DISSERTATION

INHIBITION OF HT-29 COLON CANCER CELL CULTURES BY EXTRACTS FROM BIODIVERSE GERMPLASM SOURCES OF *SOLANUM TUBEROSUM* L.

Submitted by

Tatiana Zuber

Department of Horticulture and Landscape Architecture

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Doctoral Committee:

Advisor: Cecil Stushnoff

Patrick F. Byrne
David G. Holm
Henry J. Thompson

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

INHIBITION OF HT-29 COLON CANCER CELL CULTURES BY EXTRACTS FROM BIODIVERSE GERMPLASM SOURCES OF *SOLANUM TUBEROSUM* L.

Potato *(Solanum tuberosum* L.*)* is the fourth largest consumed staple food crop in the world (Kang & Priyadarshan, 2007). Potentially bioactive classes of metabolites in potato, found by others to induce inhibitory activity in cancer cells *in vivo* and/or *in vitro* include dietary fiber, resistant starch, vitamin C, glycoalkaloids, flavonoids and polyphenolic acids. Colorectal carcinoma, cancer of the colon and rectum, is the third leading cause of cancer death in both men and women in the United States, the second leading cause of cancer death worldwide in women and the third leading cause of cancer death worldwide in men. Accordingly, high prevalence of potato consumption worldwide, as well as previous research that suggested bioactivity and possible inhibitory properties against colorectal cancer, rationalized this research as worthy of further investigation with great potential benefit for many.

A total of 13 clones of *S. tuberosum* L. were obtained from the Colorado Potato Breeding Program. The objectives of this PhD dissertation were to utilize these 13 clones obtained during several developmental stages in both raw and cooked forms to: (1) quantify the free radical scavenging capacity and total phenolic content; (2) compare HT-29 colon cancer cell culture inhibition efficacy of aqueous samples from freeze dried and cryogenically extracted samples of *Solanum tuberosm* L. *in vitro*; (3) develop a

novel method for quantitatively expressing cell viability based on a known reference standard curve; (4) screen tubers, flesh, peels and flowers for inhibitory activity against HT-29 colon cancer cells *in vitro*; (5) determine if the mechanism of inhibitory activity is related to apoptotic events *in vitro*; (6) explore possible explanations for observed enhanced inhibition associated with developmental stage in immature tubers by examining whether inhibitory metabolites exist only at the outset of tuber initiation and early development, and/or if mechanical and chemical vine kill practices prior to harvest are responsible for degradation of inhibitory metabolites; (7) gain insight as to what class of metabolites may be responsible for inhibitory activity of HT-29 colon cancer cells *in vitro* and to evaluate if the tuber matrix serves to protect these inhibitory metabolites.

Three to five biological replicates (median slices of peel and flesh from tubers and plant components from separate plants) from each clone at different developmental stages and in both raw and cooked modes were freeze dried (cryogenically ground in liquid nitrogen to meet objective in chapter 3), extracted in organic solvent and vacufuged to dryness to attain 60 $mg \cdot mL^{-1}$ potato extracts that were stored at -20 °C until needed for analyses. Total phenolic content was determined by the Folin-Ciocalteu method. Radical scavenging capacity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega was used to measure the number of viable HT-29 colon cancer cells in all cell culture assays. Apoalert® Caspase-3 Colorimetric Assay Kit is a colorimetric microplate assay that was utilized to detect caspase-3 protease activity. Analyses of

variance (ANOVA) were used to evaluate the main effects followed by Tukey-Kramer test to assess separation of means using SAS software.

Reporting results based on the devised acetylsalicylic acid standard curve allows for an easier, standardized and novel quantitative method for expressing number of viable HT-29 colon cancer cells in a colorimetric assay. Raw, pigmented and immature clones generally contained higher levels of phenolic antioxidants and radical scavenging capacity. Selections exhibiting the strongest inhibitory activity were obtained from raw, immature tuber extracts of CO97226-2R/R, CO97216-1P/P and CO04058-3RW red tissues at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium. Another potent source of inhibitory activity was obtained from potato peels and flowers of strongly inhibiting clones in addition to normally weak to nil inhibiting clones at extract concentrations of less than 3.6 $mg \cdot mL^{-1}$ cell culture medium. Extracts prepared via cryogenic grinding inhibited more strongly than extracts prepared via freeze drying preparation methods. Results of investigations of harvest/topkill treatment depended upon which medicinal attribute one wished to maximize, antioxidant or inhibitory properties. Inhibition in strongly inhibiting clones can be attributed to varying levels of caspase-3 protease activities depending on selection. Full to partial inhibitory activity retained in heated tubers at high temperatures of strongly inhibiting clones may be a result of heat stable glycoalkaloids. Reports regarding the presence of salicylic acid in potato leaves in addition to our recent finding regarding the similarities in both slope of inhibitory responses and range of extract concentrations inducing inhibitory responses shared by both acetylsalicylic acid and potato may suggest that salicylic acid or a compound similar to salicylic may exist in tubers and may also be responsible for

inhibitory responses. Higher inhibitory activity found in heated tubers verses heated extracts of strongly inhibiting clones may suggest that the tuber matrix may also have served to protect additional heat sensitive inhibitory metabolites from thermal degradation whereas inhibitory metabolites alone found in heated extracts did not have a tuber matrix to protect heat sensitive activity.

This study has acknowledged the significance of sample preparation methods, germplasm source, developmental stage, dosage, cooking, harvest/top kill methods, novel and successful cell viability quantification method and potential sources of bioactive compounds that exist in the worlds' fourth largest staple crop as it relates to cancer cell culture inhibition, antioxidant capacity and total phenolic content.

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unconditionally loving me every chance they could, I am most grateful to have you as my companions and best friends throughout my life.

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Chapter 1

INTRODUCTION

Cancer. Cancer is an epidemic affecting every region, race, and socioeconomic class across the globe (American Cancer Society, 2011a). The American Cancer Society estimates that in 2030 about 21.4 million new cancer cases will arise globally and 13.2 million of these cases are expected to result in death (American Cancer Society, 2011a). The American Cancer Society estimates that in the year 2011, about 1,596,670 Americans are expected to be diagnosed with cancer and 571,950 of these are expected to die of cancer (American Cancer Society, 2011a). Cancer accounts for 1 in every 8 deaths worldwide while in the United States alone cancer accounts for 1 in every 4 deaths making cancer the second leading cause of death in the United States after heart disease (American Cancer Society, 2011a).

Cancer can crudely be defined as a manifold of diseases where abnormal cells ensue, spread and proliferate uncontrollably (American Cancer Society, 2011a). If proliferation of these abnormal cells is not stopped, the irreversible fate of death is a great possibility (American Cancer Society, 2011a). In order for one to gain insight about cancer, it is crucial that one comprehends normal cell physiology, variations of genetic mutations, stages of tumor development, internal and external risk factors associated with cancer and the “hallmarks of cancer”.

The body is composed of an array of cells that are the basic building units of tissues, organs and living systems. As cells age or become damaged, cells must give rise to genetically identical new cells in order to maintain a healthy living system. This

occurs by means of the cell cycle, composed of four distinct phases (G1, S, G2, M) and three checkpoints where the first checkpoint (G1) ensures that the genetic material of the cell is not damaged and ready for DNA synthesis, the second checkpoint (G2) ensures that DNA is replicated properly and the last checkpoint (M) ensures that the spindle fibers have assembled and chromosomes have aligned (Liber, 2009).

Carcinogenesis is the process by which a normal cell undergoes a series of genetic mutations and is ultimately transformed into a cancer cell (National Cancer Institute, 2006a). Liber (2009) defines and describes characteristics and consequences of a mutation. A mutation may be defined as any change in the linear sequence of DNA. Mutations may be genetic, chromosomal or spontaneous. A genetic mutation may result from a transition or transversion type point mutations and/or an insertion or deletion of three or more nucleotides. Consequences that may culminate as a result include an amino acid substitution, splicing errors, frame shift mutations, regulatory changes, no detectable change and lastly the formation of premature termination codons ending mRNA transcripts too early, thus affecting the function of the final protein product. Types of chromosomal mutations include multilocus deletions, translocations and interchromosomal homologous recombination. Consequences of multilocus deletions include gene dosage effects or the unmasking of recessive alleles. Translocations may lead to chromosomal rearrangement and non-normal fusion resulting in karyotype alterations. Interchromosomal homologous recombination can unmask recessive alleles which may lead to the loss of heterozygosity or what is understood to be the “two hit theory” explained in detail later under combined internal and external risk factors section (Fig. 1.2). Severe chromosomal aberrations can be

lethal. Spontaneous mutations may arise from tautomeric shifts where nucleic acid bases shift between keto and enol forms and/or amino and imino forms or spontaneous isomerization of a nitrogen base to an alternative hyrdogen-bonding form may occur.

The National Cancer Institute of Health (1999) describes tumor development and staging. Tumor development is believed to be monoclonal in nature where one single cell undergoes a genetic mutation and all of the cells within that tumor are direct progenies of that specific cell (Fig. 1.1). The stages of tumor development begin when a cell inherits a genetic mutation (Fig. 1.1). This single genetically altered cell then proliferates into a stage termed hyperplasia (Fig. 1.1). A single hyperplastic cell may then undergo another genetic mutation initiating these cells to proliferate and appear morphologically abnormal (Fig. 1.1). This stage of tumor development is termed dysplasia (Fig. 1.1). The final stage of tumorogenesis is termed either *in situ* cancer or an invasive cancer. This final stage is reached when a single dysplastic cell undergoes yet another genetic mutation, which exhibits extremely abnormal characteristics both morphologically and in the way it rapidly proliferates (Fig. 1.1). If the tumor that has descended from a dysplatic cell that is still located within the tissue where it originated then it is termed *in situ* cancer (Fig. 1.1). If the tumor that has descended from a dysplastic cell has undergone additional mutations enabling angiogenesis and metastasis (defined later in Chapter 1), then the stage of tumor development is termed a malignant and/or invasive cancer (Fig. 1.1).

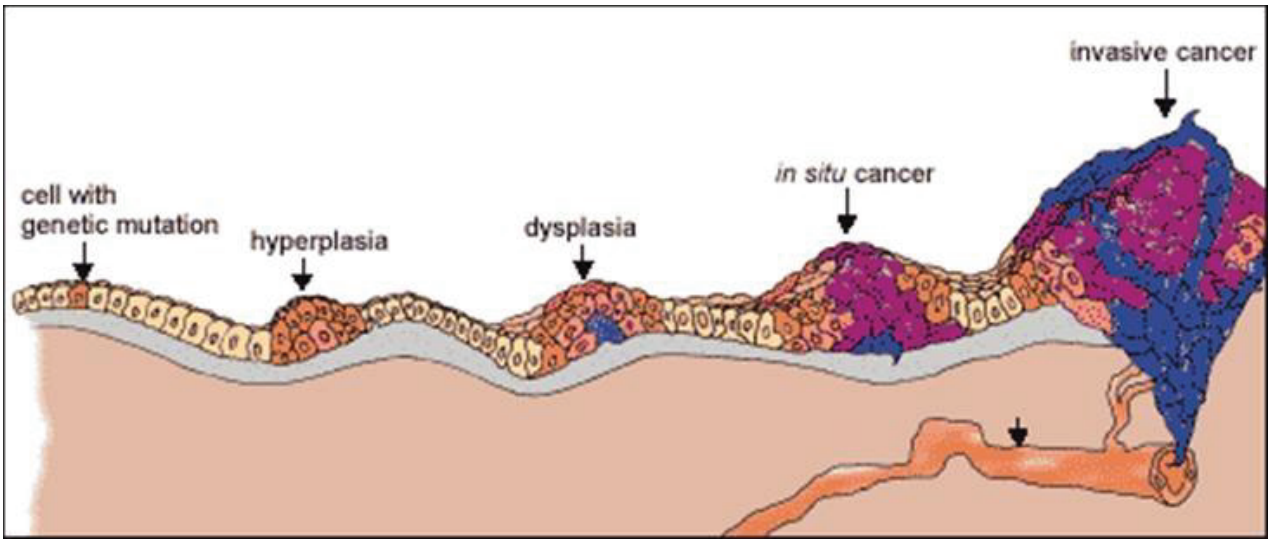

Figure 1.1 The stages of tumor development (National Cancer Institute of Health, 1999).

The causal agents that induce genetic mutations and lead to the induction of invasive cancers can be a result of external or internal factors or a combination of both. The following information is an overview of external and internal factors that have been implicated to be cancer causing agents. Types of external factors include but are not limited to: foods, chemicals/environment, infectious agents, radiation, tobacco, physical inactivity and obesity. Types of internal factors include but are not limited to both familial traits and genetic predispositions.

Certain chemicals found in foods serve as vehicles for the introduction of carcinogens into our body**.** Heterocyclic amines and polycyclic aromatic hydrocarbons are chemicals formed when muscle meat is cooked at high temperatures proven to be mutagenic (National Cancer Institute, 2010a). Certain types of foods heated to temperatures above 120 °C and cigarette smoke are the major sources of exposure to the mutagen acrylamide which was found to be a “mutagen” and “probable human

carcinogen" but only in laboratory animal studies (National Cancer Institute, 2008b). It is unknown if acrylamide levels found in certain foods are actually harmful to humans (National Cancer Institute, 2008b). Fluoridated water and artificial sweeteners have both been implicated in the past as being potentially carcinogenic but overall studies executed on laboratory animals and humans provide no clear evidence (National Cancer Institute, 2008a; National Cancer Institute, 2012c).

Exposures to specific chemicals and the environment also are the cause of many cancers. The U.S. Department of Health and Human Services, the EPA, and the International Agency for Research on Cancer have characterized asbestos to be a human carcinogen (National Cancer Institute, 2012a). Exposure to certain pesticides such as imazethapyr, the insecticide dieldrin, the fumigant mixture carbon-tetrachloride/carbon disulfide, the fungicide chlorothalonil, and the herbicide atrazine suggest an increased risk of attaining cancer (National Cancer Institute, 2011a). The Department of Health and Human Services (DHHS) has found that long term exposure to benzene in the air may result in leukemia (Center for Disease Control and Prevention, 2006). International Agency for Research on Cancer has classified formaldehyde as a known human carcinogen and the U.S. Environmental Protection Agency has classified it as a probable human carcinogen (National Cancer Institute, 2011b).

Infectious agents include specific parasites, viruses or bacteria that may cause prolonged inflammation, suppression of the immune system or directly affect DNA increasing the risk factors for certain types of human cancers (American Cancer Society, 2011c). The Human Papilloma Virus (HPV) is a group of more than 150 related

viruses that is often a sexually transmitted disease that may cause warts or papillomas, which are benign tumors (National Cancer Institute, 2011d). Low risk and high risk types of HPV both exist, but only persistent infections with high risk types of HPV can cause cell abnormalities that can lead to the development of certain cancers (National Cancer Institute, 2011d). *Helicobacter pylori* are bacterium that may be transmitted to the mucus of the stomach via consumption of contaminated food and water and direct mouth to mouth contact (National Cancer Institute, 2011c). *H. pylori* has been classified as a carcinogen by the International Agency for Research on Cancer and epidemiological studies have confirmed that infection with this bacterium leads to increased risk factors for gastric type cancers (National Cancer Institute, 2011c).

Radiation may simply be defined as “energy traveling through space” (Occupational Safety and Health Administration, 2008). Radiation can be broken up into two primary categories, ionizing radiation and nonionizing radiation (Occupational Safety and Health Administration, 2008). Types of damaging ionizing radiation may include radioactive fallout, radon gas or x-ray’s that carry sufficient energy that may ionize atoms and cause destabilization of molecules in cells leading to cell death, changes in cell reproduction and division, or induction of mutations that may ultimately cause increased risks for certain types of cancers (Occupational Safety and Health Administration, 2008; Curtis, 1999; National Cancer Institute, 2006b). Nonionizing radiation spans the electromagnetic spectrum beginning with extremely low frequency (ELF) and ending at ultraviolet (UV) radiation (Occupational Safety and Health Administration, 2008). Exposure to ultraviolet radiation can cause premature aging of the skin or damage to the skin that may lead to cancers of the skin (Curtis, 1999).

Unhealthy lifestyle choices appear to be the culprit of the development of many types of cancers. Lung cancer is the number one cause of death in both men and women (National Cancer Institute, 2010b). Approximately 90% of men and 80% of women in the United States who have died of lung cancer, have died as a result of smoking tobacco (National Cancer Institute, 2010b). About 3,400 lung cancer deaths annually are attributed to secondhand smoke in the United States (Center for Disease Control and Prevention, 2012). Smokeless tobacco comprises 28 different types of carcinogens and increases the risk of developing cancer of the oral cavity (Center for Disease Control and Prevention, 2012). A lack of physical activity and obesity has also shown a strong correlation toward the development of several types of cancer to include cancer of the breast, endometrium, colon, kidney and esophagus (National Cancer Institute, 2011f). Approximately 14% of men and 20% of women in the United States were recently reported to die of cancer as a result of excess weight and obesity (National Cancer Institute, 2011f).

Familial traits and genetic predispositions to cancer are both internal type factors that may contribute to invasive malignant cancers (National Cancer Institute, 2012b). Familial traits as they relate to cancer may have either a genetic or nongenetic etiology and are found to occur in larger frequencies within a particular family than the overall population (National Cancer Institute, 2012b). A genetic predisposition with regards to cancer may be defined as an increased probability that a person will develop a cancer attributable to the existence of one or more gene mutations and/or a family history indicative of having higher frequencies of cancer (National Cancer Institute, 2012b). A genetic predisposition to cancer and/or familial cancers may ensue via three patterns of

genetic inheritance within families (National Cancer Institute, 2012b; University of Vermont, 2001). Autosomal dominant inheritance refers to the heterozygous genetic condition where a person carries a mutation in only one copy of a certain gene (National Cancer Institute, 2012b; University of Vermont, 2001). Thus affected males and females have a 50% probability of passing on the mutation to their offspring (University of Vermont, 2001). Autosomal recessive inheritance refers to either the homozygous genetic condition where a person carries a mutation in both copies of a certain gene or the compound heterozygous condition where a person carries two different mutations within the same gene (National Cancer Institute, 2012b; University of Vermont, 2001). Thus, when two carriers mate, their progeny have a 25% chance of being homozygous wild-type (unaffected), a 25% chance of being homozygous mutant (affected) or a 50% chance of being heterozygous (unaffected carrier) (University of Vermont, 2001). X-linked trait inheritance may be recessive or dominant and refers to the genetic condition where a mutation occurs on a gene whose locus resides on the X-chromosome (University of Vermont, 2001). X-linked recessive traits are not expressed when one wild type copy of the gene is present. X-linked dominant traits in contrast are expressed when one copy of the mutant allele is present (University of Vermont, 2001).

External and internal factors may combine to display what is termed a multifactorial inheritance pattern where genetic factors and environmental factors work together to manifest the onset of an invasive malignant cancer. This multifactorial inheritance can be best explained by Alfred G. Knudson's Jr., MD, PhD, "two hit theory" of cancer causation (Fox Chase Cancer Center, 2004) (Fig. 1.2).

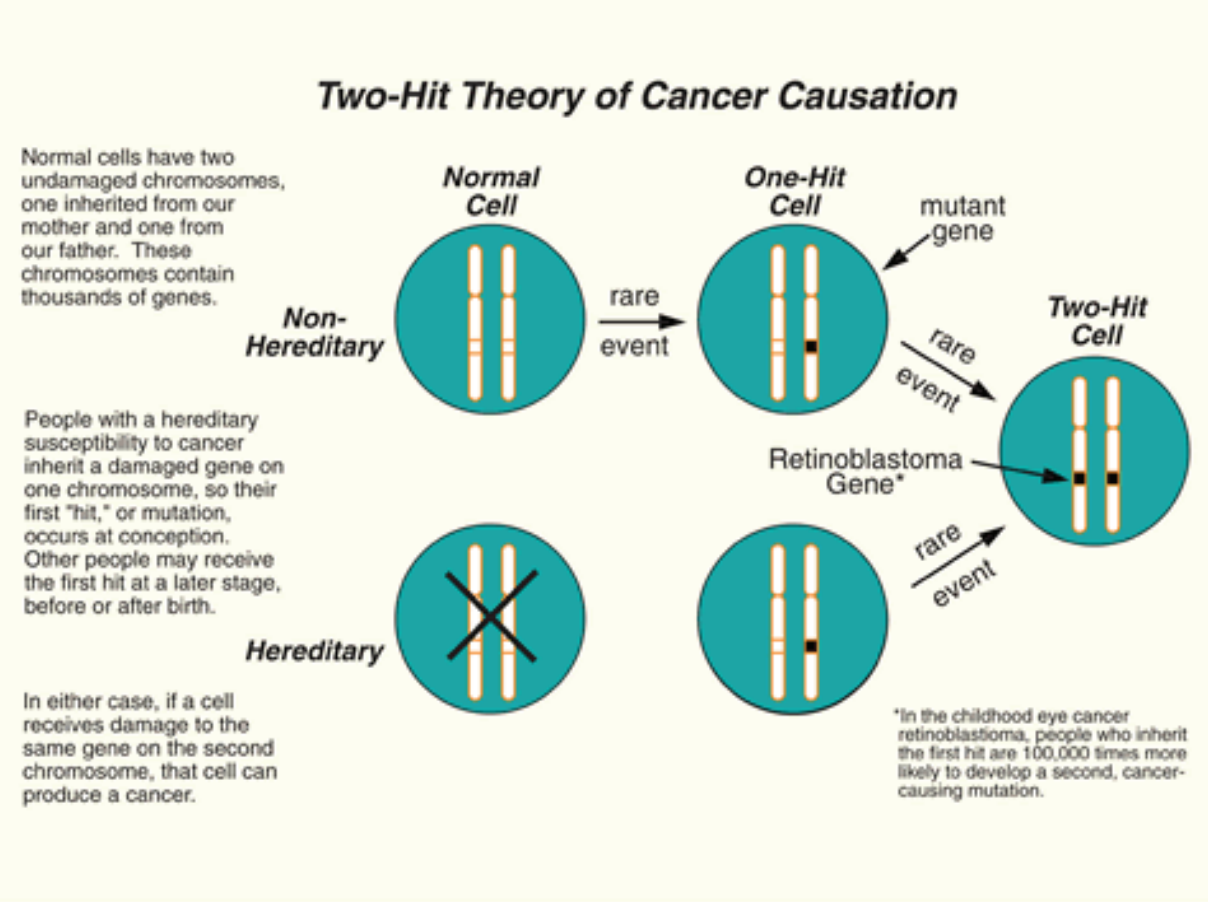

Figure 1.2 Two-hit theory of cancer causation (Fox Chase Cancer Center, 2004).

The comprehension of normal cell physiology, variations of genetic mutation, stages of tumor development and causal agents of cancer finally allow for a more in depth examination at the progression of carcinogenesis. Douglas Hanahan and Robert A. Weinberg suggest that there exist six modifications in cell physiology that collectively, but not necessarily in chronological order, dictate malignant growth entitled the "Hallmarks of Cancer" (Hanahan & Weinberg, 2000).

The first acquired capability Hanahan & Weinberg (2000) describe is "Self Sufficiency in Growth Signals". Three molecular mechanisms for achieving this

acquired capability include the alteration of extracellular growth signals, the alteration of transcellular transducers of those growth signals and lastly the alteration of the intracellular circuits that translate those signals into action. A specific example depicting this acquired capability involves cell surface receptors composed of tyrosine kinase domains which serve to carry growth factor signals into a cell's interior. These domains have been found to be overexpressed in cancer cells allowing cancer cells to become overly responsive to ambient levels of growth factors in the extracellular matrix.

The second acquired capability Hanahan & Weinberg (2000) describe is "Insensitivity to Antigrowth Signals". Antigrowth signals include soluble growth signal inhibitors and immobilized inhibitors that can be found in the extracellular matrix as well as on the surface of neighboring cells that function to keep cells in the quiescent state or in the post mitotic state of the cycle. A specific example of how a cancer cell is able to evade antigrowth signals is by allowing the soluble signaling molecule TGFβ to disrupt the phosphorylation of retinoblastoma protein (pRB) which under normal circumstances when hyperphosphorylated functions to block E2F transcription factors that control genes that allow a cell to advance from G1 into S phase of the cell cycle.

The third acquired capability Hanahan & Weinberg (2000) describe is "Evading Apoptosis". Apoptosis, as it is defined by the Dorland's Medical Dictionary, is "a pattern of cell death affecting single cells, marked by shrinkage of the cell, condensation of chromatin, and fragmentation of the cell into membrane-bound bodies that are eliminated by phagocytosis; often used synonymously with programmed cell death" (Saunders, 2007). Hanahan & Weinberg (2000) explain that sensors and effectors are the contrivances involved in the initiation and execution of apoptosis. Sensors are

receptors that function to supervise anomalies that may exist within the extracellular and intracellular environment of the cell and ultimately determine if a cell is robust or damaged and must die (Hanahan & Weinberg, 2000). Consequently, when a molecule, favorable or unfavorable to the cell, attempts to cross the plasma membrane, an extracellular sensor will respond by signaling for either a survival or a death ligand to bind to its corresponding cell surface receptor and elicit a response to live or die (Hanahan & Weinberg, 2000). Specific survival ligand/receptor pairs include IGF-1/IGF-2 ligands binding to IGF-1R receptor and IL-3 ligand binding to its corresponding receptor IL-3R (Hanahan & Weinberg, 2000). Specific death ligand/receptor pairs include FAS ligand binding to FAS receptor and TNFα ligand binding to TNF-R1 receptor (Hanahan & Weinberg, 2000). Conversely, when an anomaly is detected within the cell, such as DNA damage or hypoxia for example, an intracellular nuclear receptor will signal the apoptotic pathway (Hanahan & Weinberg, 2000; Weinberg, 2007). The apoptotic signals elicited from the sensors are then transmitted to the second contrivances, the effectors, which are the regulatory proteins and enzymes that function to execute apoptosis either indirectly through the mitochondria or via direct signal transduction (Hanahan & Weinberg, 2000). Many of the sensor signals that are transduced to the effectors occur indirectly through the mitochondria where either increased membrane permeability activates SMAC, or proapoptotic regulatory proteins activate the release of cytochrome C, where in both scenarios catalyzes an array of caspase enzymes to kill the cell or contrariwise antiapoptotic regulatory proteins to prevent the activation of the caspase enzymes allowing the cell to live (Hanahan & Weinberg, 2000; Liber, 2009; Weinberg, 2007). Direct signal transduction occurs via

the sensors where the binding of the FAS death ligand binds to the FAS receptor activating the release of the FADD protein or the TNFα death ligand binding to the TNF-R1 receptor activating the release of the TRADD protein which activates the release of the FADD protein, where in both scenarios catalyze the release of the caspase 8 enzyme to kill the cell (Liber, 2009; Weinberg, 2007). Cancer cells have acquired the capability to evade apoptosis through a variety of mechanisms to include loss of function to the ligand/ receptor sensors of survival signals, death signals and most notably the loss of function of the regulatory protein p53 tumor suppressor gene inhibiting the release of cytochrome C thus inhibiting the release of caspase enzymes (Hanahan & Weinberg, 2000).

The fourth acquired capability Hanahan & Weinberg (2000) describe is “Limitless Replicative Potential”. Robert A. Weinberg, author of The Biology of Cancer, defines telomeres as protective nucleoprotein structures at the end of a eukaryotic chromosome that protects this end from degradation and from fusion with other chromosomes (Weinberg, 2007). Telomeres are composed of hexanucleotide DNA sequences that are tandemly repeated thousands of times (Weinberg, 2007). One strand of the telomere is composed of the following guanosine rich DNA sequence 5’-TTAGGG-3’ while the complimentary strand is composed of the following cytosine rich DNA sequence 5’-CCCTAA-3’ (Weinberg, 2007). The guanosine rich strand is slightly longer by approximately one hundred to several hundred nucleotides than the cytosine rich strand resulting in a single strand overhang forming a unique T-loop molecular configuration that may serve to shield the ends of linear DNA by inserting the end of the single stranded overhang region into the double strand region positioning it out of

harm's way (Weinberg, 2007). Specific proteins bind to hexanucleotide specific domains found on telomeric DNA forming the nucleoprotein complexes known as telomeres (Weinberg, 2007). TRF1 complexes are nucleoprotein complexes involved in controlling telomere length (Weinberg, 2007). TRF2 complexes are nucleoprotein complexes involved in the protection of telomeric DNA and are known to play a part in the reparation of genomic damage (Weinberg, 2007). When a normal cell is in need of replication it enters the cell cycle and during the S phase the DNA is replicated (Weinberg, 2007; Walrond, 2008). In order for DNA replication to commence, RNA primase synthesizes the first few nucleotides on the leading strand a substantial number of bases from the parental strand 3'end providing a free 3'-hydroxyl end for DNA polymerase to bind to and add the complimentary bases synthesizing a continuous new leading strand in the 5' to 3' direction (Weinberg, 2007; Walrond, 2008). The lagging strand grows discontinuously creating okazaki fragments in the opposite direction (Weinberg, 2007; Walrond, 2008). Synthesis of the telomeric DNA in the lagging strand is completed without problems but synthesis of the telomeric DNA on the leading strand synthesis presents two major problems known as the end-replication problem (Weinberg, 2007). The first problem encountered during leading strand synthesis occurs when RNA primase synthesizes the primer a considerable number of bases form the parental 3'end resulting in under replication of the parental strand (Liber, 2009; Weinberg, 2007). The second problem that is encountered exists at the completion of the leading strand elongation where the RNA primer that was used to initiate leading strand synthesis is degraded after it has served its purpose resulting once again in under replication of the parental strand (Liber, 2009; Weinberg, 2007). Thus, every time

a normal cell passes through the cell cycle, it results in the shortening of the telomeric DNA, consequently making mammalian cells mortal (Liber, 2009; Weinberg, 2007). Cancer cells have acquired the ability to evade mortality via two mechanisms in mammalian cells. The first mechanism is termed ALT (Alternatively Lengthening Telomeres) where telomere length is maintained via recombination-based interchromosomal exchanges of sequence information (Hanahan & Weinberg, 2000). The second mechanism is via the enzyme telomerase whose function is to extend telomeric DNA (Weinberg, 2007). Telomerase holoenzyme is composed of two essential subunits, hTERT and hTR (Liber, 2009; Weinberg, 2007). hTERT is the Reverse Transcriptase Catalytic Subunit that is only expressed under normal cellular conditions in germ lines and highly proliferative tissues (Liber, 2009; Weinberg, 2007). hTR is the subunit that under normal cellular conditions is the RNA template of the holoenzyme that is highly expressed in all cells (Liber, 2009; Weinberg, 2007). Normal human cells are mortal because they carry the genes for telomerase enzyme but lack significant expression of hTERT subunit (Liber, 2009; Weinberg, 2007). Thus, cancer cells have acquired the capability to express both essential subunits of telomerase holoenzyme or alternatively use the ALT mechanism, permitting cancer cells to retain the ability to infinitely protect its telomeric DNA, subsequently resulting in “immortality” and/or “Limitless Replicative Potential” (Hanahan & Weinberg, 2000; Liber, 2009; Weinberg, 2007).

The fifth acquired capability Hanahan & Weinberg (2000) describe is “sustained angiogenesis”. The vasculature system provides oxygen and nutrients essential for a cells continued existence and also allows for the elimination of metabolic waste

products and carbon dioxide from cells (Hanahan & Weinberg, 2000; Weinberg, 2007). Cells must exist within 200 μm of a capillary blood vessel, the distance limit for oxygen diffusion to transpire, or they will become hypoxic and in turn necrotic signaling apoptotic death pathways (Thamm, 2009; Weinberg, 2007). Research studies have proven that tumors are unable to continue to grow if they reside more than 1-2 mm away from capillary blood vessels (Thamm, 2009; National Cancer Institute, 2006c). Angiogenesis may be defined as the process by which new blood vessels are formed from preexisting ones (Weinberg, 2007; Thamm, 2009). Tumor angiogenesis is initiated when tumor cells release activator signaling molecules activating genes in the surrounding host tissue to promote the in growth of blood vessels into tumor cells (National Cancer Institute, 2006c). Approximately a dozen activator and inhibitor signaling molecules have been identified and are listed in the figures below, but this list is not exhaustive (National Cancer Institute, 2006c) (Fig. 1.3). The balance between the concentration of activator signaling molecules versus the conentration of inhbitor signaling molecules dictates the fate of the anigogenic process (Thamm 2009; National Cancer Institute, 2006c).

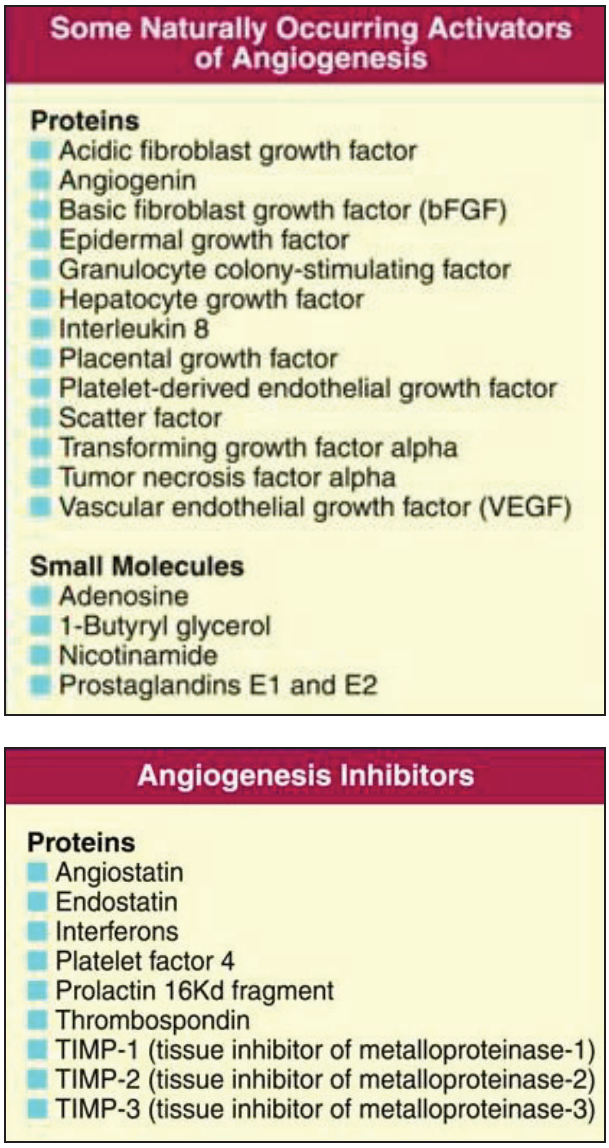

Figure 1.3 Activators and inhibitors of angiogenesis (National Cancer Institute, 2006c).

An example of how high concentrations of activator molecules allow tumor angiogenesis to proceed is as follows. Von Hippel-Lindau syndrome is a genetic predisposition that gives rise to a variety of tumors as the direct result of a germ line mutation in the VHL gene allowing HIF-1 subunits to activate angiogenic activator signaling molecules (Weinberg, 2007). The VHL gene produces pVHL, which is

responsible for the degradation of the alpha subunit of hypoxia-inducible factor-1 (HIF-1) (Weinberg, 2007). HIF-1, composed of alpha and beta subunits, is a transcription factor that is able to bind to genes that endcode for angiogenic activator singaling molecules, VEGF, PDGF and TGF-α activator (Weinberg, 2007). When the VHL gene is not mutated and normoxic conditions exist within a cell, the HIF-1 transcription factor is degraded, thus activation of angiogenic activator signaling molecules are hindered (Weinberg, 2007). Under hypoxic conditions or when the VHL gene is mutated, HIF-1 transcritption factors become highly concentrated and angiogenic activator signaling molecules are generated (Weinberg, 2007). VEGF, activated by HIF-1, and bFGF have been identified as the two most prominent proteins for upregulating angiognesis within numerous types of cancer cells as well as in normally functioning cells (National Cancer Institute, 2006c). Hypoxic tumor cells synthesize and release VEGF and bFGF proteins into the surrounding host tissue and bind to tyrosine kinase receptors located on the surface of endothelial cells (National Cancer Institute, 2006c). This binding activates the transcription, translation and release of matrix metalloproteases (MMP) into the extracellular matrix breaking down proteins and polysaccharides creating a paved pathway for edothelial cells to escape and migrate toward the hypoxic tumor cells (Thamm, 2009; National Cancer Institute, 2006c). Endothelial cells are then able to divide and organize into hollow tubes with the help of Ang-1 and Ang-2 eventually creating a vasculature system that can supply previously hypoxic tumor cells (Thamm, 2009; National Cancer Institute, 2006c). Thus, when angiogenic activator signaling molecules are able to tip the scales and supercede that of inhibitor signaling molecules, sustained angiogenesis will prevail (Thamm, 2009; National Cancer Institute, 2006c).

The sixth acquired capability Hanahan & Weinberg (2000) describe is “tissue invasion and metastasis”. This involves the movement of primary tumor cells to new parts of the body which typically progresses in the subsequent order: (1) invasion of local tissues; (2) intravasation of local lymph and blood vessel cell walls; (3) journey through cirulatory and lymphatic vessels to distant parts of body; (4) stopover in capillary followed by extravasation of capillary cell walls and migration of primary cancer cells into local tissue; (5) cell proliferation; (6) angiogenesis (National Cancer Institute, 2011e). The mechanisms governing tissue invasion and metastasis are extremely complex and not completely understand (Hanahan & Weinberg, 2000). The two most commonly identified mechanisms governing tissue invasion and metastasis involve: (1) the activation of extracellular proteases which allow cancer cells to invade local stroma, vessel walls and normal epithelial cell walls; (2) alterations in expression of anchoring junction transmembrane cell adhesion molecules (CAMs), particularly those that link cells to cells (cadherins and immunogloblins) and those that link cells to the extracellular matrix (integrins) (Fig. 1.4) (Walrond, 2007; Hanahan & Weinberg, 2000). Once a metastatic cancer arrives at a distant location it may lie dormant for several years and/or never commence proliferation. On the other hand, tissue invasion and metastasis has been the final aquired capability that has contributed to 90% of human cancer deaths (National Cancer Institute, 2011e; Hanahan & Weinberg, 2000).

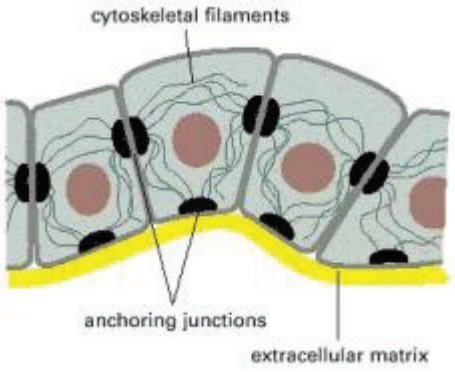

Figure 1.4 Simplified depiction of how anchoring junctions join cytoskeletal filament from cell to cell and from cells to the extracellular matrix (Alberts et al., 2002).

Colorectal Cancer. Colorectal carcinoma is the gastrointestinal cancer of the colon and rectum (American Cancer Society, 2011b). The digestive system is composed of several constituents whose primary function is to process food for energy and liberate the body of solid waste (American Cancer Society, 2011b). The journey of food begins via mastication in the mouth that continues its travels through the esophagus, stomach and small intestine which attaches to the first section of the colon, the ascending colon or large intestine (American Cancer Society, 2011b). The transverse colon occupies the second section of the colon which crosses the body from the right to the left side (American Cancer Society, 2011b). The descending colon is the third proceeding section of the colon that continues downward onto the left side of the human body (American Cancer Society, 2011b). The fourth and final section is the "S" shaped sigmoid colon that joins the rectum which links to the anus (American Cancer Society, 2011b).

Colorectal carcinoma is the third leading cause of cancer death in both men and women in the United States (American Cancer Society, 2011b). In 2011, the American Cancer Society estimates that approximately 141,210 new diagnoses will arise and 49,380 people are expected to die of this potentially preventable disease by means of cancer prevention awareness and early detection through routine screening tests (American Cancer Society, 2011b). The American Cancer Society statistics have shown that 90% of newly diagnosed colorectal cancer cases comprise individuals of the age of 50 or more (American Cancer Society, 2011b). The occurrence of colorectal cancer is 35% to 40% more frequent in men than in women, and is most prevalent in both African American men and women (American Cancer Society, 2011b). Approximately 5% of individuals affected with colorectal cancer have inherited one of two hereditary forms to include familial adenomatous polyposis (FAP) or hereditary non-polyposis colorectal cancer (HNPCC) (National Human Genome Research Institute, 2012). Those diagnosed with FAP have a nearly100% chance of developing colon cancer at an onset before the age of 40 (National Human Genome Research Institute, 2012). Those diagnosed with HNPCC have a nearly 80% risk probability of developing colorectal cancer throughout their lifetime (National Human Genome Research Institute, 2012).

There are a number of internal and external risk factors explicitly correlated with colorectal cancer, some of which are modifiable. Modifiable risk factors include physical inactivity, obesity, red meat consumption, smoking, alcohol (more than one drink per day), absence of supplemental multivitamin containing folic acid, absence of supplemental aspirin, lack of cancer screening and inadequate vegetable and fruit consumption (5 or more servings per day) (Liber, 2009; Stein & Colditz, 2004). Non-

modifiable risk factors include family history (first degree relative), inflammatory bowel disease (Crohn's disease, ulcerative colitis or pancolitis) and use of oral contraceptives or estrogen for five years or more (Liber, 2009).

Colorectal adenocarcinoma accounts for 96% of colorectal carcinomas where adenomatous polyps (adenomas), benign protrusions of the colonic mucosa, develop from glandular tissue into carcinomas (On-On Chan, 2002a; American Cancer Society, 2011b). A colonic adenoma may be morphologically designated as tubular, villous or tubulovillous (On-On Chan, 2002a). Tubular adenomas account for approximately 2-3% of invasive cancers (On-On Chan, 2002a). Tubulovillous adenomas account for 6-8% of invasive cancers (On-On Chan, 2002a). Villous adenomas account for the highest percentage of invasive cancers at approximately 10-18% (On-On Chan, 2002a). The manner that colorectal carcinomas invade the colon begins often by the formation of an adenomatous polyp in the large intestine which if not detected may transition from an adenoma to a carcinoma and proliferate through the mucosa, submucosa, muscles layers and serosa of the colon or rectum (American Cancer Society, 2011b). Complete penetration the colonic wall allows carcinomas to infiltrate blood vessels and lymphnodes gaining the acquired capability to induce angiogenic activities and metastasize colon cancer cells to remote segments of the body such as the liver, lungs abdominal cavity and so forth (American Cancer Society, 2011b).

The magnitude of the spread of the colorectal carcinoma within the body may be defined via two types of staging systems, TNM and SEER. The SEER staging system (Surveillance, Epidemiology and End Results) is normally used for descriptive and statistical analysis of tumor registry data (American Cancer Society, 2011b). The details

of this staging system have been directly quoted below from the American Cancer Society (2011b):

- **In situ**: Cancers that have not yet begun to invade the wall of the colon or rectum; these preinvasive lesions are not counted in cancer statistics.
- **Local**: Cancers that have grown into the wall of the colon and rectum but have not extended through the wall to invade nearby tissues.
- **Regional**: Cancers that have spread through the wall of the colon or rectum and have invaded nearby tissue, or that have spread to nearby lymph nodes.
- **Distant**: Cancers that have spread to other parts of the body, such as the liver or lung.

The (AJCC) TNM staging system is normally used in clinical settings and was developed by the American Joint Committee on Cancer (AJCC). The details of this alternative staging system have also been directly quoted by the American Cancer Society (2012):

- The **T** describes how far the main (primary) tumor has grown into the wall of the intestine and whether it has grown into nearby areas.
- **N** describes the extent of spread to nearby (regional) lymph nodes. Lymph nodes are small bean-shaped collections of immune system cells that are important in fighting infections.
- **M** indicates whether the cancer has spread (metastasized) to other organs of the body. (Colorectal cancer can spread almost anywhere in the body, but the most common sites of spread are the liver and lungs.)

Numbers or letters appear after T, N, and M to provide more details about each of these factors. The numbers 0 through 4 indicate increasing severity.

The carcinogenic process of colorectal cancer is driven by nonhereditary and acquired genetic alterations that transform normal healthy cells to abnormally

proliferating cancer cells. The derivation of genetic and epigenetic alterations attributing to colorectal cancer may be explained by tumor suppressor genes, oncogenes, mismatch repair genes, microsatellite instabilities and CpG island methylation (On-On Chan, 2002b). Collectively these epigenetic and genetic alterations converge to assemble the loss of heterozygosity (LOH) and replication error (RER) pathways of colorectal cancer (Fig. 1.5).

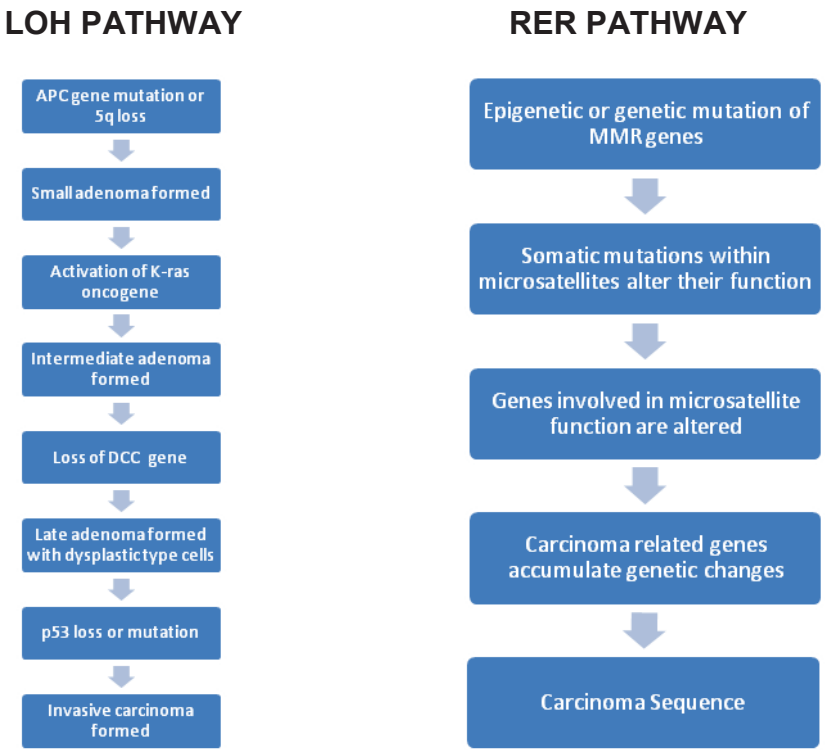

Figure 1.5 Tumor suppressor genes, oncogenes, mismatch repair genes, microsatellite instabilities and CpG island methylation constitute epigenetic and genetic alterations that may collectively converge to form the loss of heterozygosity (LOH) and replication error (RER) pathways resulting in colorectal cancer (Liber, 2009).

Tumor suppressor genes are responsible for keeping damaged cells from proceeding through the cycle and/or activating apoptotic pathways (Program on Breast Cancer and Environmental Risk Factors in New York State, 1997). Tumor suppressor genes no longer function as they should and may contribute to the progression of colorectal carcinomas when they undergo chromosomal mutations, experience a loss of heterozygosity or are deleted and/or inactivated (On-On Chan, 2002b). The imperative tumor suppressor genes that dictate progression in colorectal cancers include the loss of function of the APC (5q), p53 (17p), DCC/DPC/JV18 (18q) and MCC (5q) (On-On Chan, 2002b). APC gene mutations have been identified as being inherited as a dominant germline mutation that genetically predisposes an individual to colorectal cancer, particularly evident in individuals diagnosed with Familial Adenomatous Polyposis (FAP) (Liber, 2009; On-On Chan, 2002b). Chromosomal and genetic mutations of the vital p53 tumor suppressor gene, involved in stimulating apoptosis during the G1 stage of the cell cycle when it recognizes irreparable DNA, has been identified in nearly 75% of colorectal carcinomas to show a deletion of chromosome 17p on one allele and an inactivation point mutation on the p53 gene on the opposite allele (Liber, 2009; On-On Chan, 2002b). Chromosomal mutations of the DCC tumor suppressor genes have been identified in nearly 70% of colorectal adenocarcinomas and 50% of large adenomas to have a segment of chromosome 18 absent (Liber, 2009; On-On Chan, 2002b). Genetic mutations of the MCC tumor suppressor gene located on chromosome 5q have led to loss of function and have been identified in 20-50% of colorectal adenocarcinomas and 30% of adenomas (On-On Chan, 2002b).

Oncogenes are genes that have the potential to transform a cell and render it cancerous (Weinberg, 2007). K-ras is the principal oncogene associated in contributing to colorectal cancer. K-ras typically subsists in an unexpressed state but when activated stimulates abnormal cell proliferation (On-On Chan, 2002b). Genetic mutations have been identified in 50% of large adenomas and colorectal adenocarcinomas where a point mutation activates the expression of K-ras oncogene (Liber, 2009; On-On Chan, 2002b).

Microsatellites are simple repetitive sequences of DNA distributed throughout the genome that are stably inherited and vary from one person to the next (On-On Chan, 2002b). Accurate replication of these microsatellite sequences is imperative as the repercussions of a mutation in these microsatellite sequences can result in gene mutations altering gene function. Mutations that may exist within microsatellites have a relatively low rate of occurring but when this does occur it is termed as microsatellite instability (MIN) (On-On Chan, 2002b). Fortunately, there is a system in place designated as the DNA mismatch repair (MMR) system which comprises of MMR genes that are responsible for recognizing and repairing the incorporation of an incorrect base in any newly synthesized DNA strand (On-On Chan, 2002b; Weinberg, 2007). Undesirable genetic mutations have been detected in MMR genes hMSH2, hMLH1, hPMS2 generating microsatellite instabilities that have been strongly associated with hereditary nonpolyposis colorectal cancer (HNPCC) (On-On Chan, 2002b). Undesirable epigenetic changes have also been observed in MMR genes where hypermethylation of CpG islands located within the hMLH1 gene resulted in gene silencing generating microsatellite instabilities, wherein this scenario has been

associated with the development of sporadic colorectal cancer (On-On Chan, 2002b; Issa, 2008). Thus, faulty MMR systems and MIN's together increase the occurrence of frame shift mutations in cancer related genes expediting the carcinogenic process in genes containing repetitive sequences associated with colon cancer (On-On Chan, 2002b). In addition, the molecular mechanism, genetic or epigenetic, that induces the mutation of a specific MMR gene, such as hMLH1, appears to govern the type of colorectal cancer that will ensue (Issa, 2008; On-On Chan, 2002b).

***Solanum tuberosum* L.** Potato *(S. tuberosum* L.*)* is the fourth largest consumed staple food crop in the world and is the world's most important non-grain food crop. While the USDA has recently suggested a questionable recommendation, to remove potato from the list of recommended foods for school lunches, the United Nations officially declared the year 2008 as the International Year of the Potato to raise its profile in developing nations, calling the crop a "hidden treasure." The subsequent information shall provide an informative background on potato focusing on the historical introduction, taxonomy, modes of consumption, genetics, breeding methods and valuable bioactive compounds that have been found to inhibit cancer cells *in vivo* and *in vitro*.

Historical Introduction and Taxonomy of Potato. Molecular taxonomic evidence reveals that farmers of the Andes of southern Peru and northern Bolivia gave rise to a single domestication from the northern group of members of the *Solanum brevicaule* complex of species more than 7,000 years ago (Kang & Priyadarshan, 2007). Shortly after, arose the domestication of *Solanum stenotomum*, which led to the six most widely cultivated species in South America to include *Solanum tuberosum*,

which itself can be further classified into two genetically distinct subspecies *Andigena* and *Chilean tuberosum* (Kang & Priyadarshan, 2007). In 1537, the Conquistadors invaded Peru and in 1570 the European continent would obtain its first introduction of potato believed to have been from both the Andes and Chile, although debatable (Kang & Priyadarshan, 2007). Potato tubers adapted and produced well in Europe, but unfortunately they carried a dire reputation that it would be a food for the underclass and used primarily to feed hospital inmates (Rayment, 2012). In the British Isles during 1786, Anderson proposed the first approach for evaluating potato varieties in a paper to the Bath and West of England Society. Unfortunately, Anderson's selection proposals were not utilized and unscreened cultivars such as Lumper, Cups, Ashleaf, Fortyfold, Regent, Rock and Fluke were introduced to British farmers (Promar International, 2003). In the 1780's, the potato was a success with the people of Ireland leading to a population explosion followed by a population plummet as a result of emigration and famine in the 1840's when potato cultivars such as Lumper and Cups were infected by the fungus *Phytophthora infestans* (Promar International, 2003; Rayment, 2012). While in France, Antoine Augustine Parmentier, a French farmer, chemist, pharmacist and employee of Lois XV, recognized the overall beneficial traits of the potato and used clever tactics to help the French people overcome their resistance towards the potato (Rayment, 2012). Soon thereafter, the potato was embraced across Europe and North America and is now a common and successful staple worldwide (Rayment, 2012).

Potato Consumption Products. Potatoes can be purchased fresh and prepared in the microwave, fried, baked, roasted, boiled or steamed for homemade consumption (Vreughenhil & Bradshaw, 2007). Potatoes may also be purchased processed, which

accounts for 60% of developing nation's mode of consumption (Vreughenhil & Bradshaw, 2007). This section will discuss the most popular modes of potato consumption worldwide (Vreughenhil & Bradshaw, 2007).

The potato chip originated in a hotel kitchen in Saratoga Springs, New York in 1853 (Vreughenhil & Bradshaw, 2007). This popular potato product can be prepared using two different methods (Vreughenhil & Bradshaw, 2007). The first method involves deep-frying thinly sliced potatoes in a vegetable oil to possess a final moisture content of 1.3-1.5% (Vreughenhil & Bradshaw, 2007). The second method involves using dehydrated potato to form dough that will be either baked or flash-fried into a potato chip (Vreughenhil & Bradshaw, 2007). Potato chip flavors are often enhanced by adding salt, salt and vinegar, cheese, paprika, onion and lemon to name a few (Vreughenhil & Bradshaw, 2007).

Pommes frites, the french fry, originated in France and the invention of the industrial process for french fry production is generally attributed to Jack Simplot of the J.R. Simplot Company in Idaho (Vreughenhil & Bradshaw, 2007). French fries are another popular potato product worldwide (Vreughenhil & Bradshaw, 2007). The steps involved in producing french fries begin by cutting the potato into thin strips (Vreughenhil & Bradshaw, 2007). Next they are washed briefly in cold water and partially dried to remove any surface water (Vreughenhil & Bradshaw, 2007). The final step is to deep fry the potato strips in vegetable oil until they turn a light golden color (Vreughenhil & Bradshaw, 2007). The frozen market processes frozen fries cut in a variety of shapes and can be shipped raw, par-fried, or partially cooked and drizzled

with oil to be either deep fat fried, baked or microwaved (Vreughenhil & Bradshaw, 2007).

Potatoes can be processed to develop other products comprising the global market in developing nations (Vreughenhil & Bradshaw, 2007). Common processed frozen potato products other than the french fry include waffles, wedges, hashed brown potatoes, rosti, preformed mashed potatoes, patties, potato rounds, diced potatoes, bay roasts and a variety of shaped potato products with child appeal (Vreughenhil & Bradshaw, 2007). Potatoes are also processed into canned goods that are popular with restaurants and catering businesses (Vreughenhil & Bradshaw, 2007). Potatoes can be used to feed livestock. Potatoes can be used primarily as a starch source as well (Sleper & Poehlman, 2006). Lastly, potatoes can be processed for the production of alcohol (Sleper & Poehlman, 2006).

Potato Genetics. Most tuber bearing species belong to the genus *Solanum*, subgenus *Potatoe* and section *Petota* (Vreugdenhil & Bradshaw, 2007). *Petota* can be further subdivided into 21 series containing 228 wild and 7 cultivated species where all species contain the basic chromosome number (x=12) but contain varying polyploid levels to include diploid (2n=2x=24), triploid (2n=3x=36), tetraploid (2n=4x=48), pentaploid (2n=5x=60) and hexaploid (2n=6x=72) (Vreugdenhil & Bradshaw, 2007). *S. tuberosum*, the most widely cultivated potato species in the world, is an autotetraploid (2n=4x=48) (Vreugdenhil & Bradshaw, 2007).

Autotetraploids, generally demonstrate high frequency of multivalents at metaphase I, irregular meiosis, sterility and/or very low fertility resulting typically in maintenance via vegetative propagation (Vreugdenhil & Bradshaw, 2007). In an

autotetrapolid there are five tetrasomic conditions possible at any single locus ((a_1 a_1 a_1 a_1) (a_1 a_1 a_1 a_2) (a_1 a_1 a_2 a_2) (a_1 a_1 a_2 a_3) (a_1 a_2 a_3 a_4)) (Sleper & Poehlman, 2006). The monoallelic condition (a_1 a_1 a_1 a_1) contains zero interactions possible because all alleles are identical (Sleper & Poehlman, 2006). In contrast, the tetrallelic condition (a_1 a_2 a_3 a_4) boasts the maximum amount, 11 different interactions that can occur, because all of the alleles are different (Sleper & Poehlman, 2006). Although, autotetraploids clearly create more complex inheritance patterns and present challenges for breeders, increased ploidy levels lead to the possibility for more diverse alleles and greater number of interactions within a locus which may lead to higher heterozygosity, especially in the tetra-allelic condition (Sleper & Poehlman, 2006). In turn, higher heterozygosity results in increased heterosis, which is important in improving desirable traits in cultivated potato (Sleper & Poehlman, 2006).

The Potato Genome Sequencing Consortium (PGSC) comprising 39 scientists from 16 international research groups attempted to complete the genome sequencing of two potato varieties representative of potato genomic biodiversity: DM1-3 516R44 (DM) and RH89-039-16 (RH) (The Potato Genome Consortium*, 2011). DM was established by doubling the chromosomes of a monoploid anther culture of a heterozygous diploid *S. tuberosum* group Phureja (PI 225669), a fingerling type tuber, resulting in a homozygous double monoploid clone (The Potato Genome Consortium*, 2011). RH was established by crossing a dihaploid *S. tuberosum* (SUH2293) with a diploid clone (BC1034) derived by two *S. tuberosum* group Phureja hybrids resulting in a heterozygous diploid, a more representative selection of a commercially cultivated tetraploid potato (The Potato Genome Consortium*, 2011). Sizeable segments of the

RH genome have been sequenced but due to the complexities that exist with the heterozygous condition, physical mapping and assembly of genome sequence has not been completed (The Potato Genome Consortium*, 2011). On the other hand, the simplicity of the homozygous condition of the DM variety has led to the successful sequencing of the DM genome uncovering 12 chromosomes, with a haploid length of 844 mega base pairs and 39,031 protein-coding genes giving way to a medium sized genome, one quarter the size of the human genome (The Potato Genome Consortium*, 2011). The sequencing of the potato genome in conjunction with mapping techniques has provided insight about the evolution of the potato genome, biology of tubers and unraveled its complex underlying genetics permitting for more rapid improvements of quantitative traits in this vital global staple (The Potato Genome Consortium*, 2011).

Potato Breeding Methods. The maintenance of cultivated *S. tuberosum* and hybridization of novel potato parents to reveal desired traits can be accomplished using various methods including but not limited to sexual reproduction, asexual reproduction, conventional hybridization breeding, sexual polyploidization and genetic transformation. The following information will explain how these methods are employed in the breeding of potatoes as well as the advantages and disadvantages each may possess.

Sexual reproduction of potato involves the union of gametes (*n*) resulting in the formation of true potato seed (TPS) (Sleper & Poehlman, 2006). In nature, self pollination is the primary means of sexual reproduction although cross pollination is often accomplished by insects, particularly bumblebees (Sleper & Poehlman, 2006). Potato breeders may also employ crossing techniques to maintain and/or derive novel potato hybrids with desirable traits important to both the breeder and consumer. The

first step in executing crossing techniques is to select desirable parents (Sleper & Poehlman, 2006). Intraploid and interploid crosses require the selected desirable parents to be compatible in their ability to properly develop viable endosperm so TPS can form successfully (Sleper & Poehlman, 2006). According to the endosperm balance number (EBN) hypothesis, normal endosperm development ensues only when the ratio for maternal to paternal EBN contribution is 2:1 (Sleper & Poehlman, 2006). Any type of departure from this EBN ratio will result in shriveled and/or aborted seeds (Sleper & Poehlman, 2006). The maternal EBN contribution assigned to their progeny is two and the paternal EBN contribution assigned to their progeny is one (Sleper & Poehlman, 2006). In the case where unreduced gametes exist, both paternal contributions are doubled (Sleper & Poehlman, 2006). Next, mature, plump flower buds from the selected male parent are removed to prevent self pollination and/or contamination (Sleper & Poehlman, 2006). The flowers are bagged, dried out overnight and then dexterously emasculated (Sleper & Poehlman, 2006). The desiccated pollen is stored in gelatin capsules in the refrigerator at 4 °C for 1-2 weeks or in the freezer at -20 °C for 6 months to 2 years (Wakefield, 1970; Sleper & Poehlman, 2006). Pollen may also be collected by excising the flower at the stem and placing it in a jar of water with an antibacterial agent in order to reduce the possibility of contamination (Kang & Priyadarshan, 2007). Pollination involves dipping the stigma of a potato flower grown in soil into the gel capsule and then placing a bag over the flower until the fruit is harvested (Sleper & Poehlman, 2006). Germination of pollen tubes occurs within 30 minutes and fertilization of the ovary within 12 hours leading to the ultimate production

of a berry type fruit, dubbed a seed ball or apple, that will produce anywhere from 50-200 true potato seeds per fruit, depending on the cultivar (Sleper & Poehlman, 2006).

The advantages of sexual reproduction of potato include but are not limited to: increased genetic diversity leading to heterosis, reduced storage problems, lower shipping costs because TPS is light weight and it only requires 100g TPS to seed a hectare where it requires 2000kg of seed tuber to seed the same area, and lastly, is generally virus free, as seeds do not transmit viruses (Vreugdenhil & Bradshaw, 2007).

The disadvantages of using sexual reproduction are unfortunately many. Obstacles encountered in obtaining TPS include but are not limited to the following: failure of flowers to form, dropping of buds and flowers either before or after fertilization, low or poor-quality pollen production, failure to produce viable pollen, male sterility as a result of deformed flower with indehiscent anthers or shriveled microspores which do not separate, male sterility as a result of an inherent characteristic where sterility is dominant to fertility, failure to meet (2EBN maternal:1EBN paternal) ratio requirements for normal endosperm balance number production resulting in degenerative endosperm and thus no seed set. The gametophytic system of incompatibility controlled by *S* alleles is modified by an *R* allele in the style preventing fertilization from a pollen tube carrying *S* alleles may also prevent seed set. Other issues include: the presence of a tetrasomic gene in the homozygous or heterozygous condition proven to be fully or partially lethal; great technical skill, and intensive labor is required to ensure successful execution of sexual production methods; and lastly potato plants obtained from TPS are often highly heterozygous leading to high variability from one generation to the next (Kang & Priyadarshan, 2007; Sleper & Poehlman, 2006; Vreugdenhil & Bradshaw, 2007).

Asexual reproduction of potato does not involve the union of gametes (Sleper & Poehlman, 2006). The asexual vegetative propagation of potato can be possible by rooting stem cuttings or most commonly by planting seed tubers (Sleper & Poehlman, 2006). Seed tubers are either cut so that one or two eyes are left on the surface of the potato and/or planted whole before the last killing frost of spring (Rayment, 2012). The buds in the eye of the tuber germinate and produce sprouts giving rise to a whole new potato plant (Sleper & Poehlman, 2006).

The primary advantage of asexual reproduction of potato is the ability to maintain desirable genetically identical potato clones with consistent uniformity from year to year (Sleper & Poehlman, 2006). Another great advantage involves the production of certified seed tubers which allow desirable lines of cultivated potato to be maintained year after year that are high quality, high yielding and most importantly disease free by means of meristem culture, thermotherapy, chemotherapy and micropropagation techniques. However, a number of disadvantages associated with the asexual reproduction of potato include the susceptibility of seed tubers to accumulate and transmit viral and/or other diseases, premature sprouting of tubers where cold storage facilities do not exist and expenses related to transport of seed tubers (Kang & Priyadarshan, 2007).

Conventional hybridization breeding is the traditional method used amongst breeders which combines sexual and asexual reproduction strategies in improving the cultivated potato and this process is described in further detail by Sleper & Poehlman (2006). The first step in this process is the selection of parents carrying desirable traits that interest both breeder and consumer. Parent plants selected for conventional

hybridization breeding are produced from seed tubers and are heterozygous. Sexual reproduction crossing techniques are executed during hybridization of selected parents as described above. The F1 generation will divulge the segregation of characters in the new hybrid potatoes. Selected F1 hybrid tubers will be planted via asexual means in rows corresponding to the F1 plant it was derived from for further evaluation and increase of seed tubers. Clones that show both promise and have been sufficiently increased are then tested for performance at multiple sites with regards to yield, disease and insect resistance as well as genotype vs. environment interaction.

Unilateral sexual polyploidization refers to the formation of a polyploidy plant where unreduced gametes (2n) are produced by one parent (Sleper & Poehlman, 2006). Bilateral sexual polyploidization refers to the formation of a polyploid plant where unreduced gametes (2n) are produced by both parents (Sleper & Poehlman, 2006). Unreduced gametes (2n), such as dihaploids and naturally occurring diploids, do not have a reduction in chromosome number following normal meiosis. These unreduced gametes have the remarkable ability to transfer larger portions of heterozygosity from the 2x parent to the 4x progeny as opposed to events in normal meiotic events where reduced gametes (n) are only capable of passing additive gene effects from the 2x parent to the 4x progeny (Sleper & Poehlman, 2006). Dihaploids can be created via first division and/or second division restitution mechanisms. First division restitution mechanisms produce unreduced gametes as a result of chromosomes in anaphase I failing to separate to opposite poles (Sleper & Poehlman, 2006). Second division restitution mechanisms produce unreduced gametes as a result of chromosomes in anaphase II failing to separate to opposite poles (Sleper & Poehlman, 2006). Sexual

polyploidization has been an extremely successful technique in generating tetraploid potatoes with resistance to bacterial wilt (*Pseudomonas solanacearum*), early blight, common scab, potato cyst nematode, *Verticillium* wilt, soft rot and crossed root knot nematode-resistance in *S. tuberosum* 'Atzimba' (Vreugdenhil & Bradshaw, 2007). Unilateral and/or bilateral sexual polyploidization procedures collectively with first division and/or second division restitution mechanisms has proven to be a valuable application in potato breeding programs in that it introduces favorable unadapted germplasm into the tetraploid gene pool to augment the incidence of tetra-allelic loci with an aim to increase heterozygosity with an end purpose to improve the cultivated potato (Sleper & Poehlman, 2006).

Genetic transformation refers to the introduction of foreign DNA and can be accomplished using electroporation, particle bombardment or *Agrobacterium tumefaciens* mediated gene transfer (Pena, 2005). The commonality in all of these methods is that the transforming DNA must penetrate the cell wall, plasma membrane, travel to the nucleus and integrate into the chromosome (Pena, 2005). Electroporation uses an electric current to drive DNA or other molecules into a cell (Meneely, 2009). Particle bombardment commonly involves the use of a gene gun to deliver DNA particles into a cell culture reliably and efficiently (Meneely, 2009). The most widely used transformation method is *Agrobacterium tumefaciens* mediated transformation because it is the most straightforward and generates the most success in dicots (Pena, 2005). The first step in *Agrobacterium tumefaciens* mediated transformation with regards to potato is to clone a gene of interest and incorporate it into a Ti plasmid with a promoter and a selectable marker replacing the T-DNA (tumor inducing gene) (Pena,

2005). Freshly cut (wounded) tuber discs, leaf or internode explants of the potato are then co-cultured with the *Agrobacterium* (Sleper & Poehlman, 2006). *Agrobacterium* is attracted to the amino acids, sugars and organic acids released from wounded plant tissues and binds to the wounded plant cell wall by a polar attachment mechanism (Pena, 2005). Fresh cuts (wounding) cause the plant tissue to release phenolic compounds and monosaccharides (Pena, 2005). This indirectly or directly causes autophosphorylation of virulence genetic operon *virA* in turn phosphorylating *virG* genetic operons activating *virB*, *virC*, *virD* and *virE* on the Ti-plasmid to ultimately release the T-DNA insert and integrate it into the host genome via nonhomologous recombination (Pena, 2005). Tissue culture containing the selectable agent is then used to generate shoots and identify successful transformants (Sleper & Poehlman, 2006). Transformed potato plants generated from tissue culture are then planted in the field and/or greenhouse for the evaluation of 3 key components (Vreugdenhil & Bradshaw, 2007). First, it will be determined if the transgene maintains the desired phenotype in field and/or greenhouse conditions (Vreugdenhil & Bradshaw, 2007). Second, transgenic lines will be identified that retain all of the phenotypic characteristics of the parent cultivar (Vreugdenhil & Bradshaw, 2007). Third, safety concerns of the transgenic line will be assessed with regards to food safety and their effects on the environment (Vreugdenhil & Bradshaw, 2007).

Vreugdenhil & Bradshaw (2007) cite several examples how genetic transformation techniques have proven to be successful in improving various facets of potato. Transgenic resistance to the Colorado potato beetle (*Leptinotarsa decemLineata Say*) (CPB) was successfully achieved in potato with the introduction of a

gene from *B. thuringiensis* var. *tenebrionis* that encodes the protein Cry3A under the control of the constitutive CaMV 35S promoter. Transgenic resistance to potato tuber moth (*Phthorimaea operculella*) was achieved in potato with the introduction of the *cry*5 gene and by the *cry*1Ac9 gene encoding the Bt protein under the control of the constitutive CaMV 35S promoter. Resistance status to potato cyst nematodes in Great Britain was raised from partial to full resistance with the introduction of transgenic constructs encoding cysteine proteinase inhibitor (cystatin) from sunflower, protein-engineered rice and chicken egg whites under the control of the constitutive CaMV 35S promoter. Transgenic resistance to potato leafroll virus (PLRV) and CPB was derived using a single construct designed to prevent virus replication under the control of the FMV promoter. Transgenic resistance to potato Y (PVY) and CPB was also derived using a single construct under the control of the FMV promoter. The gene *chly* encoding the enzyme lysozyme from chicken has been introduced into the cultivar 'Desiree' and shown to enhance resistance to black leg and soft rot. Undesirable pigments produced in bruising of potatoes can be transgenically solved by the down regulation of PPO gene expression that encodes the PPO protein responsible for bruise color. Glycoalkaloid levels above 20 mg per 100 g fresh weight are considered unsuitable for human consumption. Glycoalkaloid content in potato can be reduced by down-regulating a gene encoding a sterol alkaloid glycosyltransferase (*Sgt1*). Transformation of potato with a non-allergenic seed albumin gene (*AmA1*) from *Amaranthus hypochondriacus* was found to significantly increase levels of methionine, lysine, cysteine and tyrosine in protein amino acids and a 35-45% increase was achieved in total protein content. It has been found that the down-regulation of the

zeaxanthin epoxidase gene in *S. tuberosum* increased not only the accumulation of zeaxanthin but also the total carotenoid levels by 5.7 fold. Two other studies incorporating *Erwinia uredovora crtB* gene encoding phytoene synthase proved to increase total carotenoid levels up to 35 and 78 $\mu g \cdot g^{-1}$ DW.

Vreugdenhil and Brandshaw (2007) describe the techniques and problems associated with preventing premature sprouting. Low temperatures are required to store tubers to minimize premature sprouting. In turn, low temperatures cause an increase in reducing sugars, which are responsible for unfavorable non-enzymatic browning when generating potato chips and french fries. Vreugdenhil and Brandshaw (2007) further explain how transgenic expression of an *E.coli glgC16* mutant gene that encodes for the enzyme ADPglucose pyrophosphorylase, has increased tuber starch content and ADPglucose while decreasing undesirable reducing sugars. The transgenic expression of a nonvascular invertase inhibitor protein from tobacco under the control of CaMV35S promoter has also proven to be successful in minimizing of reducing sugars. These are just a few examples illustrating the strides genetic transformation has made in improving desirable traits in potato.

Vreugdenhil & Bradshaw (2007) talk about several advantages for using genetic transformation techniques to make advances in potato improvement. Transgenic potato plants are excellent models to study gene expression and function. The germplasm pool is infinitely full of possibilities allowing the introduction of novel biochemical and desirable traits into cultivated potato. Desirable genes can be transferred directly into elite cultivars without many generations of additional crosses. New genes of interest can also be solely transferred into the potato genome without the introduction of closely

linked genes that are often associated with negative traits. Genes controlling the expression of desirable traits in potato can now be overexpressed and undesirable traits can be silenced via RNAi methods for example. The relative ease of transformation of the potato crop when compared to the limitations associated with traditional potato breeding open up a multitude of opportunities for improvement as well.

Unfortunately, Vreugdenhil & Bradshaw (2007) also explain several disadvantages to improving potato via genetic transformation as well. Using tissue culture to regenerate plants can often lead to high somaclonal variation increasing the occurrence of off-types in the field. Transgenic lines have also been found to frequently fail to exhibit the expected phenotype under field conditions. Consumer perception globally of transgenic crops has limited commercial success and as a result left the majority of these extraordinary advances at the research level. Transgenic crops have also raised a range of important questions regarding biosafety issues. What is the potential of pollen-mediated gene flow to other potatoes and related species? What is the impact on symbiotic relationships of invertebrates and microflora in the field important to the cultivation of potato? Is there a possibility that pest and disease populations will turn more virulent as a result of transgenics? Are transgenically modified potatoes really safe for human consumption? What impact does transgenically altering potatoes actually have on the nutritional composition and phytochemical content?

Potato & Cancer. Studies have demonstrated that bioactive compounds and/or antioxidants present in fruits, vegetables, tea and wine are important in the maintenance of human health and prevention of lifestyle induced and chronic disease (Liu, 2004; Chu

et al., 2002; Prakash, 2001). Potatoes are rich in essential nutrients important for the maintenance of human health and comprise phytochemicals that may offer protection against free radical induced lifestyle diseases. Potatoes have been found to contain significant amounts of antioxidants including vitamins C and E, carotenoids, flavonoids and phenolic acids. Antioxidants are molecules that are capable of inhibiting the oxidation of other molecules (Halliwell & Gutteridge, 1999). They may act as agents that catalytically remove free radicals and other reactive species (Halliwell & Gutteridge, 1999). They may act as proteins that diminish the number of pro-oxidants (Halliwell & Gutteridge, 1999). They may act as proteins that guard important biomolecules against damage (Halliwell & Gutteridge, 1999). They can also serve as low molecular mass agents that scavenge ROS and RNS (Halliwell & Gutteridge, 1999).

The WCRF/AICR report (2007b) has found that an average preventability of 35% in the United States, 37% in the United Kingdom, 30% in Brazil and 27% in China of the 12 most prevalent cancers (mouth, pharynx, larynx; oesophegus, lung, stomach, pancreas, gallbladder, liver, colorectum, breast, endometrium, prostate and kidney). The WCRF/AICR report (2007a) has also reported recommendations for preventing cancer to include: maintaining a healthy body weight; being physically active 30 minutes a day; limiting consumption of energy dense foods, eating a variety of vegetables, fruits, whole grains and pulses; limiting consumption of red and processed meats; limiting alcohol consumption; limiting salt intake and sodium processed foods; and eliminating high nutrient supplements (WCRF/AICR report, 2007a, 2007b). In opposition to the recommendation regarding fruit and vegetable consumption, a meta-analysis of 19 prospective studies by Aune et al. (2011) has recently reported, “a weak but statistically

significant nonlinear inverse association between fruit and vegetable intake and colorectal cancer risk". However, several studies have implicated classes of secondary metabolites in addition to nutritional components found in potato with inhibitory activity, apoptotic induction mechanisms and the ability to upregulate detoxifying phase 2 enzymes. The subsequent information is intended to describe the key findings of these studies.

Potatoes are an important source of dietary fiber where 8% of the recommended daily value for dietary fiber can be attained by consuming one medium sized potato with the skin (Vreugdenhil & Bradshaw, 2007). A study conducted by Harris investigated the theory that dietary fiber may offer protection against colon cancer by studying the adsorption of the hydrophobic mutagen 1,8-dinitropyrene (DNP) to the skin of potato tubers and flesh of potato tubers (Harris et al., 1991). Results showed that dietary fiber obtained from potato skins, which are composed mainly of α-cellulose, were able to bind DNP and thus this source can be considered as potentially protective against colon cancer (Harris et al., 1991). Dietary fiber obtained from potato flesh, which is composed mainly of pectic polysaccharides, did not bind DNP well and therefore may not offer any protection against colon cancer (Harris et al., 1991). This study stressed the importance of the source and/or type of plant cells walls, dietary fibers, in their ability to bind and reduce mutagen and cancer promoter contact with mucosal cells of the colon (Harris et al., 1991).

Starch, the main energy source in potato, constitutes 75% of total dry matter content (Vreugdenhil & Bradshaw, 2007). Starch is a polymer of the glucose monomers amylose and amylopectin (Vreugdenhil & Bradshaw, 2007). A small portion of starch

found in potato called resistant starch (RS), is the source of many health benefits in the colon. RS is resistant to enzymatic digestion in the small intestine by α-amylase and consequently is fermented in the large intestine (United States Potato Board, 2010; Liu & Xu, 2008). RS causes a fermentation mediated increase in the concentrations of short chain fatty acids, particularly butyrate, that exist in the large intestine (Liu & Xu, 2008). Butyrate has been found to halt growth, induce apoptosis and regulate expression of proteins associated with cellular dedifferentiation in several cancer cell culture lines including colon cancer (Liu & Xu, 2008). In a study conducted by Liu and Xu (2008), RS was administered to azoxymethane (AOM) induced colonic aberrant crypt foci (ACF), markers of preneoplastic lesions, of Wistar rats where it was confirmed that RS suppressed AOM-induced ACF formation at the promotion stage but promoted the formation of ACF at the preinitiation stage. RS is also responsible for laxation enhancement, increases in beneficial bacteria, cecal and large intestinal contents and decreases in pH and concentration of ammonia, phenols and secondary bile acids in the colon (United States Potato Board, 2010; Liu & Xu, 2008).

Glycoalkaloids are secondary metabolites that exist in the tubers, leaves, roots and sprouts of potato (Friedman, 2006). Glycoalkaloids are composed of a trisaccharide chain attached to an aglycon (Friedman, 2006). These glycoalkaloids are mostly positioned around the eyes located on the outer layer of the tuber and within the first 1mm from the exterior surface of the tuber decreasing toward the interior of the tuber (Friedman, 2006). The prominent glycoalkaloids that exist in cultivated potato are α-chaconine and α-solanine (Friedman, 2006). The initial concentration and configuration of these prominent glycoalkaloids in potato is largely dependent on

genotype (Friedman, 2006). Concentrations may be effected by environmental factors such as soil and climate and generally decrease with increasing tuber size (Friedman, 2006). Postharvest management can also cause concentration increases as a result of storage, light, heat, cutting, slicing, sprouting and exposure to phytopathogens (Friedman, 2006).

Glycoalkaloids have been associated with producing an undesirable bitter astringent taste (Vreugdenhil & Bradshaw, 2007). At certain concentrations they are reported to be toxic to bacteria, fungi, viruses, insects, animals and humans (Friedman, 2006). Of the two prominent glycoalkaloids found in potato, α-chaconine has been reported to be more toxic than α-solanine in several studies (Friedman, 2006). Ingestion of glycoalkaloids by humans in the form of potato berries, potato leaves, and blighted, greened and sprouted tubers has been reported to cause death (Friedman, 2006). Human feeding studies revealed that ingestion of potatoes varying in concentration between 1.0-2.6 $mg \cdot kg^{-1}$ body weight resulted in light to severe nausea, burning sensation in the mouth and diarrhea (Friedman, 2006). Morris and Lee et al. reported in a review paper that 2-5 $mg \cdot kg^{-1}$ of body weight is considered a toxic dose to humans and 3-6 $mg \cdot kg^{-1}$of body weight is considered to be a fatal dose to humans (Morris & Lee, 1984). Thus the maximum safe allowance for human consumption of glycoalkaloids is reported to be 200 $mg \cdot kg^{-1}$of fresh weight of potato (Vreugdenhil & Bradshaw, 2007).

Although glycoalkaloids are reported to produce toxic effects they have also been implicated as chemopreventives (Friedman et al., 2005; Friedman, 2006). The glycoalkaoids, α-chaconine and α-solanine, isolated from Dejima potatoes, were

identified as the key metabolites involved in the inhibition of the growth of several human cell lines in culture including cervical (HeLa), liver (HegG2), lymphoma (U937) and stomach (AGS and KATO III) (Friedman et al., 2005). α-chaconine has been reported to inhibit ERK ½ phosphorylation triggering caspase-3 activation generating the induction of apoptosis of human HT-29 colon cancer cells in culture in a time and concentration dependent manner (Yang et al., 2006). Methanol extracts of *S. tuberosum* callus containing higher levels of solanidin, α-chaconine and α-solanine than in tubers induced cytotoxic effects against human carcinoma cell culture lines of breast, lymphoplastic leukemia, larynx, liver, cervix, colon and brain (Al-Ashaal, 2010). Mandimika (2007) attempted to explain the effects of different amalgamations of α-chaconine and α-solanine treatments together and alone on Caco-2 colon human intestinal epithelial cell culture lines. Lactase dehydrogenase leakage assay executed in this study informed that cytotoxicity of Caco-2 cells in culture occurred in a concentration dependent manner in all treatments except α-solanine alone (Armstrong, 2011). Gene expression studies utilizing α-chaconine and α-chaconine/α-solanine treatments on Caco-2 cells indicated significant upregulation of genes involved in cholesterol biosynthesis (Mandimika, 2007). All treatment combinations showed gene expression changes in growth signaling, lipid and amino acid metabolism, mitogen activated protein kinase (MAPK) and NF-κB cascades, cell cycle and apoptosis. Late apoptotic induction of Caco 2 cells was found to occur in a concentration dependent manner by α-chaconine (Mandimika, 2007). Cell cycle analysis revealed an accumulation of Caco 2 cells during G_2/M phase induced by α-chaconine (Mandimika, 2007). Conversely, another study revealed that HT-29 colon cancer cells in culture

exposed to 5ug/mL α-chaconine and 10μg/mL α-solanine resulted in an accumulation of HT-29 cells during sub-G_0/G_1 phase of the cell cycle (Yang et al., 2006).

Vitamin C is a hydrophilic compound that acts as an antioxidant (Spencer & Saul, 2010). Vitamin C is the main vitamin in potato and contributes up to 40% of daily recommended intake of the global diet (Vreugdenhil & Bradshaw, 2007). The concentration and state of reduced ascorbic acid and oxidized dehydroascorbic acid is dependent on both potato germplasm and storage (Vreugdenhil & Bradshaw, 2007). A recent study showed that intraperitoneal pharmacological doses of vitamin C reduced tumor size and incidence in rats induced with hormone-refractory prostate cancer PA III cells (Pollard et al., 2010). The reduced state of ascorbic acid has been found to effectively kill malignant mesothelioma cells in both test tube studies and mice experiments (Pollard et al., 2010). Angiogenesis has been found to be impeded when high levels of vitamin C are present in the blood both *in vivo* and *ex vivo* (Mikirova et al., 2010). Numerous other research studies have established the value of vitamin C, thus this essential vitamin should be viewed as a powerful bioactive agent important in the intervention of many diseases.

Carotenoids are fat soluble tetraterpenoids existent in the flesh of white, yellow and orange potatoes (Brown, 2005). The most prominent carotenoids emanate from the zanthophyll subclass, oxygenated tetraterpenoids, and include lutein 5,6 epoxide, zeaxanthin, violaxanthin (Brown, 2005). Other carotenoids identified in potato in trace amounts emanate from the carotene subclass, tetraterpenoids without oxygen, and include β-carotene and α-carotene. Potato is not a worthy source of these pro-vitamin α-carotenes (Brown, 2005). Oxygen radical absorbance capacity of total carotenoids

from potato flesh have been found to range between 4.6 - 15.3 nmoles α-tocopherol equivalents per 100g FW (Brown, 2005). Carotenoids are a powerful source of antioxidants and have been associated with a reduced risk of certain cancers (International Carotenoid Society, 2011).

Potatoes contain significant amounts of flavonoids that are polyphenolic compounds structurally composed of three aromatic benzene rings containing one or more hydroxyl groups (International Carotenoid Society, 2011). Flavonoids have been acknowledged to produce antiproliferative, anticarcinogenic and antioxidative effects (Harborne, 1993; Kuhanu, 1976; Sharma et al., 1994; Rice-Evans et al., 1996; Robards et al., 1999). The prominent flavonoids found in potato are anthocyanins, catechin and epicatechin, but erodictyol, kaempferol and narigenin are also present in potato (Brown, 2005). Specific anthocyanins abundant in potato that are responsible for producing red and purple pigments in potato include the acylated anthocyanidin glucosides pelargonidin, petunidin, malvidin and peionidin (Brown, 2005 ; Stushnoff et al., 2010). Anthocyanins isolated from pigmented potatoes, have been reported to be responsible for the induction of apoptosis in KATO III human stomach cancer cell lines in culture and suppressed stomach cancer in mouse (Hayashi et al., 2006). Anthocyanins, present in red and purple steamed potatoes, also suppressed stomach cancer in mouse in the preceeding study. Specialty potato extracts and anthocyanin fractions obtained from a specialty potato genotype both initiated mitochondrial release of the proteins Endo G and AIF resulting in the induction of apoptosis and inhibited growth of LNCaP (androgen dependent) and PC-3 (androgen independent) prostate cancer cells in culture (Reddivari et al., 2007). Cyanidin and delphinidin, and to a lesser extent

malvidin, have been found to inhibit epidermal growth factor receptor in cancer cells (Sterling R.D., 2001).

Phenolic acids are hydroxylated derivatives of benzoic and hydrocinammic acids (Herrmann, 1989). The most profuse phenolic acid present in potato is chlorogenic acid but protocatechic acid, vanillic acid and p-coumaric acid also exist (Brown, 2005). Chlorogenic acid, an antioxidant abundant in pigmented potato (Stushnoff et al., 2010) has been reported to inhibit the proliferation of lung cancer cells and TPA-induced neoplastic transformation and may be a potential stimulant of NQO1 and GST enzymatic activities (Feng et al., 2005). A red pigmented cultivar, Mountain Rose (MR), comprised of higher levels of chlorogenic acid and anthocyanins resulted in a reduction in breast cancer carcinogenesis incidence and multiplicity in rats that were fed freeze dried baked MR potato (Thompson et al., 2009). Extracts obtained from *Solanum jamesii*, a wild tuber species, were found to inhibit the proliferation of HT-29 colon and LNCaP prostate cancer cells culture lines in vitro, but no evidence of cytotoxicity to cells was observed and no significant correlations to glycoalkaloid content, total phenolic content, nor antioxidant capacity were detected (Nzaramba et al., 2009).

Rationale, Cultivars and Clonal Selections Utilized in this Research. The high prevalence of potato consumption worldwide suggests that further investigation of bioactive phytochemicals and possible inhibitory metabolites that exist within potato could be of great benefit to many. A total of 13 clones of *S. tuberosum* were obtained from the Colorado Potato Breeding Program, grown under essentially commercial

production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado (Table 1.1).

Table 1.1 Cultivars and clonal selections from the Colorado Potato Breeding Program utilized in this research.

Cultivars and Clonal Selections	Female Parent	Male Parent	Skin	Flesh
CO95172-3RU	Russet Nugget	AC88165-3	Russet	White
CO97216-1P/P	CO94163-1	CO94214-1	Purple	Purple
CO97216-3P/PW	CO94163-1	CO94214-1	Purple	Purple & White
CO97226-2R/R	Mountain Rose	CO94214-1	Red	Red
CO97227-2P/PW	Mountain Rose	CO94215-1	Purple	Purple & White
CO04056-7P/PW	CO97216-1P/P	CO97227-2P/PW	Purple	Purple & White
CO04058-3RW/RW	CO97216-3P/PW	CO97216-1P/P	Red & White	Red & White
CO04061-1R/RW	CO97222-1R/R	CO97306-1R/R	Red	Red & White
Colorado Rose	NDTX9-1068-11R	DT6063-1R	Red	White
Mountain Rose	All Red	ND2109-7	Red	Red
Purple Majesty	All Blue	ND2008-2	Purple	Purple
Rio Grande Russet	Butte	A8469-5	Russet	White
Russet Nugget	Krantz	AND71609-1	Russet	White

The objectives of this PhD dissertation were to utilize these 13 cultivars and clonal selections obtained throughout several developmental stages in both raw and cooked forms to: (1) quantify the free radical scavenging capacity and total phenolic content; (2) fine-tune current methods utilized to preserve *S. tuberosm* L. for extraction and reconstitution in aqueous cell culture medium by evaluating aqueous extracts from freeze dried tuber preparation methods versus tubers that underwent cryogenic grinding preparation methods against HT-29 colon cancer cells *in vitro*; (3) develop a novel method for quantitatively expressing cell viability based on a known reference standard curve; (4) screen tubers, flesh, peels and flowers for inhibitory activity against HT-29 colon cancer cells *in vitro*; (5) determine if the mechanism of inhibitory activity is related

to apoptotic events *in vitro*; (6) explore possible explanations for observed enhanced inhibition associated with developmental stage in immature tubers by examining whether inhibitory metabolites exist only at the outset of tuber initiation and early development, and/or if mechanical and chemical vine kill practices prior to harvest are responsible for degradation of inhibitory metabolites; (7) gain insight as to what class of metabolites may be responsible for inhibitory activity of HT-29 colon cancer cells *in vitro* and to evaluate if the tuber matrix serves to protect these inhibitory metabolites.

Chapter 2

QUANTIFICATION OF TOTAL PHENOLIC CONTENT AND RADICAL SCAVENGING CAPACITY OF THIRTEEN *SOLANUM TUBEROSUM* L. GENOTYPES WITH VARYING BIOCHEMICAL PROFILES AT SEVERAL DEVELOPMENTAL STAGES IN BOTH RAW AND COOKED TUBERS

SUMMARY

Studies have demonstrated that bioactive compounds and/or antioxidants present in fruits, vegetables, tea and wine are important in the maintenance of human health and prevention of lifestyle induced and chronic disease (Liu, 2004; Chu et al., 2002; Prakash, 2001), however, some recent studies have found opposing evidence with regards to fruit and vegetable consumption and colorectal cancer (Aune et al., 2011). The main objective of this study was to investigate the total phenolic content and radical scavenging capacity of thirteen elite genotypes of aqueous *S. tuberosum* L. extracts with different pigments obtained throughout several developmental stages in both raw and cooked forms. Total phenolic content was determined by the Folin-Ciocalteu method. Radical scavenging capacity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The relationship between phenolic content and radical scavenging capacity was also statistically investigated. Both red and purple pigmented genotypes generally contained higher levels of phenolic antioxidants and radical scavenging capacity when compared to white-fleshed genotypes. Phenolic antioxidants and radical scavenging capacity were overall highest in raw tubers versus cooked tubers. Some genotypes harvested as immature tubers, tended to contain elevated

levels of phenolic antioxidants and total radical scavenging capacity. Boiling certain genotypes of immature tubers increased phenolic antioxidants and radical scavenging capacity even more than their raw counterparts. Phenolic antioxidants and radical scavenging capacity were elevated in particular genotypes of both raw and baked mature tubers that were stored for 6 months when compared to raw and baked mature tubers obtained during harvest stage. Highly significant correlations between total phenolic content and DPPH radical scavenging capacity in 10 of the clones suggest that polyphenolic compounds play an important, but not exclusive role as radical scavengers. On the basis of the results obtained immature and pigmented genotypes seem to be the most promising stages for further investigation of antioxidant properties and their possible links to disease prevention roles.

INTRODUCTION

Studies have demonstrated that bioactive compounds and/or antioxidants present in fruits, vegetables, tea and wine are important in the maintenance of human health and prevention of lifestyle induced and chronic disease (Liu, 2004; Chu et al., 2002; Prakash, 2001), however, some recent studies have found opposing evidence with regards to fruit and vegetable consumption and colorectal cancer (Aune et al., 2011). In order to recognize the importance of this statement and these studies one should comprehend the chemistry of how antioxidants may counteract diseases.

Atoms are the smallest particles of an element that can exist either alone or in combination (Merriam-Webster, 2011). Atoms are considered most stable or grounded when all electrons in the outermost shell exist in pairs (Helwig, 2011). Free radicals

may be defined as any atom or species containing one or more unpaired electrons capable of independent existence (Merriam-Webster, 2011; Halliwell & Gutteridge, 1999). Free radicals are a common byproduct of the normal chemical reactions that occur within our cells. Free radicals may also be a direct product formed as a result of exposure to alcohol, insecticides, chemicals, radiation, excess sunlight, tobacco products and/or high fat diets, as a few common examples. The most prevalent species of free radicals often contain oxygen, nitrogen and sulfur atoms and belong to groups of molecules known as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS) (Lu et al., 2010). Reactivity of a free radical is dependent upon the radical itself and what the radical reacts with (Halliwell & Gutteridge, 1999). For example, when two free radicals react with each other they form a covalent bond with their unpaired electrons establishing stable, grounded and more importantly unreactive non-radicals (Halliwell & Gutteridge, 1999). Conversely, when a free radical reacts with a non-radical, the free radical is inclined to either donate or accept an electron from the non-radical in an attempt to stabilize itself (Halliwell & Gutteridge, 1999). This may adversely result in the formation of a new free radical and initiation of a chain of these highly reactive and dangerous free radical reactions to continuously occur (Cheung, 2011).

Antioxidants may be defined as “any substance that when present at low concentrations compared with those of an oxidizable substrate (i.e. every molecule found *in vivo*), significantly delays or prevents oxidation of that substrate (Halliwell & Gutteridge, 1999). They ultimately serve to neutralize the unpaired electron condition of free radicals by either accepting or donating an electron (Lu et al., 2010; Best, 1990). In

doing so, the antioxidant comes to be a free radical itself but is considered less reactive and hazardous than the free radical it neutralized (Best, 1990; Lu et al., 2010). Antioxidants may relieve their free radical status by delocalizing the electron it accepted, encountering another antioxidant that will neutralize its' unpaired condition or via alternate mechanisms that may exist that will assist in terminating the antioxidants free radical status (Lu et al., 2010).

Four primary antioxidant defense systems exist that are vital *in vivo* (Halliwell & Gutteridge, 1999). The first antioxidant defense system encompasses enzymes that catalytically eliminate free radicals and other reactive species such as superoxide dismutase, catalase, peroxidase and thiol-specific antioxidants (Halliwell & Gutteridge, 1999). The second antioxidant defense system includes proteins such as transferrins, haptoglobins, haemopexin and metallothionein that diminish the accessibility of pro-oxidants such as iron ions, copper ions and haem for example (Halliwell & Gutteridge, 1999). The third antioxidant defense system includes proteins that guard biomolecules against injury such as heat shock proteins (Halliwell & Gutteridge, 1999). The fourth and last antioxidant defense system comprises of low molecular mass molecules that scavenge ROS, RNS and RSS such as glutathione which can be synthesized by the body or ascorbic acid, carotenoids and polyphenols which may be obtained through plant food sources (Halliwell & Gutteridge, 1999).

Healthy aerobic organisms are typically able to maintain a comparable balance between antioxidant defenses and ROS, RNS and RSS (Halliwell & Gutteridge, 1999). Unhealthy aerobic organisms undergoing environmental stress or cellular dysfunction encounter a condition known as oxidative stress where "a disturbance in the pro-oxidant

- antioxidant balance in favor of the former leading to potential damage" (Halliwell & Gutteridge, 1999). Initial consequences of oxidative stress include harmful attacks on bases of nucleic acids, side chains of amino acids and double bonds of unsaturated fatty acids (Lu et al., 2010). The long term ramifications of oxidative stress include the pathogenesis of many diseases such as inflammatory disease, cardiovascular disease, cancer, diabetes, Alzheimer's disease, cataracts, autism and aging to name a few (Lu et al., 2010). Thus, the significance of the findings of the aforementioned studies pertaining to a correlation in the reduction in lifestyle enhanced diseases via an antioxidant rich fruit and vegetable diet should now be understood.

Solanaceae is a unique family containing *Solanum tuberosum L.*, more commonly known as the cultivated potato (Kang & Priyadarshan, 2007). The cultivated potato is the fourth most consumed staple crop in the world (Kang & Priyadarshan, 2007). Potatoes have been found to contain significant amounts of antioxidants including carotenoids, phenolic compounds and vitamins C and E (Brown, 2005). The most prominent carotenoids include lutein, zeaxanthin, violaxanthin, β-carotene and α-carotene (Brown, 2005). The most prominent phenolic acid in potato is chlorogenic acid (Brown, 2005). The most prominent flavanols in potato include catechin and epicatechin. The most prominent anthocyanidins include the acylated anthocyanidin glucosides pelargonidin, petunidin, malvidin and peionidin (Brown, 2005; Stushnoff et al., 2010). The high consumption of potato worldwide suggest that further investigation of antioxidant capacity and radical scavenging capacity of cultivars and selections could lead to a reduction in oxidative stress induced diseases worldwide. The main objective of this study was to investigate the antioxidant capacity of thirteen elite genotypes of

aqueous *S. tuberosum* extracts with different pigments obtained throughout several developmental stages in both raw and cooked forms.

MATERIALS & METHODS

Clones. A total of 13 clones of *S. tuberosum* were obtained from the Colorado Potato Breeding Program, grown under essentially commercial production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado. Clones beginning with CO are in an experimental phase of release and are designated as selections (Table 2.1). Clones with names have been released to the public and are designated as cultivars (Table 2.1). Selections ending in –P/PW, -R/RW and -RW/RW are selections containing pigmented and white sectors of tissue that are otherwise of the same genotype and not confounded by environmental and developmental differences (Fig. 2.1; Table 2.1). In all selections, the first letter before the slash is indicative of tuber skin color (P=purple, R=red, W=White, RU=Russet) (Table 2.1). In all selections, the letters following the slash are indicative of tuber flesh colors (P=purple, R=red, W=White) (Table 2.1).

Figure 2.1 Sectorial expression of pigments illustrating a model system that permits sampling of tissues that differ only in pigment expression and are not confounded by environmental factors or genetic segregation.

Table 2.1 Cultivars and clonal selections from the Colorado Breeding Program utilized in this research.

Cultivars and Clonal Selections	Female Parent	Male Parent	Skin	Flesh
CO95172-3RU	Russet Nugget	AC88165-3	Russet	White
CO97216-1P/P	CO94163-1	CO94214-1	Purple	Purple
CO97216-3P/PW	CO94163-1	CO94214-1	Purple	Purple & White
CO97226-2R/R	Mountain Rose	CO94214-1	Red	Red
CO97227-2P/PW	Mountain Rose	CO94215-1	Purple	Purple & White
CO04056-7P/PW	CO97216-1P/P	CO97227-2P/PW	Purple	Purple & White
CO04058-3RW/RW	CO97216-3P/PW	CO97216-1P/P	Red & White	Red & White
CO04061-1R/RW	CO97222-1R/R	CO97306-1R/R	Red	Red & White
Colorado Rose	NDTX9-1068-11R	DT6063-1R	Red	White
Mountain Rose	All Red	ND2109-7	Red	Red
Purple Majesty	All Blue	ND2008-2	Purple	Purple
Rio Grande Russet	Butte	A8469-5	Russet	White
Russet Nugget	Krantz	AND71609-1	Russet	White

All cultivars and selections were examined for total phenolic content and radical scavenging capacity obtained throughout several developmental stages (Table 2.2) in both raw and cooked forms for two to four consecutive harvests (2007 - 2010): (1) raw potatoes at immature and intermediate developmental stages; (2) boiled potatoes (97 °C for 20 minutes) at immature and intermediate developmental stages; (3) raw potatoes at mature tuber stages; (4) baked potatoes (170 °C for 1 hour) at mature developmental stages; (5) boiled potatoes (97 °C for 20 minutes) at mature developmental stages; (6) mature raw potatoes stored for 6 months (4 °C in dark conditions); (7) mature potatoes stored for 6 months (4 °C in dark conditions) and baked (170 °C for 1 hour).

Table 2.2 Planting dates, harvest dates, days to maturity, and growing degree days by year (2007-2010) defining developmental stages utilized in this research. Data are not available for n/a because not tested.

	2007				2008				2009				2010			
Developmental Stage	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days
Stage I (Immature)	n/a	n/a	n/a	n/a	15th of May	30th of July	48	942.0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Stage II (Immature)	n/a	n/a	n/a	n/a	15th of May	9th of August	38	1126.5	1st of June	8th of August	33	852.5	18th of May	25th of July	50	945.5
Stage III (Intermediate)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18th of May	31st of July	44	1053.0
Stage IV (Intermediate)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18th of May	7th of August	37	1163.5
Stage V (Mature)	18th of May	6th of September	0	1164.5	15th of May	17th of September	0	1538.5	1st of June	11th of September	0	1284.0	18th of May	14th of September	0	1618.5
Stage V - 6 m (Mature - 6 Months Storage)	18th of May	6th of February	+6 months	1164.5	15th of May	17th of February	+6 months	1538.5	1st of June	11th of September	+6 months	1284.0	18th of May	14th of February	+6 months	1618.5

Extraction. Three to five biological replicates (median slices of tuber flesh and peel from separate plants) from each clone obtained throughout several developmental stages in both raw and cooked forms were freeze dried using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a fine sieve (100 mesh). Freeze dried powder was weighed out in 600 mg samples, added to 15 mL conical tubes in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in a refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1mL aliquots and vacufuged to dryness at 45 °C in organic mode using a speed vacuum. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for total phenolic content and DPPH radical scavenging capacity.

Quantification of Total Phenolics. Phenolic phytochemicals are a class of secondary metabolites that contain one or more acidic hydroxyls attached to one or more aromatic arene (phenyl) rings (Ainsworth & Gillespie, 2007). Phenolic compounds are an important class of potent antioxidants because of their ability to quench free radicals due to their acidity. In turn, phenolic compounds are rendered radicals that are

able to remain relatively stable attributable to their ability to delocalize electrons, a characteristic of their benzene ring structure (Cheung, 2011).

The Folin-Ciocalteau microplate based colorimetric assay, an adapted method from Spanos and Wrolstad (1990), based on the original method of Singleton and Rossi (1965), was utilized to quantify total phenolics. The amount of sample extracts containing phenolic and polyphenolic antioxidants required to reduce or inhibit the oxidation of phosphomolybdate-phosphotungstate acid complexes is measured in this colorimetric assay. The amount and/or intensity of blue complexes produced, measured by the amount of absorbance at 765 nm, are indicative of a strong antioxidant.

Three to five biological replicates of 60 $mg \cdot mL^{-1}$ potato extracts from each clone obtained throughout several developmental stages in both raw and cooked forms were reconstituted in 1 mL of 80% acetone. In a new 1.5 mL Eppendorf tube, 100 µL of reconstituted extract was diluted with 900 µL of nanopure water. In a 96 well microplate, 35 µL of each diluted sample was pipetted in triplicate replication. Utilizing a multichannel pipette, 150 µL of 0.2 M Folin-Ciocalteau reagent was pipetted into all wells. The prepared microplate was placed on a shaker, agitated for 30 seconds at 400 rpm and held for 5 minutes at room temperature. Utilizing a multichannel pipette, 115 µL of 7.5% (w/v) Na_2CO_3 was pipetted into all wells. The prepared microplate was placed on a shaker, agitated for 30 seconds at 400 rpm, incubated at 45 °C for 30 minutes and cooled to room temperature for 1 hour. The absorbance was read at 765 nm utilizing a Spectromax 640 microplate spectrophotometer. Total phenolic content

was calculated by regression based on a gallic acid standard curve expressed as mg GAE·g^{-1} DW.

Statistical analyses of total phenolic content was based on three to five biological replicates from two to four years of harvest, depending upon clone, where each Folin-Ciocalteau experiment was conducted one time. Dependent upon the clone either three-way or four-way analyses of variance (ANOVA) was used to evaluate the main effects of year, cooking, developmental stages, and flesh sector color (white vs. red or white vs. purple) followed by Tukey-Kramer test to assess separation of means using SAS software.

Quantification of DPPH Radical Scavenging Capacity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) decolorization microplate based assay used a method based on Brand-Williams, Cuvelier and Berset (1995) to quantify total antioxidant capacity or radical scavenging capacity. The amount of sample extract containing radical scavenging antioxidants required to reduce the DPPH radical to DPPH-H is measured in this colorimetric assay (Prakash et al., 2001). The amount of decolorization from dark purple to yellow produced, measured by the amount of absorbance at 515 nm, is indicative of a decrease in molar absorptivity of the DPPH molecule as a free radical scavenging antioxidant donates a hydrogen forming the reduced DPPH-H and is stoichiometric to the number of electrons seized (Prakash et al., 2001).

DPPH stock solution was prepared in advance by adding 7.85 mg 1,1-diphenyl-2-picrylhydrazyl (DPPH) to 1L of 100% methanol in a light protected flask, agitated on a shaker at 400 rpm for 3 hours and stored at 4 °C for no longer than 30 days. Trolox stock solution was prepared in advance by adding 12.52 mg Trolox to 50 mL of 5 mmol

phosphate buffered saline (PBS) and stored at -20 °C for no longer than 30 days. Three to five biological replicates of 60 $mg \cdot mL^{-1}$ potato extracts from each clone obtained throughout several developmental stages in both raw and cooked forms were reconstituted in 1 mL of 5 mM PBS. In a 96 well microplate, 15 µL of each reconstituted sample was pipetted in triplicate replication. The absorbance of DPPH stock solution was adjusted to 0.90 to 0.95 absorbance units (AU) by adding approximately 12 mL of 100% methanol to 18.0 mL of prepared DPPH stock solution while reading at 515 nm using a Spectromax 640 microplate spectrophotometer. Utilizing a multichannel pipette, 285 µL of adjusted DPPH stock solution was pipetted into all 96 microplate wells and held for 3 minutes at room temperature. The absorbance was read at 515 nm. Radical scavenging capacity was calculated based on a Trolox standard curve expressed as µM $TEAC \cdot g^{-1}$ DW.

Statistical analyses of DPPH radical scavenging capacity was based on three to five biological replicates from two to four years of harvest dependent upon clone where each DPPH radical scavenging capacity experiment was conducted one time. Depending upon the clone either three-way or four-way analyses of variance (ANOVA) was used to evaluate the main effects of year, cooking, developmental stages, and flesh sector color (white vs. red or white vs. purple) followed by Tukey-Kramer test to assess separation of means using SAS software.

Pearson Correlation: Total Phenolic & DPPH Radical Scavenging Capacity. Side by side biological replicate Pearson correlations of total phenolic levels were computed alongside of DPPH/TEAC levels both based on a dry weight basis utilizing SAS software.

RESULTS

Quantification of Total Phenolics. Analysis of total phenolic (TP) levels present in aqueous extracts of freeze dried potato obtained throughout several developmental stages in both raw and cooked forms, tested for two to four consecutive years, (depending on the cultivar or selection) revealed differences in total phenolic content (main effects and interactions) (Table 2.3). The four factors that affected total phenolic content responses were due to: germplasm source (cultivars and selections with pigmented versus white tissue from whole tubers and from pigmented sectors); stage of tuber development (immature tubers versus intermediate tubers versus mature tubers versus mature tubers stored for 6 months); extraction from raw, baked and boiled tuber tissues; and year (Table 2.3).

Table 2.3 Summary of statistical significance (main effects and interactions) based on ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from 13 potato cultivars and selections. Raw and cooked tuber samples grown for two to four consecutive years (2007-2010) were obtained from five developmental stages dependent on clone. These clones included russet, red and purple skins with white, red, purple solid and sectored flesh tissues. Data are not available for n/a because not tested. Data are not significant at P=0.05 for n/s.

Cultivars & Selections	Year (Y)	Sector (S)	Developmental Stage (DS)	Process-Raw & Cook (P)	DS x P	DS x Y	DS x P x Y	DS x S x Y	P x Y	P x S
RUSSET NUGGET	*	n/a	***	n/s	n/s	**	n/s	n/a	n/s	n/a
RIO GRANDE RUSSET	***	n/a	***	n/s	n/s	n/s	n/s	n/a	n/s	n/a
CO95172-3RU	***	n/a	***	n/s	n/s	n/s	n/s	n/a	n/s	n/a
COLORADO ROSE	***	n/a	***	n/s	n/s	***	**	n/a	n/s	n/a
MOUNTAIN ROSE	***	n/a	n/s	***	**	***	**	n/a	***	n/a
CO97226-2R/R	***	n/a	n/s	***	n/s	n/s	*	n/a	n/s	n/a
CO04058-3RW/RW	n/s	***	***	***	***	**	n/s	n/s	**	***
CO04061-1R/RW	n/s	***	***	***	**	n/s	n/s	n/s	n/s	n/s
CO97216-1P/P	***	n/a	n/s	***	**	***	**	n/a	n/s	n/a
PURPLE MAJESTY	***	n/a	*	***	n/s	n/s	n/s	n/a	n/s	n/a
CO97227-2P/PW	***	*	***	***	n/s	***	n/s	n/s	n/s	n/s
CO04056-7P/PW	n/s	***	**	***	*	***	n/s	n/s	**	n/s
CO97216-3P/PW	***	***	*	**	n/s	n/s	n/s	*	n/s	n/s

ANOVA = analysis of variance.

Significance level denoted as * .05, **.01, ***.001.

See Appendix A.14-A.26 for degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Quantification of Total Phenolics: Germplasm Source (Cultivars and Selections with Pigmented versus White Tissue from Whole Tubers and Pigmented Sectors). Thirteen cultivars and selections of potato obtained from the Colorado Breeding Program were evaluated for gallic acid equivalents of total phenolic content contained one obvious difference, pigmentation (Fig. 2.2, Fig. 2.3, Fig. 2.4, Fig. 2.5; Table 2.4). CO97226-2R/R and Mountain Rose are solid red skin and flesh genotypes. CO97216-1P/P and Purple Majesty are solid purple skin and flesh genotypes. Russet Nugget, Rio Grande Russet, CO95172-3RU and Colorado Rose are white-fleshed genotypes that differ only in their skin types. CO04058-3RW/RW, CO04061-1R/RW, CO97216-3P/PW, CO97226-2P/PW and CO4056-7P/PW are sectorial expressing selections that provide an excellent model system for analysis of tissues that differ only in pigment expression that are not confounded by environmental factors or genetic segregation (Fig. 2.1).

Aqueous extracts of red and purple pigmented genotypes contained higher levels of TP when compared to white non-pigmented genotypes (Fig. 2.2, Fig. 2.3, Fig. 2.4, Fig. 2.5, and Table 2.4). This observation was significantly pronounced in pigmented sectors of two raw sectorial expressing selections, CO04058-3RW/RW and CO04056-7P/PW, throughout all developmental stages with the exception of raw, immature CO04056-7P/PW tubers obtained during stage III (Table 2.3, 2.4). CO04061-1R/RW also had higher levels of TP in red tissues when compared to white tissues but only in raw tubers obtained during harvest stage stored for 6 months (Table 2.3, 2.4).

Table 2.4 Summary of statistical significance based on Tukey-Kramer HSD for gallic acid equivalents of total phenolic content of statistically significant differences (P=0.05) that exist between white tuber tissue and purple/red tuber tissues from five sectorial expressing selections during five developmental stages. Raw and baked (170 °C for 1 hour) tuber data are based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on selection. Boiled (97 °C for 20 minutes) tuber data are based on three biological replicates for one year of harvest (2010). Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

HARVEST STAGE	Stage II (Immature)	Stage III (Intermediate)	Stage IV (Intermediate)	Stage V (Mature)	Stage V (Mature - 6 Months Storage)
RAW SELECTIONS					
Significant increase in gallic acid equivalents of TP from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	2009, 2010	2010	2010	2009, 2010	2009
CO04061-1R/RW	-	-	-	-	2009
CO97227-2P/PW	-	n/a	n/a	n/a	n/a
CO04056-7P/PW	2009	-	2010	2009	2009
CO97216-3P/PW	-	-	-	-	n/a
BAKED (170 °C for 1 Hour) SELECTIONS					
Significant increase in gallic acid equivalents of TP from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	n/a	n/a	n/a	-	n/a
CO04061-1R/RW	n/a	n/a	n/a	-	n/a
CO97227-2P/PW	n/a	n/a	n/a	n/a	n/a
CO04056-7P/PW	n/a	n/a	n/a	-	-
CO97216-3P/PW	n/a	n/a	n/a	-	-
BOILED (97 °C for 20 minutes) SELECTIONS					
Significant increase in gallic acid equivalents of TP from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	-	2010	-	-	n/a
CO04061-1R/RW	-	-	n/a	-	n/a
CO97227-2P/PW	n/a	n/a	n/a	n/a	n/a
CO04056-7P/PW	-	-	n/a	n/a	n/a
CO97216-3P/PW	-	-	-	-	n/a

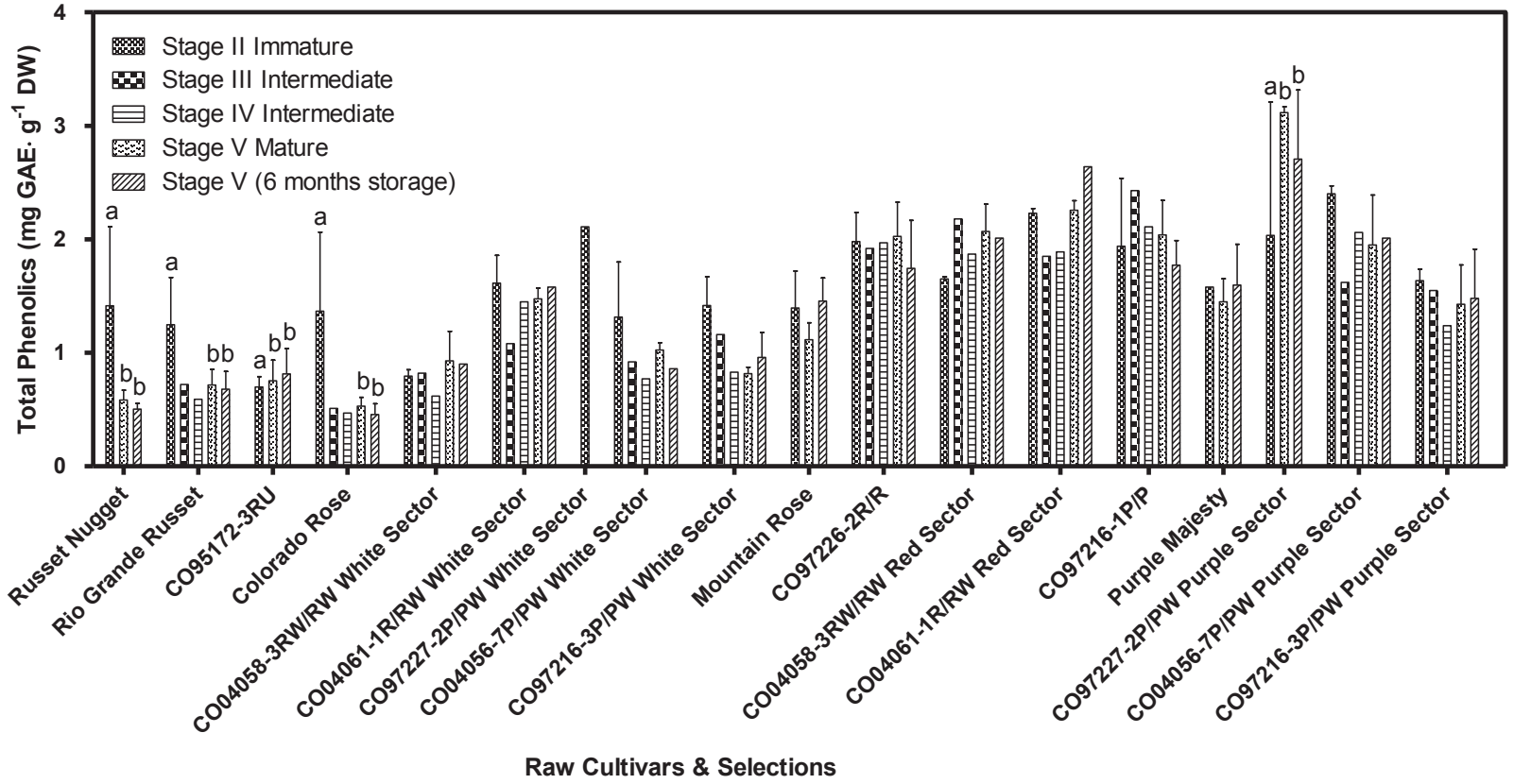

Figure 2.2 Gallic acid equivalents of total phenolics for 13 raw tuber clones for five developmental stages. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection. Letters denote statistically significant changes at P=0.05 in gallic acid equivalents of TP with increasing raw tuber maturity that occurred within a cultivar or selection based on Tukey-Kramer HSD. See Appendix Table A.28 for specific years that statistically significant changes occurred at P=0.05 in gallic acid equivalents of TP with increasing raw tuber maturity within a cultivar or selection based on Tukey-Kramer HSD.

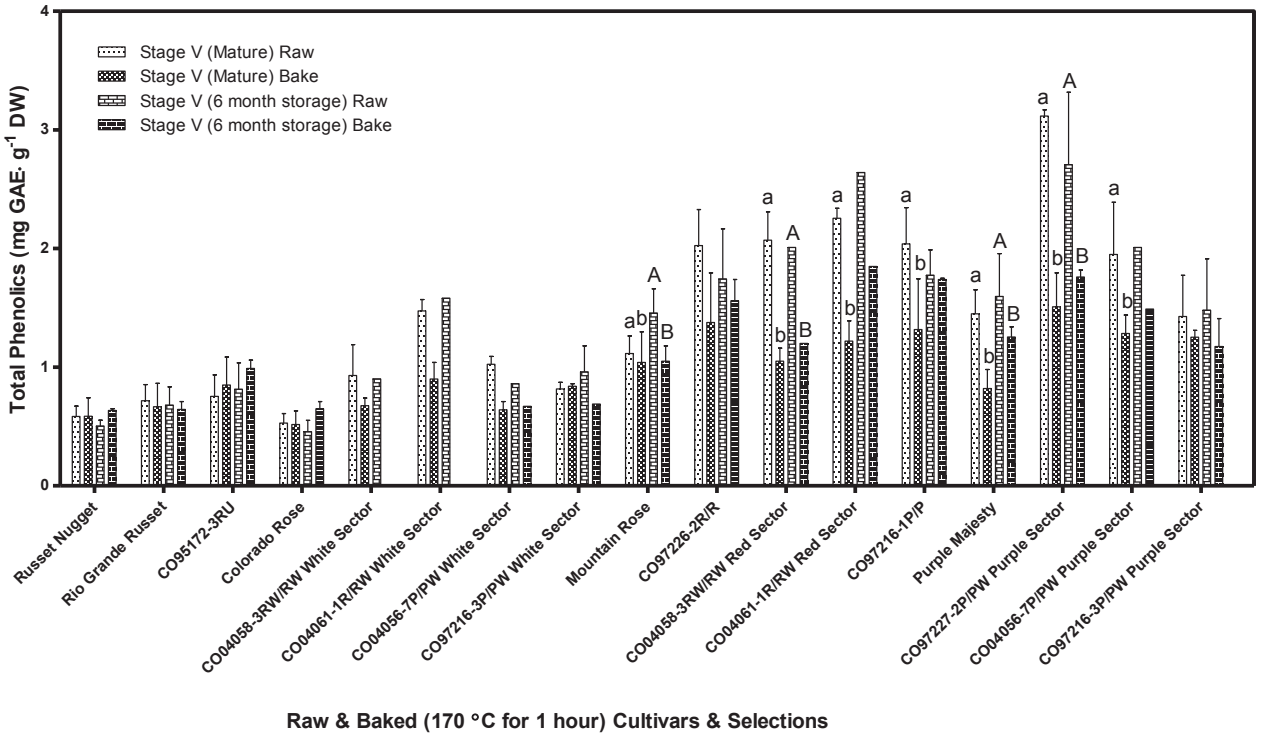

Figure 2.3 Gallic acid equivalents of total phenolics for 13 raw and baked (170 °C for 1 hour) tuber clones for two developmental stages. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process. Lower case letters denote statistically significant changes (P=0.05) in gallic acid equivalents of TP from raw to baked tubers within a cultivar or selection obtained during stage V harvest during one or more harvest years (2007-2010) based on Tukey-Kramer HSD. Upper case letters denote statistically significant changes at P=0.05 in gallic acid equivalents of TP from raw to baked tubers within a cultivar or selection obtained during stage V harvest and stored for six months at 4 °C that occurred during one or more harvest years (2007-2010) based on Tukey-Kramer HSD. See Appendix Table A.29 for specific years that statistically significant changes occurred at P=0.05 in gallic acid equivalents of TP from raw to baked tubers within a cultivar or selection within a developmental stage.

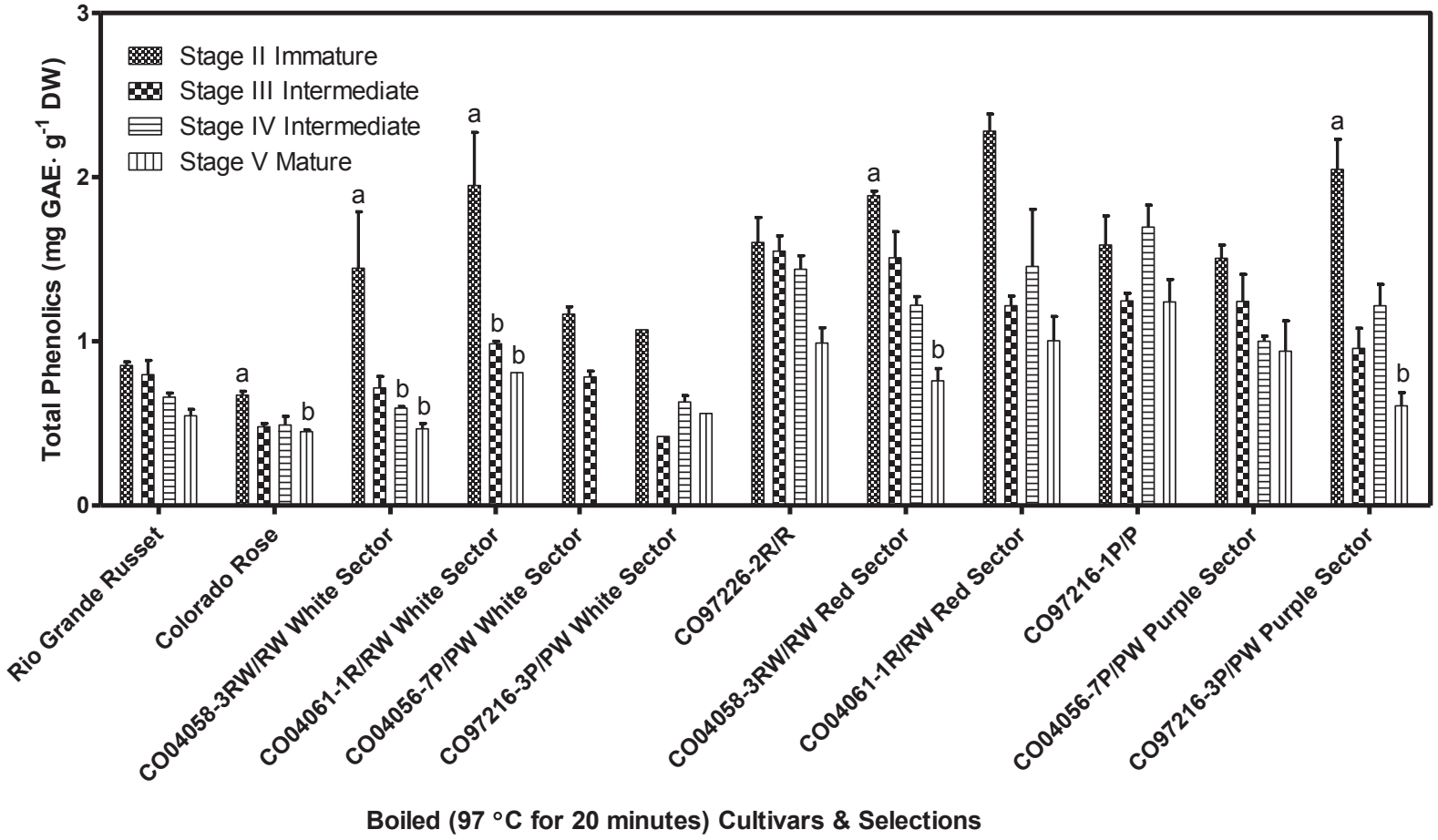

Figure 2.4 Gallic acid equivalents of total phenolics for eight boiled (97 °C for 20 minutes) tuber clones during four developmental stages. Boiled tuber data are means and SEM based on three biological replicates for one year of harvest (2010). Raw tuber data are means and SEM based on three biological replicates for two to four consecutive harvest years (2007-2010) dependent on cultivar or selection. Letters denote statistically significant changes at P=0.05 in gallic acid equivalents of TP with increasing boiled tuber maturity during 2010 harvest year within a cultivar or selection based on Tukey-Kramer HSD.

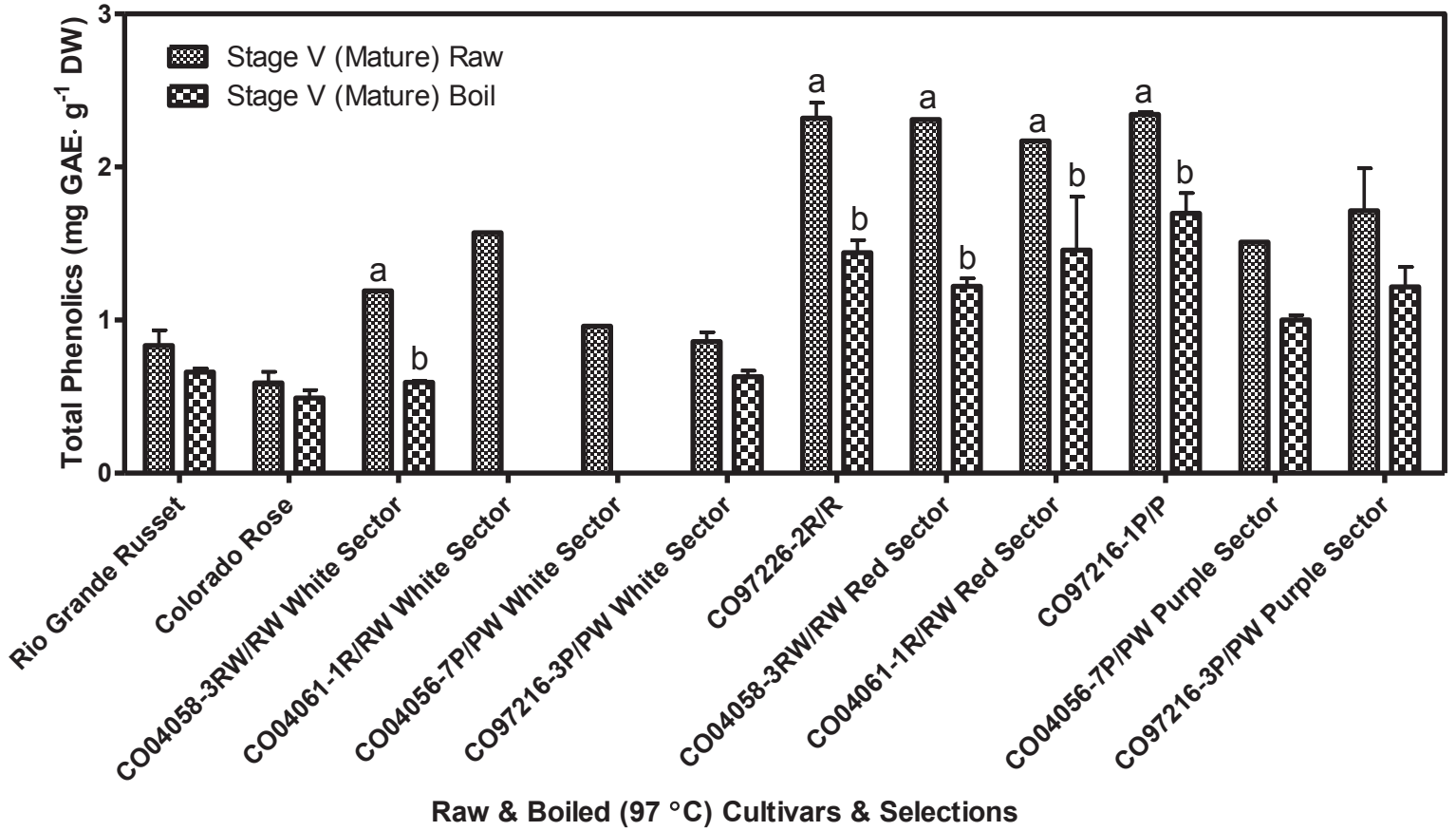

Figure 2.5 Gallic acid equivalents of total phenolics for eight clones of raw and boiled (97 °C for 20 minutes) mature tuber clones obtained during stage V. Raw tuber data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection. Boiled tuber data are means and SEM based on three biological replicates for one year of harvest (2010). Letters denote statistically significant changes at P=0.05 in gallic acid equivalents of TP from raw to boiled tubers within a cultivar or selection obtained during harvest stage during 2010 based on Tukey-Kramer HSD. See Appendix Table A.30 for all developmental stages that statistically significant changes occurred at P=0.05 in gallic acid equivalents of TP from raw to boiled tubers within a cultivar or selection.

Quantification of Total Phenolics: Developmental Stage & Cooking & Year. Total phenolic content was assayed during five developmental stages and after cooking for two to four consecutive harvests, in most cultivars and selections studied. Aqueous extracts obtained throughout several developmental stages differed significantly in ten of thirteen cultivars including: Russet Nugget, Rio Grande Russet, CO95172-3RU, Colorado Rose, CO04058-3RW/RW, C004061-1R/RW, Purple Majesty, CO97227-2P/PW, CO04056-7P/PW, and CO97216-3P/PW (Table 2.3). Aqueous extracts from cooked tuber tissues of white-fleshed cultivars did not differ significantly among Russet Nugget, Rio Grande Russet and Colorado Rose. Conversely, aqueous extracts from cooked tuber tissues of all pigmented genotypes were significantly different among Mountain Rose, CO97226-2R/R, CO04058-3RW/RW, CO04061-1R/RW, CO97216-1P/P, Purple Majesty, CO97227-2P/PW, CO040456-7P/PW and CO97216--3P/PW (Table 2.3).

Raw tubers generally had higher TP levels when compared to boiled and baked tubers during all developmental stages with a few exceptions (Fig. 2.3, 2.4). TP levels expressed as gallic acid equivalents were elevated during 2008 in aqueous extracts from the white fleshed cultivars Russet Nugget, Rio Grande Russet and Colorado Rose harvested as raw immature tubers during stage II when compared to all other developmental stages approaching maturity (Fig. 2.2). Conversely, aqueous extracts from the white fleshed and russet skinned cultivar CO95172-3RU and aqueous extracts from CO97227-2P/PW, displayed the opposite effect where TP gallic acid equivalents increased at maturity and after storage in 2008 (Fig. 2.2).

Nearly all aqueous extracts from pigmented and sectorial expressing mature genotypes obtained at harvest, and subjected to baking had lower TP levels when compared to raw tubers (Fig. 2.3). Mountain Rose, red sectors of CO04058-3RW/RW and CO04061-1R/RW, CO97216-1PP, Purple Majesty, purple sectors of CO97226-2P/PW and CO04061-7P/PW declined in TP levels at the harvest developmental stage, depending on year (Fig. 2.3). Aqueous extracts from Mountain Rose, red sectors of CO04058-3RW/RW, Purple Majesty and purple sectors of CO97227-2P/PW tubers stored for six months at 4 °C and subjected to baking declined in TP levels when compared to raw tubers stored for six months at 4 °C, depending on year (Fig. 2.3). No significant changes in TP levels were observed in baked tubers with increasing tuber maturity (Fig. 2.3).

Aqueous extracts from five of eight cultivars and selections subjected to boiling at 97 °C for 20 minutes during 2010 had lower TP levels than their raw counterparts where significant declines of TP levels were mostly observed during harvest stage (Fig. 2.5). Higher TP levels were observed in aqueous extracts from boiled immature tubers during stage II of Colorado Rose, red and white sectors of CO04058-3RW/RW, red sectors of CO04061-1R/RW and purple sectors of CO97216-3P/PW when compared to boiled tubers approaching maturity (Fig. 2.4).

Quantification of DPPH Radical Scavenging Capacity. The same four factors that affected total phenolic content also influenced DPPH/TEAC levels, namely: germplasm source (cultivars and selections with pigmented versus white tissue from whole tubers and from pigmented sectors); stage of tuber development (immature

tubers versus intermediate tubers versus mature tubers versus mature tubers stored for 6 months); extraction from raw, baked and boiled tuber tissues; and year (Table 2.5).

Table 2.5 Summary of statistical significance (main effects and interactions) based on ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from 13 potato cultivars and selections. Raw and cooked tuber samples grown for two four consecutive years (2007-2010) were obtained from 5 developmental stages, dependent on clone. These clones included russet, red and purple skins with white, red, purple solid and sectored flesh tissues. Data are not available for n/a because not tested. Data are not significant at P=0.05 for n/s.

Cultivars & Selections	Year (Y)	Sector (S)	Developmental Stage (DS)	Process-Raw & Cook (P)	P x Y	DS x Y	DS x P	DS x P x Y	DS x S	DS x S x Y	S x Y	P x S
RUSSET NUGGET	***	n/a	***	n/s	n/s	***	n/s	*	n/a	n/a	n/a	n/a
RIO GRANDE RUSSET	*	n/a	***	n/s	n/s	*	n/s	n/s	n/a	n/a	n/a	n/a
CO95172-3RU	***	n/a	***	n/s	***	n/s	***	***	n/a	n/a	n/a	n/a
COLORADO ROSE	n/s	n/a	***	n/s	n/s	n/s	n/s	n/s	n/a	n/a	n/a	n/a
MOUNTAIN ROSE	***	n/a	n/s	***	*	n/s	n/s	n/s	n/a	n/a	n/a	n/a
CO97226-2R/R	***	n/a	**	***	n/s	*	**	***	n/a	n/a	n/a	n/a
CO04058-3RW/RW	n/s	***	***	***	**	n/s	***	n/s	*	n/s	n/s	**
CO04061-1R/RW	***	***	***	***	*	n/s	***	n/s	n/s	n/s	n/s	n/s
CO97216-1P/P	***	n/a	n/s	***	*	***	n/s	*	n/a	n/a	n/a	n/a
PURPLE MAJESTY	***	n/a	n/s	***	***	*	**	n/s	n/a	n/a	n/a	n/a
CO97227-2P/PW	***	n/a	**	***	**	n/s	n/s	*	n/a	n/a	n/a	n/a
CO04061-7P/PW	n/s	***	***	***	***	n/s	*	*	n/s	**	**	n/s
CO97216-3P/PW	***	***	***	***	n/s	***	***	n/s	*	***	n/s	n/s

ANOVA = analysis of variance.

Significance level denoted as * .05, **.01, ***.001.

See Appendix A.1-A.13 for degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Quantification of DPPH Radical Scavenging Capacity: Germplasm Source (Cultivars and Selections with Pigmented versus White Tissue from Whole Tubers and Pigmented Sectors). Pigmentation was the primary difference observed among thirteen cultivars and selections of potatoes evaluated for DPPH Trolox equivalents antioxidant capacity (DPPH/TEAC) (Fig. 2.6, Fig. 2.7, Fig. 2.8, Fig 2.9; Table 2.5). Most aqueous extracts of red and purple pigmented genotypes had higher DPPH/TEAC when compared to white-fleshed tubers during all developmental stages and in both raw and cooked forms (Fig. 2.6, Fig. 2.7, Fig. 2.8, Fig. 2.9; Table 2.5). All sectorial expressing selections, CO04058-3RW/RW, CO04061-1R/RW, CO04056-7P/PW and CO97216-3P/PW revealed higher levels of DPPH/TEAC in red and purple tissues compared to white tissues throughout most developmental stages in both raw and cooked forms with some variations among years (Table 2.5, 2.6).

Table 2.6 Summary of statistical significance based on Tukey-Kramer HSD for Trolox equivalents antioxidant capacity of statistically significant differences (P=0.05) that exist between white tuber tissue and purple/red tuber tissues from five sectorial expressing selections during five developmental stages. Raw and baked (170 °C for 1 hour) tuber data are based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on selection. Boiled (97 °C for 20 minutes) tuber data are based on three biological replicates for one year of harvest (2010). Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

HARVEST STAGE	Stage II (Immature)	Stage III (Intermediate)	Stage IV (Intermediate)	Stage V (Mature)	Stage V (Mature - 6 Months Storage)
RAW SELECTIONS					
Significant increase in trolox equivalents antioxidant capacity from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	2009, 2010	2010	2010	2009, 2010	2009
CO04061-1R/RW	2009	2010	-	2010	2009
CO04056-7P/PW	2009	-	-	-	-
CO97216-3P/PW	-	-	2010	2010	2008
BAKED (170 °C for 1 Hour) SELECTIONS					
Significant increase in trolox equivalents antioxidant capacity from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	n/a	n/a	n/a	2009	n/a
CO04061-1R/RW	n/a	n/a	n/a	2009, 2010	n/a
CO04056-7P/PW	n/a	n/a	n/a	2009, 2010	2009
CO97216-3P/PW	n/a	n/a	n/a	2009, 2010	-
BOILED (97 °C for 20 minutes) SELECTIONS					
Significant increase in trolox equivalents antioxidant capacity from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	-	2010	2010	2010	n/a
CO04061-1R/RW	-	-	n/a	-	n/a
CO04056-7P/PW	-	2010	n/a	2010	n/a
CO97216-3P/PW	-	-	2010	-	n/a

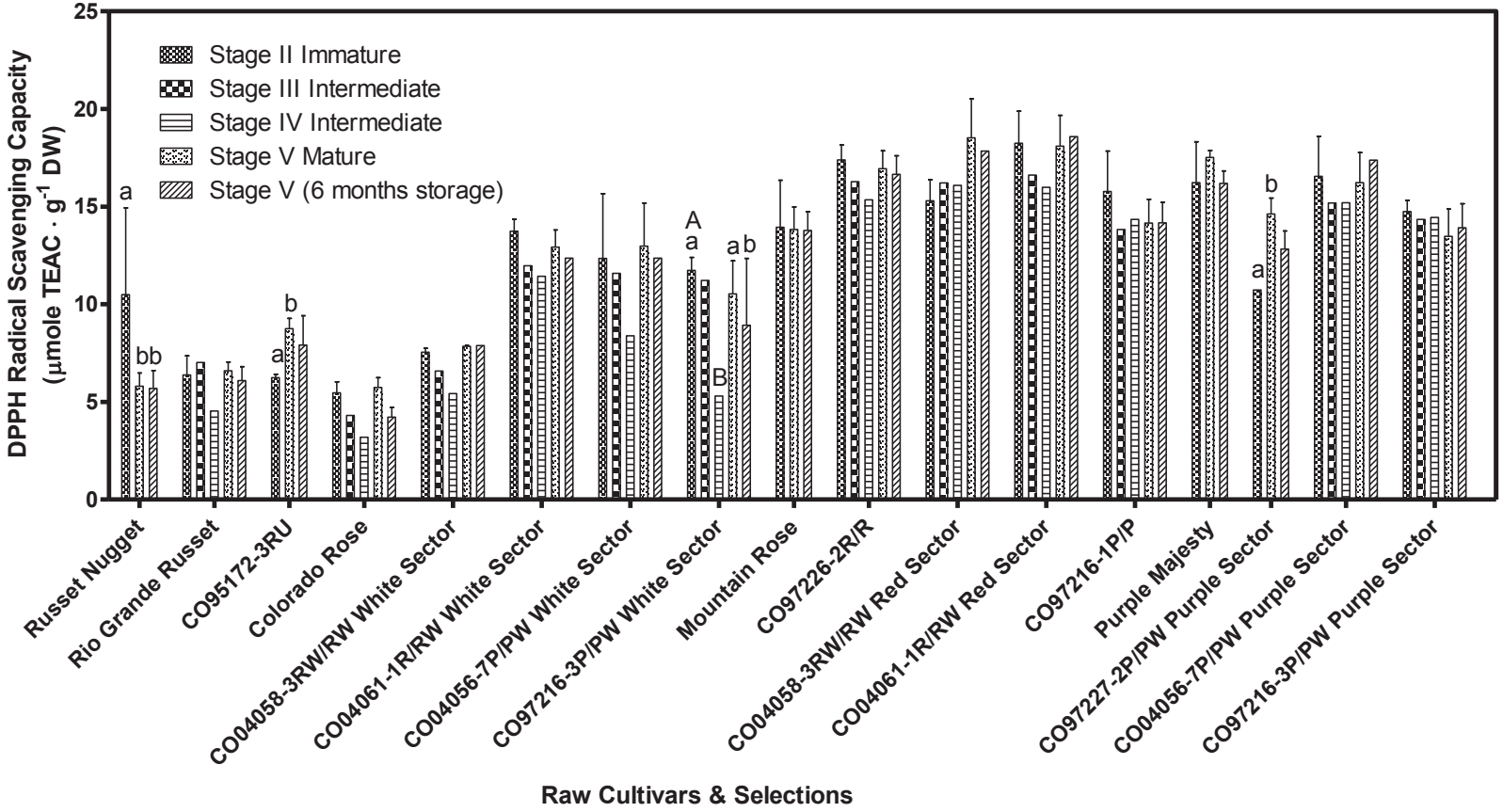

Figure 2.6 DPPH Trolox equivalents of antioxidant capacity for thirteen raw tuber clones during five developmental stages. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010). Letters denote statistically significant changes at P=0.05 in DPPH/TEAC with increasing raw tuber maturity that occurred during one or more harvest years (2007-2010) within a cultivar or selection based on Tukey-Kramer HSD. See Appendix Table A.32 for specific years that statistically significant changes occurred at P=0.05 in DPPH/TEAC with increasing raw tuber maturity within a cultivar or selection based on Tukey-Kramer HSD.

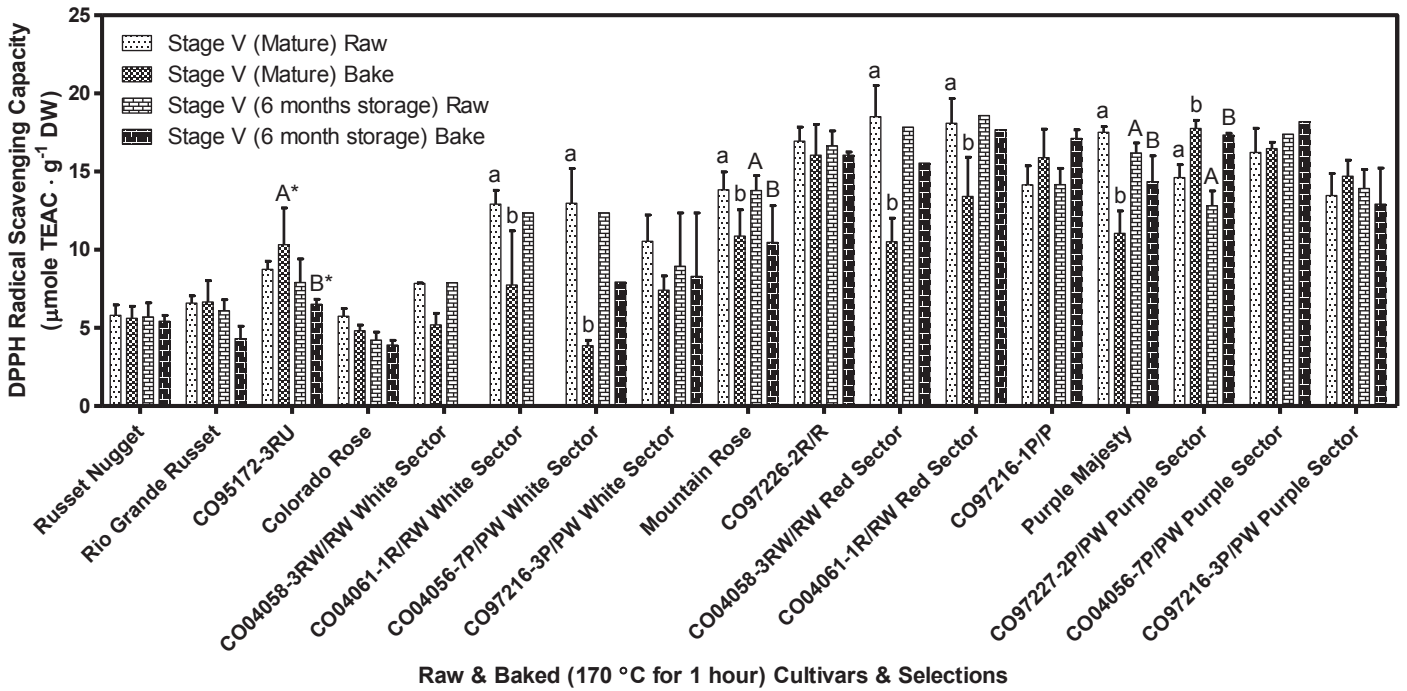

Figure 2.7 DPPH Trolox equivalents of antioxidant capacity for thirteen baked (170 °C for 1 hour) tuber clones during two developmental stages. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process. Lower case letters denote statistically significant changes (P=0.05) in DPPH/TEAC from raw to baked tubers within a cultivar or selection obtained during harvest stage during one or more harvest years (2007-2010) based on Tukey-Kramer HSD. Upper case letters denote statistically significant changes at P=0.05 in DPPH/TEAC from raw to baked tubers within a cultivar or selection obtained during harvest stage and stored for six months at 4 °C that occurred during one or more harvest years (2007-2010) based on Tukey-Kramer HSD. Upper case letters with asterisks denote statistically significant changes at P=0.05 in DPPH/TEAC with increasing baked tuber maturity within a cultivar or selection that occurred during one or more harvest years (2007-2010) based on Tukey-Kramer HSD. See Appendix Table A.33 for specific years that statistically significant changes occurred at P=0.05 in DPPH/TEAC from raw to baked tubers within a cultivar or selection within a developmental stage.

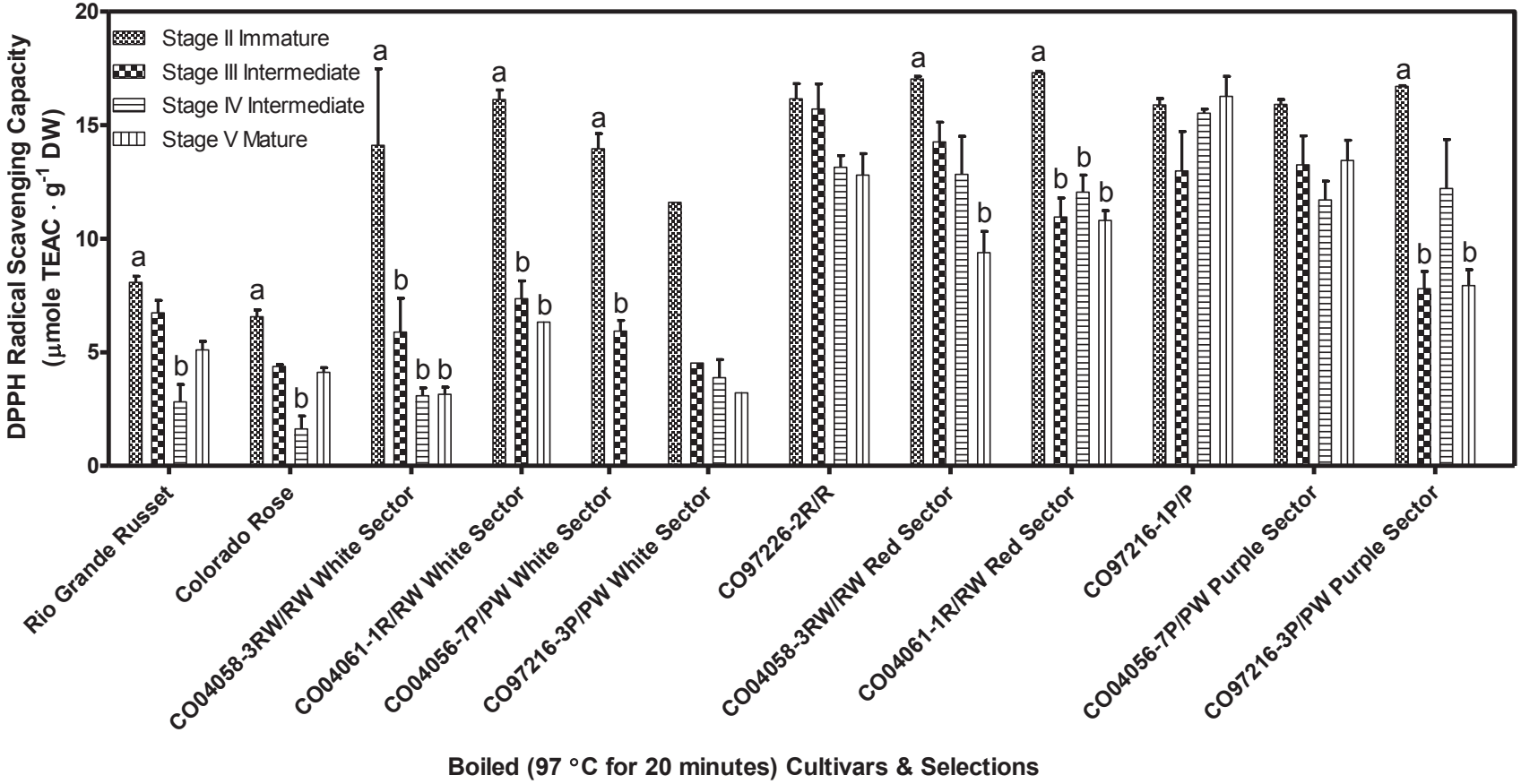

Figure 2.8 DPPH Trolox equivalents of antioxidant capacity for eight boiled (97 °C for 1 hour) tuber clones during four developmental stages. Data are means based on three biological replicates for one harvest year (2010). Boiled tuber data are means and SEM based on three biological replicates for one year of harvest (2010). Raw tuber data are means and SEM based on three biological replicates for two to four consecutive harvest years (2007-2010) dependent on cultivar or selection. Letters denote statistically significant changes at P=0.05 in DPPH/TEAC with increasing boiled tuber maturity during 2010 harvest year within a cultivar or selection based on Tukey-Kramer HSD.

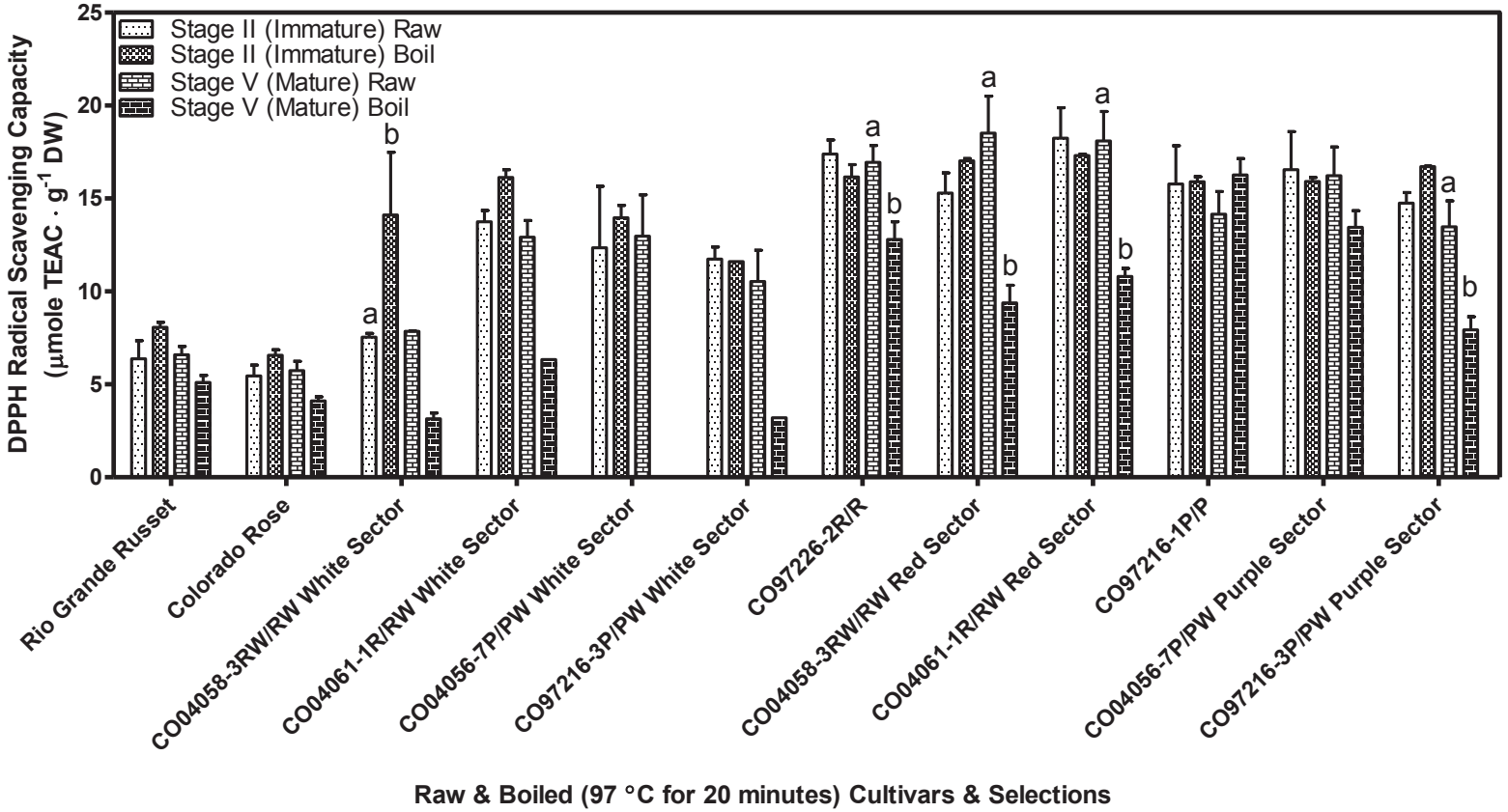

Figure 2.9 Trolox equivalent antioxidant capacity for eight clones of raw and boiled (97 °C for 20 minutes) immature tuber clones obtained during two developmental stages. Raw tuber data are means and SEM based on three biological replicates for two to four consecutive harvest years (2007-2010) dependent on cultivar or selection. Boiled tuber data are means and SEM based on three biological replicates for one harvest year (2010). Letters denote statistically significant changes at P=0.05 in DPPH/TEAC from raw to boiled tubers within a cultivar or selection obtained as in immature tuber (stage II) and harvest stage during 2010 based on Tukey-Kramer HSD. See Appendix Table A.34 for all developmental stages that statistically significant changes occurred at P=0.05 in DPPH/TEAC from raw to boiled tubers within a cultivar or selection.

Quantification of DPPH Radical Scavenging Capacity: Developmental Stage & Cooking & Year. DPPH radical scavenging capacity was assayed during five developmental stages and after cooking for 2-4 consecutive harvests, in most cultivars and selections tested. Aqueous extracts obtained throughout several developmental stages differed in 10 of the 13 cultivars (Table 2.5). Aqueous extracts from cooked tuber tissues of white-fleshed cultivars did not differ among Russet Nugget, Rio Grande Russet or Colorado Rose. Aqueous extracts from cooked tuber tissues of all pigmented and sectorial expressing genotypes differed among Mountain Rose, CO97226-2R/R, CO04058-3RW/RW, CO04061-1R/RW, CO97216-1P/P, Purple Majesty, CO97227-2P/PW, CO040456-7P/PW and CO97216--3P/PW (Table 2.5).

Raw tubers obtained throughout several developmental stages from different years revealed two trends. The first trend observed was an elevation in levels of DPPH/TEAC in aqueous extracts from Russet Nugget, purple sectors of CO97227-2P/PW and white sectors of CO97216-3P/PW harvested as raw immature tubers during stage II during one or more years when compared to all other developmental stages approaching maturity (Fig. 2.6). A second trend detected was the reverse effect where levels of DPPH/TEAC were elevated in aqueous extracts from CO95172-3RU harvested as raw mature tubers at harvest when compared to immature tubers harvested during stage II (Fig. 2.6).

Aqueous extracts of tubers subjected to boiling from different developmental stages displayed some striking tendencies. Aqueous extracts from boiled immature white tissue of CO04058-3RW/RW tubers during stage II increased in DPPH/TEAC compared to their raw counterparts (Fig. 2.9). Decreases in DPPH/TEAC were

observed in most clones of boiled tubers particularly during harvest when compared to their raw complement (Fig. 2.9). Higher DPPH/TEAC levels were observed in aqueous extracts from boiled immature tubers during stage II of most clones when compared to boiled tubers approaching maturity (Fig. 2.8).

Aqueous extracts of tubers subjected to baking from different developmental stages during different years also displayed some interesting tendencies. Aqueous extracts from Mountain Rose, red sectors of CO04058-3RW/RW, red and white sectors of CO04061-1R/RW, Purple Majesty and white sectors of CO0456-7P/PW subjected to baking at harvest decreased in DPPH/TEAC compared to raw mature tubers at harvest (Fig. 2.7). Aqueous extracts from mature clones stored for 6 months of Mountain Rose and Purple Majesty subjected to baking decreased in DPPH/TEAC when compared to raw mature tubers stored for 6 months (Fig. 2.7). Aqueous extracts from mature baked tubers of CO97227-2P/PW obtained at harvest and mature tubers stored for 6 months, displayed the opposite effect where an increase in DPPH/TEAC was observed when compared to its raw counterparts for two consecutive years (Fig. 2.7). Aqueous extracts from baked CO95172-3RU tubers obtained at harvest had higher DPPH/TEAC than baked tubers obtained at harvest and stored for six months (Fig. 2.7).

Pearson Correlation: Total Phenolic & DPPH Radical Scavenging Capacity. TP and DPPH/TEAC levels were positively correlated in all cultivars and selections at a high level of significance with the exception of CO97227-2P/PW (Table 2.7).

Table 2.7 Summary of statistical significance at P=0.05 based on Pearson Correlation for degree of linear association that exists between DPPH radical scavenging capacity and total phenolic antioxidant content. Sample size = n. Correlation coefficient = r. Level of significance = p.

CULTIVARS & SELECTIONS	n	r	p	DPPH	TP
RIO GRANDE RUSSET	63	0.377	0.0023	X	X
COLORADO ROSE	63	0.289	0.0217	X	X
RUSSSET NUGGET	42	0.855	<.0001	X	X
CO95172-3RU	39	0.572	0.0001	X	X
CO97226-2R/R	63	0.607	<.0001	X	X
MOUNTAIN ROSE	42	0.755	<.0001	X	X
CO04058-3RW/RW	78	0.908	<.0001	X	X
CO04061-1R/RW	65	0.846	<.0001	X	X
CO97216-1P/P	63	0.469	0.0001	X	X
CO04056-7P/PW	66	0.551	<.0001	X	X
CO97216-3P/PW	105	0.663	<.0001	X	X
CO97227-2P/PW	39	-0.116	0.4830	-	-
PURPLE MAJESTY	45	0.707	<.0001	X	X

DISCUSSION

This study has made evident the importance of clones with pigmented tissues. Both red and purple pigmented genotypes generally contained higher levels of phenolic antioxidants and total antioxidant capacity when compared to white genotypes. These observations may be attributable to the anthocyanidin class of phenolic metabolites, such as the acylated anthocyanidin glucosides pelargonidin, petunidin, malvidin and peionidin for example, that exist in the vacuoles of red and purple pigmented tuber cells.

This study indicated that cooking generally reduced antioxidant levels. Phenolic antioxidants and total antioxidant capacity are highest in raw tubers. Unfortunately, we as humans typically do not consume raw tubers. Although considerably lower in phenolic content and DPPH radical scavenging capacity than pigmented types, this study has uncovered that white fleshed tubers may be less susceptible to cooking processes as well but because levels are considerably lower the cooking consequence has little impact. Thus, recognizing the best genotype and cooking method is critical if we are to reap the benefits of consuming disease preventing antioxidants in potato.

The results of this study have also demonstrated that harvesting raw tubers and consuming cooked tubers at the appropriate developmental stage is also critical if we are to reap the benefits of consuming disease preventing antioxidants in potato. A few common trends observed with regards to levels of phenolic antioxidants and total antioxidant capacities in potato were revealed in this study. Perhaps the most important discovery from this work was that some genotypes harvested as immature tubers during stage II, tend to contain elevated levels of phenolic antioxidants and total antioxidant capacity. Another trend showed that boiling certain genotypes of immature tubers

obtained during stage II increased phenolic antioxidants and total antioxidant capacity even more than their raw counterparts. A final common trend observed revealed that phenolic antioxidants and total antioxidant capacity were elevated in particular genotypes of both raw and baked mature tubers that were stored for 6 months when compared to raw and baked mature tubers obtained during harvest stage. This was clearly evident when comparing red and purple segments to non-pigmented tissues in the sectorial expressing genotypes.

Highly significant correlations between TP and DPPH/TEAC in ten of the clones suggest that polyphenolic compounds play an important, but not exclusive role as radical scavengers. The low r values in two of the significant correlations also suggest that some other bioactive compound(s) besides polyphenols, may act as free radical scavengers in 'Rio Grande Russet' and 'Colorado Rose.'

The overall purpose of this study was to better understand the complexities and to quantify levels of radical scavenging capacity of phenolic antioxidants and total antioxidants that exist in various genotypes of potato developed by the Colorado Potato Breeding Program in an effort to prevent and/or reduce the prevalence of oxidative stress type induced diseases via dietary intervention. This work has demonstrated important nutritional differences and consequences of cooking of these unique cultivars and selections developed by the Colorado Potato Breeding Program. This work has also demonstrated the significance of developmental stage and genotype important to growers interested in developing high market value tubers. In conclusion, this study has revealed some key findings important to both consumers and growers of potato.

Chapter 3

FINE-TUNING MICROPLATE BASED CANCER SCREENING METHODS: (1) EXTRACTION OF *SOLANUM TUBEROSUM* L. FOR ANALYSIS AGAINST HT-29 COLON CANCER CELL CULTURES; (2) QUANTITATIVE EXPRESSION OF VIABLE CELLS IN COLON CANCER COLORIMETRIC CELL VIABILITY ASSAYS VIA DEVELOPMENT OF AN ACETYLSALICYLIC ACID STANDARD CURVE

SUMMARY

Numerous milestones in tissue and cell culture have led to the development of thousands of cancer cell lines available today utilized as *in vitro* models for studying cancer. Research studies testing the efficacy of fresh fruit and vegetable extracts for bioactivity against cancer cell cultures necessitates the preservation of the integrity of the biochemical, biological and immunological properties typically upon harvest. Two commonly utilized methods for preparing fresh fruits and vegetables for extraction in organic solvent include freeze drying and cryogenic grinding. The first objective of this study was to fine-tune current methods utilized to prepare *Solanum tuberosum* L. for extraction and reconstitution in aqueous cell culture medium by evaluating aqueous extracts from freeze dried tuber preparation methods versus tubers that underwent cryogenic grinding preparation methods against HT-29 colon cancer cells in culture. While all aqueous extracts from the two tuber selections tested inhibited HT-29 colon cancer cell cultures, inhibition was significantly enhanced ($P=0.002$) in purple pigmented tubers from cryogenically ground tubers when compared to samples from freeze dried ground tubers. A valuable result from this study is the importance of investigating what

extraction preparation method is best used prior to screening biological materials for inhibitory activity against cancer cell cultures.

Cancer activity in microplate based cell viability assays has been expressed in various ways including percent growth inhibition based on control, percent cell survival based on control and cell number versus time to name a few. The second objective of this study was to develop a novel method for quantitatively expressing cell viability based on a known reference standard curve. After various attempts, five concentrations of 0, 0.19, 0.38, 0.75 and 1.5 mg acetylsalicylic acid (ASA)·mL^{-1} aqueous cell culture medium) were designated as known reference standards to structure the fitted linear regression model to express the number of viable cells in an unknown sample when utilizing the CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega. The ASA standard curve linearly inhibits colon cancer cells in culture with an acceptable correlation coefficient, *r*, and coefficient of determination, R^2. The ASA standard curve also spans the concentrations of unknown samples and accurately interpolates and extrapolates cell viability in unknown samples. Reporting results based on the devised acetylsalicylic acid standard curve allows for an easier, standardized and novel quantitative method for expressing number of viable HT-29 colon cancer cells in a colorimetric assay.

INTRODUCTION

History of Cell Culture. Langdon (2004) explains milestones in history that led to the development of current cell and tissue culture methods. The establishment of culture type techniques dates back to 1885 when Wilhem Roux maintained tissue from

the medullary plate of chick embryos in saline solution for several days *in vitro* for the first time. Langdon (2004) defines tissue culture as the "maintenance of tissue explants in culture". In 1911 Warren Lewis investigated the necessity of factors in growth medium which led to the first culture of cancer cells in 1914 and first culture of epithelial cells in 1922 by Albert Ebeling. Langdon (2004) defines cell culture as the "maintenance of dissociated cells in culture". The first continuous cell line of rodent cells was established by Wilton Earle in 1943. Langdon (2004) defines a cell line as "a culture that is subcultured beyond the initial primary culture phase". Langdon (2004) defines a continuous cell line as "a cell line that is essentially immortal and continues indefinitely". The first human cancerous continuous cell line, HeLa, was established by George Gey in 1951. During the 1950-60's several investigators contributed to the refinement of nutritional requirements necessary for cell culture maintenance. All of these milestones in tissue and cell culture have led to the development of thousands of cancer cell lines available today utilized as *in vitro* models for studying cancer.

Uses of Cell Culture. The applications in science and medicine of cell culture are numerous. Cell culture may be utilized to explore normal cell physiology, biology, biochemistry and genetics (Chaudry, 2004). Cell models are utilized to study cytostatic responses, cytotoxic responses, induction of apoptosis and dosage requirements of both natural and chemical candidates on normal and cancerous cells and is an initial assay required prior to advancement of testing the efficacy of these treatment candidates on whole organisms (Chaudry, 2004; Langdon, 2004). Cell cultures may be utilized to investigate tissue engineering for production of artificial tissues and artificial

skin as well as to synthesize cell products, proteins, bacteria and viruses for example that are only available via propagation of animal cell cultures (Chaudry, 2004).

Advantages and Disadvantages of Cancer Cell Culture. Adamson (2004) explains that cancer cell culture methods are advantageous for several reasons. Cell culture assays are not very labor intensive and are cost effective. Cell culture assays produce consistent and reproducible results. Cell culture models provide a moderate throughput capability to screen natural and chemical treatments individually and/or synergistically on multiple cell lines in a short amount of time. In addition, if a drug does not elicit a response against cancer cells *in vitro*, there is a probability that it will not illicit a response *in vivo*. Accordingly, cell culture methods provide screening for potential future chemotherapeutic drug candidates and further comprehension of cancer biology and drug interactions at the cellular level.

Although *in vitro* cell culture methods have many advantages there are several limitations as well. Cancer cell lines are derived from primary cultures which may be defined as “the initial culture established from an individual, representing the situation most closely related to the original tissue” (Langdon, 2004). This primary culture may contain various cell types where certain cells within the mix may grow faster under culture conditions and outgrow slower non-proliferating cells resulting in a loss of heterogeneity (Langdon, 2004). Thus, if the cell type of interest is the population of cells growing rapidly then this gain in homogeneity is an advantage (Langdon, 2004). Conversely, if the cell type of interest is the population of cells proliferating slowly then this gain in homogeneity is clearly a disadvantage (Langdon, 2004). In addition, the development of cell lines must undergo a transformation that requires a homogeneous

population of cells to materialize via selective growth methods thus creating a cell line lacking the heterogeneous and diverse cell populations that truly represent the primary culture (Adamson, 2004; Langdon, 2004).

The differences in tumor microenvironment in whole organisms versus cell cultures alone present another limitation in cell culture. Cell culture models deviate from whole organism systems by lacking vascularization, organized tissue and structure, and cell to cell contact as cultures are never left to grow to 100% confluency (Adamson, 2004). A solid tumor, *in vivo* microenvironment, experiences hypoxic conditions due to disruptions in structural and functional microcirculation (Hockel & Vaupel, 2001). Hypoxic conditions in tumors *in vivo* have been found to be strongly associated with tumor propagation, malignant progression, resistance to therapy and upregulation of many of the angiogenic growth factors, including VEGF, PDGF, PlGF, and HGF (Sigma Life Science, 2011). Cell culture *in vitro* microenvironments typically experience oxygen conditions greater than 1% (BioSpherix, Ltd., 2012). Controlling for hypoxic conditions *in vitro* can be difficult but not impossible (BioSpherix, Ltd., 2012). Consequently, the presence of normoxic conditions in cell culture models may misrepresent actual cell culture based assay results.

The effects of chemotherapeutic drugs *in vivo* may differ from inhibitory effects produced on *in vitro* cells in culture. Some chemotherapeutic drugs may require metabolic activation to elicit a response available only *in vivo* (Alley et al., 1984). Also, the manner in which a chemotherapeutic drug binds plasma proteins in the blood dictates the bioavailability of the drug also determined only *in vivo* (Adamson, 2004). In addition, if a drug elicits an inhibitory response against cancer cells *in vitro* and/or

requires suprapharmacological concentrations to illicit a response, there is a probability that it will not illicit a response *in vivo* (Adamson, 2004). As a result, *in vitro* cell culture cannot be utilized solely to predict the response of a drug against cancer cells *in vivo* (Adamson, 2004).

Preparation of Extracts for Cancer Cell Culture: Fruits & Vegetables. Fresh fruits and vegetables constitute classes of phytochemicals such as allylic compounds, isothiocyanates, indoles, monoterpenes, vitamins, carotenoids, chlorophyll, flavonoids and cinnamic acids that have been found to inhibit the carcinogenic process via induction of phase I and phase II detoxification enzymes, modulation of phase I and other enzyme activities, antioxidant activity, electrophile scavenging activity, inhibition of nitrosation, and/or modulation of oncogene or proto-oncogene expression or function (Huang et al., 1994). Research studies testing the efficacy of fresh fruits and vegetables extracts as bioactive agents against cancer cells in culture necessitates the preservation of the integrity of the biochemical, biological and immunological properties typically upon harvest. Two commonly utilized methods for preparing fresh fruits and vegetables for extraction in organic solvent include freeze drying and cryogenic grinding.

Freeze-drying may be defined as a process that “removes a solvent (usually water) from dissolved or dispersed solids” (Oregon Freeze Dry Inc., 2007). Labconco Corporation (2010) explains that the freeze drying process comprises of three stages. During the first stage, prefreezing, the product is frozen below its eutectic temperature allowing for separation of solvent from solids. During the second stage, primary drying, both pressure and temperature play a role in removing all unbound water via

sublimation. A pressure gradient is created by utilizing a vacuum pump to lower the vapor pressure surrounding the product so that it is lower than the vapor pressure of the product causing frozen solvent molecules to migrate out of the product from a high vapor pressure area to a low vapor pressure area. During primary drying, a temperature gradient is also created by adding heat so that the product temperature is warmer than that of the collector which condenses the solvent vapor that was removed from the product. During the third stage, secondary drying, warmer temperatures are applied to the drying chamber still under vacuum causing residual bond water remaining to be desorbed from the product. After the freeze drying process is complete the freeze dried product may be ground into a fine powder and extracted in organic solvent for analysis for boactivity against cancer cell cultures.

There are numerous benefits for utilizing freeze drying prior to extraction. The slow rate of freezing during the prefreezing stage allows sufficient time for cells to expel their intracellular water into the extracellular matrix resulting in a dehydrated cell that does not undergo intracellular freezing which is typically lethal (Mazur, 1984). Other major advantages of freeze drying include longer shelf life; reduced weight for storage, shipping and handling; preservation of morphological, biochemical and immunological properties; high viability; and drying methods that occur at low temperatures and low oxygen levels (Oregon Freeze Dry Inc., 2007).

Conversely, there are also disadvantages of freeze drying prior to extraction. The slow rate of freezing during the prefreezing stage renders fewer nucleation sites to be initiated causing large ice crystals to form in the extracellular matrix of cells which can result in mechanical damage to delicate cell structures and may also result in

cellular dehydration (Mazur, 1984). In addition the accumulation of solutes from pure water during the prefreezing stage and/or a pH change may result in protein denaturation (Brennan & Grandison, 2012). The porous structure of the final freeze dried products is prone to both oxidation and water vapor adsorption if not adequately contained (Brennan & Grandison, 2012). The freezing temperature just below the eutectic temperature of many products may be above temperatures of -130 °C which is the approximate temperature where biological activity is significantly reduced (Mazur, 1984); therefore there is a possibility for degradation if freezing at temperatures above -130 °C. As a final point, the cost of freeze drying can be expensive.

Cryogenic grinding is a process where a product undergoes low temperature freezing via addition of liquid nitrogen (-195.79 °C), liquid argon (-185.85 °C) or liquid carbon dioxide (-57 °C) to facilitate breaking a product into small particles (Bentor, 2009; wiseGEEK, 2012). Immediately after the cryogenic grinding product process is complete the fine powder is extracted in organic solvent for analysis for bioactivity against cancer cell cultures.

There are numerous benefits of utilizing cryogenic grinding prior to extraction. The rapid rate of freezing by supercooling liquids renders numerous nucleation sites to be initiated causing small ice crystals to form in the extracellular matrix of cells which may impose zero or less damage to delicate cellular structures when compared to the large crystals formed during the slow rate of freezing during the freeze drying process (Fuller et al., 2004). Biological activity is significantly reduced at temperatures below -130 °C where the only physical states that subsist are crystalline and or glassy (Mazur, 1984). While at temperatures of -196 °C, there is inadequate thermal energy for

chemical reactions to persist and only photophysical events such as free radical formation for example may occur (Mazur, 1984). Accordingly, liquid nitrogen (-196 °C), is typically utilized for the infinite preservation of complex biological molecules (Baust & Baust, 2007). For example, liquid nitrogen is commonly used to cryogenically grind biological products in fine powder as to break down cell walls and increase surface area allowing for extraction of nucleic acids at the same time as degrading nucleases are rendered inactive at sub zero temperatures. Other advantages associated with use of cyrogenics include a reduced risk of microbial contaminations; infinite shelf life if products remain frozen at -196 °C; high viability; economical; and no heat is involved thus low potential for thermal degradation.

There are also some clear disadvantages for utilizing cryogenic grinding prior to extraction. The rapid freezing of product causes extracellular ice formation and separation of solute concentration to occur more rapidly than exosmosis can occur which may result in intracellular ice nucleation which is typically lethal (Mazur, 1984). Another problem that may be encountered involves the time period that follows the cryogenic grinding where if biological materials are subject to thawing at temperatures of ~ -15 °C to -60 °C (Mazur, 1984) they may be also subject to degradation.

Expression of Cell Viability Utilizing an Acetyl Salicylic Acid Standard Curve. In the early 1800's a bitter white crystalline glycoside found in Saliaceous plant species was extracted from the bark of the willow tree and identified as salicin, which has been utilized by many civilizations for ages as an antipyretic, antirheumatic, analgesic and tonic (Merriam-Webster, 2011; Mahdi et al., 2006). In 1835, experiments involving the acidic hydrolysis of salicin led to the discovery that salicin was composed

of D-glucose and salicyl alcohol moieties (Mahdi et al., 2006). During the mid 1800's a compound known as salicylic acid, a phenolic acid that is the ortho form of hydroxybenzoic acid, was synthetically synthesized in the laboratory via oxidation of salicyl alcohol (saligenin), oxidation of salicyl aldehyde, hydrolysis of methylsalicyl ester and also naturally extracted from *Spirea ulmaria* (Merriam-Webster, 2011; Mahdi et al., 2006). Salicylic acid has been found to inhibit the enzyme prostaglandin endoperoxide synthase (a.k.a. cyclooxygenase) which converts arachidonic acid to prostaglandins (PGs), a class of horomones generally responsible for inducing inflammatory type responses, gene expression, growth and differentiation (Pierpont, 1997; Mahdi et al., 2006). Thus, salicylic acid was found to be a powerful anti-inflammatory and medicinally useful as an antiseptic and disinfectant for treating skin disorders, a keratolytic agent for skin exfoliation, an analgesic, antipyretic and antirheumatic (Merriam-Webster, 2011). Adversely, consumption of salicylic acid was also found to irritate the gastric mucosae causing bleeding due to interference of prothrombrin synthesis causing prothrombin deficiency (Pierpont, 1997). In the 1890's, it was discovered that acetylation of salicylic acid reduced these adverse irritations of the gastric mucosae accordingly leading to the pharmacological development of acetylsalicylic acid (ASA), commonly known as aspirin (Mahdi et al., 2006).

Research has since found many beneficial effects of aspirin on human health. Analgesic and anti-inflammatory effects of aspirin have been utilized to treat rheumatism, lumbago and neuralgia (Mahdi et al., 2006). A reduced risk of myocardial infarctions has been observed with consistent aspirin regiment (Mahdi et al., 2006). Aspirin has been found to prevent recurrent transient ischemic attacks in men and is

now used as a regiment for those with a history of stroke (Mahdi et al., 2006). An observational analysis reviewed eight studies consisting of more than 25,000 participants and found a 20 percent reduction in death by certain cancers due to aspirin expenditure (Cheng, 2010). Long-term aspirin regiment has resulted in a greater reduced incidence of colorectal cancers (Mahdi et al., 2006). In addition, aspirin offers greater protection over colorectal cancers than non-gastrointestinal cancers (Mahdi et al., 2006).

Various mechanisms behind aspirin chemoprevention to date have been discovered. Several types of cancer tend to over generate the production of PGs ensuing in cellular proliferation, resistance to apoptosis and increased angiogenesis (Mahdi et al., 2006). Aspirin has been found to interfere with PG dependent pathways by inhibiting two isoforms of the enzyme cycloxygenase, COX-1 and COX-2, as aforementioned, leads to the production of PGs (Mahdi et al., 2006). Aspirin also interferes with several types of cancers via various non PG dependent pathways as well to include but not limited to: acting as an antioxidant offering cellular protection against oxidative damage (Mahdi et al., 2006); interfering with the cell cycle and/or cellular regulatory molecules (Mahdi et al., 2006); acetylation and posttranslational modification of cellular proteins resulting in inhibition of ribonucleotide synthesis (Marimuthu et al., 2011); a novel NOSH-aspirin that has been designed to produce both nitric oxide which provides protection to stomach lining and hydrogen sulfide which boosts aspirins chemopreventive effects has been found to inhibit proliferation of 11 cancer cell lines in culture to include colon, pancreatic, lung, prostate, breast and leukemia without harming normal cells (The City College of New York, 2012).

Aspirin has been found to be effective in inhibiting cell proliferation of the following cancer cell lines *in vitro* in a dose dependent manner ranging from acetylsalicylic acid concentrations of 3 mM to 20 mM: colon cancer cell lines (HT-29, HCT116, HCT116 + chr3 and SW480); pancreatic cancer cell lines (BxPC3 and Panc-1); gastric cancer cell line (AGS and MKN-28); Ishikawa human endometrial tumor cells; OVCAR-3 ovarian tumor cells; and human epithelial cell line (HeLa TG) (Mahdi et al., 2006).

CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega is a colorimetric assay that may be used to measure the number of viable cells in cell culture assays. The principal source of this assay involves the use of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is a water soluble salt in combination with (phenazine methosulfate; PMS) an electron coupling agent reagent. Dehydrogenase enzymes present in metabolically active cells cleave the tetrazolium ring and convert MTS into a colored formazan product that is soluble in tissue culture medium. The amount of formazan product produced, measured by the amount of absorbance at 490 nm, is directly proportional to the number of viable cells in culture.

Cancer activity in microplate based cell viability assays has been expressed in various ways including percent growth inhibition based on control, percent cell survival based on control and cell number versus time to name a few. An easier way to present cell viability in an unknown sample would be to reference cell viability of a commonly known reference standard in a colorimetric cell viability assay. Developing this method for expressing cell viability requires establishing a standard curve where absorbance of

a known substance at 590 nm is related to concentration of solute of a known substance spanning the range of absorbance values you would expect unknown absorbance values of future samples to fall and fitting this curve to a regression model.

The first objective of this study was to fine-tune current methods utilized to preserve *Solanum tuberosum* for extraction and reconstitution in aqueous cell culture medium by evaluating aqueous extracts from freeze dried tuber preparation methods versus tubers that underwent cryogenic grinding preparation methods against HT-29 colon cancer cells in culture. The second objective of this study was to develop a novel method for expressing cell viability based on a known reference standard curve utilizing ASA.

MATERIALS & METHODS

Clones Utilized for Freeze Dried vs. Cryogenically Ground Prepared Analysis.

Two clones of *S. tuberosum* (CO97226-2R/R and CO97216-1P/P) were obtained from the Colorado Potato Breeding Program, grown under essentially commercial production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado (Table 3.1). Both of these clones are in an experimental phase of release and are designated as selections. The first letter before the slash is indicative of tuber skin color (P=purple and R=red) (Table 3.1). The letters following the slash are indicative of tuber flesh colors (P=purple and R=red) (Table 3.1). Both selections were examined for inhibitory activity as raw potatoes harvested as immature tubers (growing degree days [GDD] = 946) during 2010 and stored for six months at 4 °C in dark conditions. Immature tubers were selected because they were found in

earlier studies to provide the highest and most consistent level of inhibitory bioactivity against HT-29 colon cancer cells in culture.

Clones Utilized for Analysis of Acetylsalicylic Acid as a Reference Standard. Two clones of *S. tuberosum* (CO97226-2R/R peel and CO04058-3RW/RW peel) were obtained from the Colorado Potato Breeding Program, grown under essentially commercial production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado (Table 3.1). Both of these clones are in an experimental phase of release and are designated as selections. The selection ending in -RW/RW contains pigmented and white sectors of tissue that are otherwise of the same genotype and not confounded by environmental and developmental differences (Table 3.1). The first letter before the slash in both selections is indicative of tuber skin color (R=red and W=White) (Table 3.1). The letters following the slash in both selections are indicative of tuber flesh colors (R=red and W=White) (Table 3.1). Both selections were examined for inhibitory activity as raw potatoes peels harvested from immature tubers (growing degree days [GDD] = 946) in 2010 based on previous evidence of high inhibitory bioactivity.

Table 3.1 Clonal selections from the Colorado Breeding Program utilized in this research.

Clonal Selections	Female Parent	Male Parent	Skin	Flesh
CO97216-1P/P	CO94163-1	CO94214-1	Purple	Purple
CO97226-2R/R	Mountain Rose	CO94214-1	Red	Red
CO04058-3RW/RW	CO97216-3P/PW	CO97216-1P/P	Red & White	Red & White

Extraction from Freeze Dried Prepared Tuber Tissues. Three to five biological replicates (median slices of peel and flesh from tubers from separate plants) from each clone were freeze dried using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a fine sieve (100 mesh). Freeze dried powder was weighed out in 600 mg samples, added to a 15 mL conical tube in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in a refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1mL aliquots and vacufuged to dryness at 45 °C in organic mode using a speed vacuum. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for microculture cell viability assays.

Extraction from Cryogenically Ground Prepared Tuber Tissues. Three to five biological replicates (median slices of peel and flesh from tubers from separate plants) from each selection were weighed out in 3 g samples and ground into a very fine powder using a mortar and pestle and addition of liquid nitrogen. Immediately after all 3 g of fresh sample were ground into a fine powder, 10 mL of organic solvent, HPLC grade 80% acetone, was added to powder in mortar and pestle, all contents transferred to a 15 mL conical tube, and rotated in a refrigerator for 1 hour at 4 °C. Conical tubes containing the liquid nitrogen derived powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1 mL aliquots and vacufuged to dryness at 45 °C in organic mode using a speed

vacuum. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for microculture cell viability assays.

Extraction from Clones Utilized for Analysis of Salicylic Acid as a Reference Standard. One biological replicate (peel obtained from tuber) from each clone was freeze dried using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a fine sieve (100 mesh). Freeze dried powder was weighed out in 600 mg samples, added to a 15 mL conical tube in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1 mL aliquots and vacufuged to dryness at 45 °C in organic mode using a speed vacuum. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for microculture cell viability assays.

Cell Lines and Cell Cultures. Human colorectal adenocarcinoma HT-29 cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were received on dry ice, placed in a 37 °C water bath for 3 minutes and immediately transferred to a sterile 15 mL conical tube containing 9 mL of warm McCoys 5A, 1x medium supplemented with 10% fetal bovine serum under sterile conditions using a sterile transfer hood. Sterile centrifuge tubes containing cells and medium were immediately centrifuged at 125 xg for 5 - 10 minutes. Under sterile condition using a sterile transfer hood, the supernatant was removed and the cell pellet was reconstituted in 10 mL of McCoys 5A, 1x medium supplemented with 10% fetal

bovine serum. Contents were transferred to a sterile 75 cm^2 cell culture flask with a vent cap where an additional 5 mL McCoys 5A, 1x medium was added to bring total flask contents up to a total of 15 mL. Flasks were incubated at 37 °C in a humidified 5% CO_2 incubator and allowed to grow to 80% confluence until cells needed to be subcultured.

First generation cells received from ATCC were grown in flasks for several days and then prepared for cryogenic preservation for future use utilizing the subsequent method. Sterile cryogenic vials comprising 2 x 10^5 - 5 x 10^6 cells·1 mL^{-1} ampule in McCoys 5A, 1x medium containing 10% fetal bovine serum and 5% DMSO were transferred inside a 1-inch foam-insulated box and kept at - 80 °C for 12 hours and stored in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Cells used in the execution of all cell culture experiments were maintained in the incubator for no more than five generations before first generation cells were retrieved from cryopreservation storage in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Microculture Tetrazolium Assay: Cell Seeding. All methods to follow were conducted using sterile materials and under sterile conditions using a sterile transfer hood. HT-29 colon cells were seeded into a 96 well plate at a rate of 5 x 10^4 cells in 50 µL per well and incubated at 37 °C in a humidified, 5% CO_2 incubator chamber for 24 hours to allow cells to adhere to the plates. Trypan blue was used in a 1:1 ratio to distinguish viable cells from nonviable cells. A hemacytometer and inverted microscope were used to facilitate the cell count during this initial seeding step. It should be noted

that no wells on the outer perimeter of the microplate were seeded with cells in order to minimize any variation that may occur in these wells.

Microculture Tetrazolium Assay: Potato Extract Treatment. Sixty $mg \cdot mL^{-1}$ potato extracts from each clone were reconstituted in 1 mL of aqueous McCoys 5A, 1x medium containing 10% fetal bovine. Reconstituted potato extracts were filter sterilized using sterile 25 mm syringe filters w/ 0.2 µm cellulose acetate membrane and 3 mL sterile syringes and transferred into sterile 1.5 mL Eppendorf tubes. Serial dilutions of 1.8 to 28.8 $mg \cdot mL^{-1}$ potato extract concentrations were made out of each of the 60 $mg \cdot mL^{-1}$ potato extracts reconstituted in cell culture medium in a 96 well plate. Fifty uL of potato extract concentrations ranging from 0.9 to 28.8 $mg \cdot mL^{-1}$ diluted in McCoys 5A, 1x medium containing 10% fetal bovine was added in triplicate replication to individual wells of a 96 well microplate containing seeded cells to achieve a final range of respective treatment extract concentrations of 0.5 to 14.4 $mg \cdot mL^{-1}$ potato extract in cell culture medium. All treated 96 well microplates were incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for potential inhibitory interactions to occur.

Microculture Tetrazolium Assay: ASA Treatment. One 81 mg (NSAID) chewable baby aspirin tablet (Bayer Healthcare LLC; distributed by Safeway Inc.) was dissolved into 27 mL McCoys 5A, 1x medium containing 10% fetal bovine serum to establish an initial stock solution of 3 mg $ASA \cdot mL^{-1}$ cell culture medium in a sterile 50 mL conical centrifuge tube. Serial dilutions as low as 0.05 mg $ASA \cdot mL^{-1}$ cell culture medium were made out of the 3 mg $ASA \cdot mL^{-1}$ cell culture medium stock solution in sterile 15 mL conical centrifuge tubes. Individual wells containing seeded cells were

treated with 50 µL of each of 7 concentrations of 0.05, 0.09, 0.19, 0.38, 0.75, 1.5 and 3 mg ASA·mL^{-1} cell culture medium in triplicate replication to achieve a final respective treatment concentration of 0.025, 0.05, 0.09, 0.19, 0.38, 0.75 and 1.5 mg ASA·mL^{-1} cell culture medium. All treated 96 well microplates were incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for potential inhibitory interactions to occur.

Microculture Tetrazolium Assay: Ethanol Treatment. Ethanol was dissolved into McCoys 5A, 1x medium containing 10% fetal bovine serum to establish various concentrations of % ethanol solutions in sterile 15 mL conical centrifuge tubes. Individual wells containing seeded cells were treated with 50 µL of each of 4 concentrations of 100, 75, 50 and 25% ethanol (v/v) in triplicate replication to achieve a final respective treatment concentration of 50, 37.5, 25 and 12.5 % ethanol (v/v). All treated 96 well microplates were incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for potential inhibitory interactions to occur.

Microculture Tetrazolium Assay: Control Treatment. Nine individual wells containing seeded cells on each microplate were each treated with 50 µL of McCoys 5A, 1x medium containing 10% fetal bovine serum and incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber. These untreated cells were designated as negative controls for calculating percent growth inhibition of treated cells residing on the same microplate.

Microculture Tetrazolium Assay: Cell Viability Assay. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega is a colorimetric assay that was used to measure the number of viable cells in all cell culture assays. The principal

source of this assay involves the use of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is a water soluble salt in combination with (phenazine methosulfate; PMS) an electron coupling agent reagent. Dehydrogenase enzymes present in metabolically active cells cleave the tetrazolium ring and convert MTS into a colored formazan product that is soluble in tissue culture medium. The amount of formazan product produced, measured by the amount of absorbance at 490 nm, is directly proportional to the number of viable cells in culture.

Exactly 24 hours after cells were treated with negative controls, potato extracts, ethanol and ASA, 20 µL of the prepared MTS/PMS solution was added to each well and incubated for 1 hour at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for dehydrogenase enzymes in any viable cells to convert MTS to formazan. The absorbance was read at 490 nm using a Spectromax 640 microplate spectrophotometer.

Microculture Tetrazolium Assay Quantification and Statistical Methods Based on % Growth Inhibition. Absorbance of the treated cells was compared to the absorbance of the untreated control cells from each individual plate and % growth inhibition was calculated using the following calculation (((control-treated) / control) x 100). Standard deviations and coefficients of variations were calculated among triplicate replications. Commonly, coefficients of variations were below 5%.

Ratio transformations of (treatment/control) were used in the statistical analysis to stabilize normal distributions and equal variance. Dependent upon the clone either two-way or three-way analyses of variance (ANOVA) was used to evaluate the main

effects of extract concentration and preparation methods followed by Tukey-Kramer test to assess separation of means using SAS software.

Statistical analyses conducted on results expressed in terms of % growth inhibition for freeze dried versus cryogenic grinding experiments are based on three to five biological replicates from one year of harvest where microculture tetrazolium experiments were repeated two separate times. Statistical analyses conducted on results expressed in terms of % growth inhibition for ASA treated cells are based on microculture tetrazolium experiments repeated three separate times. Statistical analyses conducted on results expressed in terms of % growth inhibition for both potato peel extracts and ethanol treated cells are based on microculture tetrazolium experiment conducted one time with each treatment.

Microculture Tetrazolium Assay Quantification and Statistical Methods Based on Acetylsalicylic acid as a Reference Standard. The inhibitory effects of various concentrations of ASA dissolved in McCoys 5A, 1x medium containing 10% fetal bovine serum on HT-29 colon cancer cells in culture were investigated for its consistency and linearity in an effort to establish a known reference standard for expressing the number of viable cells of an unknown sample when utilizing the CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega. Three separate microplates containing HT-29 colon cancer cells were each treated with various concentrations of ASA and one unknown treatment (CO97226-2R/R potato peel aqueous extract, CO04058-3RW/RW potato peel aqueous extract or ethanol) to assess the efficacy of expressing cell viability in culture based on a ASA standard curve expressed as mg ASA·mL^{-1} cell culture medium. The absorbance reading of cells

treated with potato peel extracts and ethanol were compared to the known reference standard curve where cell viability was calculated based on an ASA standard curve expressed as mg ASA·mL^{-1} cell culture medium using the following calculation ((absorbance of treated sample- y-intercept of ASA standard curve) / slope of ASA standard curve)). Standard deviations, coefficients of variations and standard error of the mean were calculated among triplicate replications of treated cells.

RESULTS

Freeze Dried vs. Cryogenic Grinding Preparation. While all aqueous extracts from the two tuber selections tested inhibited HT-29 colon cancer cell cultures, only CO97216-1P/P was significantly affected (P=0.002) by preparation processes utilized prior to extraction in organic solvent (Fig. 3.1). Aqueous extracts from immature CO97216-1P/P tubers that were prepared via cryogenic grinding methods prior to extraction in organic solvent inhibited cell cultures 39 and 55% more at a potato extract concentration of 7.2 and 14.4 mg·mL^{-1} cell culture medium when compared to aqueous extracts from immature tubers that were prepared via freeze drying prior to extraction in organic solvent (Fig 3.1). Aqueous extracts from CO97226-2R/R were not significantly different at P=0.05 with regards to preparation methods prior to extraction in organic solvent (Fig 3.2).

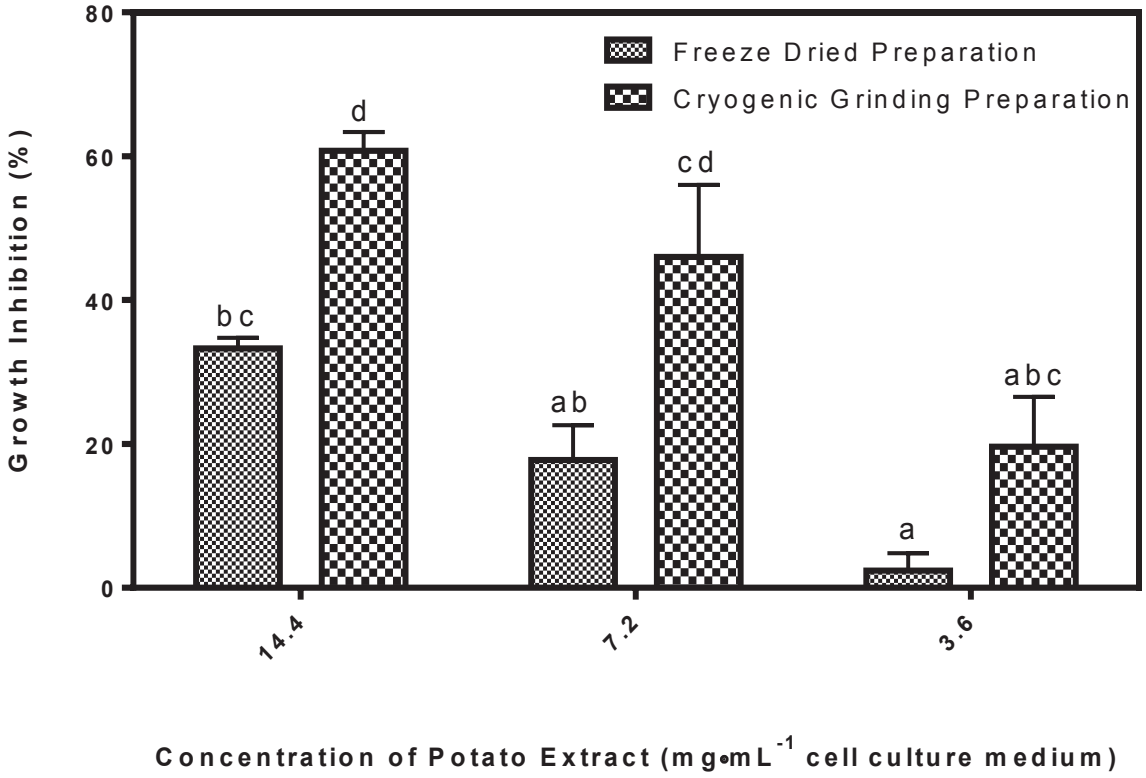

Figure 3.1 % Growth inhibition of HT-29 colon cancer cells after 24 hours by extracts reconstituted in cell culture medium from raw immature CO97216-1P/P tubers tissues that were prepared either cryogenically or by freeze drying methods. Data are means of experiment repeated twice ± standard errors of three to five biological replicates for one year of harvest (2010). Letters denote statistically significant differences between extract concentrations and preparation methods. See Appendix B.1 for analysis of variance (ANOVA) containing degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

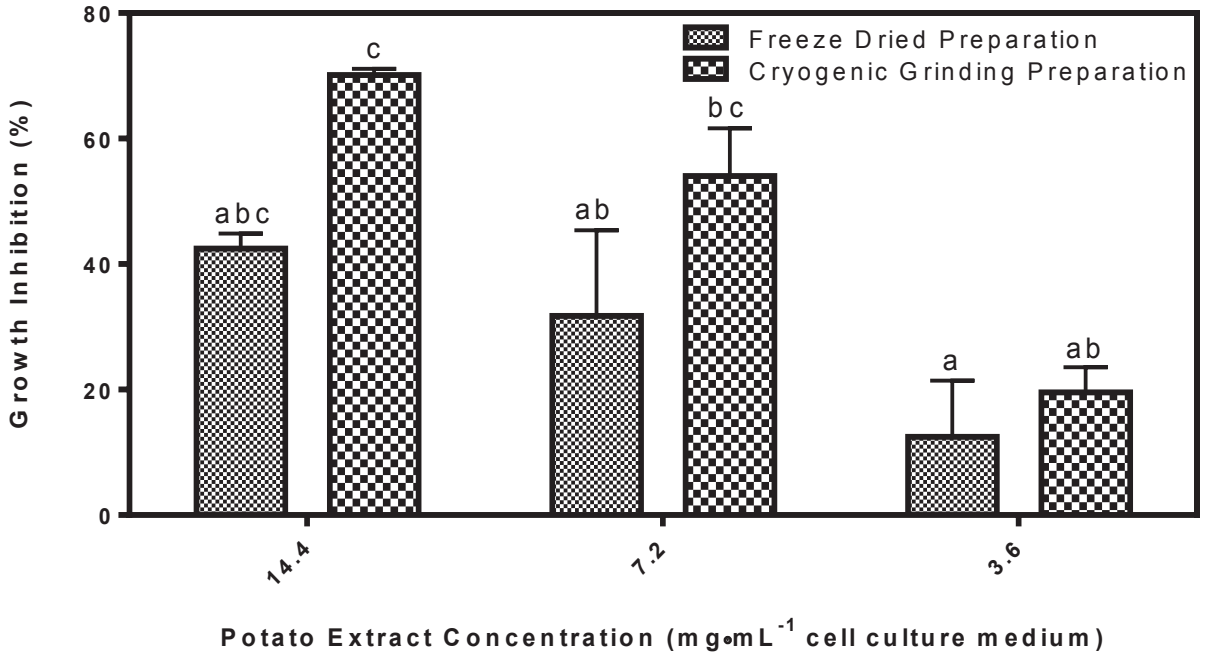

Figure 3.2 % Growth inhibition of HT-29 colon cancer cells after 24 hours by extracts reconstituted in cell culture medium from raw immature CO97226-2R/R tubers tissues that were prepared either cryogenically or by freeze drying methods. Data are means of experiment repeated twice ± standard errors of three to five biological replicates for one year of harvest (2010). Letters denote statistically significant differences between extract concentrations and preparation methods. See Appendix B.2 for analysis of variance (ANOVA) containing degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Analysis of Acetylsalicylic Acid as a Reference Standard. After various trials and errors, five concentrations (0, 0.19, 0.38, 0.75 and1.5 mg ASA·mL^{-1} cell culture medium) were designated as known reference standards to structure the fitted linear regression model to express the number of viable cells in an unknown sample when utilizing the CellTiter 96 Aq$_{ueous}$ Non-Radioactive Cell Proliferation Assay by Promega (Fig. 3.3, 3.4). Three separate trial experiments utilizing these five concentrations of ASA dissolved in aqueous cell culture medium revealed strong linear correlation coefficients, *r,* all greater than 0.93 and relatively high coefficients of determination, R^2, all greater than 0.86 (Fig. 3.3). Triplicate replicate absorbance values obtained from cells treated with each of five concentrations of ASA during each trial experiment revealed coefficients of variations of less than 6%, standard deviations of less than 0.03 and standard error of the mean less than 0.19 (Fig. 3.3).

Various concentrations of two different aqueous extracts of freeze dried *S. tuberosum* and ethanol applied against HT-29 colon cancer cells in culture were utilized to assess the efficacy of expressing cell viability in culture based on a ASA standard curve expressed as mg ASA·mL^{-1} cell culture medium proved to be successful (Fig. 3.4, 3.5, 3.6). The first trial experiment where colon cancer cells were treated with potato peel extract concentrations of 14.4, 7.2, 3.6, 1.8 and 0.9 mg·mL^{-1} cell culture medium from CO04058-3RW/RW revealed 30.4, 55.6, 68.6, 75.5 and 78.2 % growth inhibition and were interpolated as 0.21, 0.69, 0.93, 1.06 and 1.11 mg ASA·mL^{-1} cell culture medium based on the ASA standard curve executed on the same microplate exhibiting an acceptable coefficient of determination, R^2, of 0.86 (Fig. 3.4). The second trial experiment where colon cancer cells were treated with 50, 37.5, 25 and 12.5% aqueous

extracts of ethanol (v/v) revealed 106.7, 102.1, 94.4 and 89.6% growth inhibition and were extrapolated as 1.82, 1.74, 1.61, 1.53 mg ASA·mL^{-1} cell culture medium based on the ASA standard curve executed on the same microplate exhibiting a high coefficient of determination, R^2, of 0.95 (Fig. 3.5). The third trial experiment where colon cancer cells were treated with potato peel extract concentrations of 7.2, 3.6, 1.8 and 0.9 mg·mL^{-1} cell culture medium from CO97226-2R/R revealed 9.9, 40.5, 50.5 and 17.2% growth inhibition and were interpolated as 0.11, 0.67, 0.86 and 0.25 mg ASA·mL^{-1} cell culture medium based on the ASA standard curve executed on the same microplate exhibiting the highest coefficient of determination, R^2, of 0.97 (Fig. 3.6).

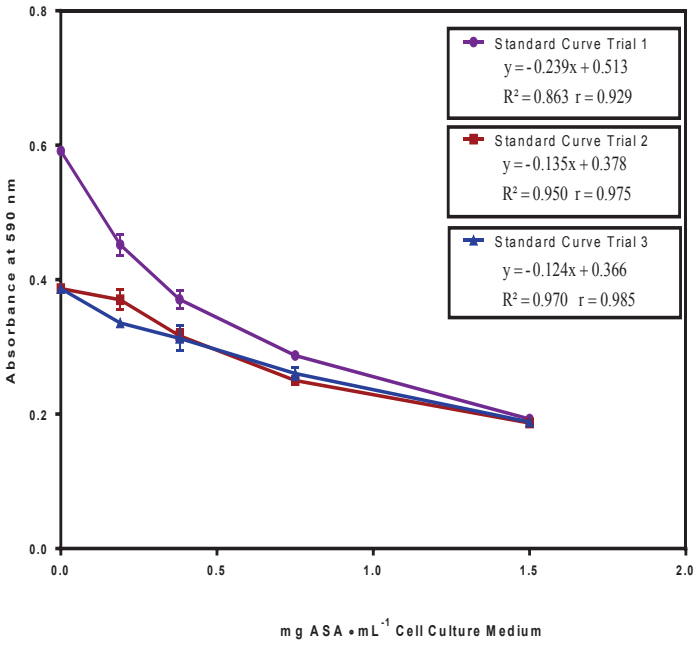

Figure 3.3 Absorbance at 590 nm after 24 hours of HT-29 colon cancer cells treated with 1.5, 0.75, 0.38, 0.19 and 0 mg ASA·mL^{-1} cell culture medium designated as known reference standards utilized to structure the fitted linear regression model during three individual experiments. Data are means of seeded cells treated at each concentration during each individual experiment in triplicate replication ± standard errors of the mean. Linear regression equation, y=mx+b, coefficient of determination, R^2, and coefficient of variation, *r*, of each standard curve experiment are listed for each individual experiment.

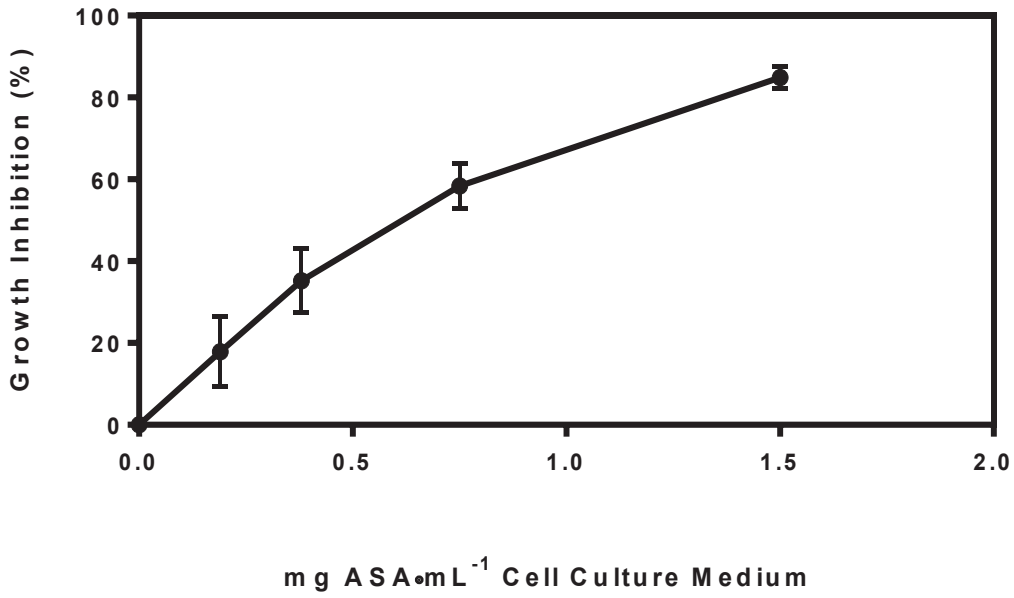

Figure 3.4 % Growth inhibition of HT-29 colon cancer cells after 24 hours treated by 1.5, 0.75, 0.38, 0.19 and 0 mg ASA·mL^{-1} cell culture medium designated as known reference standards. Data are means of standard curve trial experiment repeated three times ± standard errors of the mean.

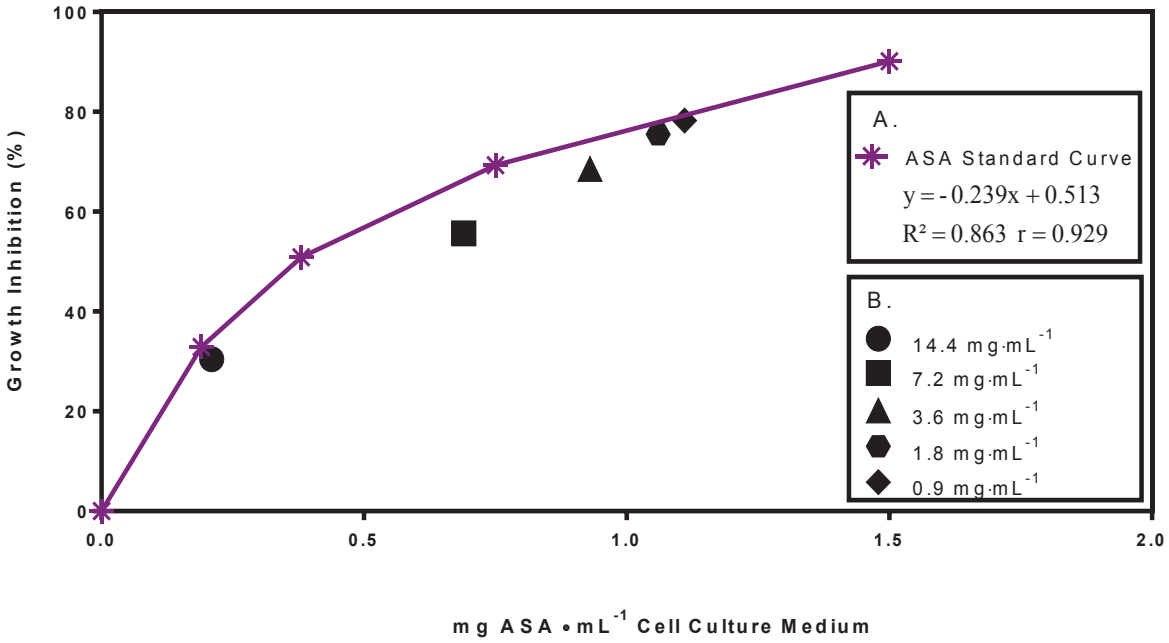

Figure 3.5 A-B (A) % Growth inhibition of HT-29 colon cancer cells after 24 hours treated by 1.5, 0.75, 0.38, 0.19 and 0 mg ASA·mL^{-1} cell culture medium designated as known reference standards of ASA standard curve. Data are means of seeded cells treated at each concentration in triplicate replication ± standard errors of the mean. Linear regression equation, y=mx+b, coefficient of determination, R^2, and coefficient of variation, *r*, of each standard curve experiment are listed for each individual experiment. **(B)** % Growth inhibition of HT-29 colon cancer cells after 24 hours by immature CO04058-3RW/RW potato peel extract concentrations of 14.4, 7.2, 3.6, 1.8 and 0.9 mg·mL^{-1} cell culture medium also expressed in mg ASA·mL^{-1} cell culture medium. Data are means of seeded cells treated at each concentration in triplicate replication.

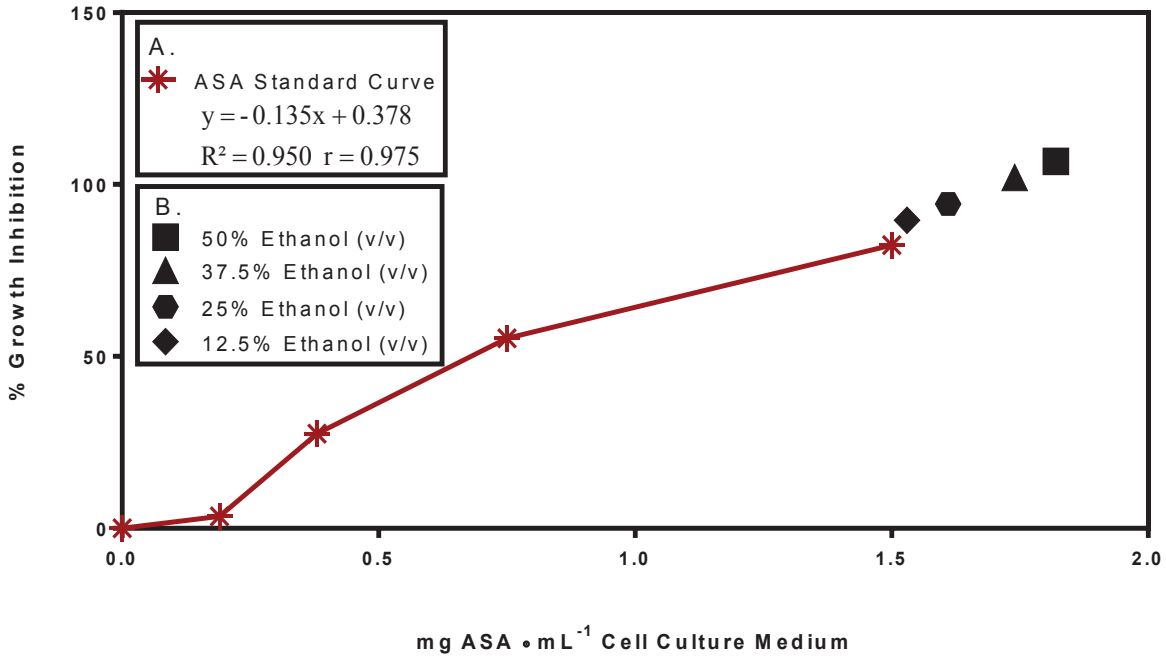

Figure 3.6 A-B (A) % Growth inhibition of HT-29 colon cancer cells after 24 hours treated by 1.5, 0.75, 0.38, 0.19 and 0 mg ASA·mL^{-1} cell culture medium designated as known reference standards of ASA standard curve. Data are means of seeded cells treated at each concentration in triplicate replication ± standard errors of the mean. Linear regression equation, y=mx+b, coefficient of determination, R^2, and coefficient of variation, *r*, of each standard curve experiment are listed for each individual experiment. **(B)** % Growth inhibition of HT-29 colon cancer cells after 24 hours by 50, 37.5, 25 and 12.5% ethanol in cell culture medium (v/v) also expressed in mg ASA·mL^{-1} cell culture medium. Data are means of seeded cells treated at each concentration in triplicate replication.

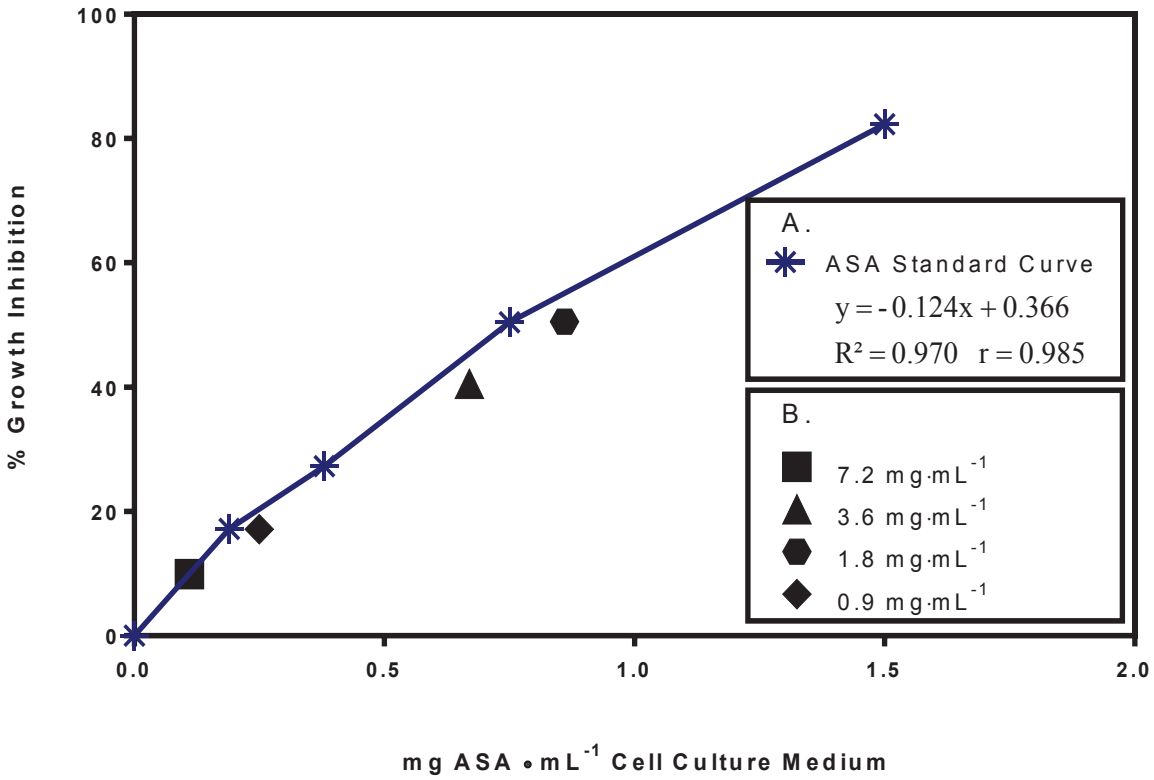

Figure 3.7 A-B (A) % Growth inhibition of HT-29 colon cancer cells after 24 hours treated by 1.5, 0.75, 0.38, 0.19 and 0 mg ASA·mL^{-1} cell culture medium as known reference standards of ASA standard curve. Data are means of seeded cells treated at each concentration ± standard errors of the mean. Linear regression equation, y=mx+b, coefficient of determination, R^2, and coefficient of variation, *r*, of each standard curve experiment are listed for each individual experiment. **(B)** % Growth inhibition of HT-29 colon cancer cells after 24 hours by immature CO97226-2R/R potato peel extract concentrations of 7.2, 3.6, 1.8 and .9 mg·mL^{-1} cell culture medium also expressed in mg ASA·mL^{-1} cell culture medium. Data are means of seeded cells treated at each concentration in triplicate replication.

DISCUSSION

Freeze Dried vs. Cryogenic Grinding Preparation. Inhibition was significantly enhanced in purple pigmented tubers from cryogenically ground tubers compared to freeze dried tubers. Explanations for degradation of bioactive agents inhibiting cancer cell cultures are difficult to pinpoint as the culprit of inhibition is unknown. One could speculate that the formation of large ice crystals in freeze dried tubers and/or formation of small ice crystals in cryogenically ground tubers could have either induced in the case of freeze dried material or prevented in the case of cryogenically ground material mechanical damage of cellular structures, cellular dehydration and / or solute toxicity or a pH change causing denaturation of proteins. Another possibility may be a result of prefreezing temperature of -40 °C utilized during freeze drying where it has been established that biological activity is significantly reduced at -130 °C allowing for biological compounds to degrade at temperatures above -130 °C. For example, if a nucleic acid was the cell culture inhibitor, could an active nuclease at -40 °C have been the degrading enzyme? Oxidation of freeze dried tubers may have also played a role in the degradation of the bioactive agent inhibiting cancer cell cultures. Another interesting factor was that only freeze dried purple pigmented tubers declined in inhibitory response while red pigmented tubers declined but were not significant. Anthocyanins are a class of flavonoids stored within plant vacuoles known to induce apoptosis *in vivo* and *in vitro* and inhibit cell proliferation *in vitro*. Perhaps purple pigments are more sensitive to osmotic dehydration and/or degradation than red pigments if they are in fact the inhibitors of cancer cell cultures.

During this study only two clones were tested, thus it is very likely that other untested genotypes evaluated in past studies may also possess elevated inhibitory responses from cryogenically ground prepared tuber extracts that could provide valuable insight in identification of the inhibitors of colon cancer cell cultures. A valuable result of this study is the importance of investigating what preparation method is best used prior to screening biological materials. This portion of this study requires considerably more research.

Analysis of Acetylsalicylic Acid as a Reference Standard. Beer's Law states "that if a solute absorbs light of a particular wavelength, the absorbance is directly proportional to the concentration of substance in solution" (Caprette, 2008). Many biological assays utilize the principle behind Beer's law to devise standard curves that will allow for the quantitative identification of the concentration of solute in an unknown sample. Colorimetric cell viability assays have not taken advantage of this quantitative tool to date which has led to the development of the ASA standard curve utilized in this study. This study has shown that ASA linearly inhibits colon cancer cells in culture with an acceptable correlation coefficient, *r*, and coefficient of determination, R^2. This linear response has also proven to be repeatable with low SEM, SD and % coefficient of variations amongst triplicate replications within an each assay. This study has shown how the ASA standard curve spans the concentrations of unknown samples and how it can be used to interpolate and extrapolate cell viability in unknown samples. To ensure accuracy of this method, preparation of standards and unknowns must undergo all the same conditions throughout the assay. In addition, unknown samples and standard must be contained within the same microplate for accurate interpolation. In conclusion,

reporting results based on the devised acetylsalicylic acid standard curve allows for an easier, standardized and novel quantitative method for expressing number of viable HT-29 colon cancer cells in a colorimetric assay.

Chapter 4

INHIBITORS OF MAMMALIAN HT-29 COLORECTAL ADENOCARCINOMA CELL CULTURES BY ELITE *SOLANUM TUBEROSUM* L. GERMPLASM APPEAR TO BE MOST PRONOUNCED IN PURPLE AND RED FLESHED CLONES HARVESTED AT AN IMMATURE DEVELOPMENTAL GROWTH STAGE

SUMMARY

Potatoes are rich in nutrients and phytochemicals that may offer protection against cancer and other free radical induced lifestyle diseases. The main objective of this study was to examine the inhibitory nature and mechanism of inhibition of 13 elite genotypes of aqueous *Solanum tuberosum* extracts with different pigments, antioxidant activity, phenolic and glycoalkaloid content obtained throughout several developmental stages in both raw and cooked forms on colon (HT-29) cancer cell lines *in vitro*. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega was used to measure the number of viable HT-29 colon cancer cells in all cell culture assays. Germplasm source (cultivars and selections with pigmented versus white tissue from whole tubers and from pigmented sectors); stage of tuber development (immature tubers versus intermediate tubers versus mature tubers); extraction from uncooked or cooked tuber tissues; and extract concentration ($mg \cdot mL^{-1}$ cell culture medium) obtained from 13 cultivars and selections all impacted inhibition of HT-29 colon cancer cell cultures. Two solid pigmented skin and flesh selections, a red selection CO97226-2R/R and a purple selection CO97216-1P/P, had high and consistent inhibitory activity when harvested as immature tubers. A unique sectored selection, CO04058- 3RW/RW, that

has distinct red pigmented sectors in the same tuber as adjacent white sectors, also had high inhibitory properties, but only in the red sectors and only when harvested as immature tubers. A shared commonality amongst all clones was the importance of dosage concentration at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium required to induce consistent colon cancer growth inhibition. Overall, uncooked tubers retained inhibitory properties better than cooked tubers. The mechanism of inhibition can be attributed at least in large part to apoptosis in red sectors of CO04058-3RW/RW. While, other inhibitory selections tested did not reveal high caspase -3 protease activities, some protease activity was detected thus concluding that apoptosis may likely be the source of their inhibition as well. This study has note the significance of developmental stage for the first time in addition to germplasm source, dosage, cooking, and potential source of bioactive compounds that exist in the world's fourth largest staple crop as it relates to colon cancer.

INTRODUCTION

Potato *(Solanum tuberosum* L.*)* is the fourth largest consumed staple food crop in the world (Kang & Priyadarshan, 2007). Potatoes are rich in nutrients and phytochemicals that may offer protection against chronic diseases. Cancer is an epidemic affecting every region, race, and socioeconomic class across the globe (American Cancer Society, 2011a). The American Cancer Society (2011a) estimates that in 2030 about 21.4 million new cancer cases will arise globally and 13.2 million of these cases are expected to result in death. Colorectal carcinoma, cancer of the colon and rectum, is the third leading cause of cancer death in both men and women in the

United States (American Cancer Society, 2011b), the second leading cause of cancer death worldwide in women and the third leading cause of cancer death worldwide in men (American Cancer Society, 2011b). In 2011, the American Cancer Society estimated that approximately 141,210 new colorectal cancer diagnoses will arise in the US and 49,380 people are expected to die of this disease (American Cancer Society, 2011b). The Globocan Project reported that an estimated 1,233,700 new colorectal cancer cases arose in 2008 worldwide and that an estimated 595,700 individuals died of this potentially preventable disease (Ferlay et al., 2010).

A Danish cohort study recently found that approximately one-quarter of colorectal cancers could be prevented by maintaining a healthy abdominal weight, eating a healthy diet, being physically active 30 minutes per day and by not consuming tobacco products or excessive amounts of alcohol (Kirkegaard et al., 2010). The WCRF/AICR report (2007b) has found that an average preventability of 35% in the United States, 37% in the United Kingdom, 30% in Brazil and 27% in China of the 12 most prevalent cancers (mouth, pharynx, larynx; oesophegus, lung, stomach, pancreas, gallbladder, liver, colorectum, breast, endometrium, prostate and kidney). The WCRF/AICR report (2007a) has also reported recommendations for preventing cancer to include: maintaining a healthy body weight; being physically active 30 minutes a day; limiting consumption of energy dense foods, eating a variety of vegetables, fruits, whole grains and pulses; limiting consumption of red and processed meats; limiting alcohol consumption; limiting salt intake and sodium processed foods; and eliminating high nutrient supplements (WCRF/AICR report, 2007a, 2007b). In opposition to the recommendation regarding fruit and vegetable consumption, a meta-analysis of 19

prospective studies by Aune et al. (2011) has recently reported, “a weak but statistically significant nonlinear inverse association between fruit and vegetable intake and colorectal cancer risk”. However, several studies have implicated classes of secondary metabolites in addition to nutritional components found in potato with inhibitory activity, apoptotic induction mechanisms and the ability to upregulate detoxifying phase 2 enzymes. The subsequent information is an attempt to explain the key findings of these studies as they relate to potato.

Potatoes are an important source of dietary fiber where 8% of the recommended daily value for dietary fiber can be attained by consuming one medium sized potato with the skin (Vreugdenhil & Bradshaw, 2007). A study conducted by Harris et al. (1991) investigated the theory that dietary fiber may offer protection against colon cancer by studying the adsorption of the hydrophobic mutagen 1,8-dinitropyrene (DNP) to the skin of potato tubers and flesh of potato tubers. Results showed that dietary fiber obtained from potato skins, which are composed mainly of α-cellulose, were able to bind DNP and thus this source can be considered as potentially protective against colon cancer. Conversely, dietary fiber obtained from potato flesh, which is composed mainly of pectic polysaccharides, did not bind DNP well and therefore may not offer any protection against colon cancer. This study stressed the importance of the source and/or type of plant cells walls, dietary fibers, in their ability to bind and reduce mutagen and cancer promoter contact with mucosal cells of the colon.

Starch, the main energy source in potato, constitutes 75% of total dry matter content (Vreugdenhil & Bradshaw, 2007). Starch is a polymer of the glucose monomers amylose and amylopectin (Vreugdenhil & Bradshaw, 2007). A small portion of starch

found in potato called resistant starch (RS), is the source of many health benefits in the colon. RS is resistant to enzymatic digestion in the small intestine by α-amylase and consequently is fermented in the large intestine (United States Potato Board, 2010; Liu & Xu, 2008). RS causes a fermentation mediated increase in the concentrations of short chain fatty acids, particularly butyrate, that exist in the large intestine (Liu & Xu, 2008). Butyrate has been found to halt growth, induce apoptosis and regulate expression of proteins associated with cellular dedifferentiation in several cancer cell culture lines including colon cancer (Liu & Xu, 2008). In a study conducted by Liu and Xu (2008), RS was administered to azoxymethane (AOM) induced colonic aberrant crypt foci (ACF), markers of preneoplastic lesions, of Wistar rats where it was confirmed that RS suppressed AOM-induced ACF formation at the promotion stage but promoted the formation of ACF at the preinitation stage. RS is also responsible for laxation enchancement, increases in beneficial bacteria, cecal and large intestinal contents and decreases in pH and concentration of ammonia, phenols and secondary bile acids in the colon (United States Potato Board, 2010; Liu & Xu, 2008).

Friedman (2006) explains various aspects of glycoalkaloids. Glycoalkaloids are secondary metabolites that exist in the tubers, leaves, roots and sprouts of potato. Glycoalkaloids are composed of a trisaccharide chain attached to an aglycon. These glycoalkaloids are mostly positioned around the eyes located on the outer layer of the tuber and within the first 1mm from the exterior surface of the tuber decreasing toward the interior of the tuber. The prominent glycoalkaloids that exist in cultivated potato are α-chaconine and α-solanine. The initial concentration and configuration of these prominent glycoalkaloids in potato is largely dependent on genotype. Concentrations

may be effected by environmental factors such as soil and climate and generally decrease with increasing tuber size. Postharvest management can also cause concentration increases as a result of storage, light, heat, cutting, slicing, sprouting and exposure to phytopathogens.

Glycoalkaloids have been associated with producing an undesirable bitter astringent taste (Vreugdenhil & Bradshaw, 2007). At certain concentrations they are reported to be toxic to bacteria, fungi, viruses, insects, animals and humans (Friedman, 2006). Of the two prominent glycoalkaloids found in potato, α-chaconine has been reported to be more toxic than α-solanine in several studies (Friedman, 2006). Glycoalkaloids ingested by humans in the form of potato, potato berries, potato leaves, and blighted, greened and sprouted tubers has resulted in death (Friedman, 2006). Human feeding studies revealed that ingestion of potatoes varying in concentration between 1.0-2.6 $mg \cdot kg^{-1}$ body weight resulted in light to severe nausea, burning sensation in the mouth and diarrhea (Friedman, 2006). Morris and Lee (1984) reported in a review paper that 2-5 $mg \cdot kg^{-1}$ of body weight is considered a toxic dose to humans and 3-6 $mg \cdot kg^{-1}$ of body weight is considered to be a fatal dose to humans (Morris & Lee, 1984). Thus the maximum safe allowance for human consumption of glycoalkaloids is reported to be 200 $mg \cdot kg^{-1}$ of fresh weight of potato (Vreugdenhil & Bradshaw, 2007).

Although glycoalkaloids are reported to produce toxic effects they have also been implicated as chemopreventives (Friedman et al., 2005, Friedman et al., 2006). The glycoalkaoids, α-chaconine and α-solanine, isolated from Dejima potatoes, were identified as the key metabolites involved in the inhibition of the growth of several

human cell lines in culture including cervical (HeLa), liver (HegG2), lymphoma (U937) and stomach (AGS and KATO III) (Friedman et al., 2005). α-chaconine has been reported to inhibit of ERK ½ phosphorylation triggering caspase-3 activation generating the induction of apoptosis of human HT-29 colon cancer cells in culture in a time and concentration dependent manner (Yang et al., 2006). Methanol extracts of *S. tuberosum* callus containing higher levels of solanidin, α-chaconine and α-solanine than in tubers induced cytotoxic effects against human carcinoma cell culture lines of breast, lymphoplastic leukemia, larynx, liver, cervix, colon and brain (Al-Ashaal, 2010). Mandimika attempted to explain the effects of different amalgamations of α-chaconine and α-solanine treatments together and alone on Caco-2 colon human intestinal epithelial cell culture lines (Mandimika, 2007). Lactase dehydrogenase leakage assay executed in their study informed that cytotoxicity of Caco-2 cells in culture occurred in a concentration dependent manner in all treatments except α-solanine alone (Armstrong, 2011). Gene expression studies utilizing α-chaconine and α-chaconine/α-solanine treatments on Caco-2 cells indicated significant upregulation of genes involved in cholesterol biosynthesis (Mandimika, 2007). All treatment combinations showed gene expression changes in growth signaling, lipid and amino acid metabolism, mitogen activated protein kinase (MAPK) and NF-κB cascades, cell cycle and apoptosis. Late apoptotic induction of Caco 2 cells was found to occur in a concentration dependent manner by α-chaconine (Mandimika, 2007). Cell cycle analysis revealed an accumulation of Caco 2 cells during G_2/M phase induced by α-chaconine (Mandimika, 2007). Conversely, another study revealed that HT-29 colon cancer cells in culture

exposed to 5 ug/mL α-chaconine and 10 μg·mL^{-1} α-solanine resulted in an accumulation of HT-29 cells during sub-G_0/G_1 phase of the cell cycle (Yang et al., 2006).

Vitamin C is a hydrophilic compound that acts as an antioxidant (Spencer & Saul, 2010). Vitamin C is the main vitamin in potato and contributes up to 40% of daily - recommended intake of the global diet (Vreugdenhil & Bradshaw, 2007). The concentration and state of reduced ascorbic acid and oxidized dehydroascorbic acid is dependent on both potato germplasm and storage (Vreugdenhil & Bradshaw, 2007). A recent study showed that intraperitoneal pharmacological doses of vitamin C reduced tumor size and incidence in rats induced with hormone-refractory prostate cancer PA III cells (Pollard et al., 2010). The reduced state of ascorbic acid has been found to effectively kill malignant mesothelioma cells in both test tube studies and mice experiments (Pollard et al., 2010). Angiogenesis has been found to be impeded when high levels of vitamin C are present in the blood both *in vivo* and *ex vivo* (Mikirova et al., 2010). Numerous other research studies have established the value of vitamin C, thus this essential vitamin should be viewed as a powerful bioactive agent important in the intervention of many diseases.

Carotenoids are fat soluble tetraterpenoids existent in the flesh of white, yellow and orange potatoes (Brown, 2005). The most prominent carotenoids emanate from the zanthophyll subclass, oxygenated tetraterpenoids, and include lutein 5,6 epoxide, zeaxanthin, violaxanthin (Brown, 2005). Other carotenoids identified in potato in trace amounts emanate from the carotene subclass, tetraterpenoids without oxygen, and include β-carotene and α-carotene. Potato is not a worthy source of these pro-vitamin α-carotenes (Brown, 2005). Oxygen radical absorbance capacity of total carotenoids

from potato flesh have been found to range between 4.6 - 15.3 nmoles α-tocopherol equivalents per 100g FW (Brown, 2005). Carotenoids are a powerful source of antioxidants and have been associated with a reduced risk of certain cancers (International Carotenoid Society, 2011).

Potatoes contain significant amounts of flavonoids which are polyphenolic compounds structurally composed of three aromatic benzene rings containing one or more hydroxyl groups (International Carotenoid Society, 2011). Flavonoids have been acknowledged to produce antiproliferative, anticarcinogenic and antioxidative effects (Harborne, 1993; Kuhanu, 1976; Sharma et al., 1994; Rice-Evans et al., 1996; Robards et al., 1999). The prominent flavonoids found in potato are anthocyanins, catechin and epicatechin but erodictyol, kaempferol and narigenin are also present in potato (Brown, 2005). Specific anthocyanins abundant in potato that are responsible for producing red and purple pigments in potato include the acylated anthocyanidin glucosides pelargonidin, petunidin, malvidin and peionidin (Brown, 2005 ; Stushnoff et al., 2008; Stushnoff et al., 2010). Anthocyanins isolated from pigmented potatoes, have been reported to be responsible for the induction of apoptosis in KATO III human stomach cancer cell lines in culture and suppressed stomach cancer in mouse (Hayashi et al., 2006). Anthocyanins, present in red and purple steamed potatoes, also suppressed stomach cancer in mouse in the same study by Hyashi (2006). Specialty potato extracts and anthocyanin fractions obtained from a specialty potato genotype both initiated mitochondrial release of the proteins Endo G and AIF resulting in the induction of apoptosis and inhibited growth of LNCaP (androgen dependent) and PC-3 (androgen independent) prostate cancer cells in culture (Reddivari et al., 2007). Cyanidin and

delphinidin, and to a lesser extent malvidin, have been found to inhibit epidermal growth factor receptor in cancer cells (Sterling, 2001).

Phenolic acids are hydroxylated derivatives of benzoic and hydrocinammic acids (Herrmann, 1989). The most profuse phenolic acid present in potato is chlorogenic acid but protocatechic acid, vanillic acid and p-coumaric acid also exist (Brown, 2005). Chlorogenic acid, an antioxidant abundant in pigmented potato (Stushnoff et al., 2008; Stushnoff et al., 2010) has been reported to inhibit the proliferation of lung cancer cells and TPA-induced neoplastic transformation and may be a potential stimulant of NQO1 and GST enzymatic activities (Feng et al., 2005). A red pigmented cultivar, Mountain Rose (MR), comprised of higher levels of chlorogenic acid and anthocyanins resulted in a reduction in breast cancer carcinogenesis incidence and multiplicity in rats that were fed freeze dried baked MR potato (Thompson et al., 2009). Extracts obtained from *Solanum jamesii*, a wild tuber species, were found to inhibit the proliferation of HT-29 colon and LNCaP prostate cancer cells culture lines in vitro, but no evidence of cytotoxicity to cells was observed nor significant correlations to glycoalkaloid content, neither total phenolic content nor antioxidant capacity were detected (Nzaramba et al., 2009).

The high prevalence of potato consumption worldwide suggests that further investigation of bioactive phytochemicals and possible inhibitory metabolites against cancer that exist within potato could be of great benefit to many. The main objective of this study was to examine the inhibitory nature and mechanism of inhibition of thirteen elite genotypes of aqueous *S. tuberosum* extracts with different pigments, mineral content, antioxidant activity, phenolic and glycoalkaloid content obtained throughout

several developmental stages in both raw and cooked forms on colon (HT-29) cancer cell lines *in vitro*.

MATERIALS AND METHODS

Clones. A total of 13 clones of *S. tuberosum* were obtained from the Colorado Potato Breeding Program, grown under essentially commercial production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado. Clones beginning with CO are in an experimental phase of release and are designated as selections (Table 4.1). Clones with names have been released to the public and are designated as cultivars (Table 4.1). Selections ending in –P/PW, -R/RW and -RW/RW are selections containing pigmented and white sectors of tissue that are otherwise of the same genotype and not confounded by environmental and developmental differences (Fig. 4.1; Table 4.1). In all selections, the first letter before the slash is indicative of tuber skin color (P=purple, R=red, W=White, RU=Russet) (Table 4.1). In all selections, the letters following the slash are indicative of tuber flesh colors (P=purple, R=red, W=White) (Table 4.1).

Figure 4.1 Sectorial expression of pigments illustrating a model system that permits sampling of tissues that differ only in pigment expression and are not confounded by environmental factors or genetic segregation.

Table 4.1 Cultivars and clonal selections from the Colorado Breeding Program utilized in this research.

Cultivars and Clonal Selections	Female Parent	Male Parent	Skin	Flesh
CO95172-3RU	Russet Nugget	AC88165-3	Russet	White
CO97216-1P/P	CO94163-1	CO94214-1	Purple	Purple
CO97216-3P/PW	CO94163-1	CO94214-1	Purple	Purple & White
CO97226-2R/R	Mountain Rose	CO94214-1	Red	Red
CO97227-2P/PW	Mountain Rose	CO94215-1	Purple	Purple & White
CO04056-7P/PW	CO97216-1P/P	CO97227-2P/PW	Purple	Purple & White
CO04058-3RW/RW	CO97216-3P/PW	CO97216-1P/P	Red & White	Red & White
CO04061-1R/RW	CO97222-1R/R	CO97306-1R/R	Red	Red & White
Colorado Rose	NDTX9-1068-11R	DT6063-1R	Red	White
Mountain Rose	All Red	ND2109-7	Red	Red
Purple Majesty	All Blue	ND2008-2	Purple	Purple
Rio Grande Russet	Butte	A8469-5	Russet	White
Russet Nugget	Krantz	AND71609-1	Russet	White

All cultivars and selections were examined for inhibitory activity obtained throughout several developmental stages (Table 4.2) in both raw and cooked forms for two to three consecutive harvests (2008 - 2010): (1) raw potatoes at immature and intermediate tubers stages; (2) boiled potatoes (97 °C for 20 minutes) at immature and intermediate tuber stages; (3) raw potatoes at mature tuber stages; (4) baked potatoes (170 °C for 1 hour) at mature tuber stages; (5) boiled potatoes (97 °C for 20 minutes) at mature tuber stages; (6) mature raw potatoes stored for 6 months (4 °C in dark conditions); (7) mature potatoes stored for 6 months (4 °C in dark conditions) and baked (170 °C for 1 hour).

Table 4.2 Planting dates, harvest dates, days to maturity and growing degree days by year (2008-2010) defining developmental stages utilized in this research.

	2008				2009				2010			
Developmental Stage	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days	Planting Date	Harvest Date	Days to Maturity	Growing Degree Days
Stage I (Immature)	15th of May	30th of July	48	942.0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Stage II (Immature)	15th of May	9th of August	38	1126.5	1st of June	8th of August	33	852.5	18th of May	25th of July	50	945.5
Stage III (Intermediate)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18th of May	31st of July	44	1053.0
Stage IV (Intermediate)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	18th of May	7th of August	37	1163.5
Stage V (Mature)	15th of May	17th of September	0	1538.5	1st of June	11th of September	0	1284.0	18th of May	14th of September	0	1618.5
Stage V - 6 m (Mature - 6 Months Storage)	15th of May	17th of February	+6 months	1538.5	1st of June	11th of September	+6 months	1284.0	18th of May	14th of February	+6 months	1618.5

Extraction. Three to five biological replicates (median slices of tuber flesh and peel from separate plants) from each clone obtained throughout several developmental stages in both raw and cooked forms were freeze dried using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a fine sieve (100 mesh). Freeze dried powder was weighed out in 600 mg samples, added to 15 mL conical tubes in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in a refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1 mL aliquots and vacufuged to dryness at 45 °C in organic mode using a speed vacuum. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for cell viability assays, total phenolic content and DPPH radical scavenging capacity.

Cell lines and Cell Cultures. Human colorectal adenocarcinoma HT-29 cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were received on dry ice, placed in a 37 °C water bath for 3 minutes and immediately transferred to a sterile 15 mL conical tube containing 9 mL of warm

McCoys 5A, 1x medium supplemented with 10% fetal bovine serum under sterile conditions using a sterile transfer hood. Sterile centrifuge tubes containing cells and medium were immediately centrifuged at 125 xg for 5 - 10 minutes. Under sterile condition using a sterile transfer hood, the supernatant was removed and the cell pellet was reconstituted in 10 mL of McCoys 5A, 1x medium supplemented with 10% fetal bovine serum. Contents were transferred to a sterile 75 cm^2 cell culture flask with a vent cap where an additional 5 mL McCoys 5A, 1x medium was added to bring total flask contents up to a total of 15 mL. Flasks were incubated at 37 °C in a humidified 5% CO_2 incubator and allowed to grow to 80% confluence until cells needed to be subcultured.

First generation cells received from ATCC were grown in flasks for several days and then prepared for cryogenic preservation for future use utilizing the subsequent method. Sterile cryogenic vials comprising 2 x 10^5 - 5 x 10^6 cells·1 mL^{-1} ampule in McCoys 5A, 1x medium containing 10% fetal bovine serum and 5% DMSO were transferred inside a 1-inch foam-insulated box and kept at - 80 °C for 12 hours and stored in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Cells used in the execution of all cell culture experiments were maintained in the incubator for no more than five generations before first generation cells were retrieved from cryopreservation storage in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Microculture Tetrazolium Assay: Cell Seeding. All methods to follow were conducted using sterile materials and under sterile conditions using a sterile transfer

hood. HT-29 colon cells were seeded into a 96 well plate at a rate of 5 x 10^4 cells in 50 µL per well and incubated at 37 °C in a humidified, 5% CO_2 incubator chamber for 24 hours to allow for cells to adhere to the plates. Trypan blue was used in a 1:1 ratio to distinguish viable cells from nonviable cells. A hemacytometer and inverted microscope were used to facilitate the cell count during this initial seeding step. It should be noted that no wells on the outer perimeter of the microplate were seeded with cells in order to minimize any variation that may occur in these wells.

Microculture Tetrazolium Assay: Potato Extract Treatment. Three to five biological replicates of 60 mg·mL^{-1} potato extracts from each clone obtained throughout several developmental stages in both raw and cooked forms were reconstituted in 1 mL of aqueous McCoys 5A, 1x medium containing 10% fetal bovine. Reconstituted potato extracts were sterile filtered using sterile 25 mm syringe filters w/ 0.2 µm cellulose acetate membrane and 3 mL sterile syringes and transferred into sterile 1.5 mL Eppendorf tubes. Serial dilutions of potato extract concentrations (7.2, 14.4 and 28.8 mg·mL^{-1} cell culture medium) were made out of each of the 60 mg·mL^{-1} potato extracts reconstituted in cell culture medium in a 96 well plate. Individual wells containing seeded cells were treated with 50 µL of each of three potato extract concentrations (7.2, 14.4 and 28.8 mg·mL^{-1} cell culture medium) in triplicate replication to achieve a final respective potato extract concentration of 3.6, 7.2 abd 14.4 mg·mL^{-1} cell culture medium. All treated 96 well microplates were incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for potential inhibitory interactions to occur.

Microculture Tetrazolium Assay: Control Treatment. Nine individual wells containing seeded cells on each microplate were each treated with 50 µL of McCoys 5A, 1x medium containing 10% fetal bovine serum and incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber. Untreated cells were designated as negative controls for calculating percent growth inhibition of treated cells residing on the same microplate.

Microculture Tetrazolium Assay: Cell Viability Assay. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega is a colorimetric assay that was used to measure the number of viable cells in all cell culture assays. The principal source of this assay involves the use of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is a water soluble salt in combination with (phenazine methosulfate; PMS) an electron coupling agent reagent. Dehydrogenase enzymes present in metabolically active cells cleave the tetrazolium ring and convert MTS into a colored formazan product that is soluble in tissue culture medium. The amount of formazan product produced, measured by the amount of absorbance at 490 nm, is directly proportional to the number of viable cells in culture. Exactly 24 hours after cells were treated with potato extracts, 20 µL of the prepared MTS/PMS solution was added to each well and incubated for 1 hour at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for dehydrogenase enzymes in any viable cells to convert MTS to formazan. The absorbance was read at 490 nm using a Spectromax 640 microplate spectrophotometer.

Microculture Tetrazolium Assay Quantification and Statistical Methods Based on % Growth Inhibition. Absorbance of the treated cells was compared to the absorbance of the untreated control cells from each individual plate and % growth inhibition was calculated using the following calculation (((control-treated) / control) x 100). Standard deviations and coefficients of variations were calculated among triplicate replications. Commonly, coefficients of variations were below 5%.

Statistical analyses conducted on results expressed in terms of % growth inhibition for all potato extract treatments was based on three to five biological replicates from two to three harvest years dependent upon clone where each microculture tetrazolium experiment were conducted only one time.

Ratio transformations of (treatment/control) were used in the statistical analysis to stabilize normal distributions and equal variance. Dependent upon the clone either four-way or five-way analyses of variance (ANOVA) was used to evaluate the main effects of year, cooking, sector-flesh color, developmental stage and extract concentration followed by Tukey-Kramer test to assess separation of means using SAS software.

Pearson Correlation: Cancer Inhibition & Antioxidants. Side by side biological replicate Pearson correlations of ratio transformed % growth inhibition data were computed along side of gallic acid equivalents of total phenolic data obtained from Folin Ciocalteu assay and DPPH Trolox equivalents antioxidant capacity (DPPH/TEAC) obtained from DPPH assay from previous studies both based on a dry weight basis utilizing SAS software.

Phenolic phytochemicals are a class of secondary metabolites that contain one or more acidic hydroxyls attached to one or more aromatic arene (phenyl) rings (Ainsworth & Gillespie, 2007). Phenolic compounds are an important class of potent antioxidants because of their ability to quench free radicals due to their acidity. In turn, phenolic compounds are rendered radicals that are able to remain relatively stable attributable to their ability to delocalize electrons, a characteristic of their benzene ring structure (Cheung, 2011).

The Folin-Ciocalteau microplate based colorimetric assay, an adapted method from Spanos and Wrolstad (1990), based on the original method of Singleton and Rossi (1965), was utilized to quantify total phenolics. The amount of sample extracts containing phenolic and polyphenolic antioxidants required to reduce or inhibit the oxidation of phosphomolybdate-phosphotungstate acid complexes is measured in this colorimetric assay. The amount and/or intensity of blue complexes produced, measured by the amount of absorbance at 765 nm, are indicative of a strong antioxidant.

Three to five biological replicates of 60 $mg \cdot mL^{-1}$ potato extracts from each clone obtained throughout several developmental stages in both raw and cooked forms were reconstituted in 1 mL of 80% acetone. In a new 1.5 mL Eppendorf tube, 100 µL of reconstituted extract was diluted with 900 µL of nanopure water. In a 96 well microplate, 35 µL of each diluted sample was pipetted in triplicate replication. Utilizing a multichannel pipette, 150 µL of 0.2 M Folin-Ciocalteau reagent was pipetted into all wells. The prepared microplate was placed on a shaker, agitated for 30 seconds at 400 rpm and held for 5 minutes at room temperature. Utilizing a multichannel pipette, 115

µL of 7.5% (w/v) Na_2CO_3 was pipetted into all wells. The prepared microplate was placed on a shaker, agitated for 30 seconds at 400 rpm, incubated at 45 °C for 30 minutes and cooled to room temperature for 1 hour. The absorbance was read at 765 nm utilizing a Spectromax 640 microplate spectrophotometer. Total phenolic content was calculated by regression based on a gallic acid standard curve expressed as mg GAE·g^{-1} DW.

Statistical analyses of total phenolic content was based on three to five biological from two to three harvest years dependent upon clone where each Folin-Ciocalteau experiment was conducted only one time. Dependent upon the clone either three-way or four-way analyses of variance (ANOVA) was used to evaluate the main effects of year, cooking, developmental stages, and flesh color (purple vs. red vs. white vs. white or red vs. white or purple) followed by Tukey-Kramer test to assess separation of means using SAS software.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) decolorization microplate based assay used a method based on Brand-Williams, Cuvelier and Berset (1995) to quantify total antioxidant capacity or radical scavenging capacity. The amount of sample extract containing radical scavenging antioxidants required to reduce the DPPH radical to DPPH-H is measured in this colorimetric assay (Prakash et al., 2001). The amount of decolorization from dark purple to yellow produced, measured by the amount of absorbance at 515 nm, is indicative of a decrease in molar absorptivity of the DPPH molecule as a free radical scavenging antioxidant donates a hydrogen forming the reduced DPPH-H and is stoichiometric to the number of electrons seized (Prakash et al., 2001).

DPPH stock solution was prepared in advance by adding 7.85 mg 1,1-diphenyl-2-picrylhydrazyl (DPPH) to 1L of 100% methanol in a light protected flask, agitated on a shaker at 400 rpm for 3 hours and stored at 4 °C for no longer than 30 days. Trolox stock solution was prepared in advance by adding 12.52 mg Trolox to 50 mL of 5 mmol phosphate buffered saline (PBS) and stored at -20 °C for no longer than 30 days. Three to five biological replicates of 60 $mg \cdot mL^{-1}$ potato extracts from each clone obtained throughout several developmental stages in both raw and cooked forms were reconstituted in 1 mL of 5 mM PBS. In a 96 well microplate, 15 µL of each reconstituted sample was pipetted in triplicate replication. The absorbance of DPPH stock solution was adjusted to 0.90 to 0.95 absorbance units (AU) by adding approximately 12 mL of 100% methanol to 18.0 mL of prepared DPPH stock solution while reading at 515 nm using a Spectromax 640 microplate spectrophotometer. Utilizing a multichannel pipette, 285 µL of adjusted DPPH stock solution was pipetted into all 96 microplate wells and held for 3 minutes at room temperature. The absorbance was read at 515 nm. Radical scavenging capacity was calculated by regression based on a Trolox standard curve expressed as µM $TEAC \cdot g^{-1}$ DW.

Statistical analyses of DPPH radical scavenging capacity was based on three to five biological from two to three harvest years dependent upon clone where each DPPH radical scavenging capacity experiments was conducted only one time. Dependent upon the clone either three-way or four-way analyses of variance (ANOVA) was used to evaluate the main effects of year, cooking, developmental stages, and flesh color (purple vs. red vs. white vs. white or red vs. white or purple) followed by Tukey-Kramer test to assess separation of means using SAS software.

Determination of Apoptosis. Apoptosis, programmed cell death, is initiated via a sequential cascade of caspase proteolytic activity by one or more caspase proteases (caspases -2, -3, -6, -7, -8, -9, -10 and -12) (Peterson et al., 2010). Induction of apoptotic activity may occur either extrinsically or intrinsically (Peterson et al., 2010). Mitochondrial release of cytochrome C results in the formation of an apoptosome intrinsically activating caspase-9 which catalyzes the hydrolysis of caspase -3 and -7 (Peterson et al., 2010). Binding of death ligands to death receptors extrinsically activates caspase -8 and caspase -10 to catalyze the hydrolysis of capsase -3 and caspase -7. Caspase -3, and less significant caspase -7, are responsible for the proteolytic cleavage of more than 100 different protein substrates essentially destroying the cell ensuing in apoptosis (Peterson et al., 2010). Thus, capase detection may be used as an indicator of apoptotic cell death. The Apoalert® Caspase-3 Colorimetric Assay Kit is a colorimetric microplate assay that was utilized to detect caspase-3 protease activity (Takara Bio Company, 2011). Capase -3 catalyzes the hydrolysis of Ac-DEVD-p-nitroanilide (pNA) releasing the pNA chromophore in mammalian cells that have been induced by apoptosis (Takara Bio Company, 2011). The amount of pNA chromophore liberated from Ac-DEVD can be monitored colorimetrically as the amount of absorbance at 405 nm (Takara Bio Company, 2011).

All methods to follow were conducted using sterile materials and under sterile conditions using a sterile transfer hood. HT-29 colon cells were seeded into a 6 well plate at a rate of 2.5×10^6 cells in 2 mL per well and incubated at 37 °C in a humidified, 5% CO_2 incubator chamber for 24 hours to allow for cells to adhere to the plates. Trypan blue was used in a 1:1 ratio to distinguish viable cells from nonviable cells and a

hemacytometer and inverted microscope were used to facilitate the cell count during this initial seeding step. Three biological replicates of 60 $mg \cdot mL^{-1}$ potato extracts from each raw clone harvested immaturely during stage II were reconstituted in 1 mL of aqueous McCoys 5A, 1x medium containing 10% fetal bovine. Reconstituted potato extracts were filter sterilized using sterile 25 mm syringe filters w/ 0.2 µm cellulose acetate membrane and 3 mL sterile syringes and transferred into sterile 1.5 mL Eppendorf tubes. Individual wells of a 6 well microplate containing seeded cells were treated with 2 mL of each potato extract at a concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium in triplicate replication to achieve a final extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium. All microplates wereincubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for apoptotic induction to occur. Two controls were utilized during this assay. A negative control reaction was executed on three wells containing HT-29 colon cancer cells which were left untreated and/or uninduced. A positive control was executed on seeded HT-29 cells where 2 mL of 0.01 M colchicine solubilized in McCoys 5A, 1x medium containing 10% fetal bovine was added in triplicate replication to individual wells of a 6 well microplate to achieve a final treatment concentration of 0.005 M colchicine, a known apoptosis inducer of HT-29 colon cancer cells in culture. Medium was removed exactly 24 hours after cells were treated with potato extracts and controls and 0.5 mL of Hyclone 0.25% Trypsin EDTA, 1x was added and incubated for 10 minutes in a humidified, 5% CO_2 incubator chamber to allow for enzymes to cleave adherent HT-29 cells from each well. A hemacytometer and inverted microscope were utilized to count $2x10^6$ cells from each well which were transferred to individual sterile 15 mL conical tubes and centrifuged in a microcentrifuge

at 3000 rpm for 5 minutes. The supernatant was carefully removed from each sample and the remaining cell pellets were resuspended in 50 µL of chilled Cell Lysis Buffer and incubated on ice for 10 minutes. Cell lysates were centrifuged in a microcentrifuge at 7000 rpm for 10 minutes at 4 °C and the supernatant was transferred to a new sterile Eppendorf tube and incubated on ice. Immediately before use, Reaction Buffer and DTT were mixed together in a new sterile Eppendorf tube to a final concentration of 10 mM and 50 µL of this mix was added to all samples and incubated on ice for 30 minutes. In a 96 well sterile microplate, 5 µL of 1 mM Caspase -3 Substrate DEVD-pNA was added to 95 µL of all samples and standards to obtain a final concentration of 50 µM and allowed to incubate for 1 to 3 hours in a humidified, 5% CO_2 incubator chamber. The absorbance was read at 405 nm using a Spectromax 640 microplate spectrophotometer every hour for 3 hours. Caspase -3 activity was calculated based on a pNA standard curve expressed as nmole pNA.

Two-way analysis of variance (ANOVA) was used to evaluate the main effects of treatment and incubation time followed by Tukey-Kramer test to assess separation of means using SAS software.

Statistical analyses conducted on results expressed in terms of nmole pNA for all treatments was based on three biological replicates of each potato extract treatment and triplicate replication of positive and negative controls where the Apoalert® Caspase-3 Colorimetric assay was conducted only once.

RESULTS

While not all aqueous extracts from the 13 selections and cultivars tested inhibited HT-29 colon cancer cell cultures, several factors (main effects) did significantly inhibit HT-29 colon cancer cell cultures (Table 4.3). The four factors that impacted inhibitory responses of individual cultivars and selections were: germplasm source (cultivars and selections with pigmented versus white tissue from whole tubers and from pigmented sectors); stage of tuber development (immature tubers versus intermediate tubers versus mature tubers); extraction from uncooked or cooked tuber tissues; and extract concentration ($mg \cdot mL^{-1}$ cell culture medium) that were used to challenge the HT-29 colon cancer cell cultures.

Table 4.3 Summary of statistical significance (main effects) based on ANOVA for % growth inhibition of HT-29 colon cancer cells in culture by aqueous potato extract concentrations ($mg \cdot mL^{-1}$ cell culture medium) from 13 potato cultivars and selections. Raw and cooked tuber samples grown for two to three consecutive years (2008-2010) were obtained from four developmental stages dependent on clone and year. These clones included russet, red and purple skins with white, red, purple solid and sectored flesh tissues. Data are not available for n/a because not tested. Data are not significant for n/s at P=0.05.

Source of Variation	Year	Sector	Developmental Stage	Cooking	Extract Concentration
RUSSET NUGGET	***	n/a	***	n/a	***
RIO GRANDE RUSSET	n/s	n/a	n/s	*	***
CO95172-3RU	n/s	n/a	*	n/a	n/s
COLORADO ROSE	n/s	n/a	n/s	n/s	n/s
MOUNTAIN ROSE	n/s	n/a	*	n/a	n/s
CO97226-2R/R	***	n/a	***	***	***
CO04058-3RW/RW	n/s	***	***	**	***
CO04061-1R/RW	n/s	n/s	n/s	n/s	n/s
CO97216-1P/P	n/s	n/a	***	***	*
PURPLE MAJESTY	n/s	n/a	*	n/a	n/s
CO97227-2P/PW	*	n/a	*	n/a	n/s
CO04056-7P/PW	***	n/s	**	n/s	***
CO97216-3P/PW	***	**	***	n/s	***

ANOVA = analysis of variance.

Significance level denoted as *.05, **.01, ***.001.

See Appendix C.1-C.13 for degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Microculture Tetrazolium Assay: Germplasm Source (Cultivars and Selections with Pigmented & White Tissue from Whole Tubers & Sectors). Based on a scale of 0 to 100%, the selections and cultivars containing any inhibitory activity greater than 40% specifies a strong inhibitor, 20-39.9% specifies a moderate inhibitor and 0.1 to 19.9% specifies a weak inhibitor of HT-29 colon cancer cells in culture. Inhibition of HT-29 colon cancer cell cultures by aqueous extracts of freeze dried potato tubers, was most strongly and consistently associated with three specific potato selections developed by the Colorado State University, San Luis Valley potato breeding program. Data collected from three consecutive years of testing 13 cultivars and selections revealed that two solid pigmented skin and flesh selections, a red selection CO97226-2R/R, and a purple selection CO97216-1P/P, had high and consistent inhibitory activity (Table 4.4, 4.5). A unique sectored selection, CO04058- 3RW/RW, that has distinct red pigmented sectors in the same tuber as adjacent white sectors, also had high inhibitory properties, but only in the red sectors (Fig. 4.1, Table 4.6 A-B).

Inhibitory activity from CO97226-2R/R averaged 49.6 and 49.3% over the three years, from stage II immature tubers for potato extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium respectively from raw tuber extracts (Table 4.5, Fig. 4.2). Strong inhibitory (46%) activity was also detected in uncooked extracts from harvest stage V tubers at a potato extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium in 2010, and in all years (50%) at a potato extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 4.5, Fig. 4.3). As well, moderate (27%) activity was detected from boiled, immature stage II, 2010 samples at a potato extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 4.5).

Inhibitory activity from CO97216-1P/P averaged 18, 45.6 and 37.4% over the three years, from stage II immature tubers for potato extract concentrations of 3.6, 7.2 and 14.4 mg·mL^{-1} cell culture medium respectively from raw tuber extracts (Table 4.4, Fig. 4.2). Weak inhibitory activity was observed in tubers obtained at intermediate maturity and full harvest maturity stages (Table 4.4, Fig. 4.2, 4.3). Weak inhibition (13%) was also detected from 2010 boiled mature tubers (stage V) at harvest at a potato extract concentration of 14.4 mg·mL^{-1} cell culture medium (Table 4.4).

Inhibitory activity from red sectors of CO04058-3RW/RW averaged 5.3, 57 and 60% over the three years, from stage II immature tubers for potato extract concentrations of 3.6, 7.2 and 14.4 mg·mL^{-1} cell culture medium respectively from raw tuber extracts. Strong inhibitory activity in red sectors at intermediate maturity and moderate inhibitory activity at full harvest maturity stages was observed but was less than that of immature stage II tubers (Table 4.6A, Fig. 4.2, 4.3). Moderate inhibition (30.3%) was also detected from boiled samples from red tissues in 2010 from potato extract concentrations of 7.2 and 14.4 mg·mL^{-1} cell culture medium in stage II tubers, and strong inhibition (47.7%) was detected from boiled samples from red tissues in 2010 at a potato extract concentration of 14.4 mg·mL^{-1} cell culture medium in harvest stage tubers (Table 4.6A).

By contrast little or no inhibition was detected from extracts taken from white tissue sectors of CO04058-3RW/RW (Table 4.6A-B). The only inhibition detected from white tissue sectors from raw tubers was from the highest potato extract concentration of 14.4 mg·mL^{-1} cell culture medium where weak to moderate inhibition ranged from 1.4 to 21.1% (Table 4.6B). One anomalous result produced moderate inhibition at 24.2%

inhibition from a boiled white tissue sector at a potato extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium during immature stage II (Table 4.6B). Accordingly, a significant increase in inhibition was observed in red tissue sectors when compared to white tissue sectors of boiled tubers at a potato extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium during stage V and of raw tubers at both potato extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium during stage II (Table 4.6 A-B, 4.7).

While three pigmented selections (red, CO97226-2R/R; purple, CO97216-1P/P; red sectors from CO04058-3RW/RW) inhibited the most strongly, not all pigmented selections and cultivars inhibited appreciably (Fig 4.2, 4.3). For example, extracts from red-fleshed Mountain Rose, purple-fleshed Purple Majesty and red sectors of CO04061-1R/RW did not inhibit to any extent (Fig 4.2, 4.3). Thus pigmented characteristics alone did not account for the inhibitory properties.

Except for Rio Grande Russet (RGR), none of the other russet skinned or white fleshed clones had notable inhibitory properties. Immature stage II samples of RGR averaged over 2008-2010 moderately inhibited HT-29 cell cultures 0.4, 8.5 and 26.9% by potato extract concentrations of 3.6, 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium. Inhibition at any concentration or developmental stage by the other white fleshed selections and cultivars tested was negligible (Fig. 4.2, 4.3).

Table 4.4 % Growth inhibition of HT-29 colon cancer cells by CO97216-1P/P extracts (mg·mL^{-1} cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested except where indicated as n = (). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05. Data are not available for n/a because not tested. Table does not reflect all significant comparisons.

Potato Extract Concentration	Raw							Boil (97 °C)						
[mg·mL^{-1} cell culture medium]	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean
Stage II Immature														
3.6	38.4 (5)	a b c	14.9 (5)	b c	0	c	18	n/a	n/a	n/a	n/a	0	c	0
7.2	32.2 (5)	a b c	57.2 (5)	a	47.3	a b	45.6	n/a	n/a	n/a	n/a	0	c	0
14.4	31.0 (5)	a b c	42.4 (5)	a b	38.8	a b c	37.4	n/a	n/a	n/a	n/a	0	c	0
Stage III Intermediate														
3.6	n/a	n/a	n/a	n/a	0	c	0	n/a	n/a	n/a	n/a	0	c	0
7.2	n/a	n/a	n/a	n/a	8.9	b c	0	n/a	n/a	n/a	n/a	0	c	0
14.4	n/a	n/a	n/a	n/a	34.5	a b c	0	n/a	n/a	n/a	n/a	0	c	0
Stage IV Intermediate														
3.6	n/a	n/a	n/a	n/a	0	c	0	n/a	n/a	n/a	n/a	0	c	0
7.2	n/a	n/a	n/a	n/a	0	c	0	n/a	n/a	n/a	n/a	0	c	0
14.4	n/a	n/a	n/a	n/a	16.3	b c	16.3	n/a	n/a	n/a	n/a	0	c	0
Stage V Mature														
3.6	0	c	0	c	0	c	0	n/a	n/a	n/a	n/a	0	c	0
7.2	0	c	0	c	1.7	c	0.6	n/a	n/a	n/a	n/a	0	c	0
14.4	0	c	4.1	c	30.1	a b c	11.4	n/a	n/a	n/a	n/a	13.4	b c	13.4
Stage V (6 month storage)														
3.6	n/a		n/a		n/a		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7.2	0	c	n/a		n/a		0	n/a	n/a	n/a	n/a	n/a	n/a	n/a
14.4	n/a		n/a		n/a		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 4.5 % Growth inhibition of HT-29 colon cancer cells by CO97226-2R/R extracts (mg·mL^{-1} cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested except where indicated as n = (). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05. Data are not available for n/a because not tested. Table does not reflect all significant comparisons.

Potato Extract Concentration	Raw							Boil (97 °C)						
[mg·mL^{-1} cell culture medium]	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean
Stage II Immature														
3.6	33.2 (5)	a b c	0 (5)	a	8.6	a b	14	n/a	n/a	n/a	n/a	0	a	0
7.2	58.3 (5)	c	29.4 (5)	a b c	61.1	c	49.6	n/a	n/a	n/a	n/a	0	a	0
14.4	53.4 (5)	c	48.6 (5)	c	45.9	b c	49.3	n/a	n/a	n/a	n/a	27.0	a b c	27.0
Stage III Intermediate														
3.6	n/a	n/a	n/a	n/a	0	a	0	n/a	n/a	n/a	n/a	0	a	0
7.2	n/a	n/a	n/a	n/a	27.4	a b c	27.4	n/a	n/a	n/a	n/a	0	a	0
14.4	n/a	n/a	n/a	n/a	39.0	a b c	39.0	n/a	n/a	n/a	n/a	2.3	a	2.3
Stage IV Intermediate														
3.6	n/a	n/a	n/a	n/a	0	a	0	n/a	n/a	n/a	n/a	0	a	0
7.2	n/a	n/a	n/a	n/a	0	a	0	n/a	n/a	n/a	n/a	0	a	0
14.4	n/a	n/a	n/a	n/a	38.9	a b c	38.9	n/a	n/a	n/a	n/a	0	a	0
Stage V Mature														
3.6	0	a	0	a	0.5	a	0	n/a	n/a	n/a	n/a	0	a	0
7.2	5.6	a	0	a	46.0	b c	17.2	n/a	n/a	n/a	n/a	0	a	0
14.4	50.9	c	45.9	b c	54.8	c	50.5	n/a	n/a	n/a	n/a	0	a	0
Stage V (6 month storage)														
3.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
14.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 4.6 A-B (A) % Growth inhibition of HT-29 colon cancer cells by CO04058-3RW/RW red sector extracts (mg·mL^{-1} cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested except where indicated as n = (). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05. Data are not available for n/a because not tested. Tables do not reflect all significant comparisons. **(B)** % Growth inhibition of HT-29 colon cancer cells by CO04058-3RW/RW white sector extracts (mg·mL^{-1} cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested except where indicated as n = (). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05. Data are not available for n/a because not tested. Tables do not reflect all significant comparisons.

A:

Potato Extract Concentration	Raw							Boil (97 °C)						
[mg·mL^{-1} cell culture medium]	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean
Stage II Immature														
3.6	n/a	n/a	0.6 (5)	a b	10	a b	5.3	n/a	n/a	n/a	n/a	2.5	a b	2.5
7.2	n/a	n/a	50.9 (5)	c d e	63.1	e	57.0	n/a	n/a	n/a	n/a	30.3	a b c d e	30.3
14.4	n/a	n/a	62.0 (5)	e	57.9	d e	60.0	n/a	n/a	n/a	n/a	37.4	a b c d e	37.4
Stage III Intermediate														
3.6	n/a	n/a	n/a	n/a	1.4	a b	1.4	n/a	n/a	n/a	n/a	0	a b	0
7.2	n/a	n/a	n/a	n/a	29.7	a b c d e	29.7	n/a	n/a	n/a	n/a	0.5	a b	0.5
14.4	n/a	n/a	n/a	n/a	40.7	a b c d e	40.7	n/a	n/a	n/a	n/a	1.8	a b	1.8
Stage IV Intermediate														
3.6	n/a	n/a	n/a	n/a	1.7	a b	1.7	n/a	n/a	n/a	n/a	0	a b	0
7.2	n/a	n/a	n/a	n/a	40.5	a b c d e	40.5	n/a	n/a	n/a	n/a	0	a b	0
14.4	n/a	n/a	n/a	n/a	47.9	b c d e	47.9	n/a	n/a	n/a	n/a	13.2	a b c	13.2
Stage V Mature														
3.6	n/a	n/a	0	a b	0	a b	0	n/a	n/a	n/a	n/a	0	a b	0
7.2	n/a	n/a	17.2	a b c d	0	a	8.6	n/a	n/a	n/a	n/a	6	a b	6.0
14.4	n/a	n/a	36.6	a b c d e	10.4	a b	23.5	n/a	n/a	n/a	n/a	47.7	b c d e	47.7
Stage V (6 month storage)														
3.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
14.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

B:

Potato Extract Concentration [mg·mL^{-1} cell culture medium]	Raw							Boil (97 °C)						
	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean	2008 (n)	*w*	2009 (n)	*w*	2010 (n)	*w*	Mean
Stage II Immature														
3.6	n/a	n/a	0 (4)	a b	0	a b	0	n/a	n/a	n/a	n/a	0 (2)	a b	0
7.2	n/a	n/a	0 (4)	a b	0	a	0	n/a	n/a	n/a	n/a	0.65 (2)	a b	0.7
14.4	n/a	n/a	5.0 (4)	a b	0	a b	2.5	n/a	n/a	n/a	n/a	24.2 (2)	a b c d e	24.2
Stage III Intermediate														
3.6	n/a	n/a	n/a	n/a	0	a b	0	n/a	n/a	n/a	n/a	0	a b	0
7.2	n/a	n/a	n/a	n/a	0	a b	0	n/a	n/a	n/a	n/a	0	a b	0
14.4	n/a	n/a	n/a	n/a	7.2	a b	7.2	n/a	n/a	n/a	n/a	14.7	a b c d	14.7
Stage IV Intermediate														
3.6	n/a	n/a	n/a	n/a	0	a b	0	n/a	n/a	n/a	n/a	0	a b	0
7.2	n/a	n/a	n/a	n/a	0.6	a b	0.6	n/a	n/a	n/a	n/a	0	a b	0
14.4	n/a	n/a	n/a	n/a	21.1	a b c d e	21.1	n/a	n/a	n/a	n/a	0	a b	0
Stage V Mature														
3.6	n/a	n/a	0 (2)	a b	0	a b	0	n/a	n/a	n/a	n/a	0	a b	0
7.2	n/a	n/a	0 (2)	a b	0	a b	0	n/a	n/a	n/a	n/a	0	a b	0
14.4	n/a	n/a	0 (2)	a b	2.8	a b	1.4	n/a	n/a	n/a	n/a	0	a b	0
Stage V (6 month storage)														
3.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
14.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 4.7 Summary of statistical significance at P=0.05 based on Tukey-Kramer HSD for differences in % growth inhibition of HT-29 colon cancer cells after 24 hours that exist between white tuber tissue and purple/red tuber tissue extracts (mg·mL^{-1} cell culture medium) from four sectorial expressing selections. Raw data are based on three biological replicates for two to three consecutive harvest years (2008-2010) dependent on selection. Boiled tuber data are based on three biological replicates for one harvest year (2010). Data (main effects) are not significant for (-) based on analysis of variance (ANOVA) for % growth inhibition of HT-29 colon cancer cells at P=0.05. Data are not available for n/a because not tested. Table does not reflect all significant comparisons.

HARVEST STAGE	Stage II (Immature)	Stage III (Intermediate)	Stage IV (Intermediate)	Stage V (Mature)
RAW SELECTIONS				
Significant increase in % growth inhibition from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage at a specific ext. conc. (mg·mL^{-1} cell culture medium)				
CO04058-3RW/RW	7.2, 14.4	-	-	-
CO04061-1R/RW	-	-	-	-
CO04056-7P/PW	-	-	-	-
CO97216-3P/PW	7.2, 14.4	-	-	-
Significant decrease in % growth inhibition from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage at a specific ext. conc. (mg·mL^{-1} cell culture medium)				
CO04058-3RW/RW	-	-	-	-
CO04061-1R/RW	-	-	-	-
CO04056-7P/PW	-	-	-	-
CO97216-3P/PW	-	14.4	-	-
BOILED (97 °C for 20 minutes) SELECTIONS				
Significant increase in % growth inhibition from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage at a specific ext. conc. (mg·mL^{-1} cell culture medium)				
CO04058-3RW/RW	-	-	-	14.4
CO04061-1R/RW	-	-	-	-
CO04056-7P/PW	-	-	-	-
CO97216-3P/PW	-	-	-	-
Significant decrease in % growth inhibition from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage at a specific ext. conc. (mg·mL^{-1} cell culture medium)				
CO04058-3RW/RW	-	-	-	-
CO04061-1R/RW	-	-	-	-
CO04056-7P/PW	-	-	-	-
CO97216-3P/PW	-	-	-	-

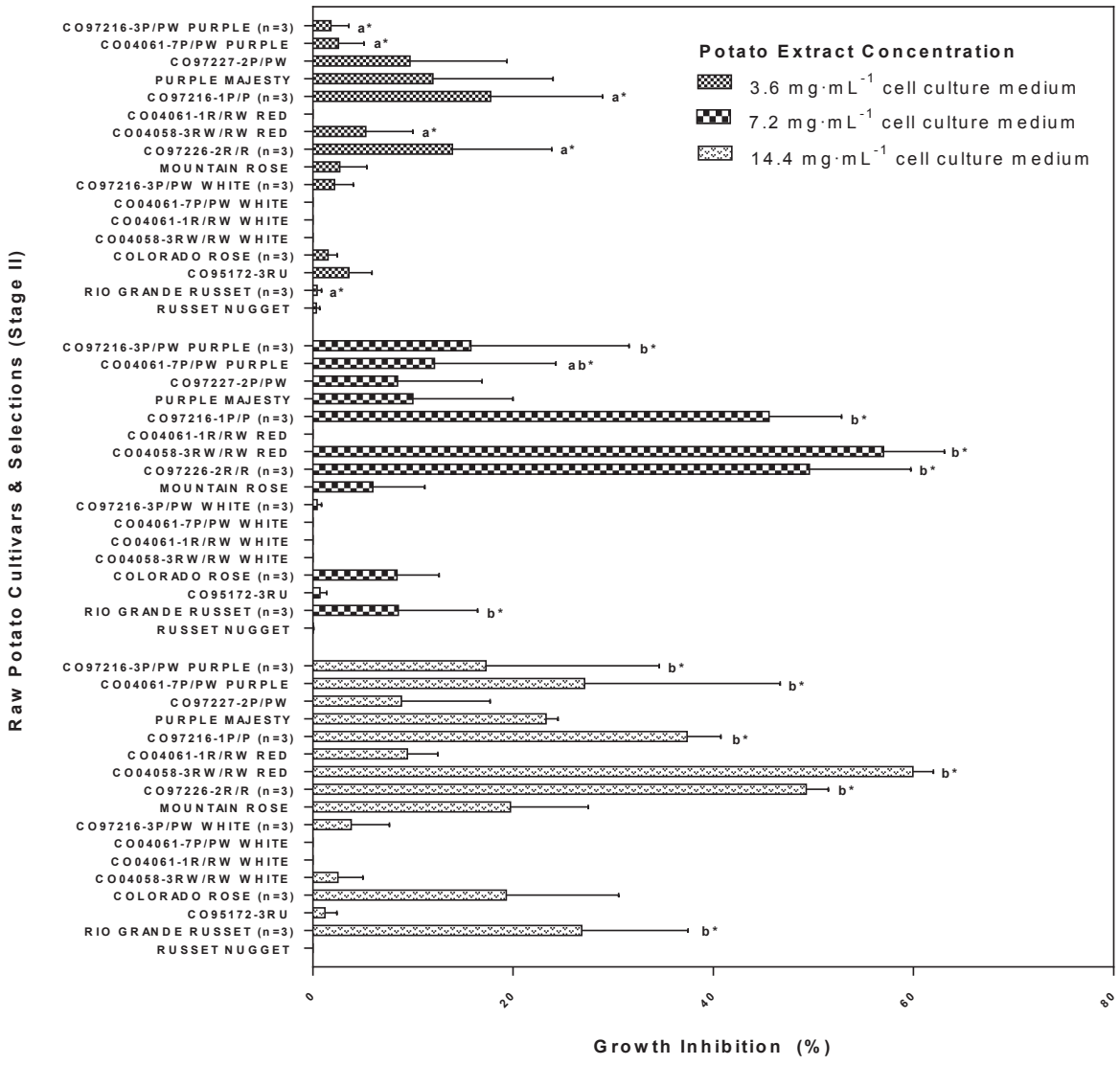

Figure 4.2 % Growth inhibition of HT-29 colon cancer cells after 24 hours for 13 clones of raw immature (stage II) tuber extracts (mg·mL^{-1} cell culture medium). Data are means ± standard errors of five biological replicates for two consecutive harvest years (2008-2010) except where indicated by n = (). Letters* denote statistically significant differences between potato extract concentrations (mg·mL^{-1} cell culture medium) within a specific clone.

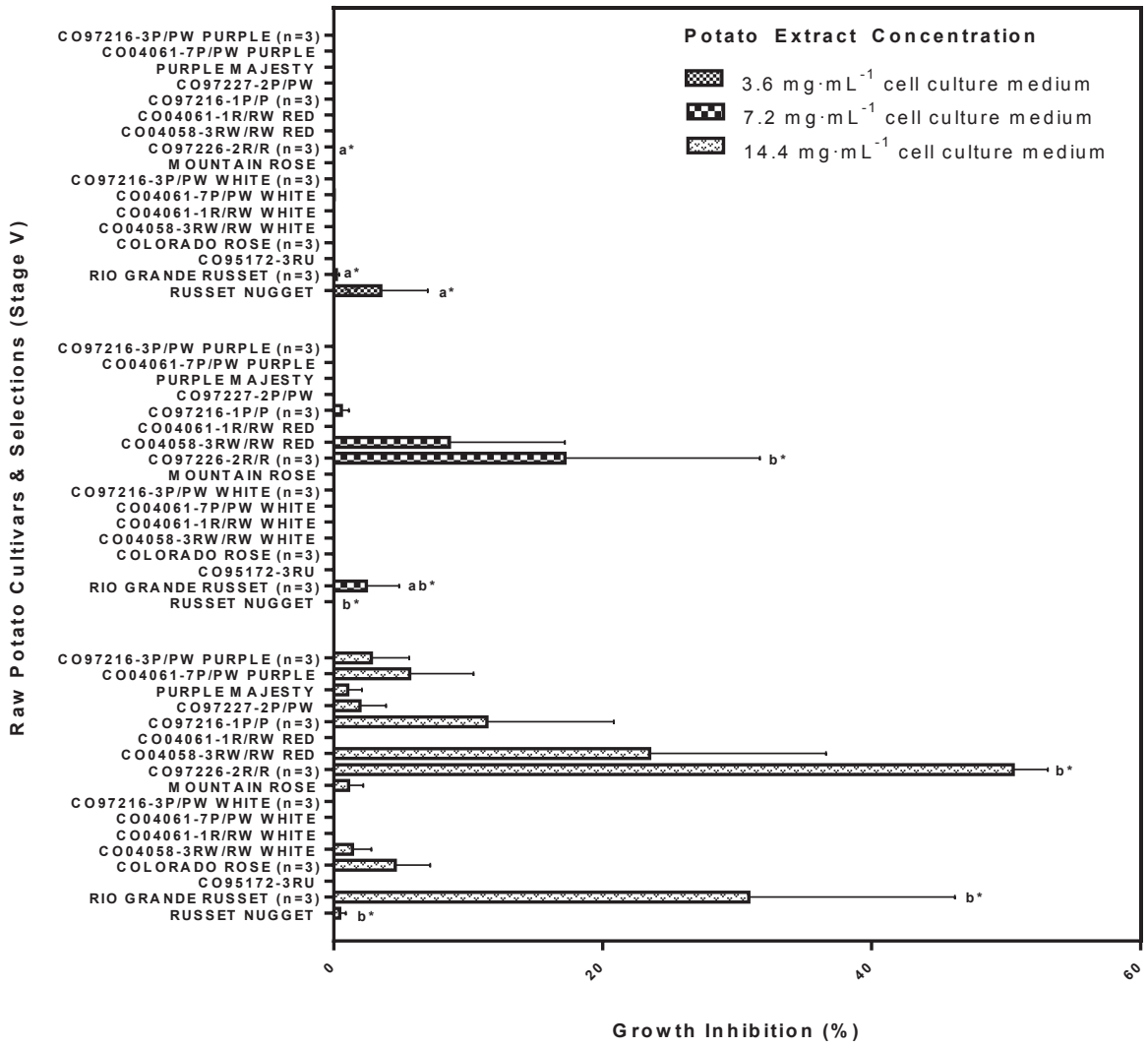

Figure 4.3 % Growth inhibition of HT-29 colon cancer cells after 24 hours for 13 clones of raw mature (stage V) tuber extracts (mg·mL^{-1} cell culture medium). Data are means ± standard errors of three to five biological replicates for two consecutive years (2008-2010) except where indicated by n=(). Letters* denote statistically significant differences between extract concentrations within a specific clone.

Microculture Tetrazolium Assay: Developmental Stage. With minor exceptions, the most effective inhibition was from immature tubers (Table 4.8, Fig 4.2, 4.3). Inhibition lessened with increasing maturity and except for the three strongly inhibitory clones, generally declined to trivial levels at normal harvest maturity (Table 4.8).

Table 4.8 Summary of statistical significance at P=0.05 based on Tukey-Kramer HSD for differences in % growth inhibition of HT-29 colon cancer cells after 24 hours that exist between developmental stages at the same extract concentration (mg·mL^{-1} cell culture medium) of a particular clone. Raw tuber data are based on three biological replicates for two to three consecutive years of harvest (2008-2010) dependent on cultivar or selection. Boiled tuber data are based on three biological replicates for one harvest year (2010). Data are not significant for (-) at P=0.05. Data are not available for n/a because not tested.

	Raw Tubers		Boiled Tubers	
Cultivars and Selections	**Developmental Stage (II - V)**	**Extract Concentration (mg·mL^{-1} cell culture medium)**	**Developmental Stage (II - V)**	**Extract Concentration (mg·mL^{-1} cell culture medium)**
Significant increase in % growth inhibition with increasing maturity between developmental stages of the same extract concentration				
Russet Nugget	$II^a < V^b$ (2008)	3.6	n/a	n/a
Rio Grande Russet	-	-	-	-
CO95172-3RU	-	-	n/a	n/a
Colorado Rose	-	-	-	-
Mountain Rose	-	-	n/a	
CO97226-2R/R	-	-	-	-
CO04058-3RW/RW Red Sector	-	-	$III^a < V^b$ (2010)	14.4
CO04058-3RW/RW White Sector	-	-	-	-
CO04061-1R/RW Red Sector	-	-	-	-
CO04061-1R/RW White Sector	-	-	-	-
CO97216-1P/P	-	-	-	-
Purple Majesty	-	-	n/a	n/a
CO97227-2P/PW Purple Sector	-	-	n/a	n/a
CO04056-7P/PW Purple Sector	-	-	-	-
CO04056-7P/PW White Sector	II^a,$III^a < IV^b > V^a$ (2010)	14.4	-	-
CO97216-3P/PW Purple Sector	-	-	-	-
CO97216-3P/PW White Sector	$II^a < III^b$ (2010)	14.4	-	-
Significant decrease in % growth inhibition with increasing maturity between developmental stages of the same extract concentration				
Russet Nugget	-	-	n/a	n/a
Rio Grande Russet	-	-	-	-
CO95172-3RU	-	-	n/a	n/a
Colorado Rose	-	-	-	-
Mountain Rose	-	-	n/a	n/a
CO97226-2R/R	$II^c > IV^a < V^{bc}$ (2010)	7.2	-	-
CO97226-2R/R	$II^a > V^b$ (2008)	7.2	-	-
CO04058-3RW/RW Red Sector	II^a, III^a, $IV^a > V^b$ (2010)	7.2, 14.4	-	-
CO04058-3RW/RW White Sector	-	-	-	-
CO04061-1R/RW Red Sector	-	-	-	-
CO04061-1R/RW White Sector	-	-	-	-
CO97216-1P/P	$II^a > V^b$ (2008)	3.6, 7.2, 14.4	-	-
CO97216-1P/P	$II^a > V^b$ (2009)	7.2, 14.4	-	-
CO97216-1P/P	$II^a > IV^b$, V^b (2010)	7.2	-	-
Purple Majesty	-	-	n/a	n/a
CO97227-2P/PW Purple Sector	-	-	n/a	n/a
CO04056-7P/PW Purple Sector	$II^a > V^b$ (2009)	14.4	-	-
CO04056-7P/PW White Sector	-	-	-	-
CO97216-3P/PW Purple Sector	$II^a > V^b$ (2008)	7.2, 14.4	-	-
CO97216-3P/PW White Sector	$III^a > IV^b$, V^b (2010)	14.4	-	-

Microculture Tetrazolium Assay: Extract Concentration. While potato extract concentrations of 3.6, 7.2 and 14.4 mg·mL^{-1} cell culture medium were most effective, and some interactions were evident, 7.2 mg·mL^{-1} cell culture medium appeared to be the lowest potato extract concentration that provided high levels of inhibition (Fig 4.2, 4.3; Table 4.7, 4.8). Accordingly, it seems most prudent to emphasize comparative responses at a potato extract concentration of 7.2 mg·mL^{-1} cell culture medium.

Microculture Tetrazolium Assay: Extraction from Uncooked and Cooked Tubers. Samples prepared from uncooked tubers were overall more effective inhibitors than boiled samples (Fig 4.2, 4.4). Boiling of tubers resulted in a significant decrease in inhibitory activity in CO97216-1P/P and CO97226-2R/R immature tubers obtained during stage II at a potato extract concentration of 7.2 mg·mL^{-1} cell culture medium and in CO97226-2RR mature tubers during harvest stage V at both potato extract concentrations of 7.2 and 14.4 mg·mL^{-1} cell culture medium (Fig 4.2, 4.4; Table 4.4, 4.5). A few notable exceptions were detected where boiled tubers retained moderate to strong inhibitory activity. For example, boiled stage II tubers of CO97226-2R/R harvested in 2010 inhibited 27% of HT-29 cells in culture at a potato extract concentration of 14.4 mg·mL^{-1} cell culture medium (Table 4.5, Fig 4.4). Boiled red sectors of CO04058-3RW/RW harvested during immature stage II in 2010 inhibited 30.3 and 37.4%, from both potato extract concentrations of 7.2 and 14.4 mg·mL^{-1} cell culture medium, while mature boiled red sectors during stage V harvest inhibited 47.7% at a potato extract concentration of 14.4 mg·mL^{-1} cell culture medium (Table 4.6A, Fig 4.4). These exceptions are in contrast to data from the other clones tested, that did not inhibit HT-29 cell cultures after cooking (Fig 4.4).

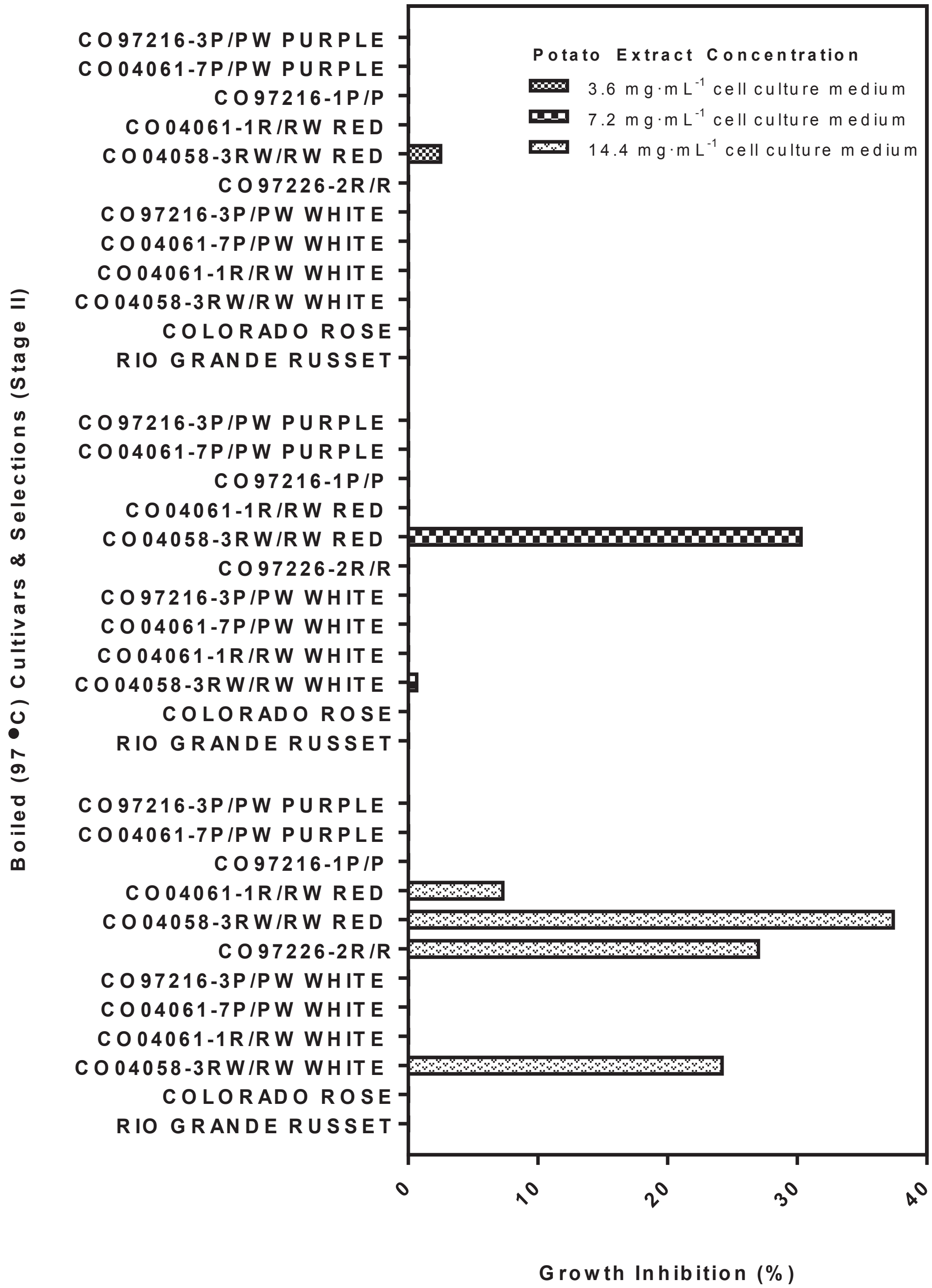

Figure 4.4 % Growth inhibition of HT-29 colon cancer cells after 24 hours for 13 clones of boiled (97 °C for 20 miutes) immature (stage II) tuber extracts (mg·mL^{-1} cell culture medium). Data are means ± standard errors of three to five biological replicates for one harvest year (2010).

Pearson Correlation: Cancer Growth Inhibition & Antioxidants. Most statistically significant comparisons of antioxidant TP or DPPH/TEAC with HT-29 colon cell culture inhibition were negatively correlated with cancer inhibition (Table 4.9). Negative correlations associated with both DPPH radical scavenging capacity and total phenolic content against cancer inhibition were found to exist within the three strongest inhibiting clones at both potato extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium. Notable exceptions with purple pigmented clones include a positive correlation detected at a potato extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium for TP levels in CO97216-1P/P tubers (Table 4.9) as well as positive correlations for inhibition with extracts from CO97227-2P/PW and Purple Majesty with DPPH radical scavenging capacity. One other notable exception was a positive correlation for the russet, white fleshed clone, CO95172-3RU with both DPPH radical scavenging capacity and total phenolic content (Table 4.9).

Table 4.9 Summary of statistical significance at P=0.05 based on Pearson Correlation for degree of linear association that exists between cancer inhibition activity, total antioxidant capacity based on DPPH radical scavenging capacity assay and total phenolic antioxidant content based on Folin Ciocalteu reagent assay. Correlations were computed for each clone based on potato extract concentration (3.6, 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium) utilized in microculture tetrazolium assays. Sample size = n. Correlation coefficient = r. Level of significance = p.

CULTIVARS & SELECTIONS	EXTRACT CONCENTRATION ($mg \cdot mL^{-1}$ cell culture medium)	n	r	p	DPPH	TP	CANCER INHIBITION
RIO GRANDE RUSSET	7.2	36	-0.385	0.0204		X	X
RIO GRANDE RUSSET	14.4	36	-0.411	0.0128		X	X
RUSSET NUGGET	3.6	12	-0.564	0.0559	X		X
RUSSET NUGGET	14.4	12	-0.686	0.0137		X	X
COLORADO ROSE	7.2	36	-0.600	0.0001		X	X
COLORADO ROSE	7.2	36	-0.730	<.0001	X		X
COLORADO ROSE	14.4	36	-0.470	0.0038		X	X
COLORADO ROSE	14.4	36	-0.632	<.0001	X		X
CO04056-7P/PW	14.4	49	-0.326	0.0223		X	X
CO04058-3RW/RW	7.2	58	-0.505	<.0001		X	X
CO04058-3RW/RW	7.2	58	-0.464	0.0002	X		X
CO04058-3RW/RW	14.4	58	-0.478	0.0001		X	X
CO04058-3RW/RW	14.4	58	-0.494	<.0001	X		X
CO95172-3RU	3.6	12	0.655	0.0209		X	X
CO95172-3RU	3.6	12	0.779	0.0028	X		X
CO95172-3RU	7.2	12	0.578	0.0491	X		X
CO97226-2R/R	7.2	36	-0.437	0.0078	X		X
CO97226-2R/R	14.4	36	-0.509	0.0015		X	X
CO97226-2R/R	14.4	36	-0.637	<.0001	X		X
CO97227-2P/PW	3.6	9	0.694	0.0380	X		X
CO97227-2P/PW	7.2	9	0.694	0.0380	X		X
CO04061-1R/RW	14.4	48	-0.298	0.0398		X	X
CO04061-1R/RW	14.4	48	-0.311	0.0314	X		X
CO97216-1P/P	3.6	36	0.433	0.0083		X	X
CO97216-1P/P	7.2	36	-0.327	0.0283		X	X
PURPLE MAJESTY	7.2	12	0.670	0.0171	X		X
PURPLE MAJESTY	14.4	12	0.733	0.0067	X		X

Determination of Apoptosis. Extracts from immature tubers of CO97216-1P/P, CO97226-2R/R and CO04058-3RW/RW from developmental stage II at a potato extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium were determined to be the most effective inhibitors of HT-29 colon cancer cells culture. Thus, three biological replicates from these 3 clones in addition to colchicine, a known inducer of apoptosis of HT-29 colon cancer cells at 0.005 M concentration, were evaluated for caspase -3 protease activities to establish if an apoptosis signaling pathway was the source of cancer cell growth inhibition (Cohen et al., 1999).

Absorbance was read every 1 hour for 3 hours to determine the best incubation time point for reporting Caspase -3 reactions results utilizing Apoalert caspase -3 colorimetric assay kit. Based on Tukey's HSD, microplate incubation after either two or three hours are both statistically appropriate time points for maximizing caspase -3 protease reactions (Fig. 4.5). Results will be referenced based on nmoles of pNA at the time point of three hours.

Colchicine, a known inducer of apoptosis, produced the highest level of caspase -3 activities with an average of 22.5 nmole pNA (Fig. 4.5). Red tissue sectors from CO04058-3RW which strongly inhibited cancer cell cultures on average 57% over two years produced the second highest level of caspase -3 activities with an average of 12.9 nmole pNA (Table 4.6A, Fig 4.5). Conversely, white tissue sectors from this sectorial expressing selection which was a weak inhibitor of cancer cell cultures produced statistically the same amount of caspase-3 activity as the untreated control averaging 6.3 nmole pNA (Table 4.6B, Fig. 4.5). CO97216-1P/P and CO97216-2R/R which were both strong inhibitors of cancer cell cultures averaging 45.6% and 49.6% over three

years were statistically classified the same as the white sectors of CO04058-3RW/RW producing 9.5 and 11.4 nmoles pNA (Table 4.4, 4.5, Fig 4.5).

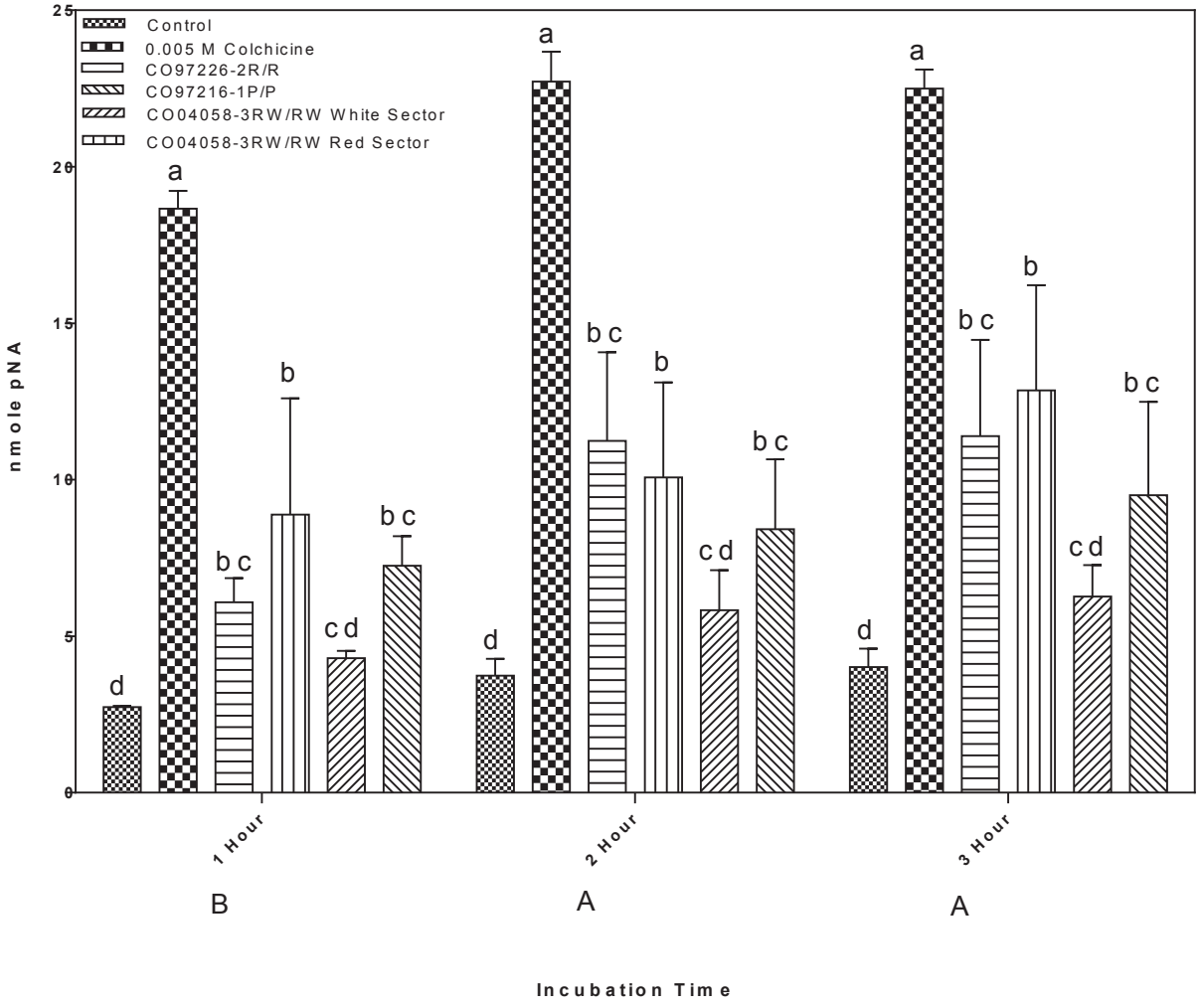

Figure 4.5 Caspase -3 protease activity measured after 24 hours of treatment expressed in nmole pNA by aqueous extracts (7.2 mg·mL^{-1} cell culture medium) of three strong inhibitory clones (CO97226-2R/R, CO97216-1P/P, CO04058-3RW/RW red sector) and one weak inhibitory clones (CO04058-3RW/RW white sector) obtained during stage II in addition to a negative control (untreated cells) and positive control (0.005 M colchicine). Data are means ± standard errors of 3 biological replicates. Lower case letters denote statistically significant differences in caspase -3 protease activities. Upper case letters denote statistically significant differences between apoptosis experimental incubation reaction times.

DISCUSSION

Microculture Tetrazolium Assay: Germplasm Source (Cultivars and Selections with Pigmented & White Tissue from Whole Tubers & Sectors). Plant explorations and taxonomic systematic research many decades ago established existence of extensive biodiversity among species and land races of potato in the Andes (Valvilov, 1951). While European plant explorers were instrumental in introducing potatoes as a valuable food staple, especially suited to cool climates, clones with red and purple flesh were ignored or selected against in favor of white fleshed tubers. Recent research (Stushnoff et al., 2008, 2010; Brown, 2005) has discovered that the pigmented genotypes possess several multiples of antioxidant content and radical scavenging capacity, primarily attributed to their much higher polyphenolic anthocyanins and related simple phenolic acid content. Thus a logical next step was to explore if these unique genotypes may or may not possess inhibitory properties when exposed to colon cancer cells in culture. Results from this research revealed that while maximum inhibition was detected in clones with red and purple fleshed tubers, not all pigmented clones have inhibitory properties. While a few white fleshed tubers inhibited cell cultures, percent growth inhibition was much less pronounced than that from pigmented tissues.

Selections that express both red and white tissues in the same tuber, such as CO04058-3RW/RW, and display inhibitory properties provide an ideal model to study genomic expression. These genotypes eliminate genotype by environment, genotype by developmental stage, genotype by nutrient content and soil nutrient plant to plant variation. Expression thus depends on activity of the transcriptome in each type of

tissue and greatly sharpens capacity to interpret gene action (Stushnoff et al., 2010). Inhibition by red tissues exceeded that of white tissues, reinforcing the possibility that some metabolite(s) closely associated with the phenyalanine (PAL) pathway are responsible. Because some inhibition was detected from white tissue sectors and tubers, including RGR, inhibition cannot be exclusively attributed to a pigment compound. It may be due to one or more than one compound(s) associated with the PAL pathway.

Only a few clones were tested in this study, thus it is very likely that other untested cultivars and selections may also possess inhibitory properties that could provide valuable dietary intervention to guard against colon cancer. This supposition requires considerably more research.

Microculture Tetrazolium Assay: Developmental Stage. A major factor contributing to inhibition, reported here for the first time, is the unique property of enhanced inhibition associated with developmental stage. Possible explanations include: upregulation of key inhibitory metabolites found only at the outset of tuber initiation and early development, degradation of the bioactive agent with tuber maturity for various reasons and RNA upregulation along with many other transcriptome regulated metabolites. Ramifications of this finding are important and interesting for both consumers and growers of potato should the phenomenon that exists between cancer inhibition and immature harvested potatoes be discerned. A very optimistic view may be a reduced incidence of colon cancer as a dietary intervention wave of the future simply by consumption of immature cancer inhibiting tuber clones and/or development of natural cures creating niche markets for growers and medical professionals.

While it is clear that the earliest developmental stages provide tubers with the highest inhibitory bioactivity, it must also be recognized that modern potato production practices in the San Luis Valley attempt to control harvest date by employing top kill practices to induce tuber maturity. Thus the true mature stage may vary from year to year as a function of when growers apply top kill treatments to ensure harvest in a timely manner before frost killing temperatures ensue. Accordingly, the impact of maturity becomes more difficult to pin point than identifying an active inhibitory phase in immature and intermediate maturity tubers.

Microculture Tetrazolium Assay: Extract Concentration. A shared commonality amongst all clones was the importance of dosage concentration at potato extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium required to induce consistent colon cancer growth inhibition. Identifying this threshold is critical as it provides a starting point for screening novel clones that may too comprise valuable and potentially greater defenses against cancer. In addition, knowledge of this threshold provides a dietary guideline of how much potato product one should consume should future research demonstrate that inhibitory compounds in potato are successfully delivered to the colon undamaged by the digestive process and guarding the colon against colon cancer.

Microculture Tetrazolium Assay: Extraction from Uncooked and Cooked Tubers. Cooking was a major factor affecting inhibition. Overall uncooked tubers retained inhibitory properties better than cooked tubers. In spite of this fact, immature red sectors of CO04058-3RW/RW and CO97226-2R/R managed to retain half of their inhibitory activity after cooking when compared to their raw counterparts during stage II.

This finding is important for several reasons. Potatoes are typically consumed as a cooked product. In order for a consumer to reap the potential protective benefits against colon cancer, inhibitory compounds must be delivered to the colon undamaged. Thus, finding a tuber that possesses inhibitory properties against colon cancer that can withstand cooking is essential for a consumer to reap these benefits as well as for a grower to market these benefits.

This finding has also unearthed the idea that there exists more than one bioactive compound responsible for inhibition of colon cancer cells in culture. Glycoalkaloids are heat stable compounds that exist in potato suspected for partial inhibition of colon cancer cells, confirmed in several studies to successfully inhibit HT-29 colon cancer cells in culture. One could infer that the partial inhibitory activity retained in these two selections after cooking may be a result of heat stable glycoalkaloids. While, the remaining inhibitory activity that has been lost to cooking suggests that glycoalkaloids may not be the sole inhibitors of colon cancer.

Pearson Correlation: Cancer Growth Inhibition & Antioxidants. Negative correlations that exist between DPPH radical scavenging capacity and phenolic antioxidants against cancer inhibition detected in inhibitory clones at critical inhibitory potato extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium suggest that polyphenolic compounds that are responsible for high TP and DPPH/TEAC values are not likely responsible for inhibiting HT-29 colon cancer cell cultures. This does not rule out activity by compounds found in pigmented tubers that do not react with TP and DPPH assays. Positive significant correlations with inhibitory clones suggest the presence of an elusive inhibitory factor not yet identified in this investigation. While for

one reason or another the most potent inhibitory clones appear to have either red or purple skin and tuber flesh color, which are also high in phenolic based antioxidants, the data from correlation analyses does not support a strong role for anthocyanidin polyphenolic antioxidants based upon assays used here.

Another possible confounding factor is that potatoes are known to contain elemental constituents, vitamins and a good balance of amino acids that are necessary for supporting growth of in vitro cell cultures. Some clones may be very useful in optimizing cell culture growth of HT-29 colon cancer cells. A combination of promotion factors in the absence or presence of endogenous inhibitors may also contribute to less than compelling correlation data.

Determination of Apoptosis. This study concludes that the mechanism of inhibition can be attributed at least in large part to apoptosis in red sectors of CO04058-3RW/RW. While, other inhibitory selections tested did not reveal high caspase -3 activities, some protease activity was detected thus concluding that apoptosis may likely be the source of their inhibition as well. A possible explanation for this observed reduction in apoptotic activity in strong inhibiting clones as well as a possible shortcoming of this study is that the assay utilized only detects the presence of caspase -3 activities. Although caspase -3 is a crucial protease implicated in the execution phase of apoptosis, several other caspases exist throughout the apopotic cascade that also play key roles in initiation and execution of apoptosis that may be more readily induced by strongly inhibiting tuber clones.

Future Research Aims. This study has acknowledged the significance of pigmented and white fleshed clones, developmental stage, dosage, cooking and

potential source of bioactive compounds that exist in potato as it relates to colon cancer. It has also has answered some questions and perhaps generated more new questions in the quest for finding for a cure and/or dietary intervention/prevention vehicle for colon cancer.

Several future research aims need to be investigated based on conclusions and inconclusiveness of this study. Continuous screening of potato germplasm needs to be executed to find new potential sources of cancer inhibition. The phenomenon that exists between cancer inhibition and immaturely harvested potatoes must be addressed. Differences in metabolites and upregulation of genes that exist in raw and cooked immature and mature tubers need to be identified and quantified in an effort to pinpoint the source of inhibition at both the transcriptome and metabalome level. This study only evaluated the effect on inhibitory properties in one facet of cooking and thus future work needs to address other common modes of cooking such as microwave, baking and frying. *In vivo* studies involving feeding and intraperitoneal injection of animals need to be executed to determine ramifications of inhibitory clones on gastrointestinal system *in vivo* in hopes of moving studies up into human clinical trials. Further analysis of apoptosis utilizing various caspases need to be conducted to conclude mechanism of inhibition in inhibitory clones demonstrating lower capase -3 activity.

Chapter 5

ASSESSMENT OF ANTIOXIDANT CAPACITY, TOTAL PHENOLIC CONTENT AND INHIBITION RESPONSE OF HT-29 COLON CANCER CELLS *IN VITRO* BY *SOLANUM TUBEROSUM* L. TUBERS, PEELS, FLESH AND FLOWERS SUBJECTED TO VARIOUS HARVEST/TOP KILL TREATMENTS DURING SEVERAL DEVELOPMENTAL STAGES OF TUBER MATURITY

SUMMARY

Our previous research revealed that two solid pigmented skin and flesh selections; a red selection CO97226-2R/R, a purple selection, CO97216-1P/P; and a unique sectored selection, CO04058- 3RW/RW that has distinct red pigmented sectors in the same tuber as adjacent white sectors had high and consistent inhibitory activity against mammalian HT-29 colon cancer cells in culture when harvested as immature tubers. Prior to tuber harvest, vine kill methods (chemical sprays, mechanical or both) are widely exercised to remove the above ground plant foliage to induce tuber maturity, accelerate skin set, reduce incidence of fungal disease, reduce insect infestations, control tuber sizing, stabilize tuber solids and reduce trash related harvest problems. The primary objective of this study was to explore possible explanations for observed enhanced inhibition associated with developmental stage in immature tubers by examining whether inhibitory metabolites exist only at the outset of tuber initiation and early development, and/or if mechanical and chemical vine kill practices prior to harvest are responsible for degradation of inhibitory metabolites. Total phenolic content, DPPH radical scavenging capacity and inhibitory activity against HT-29 colon cancer cell

culture *in vitro* were assessed utilizing aqueous extracts from flowers, peels, flesh and whole tubers obtained throughout several developmental stages that were collected after no treatment, after vine tops were cut to 10 cm in height and after chemical (95% sulfuric acid and Reglone) removal of vine tops. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega was used to measure the number of viable HT-29 colon cancer cells in all cell culture assays. Total phenolic content was determined by the Folin-Ciocalteu method. Radical scavenging capacity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. In every situation where significant differences were observed within a developmental stage, regardless of which harvest/top kill treatment was utilized, the same pattern was observed where higher DPPH/TEAC, TP and inhibition levels occurred in immature tubers compared to tubers with increasing maturity. Total phenolic antioxidants and DPPH radical scavenging antioxidants were best conserved in immature tubers where the tops were not killed by any treatment. Utilizing no treatments or no top cutting was also more favorable for conserving these metabolites in mature tubers. Acid treated mature CO97216-1P/P declined in inhibitory activity when compared to Reglone. CO97226-2R/R tubers increased in inhibitory activity in top cut immature tubers, but decreased in inhibitory activity in top cut mature tubers. TP and DPPH/TEAC levels were higher in extracts obtained from potato peels when compared to potato flesh and whole tuber extracts. Aqueous flower extracts proved to be an exceptional source of cancer cell culture inhibitory metabolites inhibiting cell cultures with extract concentrations between 0.5 and 3.6 $mg \cdot mL^{-1}$ cell culture medium. Aqueous peel extracts also were a potent source of cancer cell culture inhibitory metabolites where known strong inhibitory clones inhibited

cell cultures best at extract concentrations ranging between 0.9 and 3.6 mg·mL^{-1} cell culture medium. Clones that typically produce nil to weak inhibitory activity when utilizing extracts from whole tubers and/or flesh, however peels inhibited cell cultures best at extract concentrations ranging between 3.6 and 14.4 mg·mL^{-1} cell culture medium. Aqueous peel extracts were superior to flesh and whole tuber extracts with the exception of CO97216-1P/P where flesh only extracts were superior to aqueous flower, peel and whole tuber extracts at extract concentrations ranging between 3.6 and 14.4 mg·mL^{-1} cell culture medium. CO04058-3RW/RW and CO97226-2R/R that typically display strong inhibitory activity against cancer cell cultures at higher extract concentrations of 7.2 and 14.4 mg·mL^{-1} cell culture medium from whole tuber extracts had strong peel inhibitory activity at extract concentrations as low as 0.9 and 1.8 mg·mL^{-1} cell culture medium, with some year to year variation that existed in CO04058-3RW/RW. The significance of plant components and immature tubers from elite germplasm as sources of inhibitory bioactive compounds against cancer cell cultures has been elucidated in this study. In addition, the importance of selecting the appropriate harvest/top kill treatment as it relates to cancer cell culture inhibition, antioxidant capacity and total phenolic content has also been revealed in this study.

INTRODUCTION

Potato *(Solanum tuberosum* L.*)* is the fourth largest consumed staple food crop in the world (Kang & Priyadarshan, 2007). Studies have demonstrated that bioactive compounds and/or antioxidants present in fruits, vegetables, tea and wine are important

in the maintenance of human health and prevention of lifestyle induced and chronic disease (Liu, 2004; Chu et al., 2002; Prakash, 2001), however, some studies have found disagreeing evidence with regards to fruit and vegetable consumption and colorectal cancer (Aune et al., 2011). Potatoes are rich in nutrients and phytochemicals as they provide an excellent source of vitamin C, a good source of vitamin B6, copper, potassium, manganese, and dietary fiber, and also contain an abundance of secondary metabolites that may serve as bioactive agents (Stushnoff et al., 2010; 2008; Thompson et al., 2009; Vreugdenhil & Bradshaw, 2007). The high prevalence of potato consumption in addition to possible agents that may offer protection against chronic diseases suggests that further investigation of possible inhibitory metabolites against cancer would be of great benefit to many.

Several studies have implicated classes of secondary metabolites found in potato with antiproliferative activity, apoptotic induction mechanisms and the ability to upregulate detoxifying phase 2 enzymes. Friedman et al., (2005) reported that two important glycoalkaloids, α-chaconine and α-solanine, isolated in 'Dejima' potatoes were responsible for growth inhibition of several human cell lines in culture including cervical (HeLa), liver (HegG2), lymphoma (U937) and stomach (AGS and KATO III). Hayashi et al., (2006) reported that anthocyanins from pigmented potatoes were responsible for the induction of apoptosis in KATO III human stomach cancer cell lines in culture, as well as suppression of stomach cancer in mouse. Feng et al., (2005) found that chlorogenic acid inhibited proliferation of lung cancer cells and TPA-induced neoplastic transformation and may be a potential stimulant of NQO1 and GST enzymatic activities. A study conducted at CSU using the same potato genotypes we

are utilizing, indicated a reduction in breast cancer carcinogenesis incidence and multiplicity in rats (Thompson et al., 2009).

Increasing numbers of people around the world are developing cancers. It is an epidemic affecting every region, race, and socioeconomic class across the globe (American Cancer Society, 2011a). Cancer accounts for 1 in every 8 deaths worldwide while in the United States alone cancer accounts for 1 in every 4 deaths making cancer the second leading cause of death in the United States after heart disease (American Cancer Society, 2011a). Colorectal carcinoma is the third leading cause of cancer death in both men and women as well as the third most commonly diagnosed cancer in the United States (American Cancer Society, 2011b). In 2011, the American Cancer Society (2011b) estimates that approximately 141,210 new colorectal cancer diagnoses will arise and 49,380 people are expected to die of this potentially preventable disease by means of cancer prevention awareness and early detection through routine screening tests.

Previous research (i.e. Chapter 4 of dissertation) investigated the inhibitory nature of 13 elite genotypes of aqueous *S. tuberosum* extracts from the Colorado Potato Breeding Program with different pigments, antioxidant activity, phenolic and glycoalkaloid contents obtained throughout several developmental stages in both raw and cooked forms on colon (HT-29) cancer cell lines *in vitro*. Data collected from three consecutive years of testing these 13 cultivars and selections, grown under essentially commercial production conditions, at the Colorado State University San Luis Valley Research Center, Center, CO revealed that two solid pigmented skin and flesh selections, a red selection CO97226-2R/R and a purple selection CO97216-1P/P, had

high and consistent inhibitory activity when harvested as immature tubers. A unique sectored selection, CO04058- 3RW/RW, that has distinct red pigmented sectors in the same tuber as adjacent white sectors, also had high inhibitory properties, but only in the red sectors and when harvested as immature tubers. Selections that express both red and white tissues in the same tuber and display inhibitory properties, such as CO04058-3RW/RW, provide an ideal model to study genomic expression. These genotypes eliminate genotype by environment, genotype by developmental stage, genotype by nutrient content and soil nutrient plant to plant.

A major factor contributing to inhibition in addition to germplasm source and extract concentration, reported for the first time in this previous study (i.e. Chapter 4 of dissertation), was the unique property of enhanced inhibition associated with developmental stage. One possible explanation for this phenomenon could be related to an upregulation of key inhibitory metabolites found only at the outset of tuber initiation and early development. Or perhaps, the bioactive metabolites responsible for inhibitory responses in these clones are degraded for example before they can reach maturity.

Vine kill also referred to as top kill, involves removal of the above ground plant foliage prior to tuber harvest (Prairie Agricultural Machinery Institute, 2003). Vine kill is a widespread and advantageous practice as it induces tuber maturity, accelerates skin set, reduces incidence of fungal disease, reduces insect infestations, controls tuber sizing, stabilizes tuber solids and reduces trash related harvest problems (Prairie Agricultural Machinery Institute, 2003). Several vine removal methods exist including the use of chemical sprays, mechanical methods or combinations of both (Mosley, 2011). Common mechanical methods include chopping and roto-beating, flaming and

sometimes frost. Chemical desiccants are commonly applied to vines approximately 2 to 3 weeks prior to harvest depending on which type is utilized (Mosley, 2011). Diquat, (1,1-Ethylene 2,2-dipyridylium dibromide) brand name Reglone, is a nonvolatile, nonselective, non-residual, contact herbicide that desiccates vegetative portions of plants without affecting the roots (Helm Agro US, 2008; Mosley, 2011). Endothall (7-Oxabicyclo [2.2.1] heptane-2,3-dicarboxylic acid) is also a fast acting nonselective contact herbicide utilized to desiccate vegetative portions of plants that typically does not affect the roots (Commonweatlh of Massachusetts, 2012; Mosley, 2011). Sulfuric acid (93%) is a caustic desiccant that works quickly to kill vines (Mosley, 2011). Monocarbamide dihydrogen sulfide is a corrosive herbicide utilized to desiccate vines (Mosley, 2011). Consequently, if inhibitory metabolites synthesized in tubers are not an outcome of immaturity then application of chemical desiccants at vine kill may possibly be the culprit degrading these inhibitory metabolites.

The results from the aforementioned study suggest that the investigation of the synthesis of inhibitory metabolites in immature tubers could be of great benefit to both farmer and consumer. The primary objective of this study was to explore possible explanations for observed enhanced inhibition associated with developmental stage in immature tubers by examining whether inhibitory metabolites exist only at the outset of tuber imitation and early development or if chemical top kill practices prior to harvest are responsible for degradation of inhibitory metabolites. Total phenolic content, DPPH radical scavenging capacity and inhibitory activity against HT-29 colon cancer cell culture was assessed utilizing aqueous extracts from peels, flesh, flowers and whole tubers obtained throughout several developmental stages that were collected after no

treatment, after vine tops cut to 10 cm in height and chemical (95% sulfuric acid and Reglone) removal of vine tops.

MATERIALS AND METHODS

Clones. A total of seven clones of *S. tuberosum* were obtained from the Colorado Potato Breeding Program, grown under essentially commercial production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado (Table 5.1). Clones beginning with CO are in an experimental phase of release and are designated as selections (Table 5.1). Clones with names have been released to the public and are designated as cultivars (Table 5.1). The selection ending in -RW/RW contains pigmented and white sectors in both peel and flesh that is otherwise of the same genotype and not confounded by environmental and developmental differences (Fig. 5.1; Table 5.1). In all selections, the first letter before the slash is indicative of tuber skin color (P=purple, R=red, W=White) (Table 5.1). In all selections, the letters following the slash are indicative of tuber flesh colors (P=purple, R=red, W=White) (Table 5.1).

Figure 5.1 Sectorial expression of pigments illustrating a model system that permits sampling of tissues that differ only in pigment expression and are not confounded by environmental factors or genetic segregation.

Table 5.1 Cultivars and clonal selections from the Colorado Breeding Program utilized in this research.

Cultivars and Clonal Selections	Female Parent	Male Parent	Skin	Flesh
CO97216-1P/P	CO94163-1	CO94214-1	Purple	Purple
CO97216-3P/PW	CO94163-1	CO94214-1	Purple	Purple & White
CO97226-2R/R	Mountain Rose	CO94214-1	Red	Red
CO04058-3RW/RW	CO97216-3P/PW	CO97216-1P/P	Red & White	Red & White
Mountain Rose	All Red	ND2109-7	Red	Red
Purple Majesty	All Blue	ND2008-2	Purple	Purple
Russet Nugget	Krantz	AND71609-1	Russet	White

All cultivars and selections were examined for inhibitory activity, total phenolic content and DPPH radical scavenging capacity throughout several developmental stages and after application of harvest of vine tops for one harvest in 2011. The following harvest/top kill treatments were applied (Table 5.2): (1) untreated immature tubers and plant components (whole tuber, peel only, flesh only) harvested on the 10th of August, 2011, 29 days before maturity (immature, day 1, no treatment; immature d1NT); (2) vine tops were mechanically sheared to 10 cm. in height on the 10th of August, 2011, 29 days before maturity and immature tubers harvested on the 18th of August, 2011, 21 days before maturity (immature, day 9, tops cut; immature d9TC); (3) vine tops were mechanically sheared to 10 cm. in height and 95% sulfuric acid applied to all stem tops utilizing a micropipette on the 10th of August, 2011, 29 days before maturity and immature tubers harvested on the 18th of August, 2011, 21 days before maturity (immature, day 9, acid treated; immature d9AT); (4) untreated tubers harvested on the 18th of August, two days before top kill and 19 days before maturity (intermediate, day 9, not treated; intermediate d9NT); (5) vine tops were mechanically sheared to 10 cm in height on the 18th of August, 2011, 21 days before maturity and

mature tubers harvested on the 8th of September, 2011, at maturity stage (mature, day 30, tops cut; mature d30TC); (6) vine tops were mechanically sheared to 10 cm in height and 95% sulfuric acid applied to all stem tops utilizing a micropipette on the 18th of August, 2011, 21 days before maturity and mature tubers harvested on the 8th of September, 2011, at maturity stage (mature, day 30, acid; mature d30AT); (7) vine tops were chemically field sprayed with Reglone on the 23rd of August, 2011, 16 days before maturity and mature tubers harvested on the 8th of September, 2011, at maturity stage (mature, day 30, Reglone; mature d30RT).

Table 5.2 Summary of treatments, treatment abbreviations, planting dates, treatment dates, harvest dates and defining of developmental stage in terms of days to maturity and growing degree days that were utilized to execute harvest/top kill treatments in this research. Data not applicable for n/a because no treatment was applied.

Harvest/Top Kill Treatment	Harvest/Top Kill Treatment Abbreviation	Planting Date	Harvest/Top Kill Treatment Date	Days to Maturity (Harvest/Top Kill Treatment)	Harvest Date	Days to Maturity (Harvest)	Growing Degree Days (Harvest)
immature, day 1, no treatment	immature d1NT	25th of May	n/a	29	10th of August, 2011	29	1289.5
immature, day 9, tops cut	immature d9TC	25th of May	10th of August, 2011	29	18th of August, 2011	21	1439.5
immature, day 9, acid treated	immature d9AT	25th of May	10th of August, 2011	29	18th of August, 2011	21	1439.5
intermediate, day 9, no treatment	intermediate d9NT	25th of May	n/a	21	18th of August, 2011	21	1439.5
mature, day 30, tops cut	mature d30TC	25th of May	18th of August, 2011	21	8th of September, 2011	0	1794.0
mature, day 30, acid treated	mature d30AT	25th of May	18th of August, 2011	21	8th of September, 2011	0	1794.0
mature, day 30, Reglone treated	mature d30RT	25th of May	23rd of August, 2011	16	8th of September, 2011	0	1794.0

Extraction. Three to four biological replicates (whole tubers (median slices of peel and flesh), potato peels, potato flesh and flowers from separate plants) from each clone throughout several developmental stages and after harvest/top kill treatments were freeze dried using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a fine sieve (100 mesh). Freeze dried powder was weighed out in 600 mg samples, added to 15 mL conical tubes in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in a refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1mL aliquots and vacufuged to dryness at 45 °C in organic mode using a speed vacuum. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for cell viability assays, total phenolic content and DPPH radical scavenging capacity.

Quantification of Total Phenolics. Phenolic phytochemicals are a class of secondary metabolites that contain one or more acidic hydroxyls attached to one or more aromatic arene (phenyl) rings (Ainsworth & Gillespie, 2007). Phenolic compounds are an important class of potent antioxidants because of their ability to quench free radicals due to their acidity. In turn, phenolic compounds are rendered radicals that are able to remain relatively stable attributable to their ability to delocalize electrons, a characteristic of their benzene ring structure (Cheung, 2011).

The Folin-Ciocalteau microplate based colorimetric assay, an adapted method from Spanos and Wrolstad (1990), based on the original method of Singleton and Rossi (1965), was utilized to quantify total phenolics. The amount of sample extracts

containing phenolic and polyphenolic antioxidants required to reduce or inhibit the oxidation of phosphomolybdate-phosphotungstate acid complexes is measured in this colorimetric assay. The amount and/or intensity of blue complexes produced, measured by the amount of absorbance at 765 nm, are indicative of a strong antioxidant.

Three biological replicates of 60 $mg \cdot mL^{-1}$ whole tuber, potato peel, potato flesh and flower extracts from each clone throughout several developmental stages and after harvest/top kill treatments were reconstituted in 1 mL of 80% acetone. In a new 1.5 mL Eppendorf tube, 100 µL of reconstituted extract was diluted with 900 µL of nanopure water. In a 96 well microplate, 35 µL of each diluted sample was pipetted in triplicate replication. Utilizing a multichannel pipette, 150 µL of 0.2 M Folin-Ciocalteau reagent was pipetted into all wells. The prepared microplate was placed on a shaker, agitated for 30 seconds at 400 rpm and held for 5 minutes at room temperature. Utilizing a multichannel pipette, 115 µL of 7.5% (w/v) Na_2CO_3 was pipetted into all wells. The prepared microplate was placed on a shaker, agitated for 30 seconds at 400 rpm, incubated at 45 °C for 30 minutes and cooled to room temperature for 1 hour. The absorbance was read at 765 nm utilizing a Spectromax 640 microplate spectrophotometer. Total phenolic content was calculated by regression based on a gallic acid standard curve expressed as mg $GAE \cdot g^{-1}$ DW.

Statistical analyses of total phenolic content was based on three biological replicates from one or two harvest years (2010-2011) dependent upon clone where each Folin-Ciocalteau experiment was conducted only one time. Dependent upon the clone either three-way or four-way analyses of variance (ANOVA) was used to evaluate

the main effects of harvest/topkill treatment, developmental stages, plant component, sector color (purple vs. white or red vs. white) followed by Tukey-Kramer test to assess separation of means using SAS software.

DPPH Radical Scavenging Capacity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) decolorization microplate based assay used a method based on Brand-Williams, Cuvelier and Berset (1995) to quantify total antioxidant capacity or radical scavenging capacity. The amount of sample extract containing radical scavenging antioxidants required to reduce the DPPH radical to DPPH-H is measured in this colorimetric assay (Prakash et al., 2001). The amount of decolorization from dark purple to yellow produced, measured by the amount of absorbance at 515 nm, is indicative of a decrease in molar absorptivity of the DPPH molecule as a free radical scavenging antioxidant donates a hydrogen forming the reduced DPPH-H and is stoichiometric to the number of electrons seized (Prakash et al., 2001).

DPPH stock solution was prepared in advance by adding 7.85 mg 1,1-diphenyl-2-picrylhydrazyl (DPPH) to 1L of 100% methanol in a light protected flask, agitated on a shaker at 400 rpm for 3 hours and stored at 4 °C for no longer than 30 days. Trolox stock solution was prepared in advance by adding 12.52 mg Trolox to 50 mL of 5 mmol phosphate buffered saline (PBS) and stored at -20 °C for no longer than 30 days. Three biological replicates of 60 $mg \cdot mL^{-1}$ whole tuber, potato peel, potato flesh and flower extracts from each clone throughout several developmental stages and after harvest/top kill treatments were reconstituted in 1 mL of 5 mM PBS. In a 96 well microplate, 15 µL of each reconstituted sample was pipetted in triplicate replication. The absorbance of DPPH stock solution was adjusted to 0.90 to 0.95 absorbance units

(AU) by adding approximately 12 mL of 100% methanol to 18 mL of prepared DPPH stock solution while reading at 515 nm using a Spectromax 640 microplate spectrophotometer. Utilizing a multichannel pipette, 285 µL of adjusted DPPH stock solution was pipetted into all 96 microplate wells and held for 3 minutes at room temperature. The absorbance was read at 515 nm. Radical scavenging capacity was calculated by regression based on a Trolox standard curve expressed as µM TEAC·g^{-1} DW.

Statistical analyses of DPPH radical scavenging capacity was based on three biological replicates from one to two harvest years (2010-2011) dependent upon clone where each DPPH radical scavenging capacity experiments was conducted only one time. Dependent upon the clone either three-way or four-way analyses of variance (ANOVA) was used to evaluate the main effects of harvest/topkill treatment, developmental stages, plant component, sector color (purple vs. white or red vs. white) followed by Tukey-Kramer test to assess separation of means using SAS software.

Cell lines and Cell Cultures. Human colorectal adenocarcinoma HT-29 cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were received on dry ice, placed in a 37 °C water bath for 3 minutes and immediately transferred to a sterile 15 mL conical tube containing 9 mL of warm McCoys 5A, 1x medium supplemented with 10% fetal bovine serum under sterile conditions using a sterile transfer hood. Sterile centrifuge tubes containing cells and medium were immediately centrifuged at 125 xg for 5 - 10 minutes. Under sterile condition using a sterile transfer hood, the supernatant was removed and the cell pellet was reconstituted in 10 mL of McCoys 5A, 1x medium supplemented with 10% fetal

bovine serum. Contents were transferred to a sterile 75 cm^2 cell culture flask with a vent cap where an additional 5 mL McCoys 5A, 1x medium was added to bring total flask contents up to a total of 15 mL. Flasks were incubated at 37 °C in a humidified 5% CO_2 incubator and allowed to grow to 80% confluence until cells needed to be subcultured.

First generation cells received from ATCC were grown in flasks for several days and then prepared for cryogenic preservation for future use utilizing the subsequent method. Sterile cryogenic vials comprising 2 x 10^5 - 5 x 10^6 cells·1 mL^{-1} ampule in McCoys 5A, 1x medium containing 10% fetal bovine serum and 5% DMSO were transferred inside a 1-inch foam-insulated box and kept at - 80 °C for 12 hours and stored in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Cells used in the execution of all cell culture experiments were maintained in the incubator for no more than five generations before first generation cells were retrieved from cryopreservation storage in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Microculture Tetrazolium Assay: Cell Seeding. All methods to follow were conducted using sterile materials and under sterile conditions using a sterile transfer hood. HT-29 colon cells were seeded into a 96 well plate at a rate of 5 x 10^4 cells in 50 µl per well and incubated at 37 °C in a humidified, 5% CO_2 incubator chamber for 24 hours to allow for cells to adhere to the plates. Trypan blue was used in a 1:1 ratio to distinguish viable cells from nonviable cells. A hemacytometer and inverted microscope were used to facilitate the cell count during this initial seeding step. It should be noted

that no wells on the outer perimeter of the microplate were seeded with cells in order to minimize any variation that may occur in these wells.

Microculture Tetrazolium Assay: Potato/Plant Component Extract Treatment. Three biological replicates of 60 $mg \cdot mL^{-1}$ whole tuber, potato peel, potato flesh and flower extracts from each clone throughout several developmental stages and after harvest/top kill treatments were reconstituted in 1 mL of aqueous McCoys 5A, 1x medium containing 10% fetal bovine. Reconstituted potato extracts were filter sterilized using sterile 25 mm syringe filters w/ 0.2 µm cellulose acetate membrane and 3 mL sterile syringes and transferred into sterile 1.5 mL Eppendorf tubes. Dependent upon plant component, serial dilutions of plant component extract concentrations ranging from 0.9 to 28.8 $mg \cdot mL^{-1}$ were made out of each of the 60 $mg \cdot mL^{-1}$ plant component extracts reconstituted in cell culture medium in a 96 well plate. Dependent upon plant component extract, individual wells containing seeded cells were treated with 50 µL of each extract concentration ranging from 0.9 to 28.8 $mg \cdot mL^{-1}$ cell culture medium in triplicate replication to achieve a final respective extract concentration ranging from 0.5 to 14.4 $mg \cdot mL^{-1}$ cell culture medium. All treated microplates were incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for potential inhibitory interactions to occur.

Microculture Tetrazolium Assay: Control Treatment. Nine individual wells containing seeded cells on each microplate were each treated with 50 µL of McCoys 5A, 1x medium containing 10% fetal bovine serum and incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber. Untreated cells were designated as

negative controls for calculating percent growth inhibition of treated cells residing on the same microplate.

Microculture Tetrazolium Assay: Cell Viability Assay. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega is a colorimetric assay that was used to measure the number of viable cells in all cell culture assays. The principal source of this assay involves the use of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is a water soluble salt in combination with (phenazine methosulfate; PMS) an electron coupling agent reagent. Dehydrogenase enzymes present in metabolically active cells cleave the tetrazolium ring and convert MTS into a colored formazan product that is soluble in tissue culture medium. The amount of formazan product produced, measured by the amount of absorbance at 490 nm, is directly proportional to the number of viable cells in culture. Exactly 24 hours after cells were treated with potato extracts, 20 µL of the prepared MTS/PMS solution was added to each well and incubated for 1 hour at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for dehydrogenase enzymes in any viable cells to convert MTS to formazan. The absorbance was read at 490 nm using a Spectromax 640 microplate spectrophotometer.

Microculture Tetrazolium Assay Quantification and Statistical Methods Based on % Growth Inhibition. Absorbance of the treated cells was compared to the absorbance of the untreated control cells from each individual plate and % growth inhibition was calculated using the following calculation (((control-treated) / control) x

100). Standard deviations and coefficients of variations were calculated among triplicate replications. Commonly, coefficients of variations were below 5%.

Statistical analyses conducted on results expressed in terms of % growth inhibition for all extract treatments was based on three biological replicates from one or two harvest years dependent upon clone where each microculture tetrazolium experiment was conducted only one time.

Ratio transformations of (treatment/control) were used in the statistical analysis to stabilize normal distributions and equal variance. Dependent upon the clone either five-way or six-way analyses of variance (ANOVA) was used to evaluate the main effects of year, plant component, harvest/topkill treatment, developmental stage, extract concentration and sector flesh color (purple vs. white or red vs. white) followed by Tukey-Kramer test to assess separation of means using SAS software.

Pearson Correlation: Cancer Inhibition & Antioxidants. Side by side biological replicate Pearson correlations of ratio transformed % growth inhibition data were computed alongside of gallic acid equivalents of total phenolic data obtained from Folin Ciocalteu assay and DPPH Trolox equivalents antioxidant capacity (DPPH/TEAC) obtained from DPPH assay both based on a dry weight basis utilizing SAS software.

RESULTS

Total phenolic (TP) levels and DPPH Trolox equivalents antioxidant capacity (DPPH/TEAC) present in aqueous extracts of freeze dried whole tuber, peel and flesh extracts from different sources of germplasm (cultivars and selections with pigmented

versus white tissue from whole tubers and from pigmented sectors), from several developmental stages, and after harvest/top kill treatments revealed differences in total phenolic content and DPPH radical scavenging capacity (main effects) (Table 5.3, 5.4).

While not all aqueous extracts from the seven selections and cultivars tested inhibited HT-29 colon cancer cell cultures, several factors (main effects) significantly inhibited HT-29 colon cancer cell cultures (Table 5.5). Five factors affected inhibitory activity of HT-29 cancer cells in culture: germplasm source (cultivars and selections with pigmented versus white tissue from whole tubers and from pigmented sectors); stage of tuber development (immature tubers versus intermediate tubers versus mature tubers); extract concentrations ($mg \cdot mL^{-1}$ cell culture medium); harvest/top kill treatments (tubers harvested after no treatment, vine tops cut to 10 cm in height and chemical removal (95% sulfuric acid and Reglone) of vine top); and plant component, (peel versus flesh versus whole tuber versus flower) (Table 5.5).

Table 5.3 Summary of statistical significance (main effects) based on ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts of potato and/or plant component from seven cultivars and selections. Immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops. These clones included russet, red and purple skins with white, red, purple solid and sectored flesh tissues. Data are not available for n/a because not tested. Data are not significant for n/s at P=0.05.

Source of Variation	Sector	Harvest/Top Kill Treatment	Plant Component	Developmental Stage
RUSSET NUGGET	n/a	n/s	***	n/s
MOUNTAIN ROSE	n/a	***	***	***
CO97226-2R/R	n/a	**	***	***
CO04058-3RW/RW	***	*	***	***
CO97216-3P/PW	***	n/s	n/a	***
CO97216-1P/P	n/a	n/s	*	**
PURPLE MAJESTY	n/a	n/s	**	**

ANOVA = analysis of variance.

Significance level denoted as *.05, **.01, ***.001.

See Appendix D.8-D.14 for degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Table 5.4 Summary of statistical significance (main effects) based on ANOVA for Trolox equivalent antioxidant capacity of aqueous extracts of potato and/or plant component from seven cultivars and selections. Immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height and chemical (95% sulfuric acid and Reglone) removal of vine tops. These clones included russet, red and purple skin with white, red, purple solid and sectored flesh tissues. Data are not available for n/a because not tested. Data are not significant for n/s at P=0.05.

Source of Variation	Sector	Harvest/Top Kill Treatment	Plant Component	Developmental Stage
RUSSET NUGGET	n/a	***	***	**
MOUNTAIN ROSE	n/a	*	**	*
CO97226-2R/R	n/a	***	n/s	**
CO04058-3RW/RW	***	n/s	**	n/s
CO97216-3P/PW	***	***	n/a	n/s
CO97216-1P/P	n/a	***	**	n/s
PURPLE MAJESTY	n/a	***	n/s	**

ANOVA = analysis of variance.

Significance level denoted as *.05, **.01, ***.001.

See Appendix D.15-D.21 for degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Table 5.5 Summary of statistical significance (main effects) based on ANOVA for % growth inhibition of HT-29 colon cancer cells in culture by aqueous potato and/or plant component extract concentrations ($mg \cdot mL^{-1}$ cell culture medium) from seven cultivars and selections. Immature, intermediate and mature raw tuber samples and plant components grown for one to two years (2010-2011) dependent upon clone and plant component were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height and chemical (95% sulfuric acid and Reglone) removal of vine tops. These clones included russet, red and purple skins with white, red, purple solid and sectored flesh tissues. Data are not available for n/a because not tested. Data are not significant for n/s at P=0.05.

Source of Variation	Year	Sector	Harvest/Top Kill Treatment	Plant Component	Developmental Stage	Extract Concentration
RUSSET NUGGET	n/a	n/a	n/s	***	n/s	***
MOUNTAIN ROSE	n/a	n/a	n/s	***	n/s	***
CO97226-2R/R	n/a	n/a	***	n/s	***	***
CO04058-3RW/RW	***	***	*	***	n/s	***
CO97216-3P/PW	n/a	n/s	n/s	n/a	n/s	n/s
CO97216-1P/P	*	n/a	*	***	***	***
PURPLE MAJESTY	n/a	n/a	n/s	***	***	***

ANOVA = analysis of variance.

Significance level denoted as *.05, **.01, ***.001.

See Appendix D.1-D.7 for degrees of freedom, mean squares, F-values and level of significance for all cultivars and selections.

Developmental Stage. Inhibitory activity practically vanished in mature d30TC tubers when compared to immature d9TC tubers of two clones, CO97226-2R/R and Purple Majesty (Table 5.6, 5.7, 5.8). Aqueous extracts from CO97226-2R/R decreased from 53.8% inhibition in immature d9TC tubers to 0.9% inhibition in mature d30TC tubers (Table 5.6, 5.7). Aqueous extracts from Purple Majesty decreased from 48.9% inhibition in immature d9TC tubers to 0.3% inhibition in mature d30TC tubers (Table 5.6, 5.8).

Developmental stage affected TP levels even more so than inhibitory activity (Table 5.6). TP levels were lower in aqueous extracts from purple and red pigmented Mountain Rose, CO97226-2R/R, CO97216-1P/P and purple sectors of CO97216-3P/PW intermediate d9NT tubers when compared to immature d1NT tubers (Table 5.6, 5.9). TP levels were lower in aqueous extracts from mature d30AT CO97226-2R/R and purple sectors of CO97216-3P/PW tubers when compared to analogous immature d9AT tubers (Table 5.6, 5.9). Aqueous extracts from purple sectors of CO97216-3P/PW mature d30TC tubers also were lower in TP levels when compared to immature d9TC tubers (Table 5.6, 5.9).

DPPH radical scavenging capacity was less affected by developmental stage than TP levels (Table 5.6). Aqueous extracts from CO97226-2R/R immature d9TC tubers were lower in DPPH/TEAC levels when compared to mature d30TC tubers; a similar response was also observed with regards to cancer inhibition (Table 5.6, 5.7, 5.10). Aqueous extracts from intermediate d9NT Purple Majesty tubers had lower DPPH/TEAC levels when compared to immature d1NT tubers (Table 5.6, 5.10).

Table 5.6 Summary of statistical significance at P=0.05 based on Tukey-Kramer HSD for differences that exist between developmental stages within whole tubers (median slices of peel and flesh) of a particular clone subjected to several harvest/top kill treatments with regards to: % growth inhibition of HT-29 colon cancer cells at the same extract concentration (mg·mL^{-1} cell culture medium) after 24 hours; TP levels; and DPPH/TEAC levels. Data are based on three biological replicates for one harvest year (2010). Data are not significant for (-) at P=0.05. Data are not available for n/a because not tested.

Cultivars and Selections	Cancer Inhibition (%) Developmental Stage$^{\text{Harvest/Top Kill Treatment}}$ (Extract Concentration)	Total Phenolic Content (mg GAE·g^{-1} DW) Developmental Stage$^{\text{Harvest/Top Kill Treatment}}$	DPPH Radical Scavenging Capacity(µmole TEAC·g^{-1} DW) Developmental Stage$^{\text{Harvest/Top Kill Treatment}}$
Significant change in % cancer inhibition, total phenolic content or DPPH radical scavenging capacity with increasing maturity in whole tubers			
Russet Nugget	-	-	-
Mountain Rose	-	$I^{NT} > II^{NT}$	-
CO97226-2R/R	$I^{TC} > III^{TC}$ (7.2 mg·mL^{-1} cell culture medium)	$I^{NT} > II^{NT}$; $I^{A} > III^{A}$	$I^{TC} > III^{TC}$
CO04058-3RW/RW Red Sector	-	n/a	-
CO04058-3RW/RW White Sector	-	n/a	-
CO97216-1P/P	-	$I^{NT} > II^{NT}$	-
Purple Majesty	$I^{TC} > III^{TC}$ (14.4 mg·mL^{-1} cell culture medium)	-	$I^{NT} > II^{NT}$
CO97216-3P/PW Purple Sector	-	$I^{NT} > II^{NT}$; $I^{A} > III^{A}$; $I^{TC} > III^{TC}$	-
CO97216-3P/PW White Sector	-	-	-

I = Immature developmental stage. II = Intermediate developmental stage. III = Mature developmental stage. ^{TC} = Tops cut. ^{NT} = No treatment. ^{A} = Acid treated.

Table 5.7 % Growth inhibition of HT-29 colon cancer cells by CO97226-2R/R extracts ($mg \cdot mL^{-1}$ cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested for one year of harvest (2011). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey-Kramer HSD analysis. Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested.

Extract Concentration	Immature		Intermediate		Mature	
[$mg \cdot mL^{-1}$ cell culture medium]	2011 (n)	*w*	2011 (n)	*w*	2011 (n)	*w*
No Treatment (Peel)						
0.5	0.0	a	n/a	n/a	n/a	n/a
0.9	8.5	a b c	n/a	n/a	n/a	n/a
1.8	56.7	h	n/a	n/a	n/a	n/a
3.6	47.2	f g h	n/a	n/a	n/a	n/a
7.2	17.2	a b c d e	n/a	n/a	n/a	n/a
14.4	0.0	a	n/a	n/a	n/a	n/a
No Treatment (Flesh)						
3.6	3.9	a b	n/a	n/a	n/a	n/a
7.2	27.2	a b c d e f g	n/a	n/a	n/a	n/a
14.4	23.0	a b c d e f	n/a	n/a	n/a	n/a
No Treatment (Whole Tuber)						
3.6	10.2	a b c	12.9	a b c	n/a	n/a
7.2	31.5	b c d e f g h	48.2	d e f g h	n/a	n/a
14.4	15.9	a b c d	28.9	b c d e f g h	n/a	n/a
Tops Cut (Whole Tuber)						
3.6	22.9	a b c d e f	n/a	n/a	0	a
7.2	53.8	g h	n/a	n/a	0.9	a
14.4	44.0	e f g h	n/a	n/a	18.4	a b c d e
Acid Treated (Whole Tuber)						
3.6	0	a	n/a	n/a	0	a
7.2	15.8	a b c d	n/a	n/a	28.9	b c d e f g h
14.4	29.0	b c d e f g h	n/a	n/a	25.9	a b c d e f
Reglone Treated (Whole Tuber)						
3.6	n/a	n/a	n/a	n/a	0	a
7.2	n/a	n/a	n/a	n/a	34.1	c d e f g h
14.4	n/a	n/a	n/a	n/a	42.1	d e f g h

Table 5.8 % Growth inhibition of HT-29 colon cancer cells by Purple Majesty extracts ($mg \cdot mL^{-1}$ cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested for one year of harvest (2011). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey-Kramer HSD analysis. Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested.

Extract Concentration [$mg \cdot mL^{-1}$ cell culture medium]	Immature		Intermediate		Mature	
	2011 (n)	*w*	2011 (n)	*w*	2011 (n)	*w*
No Treatment (Peel)						
0.5	0	a	n/a	n/a	n/a	n/a
0.9	22.2	a b c d	n/a	n/a	n/a	n/a
1.8	55.5	d e	n/a	n/a	n/a	n/a
3.6	62.6	e	n/a	n/a	n/a	n/a
7.2	38.9	b c d e	n/a	n/a	n/a	n/a
14.4	0	a	n/a	n/a	n/a	n/a
No Treatment (Flesh)						
3.6	0	a	n/a	n/a	n/a	n/a
7.2	0	a	n/a	n/a	n/a	n/a
14.4	15.2	a b c	n/a	n/a	n/a	n/a
No Treatment (Whole Tuber)						
3.6	0	a	0	a	n/a	n/a
7.2	12.9	a b	0	a	n/a	n/a
14.4	25.9	a b c d	22.9	a b c d	n/a	n/a
Tops Cut (Whole Tuber)						
3.6	0	a	n/a	n/a	0	a
7.2	0	a	n/a	n/a	0	a
14.4	48.9	c d e	n/a	n/a	0.3	a
Acid Treated (Whole Tuber)						
3.6	17.0	a b	n/a	n/a	0	a
7.2	13.3	a b c	n/a	n/a	0	a
14.4	28.0	a b c d e	n/a	n/a	0.9	a
Reglone Treated (Whole Tuber)						
3.6	n/a	n/a	n/a	n/a	0	a
7.2	n/a	n/a	n/a	n/a	0	a
14.4	n/a	n/a	n/a	n/a	4.7	a b

Table 5.9 Separation of means based on Tukey-Kramer HSD for gallic acid equivalents of total phenolic content for seven clones. Data are based on three biological replicates for one harvest year (2011). Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested.

Harvest/Top Kill Treatment (Plant Component)	CO97216-1P/P mg GAE·g^{-1} DW	*w*	PURPLE MAJESTY mg GAE·g^{-1} DW	*w*	CO97216-3P/PW PURPLE mg GAE·g^{-1} DW	*w*	CO97216-3P/PW WHITE mg GAE·g^{-1} DW	*w*	RUSSET NUGGET mg GAE·g^{-1} DW	*w*	CO97226-2R/R mg GAE·g^{-1} DW	*w*	MOUTAIN ROSE mg GAE·g^{-1} DW	*w*	CO04058-3RW/RW RED mg GAE·g^{-1} DW	*w*	CO04058-3RW/RW WHITE mg GAE·g^{-1} DW	*w*
Stage I (Immature)																		
No Treatment (Peel)	3.66 (0.95) (2)	a	2.81 (0.81)	a	n/a	n/a	n/a	n/a	1.81 (0.42)	a	4.43 (0.74)	a	2.26 (0.38)	a	1.60 (0.76)	b c	n/a	n/a
No Treatment (Flesh)	2.90 (0.72)	a b	1.38 (0.88)	b	n/a	n/a	n/a	n/a	0.96 (0.02)	b	2.55 (0.42)	b	1.15 (0) (1)	b c d	3.78 (0.77)	a	n/a	n/a
No Treatment Whole Tuber)	2.51 (0.43)	a b c	1.96 (0.40)	a b	1.91 (0.20)	a	1.1 (0.10) (2)	b	0.67 (0.11)	b	2.27 (0.41)	b c	1.49 (0.14)	b	1.64 (0.14)	b c	0.90 (0.15)	c
Tops Cut (Whole Tuber)	1.55 (0.15)	c d	1.49 (0.13)	b	1.98 (0.05)	a	n/a	n/a	0.98 (0.58)	b	1.17 (0.09)	d	0.70 (0.06)	d	1.47 (0.55)	b c	0.73 (0.05)	c
Acid Treated (Whole Tuber)	2.10 (0.33)	b c d	1.46 (0.41)	b	2.20 (0.07)	a	0.88 (0.40) (2)	b	0.73 (0.03)	b	2.42 (0.15)	b	1.28 (0.18)	b c	2.68 (0.59)	a b	0.81 (0.11)	c
Stage II (Intermediate)																		
No Treatment (Whole Tuber)	1.28 (0.09)	d	0.87 (0.07)	b	1.16 (0.13)	b	0.57 (0.00) (1)	b	0.46 (0.03)	b	1.12 (0.17)	d	0.67 (0.11)	d	n/a	n/a	n/a	n/a
Stage III (Mature)																		
Tops Cut (Whole Tuber)	1.54 (0.23)	c d	0.87 (0.02)	b	0.95 (0.17)	b	0.60 (0.01) (2)	b	0.57 (0.02)	b	1.21 (0.20)	d	0.73 (0.04)	d	n/a	n/a	n/a	n/a
Acid Treated (Whole Tuber)	1.65 (0.23	c d	0.88 (0.08)	b	1.08 (0.08)	b	0.72 (0.06)	b	0.51 (0.04)	b	1.30 (0.15)	d	0.81 (0.09)	c d	n/a	n/a	n/a	n/a
Reglone Treated (Whole Tuber)	1.39 (0.06)	c d	0.85 (0.06)	b	1.02 (0.15)	b	0.71 (0.10) (2)	b	0.48 (0.04)	b	1.32 (0.07)	c d	0.85 (0.02)	c d	0.78 (0.18)	c	0.60 (0.09)	c

Table 5.10 Separation of means based on Tukey-Kramer HSD for DPPH radical scavenging capacity for seven clones. Data are based on three biological replicates for one harvest year (2011). Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested.

Harvest/Top Kill Treatment (Plant Component)	CO97216-1P/P µmole TEAC ·g^{-1} DW	w	PURPLE MAJESTY µmole TEAC ·g^{-1} DW	w	CO97216-3P/PW PURPLE µmole TEAC ·g^{-1} DW	w	CO97216-3P/PW WHITE µmole TEAC ·g^{-1} DW	w	RUSSET NUGGET µmole TEAC ·g^{-1} DW	w	CO97226-2R/R µmole TEAC ·g^{-1} DW	w	MOUTAIN ROSE µmole TEAC ·g^{-1} DW	w	CO04058-3RW/RW RED µmole TEAC ·g^{-1} DW	w	CO04058-3RW/RW WHITE µmole TEAC ·g^{-1} DW	w
Stage I (Immature)																		
No Treatment (Peel)	13.13 (1.28)	a b c	17.82 (0.27)	a	n/a	n/a	n/a	n/a	16.04 (0.55)	a	17.79 (0.18)	a	17.97 (1.31)	a	10.98 (6.34)	b c d	n/a	n/a
No Treatment (Flesh)	14.10 (1.33) (2)	a b	17.13 (0.19)	a b c	n/a	n/a	n/a	n/a	8.69 (1.11)	b	16.59 (0.96)	a	11.91 (0) (1)	a b	18.57 (0.11)	a	n/a	n/a
No Treatment Whole Tuber)	15.47 (0.73)	a	17.35 (0.09)	a b	15.22 (0.71)	a	8.32 (0.16) (2)	c d e	4.97 (0.18)	c d	16.90 (0.99)	a	13.78 (1.51)	a b	14.27 (1.44)	a b	4.83 (0.90)	d
Tops Cut (Whole Tuber)	11.92 (0.29)	c	15.87 (0.27)	c d	12.73 (0.80)	a b c	n/a	n/a	5.19 (0.24)	c d	12.16 (1.79)	b	9.27 (2.22)	b	12.27 (0.53)	a b c	5.28 (1.28)	d
Acid Treated (Whole Tuber)	13.04 (0.76)	b c	15.55 (0.66)	d	14.15 (0.74)	a b	10.22 (2.76) (2)	b c d	4.95 (0.87)	c d	16.01 (1.20)	a	10.44 (0.95)	b	15.90 (0.12)	a b	6.62 (0.74)	c d
Stage II (Intermediate)																		
No Treatment (Whole Tuber)	14.53 (0.23)	a b	15.68 (0.66)	c d	13.80 (0.36)	a b	6.48 (0.00) (1)	d e	4.72 (0.81)	c d d	15.97 (0.79)	a	11.12 (3.11)	b	n/a	n/a	n/a	n/a
Stage III (Mature)																		
Tops Cut (Whole Tuber)	13.06 (0.92)	b c	16.00 (0.10)	b c d	12.77 (1.22)	a b c	4.67 (0.06) (2)	e	6.15 (0.20)	c	15.42 (0.36)	a	11.51 (0.58)	b	n/a	n/a	n/a	n/a
Acid Treated (Whole Tuber)	13.36 (0.18)	a b c	15.42 (0.60)	d	16.22 (0.21)	a	9.06 (2.84)	c d e	6.54 (0.63)	c	16.46 (0.07)	a	12.22 (0.58)	a b	n/a	n/a	n/a	n/a
Reglone Treated (Whole Tuber)	14.77 (0.15)	a b	13.44 (0.95)	e	14.52 (1.05)	a b	7.94 (1.47) (2)	d e	3.85 (0.75)	d	15.94 (0.19)	a	10.88 (1.09)	b	12.24 (1.04)	b c	6.07 (0.90)	c d

Harvest /Top Kill Treatments. Harvest/top kill treatments affected inhibitory activity differently in cultivars and selections within a developmental stage, but only in strongly inhibitory clones (> 40% inhibition). Strong inhibitory activity was detected in 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium aqueous extracts of immature d9TC CO97226-2R/R tubers and decreased by more than half in both immature d1NT and d9AT CO97226-2R/R tubers (Table 5.7, 5.11). Conversely, 7.2 $mg \cdot mL^{-1}$ cell culture medium aqueous extract concentrations from intermediate d9NT, mature d30AT and mature d30RT CO97226-2R/R tubers inhibited higher than mature d30TC CO97226-2R/R tubers (Table 5.7, 5.11). Inhibitory activity was lower in aqueous extracts from mature d30AT CO97216-1P/P tubers when compared to mature d30RT tubers at a potato extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.11, 5.12).

TP levels were higher in red pigmented immature d1NT Mountain Rose and CO97226-2R/R tubers when compared to immature d9TC tubers (Table 5.9, 5.11).

DPPH/TEAC levels were higher in immature d1NT CO97226-2R/R, CO97216-1P/P and Purple Majesty when compared to immature d9TC tubers (Table 5.10, 5.11). DPPH/TEAC levels were also higher in purple pigmented immature d1NT CO97216-1P/P and Purple Majesty tubers when compared to immature d9AT tubers (Table 5.10, 5.11). Mature d30TC Russet Nugget tubers contained higher levels of DPPH/TEAC levels when compared to both mature d30AT and mature d30RT tubers (Table 5.10, 5.11). DPPH/TEAC levels assessed in mature d30RT Purple Majesty tubers were inferior to all other harvest/top kill treatments examined (Table 5.10, 5.11).

Table 5.11 Summary of statistical significance at P=0.05 based on Tukey-Kramer HSD for differences that exist between harvest/top kill treatment of whole tubers (median slices of peel and flesh) within a developmental stage with regards to: % growth inhibition of HT-29 colon cancer cells after 24 hours by a particular clone at the same potato extract concentration (mg·mL^{-1} cell culture medium); TP levels; and DPPH/TEAC levels. Data are based on three biological replicates for one harvest year (2011). Data (main effects) are not significant for (-) based on analysis of variance (ANOVA) at P=0.05.

	Cancer Inhibition (%)		Total Phenolic Content (mg GAE·g^{-1} DW)		DPPH Radical Scavenging Capacity (µmole TEAC·g^{-1} DW)	
Cultivars and Selections	**Harvest/Top Kill Treatment (Immature)**	**Harvest/Top Kill Treatment (Intermediate & Mature)**	**Harvest/Top Kill Treatment (Immature)**	**Harvest/Top Kill Treatment (Intermediate & Mature)**	**Harvest/Top Kill Treatment (Immature)**	**Harvest/Top Kill Treatment (Intermediate & Mature)**
Significant change in % cancer growth inhibition, total phenolic content or DPPH radical scavenging capacity within a developmental stage and extract concentration (where applicable) due to treatment						
Russet Nugget	-	-	-	-	-	TC > R; TC > A
Mountain Rose	-	-	NT > TC	-	-	-
CO97226-2R/R	$TC^{14.4} > NT^{14.4}$; $TC^{7.2} > A^{7.2}$	$NT^{7.2} > TC^{7.2}$; $A^{7.2} > TC^{7.2}$; $R^{7.2} > TC^{7.2}$	NT > TC	-	NT > TC	-
CO04058-3RW/RW Red Sector	-	-	-	-	-	-
CO04058-3RW/RW White Sector	-	-	-	-	-	-
CO97216-1P/P	-	$R^{14.4} > A^{14.4}$	-	-	NT > TC; NT > A	-
Purple Majesty	-	-	-	-	NT > TC; NT > A	NT > R; A > R; TC > R
CO97216-3P/PW Purple Sector	-	-	-	-	-	-
CO97216-3P/PW White Sector	-	-	-	-	-	-

TC = Tops cut. NT = No treatment. A = Acid treated. R = Reglone treated.

Values denoted in superscript following harvest/top kill treatments indicate potato extract concentration (mg·mL^{-1} cell culture medium) at which significant differences in harvest/topkill treatments were observed for cancer inhibition data.

Table 5.12 % Growth inhibition of HT-29 colon cancer cells by CO97216-1P/P extracts ($mg \cdot mL^{-1}$ cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested for one year of harvest (2011). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey-Kramer HSD analysis. Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested. Table does not represent all significant comparisons.

Extract Concentration	Immature					Intermediate		Mature	
[$mg \cdot mL^{-1}$ cell culture medium]	2010 (n)	*w*	2011 (n)	*w*	Mean	2011 (n)	*w*	2011 (n)	*w*
No Treatment (Flower)									
0.5	n/a	n/a	71.5	d	71.5	n/a	n/a	n/a	n/a
0.9	n/a	n/a	61.1	c d	61.1	n/a	n/a	n/a	n/a
1.8	n/a	n/a	41.6	a b c d	41.6	n/a	n/a	n/a	n/a
3.6	41.50	a b c d	7.7	a b	24.6	n/a	n/a	n/a	n/a
7.2	8.54	a b	0	a	4.3	n/a	n/a	n/a	n/a
14.4	0	a	0	a	0.0	n/a	n/a	n/a	n/a
No Treatment (Peel)									
0.5	n/a	n/a	0	a	0.0	n/a	n/a	n/a	n/a
0.9	n/a	n/a	24.4	a b c	24.4	n/a	n/a	n/a	n/a
1.8	n/a	n/a	53.2	c d	53.2	n/a	n/a	n/a	n/a
3.6	n/a	n/a	45.5	b c d	45.5	n/a	n/a	n/a	n/a
7.2	n/a	n/a	10.1	a b	10.1	n/a	n/a	n/a	n/a
14.4	n/a	n/a	0	a	0.0	n/a	n/a	n/a	n/a
No Treatment (Flesh)									
3.6	n/a	n/a	70.4	d	70.4	n/a	n/a	n/a	n/a
7.2	n/a	n/a	68.2	d	68.2	n/a	n/a	n/a	n/a
14.4	n/a	n/a	53.5	c d	53.5	n/a	n/a	n/a	n/a
No Treatment (Whole Tuber)									
3.6	n/a	n/a	3.2	a	3.2	0	a	n/a	n/a
7.2	n/a	n/a	40.7	a b c d	40.7	4.1	a	n/a	n/a
14.4	n/a	n/a	14.0	a b	14.0	0	a	n/a	n/a
Tops Cut (Whole Tuber)									
3.6	n/a	n/a	7.0	a	7.0	n/a	n/a	0	a
7.2	n/a	n/a	31.0	a b c	31.0	n/a	n/a	7.0	a
14.4	n/a	n/a	14.3	a b	14.3	n/a	n/a	9.7	a b
Acid Treated (Whole Tuber)									
3.6	n/a	n/a	0	a	0.0	n/a	n/a	0.6	a
7.2	n/a	n/a	10.8	a b	10.8	n/a	n/a	26.0	a b c
14.4	n/a	n/a	22.1	a b c	22.1	n/a	n/a	2.6	a
Reglone Treated (Whole Tuber)									
3.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0	a
7.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	15.1	a b
14.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	32.8	a b c d

Plant Component. *S. tuberosum* flowers, potato peels, potato flesh and whole tubers obtained during an immature developmental stage, subjected to no treatments, played a vital role in inhibition of cancer cell cultures within most cultivars and selections (Table 5.13). Aqueous extracts from russet peels obtained from immature d1NT Russet Nugget tubers strongly inhibited cell cultures by 81.9, 70.3 and 50.1% at potato extract concentrations of 3.6, 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium, whereas flesh alone and whole tubers revealed near 0% inhibition (Table 5.13, 5.14). Aqueous extracts from peels obtained from immature d1NT Mountain Rose tubers also strongly inhibited cell cultures by 52.8, 71.9, 62.1 and 40.1% at potato extract concentrations of 1.8, 3.6, 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium while whole tubers revealed weak to 0% inhibition (Table 5.13, 5.15). Aqueous extracts from peels obtained from immature d1NT CO97226-2R/R tubers strongly inhibited cell cultures at a peel extract concentration as low as 1.8 $mg \cdot mL^{-1}$ cell culture medium where moderate inhibition was not detected until an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium in both flesh and whole tubers and was nearly half the activity of the peels (Table 5.13, 5.7). Aqueous extracts from peels of immature d1NT Purple Majesty tubers were superior to flesh extracts at extract concentrations of 1.8 and 3.6 $mg \cdot mL^{-1}$ cell culture medium and whole tuber extracts at 3.6 $mg \cdot mL^{-1}$ cell culture medium extract concentration (Table 5.13, 5.8).

Aqueous extracts of flowers, potato peels, potato flesh and whole tubers from immature d1NT CO04058-3RW/RW tubers and plants revealed a variety of differences with regards to cancer inhibition (Table 5.13, 5.16). In 2010 peels were superior to red flesh at extract concentrations of 3.6 and 7.2 $mg \cdot mL^{-1}$ cell culture medium inhibiting cell cultures 55.1 and 28.9% (Table 5.13, 5.16). Conversely, red flesh was superior to peels

at an extract concentration of 3.6 mg·mL^{-1} cell culture medium in 2011 inhibiting cultures 55.5% (Table 5.13, 5.16). Peels superseded red flesh inhibitory activity when extract concentrations reached as high as 14.4 mg·mL^{-1} cell culture medium in 2011 (Table 5.13, 5.16). Meanwhile, whole tubers were more effective in inhibiting cell cultures when compared to peels at extract concentrations of 3.6 and 7.2 mg·mL^{-1} cell culture medium in 2011(Table 5.13, 5.16). Flower extracts were also superior to peel extracts inhibiting cell cultures as high as 73.3 and 65.4% at extract concentrations as low as 0.5 and 0.9 mg·mL^{-1} cell culture medium (Table 5.13, 5.16).

Aqueous extracts of flowers, potato peels, potato flesh and whole tubers from immature d1NT CO97216-1P/P tubers and plants also revealed a variety of differences with regards to cancer cell culture inhibition (Table 5.13). Aqueous extracts from flesh displayed stronger inhibitory activity than peel at extract concentrations of 7.2 and 14.4 mg·mL^{-1} cell culture medium (Table 5.12, 5.13). Aqueous extracts from flesh were also more effective inhibitors of cancer cell cultures than whole tubers at extract concentrations of 3.6 and 14.4 mg·mL^{-1} cell culture medium (Table 5.12, 5.13). Flower extracts inhibited cell cultures as high as 71.5, 61.1 and 41.6% at low extract concentrations of 0.5, 0.9 and 1.8 mg·mL^{-1} cell culture medium while flesh extracts behaved similarly inhibiting cell cultures 70.4, 68.2 and 53.5% but at much higher extract concentrations of 3.6, 7.2 and 14.4 mg·mL^{-1} cell culture medium. Flower extracts were also more effective inhibitors than peel extracts at 0.5 and 0.9 mg·mL^{-1} cell culture medium (Table 5.12, 5.13).

Total phenolic content was also different among plant components. Aqueous peel extracts from immature d1NT Russet Nugget, Mountain Rose, CO97226-2R/R

tubers all had higher TP levels when compared to aqueous extracts of flesh and whole tuber (Table 5.9, 5.13). Aqueous peel extracts from immature d1NT Purple Majesty tubers behaved similarly, but had higher TP levels than aqueous extracts of flesh (Table 5.9, 5.13). Aqueous extracts from red flesh sectors of immature d1NT CO04058-3RW/RW tubers had higher TP levels when compared to aqueous extracts of peel and whole tuber (Table 5.9, 5.13).

DPPH radical scavenging capacity was less affected by plant components (Table 5.13). Peel extracts from immature d1NT Russet Nugget tubers contained higher levels of DPPH/TEAC than extracts from flesh and whole tubers (Table 5.13, 5.14). Flesh extracts from immature d1NT Russet Nugget tubers contained higher levels of DPPH/TEAC than extracts from whole tubers (Table 5.13, 5.14). Red flesh extracts from immature d1NT CO04058-3RW/RW had higher DPPH/TEAC levels than in aqueous peel extracts (Table 5.13, 5.14).

Table 5.13 Summary of statistical significance at P=0.05 based on Tukey-Kramer HSD for differences that exist between plant components (peel, flesh, whole tuber, flower) obtained from immature developmental stage tubers with regards to: % growth inhibition of HT-29 colon cancer cells after 24 hours at the same extract concentration (mg·mL^{-1} cell culture medium) of a particular clone; TP levels; and DPPH/TEAC levels. Data are based on three biological replicates for one or two harvest years (2010-2011) dependent on cultivar or selection. Data are not significant for (-) at P=0.05. Data are not available for n/a because not tested.

	Cancer Inhibition (%)	Total Phenolic Content (mg GAE·g^{-1} DW)	DPPH Radical Scavenging Capacity (µmole TEAC·g^{-1} DW)
Cultivars and Selections	**Plant Component** Extract Concentration (mg·mL-1 cell culture medium)	**Plant Component**	**Plant Component**
Significant change in % cancer inhibition, total phenolic content or DPPH radical scavenging capacity due to plant component			
Russet Nugget	$P^{3.6,\ 7.2,14.4} > F^{3.6,\ 7.2,14.4}$; $P^{3.6,\ 7.2,14.4} > W^{3.6,\ 7.2,14.4}$	P > F; P > W	P > F > W
Mountain Rose	$P^{3.6,\ 7.2,14.4} > W^{3.6,\ 7.2,14.4}$	P > F; P > W	-
CO97226-2R/R	$P^{3.6} > F^{3.6}$; $P^{3.6} > W^{3.6}$	P > F; P > W	-
CO04058-3RW/RW Red Sector	$P^{14.4} > F^{14.4}$ (2011); $P^{3.6,\ 7.2} > F^{3.6,\ 7.2}$ (2010); $P^{3.6} < F^{3.6}$ (2011); $P^{3.6,\ 7.2} < W^{3.6,\ 7.2}$ (2011); $P^{0.5,\ 0.9} < FL^{0.5,\ 0.9}$(2011)	F > P; F > W	F > P
CO04058-3RW/RW White Sector	-	n/a	n/a
CO97216-1P/P	$F^{3.6,\ 7.2,\ 14.4} > Fl^{3.6,\ 7.2,\ 14.4}$; $F^{7.2,\ 14.4} > P^{7.2,\ 14.4}$; $F^{3.6,\ 14.4} > W^{3.6,\ 14.4}$; $FL^{0.5,\ 0.9} > P^{0.5,\ 0.9}$	-	-
Purple Majesty	$P^{3.6,\ 7.2} > F^{3.6,\ 7.2}$; $P^{3.6} > W^{3.6}$	P > F	-
CO97216-3P/PW Purple Sector	n/a	n/a	n/a
CO97216-3P/PW White Sector	n/a	n/a	n/a

P = Peel. F = Flesh. W = Whole Tuber. FL = Flower.

Values denoted in superscript following plant component indicate extract concentration (mg·mL^{-1} cell culture medium) at which significant differences (P=0.05) exist between plant components for cancer inhibition data.

Table 5.14 % Growth inhibition of HT-29 colon cancer cells by Russet Nugget extracts ($mg \cdot mL^{-1}$ cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested for one year of harvest (2011). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey-Kramer HSD analysis. Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested.

Extract Concentration	Immature		Intermediate		Mature	
[$mg \cdot mL^{-1}$ cell culture medium]	2011 (n)	*w*	2011 (n)	*w*	2011 (n)	*w*
No Treatment (Peel)						
0.5	5.8	a b	n/a	n/a	n/a	n/a
0.9	4.2	a b	n/a	n/a	n/a	n/a
1.8	15.8	b	n/a	n/a	n/a	n/a
3.6	81.9	d	n/a	n/a	n/a	n/a
7.2	71.3	d	n/a	n/a	n/a	n/a
14.4	50.1	c	n/a	n/a	n/a	n/a
No Treatment (Flesh)						
3.6	0	a	n/a	n/a	n/a	n/a
7.2	1.7	a	n/a	n/a	n/a	n/a
14.4	0	a	n/a	n/a	n/a	n/a
No Treatment (Whole Tuber)						
3.6	0	a	1.26	a	n/a	n/a
7.2	0	a	0	a	n/a	n/a
14.4	0	a	0	a	n/a	n/a
Tops Cut (Whole Tuber)						
3.6	0	a	n/a	n/a	0 (2)	a
7.2	0	a	n/a	n/a	0.4 (2)	a
14.4	0	a	n/a	n/a	0 (2)	a
Acid Treated (Whole Tuber)						
3.6	3.1	a	n/a	n/a	0	a
7.2	1.3	a	n/a	n/a	0	a
14.4	0	a	n/a	n/a	0	a
Reglone Treated (Whole Tuber)						
3.6	n/a	n/a	n/a	n/a	n/a	n/a
7.2	n/a	n/a	n/a	n/a	n/a	n/a
14.4	n/a	n/a	n/a	n/a	n/a	n/a

Table 5.15 % Growth inhibition of HT-29 colon cancer cells by Mountain Rose extracts ($mg \cdot mL^{-1}$ cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested for one year of harvest (2011). % Growth inhibition data represented in this table were ratio transformed to conduct Tukey-Kramer HSD analysis. Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because note tested.

Extract Concentration [$mg \cdot mL^{-1}$ cell culture medium]	Immature		Intermediate		Mature	
	2011 (n)	*w*	2011 (n)	*w*	2011 (n)	*w*
No Treatment (Peel)						
0.5	0	a	n/a	n/a	n/a	n/a
0.9	8.7	a	n/a	n/a	n/a	n/a
1.8	52.8	b c	n/a	n/a	n/a	n/a
3.6	71.9	c	n/a	n/a	n/a	n/a
7.2	62.1	b c	n/a	n/a	n/a	n/a
14.4	40.1	b	n/a	n/a	n/a	n/a
No Treatment (Flesh)						
3.6	n/a	n/a	n/a	n/a	n/a	n/a
7.2	n/a	n/a	n/a	n/a	n/a	n/a
14.4	n/a	n/a	n/a	n/a	n/a	n/a
No Treatment (Whole Tuber)						
3.6	0	a	0	a	n/a	n/a
7.2	0	a	0	a	n/a	n/a
14.4	10.6	a	8.6	a	n/a	n/a
Tops Cut (Whole Tuber)						
3.6	0	a	n/a	n/a	0	a
7.2	0	a	n/a	n/a	0	a
14.4	2.2	a	n/a	n/a	0	a
Acid Treated (Whole Tuber)						
3.6	0	a	n/a	n/a	0	a
7.2	0	a	n/a	n/a	0	a
14.4	0	a	n/a	n/a	6.7	a
Reglone Treated (Whole Tuber)						
3.6	n/a	n/a	n/a	n/a	0	a
7.2	n/a	n/a	n/a	n/a	0	a
14.4	n/a	n/a	n/a	n/a	0	a

Table 5.16 % Growth inhibition of HT-29 colon cancer cells by red sectors of CO04058-3RW/RW extracts (mg·mL^{-1} cell culture medium) after 24 hours. Data are means of all replicates (%). Three biological replicates were tested for one to two years of harvest (2010-2011) dependent on plant component. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey-Kramer HSD analysis. Means followed by the same letter are not significantly different based on Tukey-Kramer HSD, P=0.05. Data are not available for n/a because not tested. Table does not represent all significant comparisons.

Extract Concentration	Immature					Intermediate		Mature	
[mg·mL^{-1} cell culture medium]	2010 (n)	*w*	2011 (n)	*w*	Mean	2010 (n)	*w*	2011 (n)	*w*
No Treatment (Peel)									
0.5	19.5	a b c	1.8	a b	10.6	n/a	n/a	n/a	n/a
0.9	68.1	c d	0	a b	34.1	n/a	n/a	n/a	n/a
1.8	73.7	d	0	a b	36.8	n/a	n/a	n/a	n/a
3.6	68.7	c d	0	a b	34.4	n/a	n/a	n/a	n/a
7.2	55.1	b c d	11.3	a b	33.2	n/a	n/a	n/a	n/a
14.4	28.9	a b c d	48.1	b c d	38.5	n/a	n/a	n/a	n/a
No Treatment (Flesh)									
3.6	0	a b	55.5	b c d	27.8	n/a	n/a	n/a	n/a
7.2	10.2	a b	31.8	a b c d	21.0	n/a	n/a	n/a	n/a
14.4	39.6	a b c d	0.36	a b	20.0	n/a	n/a	n/a	n/a
No Treatment (Whole Tuber)									
3.6	47.9	a b c d	41.6	a b c d	44.8	0	a b	n/a	n/a
7.2	40.5	a b c d	51.5	b c d	46.0	39.3	a b c d	n/a	n/a
14.4	0	a b	31.8	a b c d	15.9	63.6	b c d	n/a	n/a
No Treatment (Flower)									
0.5	n/a	n/a	73.3	c d	73.3	n/a	n/a	n/a	n/a
0.9	n/a	n/a	65.4	b c d	65.4	n/a	n/a	n/a	n/a
1.8	n/a	n/a	47.9	a b c d	47.9	n/a	n/a	n/a	n/a
3.6	n/a	n/a	23.6	a b c d	23.6	n/a	n/a	n/a	n/a
7.2	n/a	n/a	0	a b	0	n/a	n/a	n/a	n/a
14.4	n/a	n/a	0	a b	0	n/a	n/a	n/a	n/a
Tops Cut (Whole Tuber)									
3.6	n/a	n/a	9.5	a b	9.5	n/a	n/a	n/a	n/a
7.2	n/a	n/a	46.1	a b c d	46.1	n/a	n/a	n/a	n/a
14.4	n/a	n/a	30.9	a b c d	30.9	n/a	n/a	n/a	n/a
Acid Treated (Whole Tuber)									
3.6	n/a	n/a	49.4	b c d	49.4	n/a	n/a	n/a	n/a
7.2	n/a	n/a	35.0	a b c d	35.0	n/a	n/a	n/a	n/a
14.4	n/a	n/a	8.0	a b	8.0	n/a	n/a	n/a	n/a
Reglone Treated (Whole Tuber)									
3.6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	6.1	a b
7.2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	44.4	a b c d
14.4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	48.2	b c d

Extract Concentration. Flower extracts from CO04058-3RW/RW inhibited most strongly at the lowest extract concentration levels of 0.5, 0.9 and 1.8 vs. 3.6, 7.2 and14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.16, 5.17). Flower extracts from CO97216-1P/P in 2011 also revealed highest levels of inhibition at low extract concentrations of 0.5 and 0.9 $mg \cdot mL^{-1}$ cell culture medium compared to extract concentrations of 3.6, 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.12, 5.17).

Aqueous peel extracts were more influenced by extract concentration than aqueous extracts from flesh (Table 5.17). Immature d1NT Russet Nugget peel extracts were most effective at extract concentrations of 3.6 and 7.2 $mg \cdot mL^{-1}$ cell culture medium (Table 5.14, 5.17). Immature d1NT Mountain Rose and Purple Majesty aqueous peel extracts produced the most effective inhibitory activities at extract concentrations of 1.8, 3.6 and 7.2 $mg \cdot mL^{-1}$ cell culture medium (Table 5.8, 5.15, 5.17). Immature d1NT CO97226-2R/R and CO97216-1P/P aqueous peel extracts produced the most effective inhibitory activity at extract concentrations of 1.8 and 3.6 $mg \cdot mL^{-1}$ cell culture medium (Table 5.7, 5.12, 5.17). Immature d1NT CO04058-3RW/RW aqueous peel extracts produced the most effective inhibitory activity at extract concentrations of 0.9, 1.8 and 3.6 $mg \cdot mL^{-1}$ cell culture medium in 2010 and at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium in 2011 (5.16, 5.17). Aqueous extracts from immature d1NT red flesh of CO04058-3RW/RW in 2011 revealed that an extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium produced the strongest inhibitory activity and this selection was the only clone to have flesh components influenced by extract concentration (5.16, 5.17).

Harvest/top kill treatment influenced inhibitory activity as it relates to extract concentration primarily in aqueous extracts from CO97226-2R/R tubers (Table 5.7, 5.17). Aqueous extracts from immature d9TC CO97226-2R/R tubers were most effective at inhibiting cell cultures at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium, while immature d9AT tubers were most effective at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.7, 5.17). Aqueous extracts from immature d9TC Purple Majesty tubers were also influenced by extract concentration with higher inhibitory activity at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.8, 5.17). Aqueous extracts from intermediate d9NT and mature d30AT CO97226-2R/R tubers had the highest inhibitory activity at and extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium (Table 5.7, 5.17). Aqueous extracts from mature d30RT CO97226-2R/R tubers had the highest inhibitory activity at both extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.7, 5.17). Aqueous extracts from mature d9NT CO97216-1P/P tubers also had higher inhibitory activity at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.12, 5.17).

Table 5.17 Summary of statistical significance at P=0.05 based on Tukey-Kramer HSD for differences in % growth inhibition of HT-29 colon cancer cells after 24 hours that exist between extract concentrations (mg·mL^{-1} cell culture medium) within the same harvest/top kill treatment and developmental stage of a particular clone. Data are based on three biological replicates for one or two harvest years (2010-2011) dependent on cultivar or selection or plant component. Data are not significant for (-) at P=0.05. Data are not available for n/a because not tested.

	Stage I (Immature)						Stage II (Intermediate)	Stage III (Mature)		
	No Treatment (Flower)	No Treatment (Peel)	No Treatment (Flesh)	No Treatment (Whole Tuber)	Tops Cut	Acid Treated	No Treatment	Tops Cut	Acid Treated	Reglone Treated
Significant change in % cancer inhibition with increasing extract concentration (mg·mL^{-1} cell culture medium) within a developmental stage										
Russet Nugget	n/a	0.5, 0.9, 1.8 < 3.6, 7.2, 14.4; 14.4 < 3.6, 7.2	-	-	-	-	-	-	-	-
Mountain Rose	n/a	0.5, 0.9 < 1.8, 3.6, 7.2, 14.4; 14.4 < 3.6	-	-	-	-	-	-	-	-
CO97226-2R/R	n/a	0.5, 0.9, 7.2, 14.4 < 1.8, 3.6	-	-	3.6 < 7.2	3.6 < 14.4	3.6 < 7.2	-	3.6 < 7.2	3.6 < 7.2, 14.4
CO04058-3RW/RW	0.5 > 7.2, 14.4	0.5 < 0.9, 1.8, 3.6 (2010); 14.4 < 1.8, 3.6 (2010); 0.9, 1.8, 3.6 < 14.4 (2011)	-	-	-	-	-	-	-	-
CO04058-3RW/RW Red Sector	n/a	n/a	3.6 > 14.4	-	-	-	-	-	-	-
CO04058-3RW/RW White Sector	n/a	n/a	-	-	-	-	-	-	-	-
CO97216-1P/P	3.6 > 14.4 (2010); 3.6, 7.2, 14.4 < 0.5, 0.9 (2011)	0.5, 14.4 < 1.8, 3.6	-	-	-	-	-	-	-	3.6 < 14.4
Purple Majesty	n/a	0.5, 14.4 < 1.8, 3.6, 7.2; 0.9 < 3.6	-	-	3.6, 7.2 < 14.4	-	-	-	-	-
CO97216-3P/PW Purple Sector	-	-	-	-	-	-	-	-	-	-
CO97216-3P/PW White Sector	-	-	-	-	-	-	-	-	-	-

Pearson Correlation: Cancer Inhibition & Antioxidants. Most whole tuber clones with a statistically significant Pearson correlation coefficient were negatively correlated with cancer inhibition (Table 5.18). Russet Nugget and CO97226-2R/R had positive correlations with cancer inhibition and DPPH radical scavenging capacity (Table 5.18). CO04058-3RW/RW and CO97226-2R/R also displayed positive correlations, but with cancer inhibition and total phenolic content (Table 5.18). All cultivars and selections except CO97216-1P/P displayed relatively high positive correlations between DPPH radical scavenging capacity and total phenolic content at a high level significance (Table 5.19).

Table 5.18 Summary of statistical significance at P=0.05 based on Pearson Correlation coefficients for degree of linear association that exists in whole tuber clones between cancer inhibition activity and total antioxidant capacity based on DPPH radical scavenging capacity assay or total phenolic antioxidant content based on Folin Ciocalteu reagent assay. Correlations were computed for each clone based on extract concentration ($mg \cdot mL^{-1}$ cell culture medium) utilized in microculture tetrazolium assays. Table only represents statistically significant correlations at P=0.05 that were found to exist. Sample size = n. Correlation coefficient = r. Level of significance = p.

CULTIVARS & SELECTIONS	EXTRACT CONCENTRATION	n	r	p	DPPH	TP	CANCER INHIBITION
CO04058-3RW/RW	3.6	30	-0.774	<.0001	X		X
CO04058-3RW/RW	7.2	30	-0.580	0.0008	X		X
RUSSET NUGGET	14.4	26	-0.914	<.0001	X		X
RUSSET NUGGET	7.2	26	-0.926	<.0001	X		X
RUSSET NUGGET	3.6	26	0.911	<.0001	X		X
PURPLE MAJESTY	7.2	27	-0.497	0.0083	X		X
PURPLE MAJESTY	14.4	27	-0.463	0.0150	X		X
MOUNTAIN ROSE	14.4	24	-0.798	<.0001	X		X
MOUNTAIN ROSE	7.2	24	-0.766	<.0001	X		X
MOUNTAIN ROSE	3.6	24	-0.768	<.0001	X		X
CO97226-2R/R	14.4	27	0.584	0.0014	X		X
CO04058-3RW/RW	3.6	30	-0.795	<.0001		X	X
CO04058-3RW/RW	14.4	30	0.435	0.0162		X	X
RUSSET NUGGET	14.4	26	-0.781	<.0001		X	X
RUSSET NUGGET	7.2	26	-0.799	<.0001		X	X
RUSSET NUGGET	3.6	26	-0.798	<.0001		X	X
PURPLE MAJESTY	7.2	27	-0.712	<.0001		X	X
PURPLE MAJESTY	14.4	27	-0.684	<.0001		X	X
CO97216-1P/P	3.6	26	-0.655	0.0003		X	X
MOUNTAIN ROSE	14.4	24	-0.712	<.0001		X	X
MOUNTAIN ROSE	7.2	24	-0.803	<.0001		X	X
MOUNTAIN ROSE	3.6	24	-0.811	<.0001		X	X
CO97226-2R/R	14.4	27	0.668	0.0001		X	X
CO97226-2R/R	3.6	27	-0.652	0.0002		X	X

Table 5.19 Summary of statistical significance at P=0.05 based on Pearson Correlation for degree of linear association that exists between total antioxidant capacity based on DPPH radical scavenging capacity assay and total phenolic antioxidant content based on Folin Ciocalteu reagent assay. Correlations were computed for each clone based on extract concentration utilized in microculture tetrazolium assays. Significant correlations (P=0.05) are denoted with an (X). Nonsignificant correlations (P<0.05) are denoted with (-). Sample size = n. Correlation coefficient = r. Level of significance = p.

CULTIVARS & SELECTIONS	n	r	p	DPPH	TP
CO04058-3RW/RW	30	0.820	<.0001	X	X
CO97216-1P/P	26	-0.074	0.7190	-	-
RUSSET NUGGET	26	0.815	<.0001	X	X
PURPLE MAJESTY	27	0.627	0.0005	X	X
CO97216-3P/PW	32	0.558	0.0009	X	X
MOUNTAIN ROSE	24	0.806	<.0001	X	X
CO97226-2R/R	27	0.560	0.0024	X	X

DISCUSSION

Developmental Stage. Once more, results from this study reinforced the significance of developmental stage in attaining higher levels of DPPH radical scavenging capacity, total phenolic content and inhibition against HT-29 colon cancer cells. In every situation where significant differences were observed within developmental stage, regardless of which harvest/top kill treatment was utilized, the same pattern was observed where higher DPPH/TEAC, TP and inhibition levels occurred in immature tubers compared to tubers with increasing maturity (Table 5.6). Thus, it seems critical to harvest tubers at an immature developmental stage to achieve maximum levels of beneficial metabolites.

While it is clear that the earliest developmental stages provide tubers with the highest inhibitory bioactivity, it must also be recognized that modern potato production practices in the San Luis Valley attempt to control harvest date by employing top kill practices to induce tuber maturity. Thus the true mature stage may vary from year to year as a function of when growers apply top kill treatments to ensure harvest in a timely manner before frost killing temperatures ensue. Accordingly, the impact of maturity becomes more difficult to pin point than identifying an active inhibitory phase in immature and intermediate maturity tubers.

Harvest/Top Kill Treatments. A further objective of this study was to assess the role vine kill treatments utilized prior to harvest may have on inhibitory activity, DPPH /TEAC and TP levels in tubers. Total phenolic antioxidants and DPPH radical scavenging antioxidants are best conserved in immature tubers utilizing approaches that consist of no treatment rather than top cutting and acid treatments (Table 5.11).

Harvest/top kill treatments played a much more minor role in conserving phenolic content and radical scavenging antioxidants in mature tubers whereas time of harvest seems to be more critical as mentioned earlier (Table 5.6, 5.11). Despite that, utilizing no treatments or top cutting was more favorable for conserving these valuable metabolites in mature tubers rather than application of chemical desiccants, sulfuric acid and Reglone (Table 5.11).

Of the seven clones examined only strong inhibitory clones CO97216-1P/P and CO97226-2R/R responded significantly to harvest/top kill treatments in immature tubers (Table 5.7, 5.11, 5.12). Acid treated mature CO97216-1P/P declined in inhibitory activity when compared to Reglone (Table 5.11, 5.12). Thus, Reglone top kill treatments may be more favorable in an attempt to maintain cancer cell culture inhibitory activity that may exist within certain cultivars and selections.

Acid treated immature tubers and non treated immature tubers of CO97226-2R/R declined in inhibitory activity, but only when compared to top cut vines of immature plants (Table 5.7, 5.11). An unexpected outcome observed within this selection was increased inhibitory activity in top cut immature tubers but a decrease in inhibitory activity in top cut mature tubers (Table 5.7, 5.11). TP and DPPH/TEAC levels in immature tubers of CO97226-2R/R were lower in top cut immature tubers than that of untreated immature plants (Table 5.7, 5.11). However, DPPH/TEAC levels were higher in top cut immature plants when compared to top cut mature plants in CO97226-2R/R tubers (Table 5.6, 5.7). Is it possible that DPPH radical scavenging antioxidants may be involved in inhibitory responses? Also, this finding might also suggest that it may be critical with certain cultivars and selections to shear vine tops prematurely as an attempt

to induce upregulation of key inhibitory metabolites found only at the outset of tuber initiation and early development.

Plant Component. A key finding achieved in this study is the importance of plant components during early development (Table 5.13). Consumption and/or utilization of appropriate plant components are vital in attaining valuable inhibitory metabolites and antioxidants important for the maintenance of human health.

Aqueous flower extracts proved to be an exceptional source of cancer cell culture inhibitory metabolites (Table 5.13). Flower extracts demonstrated some of the strongest inhibitory activity compared to other plant components where increasing inhibition was found with decreasing extract concentrations as low as 0.5 $mg \cdot mL^{-1}$ cell culture medium (Table 5.12, 5.13, 5.16).

Aqueous peel extracts were also a potent source of cancer cell culture inhibitory metabolites. Cultivars, such as Mountain Rose, Purple Majesty and Russet Nugget, that typically display nil or weak inhibitory responses to cancer cell cultures when utilizing extracts from whole tubers and/or flesh revealed strong inhibitory activity in extracts from peels (Table 5.8, 5.13, 5.14, 5.15). Clones such as CO04058-3RW/RW and CO97226-2R/R that typically display strong inhibitory activity against cancer cell cultures at higher extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium from whole tuber extracts demonstrated strong inhibitory activity at extract concentrations as low as 0.9 and 1.8 $mg \cdot mL^{-1}$ cell culture medium with some year to year variation that existed in CO04058-3RW/W which may be attributable to sectorial expression of pigments that exist in peels of this cultivar (Table 5.7, 5.13, 5.16).

This response observed in both peel and flower extracts may be attributable, at least partially to glycoalkaloids, which are secondary metabolites that exist in the tubers, leaves, roots and sprouts of potato that have been reported to inhibit proliferation of several cancer cell lines in culture (Friedman, 2006). Glycoalkaloids are composed of a trisaccharide chain attached to an aglycon (Friedman, 2006). The prominent glycoalkaloids that exist in cultivated potato are α-chaconine and α-solanine (Friedman, 2006). The initial concentration and configuration of these prominent glycoalkaloids in potato is largely dependent on genotype, maturity, stress and other environmental factors (Davis, 2011; Friedman, 2006). These glycoalkaloids are mostly positioned around the eyes located on the outer layer of the tuber and within the first 1 mm from the exterior surface of the tuber decreasing toward the interior of the tuber as well as metabolically active areas of the plant such as flowers, young leaves and small immature tubers (Davis, 2011;Friedman, 2006).

One selection, which behaved dissimilarly, was CO97216-1P/P, a known strong inhibitor of cancer cell cultures (Table 5.12, 5.13). Aqueous extracts from flesh only deemed to be superior to aqueous flower, peel and whole tuber extracts at extract concentrations ranging between 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.12, 5.13). This may suggest that another class of inhibitory metabolites is responsible for inhibition observed in this cultivar since glycoalkaloids have been reported to exist primarily on the exterior surface of tubers. However, this selection may be different with higher levels in the flesh, but this needs confirmation.

Most cultivars affected displayed higher levels of TP and DPPH/TEACH in extracts obtained from potato peels when compared to potato flesh and whole tuber

extracts (Table 5.9, 5.10, 5.13). Although red sectors of CO04058-3RW displayed the opposite effect rendering flesh a better source of TP and DPPH/TEAC levels (Table 5.9, 5.10, 5.13).

Extract Concentration. Effective inhibition of cancer cell culture proliferation was dependent upon extract concentration. As mentioned earlier, flowers inhibited cell cultures best at extract concentrations between 0.5 and 3.6 $mg \cdot mL^{-1}$ cell culture medium (Table 5.13). Peel extracts obtained from known strong inhibitory clones inhibited cell cultures best at extract concentrations ranging between 0.9 and 3.6 $mg \cdot mL^{-1}$ cell culture medium (Table 5.13). Peel extracts obtained from known clones that typically produces nil to weak inhibitory activity, inhibited cell cultures best at extract concentrations ranging between 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.13). Regardless of harvest/top kill treatment, both immature tubers and mature tubers inhibited cancer cell cultures best at 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 5.11). Thus, this study may set the extract concentration bench mark parameters for future screening of potato plant components.

Antioxidant Properties and Cell Culture Inhibition. If antioxidant content as in the case of TP and/or antioxidant capacity to disarm free radicals as measured by DPPH/TEAC were important contributors to HT-29 cancer cell culture inhibition, we would expect inhibition to increase with higher antioxidant values. This was not the case, indeed negative Pearson two tailed correlations predominated suggesting that inhibition is regulated by some product other than phenolic anthocyanidin antioxidants as detected by TP or DPPH. The negative association also suggests that the unknown product(s) may compete with antioxidant metabolites. Paradoxically, we cannot ignore

the fact that the most active inhibitory data were from highly pigmented clones. Perhaps a metabolite pathway that produces the inhibitors draws on carbons from the phenyl alanine pathway that is essential for high levels of pigmentation. Glycolalkaloids have been detected at higher levels in pigmented potato clones (Stushnoff et al., 2008; 2010), thus their possible inhibitory role cannot be ignored.

Conclusion. The significance of plant component, developmental stage, extract concentration as it relates to cancer inhibition and harvest/top kill treatment have been examined in this study. One other factor that played a role in all of these factors was the importance of germplasm source. Future work required includes repetition of this study to confirm suggested phenomena observed by accounting for year-to-year variation. Additionally, metabolomic and genomic studies are required to unravel differences in cancer inhibition, radical scavenging capacity and total phenolic content that exist between peels, flesh, flowers, immature tubers and mature tubers harvested after no treatment, vine tops cut to 10 cm in height and chemical (95% sulfuric acid and Reglone) removal of vine tops in hopes of discovering, conserving and/or increasing valuable inhibitory metabolites.

Chapter 6

WHOLE POTATO (*SOLANUM TUBEROSUM* L.) TUBERS PROVIDE A PROTECTIVE MATRIX EFFECT THAT STABILIZES INHIBITORY METABOLITES FROM THERMAL DEGRADATION, AND RETAINS INHIBITORY ACTIVITY AGAINST HT-29 COLON CANCER CELLS *IN VITRO*

SUMMARY

Our previous research revealed that two solid pigmented skin and flesh selections; a red selection CO97226-2R/R and a purple selection, CO97216-1P/P had high and consistent inhibitory activity against mammalian HT-29 colon cancer cells in culture when harvested as immature tubers. Potentially bioactive classes of metabolites in potato found by others to induce inhibitory activity in cancer cells *in vivo* and/or *in vitro* include dietary fiber, resistant starch, vitamin C, glycoalkaloids, flavonoids and polyphenolic acids. The objectives of this study were to assess the effect of heat degraded aqueous extracts of potato compared to aqueous extracts from heated tubers; evaluate if the tuber matrix serves to protect these inhibitory metabolites; and gain insight as to what class of metabolites may be responsible for inhibitory activity. Raw immature tubers, and extracts from raw immature tubers, were exposed to 22-24 °C (room temperature), 37 °C, 45 °C, 55-60 °C, 72 °C and 96 °C (boiling) for 20 minutes. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega was used to measure the number of viable HT-29 colon cancer cells in all cell culture assays. Most significant differences (P=0.05) in temperature sensitivity were detected at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium in cell cultures. Purple pigmented

CO97216-1P/P heated tubers did not lose inhibitory properties, but rather increased in inhibitory activity at 60 °C when compared to heated tubers at room temperature. On the other hand, red pigmented heated tubers from CO97226-2R/R behaved differently, where inhibitory activity decreased by half at boiling when compared to heated tubers at 45 and 60 °C. Purple pigmented heated extracts of CO97216-1P/P revealed weak inhibitory activity and had no significant differences upon exposure to six different temperatures at any extract concentration. Red pigmented heated extracts of CO97226-2R/R completely lost inhibitory activity upon exposure to high temperatures of 72 °C and boiling when compared to heated extracts at 37 °C. Higher inhibitory activity was found in heated tubers when compared to heated extracts at all temperatures in CO97216-1P/P and at 45 °C, 55-60 °C and 72 °C in CO9722-2R/R primarily at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium. Immature tubers studied here were stored at 4 °C for six months, and retained their valuable inhibitory properties under normal potato storage conditions. Full to partial inhibitory activity retained at high temperatures may be a result of heat stable glycoalkaloids. The tuber matrix itself may also have served to protect and conserve additional heat sensitive inhibitory metabolites from thermal degradation whereas inhibitory metabolites alone found in heated extracts did not have a tuber matrix to protect heat sensitive activity.

INTRODUCTION

Previous research (i.e. Chapter 4 of dissertation) investigated the inhibitory nature of 13 elite genotypes of aqueous *Solanum tuberosum* L. extracts from the Colorado Potato Breeding Program with different pigments, antioxidant activity, phenolic

and glycoalkaloid contents obtained throughout several developmental stages and after cooking on colon (HT-29) cancer cell lines *in vitro*. Data collected from three consecutive years of testing these 13 cultivars and selections, grown under essentially commercial production conditions, at the Colorado State University San Luis Valley Research Center, Center, CO revealed that two solid pigmented skin and flesh selections, a red selection CO97226-2R/R and a purple selection CO97216-1P/P, had high and consistent inhibitory activity when harvested as a immature tubers.

The objective of this study was to assess if heating resulted in thermal degradation of inhibitory metabolites in aqueous extracts from heated raw tubers exposed to various temperatures ranging from room temperature to boiling versus raw tubers that were first extracted in organic solvent and the resultant extracts exposed to various temperatures ranging from room temperature to boiling to determine if the tuber matrix serves to protect these inhibitory metabolites. Potentially bioactive classes of metabolites found in potato that have been implicated with inhibitory activity, apoptotic induction mechanisms and the ability to upregulate detoxifying phase 2 enzymes include dietary fiber, resistant starch, vitamin C, glycoalkaloids, flavonoids and polyphenolic acids. The subsequent information is a review of the inhibitory nature of these compounds in potato.

Potatoes are an important source of dietary fiber where 8% of the recommended daily value for dietary fiber can be attained by consuming one medium sized potato with the skin (Vreugdenhil & Bradshaw, 2007). A study conducted by Harris et al. (1991) investigated the theory that dietary fiber may offer protection against colon cancer by studying the adsorption of the hydrophobic mutagen 1,8-dinitropyrene (DNP) to the skin

of potato tubers and flesh of potato tubers. Results showed that dietary fiber obtained from potato skins, which are composed mainly of α-cellulose, were able to bind DNP and thus this source can be considered as potentially protective against colon cancer. Conversely, dietary fiber obtained from potato flesh, which is composed mainly of pectic polysaccharides, did not bind DNP well and therefore may not offer any protection against colon cancer. This study stressed the importance of the source and/or type of plant cells walls, dietary fibers, in their ability to bind and reduce mutagen and cancer promoter contact with mucosal cells of the colon.

Starch, the main energy source in potato, constitutes 75% of total dry matter content (Vreugdenhil & Bradshaw, 2007). Starch is a polymer of the glucose monomers amylose and amylopectin (Vreugdenhil & Bradshaw, 2007). A small portion of starch found in potato called resistant starch (RS), is the source of many health benefits in the colon. RS is resistant to enzymatic digestion in the small intestine by α-amylase and consequently is fermented in the large intestine (United States Potato Board, 2010; Liu & Xu, 2008). RS causes a fermentation mediated increase in the concentrations of short chain fatty acids, particularly butyrate, that exist in the large intestine (Liu & Xu, 2008). Butyrate has been found to halt growth, induce apoptosis and regulate expression of proteins associated with cellular dedifferentiation in several cancer cell culture lines including colon cancer (Liu & Xu, 2008). In a study conducted by Liu and Xu (2008), RS was administered to azoxymethane (AOM) induced colonic aberrant crypt foci (ACF), markers of preneoplastic lesions, of Wistar rats where it was confirmed that RS suppressed AOM-induced ACF formation at the promotion stage but promoted the formation of ACF at the preinitation stage. RS is also responsible for laxation

enchancement, increases in beneficial bacteria, cecal and large intestinal contents and decreases in pH and concentration of ammonia, phenols and secondary bile acids in the colon (United States Potato Board, 2010; Liu & Xu, 2008).

Friedman (2006) explains various aspects of glycoalkaloids. Glycoalkaloids are secondary metabolites that exist in the tubers, leaves, roots and sprouts of potato. Glycoalkaloids are composed of a trisaccharide chain attached to an aglycon. These glycoalkaloids are mostly positioned around the eyes located on the outer layer of the tuber and within the first 1mm from the exterior surface of the tuber decreasing toward the interior of the tuber. The prominent glycoalkaloids that exist in cultivated potato are α-chaconine and α-solanine. The initial concentration and configuration of these prominent glycoalkaloids in potato is largely dependent on genotype. Concentrations may be effected by environmental factors such as soil and climate and generally decrease with increasing tuber size. Postharvest management can also cause concentration increases as a result of storage, light, heat, cutting, slicing, sprouting and exposure to phytopathogens.

Glycoalkaloids have been associated with producing an undesirable bitter astringent taste (Vreugdenhil & Bradshaw, 2007). At certain concentrations they are reported to be toxic to bacteria, fungi, viruses, insects, animals and humans (Friedman, 2006). Of the two prominent glycoalkaloids found in potato, α-chaconine has been reported to be more toxic than α-solanine in several studies (Friedman, 2006). Glycoalkaloids ingested by humans in the form of potato, potato berries, potato leaves, and blighted, greened and sprouted tubers has resulted in death (Friedman, 2006). Human feeding studies revealed that ingestion of potatoes varying in concentration

between 1.0-2.6 $mg \cdot kg^{-1}$ body weight resulted in light to severe nausea, burning sensation in the mouth and diarrhea (Friedman, 2006). Morris and Lee (1984) reported in a review paper that 2-5 $mg \cdot kg^{-1}$ of body weight is considered a toxic dose to humans and 3-6 $mg \cdot kg^{-1}$ of body weight is considered to be a fatal dose to humans (Morris & Lee, 1984). Thus the maximum safe allowance for human consumption of glycoalkaloids is reported to be 200 $mg \cdot kg^{-1}$ of fresh weight of potato (Vreugdenhil & Bradshaw, 2007).

Although glycoalkaloids are reported to produce toxic effects they have also been implicated as chemopreventives (Friedman et al., 2005, Friedman et al.2006). The glycoalkaoids, α-chaconine and α-solanine, isolated from Dejima potatoes, were identified as the key metabolites involved in the inhibition of the growth of several human cell lines in culture including cervical (HeLa), liver (HegG2), lymphoma (U937) and stomach (AGS and KATO III) (Friedman et al., 2005). α-chaconine has been reported to inhibit of ERK ½ phosphorylation triggering caspase-3 activation generating the induction of apoptosis of human HT-29 colon cancer cells in culture in a time and concentration dependent manner (Yang et al., 2006). Methanol extracts of *S. tuberosum* callus containing higher levels of solanidin, α-chaconine and α-solanine than in tubers induced cytotoxic effects against human carcinoma cell culture lines of breast, lymphoplastic leukemia, larynx, liver, cervix, colon and brain (Al-Ashaal, 2010). Mandimika attempted to explain the effects of different amalgamations of α-chaconine and α-solanine treatments together and alone on Caco-2 colon human intestinal epithelial cell culture lines (Mandimika, 2007). Lactase dehydrogenase leakage assay executed in their study informed that cytotoxicity of Caco-2 cells in culture occurred in a

concentration dependent manner in all treatments except α-solanine alone (Armstrong, 2011). Gene expression studies utilizing α-chaconine and α-chaconine/α-solanine treatments on Caco-2 cells indicated significant upregulation of genes involved in cholesterol biosynthesis (Mandimika, 2007). All treatment combinations showed gene expression changes in growth signaling, lipid and amino acid metabolism, mitogen activated protein kinase (MAPK) and NF-κB cascades, cell cycle and apoptosis. Late apoptotic induction of Caco 2 cells was found to occur in a concentration dependent manner by α-chaconine (Mandimika, 2007). Cell cycle analysis revealed an accumulation of Caco 2 cells during G_2/M phase induced by α-chaconine (Mandimika, 2007). Conversely, another study revealed that HT-29 colon cancer cells in culture exposed to 5 ug·mL^{-1} α-chaconine and 10 μg·mL^{-1} α-solanine resulted in an accumulation of HT-29 cells during sub-G_0/G_1 phase of the cell cycle (Yang et al., 2006).

Vitamin C is a hydrophilic compound that acts as an antioxidant (Spencer & Saul, 2010). Vitamin C is the main vitamin in potato and contributes up to 40% of daily recommended intake of the global diet (Vreugdenhil & Bradshaw, 2007). The concentration and state of reduced ascorbic acid and oxidized dehydroascorbic acid is dependent on both potato germplasm and storage (Vreugdenhil & Bradshaw, 2007). A recent study showed that intraperitoneal pharmacological doses of vitamin C reduced tumor size and incidence in rats induced with hormone-refractory prostate cancer PA III cells (Pollard et al., 2010). The reduced state of ascorbic acid has been found to effectively kill malignant mesothelioma cells in both test tube studies and mice experiments (Pollard et al., 2010). Angiogenesis has been found to be impeded when

high levels of vitamin C are present in the blood both *in vivo* and *ex vivo* (Mikirova et al., 2010). Numerous other research studies have established the value of vitamin C, thus this essential vitamin should be viewed as a powerful bioactive agent important in the intervention of many diseases.

Carotenoids are fat soluble tetraterpenoids existent in the flesh of white, yellow and orange potatoes (Brown, 2005). The most prominent carotenoids emanate from the zanthophyll subclass, oxygenated tetraterpenoids, and include lutein 5,6 epoxide, zeaxanthin, violaxanthin (Brown, 2005). Other carotenoids identified in potato in trace amounts emanate from the carotene subclass, tetraterpenoids without oxygen, and include β-carotene and α-carotene. Potato is not a worthy source of these pro-vitamin α-carotenes (Brown, 2005). Oxygen radical absorbance capacity of total carotenoids from potato flesh have been found to range between 4.6 - 15.3 nmoles α-tocopherol equivalents per 100g FW (Brown, 2005). Carotenoids are a powerful source of antioxidants and have been associated with a reduced risk of certain cancers (International Carotenoid Society, 2011).

Potatoes contain significant amounts of flavonoids which are polyphenolic compounds structurally composed of three aromatic benzene rings containing one or more hydroxyl groups (International Carotenoid Society, 2011). Flavonoids have been acknowledged to produce antiproliferative, anticarcinogenic and antioxidative effects (Harborne, 1993; Kuhanu, 1976; Sharma et al., 1994; Rice-Evans et al., 1996; Robards et al., 1999). The prominent flavonoids found in potato are anthocyanins, catechin and epicatechin but erodictyol, kaempferol and narigenin are also present in potato (Brown, 2005). Specific anthocyanins abundant in potato that are responsible for producing red

and purple pigments in potato include the acylated anthocyanidin glucosides pelargonidin, petunidin, malvidin and peionidin (Brown, 2005 ; Stushnoff et al., 2008; Stushnoff et al., 2010). Anthocyanins isolated from pigmented potatoes, have been reported to be responsible for the induction of apoptosis in KATO III human stomach cancer cell lines in culture and suppressed stomach cancer in mouse (Hayashi et al., 2006). Anthocyanins, present in red and purple steamed potatoes, also suppressed stomach cancer in mouse in the same study by Hyashi (2006). Specialty potato extracts and anthocyanin fractions obtained from a specialty potato genotype both initiated mitochondrial release of the proteins Endo G and AIF resulting in the induction of apoptosis and inhibited growth of LNCaP (androgen dependent) and PC-3 (androgen independent) prostate cancer cells in culture (Reddivari et al., 2007). Cyanidin and delphinidin, and to a lesser extent malvidin, have been found to inhibit epidermal growth factor receptor in cancer cells (Sterling, 2001). Transcriptome analysis from recent potato sequence data revealed that nine genes upregulate and at least two down regulate pigment production in purple fleshed clones (Stushnoff et al., 2010).

Phenolic acids are hydroxylated derivatives of benzoic and hydrocinammic acids (Herrmann, 1989). The most profuse phenolic acid present in potato is chlorogenic acid but protocatechic acid, vanillic acid and p-coumaric acid also exist (Brown, 2005). Chlorogenic acid, an antioxidant abundant in pigmented potato (Stushnoff et al., 2008; Stushnoff et al., 2010) has been reported to inhibit the proliferation of lung cancer cells and TPA-induced neoplastic transformation and may be a potential stimulant of NQO1 and GST enzymatic activities (Feng et al., 2005). A red pigmented cultivar, Mountain Rose (MR), comprised of higher levels of chlorogenic acid and anthocyanins resulted in

a reduction in breast cancer carcinogenesis incidence and multiplicity in rats that were fed freeze dried baked MR potato (Thompson et al., 2009). Extracts obtained from *Solanum jamesii*, a wild tuber species, were found to inhibit the proliferation of HT-29 colon and LNCaP prostate cancer cells culture lines in vitro, but no evidence of cytotoxicity to cells was observed nor significant correlations to glycoalkaloid content, neither total phenolic content nor antioxidant capacity were detected (Nzaramba et al., 2009).

MATERIALS & METHODS

Clones. Two clones of *S. tuberosum* (CO97216-1P/P and CO97226-2R/R) from the Colorado Potato Breeding Program, grown under essentially commercial production conditions at the Colorado State University San Luis Valley Research Center, Center, Colorado were harvested as immature tubers (growing degree days [GDD] = 946) in 2010 and stored for six months at 4 °C in dark conditions (Table 6.1). Both of these clones are in an experimental phase of release and are designated as selections (Table 6.1). The first letter before the slash is indicative of tuber skin color (P=purple and R=red) (Table 6.1). The letters following the slash are indicative of tuber flesh colors (P=purple and R=red) (Table 6.1).

Table 6.1 Clonal selections from the Colorado Breeding Program utilized in this research.

Clonal Selections	Female Parent	Male Parent	Skin	Flesh
CO97216-1P/P	CO94163-1	CO94214-1	Purple	Purple
CO97226-2R/R	Mountain Rose	CO94214-1	Red	Red

Extraction of Heated Extracts. Three biological replicates (median slices of peel and flesh from raw immature tubers from separate plants) from each clone were stored for 6 months at 4 °C in dark conditions and freeze dried as raw tubers using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a 100 mesh sieve. Freeze dried powder was weighed out in 600 mg samples, added to a 15 mL conical tube in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in a refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in the amount of 1 mL aliquots and acetone was evaporated off at 4 °C in a Virtis Genesis 1500L cabinet style freeze drier. Dried 60 $mg \cdot mL^{-1}$ potato extracts in 1.5 mL Eppendorf tubes were reconstituted in 1 mL McCoys 5A, 1x medium supplemented with 10% fetal bovine serum under sterile conditions and subjected to six different temperature water baths (22 °C, 37 °C, 45 °C, 55 °C, 72 °C and 96 °C) for 20 minutes. Water bath temperatures were recorded every 5 minutes with a copper-constantan thermocouple to ensure extracts were subjected to designated water temperature for the entire 20 minutes. Heated extracts samples were assayed for inhibitory activity against HT-29 colon cancer cells in culture immediately after the heating procedure was executing using the microculture tetrazolium assay procedure described below. These aqueous extracts prepared in this manner will be referred to as “heated extracts”.

Extraction of Heated Tubers. An additional three biological replicates (median slices of peel and flesh from raw immature tubers from separate plants) from each clone

were stored for 6 months at 4 °C in dark conditions and subjected to 6 different temperature water baths (24 °C, 37 °C, 45 °C, 60 °C, 72 °C and 96 °C) for 20 minutes. Internal tuber temperatures were recorded every 5 minutes with a copper-constantan thermocouple that was inserted into the center of an indicator tuber to ensure internal tuber temperature coincided with water bath temperature. Heated tubers were freeze dried using a Virtis Genesis 1500L cabinet style freeze drier. Freeze dried samples were ground into a very fine powder using a mortar and pestle and sifted through a 100 mesh sieve. Freeze dried powder was weighed out into 600 mg samples, added to a 15 °C conical tube in conjunction with 10 mL of organic solvent, HPLC grade 80% acetone, and rotated in refrigerator for 1 hour at 4 °C. Conical tubes containing the freeze dried powders and acetone were centrifuged for 15 minutes at 4 °C and 6000 rpm. The supernatant was transferred to Eppendorf tubes in 1mL aliquots and acetone was evaporated off at 4 °C. Dried 60 $mg \cdot mL^{-1}$ potato extracts were stored at - 20 °C until analysis for inhibitory activity against HT-29 colon cancer cells in culture. These aqueous extracts prepared in this manner will be referred to as “heated tubers”.

Cell lines and Cell Cultures. Human colorectal adenocarcinoma HT-29 cells were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were received on dry ice, placed in a 37 °C water bath for 3 minutes and immediately transferred to a sterile 15 mL conical tube containing 9 mL of warm McCoys 5A, 1x medium supplemented with 10% fetal bovine serum under sterile conditions using a sterile transfer hood. Sterile centrifuge tubes containing cells and medium were immediately centrifuged at 125 xg for 5 - 10 minutes. Under sterile condition using a sterile transfer hood, the supernatant was removed and the cell pellet

was reconstituted in 10 mL of McCoys 5A, 1x medium supplemented with 10% fetal bovine serum. Contents were transferred to a sterile 75 cm^2 cell culture flask with a vent cap where an additional 5 mL McCoys 5A, 1x medium was added to bring total flask contents up to a total of 15 mL. Flasks were incubated at 37 °C in a humidified 5% CO_2 incubator and allowed to grow to 80% confluence until cells needed to be subcultured.

First generation cells received from ATCC were grown in flasks for several days and then prepared for cryogenic preservation for future use utilizing the subsequent method. Sterile cryogenic vials comprising 2 x 10^5 - 5 x 10^6 cells·1 mL^{-1} ampule in McCoys 5A, 1x medium containing 10% fetal bovine serum and 5% DMSO were transferred inside a 1-inch foam-insulated box and kept at - 80 °C for 12 hours and stored in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Cells used in the execution of all cell culture experiments were maintained in the incubator for no more than five generations before first generation cells were retrieved from cryopreservation storage in liquid nitrogen vapor at the National Center for Genetic Resources Preservation (NCGRP, Fort Collins, Colorado).

Microculture Tetrazolium Assay: Cell Seeding. All methods to follow were conducted using sterile materials and under sterile conditions using a sterile transfer hood. HT-29 colon cells were seeded into a 96 well plate at a rate of 5 x 10^4 cells in 50 µl per well and incubated at 37 °C in a humidified, 5% CO_2 incubator chamber for 24 hours to allow for cells to adhere to the plates. Trypan blue was used in a 1:1 ratio to distinguish viable cells from nonviable cells. A hemacytometer and inverted microscope

were used to facilitate the cell count during this initial seeding step. It should be noted that no wells on the outer perimeter of the microplate were seeded with cells in order to minimize any variation that may occur in these wells.

Microculture Tetrazolium Assay: Potato Extract Treatment. Three biological replicates of dried 60 $mg \cdot mL^{-1}$ potato extracts from each clone were reconstituted in 1 mL of aqueous McCoys 5A, 1x medium containing 10% fetal bovine. Reconstituted potato extracts were filter sterilized using sterile 25 mm syringe filters w/ 0.2 µm cellulose acetate membrane and 3 mL sterile syringes and transferred into sterile 1.5 mL Eppendorf tubes. Serial dilutions of 7.2, 14.4 and 28.8 $mg \cdot mL^{-1}$ potato extract concentrations were made out of each of the 60 $mg \cdot mL^{-1}$ potato extracts reconstituted in cell culture medium in a 96 well plate. Individual wells containing seeded cells were treated with 50 µL of each of 3 potato extract concentrations (7.2, 14.4 and 28.8 $mg \cdot mL^{-1}$ cell culture medium) in triplicate replication to achieve a final respective treatment extract concentration of 3.6, 7.2 and 14.4 and $mg \cdot mL^{-1}$ cell culture medium. All treated microplates were incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for potential inhibitory interactions to occur.

Microculture Tetrazolium Assay: Control Treatment. Nine individual wells containing seeded cells on each microplate were each treated with 50 µL of McCoys 5A, 1x medium containing 10% fetal bovine serum and incubated for 24 hours at 37 °C in a humidified, 5% CO_2 incubator chamber. Untreated cells were designated as negative controls for calculating percent growth inhibition of treated cells residing on the same microplate.

Microculture Tetrazolium Assay: Cell Viability Assay. CellTiter 96 Aq_{ueous} Non-Radioactive Cell Proliferation Assay by Promega is a colorimetric assay that was used to measure the number of viable cells in all cell culture assays. The principal source of this assay involves the use of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] which is a water soluble salt in combination with (phenazine methosulfate; PMS) an electron coupling agent reagent. Dehydrogenase enzymes present in metabolically active cells cleave the tetrazolium ring and convert MTS into a colored formazan product that is soluble in tissue culture medium. The amount of formazan product produced, measured by the amount of absorbance at 490 nm, is directly proportional to the number of viable cells in culture. Exactly 24 hours after cells were treated with potato extracts, 20 µL of the prepared MTS/PMS solution was added to each well and incubated for 1 hour at 37 °C in a humidified, 5% CO_2 incubator chamber to allow for dehydrogenase enzymes in any viable cells to convert MTS to formazan. The absorbance was read at 490 nm using a Spectromax 640 microplate spectrophotometer.

Microculture Tetrazolium Assay Quantification and Statistical Methods Based on % Growth Inhibition. Absorbance of the treated cells was compared to the absorbance of the untreated control cells from each individual plate and % growth inhibition was calculated using the following calculation (((control-treated) / control) x 100). Standard deviations and coefficients of variations were calculated among triplicate replications. Commonly, coefficients of variations were below 5%.

Statistical analyses conducted on results expressed in terms of % growth inhibition for all potato extract treatments was based on three to five biological replicates from one harvest year (2010) where each microculture tetrazolium experiment were conducted only one time.

Ratio transformations of (treatment/control) were used in the statistical analysis to stabilize normal distributions and equal variance. Three-way analyses of variance (ANOVA) was used to evaluate the main effects of temperature, extract concentration and heated extracts vs. heated tubers followed by Tukey-Kramer test to assess separation of means using SAS software.

RESULTS

Heated Tubers. Inhibitory activity at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium by CO97216-1P/P heated tubers increased from 36.5% at 24 °C to 64.3% at 60 °C (Table 6.2, Fig. 6.1). Inhibitory activity of CO97216-1P/P heated tubers was higher at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium than at an extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium at all temperatures (Table 6.2, Fig. 6.1). In addition inhibitory activity was higher in CO97216-1P/P heated tubers at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium when compared to an extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium but only at temperatures of 45, 60 and 72 °C (Table 6.2, Fig. 6.1).

Inhibitory activity at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium by CO97226-2R/R heated tubers decreased from 57.2 and 56.8% at 45 and 60 °C respectively down to 25.5% inhibitory activity at 96 °C (Table 6.2, Fig. 6.2). Inhibitory

activity was higher at an extract concentration of 7.2 mg·mL^{-1} cell culture medium when compared to an extract concentration of 3.6 mg·mL^{-1} cell culture medium at all temperatures that CO97226-2R/R heated tubers were subjected to except 96 °C (Table 6.2, Fig. 6.2). In addition inhibitory activity was higher at an extract concentration of 14.4 mg·mL^{-1} cell culture medium when compared to an extract concentration of 3.6 mg·mL^{-1} cell culture medium at all heated tuber temperatures (Table 6.2, Fig. 6.2).

Table 6.2 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated tubers extracts (mg·mL^{-1} cell culture medium) that were subjected to six different temperatures, compared to heated extracts (mg·mL^{-1} cell culture medium) subjected to similar temperatures from raw immature tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05. See Appendix E. 1 and E.2 for degrees of freedom, mean squares, F-values and level of significance for both selections based.

	CO97216-1P/P						CO97226-2R/R					
	3.6 (mg·mL^{-1} cell culture medium)	*w*	7.2 (mg·mL^{-1} cell culture medium)	*w*	14.4 (mg·mL^{-1} cell culture medium)	*w*	3.6 (mg·mL^{-1} cell culture medium)	*w*	7.2 (mg·mL^{-1} cell culture medium)	*w*	14.4 (mg·mL^{-1} cell culture medium)	*w*
Heated Tubers (% Growth Inhibition)												
24 °C	6.7	a b	36.5	c d e f g	22.6	a b c d e f	0	a	38.8	e f g h i j	42.8	g h i j
37 °C	10.2	a b c	46.3	e f g h	27.3	b c d e f	8.2	a b c d e	50.5	h i j	42.2	g h i j
45 °C	9.0	a b	60.9	g h	44.2	e f g h	7.2	a b c d	57.2	j	39.8	f g h i j
60 °C	19.8	a b c d e	64.3	h	46.9	f g h	5.2	a b c	56.8	i j	44.9	g h i j
72 °C	0	a	39.7	d e f g h	42.4	e f g h	9.0	a b c d e f	46.3	h i j	45.0	g h i j
96 °C	7.6	a b	39.1	d e f g h	23.9	a b c d e f	0	a	25.5	a b c d e f g h	35.4	c d e f g h i j
Heated Extracts (% Growth Inhibition)												
22 °C	0	a	8.9	a b	26.0	a b c d e f	0	a	31.4	a b c d e f g h i j	32.2	b c d e f g h i j
37 °C	0	a	0	a	21.8	a b c d e f	0	a	36.2	c d e f g h i j	31.1	a b c d e f g h i j
45 °C	0	a	4.9	a b	24.8	a b c d e f	0	a	25.2	a b c d e f g h	34.0	b c d e f g h i j
55 °C	0	a	6.3	a b	24.4	a b c d e f	0	a	13.9	a b c d e f g	37.2	d e f g h i j
72 °C	0	a	0	a	13.7	a b c d	0	a	3.3	a b	33.5	b c d e f g h i j
96 °C	0	a	0	a	7.0	a b	0	a	0	a	21.8	a b c d e f g h

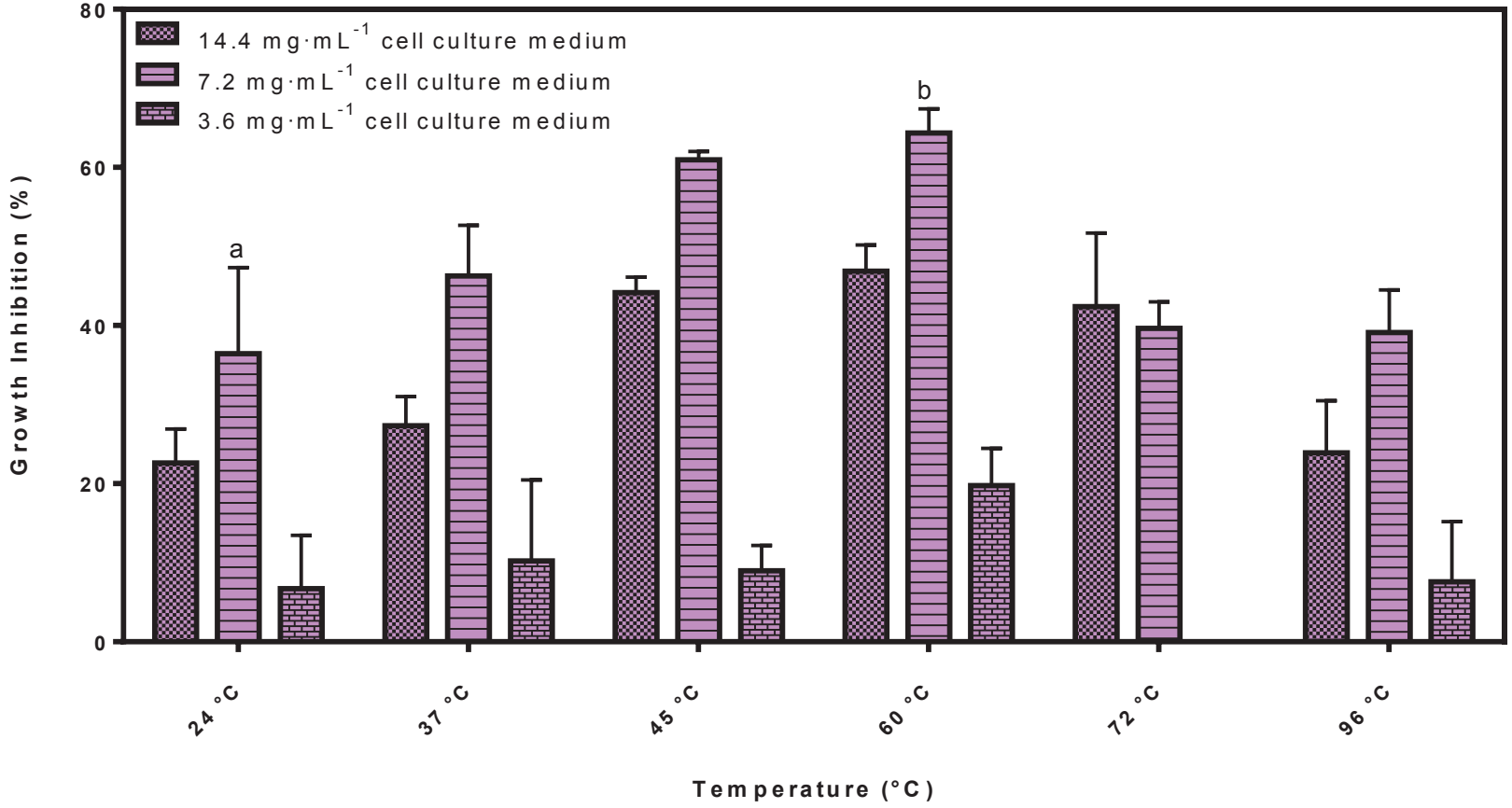

Figure 6.1 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated tuber extracts (mg·mL^{-1} cell culture medium) from immature CO97216-1P/P tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Letters denote statistically significant differences between temperatures at a specific extract concentration based on Tukey's HSD at P=0.05.

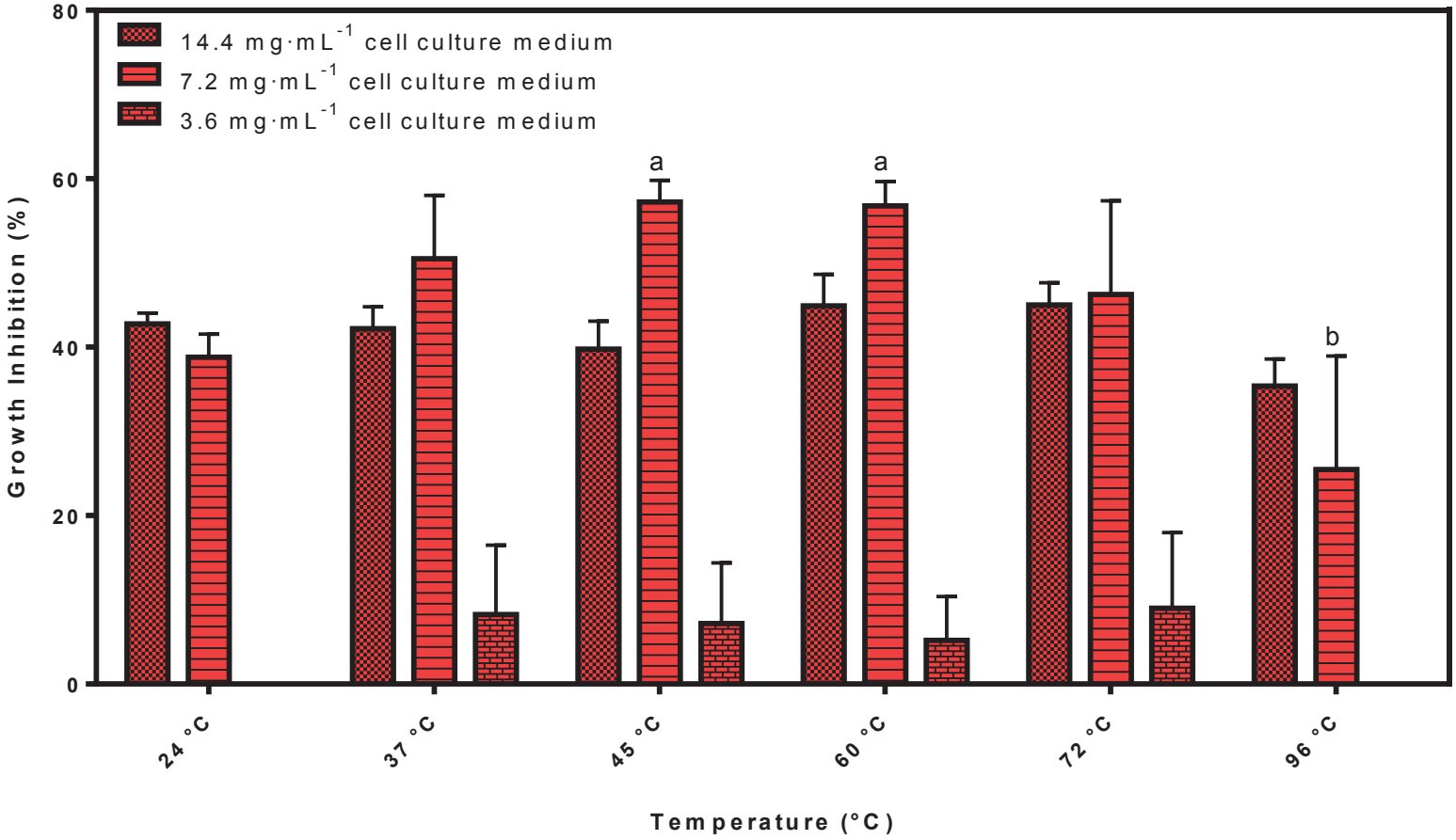

Figure 6.2 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated tuber extracts (mg·mL^{-1} cell culture medium) from immature CO97226-2R/R tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Letters denote statistically significant differences between temperatures at a specific extract concentration based on Tukey's HSD at P=0.05.

Heated Extracts. CO97216-1P/P heated extracts subjected to six different temperatures did not differ in inhibitory activity against cancer cell cultures at any extract concentration nor at any temperature (Table 6.2, Fig. 6.3).

Inhibitory activity by CO97226-2R/R heated extracts at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium decreased from 36.2% at 37 °C down to 3.3 and 0% inhibitory activity at 72 and 96 °C respectively (Table 6.2, Fig. 6.4). Inhibitory activity by CO97226-2R/R heated extracts was higher at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium when compared to at an extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium at 37 °C (Table 6.2, Fig. 6.4). Inhibitory activity by CO97226-2R/R heated extracts was also higher at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium when compared to at an extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium at 22, 45, 55 and 72 °C (Table 6.2, Fig. 6.4).

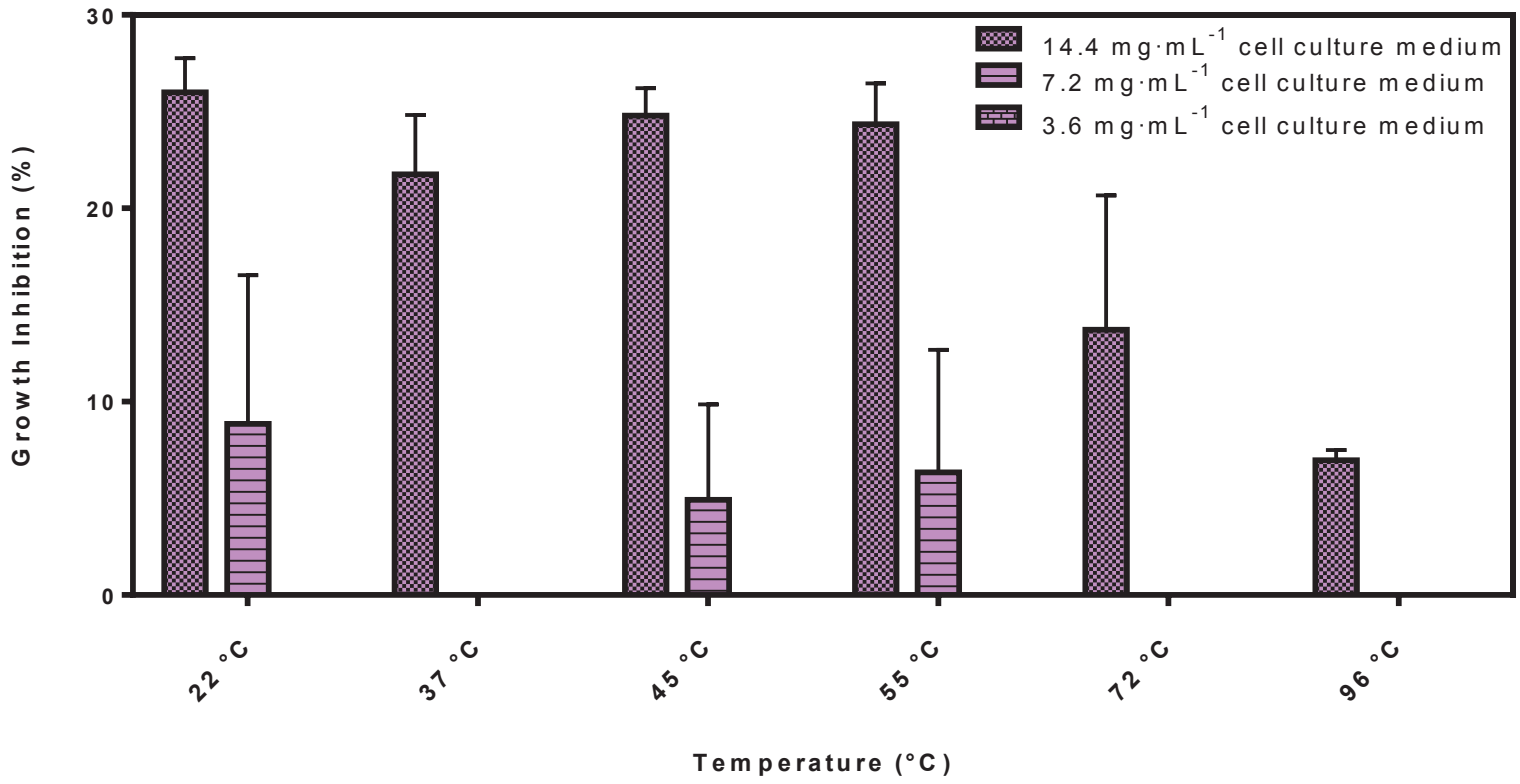

Figure 6.3 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated extracts (mg·mL^{-1} cell culture medium) from raw immature CO97216-1P/P tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05.

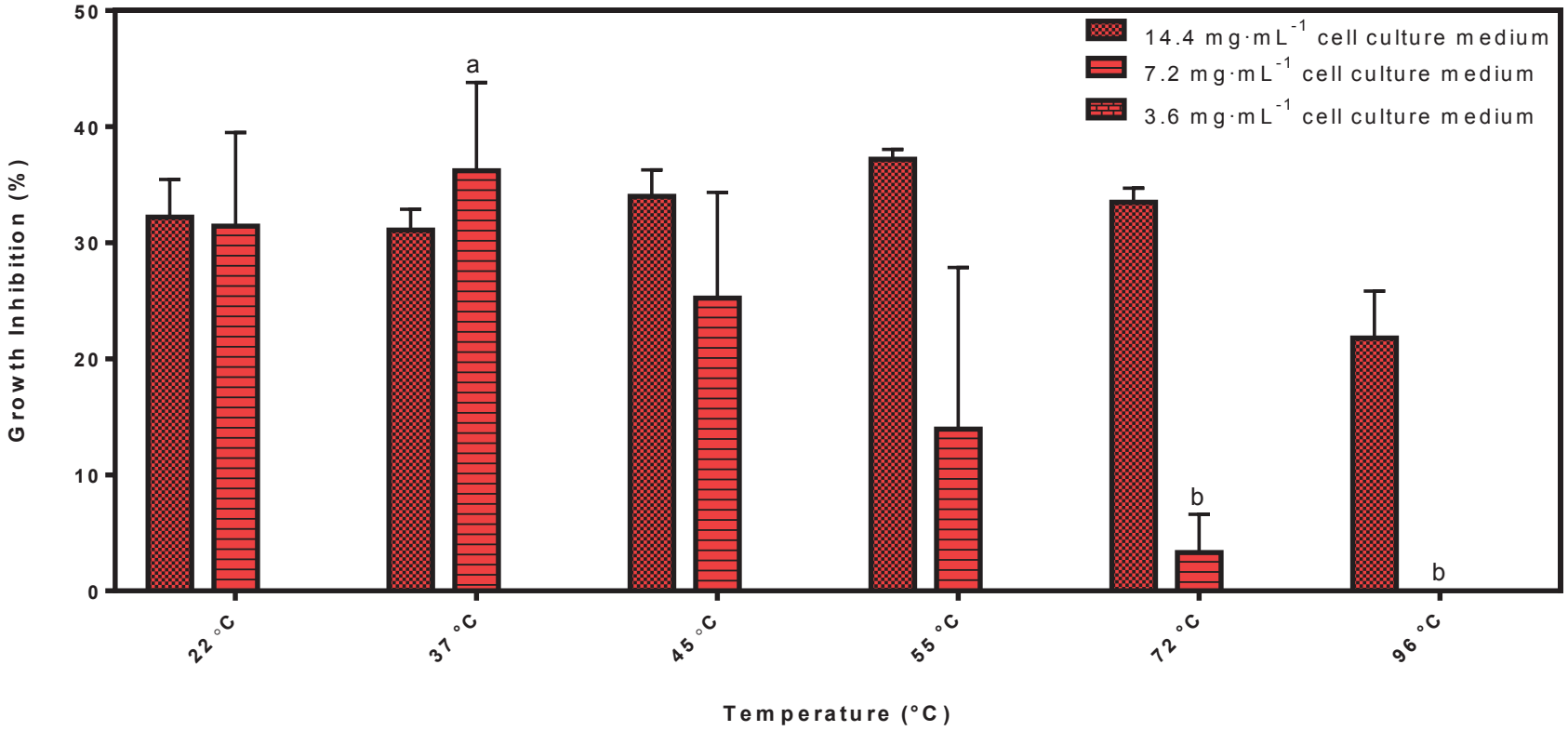

Figure 6.4 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated extracts (mg·mL^{-1} cell culture medium) from raw immature CO97226-2R/R tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Means followed by the same letter are not significantly different based on Tukey's HSD, P=0.05. Letters denote statistically significant differences between temperatures at a specific extract concentration based on Tukey's HSD at P=0.05.

Heated Tubers vs. Heated Extracts. CO97216-1P/P heated tubers that were subjected to six different temperatures had higher inhibitory activity at all temperatures at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium (and at 72 °C at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium) when compared to CO97216-1P/P heated extracts subjected to the same temperatures (Table 6.2, Fig. 6.5).

CO97226-2R/R heated tubers had higher inhibitory activity at 45, 55-60, and 72 °C at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium when compared to CO97226-2R/R heated extracts subjected to these same temperatures (Table 6.2, Fig. 6.6).

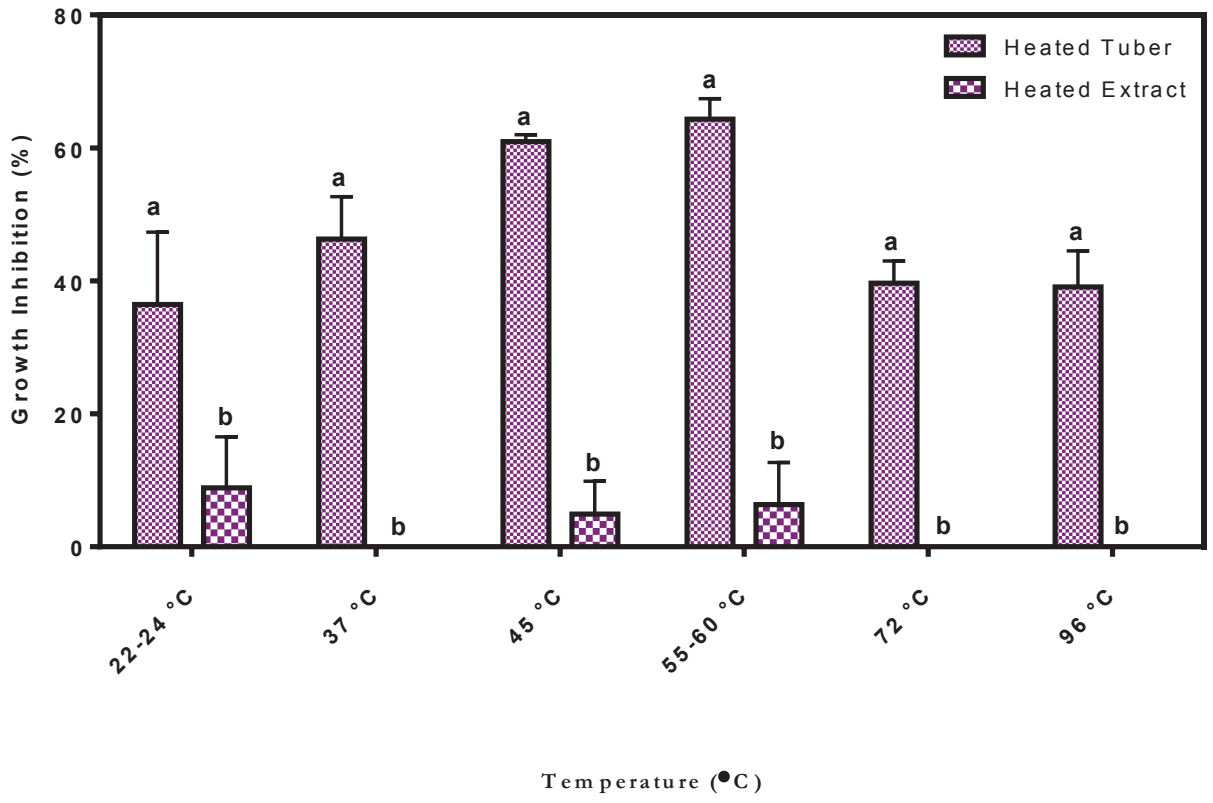

Figure 6.5 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated extracts and heated tubers (7.2 mg·mL^{-1} cell culture medium) both from immature CO97216-1P/P tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Letters denote statistically significant differences between heated tubers and heated extract treatments at an extract concentration of 7.2 mg·mL^{-1} cell culture medium.

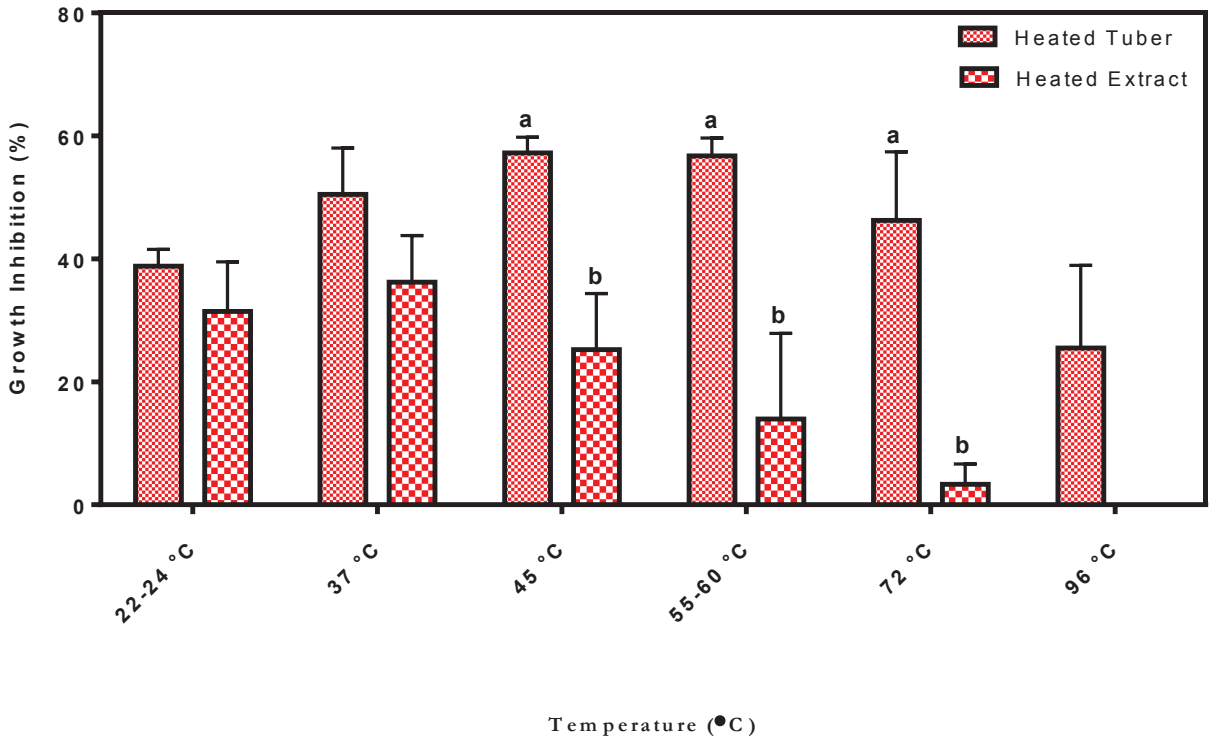

Figure 6.6 % Growth inhibition of HT-29 colon cancer cells after 24 hours by heated extracts and heated tubers (7.2 mg·mL^{-1} cell culture medium) both from immature CO97226-2R/R tubers. Data are means of all replicates (%). Three biological replicates were tested. % Growth inhibition data represented in this table were ratio transformed to conduct Tukey's HSD analysis. Letters denote statistically significant differences between heated tubers and heated extract treatments at an extract concentration of 7.2 mg·mL^{-1} cell culture medium.

DISCUSSION

Heated Tubers. Purple pigmented CO97216-1P/P heated tubers were thermally stable and did not lose any inhibitory properties, but rather increased in inhibitory activity at 60 °C compared to cooler and warmer temperatures (Table 6.2, Fig. 6.1). Potatoes worldwide are typically consumed as a cooked product. In order for a consumer to reap the potential protective benefits against colon cancer, inhibitory compounds must be delivered to the colon in a bioactive state. Thus, finding a tuber that possesses inhibitory properties against colon cancer that can withstand boiling temperatures utilized in cooking is essential for a consumer to reap these benefits as well as for a grower to market these benefits. The stability of the inhibitory nature observed at even high temperatures may suggest that glycoalkaloids, heat stable compounds, may be the culprit behind inhibition of cancer cells in culture. On the other hand as stated earlier, certain concentrations of glycoalkaloids have been reported to be toxic to humans and thus the maximum safe allowance for human consumption of glycoalkaloids is reported to be 200 $mg \cdot kg^{-1}$ of fresh weight of potato (Vreugdenhil & Bradshaw, 2007).

Red pigmented heated tubers, CO97226-2R/R behaved differently where inhibitory activity at boiling decreased by half (Table 6.2, Fig. 6.2). This finding suggests that perhaps there exists more than one bioactive compound responsible for inhibition of colon cancer cells in culture. One could infer that the partial inhibitory activity retained in this selection at high temperatures may be a result of heat stable glycoalkaloids. While, loss of the remaining inhibitory activity due to cooking suggests that another inhibitory metabolite exists that is sensitive to high temperatures.

Earlier we discovered that inhibitory properties in these two clones appeared to maximize at discreet immature stages, but decreased as tubers approached field maturity. The tubers studied here were harvested immature, but stored at 4 °C for six months, thus retaining their valuable inhibitory properties under normal potato storage conditions.

Heated Extracts. Purple pigmented heated extracts of CO97216-1P/P revealed weak inhibitory activity and had no significant differences upon exposure to six different temperatures at any extract concentration (Table 6.2, Fig. 6.3). Red pigmented heated extracts of CO97226-2R/R revealed complete loss of inhibitory activity upon exposure to high temperatures of 72 °C and boiling (Table 6.2, Fig. 6.4). This finding suggests that glycoalkaloids are not likely the inhibitory agents observed in this cultivar.

Heated Tubers vs. Heated Extracts. Higher inhibitory activity was found in heated tubers when compared to heated extracts at all temperatures in CO97216-1P/P and at 45 °C, 55-60 °C, 72 °C in CO9722-2R/R tubers at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium (Table 6.2, Fig. 6.5, 6.6). Higher inhibitory activity was also found in CO97216-1P/P heated tubers at 72 °C when compared to heated extracts at an extract concentration of 14.4 $mg \cdot mL^{-1}$ cell culture medium (Table 6.2, Fig. 6.5, 6.6). Accordingly, the tuber matrix itself may serve to protect and conserve inhibitory metabolites from thermal degradation whereas inhibitory metabolites alone found in heated extracts do not have a tuber matrix to protect activity.

Extract Concentration. Both selections had overall higher inhibitory activity at extract concentrations of 7.2 and 14.4 $mg \cdot mL^{-1}$ cell culture medium than at an extract concentration of 3.6 $mg \cdot mL^{-1}$ cell culture medium (Table 6.2, Fig. 6.1, 6.2 and 6.4).

Significant differences in temperature sensitivity were detected at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium (Table 6.2, Fig. 6.1, 6.2, 6.3, 6.4, 6.5, and 6.6). Thus it seems prudent to conduct future analyses at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium.

Chapter 7

CONCLUSIONS

Précis. In almost every situation where significant differences were observed within a developmental stage and after cooking, the same pattern was observed where higher radical scavenging capacity, total phenolic content and inhibitory activity were best conserved in raw, immature tubers when compared to cooked, mature tubers with some exceptions depending on clones. Immature tubers normally demonstrating strong inhibitory activity also retained their valuable inhibitory activity after six months storage under normal potato storage conditions.

Pigmented clones generally contained higher levels of phenolic antioxidants and radical scavenging capacity. While three of the strongest inhibitors of cancer cell cultures were pigmented, other pigmented clones were weak inhibitors of cancer cell cultures. This emphasizes the idea that not all tubers are created equal and the importance of germplasm source.

Potato peel extracts were a superior source of phenolic antioxidants, radical scavenging capacity and inhibitory activity, at extract concentrations less than 3.6 $mg \cdot mL^{-1}$ cell culture medium, when compared to analogous whole tuber and potato flesh extracts with a few exceptions depending on clones. Flower extracts also proved to be an equally potent source of cancer cell culture inhibitory metabolites at extract concentrations less than 3.6 $mg \cdot mL^{-1}$ cell culture medium. This response observed in both peel and flower extracts may be attributable, at least partially to glycoalkaloids that exist in the tubers, leaves, roots and sprouts of potato that have been reported to inhibit

proliferation of several cancer cell lines in culture, but this needs more research to confirm their specificity and quantity.

Extracts prepared via cryogenic grinding preparation methods revealed enhanced inhibitory activity compared to extracts prepared via freeze drying preparation methods. The most statistically significant and/or highest inhibitory responses against colon cancer cell cultures from whole tubers extracts were mostly found to occur at an extract concentration of 7.2 $mg \cdot mL^{-1}$ cell culture medium. Reporting results based on the devised ASA standard curve allows for an easier, standardized and novel quantitative method for expressing number of viable HT-29 colon cancer cells in a colorimetric assay. Establishing the best preparation method, dosage concentration and cell viability quantification method provides a foundation for screening potato germplasm for inhibitory activity in future studies.

Deciphering what harvest topkill treatment to use depends upon whether one wishes to maximize antioxidant, or inhibitory properties. Top cutting of vines was a superior method for increasing inhibitory activity in immature tubers and an inferior method for increasing inhibitory activity in mature tubers in affected clones. Utilizing no harvest/top kill treatment was the best method for increasing phenolic antioxidants and radical scavenging capacity levels in immature tubers while top cutting and no treatment were better methods for increasing phenolic antioxidants and radical scavenging capacity levels in mature tubers in affected clones.

Explanations for strong inhibitory responses against colon cancer cells in culture were explored. Inhibition in strongly inhibiting clones can be attributed to varying levels of caspase-3 protease activities depending on selection. Upregulation of key inhibitory

metabolites may be found only at the outset of tuber initiation and early development. Full to partial inhibitory activity retained in heated tubers at high temperatures of strongly inhibiting clones may be a result of heat stable glycoalkaloids. Higher inhibitory activity found in heated tubers versus heated extracts of strongly inhibiting clones may suggest that the tuber matrix may also have served to protect additional heat sensitive inhibitory metabolites from thermal degradation whereas inhibitory metabolites alone found in heated extracts did not have a tuber matrix to protect heat sensitive activity. Loss of partial inhibitory activity may also be a result of degradation of unidentified non-heat stable vital inhibitory compounds in strongly inhibiting clones as cooking appears to reduce inhibition of colon cancer cells. Reports regarding the presence of salicylic acid in potato leaves in addition to our recent finding regarding the similarities in both slope of inhibitory responses and range of extract concentrations inducing inhibitory responses shared by both acetylsalicylic acid and potato may suggest that salicylic acid or a compound similar to salicylic may exist in tubers and may also be responsible for inhibitory responses.

This study has acknowledged the significance of germplasm preparation methods, germplasm source, developmental stage, dosage, cooking, harvest/top kill methods, novel and successful cell viability quantification method and potential sources of bioactive compounds that exist in the worlds' fourth largest staple crop as it relates to cancer cell culture inhibition, antioxidant capacity and total phenolic content. While cell culture studies provide insight and preliminary data, subsequent animal and human studies are required. Impacts of such studies could lead to creation of novel niche markets for immature cancer fighting tubers of specific clones, pharmacological markets

of potato extracts and/or extraction of inhibitory metabolites that may deter or reduce colon cancer incidence ultimately providing great benefits for both farmer and consumer.

Limitations. First and foremost, studies that were only conducted for one year of harvest certainly require repetition to confirm phenomena and strengthen statistical analyses. Secondly, although, cell culture models utilized in this study provided a high throughput, inexpensive and straightforward method for investigating inhibitory responses, caspase 3 protease activity responses and dosage responses against HT-29 colon cancer cells, this model system did not come without disadvantages and limitations as: (1) establishment of primary cell lines may undergo transformations misrepresenting actual cell populations; (2) differences in whole organism versus cell culture tumor microenvironment; and (3) inhibitory effects against *in vivo* models may differ from inhibitory effects produced on *in vitro* cells in culture due to metabolic activation requirements and drug-plasma binding interactions governing bioavailability.

Future Research Aims. In view of these shortcomings and in addition to new questions spawned based on results from this dissertation study, possible future research aims to be considered may involve (1) investigating the efficacy of utilizing ASA standard curve to quantify viable cells on other cancer cell lines *in vitro* screening; (2) screening of oodles of immature and mature potato clones and plant components containing diverse biochemical profiles extracted in various organic solvents in raw form and after exposure to various cooking methods (bake, boil, microwave, frying) for bioactivity against all available types of cancer cell lines *in vitro*; (3) investigating whether potato clones and plant component extracts identified in this study are capable

of inducing various caspase proteases *in vitro;* (4) investigating whether strong inhibiting potato clones and plant component extracts identified in this study are capable of inducing antiangiogenic mechanisms *in vitro*; (5) investigating the efficacy of strong inhibitory potato clones and plant component extracts identified in this study on whole organisms *ex vivo* and *in vivo* is absolutely necessary; (6) utilizing HPLC, LC/MS and GC/MS to identify and quantify differences in metabolite profiles that may exist in tuber clones demonstrating significant differences in inhibitory activity in this study of raw vs. cooked tubers; immature vs. mature tubers; peels vs. flesh vs. flowers vs. whole tubers; varying harvest/topkill treatments; weakly inhibiting pigmented clones vs. strongly inhibiting pigmented clones; and nonpigmented vs pigmented tissues in sectorial expressing selections to determine the culprit of inhibition; (7) utilizing microarray analyses to identify and quantify differences in gene regulation that may exist in immature tuber clones showing significant differences in inhibitory activity of raw vs. cooked tubers; peels vs. flesh vs. flowers vs. whole tubers; varying harvest/topkill treatments; weakly inhibiting pigmented clones vs. strongly inhibiting pigmented clones; and nonpigmented vs pigmented tissues in sectorial expressing selections to identify candidate genes responsible for production of inhibitory metabolites so they may be overproduced; (8) utilizing gene silencing methodologies, knock out identified candidate genes responsible for inhibition, should any candidate genes be identified from microarray analyses in aim 7, to test the hypotheses; (9) repeating harvest/topkill treatment studies conducted for one year and incorporating more modes of commonly used harvest/topkill treatments to determine their effects on inhibitory responses; (10) further examination as to what constituents of the tuber matrix may be involved in

protecting inhibitory metabolites against thermal degradation and screening germplasm that contain this framework.

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Appendix A

Chapter 2 Supplementary Tables

Table A.1 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Mountain Rose. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	2.38	1.63	0.2139
Process (P)	1	69.45	47.52	<.0001
Year (Y)	3	70.96	48.55	<.0001
P x Y	3	5.99	4.10	0.0157
Model	13	24.80	16.97	<.0001
Error	28	1.46		
Corrected Total	41			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.2 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Colorado Rose. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	12.38	4.97	0.0023
Process (P)	2	2.65	1.06	0.3552
Year (Y)	3	2.39	0.96	0.4221
Model	20	4.70	1.89	0.0417
Error	42	2.49		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.3 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Rio Grande Russet. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	13.88	6.79	0.0003
Process (P)	2	2.68	1.31	0.2811
Year (Y)	3	6.18	3.02	0.0402
DS x Y	4	6.87	3.36	0.0179
Model	20	7.74	3.78	0.0001
Error	42	2.05		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.4 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Purple Majesty. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.86	0.73	0.4922
Process (P)	1	173.13	146.47	<.0001
Year (Y)	3	26.57	22.48	<.0001
DS x P	1	13.53	11.45	0.0020
DS x Y	3	4.04	3.41	0.0299
P x Y	3	10.58	8.95	0.0002
Model	14	26.56	22.47	<.0001
Error	30	1.18		
Corrected Total	44			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.5 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO95712-3RU. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	56.54	80.35	<.0001
Process (P)	1	2.60	3.67	0.0665
Year (Y)	2	53.96	76.29	<.0001
DS x P	1	46.03	65.07	<.0001
P x Y	2	10.97	15.50	<.0001
DS x P x Y	1	19.39	27.41	<.0001
Model	12	19.63	27.75	<.0001
Error	26	0.71		
Corrected Total	38			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.6 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO7226-2R/R. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	7.30	4.63	0.0035
Process (P)	2	18.69	11.85	<.0001
Year (Y)	3	22.74	14.42	<.0001
DS x P	4	8.61	5.46	0.0012
DS x Y	4	5.57	3.53	0.0143
DS x P x Y	1	30.42	19.28	<.0001
Model	20	10.68	6.77	<.0001
Error	42	1.58		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.7 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO7216-1P/P. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	5.26	2.46	0.0596
Process (P)	2	19.58	9.18	0.0005
Year (Y)	3	49.26	23.09	<.0001
DS x Y	4	12.32	5.78	0.0009
P x Y	2	7.32	3.43	0.0416
DS x P x Y	1	11.95	5.60	0.0227
Model	20	14.55	6.82	<.0001
Error	42	2.13		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.8 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Russet Nugget. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	35.28	25.31	<.0001
Process (P)	1	1.11	0.79	0.3801
Year (Y)	3	10.63	7.63	0.0006
DS x Y	3	37.62	26.98	<.0001
DS x P x Y	1	5.90	4.23	0.0485
Model	14	21.02	15.08	<.0001
Error	30	1.39		
Corrected Total	44			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.9 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO97227-2P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	5.09	7.48	0.0027
Process (P)	1	96.87	142.28	<.0001
Year (Y)	3	8.21	12.05	<.0001
P x Y	2	4.62	6.78	0.0043
DS x P x Y	1	4.09	6.00	0.0213
Model	12	21.48	31.56	<.0001
Error	26	0.68		
Corrected Total	38			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.10 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO97216-3P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	31.24	10.51	<.0001
Process (P)	2	64.59	21.73	<.0001
Year (Y)	3	43.18	14.53	<.0001
Sector Purple vs White (S)	1	443.37	149.16	<.0001
DS x P	4	16.47	5.54	0.0007
DS x S	4	9.88	3.32	0.0153
DS x Y	4	16.98	5.71	0.0005
DS x S x Y	3	18.44	6.20	0.0009
Model	38	40.00	13.46	<.0001
Error	66	2.97		
Corrected Total	104			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.11 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO04056-7P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	38.00	10.96	<.0001
Process (P)	2	59.76	17.24	<.0001
Sector Purple vs White (S)	1	504.03	145.38	<.0001
Year (Y)	1	13.40	3.86	0.0563
DS x P	4	10.37	2.99	0.0300
DS x S	4	10.18	2.94	0.0322
P x S	2	46.31	13.36	<.0001
S x Y	1	28.22	8.14	0.0068
DS x S x Y	1	26.31	7.59	0.0088
Model	25	37.45	10.80	<.0001
Error	40	3.47		
Corrected Total	65			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.12 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO04058-3RW/RW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	31.25	9.12	<.0001
Process (P)	2	37.34	10.90	0.0001
Sector Red vs White (S)	1	806.44	235.48	<.0001
Year (Y)	1	0.18	0.05	0.8184
DS x P	4	49.05	14.32	<.0001
DS x S	4	8.98	2.62	0.0454
P x S	2	21.96	6.41	0.0033
P x Y	1	24.91	7.27	0.0095
Model	26	76.29	22.28	<.0001
Error	51	3.43		
Corrected Total	77			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.13 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO04061-1R/RW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	27.103	16.04	<.0001
Process (P)	2	47.412	28.06	<.0001
Sector Red vs White (S)	1	207.147	122.59	<.0001
Year (Y)	1	58.728	34.76	<.0001
DS x P	4	21.579	12.77	<.0001
P x Y	1	11.714	6.93	0.0121
Model	25	32.841	19.44	<.0001
Error	39	1.690		
Corrected Total	64			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.14 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from Mountain Rose. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.00	0.05	0.9539
Process (P)	1	0.32	14.67	0.0007
Year (Y)	3	1.10	50.90	<.0001
DS x Y	2	0.12	5.56	0.0092
P x Y	3	0.22	10.28	<.0001
DS x P x Y	1	0.18	8.11	0.0081
Model	13	0.41	18.92	<.0001
Error	28	0.02		
Corrected Total	41			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.15 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from Colorado Rose. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.96	178.68	<.0001
Process (P)	2	0.00	0.51	0.6034
Year (Y)	3	1.38	256.13	<.0001
DS x Y	4	1.24	230.18	<.0001
P x Y	2	0.00	0.40	0.6707
DS x P x Y	1	0.06	11.15	0.0018
Model	20	0.75	139.88	<.0001
Error	42	0.01		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.16 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from Rio Grande Russet. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.38	18.12	<.0001
Process (P)	2	0.04	1.76	0.185
Year (Y)	3	0.85	40.54	<.0001
DS x Y	4	0.38	18.02	<.0001
Model	20	0.38	18.11	<.0001
Error	42	0.02		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.17 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from Purple Majesty. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.30	4.73	0.0164
Process (P)	1	3.22	51.05	<.0001
Year (Y)	3	1.25	19.74	<.0001
Model	14	0.65	10.26	<.0001
Error	30	0.06		
Corrected Total	44			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.18 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO95172-3RU. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.15	13.10	0.0001
Process (P)	1	0.00	0.22	0.6441
Year (Y)	2	1.14	99.00	<.0001
DS x P	1	0.07	6.01	0.0212
DS x Y	3	0.04	3.44	0.0315
DS x P x Y	1	0.18	15.28	0.0006
Model	12	0.24	20.91	<.0001
Error	26	0.01		
Corrected Total	38			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.19 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO97226-2R/R. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.02	0.12	0.9764
Process (P)	2	2.20	16.05	<.0001
Year (Y)	3	3.11	22.70	<.0001
DS x P x Y	1	0.76	5.56	0.0231
Model	20	0.81	5.92	<.0001
Error	42	0.14		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.20 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO97216-1P/P. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.07	1.30	0.2842
Process (P)	2	2.80	51.41	<.0001
Year (Y)	3	3.03	55.73	<.0001
DS x P	4	0.21	3.93	0.0085
DS x Y	4	1.08	19.89	<.0001
DS x P x Y	1	0.49	8.97	0.0046
Model	20	1.01	18.58	<.0001
Error	42	0.05		
Corrected Total	62			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.21 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from Russet Nugget. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	1.33	10.7	0.0004
Process (P)	1	0.14	0.11	0.7372
Year (Y)	3	0.51	4.07	0.0161
DS x Y	3	0.56	4.54	0.0103
Model	13	0.57	4.57	0.0004
Error	28	0.12		
Corrected Total	41			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.22 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO97216-3P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.48	3.48	0.0121
Process (P)	2	0.81	5.89	0.0044
Sector Purple vs White (S)	1	4.10	29.74	<.0001
Year (Y)	3	1.62	11.77	<.0001
DS x S x Y	3	0.51	3.67	0.0165
Model	38	0.59	4.31	<.0001
Error	67	0.14		
Corrected Total	105			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.23 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO97227-2P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	2.92	15.99	<.0001
Process (P)	1	14.96	81.83	<.0001
Sector Purple vs White (S)	1	1.24	6.75	0.0146
Year (Y)	3	4.04	22.11	<.0001
DS x Y	2	2.75	15.02	<.0001
Model	14	2.62	14.32	<.0001
Error	29	0.18		
Corrected Total	43			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.24 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO04056-7P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.38	4.25	0.0057
Process (P)	2	2.23	24.82	<.0001
Sector Purple vs White (S)	1	6.63	73.76	<.0001
Year (Y)	1	0.25	2.78	0.1030
DS x P	4	0.24	2.66	0.0459
DS x Y	1	1.34	14.87	0.0004
P x Y	1	0.60	6.71	0.0132
Model	25	0.80	8.90	<.0001
Error	41	0.09		
Corrected Total	66			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.25 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO04058-3RW/RW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.28	6.42	0.0003
Process (P)	2	1.07	24.15	<.0001
Sector Red vs White (S)	1	8.79	198.51	<.0001
Year (Y)	1	0.04	0.82	0.3680
DS x P	4	0.91	20.55	<.0001
P x S	2	0.70	15.69	<.0001
DS x Y	1	0.41	9.31	0.0036
P x Y	1	0.42	9.46	0.0033
Model	26	0.90	20.21	<.0001
Error	52	0.04		
Corrected Total	78			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table A.26 ANOVA for gallic acid equivalents of total phenolic content of aqueous extracts from CO04061-1R/RW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.84	10.28	<.0001
Process (P)	2	1.31	16.03	<.0001
Sector Red vs White (S)	1	2.56	31.38	<.0001
Year (Y)	1	0.01	0.10	0.7494
DS x P	4	0.34	4.19	0.0065
Model	25	0.67	8.18	<.0001
Error	39	0.08		
Corrected Total	64			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

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Table A.27 Summary of statistical significance based on Tukey-Kramer HSD for gallic acid equivalents of total phenolic content of statistically significant differences (P=0.05) that exist between white tuber tissue and purple/red tuber tissues from five sectorial expressing selections. Raw and baked (170 °C for 1 hour) tuber data are based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on selection. Boiled (97 °C for 20 minutes) tuber data are based on three biological replicates for one year of harvest (2010). Data are not available for n/a because not tested. Data are not significant at P=0.05 for (-).

HARVEST STAGE	Stage II (Preharvest)	Stage III (Preharvest)	Stage IV (Preharvest)	Stage V (Harvest)	Stage V (6 Months Storage)
RAW SELECTIONS					
Significant increase in gallic acid equivalents of TP from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	2009, 2010	2010	2010	2009, 2010	2009
CO04061-1R/RW	-	-	-	-	2009
CO97227-2P/PW	-	n/a	n/a	n/a	n/a
CO04056-7P/PW	2009	-	2010	2009	2009
CO97216-3P/PW	-	-	-	-	n/a
BAKED (170°C for 1 Hour) SELECTIONS					
Significant increase in gallic acid equivalents of TP from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	n/a	n/a	n/a	-	n/a
CO04061-1R/RW	n/a	n/a	n/a	-	n/a
CO97227-2P/PW	n/a	n/a	n/a	n/a	n/a
CO04056-7P/PW	n/a	n/a	n/a	-	-
CO97216-3P/PW	n/a	n/a	n/a	-	-
BOILED (96°C for 20 minutes) SELECTIONS					
Significant increase in gallic acid equivalents of TP from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	-	2010	-	-	n/a
CO04061-1R/RW	-	-	n/a	-	n/a
CO97227-2P/PW	n/a	n/a	n/a	n/a	n/a
CO04056-7P/PW	-	-	n/a	n/a	n/a
CO97216-3P/PW	-	-	-	-	n/a

Table A.28 Summary of statistical significance based on Tukey-Kramer HSD for gallic acid equivalents of total phenolic content of statistically significant differences (P=0.05) with increasing tuber maturity that exist within a process type of 13 clones. Raw and baked (170 °C for 1 hour) tuber data are based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on selection. Boiled (97 °C for 20 minutes) tuber data are based on three biological replicates for one year of harvest (2010). Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

Cultivars and Selections	Raw Tubers	Baked Tubers	Boiled Tubers
Significant increase in gallic acid equivalents of TP with increasing tuber maturity			
Russet Nugget	-	-	n/a
Rio Grande Russet	-	-	-
CO95172-3RU	$II^a < V^b$, $V(6\ m)^b$ (2008)	-	n/a
Colorado Rose	-	-	-
Mountain Rose	-	-	-
CO97226-2R/R	-	-	-
CO04058-3RW/RW Red Sector	-	-	-
CO04058-3RW/RW White Sector	-	n/a	n/a
CO04061-1R/RW Red Sector	-	-	-
CO04061-1R/RW White Sector	-	n/a	-
CO97216-1P/P	-	-	-
Purple Majesty	-	-	n/a
CO97227-2P/PW Purple Sector	$II^a < V^b$, $V(6\ m)^b$ (2008)	-	n/a
CO97227-2P/PW White Sector	n/a	n/a	n/a
CO04056-7P/PW Purple Sector	-	-	-
CO04056-7P/PW White Sector	-	-	-
CO97216-3P/PW Purple Sector	-	-	-
CO97216-3P/PW White Sector	-	-	-
Significant decrease in gallic acid equivalents of TP with increasing tuber maturity			
Russet Nugget	$II^a > V^b$, $V(6\ m)^b$ (2008)	-	n/a
Rio Grande Russet	$II^a > V^b$, $V(6\ m)^b$ (2008)	-	-
CO95172-3RU	-	-	n/a
Colorado Rose	$II^a > V^b$, $V(6\ m)^b$ (2008)	-	$II^a > V^b$ (2010)
Mountain Rose	-	-	-
CO97226-2R/R	-	-	-
CO04058-3RW/RW Red Sector	-	-	$II^a > V^b$ (2010)
CO04058-3RW/RW White Sector	-	n/a	$II^a > IV^b$, V^b (2010)
CO04061-1R/RW Red Sector	-	-	$II^a > III^b$, V^b (2010)
CO04061-1R/RW White Sector	-	n/a	-
CO97216-1P/P	-	-	-
Purple Majesty	-	-	n/a
CO97227-2P/PW Purple Sector	-	-	n/a
CO97227-2P/PW White Sector	n/a	n/a	n/a
CO04056-7P/PW Purple Sector	-	-	-
CO04056-7P/PW White Sector	-	-	-
CO97216-3P/PW Purple Sector	-	-	$II^a > V^b$ (2010)
CO97216-3P/PW White Sector	-	-	-

Table A.29 Summary of statistical significance based on Tukey-Kramer HSD for gallic acid equivalents of total phenolic content of statistically significant differences (P=0.05) that exist between raw and baked (170 °C for 1 hour) tubers from thirteen cultivars and selections. Data are based on three biological replicates for two to four consecutive harvest years (2007-2010) dependent on clone. Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

Cultivars and Selections	Stage II (Preharvest)	Stage III (Preharvest)	Stage IV (Preharvest)	Stage V (Harvest)	Stage V (6 Months Storage)
Significant increase in gallic acid equivalents of TP from raw to bake in tubers within a developmental stage during 2007-2010					
Russet Nugget	n/a	n/a	n/a	-	-
Rio Grande Russet	n/a	n/a	n/a	-	-
CO95172-3RU	n/a	n/a	n/a	-	-
Colorado Rose	n/a	n/a	n/a	-	-
Mountain Rose	n/a	n/a	n/a	2010	-
CO97226-2R/R	n/a	n/a	n/a	-	-
CO04058-3RW/RW Red Sector	n/a	n/a	n/a	-	-
CO04058-3RW/RW White Sector	n/a	n/a	n/a	-	n/a
CO04061-1R/RW Red Sector	n/a	n/a	n/a	-	-
CO04061-1R/RW White Sector	n/a	n/a	n/a	-	n/a
CO97216-1P/P	n/a	n/a	n/a	-	-
Purple Majesty	n/a	n/a	n/a	-	-
CO97227-2P/PW Purple Sector	n/a	n/a	n/a	-	-
CO97227-2P/PW White Sector	n/a	n/a	n/a	n/a	n/a
CO04061-7P/PW Purple Sector	n/a	n/a	n/a	-	-
CO04061-7P/PW White Sector	n/a	n/a	n/a	-	n/a
CO97216-3P/PW Purple Sector	n/a	n/a	n/a	-	-
CO97216-3P/PW White Sector	n/a	n/a	n/a	-	-
Significant decrease in gallic acid equivalents of TP from raw to bake in tubers within a developmental stage during 2007-2010					
Russet Nugget	n/a	n/a	n/a	-	-
Rio Grande Russet	n/a	n/a	n/a	-	-
CO95172-3RU	n/a	n/a	n/a	-	-
Colorado Rose	n/a	n/a	n/a	-	-
Mountain Rose	n/a	n/a	n/a	2008	2009
CO97226-2R/R	n/a	n/a	n/a	-	-
CO04058-3RW/RW Red Sector	n/a	n/a	n/a	2009, 2010	2009
CO04058-3RW/RW White Sector	n/a	n/a	n/a	-	n/a
CO04061-1R/RW Red Sector	n/a	n/a	n/a	2009, 2010	-
CO04061-1R/RW White Sector	n/a	n/a	n/a	-	n/a
CO97216-1P/P	n/a	n/a	n/a	2008	-
Purple Majesty	n/a	n/a	n/a	2008	2008
CO97227-2P/PW Purple Sector	n/a	n/a	n/a	2008, 2010	2008, 2009
CO97227-2P/PW White Sector	n/a	n/a	n/a	n/a	n/a
CO04061-7P/PW Purple Sector	n/a	n/a	n/a	2009	-
CO04061-7P/PW White Sector	n/a	n/a	n/a	-	n/a
CO97216-3P/PW Purple Sector	n/a	n/a	n/a	-	-
CO97216-3P/PW White Sector	n/a	n/a	n/a	-	-

Table A.30 Summary of statistical significance based on Tukey-Kramer HSD for gallic acid equivalents of total phenolic content of statistically significant differences that exist between raw and boiled (97 °C for 20 minutes) tubers from eight cultivars and selections. Data are based on three biological replicates for one harvest year (2010). Significant differences at P=0.05 are denoted with an **X**. Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

Cultivars and Selections	Stage II (Preharvest)	Stage III (Preharvest)	Stage IV (Preharvest)	Stage V (Harvest)
Significant increase in gallic acid equivalents of TP from raw to boil in tubers within a developmental stage during 2010				
Rio Grande Russet	-	-	-	-
Colorado Rose	-	-	-	-
CO97226-2R/R	-	-	-	-
CO04058-3RW/RW Red Sector	-	-	-	-
CO04058-3RW/RW White Sector	-	-	-	-
CO04061-1R/RW Red Sector	-	-	-	-
CO04061-1R/RW White Sector	-	-	n/a	-
CO97216-1P/P	-	-	-	-
CO04056-7P/PW Purple Sector	-	-	-	-
CO04056-7P/PW White Sector	-	-	n/a	n/a
CO97216-3P/PW Purple Sector	-	-	-	-
CO97216-3P/PW White Sector	-	-	-	-
Significant decrease in gallic acid equivalents of TP from raw to boil in tubers within a developmental stage during 2010				
Rio Grande Russet	-	-	-	-
Colorado Rose	-	-	-	-
CO97226-2R/R	-	-	-	**X**
CO04058-3RW/RW Red Sector	-	**X**	-	**X**
CO04058-3RW/RW White Sector	-	-	-	**X**
CO04061-1R/RW Red Sector	-	-	-	**X**
CO04061-1R/RW White Sector	-	-	n/a	-
CO97216-1P/P	**X**	**X**	-	**X**
CO04056-7P/PW Purple Sector	-	-	**X**	-
CO04056-7P/PW White Sector	-	-	n/a	n/a
CO97216-3P/PW Purple Sector	-	-	-	-
CO97216-3P/PW White Sector	-	-	-	-

Table A.31 Summary of statistical significance based on Tukey-Kramer HSD for trolox equivalents antioxidant capacity of statistically significant differences (P=0.05) that exist between white tuber tissue and purple/red tuber tissues from five sectorial expressing selections. Raw and baked (170 °C for 1 hour) tuber data are based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on selection. Boiled (97 °C for 20 minutes) tuber data are based on three biological replicates for one year of harvest (2010). Data are not available for n/a because not tested.

HARVEST STAGE	Stage II (Preharvest)	Stage III (Preharvest)	Stage IV (Preharvest)	Stage V (Harvest)	Stage V (6 Months Storage)
RAW SELECTIONS					
Significant increase in trolox equivalents antioxidant capacity from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	2009, 2010	2010	2010	2009, 2010	2009
CO04061-1R/RW	2009	2010	-	2010	2009
CO04056-7P/PW	2009	-	-	-	-
CO97216-3P/PW	-	-	2010	2010	2008
BAKED (170°C for 1 Hour) SELECTIONS					
Significant increase in trolox equivalents antioxidant capacity from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	n/a	n/a	n/a	2009	n/a
CO04061-1R/RW	n/a	n/a	n/a	2009, 2010	n/a
CO04056-7P/PW	n/a	n/a	n/a	2009, 2010	2009
CO97216-3P/PW	n/a	n/a	n/a	2009, 2010	-
BOILED (96°C for 20 minutes) SELECTIONS					
Significant increase in trolox equivalents antioxidant capacity from white tissue to red/purple tissue in sectorial expressing tubers within a developmental stage during 2007-2010					
CO04058-3RW/RW	-	2010	2010	2010	n/a
CO04061-1R/RW	-	-	n/a	-	n/a
CO04056-7P/PW	-	2010	n/a	2010	n/a
CO97216-3P/PW	-	-	2010	-	n/a

Table A.32 Summary of statistical significance at P=.05 based on Tukey-Kramer HSD for trolox equivalents of antioxidant capacity for statistically significant differences (P=0.05) with increasing tuber maturity that exist within a process type of 13 clones. Raw and baked (170 °C for 1 hour) tuber data are based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on selection. Boiled (97 °C for 20 minutes) tuber data are based on three biological replicates for one harvest year (2010). Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

Cultivars and Selections	Raw Tubers	Baked Tubers	Boiled Tubers
Significant increase in trolox equivalents antioxidant capacity with increasing tuber maturity during 2007-2010			
Russet Nugget	-	-	n/a
Rio Grande Russet	-	-	-
CO95172-3RU	$II^a < V^b$ (2008, 2009)	-	n/a
Colorado Rose	-	-	-
Mountain Rose	-	-	-
CO97226-2R/R	-	-	-
CO04058-3RW/RW Red Sector	-	-	-
CO04058-3RW/RW White Sector	-	n/a	-
CO04061-1R/RW Red Sector	-	-	-
CO04061-1R/RW White Sector	-	n/a	-
CO97216-1P/P	-	-	-
Purple Majesty	-	-	-
CO97227-2P/PW Purple Sector	-	-	n/a
CO04056-7P/PW Purple Sector	-	-	-
CO04056-7P/PW White Sector	-	-	-
CO97216-3P/PW Purple Sector	-	-	-
CO97216-3P/PW White Sector	-	-	-
Significant decrease in trolox equivalents antioxidant capacity with increasing tuber maturity during 2007-2010			
Russet Nugget	$II^a > V^b$, $V(6\ m)^b$ (2008)	-	n/a
Rio Grande Russet	-	-	$II^a > IV^b$ (2010)
CO95172-3RU	-	$V^a > V(6\ m)^b$ (2008, 2009)	n/a
Colorado Rose	-	-	$II^a > IV^b$ (2010)
Mountain Rose	-	-	-
CO97226-2R/R	-	-	-
CO04058-3RW/RW Red Sector	-	-	$II^a > V^b$ (2010)
CO04058-3RW/RW White Sector	-	n/a	$II^a > III^b, IV^b, V^b$ (2010)
CO04061-1R/RW Red Sector	-	-	$II^a > III^b, IV^b, V^b$ (2010)
CO04061-1R/RW White Sector	-	n/a	$II^a > III^b, V^b$ (2010)
CO97216-1P/P	-	-	-
Purple Majesty	-	-	-
CO97227-2P/PW Purple Sector	$II^a > V^b$ (2008)	-	n/a
CO04056-7P/PW Purple Sector	-	-	-
CO04056-7P/PW White Sector	-	-	$II^a > III^b$ (2010)
CO97216-3P/PW Purple Sector	-	-	$II^a > III^b, V^b$ (2010)
CO97216-3P/PW White Sector	$II^a, V^a > V(6\ m)^b$ (2008); $II^a > IV^b$ (2010)	-	-

Table A.33 Summary of statistical significance based on Tukey-Kramer HSD for Trolox equivalents of antioxidant capacity for statistically significant (P=0.05) that exist between raw and baked (170 °C for 1 hour) tubers from thirteen cultivars and selections. Data are based on three biological replicates for two to four harvest years (2007-2010) dependent on clone. Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

Cultivars and Selections	Stage II (Preharvest)	Stage III (Preharvest)	Stage IV (Preharvest)	Stage V (Harvest)	Stage V (6 Months Storage)
Significant increase in trolox equivalents antioxidant capacity from raw to bake in tubers within a developmental stage during 2007-2010					
Russet Nugget	-	-	-	-	-
Rio Grande Russet	-	-	-	-	-
CO95172-3RU	-	-	-	-	-
Colorado Rose	-	-	-	-	-
Mountain Rose	n/a	n/a	n/a	-	-
CO97226-2R/R	n/a	n/a	n/a	-	-
CO04058-3RW/RW Red Sector	n/a	n/a	n/a	-	-
CO04058-3RW/RW White Sector	n/a	n/a	n/a	-	n/a
CO04061-1R/RW Red Sector	n/a	n/a	n/a	-	-
CO04061-1R/RW White Sector	n/a	n/a	n/a	-	n/a
CO97216-1P/P	n/a	n/a	n/a	-	-
Purple Majesty	n/a	n/a	n/a	-	-
CO97227-2P/PW Purple Sector	n/a	n/a	n/a	2008, 2008	2008, 2009
CO04056-7P/PW Purple Sector	n/a	n/a	n/a	-	-
CO04056-7P/PW White Sector	n/a	n/a	n/a	-	-
CO97216-3P/PW Purple Sector	n/a	n/a	n/a	-	-
CO97216-3P/PW White Sector	n/a	n/a	n/a	-	-
Significant decrease in trolox equivalents antioxidant capacity from raw to bake in tubers within a developmental stage during 2007-2010					
Russet Nugget	-	-	-	-	-
Rio Grande Russet	-	-	-	-	-
CO95172-3RU	-	-	-	-	-
Colorado Rose	-	-	-	-	-
Mountain Rose	n/a	n/a	n/a	2008	2008
CO97226-2R/R	n/a	n/a	n/a	-	-
CO04058-3RW/RW Red Sector	n/a	n/a	n/a	2010	-
CO04058-3RW/RW White Sector	n/a	n/a	n/a	-	n/a
CO04061-1R/RW Red Sector	n/a	n/a	n/a	2010	-
CO04061-1R/RW White Sector	n/a	n/a	n/a	2010	n/a
CO97216-1P/P	n/a	n/a	n/a	-	-
Purple Majesty	n/a	n/a	n/a	2007, 2008, 2010	2008
CO97227-2P/PW Purple Sector	n/a	n/a	n/a	-	-
CO04056-7P/PW Purple Sector	n/a	n/a	n/a	-	-
CO04056-7P/PW White Sector	n/a	n/a	n/a	2010	-
CO97216-3P/PW Purple Sector	n/a	n/a	n/a	-	-
CO97216-3P/PW White Sector	n/a	n/a	n/a	-	-

Table A.34 Summary of statistical significance based on Tukey-Kramer HSD for Trolox equivalents of antioxidant capacity for statistically significant differences that exist between raw and boiled (97 °C for 20 minutes) tubers from eight cultivars and selections. Data are based on three biological replicates for one harvest year (2010). Significant differences at P=0.05 are denoted with an X. Data are not available for n/a because not tested. Data are not significant for (-) at P=0.05.

Cultivars and Selections	Stage II (Preharvest)	Stage III (Preharvest)	Stage IV (Preharvest)	Stage V (Harvest)	Stage V (6 Months Storage)
Significant increase in trolox equivalents antioxidant capacity from raw to boil in tubers within a developmental stage during 2010					
Russet Nugget	-	-	-	-	-
Rio Grande Russet	-	-	-	-	-
CO95172-3RU	-	-	-	-	-
Colorado Rose	-	-	-	-	-
Mountain Rose	n/a	n/a	n/a	n/a	n/a
CO97226-2R/R	-	-	-	-	n/a
CO04058-3RW/RW Red Sector	-	-	-	-	n/a
CO04058-3RW/RW White Sector	X	-	-	-	n/a
CO04061-1R/RW Red Sector	-	-	-	-	n/a
CO04061-1R/RW White Sector	-	-	n/a	-	n/a
CO97216-1P/P	-	-	-	-	n/a
Purple Majesty	n/a	n/a	n/a	n/a	n/a
CO97227-2P/PW Purple Sector	n/a	n/a	n/a	n/a	n/a
CO04056-7P/PW Purple Sector	-	-	-	-	n/a
CO04056-7P/PW White Sector	-	-	n/a	n/a	n/a
CO97216-3P/PW Purple Sector	-	-	-	-	n/a
CO97216-3P/PW White Sector	-	-	-	-	n/a
Significant decrease in trolox equivalents antioxidant capacity from raw to boil in tubers within a developmental stage during 2010					
Russet Nugget	-	-	-	-	-
Rio Grande Russet	-	-	-	-	-
CO95172-3RU	-	-	-	-	-
Colorado Rose	-	-	-	-	-
Mountain Rose	n/a	n/a	n/a	n/a	n/a
CO97226-2R/R	-	-	-	X	n/a
CO04058-3RW/RW Red Sector	-	-	-	X	n/a
CO04058-3RW/RW White Sector	-	-	-	-	n/a
CO04061-1R/RW Red Sector	-	X	-	X	n/a
CO04061-1R/RW White Sector	-	-	n/a	-	n/a
CO97216-1P/P	-	-	-	-	n/a
Purple Majesty	n/a	n/a	n/a	n/a	n/a
CO97227-2P/PW Purple Sector	n/a	n/a	n/a	n/a	n/a
CO04056-7P/PW Purple Sector	-	-	-	-	n/a
CO04056-7P/PW White Sector	-	-	n/a	n/a	n/a
CO97216-3P/PW Purple Sector	-	X	-	X	n/a
CO97216-3P/PW White Sector	-	-	-	-	n/a

Appendix B

Chapter 3 Supplementary Tables

Table B.1 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from immature CO97216-1P/P tubers preserved via freeze drying or cryogenic grinding in liquid nitrogen. Data are means and SEM based on three biological replicates for one harvests year (2010).

Source of Variation	*df*	Mean Square	*F*	*p*
Preservation Process (P)	1	0.27	28.56	0.0001
Extract Concentration (EC)	2	0.20	21.09	0.0002
Model	5	0.13	14.39	0.0001
Error	12	0.01		
Corrected Total	17			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table B.2 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from immature CO97226-2R/R tubers preserved via freeze drying or cryogenic grinding in liquid nitrogen. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Preservation Process (P)	1	0.16	9.69	0.0098
Extract Concentration (EC)	2	0.25	14.57	0.0006
Model	5	0.14	8.10	0.0015
Error	12	0.17		
Corrected Total	17			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Appendix C

Chapter 4 Supplementary Tables

Table C.1 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO04058-3RW/RW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.11	6.55	0.0004
Process (P)	1	0.15	8.93	0.0034
Extract Concentration (EC)	2	0.60	36.27	<.0001
Year (Y)	1	0.01	0.60	0.4387
Sector Red vs White (S)	1	1.19	71.81	<.0001
DS x P	3	0.09	5.24	0.0019
DS x S	3	0.07	3.91	0.0105
P x S	1	0.18	10.79	0.0013
EC x S	2	0.28	16.95	<.0001
DS x P x S	3	0.09	5.47	0.0015
Model	59	0.12	7.05	<.0001
Error	123	0.02		
Corrected Total	182			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.2 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO04056-7P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.04	5.77	0.0011
Process (P)	1	0.00	0.03	0.8654
Extract Concentration (EC)	2	0.08	12.62	<.0001
Year (Y)	1	0.08	13.39	0.0004
Sector Purple vs White (S)	1	0.01	1.88	0.1733
DS x EC	6	0.02	3.61	0.0026
DS x Y	1	0.05	7.59	0.0069
EC x Y	2	0.02	2.99	0.0544
Y x S	1	0.08	13.36	0.0004
DS x S	3	0.04	7.37	0.0002
DS x EC x S	6	0.03	4.55	0.0004
DS x Y x S	1	0.05	7.61	0.0070
EC x Y x S	2	0.02	3.00	0.0540
Model	53	0.02	3.91	<.0001
Error	108	0.01		
Corrected Total	161			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.3 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO04061-1R/RW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.002	0.93	0.4315
Process (P)	1	0.001	0.47	0.4955
Extract Concentration (EC)	2	0.002	0.81	0.4489
Year (Y)	1	0.000	0.20	0.6544
Sector Red vs White (S)	1	0.002	1.14	0.2886
Model	56	0.002	0.90	0.6605
Error	94	0.002		
Corrected Total	150			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.4 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO95172-3RU. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	1	0.004	4.88	0.034
Extract Concentration (EC)	2	0.001	1.15	0.327
Year (Y)	1	0.000	0.03	0.874
Model	11	0.001	1.84	0.083
Error	36	0.001		
Corrected Total	47			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.5 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO97216-1P/P. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	4	0.19	12.78	< .0001
Process (P)	2	0.16	10.49	< .0001
Extract Concentration (EC)	2	0.05	3.17	0.0468
Year (Y)	2	0.01	0.38	0.6855
DS x P	4	0.06	3.94	0.0108
P x EC	2	0.10	6.44	0.0024
DS x P x EC	6	0.02	1.50	0.1860
Model	38	0.11	7.33	< .0001
Error	90	0.02		
Corrected Total	128			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.6 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO97226-2R/R. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.21	14.55	<.0001
Process (P)	1	1.06	72.40	<.0001
Extract Concentration (EC)	2	0.60	41.38	<.0001
Year (Y)	2	0.15	10.00	0.0001
DS x P	3	0.04	2.77	0.0466
DS x EC	6	0.03	2.27	0.0445
P x EC	2	0.22	14.96	<.0001
DS x Y	2	0.09	5.94	0.0038
EC x Y	4	0.08	5.63	0.0005
DS x P x EC	6	0.05	3.37	0.0050
Model				
Error				
Corrected Total				

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.7 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO97227-2P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	1	0.07	4.50	0.0423
Extract Concentration (EC)	2	0.00	0.03	0.9702
Year (Y)	1	0.09	6.03	0.0201
DS x Y	1	0.07	4.50	0.0423
Model	11	0.02	1.32	0.2595
Error	30	0.02		
Corrected Total	41			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.8 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from CO97216-3P/PW. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.02	7.94	<.0001
Process (P)	1	0.01	2.46	0.1194
Extract Concentration (EC)	2	0.04	11.68	<.0001
Year (Y)	2	0.08	26.93	<.0001
Sector Purple vs White (S)	1	0.03	10.87	0.0013
DS x Y	2	0.09	30.46	<.0001
EC x Y	4	0.02	5.90	0.0002
DS x S	3	0.02	4.92	0.0029
EC x S	2	0.01	3.20	0.0442
Y x S	2	0.11	36.28	<.0001
DS x EC x S	6	0.01	2.88	0.0114
DS x EC x Y	4	0.02	6.28	0.0001
DS x Y x S	2	0.13	43.41	<.0001
EC x Y x S	4	0.02	5.63	0.0003
DS x EC x Y x S	4	0.02	7.57	<.0001
Model	71	0.03	9.62	<.0001
Error	128	0.00		
Corrected Total	199			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.9 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from Colorado Rose. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.02	1.20	0.3133
Process (P)	1	0.00	0.00	0.9769
Extract Concentration (EC)	2	0.04	1.93	0.1515
Year (Y)	2	0.05	2.72	0.0717
Model	35	0.03	1.37	0.1231
Error	84	0.02		
Corrected Total	119			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.10 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from Mountain Rose. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	1	0.09	3.95	0.0545
Extract Concentration (EC)	2	0.04	1.48	0.2408
Year (Y)	1	0.00	0.09	0.7642
Model	11	0.03	1.36	0.2351
Error	36	0.02		
Corrected Total	47			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.11 ANOVA for % growth inhibition of HT-29 colon cancer cells by aqueous extracts from Purple Majesty. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	1	0.24	5.73	0.0220
Extract Concentration (EC)	2	0.02	0.53	0.5905
Year (Y)	1	0.06	1.44	0.2385
Model	11	0.05	1.27	0.2825
Error	36	0.04		
Corrected Total	47			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.12 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from Rio Grande Russet. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	3	0.02	0.51	0.6793
Process (P)	1	0.14	4.59	0.0350
Extract Concentration (EC)	2	0.35	11.4	<.0001
Year (Y)	2	0.09	2.83	0.0645
DS x Y	2	0.14	4.60	0.0127
Model	35	0.07	2.40	0.0006
Error	84	0.03		
Corrected Total	119			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table C.13 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by aqueous extracts from Russet Nugget. Data are means and SEM based on three biological replicates for two to four consecutive harvests (2007-2010) dependent on cultivar or selection or process.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	1	0.002	21.54	<.0001
Extract Concentration (EC)	2	0.002	21.88	<.0001
Year (Y)	1	0.002	32.08	<.0001
DS x Y	1	0.002	21.54	<.0001
DS x EC	2	0.001	15.49	<.0001
Model	11	0.001	16.81	<.0001
Error	36	0.000		
Corrected Total	47			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Appendix D

Chapter 5 Supplementary Tables

Table D.1 ANOVA for % growth inhibition of HT-29 colon cancer cells by CO97216-1PP extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.09	12.96	<.0001
Treatment (T)	3	0.02	2.75	0.0503
Plant Component (PC)	3	0.55	78.10	<.0001
Extract Concentration (EC)	5	0.11	15.65	<.0001
Year (Y)	1	0.04	5.64	0.0206
DS x EC	4	0.03	3.57	0.0109
T x EC	6	0.03	3.99	0.0019
EC x PC	9	0.11	15.36	<.0001
DS x T x EC	2	0.06	7.85	0.0009
Model	38	0.13	17.71	<.0001
Error	63	0.01		
Corrected Total	101			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.2 ANOVA for % growth inhibition of HT-29 colon cancer cells by CO97226-2R/R extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.12	16.36	<.0001
Treatment (T)	3	0.06	8.2	0.0001
Plant Component (PC)	2	0.00	0.36	0.6974
Extract Concentration (EC)	5	0.16	21.04	<.0001
DS x T	1	0.31	41.11	<.0001
T x EC	6	0.02	2.36	0.0411
DS x EC	2	0.05	6.24	0.0035
EC x PC	4	0.11	14.50	<.0001
Model	29	0.09	12.06	<.0001
Error	60	0.01		
Corrected Total	89			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.3 ANOVA for % growth inhibition of HT-29 colon cancer cells by Mountain Rose extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.00	0.07	0.9348
Treatment (T)	3	0.00	0.46	0.7082
Plant Component (PC)	1	1.34	257.16	<.0001
Extract Concentration (EC)	5	0.23	44.11	<.0001
EC x PC	2	0.07	14.23	<.0001
Model	26	0.13	24.77	<.0001
Error	54	0.01		
Corrected Total	80			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.4 ANOVA for % growth inhibition of HT-29 colon cancer cells by Purple Majesty extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2010) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.15	12.33	<.0001
Treatment (T)	3	0.01	0.51	0.675
Plant Component (PC)	2	0.20	16.73	<.0001
Extract Concentration (EC)	5	0.11	9.13	<.0001
EC x PC	4	0.18	15.18	<.0001
DS x EC	4	0.05	4.16	0.0049
Model	29	0.10	8.45	<.0001
Error	60	0.01		
Corrected Total	89			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.5 ANOVA for % growth inhibition of HT-29 colon cancer cells by Russet Nugget extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.0002	0.15	0.8596
Treatment (T)	3	0.0002	0.16	0.9221
Plant Component (PC)	2	1.365	981.49	<.0001
Extract Concentration (EC)	5	0.326	234.72	<.0001
EC x PC	4	0.026	18.66	<.0001
Model	29	0.131	94.28	<.0001
Error	57	0.001		
Corrected Total	86			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.6 ANOVA for % growth inhibition of HT-29 colon cancer cells by CO04058-3RW/RW extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one or two years (2010-2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.03	2.13	0.1234
Treatment (T)	2	0.05	3.75	0.0267
Plant Component (PC)	2	0.17	12.96	<.0001
Extract Concentration (EC)	9	0.25	19.01	<.0001
Sector (S)	1	1.32	98.80	<.0001
Year (Y)	1	0.41	30.84	<.0001
EC x PC	7	0.14	10.57	<.0001
EC x T	4	0.08	5.79	0.0003
EC x Y	5	0.08	5.95	<.0001
EC x S	2	0.03	7.72	0.0007
Model	60	0.16	11.78	<.0001
Error	109	0.01		
Corrected Total	169			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.7 ANOVA for % growth inhibition of HT-29 colon cancer cells by CO97216-3P/PW extracts in aqueous cell culture medium after 24 hours. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.00	0.11	0.8999
Treatment (T)	3	0.00	0.31	0.8183
Extract Concentration (EC)	2	0.01	1.60	0.2104
Sector (S)	1	0.02	2.06	0.1563
Model	41	0.01	0.78	0.8016
Error	57	0.01		
Corrected Total	98			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.8 ANOVA for gallic acid equivalents of total phenolic content from CO97216-1PP extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	1.22	7.38	0.0049
Treatment (T)	3	0.45	2.73	0.076
Plant Component (PC)	2	0.80	4.87	0.0213
Model	8	1.63	9.88	<.0001
Error	17	0.17		
Corrected Total	25			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.9 ANOVA for gallic acid equivalents of total phenolic content from CO97226-2R/R extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	1.42	12.45	0.0004
Treatment (T)	3	0.59	5.19	0.0092
Plant Component (PC)	2	4.16	36.39	<.0001
DS x T	1	1.01	8.87	0.008
Model	8	3.56	31.17	<.0001
Error	18	0.11		
Corrected Total	26			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table D.10 ANOVA for gallic acid equivalents of total phenolic content from CO97216-3P/PW extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	1.18	54.98	<.0001
Treatment (T)	3	0.02	0.96	0.4294
Sector (S)	1	2.52	117.33	<.0001
DS x S	2	0.36	16.88	<.0001
Model	12	0.77	35.83	<.0001
Error	20	0.43		
Corrected Total	32			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table D.11 ANOVA for gallic acid equivalents of total phenolic content from CO04058-3RW/RW extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one or two years (2010-2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Developmental Stage (DS)	1	4.90	25.3	<.0001
Treatment (T)	2	0.67	3.47	0.0508
Plant Component (PC)	2	4.66	24.05	<.0001
Sector (S)	1	3.48	17.97	0.0004
Model	9	3.13	16.15	<.0001
Error	20	0.19		
Corrected Total	29			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table D.12 ANOVA for gallic acid equivalents of total phenolic content from Mountain Rose extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.58	20.4	<.0001
Treatment (T)	3	0.28	9.73	0.0007
Plant Component (PC)	2	0.67	23.63	<.0001
DS x T	1	0.18	6.49	0.0215
Model	8	0.81	28.55	<.0001
Error	16	0.03		
Corrected Total	24			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table D.13 ANOVA for gallic acid equivalents of total phenolic content from Russet Nugget extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	0.18	3.1	0.0697
Treatment (T)	3	0.05	0.81	0.506
Plant Component (PC)	2	1.05	17.83	<.0001
Model	8	0.55	9.25	<.0001
Error	18	0.06		
Corrected Total	26			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table D.14 ANOVA for gallic acid equivalents of total phenolic content from Purple Majesty extracts. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	1.42	7.13	0.0052
Treatment (T)	3	0.16	0.8	0.5096
Plant Component (PC)	2	1.56	7.84	0.0036
Model	8	1.29	6.50	0.0005
Error	18	0.20		
Corrected Total	26			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table D.15 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO97216-1P/P. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	1.47	2.63	0.1015
Treatment (T)	3	8.08	14.48	<.0001
Tuber Part (TP)	2	3.47	6.21	0.0095
Model	8	3.55	6.35	0.0007
Error	17	0.56		
Corrected Total	25			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.16 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO97226-2R/R. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	5.79	7.05	0.0055
Treatment (T)	3	11.25	13.69	<.0001
Tuber Part (TP)	2	1.16	1.41	0.2688
DS x T	1	5.92	7.21	0.0151
Model	8	7.35	8.95	<.0001
Error	18	0.82		
Corrected Total	26			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.17 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO04058-3RW/RW. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one or two years (2010-2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	1	17.95	3.78	0.0661
Treatment (T)	2	9.73	2.05	0.1551
Tuber Part (TP)	2	43.53	9.16	0.0015
Sector (S)	1	336.34	70.81	<.0001
Model	9	69.53	14.64	<.0001
Error	20	4.75		
Corrected Total	29			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.18 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from CO97216-3P/PW. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples grown for one or two years (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	2.44	1.40	0.2703
Treatment (T)	3	13.93	7.98	0.0011
Sector (S)	1	247.32	141.79	<.0001
Model	12	30.06	17.24	<.0001
Error	20	1.74		
Corrected Total	32			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.19 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Mountain Rose. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	11.32	4.26	0.0334
Treatment (T)	3	11.77	4.40	0.0194
Tuber Part (TP)	2	19.94	7.46	0.0051
Model	8	19.07	7.14	0.0005
Error	16	2.67		
Corrected Total	24			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.20 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Russet Nugget. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	2.50	5.56	0.0132
Treatment (T)	3	4.14	9.23	0.0006
Tuber Part (TP)	2	95.28	212.25	<.0001
Model	8	41.94	93.43	<.0001
Error	18	0.45		
Corrected Total	26			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Table D.21 ANOVA for DPPH/Trolox equivalent antioxidant capacity of aqueous extracts from Purple Majesty. Data are means and SEM based on three biological replicates of immature, intermediate and mature raw tuber samples and plant components grown for one year (2011) were obtained after application of harvest/top kill treatments to include no treatment controls, vine tops cut to 10 cm in height, and chemical (95% sulfuric acid and Reglone) removal of vine tops.

Source of Variation	*df*	Mean Square	*F*	*p*
Develpomental Stage (DS)	2	2.09	8.02	0.0032
Treatment (T)	3	5.42	20.81	<.0001
Tuber Part (TP)	2	0.37	1.40	0.2718
Model	8	5.11	19.60	<.0001
Error	18	0.26		
Corrected Total	26			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value. *p*=Level of significance.

Appendix E

Chapter 6 Supplementary Tables

Table E.1 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by heated extracts and heated tubers in aqueous cell culture medium both from immature CO97216-1P/P tubers. Data are means and SEM based on three biological replicates for one harvest year (2010).

Source of Variation	*df*	Mean Square	*F*	*p*
Temperature (T)	5	0.05	7.43	<.0001
Extract Concentration (EC)	2	0.58	84.50	<.0001
Heated Extract vs. Heated Tuber (H)	1	1.40	204.97	<.0001
T x H	5	0.03	4.01	0.0029
H x EC	2	0.33	47.73	<.0001
Model	35	0.11	15.80	<.0001
Error	72	0.01		
Corrected Total	107			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.

Table E.2 ANOVA for % growth inhibition of HT-29 colon cancer cells after 24 hours by heated extracts and heated tubers in aqueous cell culture medium both from immature CO97226-2R/R tubers. Data are means and SEM based on three biological replicates for one harvest year (2010).

Source of Variation	*df*	Mean Square	*F*	*p*
Temperature (T)	5	0.05	5.35	0.0003
Extract Concentration (EC)	2	1.24	133.94	<.0001
Heated Extract vs. Heated Tuber (H)	1	0.54	58.43	<.0001
T x EC	10	0.02	2.31	0.0202
H x EC	2	0.13	13.61	<.0001
Model	35	0.11	12.08	<.0001
Error	72	0.01		
Corrected Total	107			

ANOVA=Analysis of Variance. *df*=degrees of freedom. *F*=F-value.
p=Level of significance.