ABSTRACT

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This study examined chardonnay, muscadine, concord, and ruby red grape seed oil and flour extracts for antioxidant and anti-proliferative properties. The extracts were tested for total phenolic content, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging capacity and effect against HT-29 cancer cell proliferation.

All of the grape seed extracts exhibited $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical quenching activity. The flour extracts from chardonnay demonstrated the highest $ABTS^{\bullet+}$ radical quenching capacity of 186 μmoles trolox equivalents/g and $DPPH^{\bullet}$ radical quenching activity of 30 μmoles trolox equivalents/g. All of the grape seed oil and flour extracts contained significant levels of phenolics. Chardonnay grape seed flour extract displayed the most prominent dose dependent anti-proliferative effect. Muscadine and ruby red, but not concord grape seed flour extracts, also exhibited anti-proliferative activities. The collected data advocate for the potential of grape seed extracts as dietary sources of anti-proliferative and antioxidant components.

ANTIOXIDANT AND ANTI-PROLIFERATIVE PROPERTIES OF SELECTED GRAPE SEED EXTRACTS

By

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Colorectal Cancer

Normal somatic cells function as a kinship, relying on one another for sustainment. Cancer cells can be thought of as a disease of this kinship. Cancer cells function independently and may corrupt neighboring cells. These actions usually evolve from a series of free radical chain reactions (Heinmets 1966). These reactions can be linked to the onset of cancer because damaged cells are unable to perform the necessary repairs before undergoing cell division. In carcinogenesis, cell division is a rapid process (Preston-Martin *et al* 1990). The mechanisms concerned with the development of cancer are best understood by exploring the various components and the relationship between the mechanisms involved.

The National Institute of Cancer estimated that within the United States there were going to be 153, 000 new diagnosed cases of colorectal and approximately 50, 000 deaths due to this type of cancer in 2008. Globally, there are over one million new diagnosed cases annually and nearly one half of those are deadly (Ries *et al* 2007). The highest incidence of colorectal cancer occurs more in developed countries, like the United States. Lower rates of occurrence are found in underdeveloped countries, such as India, and these pockets of incidence can be linked to the environmental risk factors related to the development of colorectal cancer, specifically dietary intake (Potter 1999). However, genetic predisposition is also a risk factor, such as familial adenomatous polyposis (FAP) and hereditary

nonpolyposis colorectal cancer (HNPCC) also referred to as Lynch syndrome (Potter 1999).

Chemopreventative research is a valuable tool in the fight against colorectal cancer. Current research has explored potential preventative methods by consuming fruits and vegetables, frequent physical activity, hormone replacement therapy, and intake of nonsteroidal anti-inflammatory drugs (NSAIDs). In addition to factors contributed by the individual, the development of colorectal cancer has several molecular pathways, both inherited and acquired (Potter 1999).

Molecular Pathways

The molecular pathways related to colorectal cancer are the *APC* (adenomatous polyposis coli)-β-catenin-Tcf (T-cell factor; a transcriptional activator) pathway and the pathway involving abnormalities of DNA mismatch repair, referred to as the hereditary nonpolyposis colorectal cancer, HNPCC. As well, there are two additional pathways: the ulcerative colitis dysplasia-carcinoma sequence pathway and the hypermethylation of the estrogen receptor gene. However, these two mechanisms still need to be further investigated, but are important to include in the discussion of the mechanisms involved in the development of colorectal cancer (Potter 1999).

The *APC*- β -catenin-Tcf pathway proposes that a mutation of *APC,* adenomatosis polyposis coli, or β -catenin will impair the pathway and induce the formation of an adenoma. Colonic luminal agents may cause the adenoma to become malignant, thus generating colorectal cancer.

The mutation of *APC* is one of the first mutations in the progression of colorectal cancer and is found in approximately 80% of all colorectal growths and

cancers (Potter 1999). Due to *APC*'s ability to inhibit tumor cell growth, and its similarities with other tumor suppressor genes, *APC* is regarded as a tumor suppressor gene. The role of *APC* in the progression of colorectal cancer is due to its function in the body: induction of cellular adhesion, regulation of cell migration and systematic cell replication. The ability of *APC* to perform the aforementioned functions is due to its control over the transcriptional profile in cells and the regulation of β-catenin (Goss and Gordon 2000).

The second mechanism of colorectal cancer is the HNPCC pathway. This pathway is more complex than the other three pathways. The initiation step requires the hypermethylation of multiple genes relevant to their tissue location. Based on which genes undergo hypermethylation, the *Cyclin d-p16-Rb* pathway may become inactive. This *Cyclin d-p16-Rb* pathway is involved in the progression of a cell through the cell cycle, an important regulator of cancer cell proliferation (Potter 1999).

The instability of microsatellites, simple sequence repeats of DNA, may also be a result of hypermethylation or a mutation of the inherited mismatch repair (MMR) gene. Microsatellite instability increases the rate of minor DNA changes 1000-fold (Komarova *et al* 2002). The damage caused to microsatellites may lead to a mutation of the acquired mismatch repair genes, BAX gene or the transforming growth factor (TGF) β receptor II gene (Potter 1999). Damage to the BAX gene may result in an inability or loss of apoptosis, while sequence changes to TGF β receptor II may lead to disruption of cell differentiation, apoptosis, and/or cellular homeostasis (Potter 1999).

The third mechanism involves the hypermethylation of the estrogen receptor genes. This action may silence these genes and have a downstream effect of colorectal cancer. The pathway is caused by a reduction of circulating levels of endogenous estrogen. The lower levels of endogenous estrogen are usually related to an increase in age. These low circulating levels will disrupt the estrogen receptor gene response pathways and may eradicate activity. A study conducted by Slaterry *et al* 2001 suggests that estrogen accounts for the differences in frequency of colorectal cancer occurrence between the two genders. It was found that men are more likely to have microsatellite instability at a young age and women at an age where estrogen levels decline. However, the use of hormone replacement therapy lowered the risk of colorectal cancer that was caused by microsatellite damage. In summary, Slaterry *et al* were able to show a relationship between estrogen levels and the ability to reduce the risk of microsatellite instability; therefore estrogen therapy is a potential candidate for colorectal cancer prevention (Slattery *et al* 2001).

Fourth, the ulcerative colitis dysplasia-carcinoma sequence is initiated by the loss of colonic epithelial integrity caused by chronic inflammation in conjunction with a variety of colonical luminal agents. This loss of integrity may lead to a mutation of the *p*53 tumor suppressor gene. Aneuploidy is an abnormal number of chromosomes and the most widely recognized genetic defect in humans. Aneuploidy may create the loss of heterozygosity in the *p*53 tumor suppressor gene, resulting an uncontrolled growth of colonocytes and eventually colorectal cancer. Individuals already suffering from ulcerative colitis increase their risk of colorectal cancer 20-fold. This pathway does not require the formation of polyps and it has been found

that *APC* mutations are not likely to occur. The loss of the p53 tumor suppressor gene is a marker for this mechanism (Ju *et al* 2005).

An underlying theme to all of the mechanisms discussed is the presence of crypt cells. The epithelial layer of the human colon is comprised of one sheet of columnar cells that will move together to create millions of invaginations within the lamina propria. These invaginations, called crypts, may go all the way through to the underlying connective tissue. Crypts are the basic functional unit of the intestine.

A crypt is approximately 50 cells deep and forms the small intestine's villi, which are responsible for providing the large surface area for nutrient absorption. The lowest one third of the crypt houses the primary cells. This location is believed to be a protection mechanism against mutations. Before the cells migrate to the surface, they are no longer replicating, and have already differentiated. These cells may also be at a stage in which they may undergo apoptosis. However, blood-borne carcinogens and germ line mutations may infect the crypt, leading to mutated primary or progenitor daughter cells and potentially colorectal cancer (Humphries and Wright 2008).

As cell division and proliferation progresses, mutations may form. A mutation can lead to clonal conversions, where the daughter cells of a mutated primary or progenitor cell will continue to proliferate, replacing all of the primary or progenitor cells within the crypt with the mutated versions. As these mutated cells proliferate and commit to lineage as one of the four major types of epithelial cells of the colon: the colonocytes (absorption), goblet cells (mucus-secreting), endocrine

cells (hormone-secreting) and Paneth cells, they carry with them the beginnings of colorectal tumor and polyp formation (Humphries and Wright 2008).

Inflammation

Chronic inflammation and irritable bowel disease have been implicated as key players in the development of colorectal cancer. From the onset of colitis, the rate of colorectal cancer incidence increases 0.5-1.0% per year. However, the use of anti-inflammatory medication, (NSAIDS), have been shown to reduce the development of colorectal dysplasia as well as cancer in those with irritable bowel disease.

Patients with irritable bowel disease have an increased rate of epithelial cell turnover. This increased rate of cell turnover amplifies the potential for mutations and the proliferation of mutated DNA caused by inflammation drive perpetuates the carcinogenic process.

The process of cell division and proliferation also play an important role in the promotion of cancer in the colon and small intestine because this type of cell is the most rapidly dividing somatic cells. Colon cancer is more prevalent, about 30 times more so, than cancer of the small intestine (Chadwick *et al* 1992). One of the possible reasons is that there is a substantial increase in the concentration of bacteria between the ileum and the colon, implying that bacteria could be a key player in the progression of colon cancer.

The localized bacteria contain enzymes that can convert the pro-carcinogens taken in from the diet and the environment into carcinogens (Robertson 1993). As well, when bile acids are deconjugated, secondary bile acids are produced and these have been shown to promote tumor formation.

The mechanism of action has been proposed that bile acids act indirectly as tumor promoters through the activation of colonocyte phospholipase activity by degrading lipids, liberating arachidonate and diacyglycerol. These will initiate the activity of protein kinase C. Protein kinase C causes the stimulation of cell proliferation and oxidative bursts within neutrophils. Protein kinase C is involved in receptor desensitization, the alteration of the events that occur in the membrane structure, transcription regulation, immune responses, cell growth, as well as learning and memory. The action of protein kinase C is determined by the phosphorylation of other proteins. However, phosphorylation is substrate protein specific and dependent upon the cell. Therefore, the function of protein C kinase is specific to the cell type and when unregulated can lead to malignant transformations and tumor promotion (Mackay and Twelves 2003). Free radicals may serve as another means of mutations and initiation of colorectal cancer.

Free Radicals and Carcinogenesis

A free radical is a high-energy intermediate that requires an electron to fill an unfilled molecular valence orbital (Halliwell and Gutteridge 1990). Their level of reactivity is at such a high degree that free radicals have a lowered chemical specificity. For this reason, they often "attack" and remove electrons from the molecule in closest proximity. This mechanism renders the oxidized molecule a free radical, and may initiate a chain reaction, as the newly formed free radical will seek stability from a nearby molecule without discrimination. Once radical formation is initiated, it may cascade through a series of propagation events until finally the process terminates. To be more specific, initiation reactions are those that will result

in a net increase in the number of free radicals present. These reactions may involve the formation of free radicals from a stable species or they may involve reactions of free radicals with stable species to form more free radicals. Essentially, initiation forms the precursor free radicals, which will be amplified and modified during propagation. Termination reactions will result in a net decrease in the number of free radicals present.

Despite their negative implications in a variety of human diseases, free radicals are a necessity for the maintenance of biological life. The body uses the majority of free radicals in the immune system or inflammatory reactions. Certain cell types of the body will use free radicals to engulf bacteria or viruses. Free radicals are also necessary for the regulation of vascular tone, regulation of oxygen tension in regards to the control of ventilation and signal transduction from membrane receptors in various physiological processes (Dröge 2002). However, termination, side chain reactions and/or increased radical concentration may result in detrimental biological consequences, such as damage to DNA, which can cause mutations and may impact the potential for malignancy.

Free radicals are abundant and can arise from exogenous or endogenous sources. Exogenous sources of free radicals include ionizing radiation, industrial chemical waste, combustion and various food preparations, such as grilling and barbequing. Auto-oxidation (i.e., superoxide), chronic inflammation or infection (Wolfe and Liu 2007) and oxidative respiratory bursts account for the major source of endogenous free radicals. As to the source that is most influential in the initiation and promotion of cancer in humans, it has yet to be determined (Ames and Gold 1997).

However, there is evidence suggesting the leading human carcinogens may be the metabolites of the gases that comprise air, oxygen and nitrogen. Ironically, though these two gases are essential for the sustainment of living systems they may have devastating effects under certain conditions (Davies 1995).

Collectively, nitrogen metabolites, also termed reactive nitrogen species (RNS) enter the body as nitrates, nitrites proteins, peptides and amino acids. These molecules, through a series of propagation events, generate free radicals metabolites. Most notably in human cancers are peroxynitrate ($ONOO^-$), nitric oxide ($NO^\bullet$) and higher oxides of nitrogen such as nitrous anhydride (N_2O_3) and nitrous acid (HNO_2) (Tamir and Tannenbaum 1996). These reactive species have been linked to nitration, nitrosylation, and deamination of DNA bases, potentially leading to strand breaks and carcinogenic mutations (Feig 1994).

Free radicals produced from oxygen are jointly termed reactive oxygen species (ROS). The susceptibility of oxygen to radical formation is caused by the presence of two unpaired electrons in a separate orbital within oxygen's outer valence shell. The biologically reactive species of oxygen include oxygen radicals, oxidizing agents, and non-radicals that are easily transformed into radicals. Oxygen, as well as nitrogen, produces metabolites that have been linked to human cancers.

ROS have been shown to impact the initiation and promotion stages of carcinogenesis by causing various types of damage to DNA (Dresher and Junod 1996). Often, the damages are not repaired prior to replication, which influence the onset of cancer. Some such alterations include the enzyme-catalyzed methylation of adjacent cytosines, such as the modification of guanine into 8-hydroxyguanine. This

reaction elucidates a link between oxidation and altered methylation patterns, a known precursor to colorectal cancer (Weitzman *et al* 1994). Hydrogen peroxide, a product of superoxide, is able to permeate the cell membrane and displace transcription factors so as to interrupt the nuclear and cytoplasmic transduction pathways. Hydrogen peroxide also initiates lipid peroxidation, mostly resulting in damage to the cell membranes polyunsaturated fatty acids. Additionally, damage to proteins triggers enzyme inhibition, denaturation and protein degradation *(*Nakabeppu *et al* 2006). All of these alterations can promote cancer.

Tumors of the colon and rectum have been linked to the instability of the genome at nucleotide motifs, specifically, mono-, di- and tri-nucleotide repeats (Kunkel 1993). The contraction and extension of these sequences may be caused by the errors that result during replication due to strand slippage. These blunders cannot be corrected because ROS renders repair enzymes dysfunctional (Shibata *et al* 1993). Oxidative species can cause can cause both chemical and conformational changes to DNA. Chemical alterations may interfere with nucleotide motif patterns, thus changing replication as well as inducing differential hydrogen binding specificity. Conformational changes within the DNA template will affect the accuracy of replication (Feig *et al* 1994). DNA base deletions and insertions, for example in the case of the addition of a nitro group to a tyrosine residue that inhibits phosphorylation is a result of a conformational change induced by a radical species. As well, gene expression can become unregulated by the interference of cytosine methylation by ROS/RNS (Weitzman *et al* 1994). Cell proliferation, apoptosis, and differentiation

are also impacted by the actions of ROS/RNS. For example, the gene for avian sarcoma virus 17 is provoked by H_2O_2 (Rao *et al* 1993).

Structurally, ROS may affect DNA in a way that can damage the expression of tumor suppressor genes (Cerutti 1994) and/or enhance the expression of the pro-oncogenes (Jackson 1994) through mutation of base pairs, amplification of sequences, deletions, and insertions. Additionally, ROS can trigger DNA point mutations and chromosomal alterations, which may lead to the expression of a mutated proto-oncogene or reduction in tumor suppressor genes (Cerutti 1994). A tumor suppressor gene, sometimes referred to as an anti-ocogene, protects the cell from the steps involved in the cancer pathway. These include p53, p27 and several others that regulate cell cycle progression.

Through sequential reduction, oxygen produces an abundance of the following oxygen reactive species: superoxide anion and hydroxyl radical. Each of these radicals follows a path of initiation, propagation and termination resulting in species-specific effects.

The superoxide anion, O_2^-, is the product of the reduction of the one-electron deoxygenate. Superoxide has one unpaired electron, which constitutes it as a paramagnetic free radical. This anion has been linked to a number of important cellular mechanisms, such as damage due to oxidative stress (Stohs 1995), tumor promotion (Kozumbo *et al* 2005) and the growth and synthesis of DNA of proto-oncogenes (Irani *et al* 1997).

Although superoxide intracellular levels above 1 nM are lethal, there are several biological functions for superoxide. Phagocytes use superoxide, produced by

the enzyme NADPH oxidase in large quantities, to facilitate the oxygen-dependent elimination system of invading pathogens. Mitochondrial respiration also produces superoxide, but as a byproduct (McCormick *et al* 1998).

While not completely understood, the biological toxicity of superoxide is associated with its ability to inactivate the nearly essential iron-sulfur cluster containing enzymes. This allows for iron to be freed from the cell wall, which can undergo Fenton chemistry, generating the hydroxyl radical. Superoxide is also a mediator of the initiation of the lipid peroxidation of polyunsaturated fatty acids.

The formation of peroxy radicals can also be attributed to the superoxide anion via reactions with carbonyl compounds and halogenated carbons. The conversion of nitric oxide (NO) to form ONOO– is a result of superoxide interactions. Superoxide can also form tyrosine peroxides, which have been implicated to be a part of protein and lipid oxidation at sites of inflammation (McCormick *et al* 1998). Superoxide can also oxidize hemoglobin, forming the non-oxygen carrying met-hemoglobin. Finally, superoxide can oxidize low potential thiols, the functional group of the amino acid cysteine. There is a vast array of the negative implications of superoxide and it is well established that it is the major cause of oxidative stress. As well, superoxide's byproduct, hydrogen peroxide, is involved in oxidative stressed aimed at cell membranes. Hydrogen peroxide anions have been shown to reduce the activity of membrane-bound enzymes, increase membrane rigidity, and alter the activity of membrane receptors and permeability (Nozik-Grayck *et al* 2005).

When hydroxyl radicals react with nucleic acids they can generate strand breaks and mutations. Strand breaks most often occur when the hydroxyl radical damages the sugar portion of the base by reacting with π bonds of DNA bases and the abstract hydrogen from the deoxyribose sugars (Breen and Murphy 1995). If the break is not repaired then the cell may malfunction. However, even if the cell is repaired prior to replication there is a great possibility the original base sequence will be modified. Such modifications can result in 8-hydroxyguanine, 5-hydroxymethyluracil, and thymine glycol. The implications of 8-hydroxyguanine in carcinogenesis suggest that it maybe an initiator by causing polymerase-induced miscoding in newly transcribed DNA strands (Kuchino *et al* 1987). These types of errors have been correlated with tumor formation.

As a result of radical activity, organisms are equipped with a multi-faceted network of antioxidant metabolites and enzymes that collaborate to maintain the integrity of cellular components such as DNA, proteins and lipids against oxidative damage (Sies 1997). Antioxidants obtained from the diet can provide protection from radicals as well. Although free radicals are necessary for maintaining normal biological function, they must, in some way, have a system of checks and balances (Rhee 2006).

Antioxidants

Antioxidants provide a mechanism to combat free radicals and lessen their potential of causing biological damage. In general, an antioxidant is a chemical that has the capability, via reactions with radicals, to delay or prevent the oxidation of other molecules. Antioxidants are able to act as reducing agents through the donation

of electrons or hydrogen atoms without promoting oxidation. Antioxidants have been shown to prevent the formation of reactive oxygen/nitrogen species through sequestering metal ions, directly reacting with and scavenging reactive oxygen or nitrogen species, inhibiting oxidative enzymes (i.e. cyclooxygenases), as well as inducing antioxidant enzyme activities.

Antioxidants can be categorized broadly into one of two categories: an enzymatic antioxidant or dietary antioxidant. Enzymatic antioxidants are endogenous. The most common endogenous antioxidant systems are: superoxide dismutase, catalase, and glutathione peroxidase.

Superoxide dismutase is a group of enzymes that catalyze the degradation of the superoxide anion into oxygen and hydrogen peroxide at a rate that is approximately 10,000-fold when compared to the non-catalyzed reaction by a group of enzymes collectively called superoxide dismutases (SODs) (Zelko *et al* 2002). This conversion is beneficial because hydrogen peroxide is much less toxic to the human body than superoxide.

SOD enzymes require a metallic cofactor, either copper, manganese, zinc, or iron, which determines their location within the cell (Johnson *et al* 2008). In humans, the copper/zinc SOD is functional within the cytosol, whereas the manganese SOD is located in the mitochondrion (Bannister *et al* 1987). Additionally, in the extracellular fluid there is a third form of SOD. This SOD is made functional by having zinc and copper present in its active sites (Nozik-Grayck *et al* 2000).

Mitochondrial SODs are, by comparison, more biologically significant than those of extracellular or cytoplasmic SODs. It has been shown that mice lacking the

mitochondrial manganese SOD died shortly after birth, about 21 days, from neurodegeneration, cardiomyopathy or lactic acidosis (Melov *et al* 1998). While, mice reached maturity but were lacking cytoplasmic copper/zinc SOD encountered lowered fertility in females with increasing age, reduced lifespan, liver cancer, muscle atrophy, cataracts and hemolytic anemia (Reaume *et al* 1996; Ho *et al* 2000). In the yeast model, it was found that when both mitochondrial and cytosolic SOD are absent, yeast grows very poorly in the presence of air. However, the yeast under the same SOD conditions grew well in an anaerobic environment.

A study conducted by Muller *et al* 1997 showed implication of the role of SOD in the pathogenesis of disease as well as the aging process. Using mice lacking the CuZn-superoxide dismutase, an increase in the loss of skeletal muscle mass in correlation to an increase of age was seen. As well, these mice had less lower muscle mass and were 17 to 20% smaller than wild-type mice at 3-4 months of age. At 20 months of age the muscle mass of the hind limb in the mice lacking the CuZn superoxide dismutase was 50% lower than wild-type mice of the same age. It was also found in the skeletal muscle that the level of oxidative damage to proteins, lipids and DNA was higher in those lacking the dismutase as compared to the wild-type mice. In combination with the reduction in muscle mass and increased damage from oxidation, a 40% decrease in voluntary wheel running was noted by 6 months of age. Loss of muscle mass was also correlated with the presence of tremors and difficulty walking. Conclusively, this study illustrated the effects of the absence of the copper zinc superoxide dismutase. The results seen in the mice show a link between the aging process and the presence of absence of copper zinc superoxide dismutase

(Brand *et al* 2004). Other mechanisms of endogenous antioxidant systems are catalase and glutathione.

Catalase, found mainly in the peroxisomes organelle, furthers the SOD reaction by breaking hydrogen peroxide down into its constituents, water and oxygen. This is accomplished though the combination of catalase with a cofactor, usually iron or manganese. This enzyme is unique in that it undergoes a double displacement reaction even though its only substrate is hydrogen peroxide (Hiner *et al* 2002). Although, catalase provides a valuable service by removing hydrogen peroxide its deficiency has little effect in the human and animal model (Muller *et al* 1997; Ogata 1991).

The most abundant group of protective enzymatic antioxidants is the glutathione peroxidases, a selenium dependent family of multiple isozymes. These isozymes function to further the reaction generated by superoxide dismutase by further reducing H_2O_2 into water by acting as an electron donor. Mammalian tissues have four major types of isozymes, abbreviated as GPx. The first is the classical isozyme, GPx1 is found in red blood cells, the liver, lungs and kidney. Second is the isozyme GPx2 located in the gastrointestinal tract. Chu *et al* was able to induce ileal and colon cancer by producing a deficiency in the enzyme levels of GPx1 and GPx2 using a mouse model (Chu *et al* 2004). The third isoenzyme, GPx3 is extracellular, but most abundant in the plasma of the kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle. Last, is the phospholipid isozyme, GPx4. This isozyme is widely distributed similarly to the third isozyme of the plasma. More distinctively, each isozyme functions optimally in a specific sub-cellular location.

The classical isozyme is found in the cytosol, nucleus and mitochondria. Where as the gastrointestinal tract isozyme is located in the cytosol and the nucleus. Additionally, the plasma isozyme can be found in the cytosol exclusively, and the phospholipid isozyme is accumulated in the nucleus, cytosol, mitochondria and bound to membranes. Additionally, glutathione peroxidases also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants (Margis *et al* 2008).

Superoxide dismutases, catalase and glutathione peroxidases act as endogenous sources of antioxidants. The body also takes in antioxidants from exogenous sources found in the diet. There are two broad classifications of dietary antioxidants that are based on solubility: hydrophilic and hydrophobic. Generally, cytoplasm and blood are protected by water-soluble antioxidants and cell membranes by lipid soluble antioxidants (Sies 1997). The action of an antioxidant is dependent on concentration, radical affinity, and the proper functions of the various components in the antioxidant system. Recently, the antioxidant components of seeds obtained from common fruits and vegetables have been studied for the purposes of cancer prevention (Parry *et al* 2008).

Chemoprevention

Current Methods

The events that occur within the molecular pathways: APC-ß-catenin-Tcf pathway, HNPCC pathway, ulcerative colitis dysplasia-carcinoma sequence pathway and the hypermethylation of the estrogen receptor gene as well as those that occur in inflammation may be potential anti-cancer targets. However, as of current, the most effective treatments for cancers are early detection, invasive surgery, and

chemotherapy. Although, these treatments have been shown to be successful they can pose adverse effects, such as mouth sores, loss of taste, nausea, hair loss and diaherra. Chemotherapeutic remedies also pose the issue of drug resistance, for the patient undergoing treatment, and cell sensitivity (Meuller *et al* 2004). Considering the potential hazards imposed by current cancer treatments researchers has shifted their focus to methods of prevention. One method currently under investigation is the use of the diet to prevent colorectal cancer. It is believed that the diet harnesses important antioxidant and anti-proliferative components that may be used to target specific carcinogenic pathways.

Potential uses for the diet in the prevention of colorectal cancer have been elucidated by studies involving migrant populations. These studies further the understanding of the role of environmental factors in colorectal cancer, particularly dietary in the prevention (Levin and Dozois 1991). It has been shown that the diet influences colon cancer deaths 70-90% of the time, which is why the focus of dietary prevention is so necessary (Schatzkin and Kelloff 1995). Additional results implicating the diet as a means of cancer treatment and/or prevention may benefit the agriculture industry. A survey conducted found that two-thirds of grocery shoppers reported that their product selection is mediated by their desire to reduce risk or cope with a particular health condition (Sloan 2000). One potential way of implementing cancer treatment and prevention into the diet is through the inclusion of the seeds found in commonly available fruits and vegetables. The seed components are thought to contain properties that may combat the action promoted by the molecular

pathways, inflammation, and free radicals that have the potential to be carcinogenic (Blakeborough *et al* 1989).

Edible seed oil and flour are rich in natural antioxidants

Seeds are a superfluous byproduct of commercially available fruit (Shi *et al* 2003). They are often used for inexpensive animal feed or disposed of for a fee. Such as in the case of fruits like apples, cherries, strawberries, and peaches which can be eaten fresh or used to make jams, marmalades and other preserves. Fruits can also be used in the production of processed baked goods, frozen foods, juices and alcoholic beverages (McGee 1984). For all of these consumables, the crop value is harnessed in the flesh of the fruit. However, fruit seeds are rich in a variety of nutrients. These nutrients can be grouped as macronutrients, micronutrients and other components such as phytochemicals, which have antioxidant properties. Each of these groups has the potential to provide health benefits, which may increase the crop value of particular fruits (Parry *et al* 2005).

The beneficial properties of a seed for the purposes of cancer prevention and treatment can be better understood by examining the seeds components: oil and flour. The flour is what remains after the extraction of the seed's oil (Parry *et al* 2006).

Cold-Press Method

Previous to the development of the cold-press method, seed meal was treated with organic solvents and heat to obtain the oil. However, those conventions are no longer necessary.

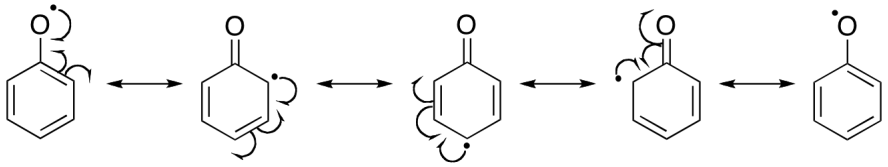
The cold-pressing method is based on pressure applied to the seed from a machine. First, the seeds are dried. Drying is followed by a separation of impurities

using a sieve. To begin the process of pressing, the purified seeds are loaded into a screw press. Using intense pressure and very low temperature the seeds are crushed. After the oil is collected the residual substance is referred to as the seed flour. A cold-press can also be achieved through an alternate pathway. Seeds can be placed into slit-lined barrels, where pressure is applied to extract the oil, which will run down the slits (Patent). After the collection of oil, the crushed seeds are collected to obtain the seed flour. Each of these components contains properties that may quench the potential carcinogenic free radicals by providing antioxidant properties.

Seed Components

Phenolics

One of the major antioxidants found in the diet are phenolics, the most abundant secondary metabolite in plants. Phenols are known to be the fourth most abundant constituent in grapes, only surpassed by their concentration of water, carbohydrates and fruit acids. Their abundance is important for the purpose of colorectal cancer prevention because phenols are able to act as free radical scavengers. As well, they possess antioxidant properties. Both of these properties can be attributed to the structure of a phenol an aromatic ring, with at least one hydroxyl group.



When the hydrogen molecule is removed from the hydroxyl group, oxygen is able to act as a nucleophile so that it may quench radicals. The nucleophile's ability

to quench free radicals is one of the key properties that allow phenolics to interfere with the initiation and propagation of free radical chain reactions implicated in the development of colorectal cancer.

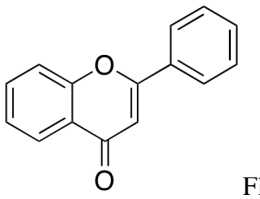
In general, "phenolics" is an umbrella term that encompasses a wide array of compounds. A variety of studies have been conducted on the study of phenolics in the prevention of colorectal cancer. Fini *et* al found that olive oil offers is rich in phenolic compounds. The study tested various phenolic compounds on several different colorectal cell lines. An increase in apoptosis via an up regulation of the ATM-p53 cascade pathway was seen. The induction of this pathway was specifically linked to the polyphenols present in olive oil (Fini *et al* 2008). For the purposes of discussing the most beneficial phenolics, flavonoids are of primary focus.

The flavonoids can be distinguished further as colored and colorless flavan-3-ols. Anthocyanins are often the primary polyphenolics in red grapes, such as muscadine, which have been reported to contain more than 40 mg/kg of anthocyanins. (Ector *et al* 1996). However, in white grapes such as chardonnay, flavan-3-ols (e.g. catechins) are the most abundant phenolic (Cantos *et al* 2002). As for the phenolic acids present in grapes, the most abundant in seeds have been found to be cinnamic, benzoic and gallic acid. Gallic acid has been shown effective in the induction of apoptosis in colon adenocarcinoma COLO 205 cell lines. (Yoshioka *et al* 2000).

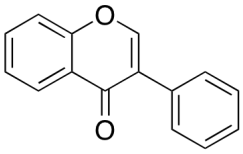
Flavonoids

A flavonoid is a secondary plant metabolite. They can be classified into three groups based their derivative using the IUPAC naming system: 1. Flavonoid: derived from 2-phenyl-1, 4-benzopyrone 2. Isoflavonoids: derived from 3-phenyl-1,4-

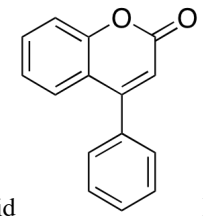
benzopyrone 3. Neoflavonoids: derived from 4-phenyl-1, 2-benzopyrone (McNaught and Wilkinson 1997). The majority of the flavonoids found in grapes are derivatives of 2-phenyl-1, 4-benzopyrone.



Flavonoid



Isoflavonoid



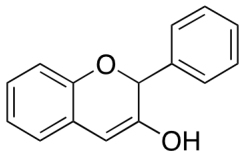
Neoflavonoid

In general, flavonoids are synthesized through the phenylpropanoid metabolic pathway. This pathway uses phenylalanine, an amino acid, to generate 4-coumaroyl-CoA. The first step involves the conversion of phenylalanine to cinnamic acid by the phenylalanine ammonia-lyase (PAL) enzyme. Cinnamic acid is converted into p-coumaric acid using the cinnamare-4-hydroxylase (C4H) enzyme. From the *p*-coumaric acid 4-coumaroyl-CoA is made via the 4-coumaroyl: CoA-ligase enzyme (4CL). 4-coumaroyl acid combined with malonyl-CoA forms the basic structure of all flavonoids. (Winkel-Shirley 2001). From this backbone, anthocyanidins, flavonols, flavanols, flavones, and flavonones are created.

Anthocyanins

Anthocyanins are a type of water-soluble pigment. Depending on the pH, pigments can range from red, purple to blue in appearance. Grape seed extract is a rich source of proanthocyanidins (Faria *et al* 2006). Using ApcMin mice and human colon cell lines, Kang *et al* conducted experiments to test the potential of anthocyanins to inhibit intestinal tumor development. The mice fed the diet rich in anthocyanins displayed significantly smaller and fewer cecal adenomas compared to the control mice. A reduced level of cell growth was also seen in the colon cancer

cell lines, HT29 and HCT116 using anthocyanins. The results generated by Kang *et al* show potential for the use of anthocyanins in the prevention of colorectal cancer (Kang *et al* 2003).



Anthocyanidins

Flavonols

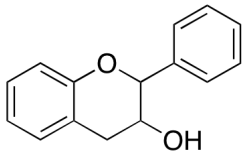
Flavonols are distinguished from the other types of flavonoids by the presence of a hydroxyl at carbon 3 of the benzene ring and a carbonyl group at carbon 4. However, substitutions may occur at the 3', 4' and 5' positions on the carbon ring. These substitutions generate the compounds within the flavonol group: kaempferol, quercetin, myricetin and fisetin. An eight-year study demonstrated the beneficial effects of kaempferol on pancreatic cancer (Nöthlings *et al* 2007).

Flavanols

Flavanols are sometimes called Flavan-3-ols and are a derivative of the 2-phenyl-3, 4-dihydro-2H-chromen-3-ol backbone. This group of compounds includes the catechins and catechin gallates.

Catechins extracted from green tea were used to study their effect on $Apc^{min/+}$ mice and HT29 in the role of colorectal cancer. Ju *et al* (2005) found that small intestine tumor formation was reduced by approximately 40% using a dose dependent application of catechins in the mice. As well, HT29 cell cultures showed increased levels of E-cadherin, a tumor suppressor gene, and decreased the levels of cyclin D

and c-Myc, both key components of the cell cycle. Catechins are a potential phenolic for the prevention of colorectal cancer (Ju *et al* 2005).



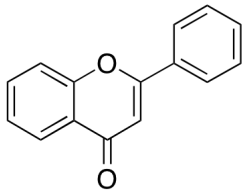
Flavanols

Flavanols are another class of flavonoids. Flavanols serve as the building blocks of proanthocyanidins. Clinical data shows that proanthocyanidin oligomers, polymer chains of flavanoids, are 20 times more potent in their antioxidant capacity than vitamin C and 50 times more potent than vitamin E. Also, phenolics have the ability to inhibit the action of some of the enzymes that are involved in the catalyzed the release of inflammatory response molecules such as histamine, serine protease, prostaglandins, leukotrienes. (Shi *et al* 2003) The antioxidant properties of grape seed oligomeric proanthocyanidins have been further elucidated through several biochemical and medical studies for last three decades. (Bagchi *et al* 2000). Together with tannins, polyphenols and polyunsaturated fatty acids display inhibitory activities against several experimental disease models, including cancer, heart failure and other disorders associated with oxidative stress (Agarwal *et al* 2002). Agullo *et al* have shown in both colorectal cell lines HT-29 and Caco-2 that the flavonol, quercetin, decreased cellular ATP and created a cytotoxic environment (Agullo *et al* 1994).

Flavones

The 2-phenyl-1, 4-benzopyrone flavonoid serves as the backbone of the flavones. The antioxidant properties of synthetically generated 24 hydroxy-flavones

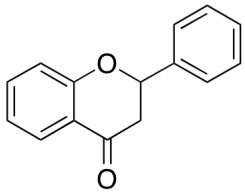
were measured by Cotelle *et al*. Using the DPPH radical scavenging assay as well as the MDA test, measuring the ability of the flavones to inhibit peroxidation, it was shown that a hydroxyl substitution at carbons 2′, 3′, 4′ of the carbon ring make a significant impact. As well, 7-hydroxy flavones showed the potential to inhibit xanthine oxidase, a part of the metabolic pathway of uric acid. These results give light to the potential for flavones to be used for the purposes of free radical scavenging, targeting the origins of cancer (Cotelle *et al* 1996).



Flavones

Flavonones

A study conducted by Borradaile *et al* demonstrated the beneficial effects of flavanones using HepG2 cells. The flavanones used were extracted form citrus fruits and applied to the cells in a dose dependent manner. A reduction in the net ApoB, a protein involved in the uptake of low density lipids, secretion was seen dose-dependently up to 81% after a period of 24 hours of incubation. ApoB secretion was reduced by 50% after only 4 hours using a 60 μg/mL dose. This study of the relationship between ApoB and flavanones elucidates one of the many uses for flavonoids in the prevention of human disease *(Borradaile et al 1999)*.



Flavonones

Grape seeds

Overview

The major phenolic compounds discussed provide insight into the French Paradox, which further unravels the potential for grapes and their constituents in the prevention of colorectal cancer. By understanding the French paradox in coronary heart disease a relationship can be correlated to grape consumption and colorectal cancer. A high mortality rate from coronary heart disease is associated to a high intake of saturated fat.

The French Paradox provides interesting insight into the health benefits of grapes. The incidence of mortality in France due to coronary heart disease is low in relation to the intake of saturated fat. This relationship is thought to be partially a result of the high level of wine consumption. The risk of cardiovascular heart disease of the French has been reduced by at least 40% with a level of 20-30 g of wine per day (Opie and Lecour 2007).

This paradox may be a result of the polyphenols present in grapes and grape seeds. Polyphenols have many benefits such as reducing susceptibility to blood vessel damage, lowering blood pressure (Sato *et al* 2002), as well as the inhibition of heart disease, cancer, degenerative nerve disease, and viral infections (Shanker *et al* 2007 and Mancuso *et al* 2007). As well, the components of grape seeds may be an

important part of inhibiting the expression and replication of HIV (Nair *et al* 2005), increasing vascular endothelial growth factor, which will accelerate wound healing (Khanna *et al* 2002), slow the growth of oral bacteria, preventing tooth decay (Smullen *et al* 2007), enhancing bone density and strength (Yahara *et al* 2005). There is a large bioavailability of polyphenols, specifically flavonoids, in the large intestine. However, to achieve these benefits as well as many other, it is believed that one glass of wine a day for women and two a day for men should be consumed (de Lang and van de Wiel 2004).

A grape seed is two celled with two ovules in each cell (Gesundh 1929). The seed oil by product in from wine manufactured in 2001 would have totaled approximately 201 tons of oil, with a value of approximately 1.6 to 2 million dollars (Beveridge *et al* 2005) According to the Food and Agriculture Organization (FAO) in 2004, there are nearly 76,000 square kilometers of the world that are devoted to grapes. Of those cultivated, approximately 71% of world grape production is for wine, 27% for fresh fruit, and 2% for dried fruit. In the United States 471,253 metric tons of grapes were cultivated in 2004 with a value of approximately 878,617,000 dollars (FAO 2004). The continued research of grape seed antioxidant and anti-proliferative properties will benefit health as well as the agricultural business. Grapes in the current study include concord, muscadine, chardonnay, and ruby red.

Muscadine Grape Seed

Muscadine grapes (*Vitis rotundifolia)* are indigenous to the southeastern portion of the United States. Their skins range in color from purple to bronze and the total phenolic content within muscadine is most prominent in the seeds followed by the skin, then leaves and finally the pulp (Pastrana-Bonilla *et al* 2003). The phenolic compounds with the highest antioxidant capacities are: tannins, quercetin, catechins, epicatechin, gallic acid, ellagic acid, myricetin, and kaempferol. Ellagic acid is a bioactive compound that naturally occurs in nuts, berries and grapes. Using Caco-2 cells treated with 100 μmol/L ellagic acid, a decrease in ATP was seen after 24 hours of incubation. Additionally, the same treatment applied to normal cells had no harmful effects, showing potential use in cancer treatment (Losso *et al* 2004). In agreement, a study conducted by Mertens-Talcott et al has shown similar anti-cancer properties of the ellagic acid (Mertens-Talcott *et al* 2006). Fractions from muscadine grape extract were tested for antiproliferative effects on HepG2 liver cancer cells. The anthocyanin fraction of the grape was the most effective at inhibiting cell growth by 50% at doses form 100-300 µg/mL (Yi *et al* 2006)

Chardonnay Grape Seed

Chardonnay is the second most cultivated grape in the world. It is a green skinned grape that is used to create a variety of white wines. This particular grape is very easy to cultivate and will thrive in various climates and soil types. Studies have shown that chardonnay extract inhibits the proliferation of the HT-29 colon cancer cell line (Parry *et al* 2006). As well, chardonnay extracts significantly reduced DPPH radical and ORAC values compared to control (Luther *et al* 2007).

Ruby Red Grape Seed

Vitis vinifera is known as the common grape. It includes a large variety of grapes, one being Ruby Red. These grapes are native to central Europe and the Mediterranean region. They are also cultivated in Germany, Iran, Morroco and Spain.

Concord Grape Seed

Concord grapes are a member of the *Vitis labrusca* species. This type of grape is most commonly used in juices and table wines. Concord grapes are not typically sold as for raw consumption because seedless varieties are often preferred. Concord grapes are very aromatic and have large seeds. The skin, which is easily separated, is typically blue or purple.

Assays for Bioactivity Determination

Cell Cycle

The development of cancer is associated with an estimated rate of 1 out of 2 x 10^7 mutations per gene cell division. To prevent the replication of mutated cells, a protective mechanism, the cell cycle, is in place. This cycle takes a cell through a series of events to ensure proper replication. As a cell prepares for replication it is taken through a series of events, G1, S, and G2, collectively termed interphase. During interphase, the cell will accumulate nutrients and replicate its DNA in preparation for the mitotic phase, M phase. Although the M phase is exists for a fraction of the cell cycle is plays 2 major functions: mitosis and cytokinesis. Cyclins and cyclin-dependent kinases are regulatory molecules that manage all of these molecular events.

In normal cell replication the G1 phase represents a "gap" between M phase and the next cycle. During this time the cell will increase its nutrient and organelle supply as well as grow in size. S phase represents chromosomal replication. G2 plays a similar role to G1 in that it prepares for the next step of replication by implementing a checkpoint into the cycle. This checkpoint will halt replication if a hazardous error, such as DNA mutations or incomplete replication is detected. Otherwise, the cell cycle will progress into M phase. Here, the cell will undergo the redistribution and separation of duplicate chromosome. Spindle fibers will shorten, pulling the chromosomes into opposite ends of the cell. Once the separation is complete the cell will experience cytokinesis, which will divide the cell into two identical daughter cells.

Progression of the cell cycle is mediated by cyclins and cyclin-dependent kinases (CDKs) as well as a series of negative feed back loops (Nasmyth 1993). The regulatory cyclin subunit and the catalytic CDK subunit form a heterodimer. This heterodimer operates to phosphorylate target proteins. The proteins targeted are specified by the components of the cyclin-CDK complex that are critical for the promotion or prevention of cell cycle progression. G1 cyclin-CDK complexes are activated by extracellular pro-mitotic signals, promote the expression of transcription factors that will initiate the phosphorylation of enzymes and pre-assembled cyclins that are necessary for a single DNA replication during S phase. The cyclin-CDK complexes necessary for M phase activate proteins important for spindle assembly and chromosome condensation. Lastly, the anaphase-promoting complex (APC)

targets mitotic cyclins for degradation. The breakdown of cyclins allows for the execution of cytokinesis that will create two identical cells (Webster 1998).

The cell cycle mitigates the proliferation of carcinogenic cells through three different mechanisms. Tumor development can occur by disturbing the cellular mitogen signals. A mitogen is a chemical substance, usually some form of a protein that encourages a cell to commence cell division, thereby triggering mitosis. Normal cells are totally dependent on these signals for their proliferation. Cells will pass through the G1 checkpoint only after extensive exposure to mitogens.

Additionally, the deletion of the *RB*gene, a gene that regulates the G1 checkpoint, and/or the interruption of the phosphorylation and functionality of CDKS can inactivate the *RB*gene cause the cycle to progress even though a cell is cancerous. These can be achieved through either loss of inhibitors or directly through the activation of CDKs (Sperandio 2000).

Uncontrolled expression of cMyc or elevated levels has been demonstrated in tumor cells. Myc is controlled by the availability of mitogen. pRB, and cMyc are needed for cell differentiation.

Apoptosis

Apoptosis is the course of events resulting in programmed cell death that occurs in multi-cellular organisms. Programmed cell death involves the occurrence of a series of biochemical events leading to a characteristic cell morphology and death. These events cause changes lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, nuclear fragmentation, chromatin condensation, and chromosomal DNA

fragmentation (Fadeel and Orrenius 2005). Generally, apoptosis is an advantageous process during an organism's life cycle. The development of the separation of fingers and toes in humans is a result of apoptosis in the womb. Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult. For an average child between the ages of 8 and 14, approximately 20 billion to 30 billion cells die a day. In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weigh (Fadeel and Orrenius 2005).

The amount of apoptosis research has grown significantly since the early 1990s. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in an extensive variety of diseases. In the event of excessive apoptosis hypotrophy can occur (Ghobrial 2005). However, an insufficient amount of apoptosis results in uncontrolled cell proliferation, such as cancer.

Apoptosis can occur due to deregulated cell expression and/or the exhaustion of the essential cell survival factors. An essential mechanism that preserves tissue integrity and function as well as removes damaged or useless cells is apoptosis (Ghobrial 2005). The active physiological process or cell self-destruction, by way of particular biochemical and morphological changes in the cytoplasm and nucleus is known as apoptosis or programmed cell death. The inhibition of malignant cells, through the induction of apoptosis may serve as an effective means of chemotherapy and chemoprevention. This theory showed promise in the use of maslinic acid isolated from olive pelt as a means of induces apoptosis in colorectal cancer cells, HT-29 and Caco-2 (Reyes *et al* 2006). It is important to note the measurements used to determine apoptosis in the maslinic study. After 48 h exposure to maslinic acid at

IC80 concentration, the amount of time for 80 percent of the total cell population to undergo apoptosis, the cells showed typical apoptotic changes, including cell shrinkage, chromatin condensation, and loss of normal nuclear architecture. At 72 h of incubation the disruption of cell-membrane integrity was more prominent. The mechanism to achieve the events described of apoptosis can be elucidated through one of two pathways.

Apoptosis can be achieved through two distinct pathways: intrinsic and extrinsic. The intrinsic pathway is initiated from within the cell as a consequence of extreme cellular stress or irregular growth and development. This pathway releases cytochrome-c pro-apoptotic proteins from the mitochondria. These proteins activate caspase enzymes, which will initiate and carry out the mechanisms along the pathway to apoptosis. (Fulda and Debatin 2006).

The intrinsic apoptotic pathway is mediated by the interactions of the super family of proteins that regulate the permeability of the mitochondrial membrane, BCL2 (Coultas 2003). The anti-apoptotic BCL2 proteins BCL2 and BCLXL achieve mitochondrial permeation. These two proteins inhibit BAX and BAK, two pro-apoptotic BCL2 proteins. (Reed 1998). The over expression of BCL2 and BCLXL have been linked to the promotion of colorectal cancer (Jiang 2003).

Cell stress initiates the intrinsic pathway through the activating transcriptional factors of specific members of the pro-apoptotic BCL2 protein family involved in the promotion of apoptosis, such as the BH3-only proteins PUMA and NOXA. PUMA and NOXA are located in the cytoplasm and act to detect cell damage or stress (Karst and Li 2007). This up regulation will activate the pro-apoptotic proteins BAX or

BAK. These will travel into the mitochondrial membrane where they will upset the function of the anti-apoptotic BCL2 proteins. This allows the mitochondrial membrane to be permeated (Henry-Mowatt *et al* 2004). Cytochrome c and the pro-apoptotic protein SMAC/DIABLO are then able to leak from the intermembrane space of the mitochondria into the cytosol (Henry-Mowatt *et al* 2004). This is why the intrinsic pathway is sometimes referred to as the mitochondrial pathway.

Cytochrome C will bind the adaptor apoptotic protease activating factor-1 (APAF1). This will form a large multi-protein structure known as the apoptosome. The apoptosome will recruit and activate caspase 9, which will in turn, activate the downstream effector caspases, including caspase 3, 6, and 7, leading to apoptosis. (Henry-Mowatt *et al* 2004).

Under normal conditions, the activity of the caspase is regulated by a protein family called inhibitor of apoptosis proteins (IAPs) (Lavrik *et al* 2005) IAPs are not directly involved in the apoptosis signaling pathways, but they are capable of preventing cell death by restraining the endogenous initiator and effector caspases. As part of the intrinsic apoptosis pathway, the SMAC/DIABLO protein released from the mitochondria promotes apoptosis by directly interacting with IAPs and disrupting their ability to inactivate the caspase enzymes (Henry-Mowatt *et al* 2004).

On the other hand, the extrinsic pathway begins outside of the cell. On the surface of the cell, pro-apoptotic ligands bind to pro-apoptotic receptors (Ashkenazi 2002). The binding activates caspases 8 and 10. These two caspases release active enzyme molecules in the cytosol and then they activate caspases 3,6, and 7 where they converge with the intrinsic pathway (Lavrik *et al* 2005).

Once activated by extracellular ligand binding, the intracellular domains of these receptors, known as the 'death domains', bind to the adaptor protein Fas-associated death domain (FADD), leading to the assembly of the death-inducing signaling complex, or DISC, and recruitment and assembly of initiator caspases 8 and 10 (Boldin *et al* 1995) Caspases 8 and 10 are stimulated and undergo self processing, releasing active enzyme molecules into the cytosol, where they activate caspases 3, 6, and 7, thereby converging on the intrinsic pathway.(Lavrik *et al* 2005).

Caspases are a family of intracellular cysteine enzymes that disassemble cellular components, mainly nucelotides, through a cascade effect though the assembly of multiprotein complexes. The cascade begins with initiator caspases. These include caspase 2, 8, 9 and 10, which become active by way of the apoptosis-signaling pathways and in turn, activate the effector caspases, caspase 3, 6, and 7 (Thornberry and Lazebnik 1998).

The equilibrium between cell proliferation and apoptosis loses balance when proto-oncogenes and/or tumor suppressor genes are mutated and influenced by genes that contribute to the development of cancer (oncogenes) and those that encode proteins that normally suppress tumor formation (tumor suppressor genes) (Vogelstein and Kinzler 2004). Oncogenes are mutated forms of normal cellular genes known as proto-oncogenes which, when activated by mutation or increased expression, increase the chance that a normal cell will become malignant.

In cancer cells, persistent and/or elevated signaling from oncogenes such as myc genes and E1A drive proliferation. In advanced malignancy, there is often selection for tumor cells with inactive p53 or over-expression of specific anti-

apoptotic factors such as Bcl2 — properties that enable the tumor cell to evade apoptosis. Increased Bcl2 protein production occurs in numerous human cancers and is linked to poor disease outcome. In addition, over-expression of the Bcl2 gene may explain cancer cell chemotherapeutic drugs resistance (Reed 1998).

As a consequence of lesions created by oncogenes, tumor cells may be innately more resistant to the induction of apoptosis. Myc is a powerful inducer of apoptosis under adverse conditions such as cellular stress, DNA damage, or when levels of survival factors are low (Evan *et al* 1992). Myc may in fact enhance tumor cells' sensitivity to apoptotic signaling via death receptors (Lutz *et al* 1998). This duality may serve as an inbuilt check on the proliferative effects of Myc, and similar observations have been reported for almost all growth-promoting proteins (Evan and Vousden 2001)

Bcl-2 proteins are layered on the surface of the mitochondria. This protein detects damage and activates a class of proteins called Bax, which punch holes in the mitochondrial membrane, causing cytochrome C to leak out. Cytochrome C then binds to Apaf-1, or apoptotic protease activating factor-1, which is free-floating in the cell's cytoplasm. Using energy from the ATP in the mitochondrion, the Apaf-1 and cytochrome C bind together to form apoptosomes. The apoptosomes binds to and activates caspase-9, another free-floating protein. The caspase-9 then cleaves the proteins of the mitochondrial membrane, causing it to break down and start a chain reaction of protein denaturation and eventually phagocytosis of the cell. PARP is a DNA binding protein that identifies breaks in DNA strands. It is cleaved by caspase-3 and this cleavage is a mark for apoptosis (Nicholson *et al* 1995).

Total Phenolic Content

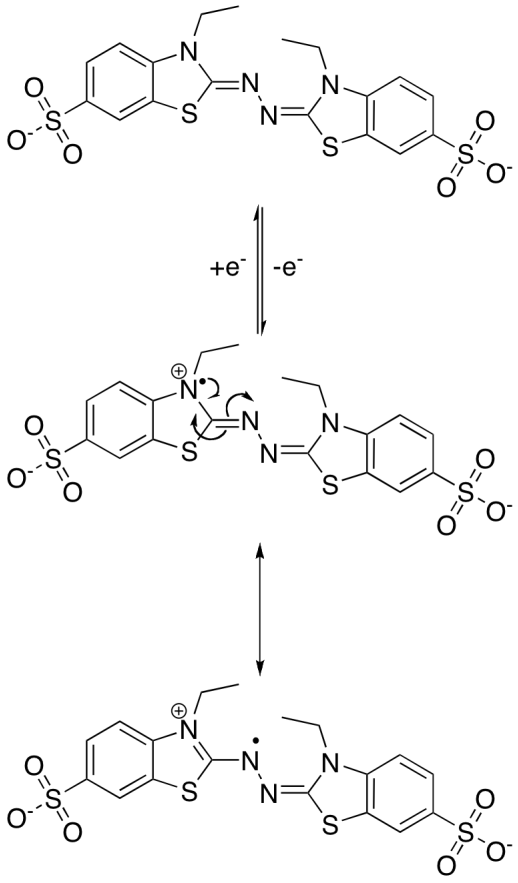
The total phenolic content (TPC) assay using Folin-Ciocalteu Reagent (FCR) was created to analyze proteins. However, due to the assays use of tyrosine residues, which contain a phenol group, TPC is now used to quantify the phenolic content of a variety of foods.

The assay begins with the preparation of the FCR by boiling its constituents: sodium tungstate, sodium molybdate, concentrated hydrochloric acid, phosphoric acid and water. The addition of lithium sulfate after the initial boiling will make the FCR a vivid yellow. When reductants are added to the FCR solution, the color will become green. However, by adding oxidants the color will return to the color yellow. It is believed that this occurs because FCR contains heteropolyphosphotunstates-molybdates.

The mechanism by which TPC is determined must be performed under basic conditions of approximately pH 10. This is because reductants are not limited to the family of phenolics, which will only react with FCR in a basic environment. Basic conditions will deprotonate a phenolic group creating a phenolate anion. This anion has the capability to reduce FCR, causing a color change from yellow to blue (Huang *et al* 2005). The total phenolic content (TPC) assay using Folin-Ciocalteu Reagent allows for phenolic content to be quantified using a standard phenolic acid for comparison. In the study of fruits, gallic acid is the most common standard phenolic acid (Khanizadeh, 2007).

ABTS$^{\bullet+}$

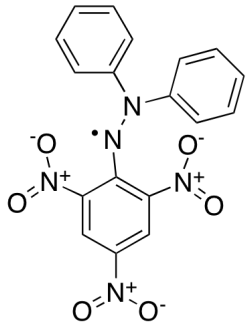
The chemically generated ABTS cation radical (ABTS$^{\bullet+}$) is used in a decolorization assay that can validate the radical quenching capacity of both lipophilic and hydrophilic antioxidants. Through antioxidant reduction ABTS$^{\bullet+}$ is transformed from a blue green color into a non-radical colorless ABTS molecule. Absorbance readings are taken at 734 nm using a spectrophotometer and compared to a standard (trolox) to determine the degree of antioxidant protection (Miller *et al* 1996).



Structure of cation ABTS radical

DPPH•

The DPPH• radical is stable due to the delocalization of a spare electron over the molecule, thus preventing dimer formation. This radical is used in the DPPH• radical scavenging capacity assay to quantify the ability of antioxidants to quench the DPPH• radical. The dark purple color of DPPH• will be lost when it is reduced to its non-radical form stable organic nitrogen centered free radical with a dark purple color which when reduced to its non-radical form by antioxidants becomes colorless. DPPH• radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. When the DPPH• radical is scavenged, the color of the reaction mixture changes from purple to yellow with decreasing of absorbance at wavelength 517nm. This method measures the antioxidant capacity at ambient temperatures to reduce the risk of molecule degradation by heat (Bondet *et al* 1995).



Structure of DPPH radical

Cell Studies

Using cells as a model of carcinogen inhibition has many advantages. Cell studies can serve as pilot studies. The cost of a cell study is relatively low in comparison to other methods such as animal models. As well they allow for

understanding of the mechanisms involved and their environment can be easily controlled.

Although using human and animals is the best method for determination of reactions from treatments, these methods are expensive, time consuming, and not suitable for pilot studies. Therefore, it is often best to use cell models. This approach allows for relatively fast way of measuring biological activity and is cost-effective (Wolfe and Liu 2007).

Objectives and Significance

Grape seeds are a superfluous byproduct of the food processing industry. The objectives of these studies were to elucidate a function of these waste products that contain beneficial health constituents and may add value to a current waste by productrevenue increasing properties thereby creating market for something that was previously useless. These studies may also accommodate the cultural shift towards consumption of functional foods.

Specific Objectives

1. To determine the radical scavenging capacities of muscadine, chardonnay, concord and ruby red grape seed extracts using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$.

2. To uncover the total phenolic content of muscadine, chardonnay, concord, and ruby red grape seed extract.

3. To elucidate the anti-proliferative properties of muscadine, chardonnay, concord and Ruby Red grape seed extracts by treating HT-29 colorectal cell line.

This research serves as a pilot study aiming at the long term goal of using the grape seed extracts studied for marketable products. The goal has been shown to be feasible as the extracts demonstrate antioxidant and *in vitro* anti-proliferative properties.

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Chapter 2: Antioxidant and Anti-proliferative Properties of Selected Grape Seed Extracts

Introduction

Free radicals have been implicated as a possible mechanism responsible for the onset and development of colorectal cancer, a globally growing health issue. It has been estimated by the National Institute of Cancer that within the United States there were 148,810 newly diagnosed cases and 49, 960 deaths due to colorectal cancer in 2008. Globally, there are over one million newly diagnosed cases annually and nearly one half of those are deadly (Ries *et al* 2007). These staggering figures warrant research in the field of colorectal cancer prevention.

Although the development of free radicals is often a natural and beneficial occurrence, when unregulated, free radicals may become biologically hazardous. Their involvement in the onset and development of colorectal cancer is widely accepted. However, it has been shown that antioxidants can quench free radicals thereby affecting the ability of the radical to progress into the propagation and termination steps to prevent biological damage. The chain reaction leading to the development of free radicals can be initiated endogenously or exogenously from a variety of sources such as pollution, chemical waste, and natural biological reactions. The steps of the chain reaction responsible for the actions of free radicals include the following: initiation, propagation, and termination. The final step, termination, is often the cause of free radical onset cancer because this step may lead to DNA damage, mutations and various other harmful effects.

Antioxidants are able to disrupt and safely terminate the chain reaction by quenching the free radicals. This mechanism of defense has previously been shown

to be effective in the treatment of the HT-29 human colorectal adenocarcinoma cell line using selected seed flour and oil extracts containing potent levels of antioxidants (Parry *et al* 2006; Wang *et* al 2007; Parry *et* al 2008). Antioxidants from other edible materials have also been shown to be an effective chemo-preventitive agent against colorectal cancer (Kaur *et al* 2006; Parry et al 2006).

It is important to note the consumer demand for natural sources of antioxidants. Culturally, we are making a shift away from synthetic products and seeking products containing natural ingredients that are able to provide a variety of health benefits. Since seeds are a waste by-product of food manufacturing, findings of their anti-carcinogenic properties would be of great benefit to the agricultural economy.

The studies explored in this thesis are intended to provide further understanding of the potential use for grape seed components in the prevention of colorectal cancer. It is believed that the grape seed extracts contain properties that may act to quench the presence of harmful or excessive free radicals. The use of cells to model carcinogenic activities, specifically growth inhibition via grape seed extract intervention has many advantages. First, cell research can serve as a pilot study and enhance the development of further research in a specific area of study. Cell studies are also able to elucidate the mechanisms of cancer formation and development as well as uncover targets for prevention. Third, the cost of conducting a cell study is relatively low in comparison to other methods. As well, the environment in which cell studies are conducted is easily controlled for confounding variables and creates a clearly defined variable. Lastly, the use of cells in data collection allows easily

produced replication. In summation, this approach allows for a relatively fast way of measuring biological activity and cost-effective (Wolfe and Liu 2007).

The present study was conducted to evaluate the selected grape seed oil and flour extracts of muscadine, chardonnay, ruby red, and concord grapes for their 1) radical scavenging capacities against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$; total phenolic content; and 3) anti- proliferative properties using the HT-29 human colorectal adenocarcinoma cell line.

Materials and Methods

Materials and preparation of extracts

HT-29 human colorectal adenocarcinoma cell line (American Type Culture Collection, Rockville, MD), Materials necessary for cell culture [Dimethyl Sulfoxide (DMSO), Fetal Bovin Serum (FBS), 1% antibiotic/antimycotic, McCoy's 5A medium modified with l-glutamine, 0.25% with 0.9 EDTA Trypsin] were purchased from Invitrogen (Carlsbad, CA). Charonnay, muscadine, ruby red, concord grape seed oil and flour extracts (Botanical Oil Innovations, Spoon, WI), ATP-Lite 1 step kit (Perkin Elmer Life and Analytical Sciences, Shelton, CT), Victor^3 multi-well plate reader (Perkin Elmer, Turku, Finland), Ultrapure water, prepared by an ELGA Purelab Ultra Genetic polishing system with < 5ppb TOC and resistivity of 18.2 mΩ water was used in all experiments requiring water (Lowell, MA). ABTS chromophore diammonium salt was purchased from Calbiochem (San Diego, CA), whatman #43 paper (Whatman International Ltd., Maidstone, England) gallic acid, 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), potassium chloride (J.T. Baker), sodium chloride (Fluka), and sodium biphosphate, monopotassium phosphate (J.T. Baker), manganese

dioxide and 6-hydroxy-2, 5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals and solvents used were of the highest commercial grade and used without further purification.

The preparation of the grape seed oil extracts was carried out according to a previously described laboratory method. Briefly, 1 gram of each of the cold-pressed seed oil samples was extracted using 3 mL of methanol (Parry *et al* 2006). This yielded a concentration of .33 grams oil equivalents/mL MeOH. The extracts were kept in the dark under nitrogen at ambient temperature until further analysis.

Defatted grape seed flours were ground to mesh size 20 before further preparation for use in the treatment of cells. Following grinding, the Soxhlet method was used to extract 10 grams of equivalents of each of the ground seed flours in 150 mL of 100% ethanol for 3 hours (Luther *et al* 2007). From this extraction solution 5 mL was removed and the ethanol was evaporated. The residue was re-dissolved in 5 mL of methanol. This yielded a final concentration of 0.066 grams flour equivalents/ mL MeOH. The extracts were stored in the dark under nitrogen at ambient until further analysis.

For use in the cell studies, the flour and oil grape seed extracts were evaporated to remove methanol and ethanol respectively. To achieve a stock concentration of 2 g/mL, 10 grams of each of the grape seed oil and flour extracts were added to 5 mL dimethysulfoxide (DMSO). The stock extracts were diluted using cell media to achieve a high dose of 6 mg extract equivalent/mL and a low dose of 0.6 mg extract equivalent/mL. To ensure consistency, all samples contained the same

amount of DMSO. Additionally to eliminate contamination all samples were filtered through a 0.2 μm pore filter.

DPPH• Radical Scavenging Capacity Assay

The free radical scavenging capacity of each grape seed flour and oil extract was estimated using the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH•) according to a previously established method (Cheng, Moore, & Yu, 2006). Briefly, the reactions were carried out in clear 96-well plates and the absorbance readings were performed using a Victor^3 multi-label plate reader (PerkinElmer, Turku, Finland). A sample reaction well contained 100 μL of the appropriate grape seed extract in addition to 100 μL of 0.2 mM DPPH• solution. The addition of DPPH• solution to each reaction well initiated the reaction between the radical and the antioxidant. Immediately after the addition of DPPH• solution absorption was read on the plate reader at 515 nm. An absorption reading at 515 nm was also established for a blank having only 200 μL of solvent, and a control containing a mixture of 100 μL of solvent and 100 μL of 0.2 mM DPPH•.

Using the following equation, the percent of DPPH• radical remaining in solution after 40 minutes was determined, % DPPH• remaining = $[(A_{sample}-A_{blank})/(A_{control}-A_{blank})]\times 100\%$. In the equation A_{sample}, A_{blank}, and $A_{control}$ represent the absorbance of sample, blank, and control reactions at 40 minutes. Absorbances were calculated according to the standard curve generated by trolox.

Total Phenolic Content (TPC)

The total phenolic content of each selected grape seed extract was measured using freshly prepared Folin-Ciocalteu reagent according to laboratory protocol (Yu *et al* 2002). Briefly, the reaction mixture contained 3 mL ultra-pure water, 50 μL sample/standard/blank, 250 μL Folin-Ciocalteu reagent, and 750 μL of 20% sodium carbonate. The grape seed extract and reaction solutions were left for two hours at ambient temperature before absorbance readings were taken. A spectrophotometer was used to read the absorbance at 765 nm. Gallic acid was used as the standard. Absorbance readings were conducted in triplicate.

$ABTS^{\bullet+}$ Radical Scavenging Capacity Assay

The $ABTS^{\bullet+}$ radical scavenging assay was performed according to a previously described method (Miller *et al* 1996). Briefly, manganese dioxide (MnO_2) was used to chemically pre-generate the ABTS radical, $ABTS^{\bullet+}$. A 5 mM stock solution of $ABTS^{\bullet+}$ was filtered through Whatman no. 43 filter paper in the presence of manganese dioxide using. The filtered $ABTS^{\bullet+}$ solution was diluted to an absorbance of 0.70 at 734 nm using phosphate buffer (PBS) pH 7.4. Following the addition of $ABTS^{\bullet+}$ to the sample, the solution was vortexed for 30 seconds. After vortexing, the tube was left to stand for 1 minute. Immediately after the rest period, the absorbance of the sample was read.

Trolox was used as the antioxidant standard. To generate a standard curve 1.25-100 μM Trolox solutions diluted with methanol were prepared from a 0.5 mM Trolox stock solution. Methanol dilutions of grape seed extracts were prepared to attain an

absorbance within the range of the Trolox standard curve.

Anti-proliferative Assay

The study of the inhibition of HT-29 human colorectal adenocarcinoma cells in response to treatment with selected grape seed extracts was carried out according to a previously described method (Wang *et al* 2007). HT-29 cells were grown to 90% confluence in 100 mm plates in a humidified atmosphere at 37 °C and 5% carbon dioxide. The culture media used was McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. Cells were removed from plates using 0.25% trypsin.

For the anti-proliferation assay, cells were seated at 2,500 cells/well in 96-well plates. Following an incubation period of 24 hours, the media was replaced with 100 μL of the appropriate treatment media. Two doses of treatment media, 6.0 or 0.6 mg seed part equivalent/mL, were used based on previous studies (Parry *et al* 2006). To ensure that any effect was not due to the use of DMSO, the control as well as all samples in both of the treatment levels contained a the same concentration of DMSO. As well, the control and treatment media were filtered through a 0.2 μm pore, retrograde cellulose filter prior to the treatment of cells.

To measure the effect of each treatment on the inhibition of HT-29, cell count was measured according to an ATP-Lite 1 step kit protocol (Perkin Elmer Life and Analytical Sciences, Shelton, CT). The ATP-Lite 1 step kit monitors adenosine triphosphate (ATP) using firefly (*Photinus pyralis)* luciferase. The level of ATP present is an indicator of cell viability and undergoes rapid decline after cell apoptosis

or necrosis. The ATP-Lite 1 step kit produces light due to the reaction of ATP with the added luciferase according to the following reaction:

$$ATP + \text{D-Luciferin} + O_2 \Rightarrow \text{Oxyluciferin} + AMP + PP_i + CO_2 + \text{Light}$$

The luminescene is proportionate to the amount of ATP present. This directly correlates to the number of living cells. Luminescence readings were taken without a filter on a Victor^3 multi-well plate reader (Perkin Elmer, Turku, Finland) immediately prior to treatment and at 4, 24, 48, and 96 hours after initial treatment. A separate plate was used for each reading. The treatment and control media were replaced every 24 hours until a reading was taken on that plate. The reaction between DMSO and the ATP-Lite 1 step kit was shown to have a minimal effect on the results.

Statistical Analysis

Triplicate measures were used to generate mean and standard deviation of data. SPSS for Windows was used to analyze differences among means one-way analysis of variance, Tukey's test, and multi-variate analysis (Rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was declared at $P < 0.05$.

Results and Discussion

Grape seeds are a waste byproduct of manufactured foods. They are typically used as an inexpensive animal feed or disposed of for a nominal fee. However, these seeds can be used to generate edible seed oils via the coldipress method. After the extraction of the seed's oil, a powder remains. This powder is referred to as the seed flour. The oil and flour grape seed extracts are believed to contain constituents that may be natural colorectal cancer prevention agents. The determination of health

benefits harnessed in the seeds of the muscadine, chardonnay, concord, and ruby red grape varieties could be economically valuable to the agriculture and food processing industries.

Total Phenolic Content

To quantify the contribution of phenolics to the antioxidant activity, total phenolic content was measured. Results are expressed as mg gallic acid equivalent per gram of seed oil or flour extract. A statistically significant difference was observed among all of the flour extracts. Chardonnay and Ruby Red grape seed flours extracts contained significantly higher amounts of TPC as compared to the muscadine and concord seed flour extracts (Figure 1). The range of total phenolic content for the grape seed flour extracts was 6-100 mg gallic acid equivalents per gram of flour extract.

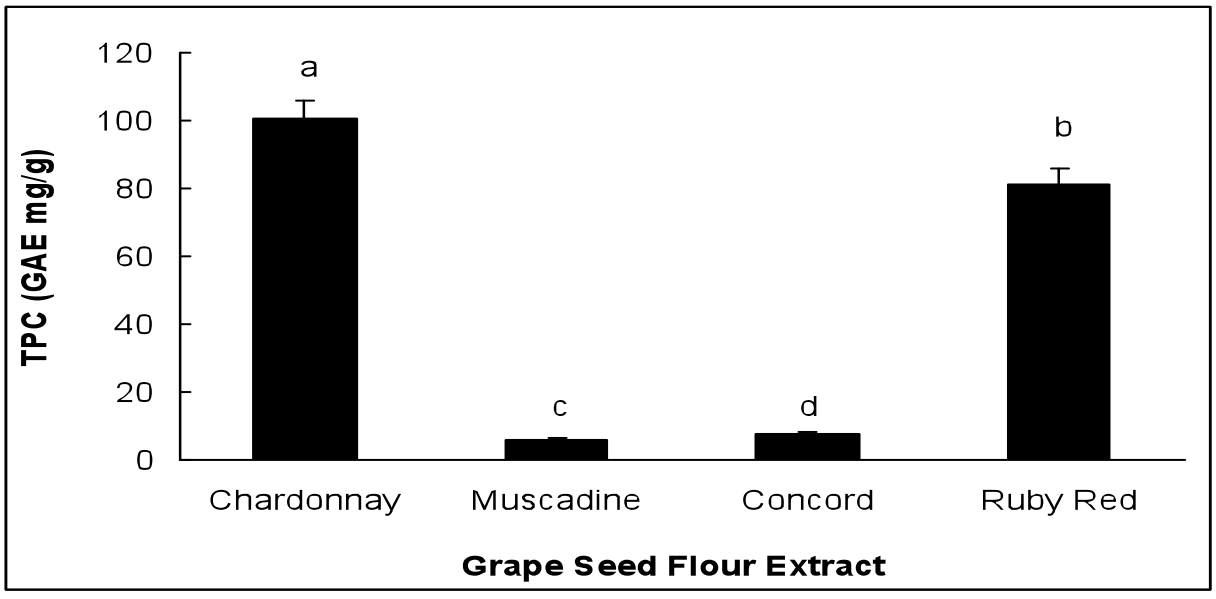

Figure 1. Total phenolic content of grape seed flour extracts. Data are expressed in gallic acid equivalents (GAE) as means ± standard deviations (n = 3). Columns marked with the same letter are not significantly different ($P < 0.05$).

The grape seed oil extracts (Figure 2.) demonstrated that muscadine contained the highest amount of phenols, followed by concord, ruby red and chardonnay muscadine and concord grape seed oil extracts were higher than chardonnay and ruby red.

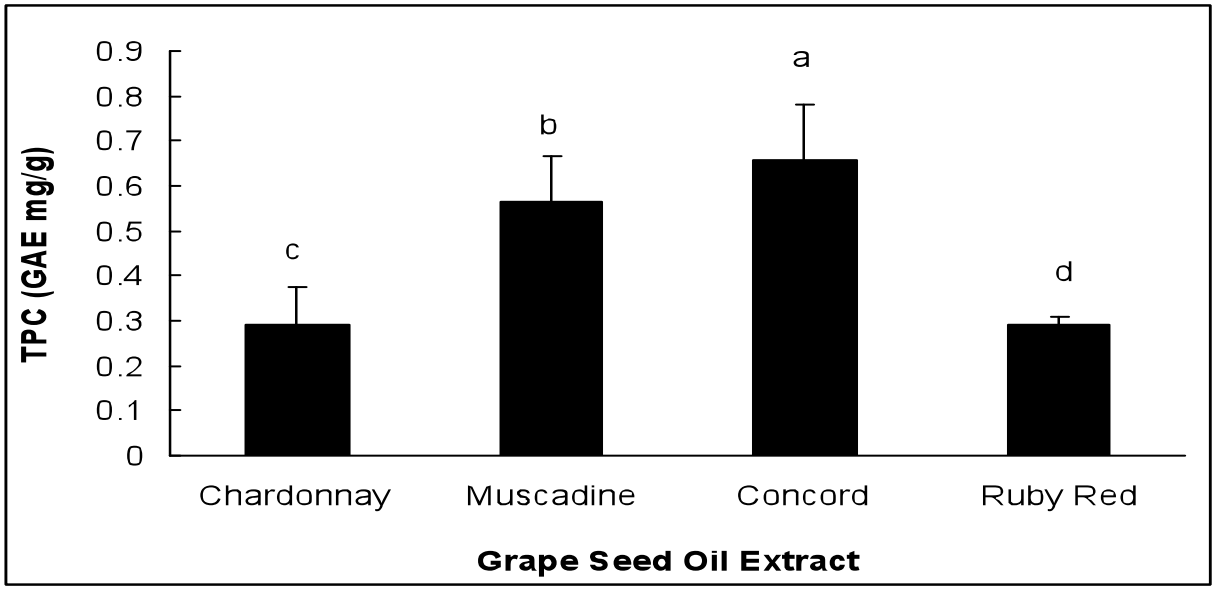

Figure 2. Total phenolic content of grape seed oil extracts. Data are expressed in gallic acid equivalents (GAE) as means ± standard deviations (n =3). Columns marked with the same letter are not significantly different ($P < 0.05$).

The comparison of phenolic content found in grape seed flour extract with grape seed oil extract clearly shows that phenolic content is much higher in the flour extracts. All flour samples were found to contain statistically different levels of TPC. This is true of the oil extracts as well. Additionally, the levels of chardonnay flour extract total phenolic content resemble previously published chardonnay flour extracts values of 99.3 mg gallic acid equivalents per gram (Luther *et al 2007)*.

ABTS$^{\bullet+}$ Radical Scavenging Activity

The ABTS$^{\bullet+}$ radical quenching activity of the selected grapeseed flour and oil extracts was tested. Results are expressed as μmoles of trolox equivalents per gram of grapeseed oil or flour extract. All of the samples were able to quench ABTS$^{\bullet+}$.

ABTS$^{\bullet+}$ quenching activity for the grape seed flour extracts is represented in Figure 3. A statistically significant difference in radical scavenging activity existed among all of the extracts. Chardonnay demonstrated the highest level of ABTS$^{\bullet+}$ quenching capacity, followed by ruby red, concord and muscadine. The scavenging activity values ranged from 11-186 μmoles of trolox equivalents per gram of grape seed flour extract.

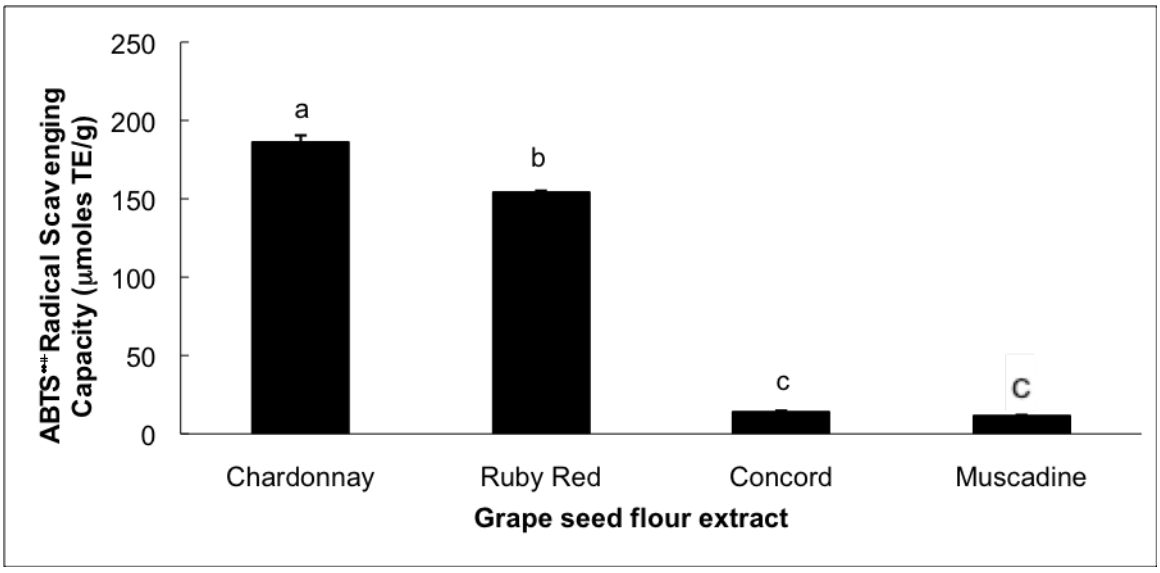

Figure 3. ABTS$^{\bullet+}$ Radical Scavenging Capacity of grape seed flour extracts.
Data are expressed as trolox equivalents as means ± standard deviations (n = 3). Values marked with the same letter are not significantly different ($P < 0.05$).

The grape seed oil extracts' ABTS$^{\bullet+}$ quenching activity is illustrated in Figure 4. The results indicate that the concord extract had the highest activity followed by

muscadine, chardonnay, and ruby red. The range of scavenging was .13-.38 μmoles of trolox equivalents per gram of grape seed oil extract.

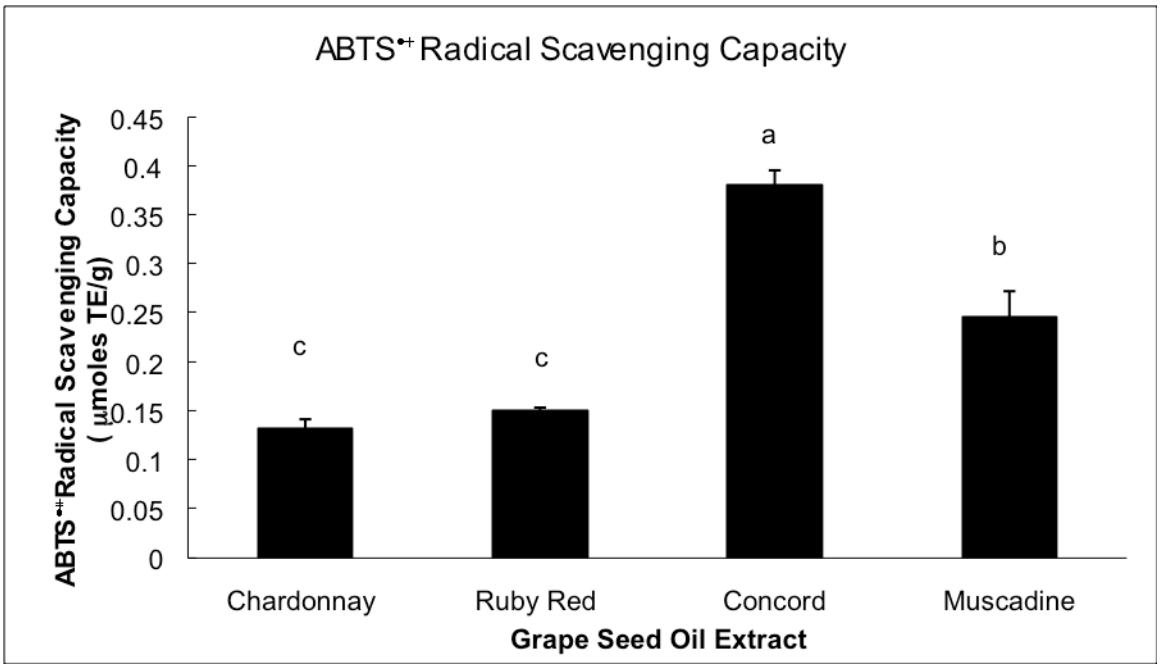

Figure 4. ABTS$^{\bullet+}$ Radical Scavenging Capacity of grapeseed oil extracts. Data are expressed as trolox equivalents as means ± standard deviations (n = 3). Values marked by the same letter in the same column are not significantly different ($P < 0.05$).

The flour extracts' radical scavenging activity far exceeds the levels obtained by the oil extracts. The data also revealed the levels of chardonnay and ruby red flour extracts to be nearly 1000 times more effective than their oil counterparts. The results of the ABTS$^{\bullet+}$ radical scavenging assay correlates to the values gathered from the total phenolic content.

DPPH$^{\bullet}$ Radical Scavenging

The selected grape seed flour and oil extracts were evaluated for DPPH$^{\bullet}$ radical quenching activity. Results are expressed as μmoles of trolox equivalents per

gram of grape seed oil or flour extract. All of the samples were able to quench the DPPH• radical.

The DPPH• scavenging activity for the grape seed flour extracts is represented in Figure 5. Statistically significant differences were shown among the different varieties for DPPH• scavenging activity. However, the concord and muscadine were similar. Chardonnay demonstrated the highest level of DPPH• radical quenching activity, followed by ruby red, concord and muscadine. The scavenging activity values ranged from 11-38 μmoles of trolox equivalents per gram of grapeseed flour extract. The flour samples had to diluted to obtain values that would fit within the trolox standard curve.

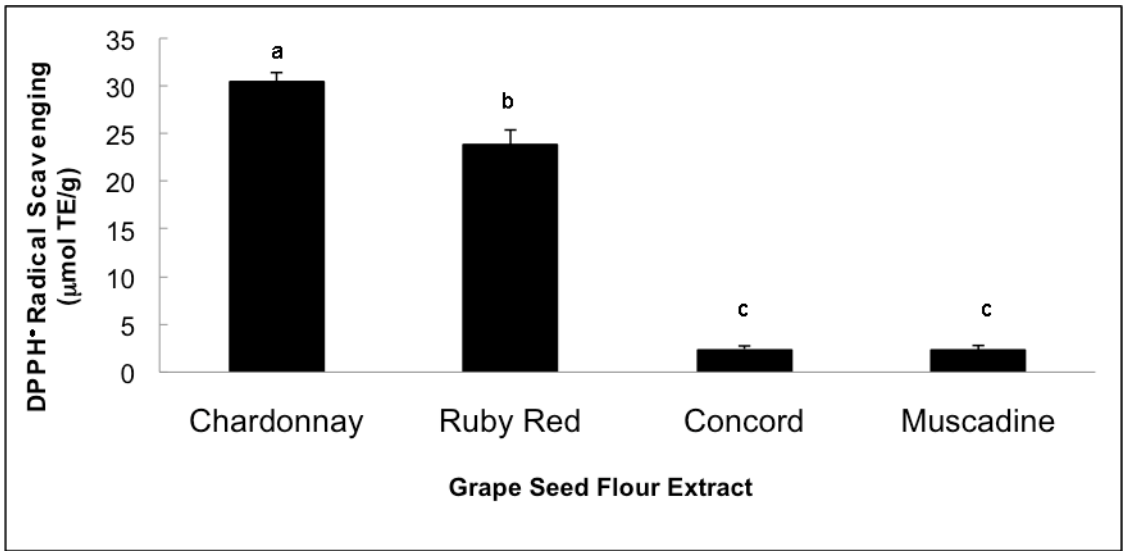

Figure 5. DPPH• Radical Scavenging Capacity of selected grape seed flour extracts. Data are expressed as trolox equivalents as means ± standard deviations (n = 3). Values marked with the same letter are not significantly different ($P < 0.05$).

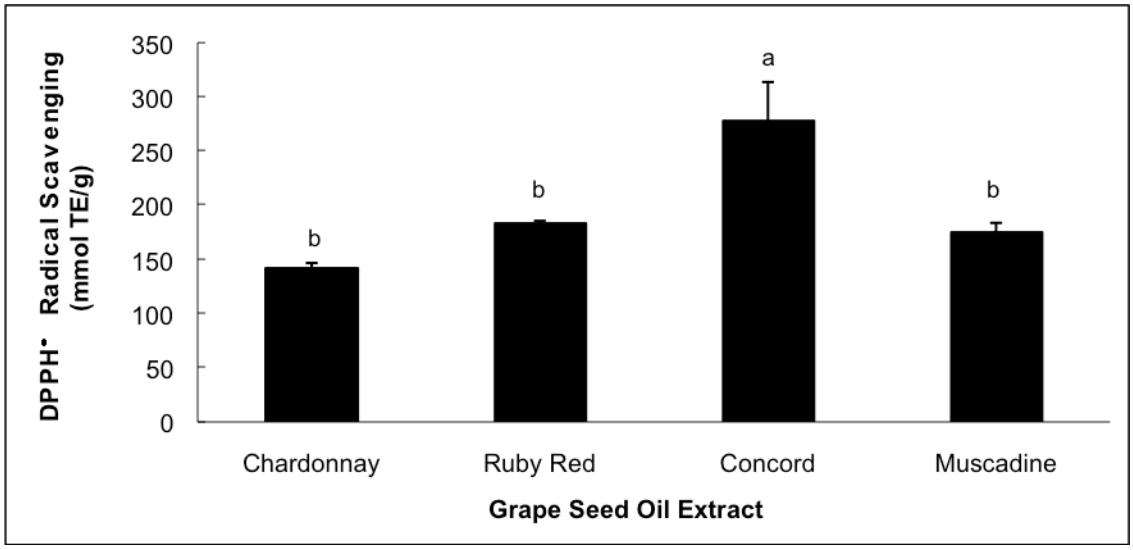

Figure 6. DPPH• Radical Scavenging Capacity of selected grape seed oils extracts. Data are expressed as trolox equivalents as means ± standard deviations (n=3). Values marked with the same letter are not significantly different ($P < 0.05$).

The results of DPPH• radical quenching activity for the grape seed oil extracts indicate that concord had the highest activity (Figure 6). Concord activity levels were followed by ruby red, muscadine and chardonnay. The range of scavenging was 141-278 μmoles of trolox equivalents per gram of grapeseed oil extract. All of the grapeseed oil extracts had to be diluted to fit within the standard curve generated by trolox.

Chardonnay flour extract was approximately 200 times more effective than its oil counterpart in quenching the DPPH• radical. The ruby red flour extract was 130 percent more effective than the ruby red oil extract in quenching the DPPH• radical.

Anti-proliferation Activity

This study was conducted to evaluate the inhibitory cell growth properties of the selected grape seed oil and flour extract. The percent of inhibition was measured by luminescence and compared to control. Higher values indicate higher cell

numbers. The percent inhibition is indicative of lower luminescence values from the extracts which is showing lower cell numbers compared to control.

At the 24 hour time point (Figure 7) with the exception of concord, all of the flour extracts inhibited HT-29 cell growth. A significant difference between percent inhibition of HT-29 cells was found to exist between the high dose of chardonnay and muscadine. No other significant differences were noted between the type of flour extract or the dose.

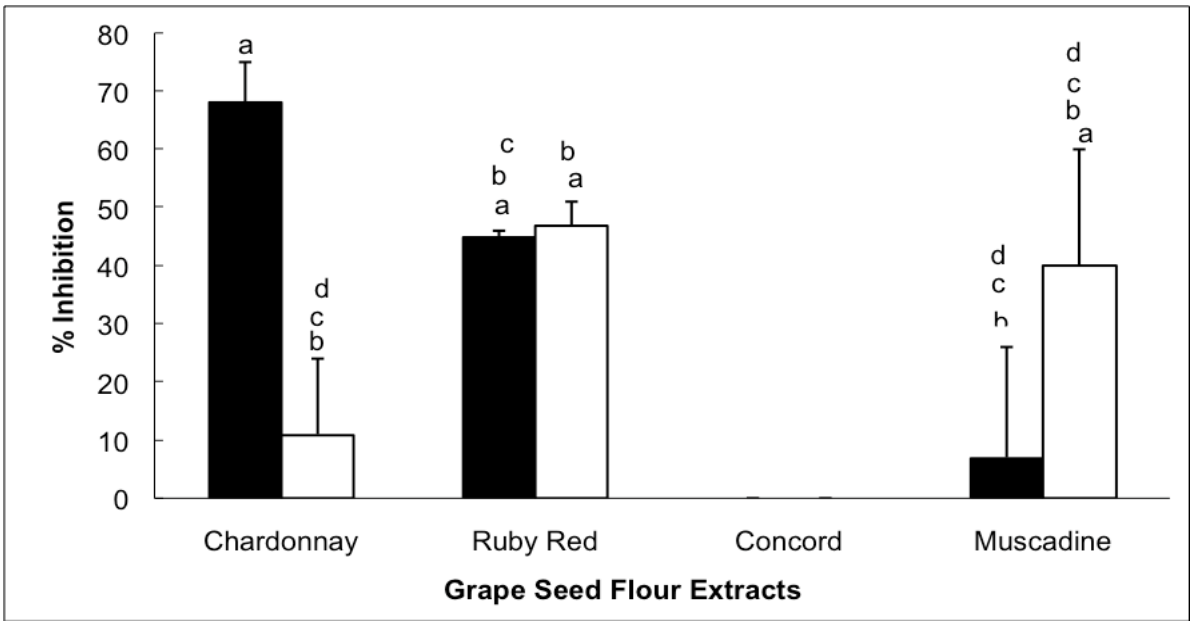

Figure 7. Anti-proliferative activity of selected grape seed flour extracts. Data are expressed in percent inhibition when compared with the control as means ± standard deviations (n = 3). Solid bar represents 0.6 mg/mL. Open bar represents 0.06 mg/mL. Values marked by the same letter are not significantly different ($P < 0.05$). The larger the bar, the greater the percent of HT-29 cell inhibition.

The chardonnay grape seed flour extract exhibited the greatest dose and time dependent inhibition against HT-29 cell proliferation under the experimental conditions (Figure 8). A statistically significant difference was demonstrated by the chardonnay flour extract at the high dose at the points 4 and 96 hours. No other significant differences were shown.

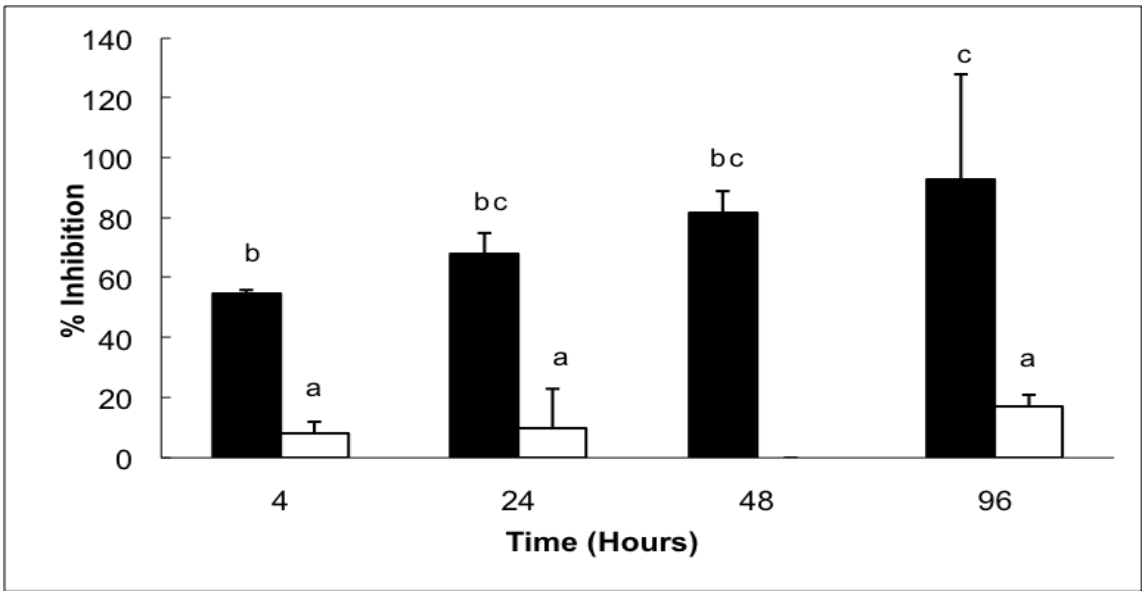

Figure 8. Percent Inhibition of Chardonnay Flour Extract. Data are expressed in percent inhibition when compared with the control as means ± standard deviations (n = 3). Values marked by the same letter in the same column are not significantly different ($P < 0.05$). Solid bars represent 0.6 mg/mL. Open bars represent 0.06 mg/mL. The larger the bar, the greater the percent of HT-29 cell inhibition.

The grape seed oil extracts showed little or no inhibition of HT-29 cell growth. Interestingly, a previous study using chardonnay grape seed extract prepared in 50% acetone showed no inhibition when treating HT-29 cells (Parry *et al* 2006).

Conclusion

Results from this study indicate that oil and flour extracts obtained from the chardonnay, ruby red, concord and muscadine grape seeds may act as a means of colorectal cancer prevention. Further testing is necessary to elucidate the specific components responsible for the cell growth inhibition. The results obtained from the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical quenching activity and HT-29 anti-proliferation activity may elucidate the importance of antioxidants in colorectal cancer prevention with the radical scavenging assay results.

The data gathered in the anti-proliferation assay shows a relationship to the data generated from the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and TPC assays. The values of percent inhibition draw a parallel with the results determined by the total phenolic content assay as well as the radical scavenging assays. It can be concluded, based on the anti-proliferation assay, the grape seed extracts containing the higher levels of antioxidants may be the most effective at inhibiting cellular growth of HT-29 human colorectal adenocarcinoma cells.

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Appendices

Glossary