevention. However, further studies are needed to elucidate the underlying mechanisms of combination effects of bioactive components in breast cancer.


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