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Anticancer Activity Of Phytolacca Americana Root Extracts And Their Fractions On Breast And Colon Cancer Cells.

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ANTICANCER ACTIVITY OF *PHYTOLACCA AMERICANA* ROOT EXTRACTS AND THEIR FRACTIONS ON BREAST AND COLON CANCER CELLS

by

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ABSTRACT

Wishon, Lisa Maness. ANTICANCER ACTIVITY OF *PHYTOLACCA AMERICANA* ROOT EXTRACTS AND THEIR FRACTIONS ON BREAST AND COLON CANCER CELLS. **(Major Advisor: Ipek Goktepe)**, North Carolina Agricultural and Technical State University.

Phytolacca americana is an herbaceous plant native to North and South America and East Asia. Pokeweed antiviral protein (PAP) has been extracted from this plant and antiviral activity toward *Chenopodium quinoa* and other viruses has been cited. Conjugated to various monoclonal antibodies, PAP has been shown to inhibit HIV-1 replication and to arrest the proliferation of B-lineage Acute Lymphoblastic Leukemia blasts. Since studies involving the activities of *P. americana* extracts on various cancer cell lines are limited, the major objectives of this study were to: 1) evaluate the antiproliferative activity of three extracts from *P. americana* against human breast (MCF-7) and colon cancer (HCT-116) cells *in vitro* and 2) investigate the changes at the protein and gene levels after exposing the HCT-116 cells to *P. americana* extracts *in vitro*.

Antiproliferative activities of crude ethanol (PRE), methanol, and water extracts of *P. americana* against HCT-116 and MCF-7 cells were determined using the MTT assay. PRE was fractionated and the fractions were tested for their antiproliferative activities. For comparison, the antiproliferative activity of PAP was also tested. Changes in levels of caspase 2, 3, 6, 8, and 9 activities were determined in HCT-116 cells exposed to PRE and its most active fraction (PREW). The Human Cancer Pathway Finder Realtime PCR Profiler was used to determine changes in activities of 84 genes in HCT-116 cells exposed to PRE and PREW.

PRE had a greater antiproliferative effect ($P \leq 0.05$) on HCT-116 cells than the methanol and water extracts. None of the extracts showed a significant antiproliferative activity against MCF-7 cells ($P \geq 0.05$). The water fraction of PRE (PREW) showed the greatest antiproliferative activity compared to the ethyl acetate and butanol fractions ($P \leq 0.05$). The effect of PAP on the proliferation of HCT-116 cells fluctuated depending on the concentration.

Caspases 6 and 9 showed increases in activity ($P \leq 0.05$) in HCT-116 cells exposed to PRE. Caspases 3, 8, and 9 had increases in activity in HCT-116 cells exposed to PREW. For the Cancer Pathway Finder, PRE at 3200 µg/ml had the most desirable gene changes in the treatment of colon cancer.

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This is to certify that the Doctoral Dissertation of

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has met the dissertation requirements of
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Greensboro, North Carolina
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DEDICATION

I dedicate this dissertation to women around the world who live in regions where they have no access to education. It is my hope to assist in this cause. In addition, this work is dedicated with love to my daughters, Casey and Madeline, who also realize the value of education.

BIOGRAPHICAL SKETCH

Lisa Maness Wishon was born on April 19, 1974 in Greensboro, NC. She received a Bachelor of Science Degree in Biology in 1996 from the University of North Carolina at Greensboro (UNCG). Lisa also earned a Bachelor of Science Degree in Medical Technology in 1997 and a Master of Science Degree in Biology in 2003 from UNCG. Before returning to obtain her Doctor of Philosophy, she was a medical technologist and a high school teacher. While attending North Carolina Agricultural and Technical State University, she became a member of the honor society of Phi Kappa Phi and Gamma Sigma Delta, honor society for agriculture, and was a Wadaran L. Kennedy 4.0 Scholar. Lisa is a candidate for the Ph.D. in Energy and Environmental Systems.

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SYMBOLS AND ABBREVIATIONS

ACS	American Cancer Society
AGS	Human Gastric Carcinoma Cells
AIF	Apoptosis Inducing Factor
ALL	Acute Lymphocytic Leukemia
APF	ATP-dependent proteolysis factor
ATP	Adenosine Triphosphate
A549	Adenocarcinomic Human Alveolar Basal Epithelial Cells
BALB/c	Albino, Laboratory Strain of the House Mouse
B43	Anti-CD19
Caco-2	Human Colon Adenocarcinoma Cells
CAM	Complementary and Alternative Medicine
cDNA	Complementary DNA
CD7	Cluster of Differentiation 7
CD19	Cluster of Differentiation 19
CIN	Chromosomal Instability
Colo205	Human Colon Cancer Cells
Colo38	Human Melanoma Cells
dATP	Deoxyadenosine Triphosphate
DFF	DNA Fragmentation Factor
DLD-1	Human Colon Carcinoma Cells

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EF-1	Elongation Factor 1
EF-2	Elongation Factor 2
E1600	*Phytolacca americana* Ethanol Extract at 1600 μg/ml Concentration
E3200	*Phytolacca americana* Ethanol Extract at 3200 μg/ml Concentration
FBS	Fetal Bovine Serum
GnRH	Gonadotropin-Releasing Hormone
GTP	Guanosine Triphosphate
HBCC	Hereditary Breast and Colorectal Cancer
HCl	Hydrochloric Acid
HCPFRPCR	Human Cancer Pathway Finder Realtime PCR
HCT-116	Colorectal (Colon) Carcinoma Cells
HCT15	Human Colorectal Carcinoma Cells
HeLa	Henrietta Lacks Cervical Cancer Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human Hepatoma Cells
Hep3B	Human Hepatoma Cells
HIV	Human Immunodeficiency Virus
HL-60	Human Promyelocytic Leukemia Cells

HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HT29	Human Colon Adenocarcinoma Cells
H1650	Non-small Cell Lung Cancer Cells
KATO-III	Human Gastric Carcinoma Cells
KB	Human Mouth Epidermal Carcinoma
K562	Myelogenous Erythroleukemia Cells
L1210	Murine Lymphocytic Leukemia Cells
L3	Ribosomal Protein
McA-RH8994	Rat Hepatoma Cell Line
MCF-7	Mammary (Breast) Adenocarcinoma Cells
MDA-231	Mammary (Breast) Adenocarcinoma Cells
MEL7	Human Melanoma Tissue
MEL14	Human Melanoma Tissue
MMR	Mismatch Repair
MSI	Microsatellite Instability
MTT	3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrazolium bromide
NADH	Glutamate Synthase
NaDOC	Sodium Deoxycholate
PAP	Pokeweed Antiviral Protein
PC-SPES	Prostate Cancer Treatment
PRE	*Phytolacca americana* Crude Root Ethanol Extract
PREW	Water Fraction of PRE

PRM	*Phytolacca americana* Crude Root Methanol Extract
PRW	*Phytolacca americana* Crude Root Water Extract
PSA	Prostate Specific Antigen
pT3/3	Tumor Stage
P388	Murine Leukemia Cells
RG3	Main Component Isolated From Gensing
RIP	Ribosome Inactivating Protein
RNAi	RNA Interference
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RSV	Resveratrol
SAOS	Sarcoma Osteogenic
SCID	Severely Combined Immunodeficient
SMAC	Second Mitochondria-Derived Activator of Caspases Protein
SW480	Human Colon Adenocarcinoma Cells
TRAPS	TNF Receptor-Associated Periodic Syndrome
t(4,11)	Chromosomal Translocation of Chromosomes 4 and 11
UDCA	Ursodeoxycholic Acid
UICC	Union for International Cancer Control
U937	Human Leukemic Monocyte Lymphoma Cells
w/v	Weight/Volume
W1600	*Phytolacca americana* Water Fraction of Ethanol Extract at 1600 μg/ml Concentration

W800	*Phytolacca americana* Water Fraction of Ethanol Extract at 800 μg/ml Concentration
XF498	Human Central Nervous System Tumor Cells
2780 AD	Human Ovarian Carcinoma Cells
28s	rRNA Component of Large Ribosomal Subunit in Eukaryotes
293T	Human Embryonic Kidney Cells

CHAPTER 1

INTRODUCTION

Cancer is the uncontrolled growth of abnormal cells in the body. Cancer tumors can occur in various parts of the body and the symptoms may vary according to the location where the tumor initially starts growing. For example, a person with colon cancer may experience diarrhea, constipation, and blood in the stool (Medline Plus, 2010). Someone with breast cancer may experience changes in breast size, bloody discharge from the nipple, or redness and peeling of the skin.

As of 2006, cancer was still the second leading cause of death in the United States, comprising 23% of all deaths, only 3% below deaths from heart diseases (American Cancer Society (ACS), 2009). The type of cancer that causes the most deaths in both men and women is lung cancer, while the most common type of cancers in men and women are prostate cancer and breast cancer, respectively. The second and third most common causes of cancer deaths in women are breast and colon cancer, respectively. In men, colon and prostate cancer are the second and third most common causes of cancer deaths while pancreatic cancer falls close behind (ACS, 2009).

Cancer may be caused by different factors, depending on the type. General causes are as follows: exposure to the sun's ultraviolet light, radon, nuclear radiation, or x-rays, use of tobacco and alcohol, the use of certain medicines or chemicals, diets high in fat content, and polluted environments.

Current cancer treatments include chemotherapy, radiotherapy, and surgery (ACS, 2009). Chemotherapy may be used when the physician believes the cancer can be cured. It involves the use of drugs, in the form of pills, shots, or intravenous injection, designed to circulate and kill cancer cells. There are side effects to chemotherapy such as bone marrow loss, hair loss, fertility problems, and fatigue.

Radiation therapy works by causing DNA mutations, resulting in an inability of cells to grow and divide (ACS, 2009). It is a local treatment and only cells around the diseased area are affected. There is a slight risk that secondary cancers from the treatment may occur 5 to 20 years later, but this is a lower risk than the patient dying from the original cancer. Risk factors must be weighed against possible outcome and other treatment options. Additionally, radiation treatment can also pose a risk to people with which the patient comes in contact.

Surgery may be used to remove benign and malignant tumors and may be curative or debulking, depending on whether all of the tumor or only part of the tumor is removed (ACS, 2009). Surgery is most often used to attempt to cure cancers that have not spread through the entire body. It may be used preventatively, diagnostically, or for palliative reasons of making the patient more comfortable. In addition to chemotherapy, radiation, and surgery, there are alternatives to these standard types of treatment options.

One group of alternative methods is complementary and alternative medicine known as CAM (Ernst, 1998). These treatments may be in the form of juices, herbal remedies, vitamins, counseling, meditation, relaxation, and diet changes. The use of CAM among cancer patients is common around the world, but the exact prevalence is

unknown due to inconsistencies in reporting and unclear definitions of what should be considered as CAM. Scientific research has been ongoing worldwide on the effects of various types of CAM, and although oncologists are not always familiar with CAM, their attitudes toward it are not as negative as had been previously reported. It has been predicted that as cancer rates increase, the use of CAM will also increase (Richardson et al., 2000).

In one study consisting of 882 cancer patients filling out a questionnaire, 99.3% of patients had heard about CAM, while 88% combined CAM with their conventional therapy, with only 61.8% discussing CAM with their physicians (Richardson et al., 2000). Seventy-six percent of patients chose to combine CAM in the form of vitamins or herbal treatments with their conventional method of treatment given by the physician. These statistics concerning the use of CAM are important, since there is evidence that there may be interactions between the conventional treatments and the herbal treatments that are often not discussed with the physician.

One study stressed the importance of physicians having knowledge of CAM use by compiling a literature review of interactions that occur among the most common herbal remedies and the most common conventional cancer therapies (Sparreboom et al., 2004). The study concluded that the majority of the common herbal remedies do have potential pharmacokinetic interactions with anticancer drugs. It is interesting to note that some of these herbal remedies have been used as treatments for centuries by native people of various countries, and scientific studies are emerging that in some cases support their antiproliferative effects. For example, traditional Chinese and American Indian

medicines are rapidly gaining acceptance in the west through increased understanding of their molecular basis of action and through successful animal and human trials (Parekh et al., 2009).

Phytolacca americana, commonly known as pokeweed, is an herbaceous perennial plant that usually grows up to 10 feet tall. The leaves are single and alternate with crinkled edges, the flowers are greenish-white, and the stems are often red or pink. The plant is most commonly recognized by its dark purple berries, which have been used as dyes and for ornamental purposes.

P. americana has been traditionally used in North and South America and in Asia as a laxative, to induce vomiting, and to treat inflammation and rashes. Anti-carcinogenic and anti-viral properties of this herb have been investigated (Schlick et al., 2000; Zarling et al., 1990). Much about the gene and protein structure of *P. americana* has been discovered, especially of pokeweed antiviral protein (PAP). However, the effects of extracts of the herb on HCT-116 colon cancer and MCF-7 breast cancer cells have not been studied.

The objectives of this study were to: 1) investigate the inhibitory activity of *Phytolacca americana* root extracts against the proliferation of HCT-116 and MCF-7 cancer cells *in vitro*; 2) test the antiproliferative effect of the fractions isolated from the most active extract of *P. americana* roots; 3) compare the inhibitory activity of the most active *P. americana* root extract against HCT-116 cells to that of pure pokeweed antiviral protein (PAP); 4) conduct apoptosis assays to understand the mechanism of action of the most potent *P. americana* root extract and its most active fraction against HCT-116 cells;

and 5) determine the changes that occur at the level of gene expression after HCT-116 cells are exposed to the most potent *P. americana* root extract and its most active fraction.

CHAPTER 2

LITERATURE REVIEW

Use of Herbal Remedies in Cancer Treatment

In searches for new cancer chemopreventive agents over the past several years, hundreds of plant extracts have been evaluated for their potential to inhibit cancer cells *in vitro*. The aqueous solution of *Astragali radix*, an ancient Chinese remedy, has been tested in the laboratory concerning its inhibitory effects on the cell growth of various cancer cell lines (Lin et al., 2003). The cell lines tested were gastric cancer cell lines AGS and KATO-III, colon cancer cell line HT29, breast cancer cell line MDA-231, and melanoma cell lines MEL7 and MEL14. The greatest inhibition of cell growth was on the gastric cancer cell lines with 68% inhibition of AGS and 62% inhibition for KATOIII. The reduction of the growth of AGS cells was shown to be concentration and time-dependent. Further, when the AGS cells were tested for evidence of apoptosis, no DNA ladder resulted after gel electrophoresis. The authors concluded that the growth inhibitory activity of *A. radix* against AGS is not due to apoptosis.

Paeoniae radix, commonly known as red peony, has been used to treat liver diseases in China for centuries without explanation of an underlying mechanism for its apparent activity. Human hepatoma cell lines HepG2 and Hep3B were inhibited after exposure to *P. radix* by the induction of apoptosis through a pathway independent of p53 (Lee et al., 2002). The inhibition was shown to be tumor protein p53 independent since HebG2 and Hep3B cell lines were both affected, and the latter cell line is deficient of p53.

Uncaria tomentosa, a species of Rubiaceae commonly called cat's claw, has been traditionally used in South America for the treatment of inflammatory conditions, arthritis, and cancer. The effects of extracts from the bark of this plant on the growth of MCF-7 breast cancer cells have been determined (Riva et al., 2001). Extraction methodology resulted in 2 fractions from the bark, both of which resulted in antiproliferative effects with up to 90% inhibition. In a separate study, inhibitions were also detected as well as increases in caspase 3 activity when the extracts of *U. tomentosa* were applied to MCF-7 cells, SAOS (sarcoma osteogenic) human osteosarcoma cells, and HeLa cells (De Martino et al., 2006). Although the inhibition of HeLa cells was greater than the inhibition of the SAOS or MCF-7 cells, all cells showed the inhibition in a dose-dependent manner. Further, apoptotic evidence was greatest in the HeLa cells.

A medicinal plant from Bangladesh, *Aegle marmelos*, has been shown to inhibit proliferation of a variety of cancer cell lines (Lampronti et al., 2003). Among the cell lines tested in this study were leukemic cell line K562, T-lymphocyte Jurkat cells, MCF-7 breast cancer cells, and melanoma Colo38 cells. Three extracts obtained from *A. marmelos* were the most promising in their antiproliferative effects on K562 leukemia cells, although all cell lines tested were inhibited by these extracts in a dose-dependent manner.

Panax quinquefolius, American Gensing, has been tested for its antiproliferative activities of colorectal cancer (Wang et al., 2008). Gensinosides including protopanaxadiol and protopanaxatriol have been found to be the most active groups. It has also been found that steam treatments may actually enhance those anticancer effects.

Silymarin is a flavonolignan from *Silybum marianum*, milk thistle, that interferes with cell cycle regulators and apoptotic proteins (Ramasamy et al., 2008). It inhibits epidermal growth factor receptor (EGFR) signaling along with suppressed expression of cyclin-dependent kinase (CDK). There is a coinciding upregulation of CDK inhibitors, CDK-interacting protein 1 (p21) and CDK inhibitor 1B (p27), and growth arrest at G1 and G2. The presence of silymarin leads to apoptosis through the mitogen-activated protein kinase (MAPK)/C-JUN N-terminal kinase (JNK) pathway. It has also shown activity against metastasis through its most active compound, silibinin. Research has shown that silymarin and silbinin are promising options in cancer prevention and treatment.

Another naturally-occurring compound, in this case a phytoalexin known as resveratrol, can be found in grapes and wine and has been found to have anticancer activity. It was found to inhibit human umbilical vein endothelial cell growth and increase gelatinolytic activities of matrix metalloproteinase-2 (Cao et al., 2005). It was also found to be an angiogenesis inhibitor in rat aorta matrix. These characteristics make herbs containing this compound ideal for further studies on cancer treatments.

Berberine, an isoquinoline alkaloid extract from the genera *Berberis*, also known as pepperidge bush, and *Coptis*, commonly called goldthread, has been reported to be a Cyclooxygenase-2 (COX-2) inhibitor, thereby restraining the growth of DLD-1 colon cancer cells. The transcription of the COX-2 gene which codes for the enzyme that plays an important role in the tumorigenesis of colon cancer cells is suppressed (Fukuda et al., 1999). It had previously been reported that the cyclooxygenase-2 (COX-2) enzyme,

which is enhanced by the expression of oncogenes such as sarcoma viral oncogene homolog (V-SRC), Harvey rat sarcoma viral oncogene homolog (V-HA-RAS), and wingless-type MMTV integration site family (WNT), is activated in colon cancer cells, resulting in an increase in prostaglandins (Sheng et al., 1997).

It has also been proposed that COX-2 inhibitors, such as berberine, could help to inhibit breast cancer cells (Howe et al., 2001). Supporting this idea are studies of another COX-2 inhibitor, nimesulide®. This hepatoxic, non-steroidal, anti-inflammatory drug was shown to result in a small decline in the incidence of tumors as well as reduce the multiplicity and size of mammary tumors in rats (Watanabe et al., 2000). Another COX-2 inhibitor, celecoxib®, was also shown to reduce incidence, volume, and multiplicity of malignant breast tumors in rats.

Mixtures of herbs have also been used in the treatment of cancer. For example, PC-SPES, is a commercially available mixture of 8 herbs: chrysanthemum, licorice, isatis, scutellaria, saw palmetto, *Panax pseudo-gensing*, *Rabdosia rubescens*, and *Ganoderma lucidum*. It was found that in patients with prostate cancer, PC-SPES decreases testosterone levels during use and that the levels increase 3 weeks after use is discontinued (DiPaola et al., 1998). In all 8 patients who participated in the study, PSA (prostate specific antigen) levels had a concomitant decrease after the first treatment of PC-SPES whether their PSA concentrations were high or low at the initial stage of the study. The concentration of prostate specific antigen increased within 3 weeks after the treatment was halted. Furthermore, PC-SPES has been shown to inhibit the growth of MCF-7 cells in cell culture (Halicka et al., 1997).

In addition to herbal extracts, mixtures of herbs, and specific compounds isolated from herbs, oils from plants have also been tested for their cancer fighting properties (Manosroi et al., 2006). One study tested 17 Thai medicinal plants against human mouth epidermal carcinoma KB cell line and murine leukemia P388 cell line. Of those 17 plants, oil from leaves of *Psidium guajava* L., guava, showed the greatest antiproliferative effect against the KB cell line, while oil from *Ocimum basilicum* L., sweet basil, had the greatest antiproliferative effect for the P388 cell line.

Phytolacca americana

Phytolacca americana is an herbaceous perennial plant, native to North and South America and East Asia, which grows up to 10 feet tall. It grows well in sun or shade and is often found in edge habitats. Although the young shoots and leaves can be cooked and eaten, the berries, roots, and mature plants are considered poisonous. The plant contains 2 primary toxins, phytolaccatoxin and phytolaccigenin, which are known to upset the gastrointestinal tract.

Native Americans used various parts of *P. americana* as a laxative to treat inflammation and rashes and to induce vomiting. More recently, the anti-carcinogenic and anti-viral properties of *P. americana* have been investigated (Schlick et al., 2000; Zarling et al., 1990). Much has been researched concerning its gene and protein structures as well.

Pokeweed Antiviral Proteins (PAP): Structure and Function

Several inhibitors of translation have been described as coming from *P. americana*. Pokeweed antiviral proteins, PAPI, PAPII, PAPIII, and PAP-S, can be extracted from the leaves during spring, early summer, and late summer, and from the seeds, respectively (Misawa et al., 1975; Ussery et al., 1977). Several studies have been done to identify and compare the structures of the various PAPs.

PAPI and PAPII have been sequenced and aligned with results indicating that there are 10 of 29 identical residues between the 2 proteins (Bjorn et al., 1984). PAP-S has been characterized as having 80% amino acid homology with PAP (Barbieri et al., 1982). Although PAP-S is similar to PAPI and PAPII, it is most similar to PAPI than PAPII. It has been found that the crystal structure of PAP-S is 1.8Å and that it is comprised of one chain of RIP along with three *N*-acetylglucosamines, each of which are linked to a different asparagine (Zeng et al., 2003). Each of the mono-sugars contains either an α-conformation or a β-conformation. It was found that there are no interactions between the polypeptide chains and the sugars with the exception of one hydrogen bond. Interestingly, the major difference between PAP-S and previously known PAPs is the differences in charge of one region of the molecules.

Similarly, the structure of PAPIII was modified by reducing the methylation of its residues of lysine, which thereby enabled X-ray diffraction studies (Kurinov and Uckun, 2003). The structures were reported and compared with the PAPI. Its active site was determined to contain Tyr69, Tyr117, Glu172, and Arg175, as in PAP1.

PAP, composed of 8 α-helices and a β sheet of 6 strands (Kurinov et al., 2000), are single chain ribosome-inactivating proteins (RIPs) that work by N-glycosidase activity to remove an adenine base from a conserved portion of the rRNA in eukaryotes and prokaryotes. The protein is an enzyme of 29,000 monomers which weakly binds to the cell wall of the leaf of the plant (Ready et al., 1986). If the cell wall and membrane are broken, the enzyme enters the cytoplasm and inhibits protein synthesis, most likely in viral defense.

RIPs impede protein synthesis by blocking the binding and activity of GTPase of the elongation factors EF-1 and EF-2 (Osborn and Hartley, 1990). There are two types of RIPs, types I and II. Type I includes pokeweed antiviral protein as well as trichosanthin, which is derived from a flowering plant. Type II includes saporin from soapwort and ricin from castor beans.

There is evidence that PAP inhibits viruses such as the cucumber mosaic viral infections of *Chenopodium quinoa,* goosefoot, (Tomlinson et al., 1974) and the brome mosaic virus directly *in vitro* (Picard et al., 2005). PAP has been shown to inhibit the replication of human viruses such as Hepatitis B virus alone and in association with the plasmid pXF3H (He et al., 2008) and herpes simplex virus type I (Barbieri et al., 1982).

It has also been reported that ribosomal inactivating proteins increase in leaves of *Phytolacca americana* that are themselves senescent (Stirpe et al., 1996). In the presence of environmental changes or natural stresses such as increases in heat, there is an increase in the release of adenine from DNA by the RIPs. Therefore, it has been suggested that

the effect of the toxin on DNA could be related to the apoptosis that is induced in plant cells.

It has been shown that the ribosomal protein L3 is required for depurination, because yeasts with mutations of this protein were found to be resistant to cytotoxic effects of PAP (Hudak et al., 1999). Those with a maintenance of killer (MAK) 8-1 allele of peptidyltransferase-linked ribosomal protein were not depurinated in the presence of PAP activation. Since the α-sarcin loop is the substrate upon which PAP targets, mutations in or near this region are likely to inhibit the function of the protein.

In other studies involving mutant alleles, PAP sequences were placed into plasmids in *Saccharomyces* cerevisiae (Hur et al., 1995). When a point mutation was induced at Glu-177, the enzyme was inactivated. However, point mutations created at the N-terminus resulted in a protein that was still active. Findings of these studies indicated that PAP toxicity involves several steps and different protein domains.

Recent work disputes the idea that antiviral activity of PAP is exclusively due to depurination of an adenine base (Hudak et al., 2000). It was shown that inhibition of translation of brome mosaic virus and potato virus X by PAP occurred without depurination. Translation was inhibited despite using mutants that are not able to depurinate tobacco or reticulocyte lysate rRNA. It was also shown that translation of capped transcripts were inhibited whereas uncapped were not, indicating that PAP can decipher the differences. The authors concluded that inhibition of translation can occur by methods other than depurination by recognizing capped rRNAs. They also noted that depurination occurs in locations other than in the sarcin/ricin loop. Whatever the

mechanism, protein inhibition that results from PAP has been found to be irreversible (Irvin and Uckun, 1992).

It was demonstrated that PAP is synthesized as a precursor and is then subjected to 2 proteolytic steps (Hur et al., 1995). There is evidence that both PAP precursors and mature forms are present in the endoplasmic reticulum (ER) in yeast (Parikh et al., 2005). The mature form is moved from the ER into the cytosol where it escapes degradation. The C-terminal signal mediates this transport of PAP to the cytosol and has itself been identified (Baykal and Tumer, 2007). It is thought that the sequences at the C-terminus responsible for this transport is similar between types I and II RIPs, since the sequence is conserved.

Although important in transport, the C-terminal end may not be necessary for depurination as was shown to be the case for trypanosome ribosomes (Ayub et al., 2008). In addition, PAP likely does not require an intact stalk to depurinate the sarcin/ricin loop (Chiou et al., 2008). Depurination eventually occurs but is delayed in mutants of the asparagine residue at position 70, although cap binding still occurs. This mutation was found to change the active state of PAP, which would allow for a decreased number of substrates and reduced toxicity.

Interestingly, it has been shown that PAP cleaves double-stranded DNA in the same active sites that are required for depurination of rRNA (Wang et al., 1999). In addition to regulating the large rRNA, it also targets its own mRNA (Parikh et al., 2002). This activity depends on the N-glycosidase activity of PAP since a mutant of the active site does not alter its own mRNA levels.

One study attempted to determine the regions of the PAP gene that are important to the function of the active protein by causing deletions of the gene and cloning into *Escherichia coli* (Xu et al., 1998). It was found that the gene coding for the mature protein cannot be expressed in *E. coli* since the protein serves to impair ribosome activity. When more than 123 codons of the N-terminal region of PAP were deleted, there was a resulting inactivation of antiviral and ribosomal inhibition, and, thus, the PAP gene could be expressed in the bacteria. When deletions were made in amino acids 7 through 107, cells did not survive. Further, it had been thought that Tyr94 and Val95 were involved in the binding of adenine bases. However, in this study it was found that the deletion of these amino acids does not lead to inactivity of PAP.

Transgenic PAP Expression

PAP expression in transgenic tobacco and potato plants was shown to render their resistance to a broad spectrum of plant viruses (Lodge et al., 1993). This method was found to be an improvement over other methods used for defending plants against viruses, because they allowed resistance only to specific viruses using coat protein genes. The low levels of PAP expression required to produce virus-resistance resulted in phenotypically normal plants.

Transgenic PAP expression has also been produced in *Agrostis palustris*, creeping bentgrass, since the grass is susceptible to fungal diseases, and PAP has been found to have antifungal activity (Dai et al., 2003). Three PAP proteins have been transformed into the bentgrass, PAP-Y and PAP-C, both of which are nontoxic mutants, and PAPII.

PAPII transformants were found to have no symptoms of toxicity, and one low-expressing line did have resistance to disease. The PAP-C transformant did not accumulate protein, while the PAP-Y transformant accumulated protein and appeared normal, but as of the time of the study, had not been field tested for resistance.

A novel ribosome-inactivating protein, termed PAP-H, was developed from the hairy roots of *P. americana* transformed from *Agrobacterium rhizogenes* (Park et al., 2002). The exudates were enhanced by ethylene elicitation and were shown to have strong antifungal activity by inhibiting fungal ribosome activity. It was shown that maximum protein accumulation occurs from the roots before the end of the exponential phase of root growth with PAP antibody cross-reactivity increasing during the early stages of growth. The N-terminal amino acid region of PAP-H was sequenced and determined to have 61% homology to PAPI and 56% homology to PAP-S.

Other Substances Isolated from *P. americana*

Various extraction methods are known to yield different products. For example, in experiments performed on *Equisetum arvense*, commonly known as horsetail, more phenolic compounds were extracted in ethanol than in the water, whereas more proteins were released from the plant into the water than into the ethanol (Nagai et al., 2005). Using water for extraction yields water-soluble extracts, while extraction with alkali solutions results in water-insoluble extracts (Mizuno, 1996). Chemically fractionating the crude extracts after processing often results in a variety of groups that have different functions from one another (Lindholm, 2002).

Other substances besides PAP have been extracted from the stems and leaves of *P. americana* through various extraction methodologies, and the products described. For example, the seeds are known to contain various compounds such as triterpenes, glycosides, and neolignans. Futhermore, several 1,4-benzo-dioxane-type compounds, such as americanoic acid methyl ester, isoamericanoic acid A methyl ester, and 9'-*O*-methylamericanol A, were isolated through methanol extraction (Takahashi et al., 2003). Complex flavonoids and free phenolic acids have been isolated from the leaves by spectroscopy, chromatography, and chemical methods (Bylka and Matlawska, 2001). Spectroscopic data allowed the structure of triterpene glycoside 1 to be described and the data from its NMR has been compared with that of triterpene glycosides 2-7 (Takahashi et al., 2001).

An antimicrobial protein known as Pa-AMP-1 was found only in seeds and not roots, leaves or stems. It has been described as having a cysteine-knot fold and hydrophobic surface (Liu et al., 2000). Mutations were introduced into each of the domains to determine the functions that are thereby lost (Peng et al., 2005). It was discovered that the hydrophobic surface allows interaction of the protein with the plasma membrane lipid raft of fungi, thus providing the seeds with their antifungal activity.

^{1}H nuclear magnetic resonance has allowed the secondary structure and cysteine pairings of a highly basic pokeweed antifungal peptide from seeds (PAFP-S) with a molecular mass of 3929 to be resolved (Gao et al., 2001). It was found to be a highly stable cationic peptide containing 38 amino acid residues with a triple-stranded antiparallel β-sheet interspersed with disulfide bridges. The antifungal activity varied

among various fungi tested and yet showed no inhibition toward *E. coli* (Shao et al., 1999).

Since the dried roots have been used as a traditional Chinese herbal remedy for treating tumors, edema, bronchitis, and abscesses, interest has been shown in isolating compounds from this part of the plant as well. Five new triterpene saponins and seven previously discovered saponins were recently isolated from the roots and their structures were characterized (Wang et al., 2008). In addition, there are at least six known lectins derived from the roots of *P. Americana*: PL-A, PL-B, PL-C, PL-D1, and PL-D2. Lectin C, which contains three chitin-binding domains, has been analyzed by crystallization and x-ray techniques and the structure has begun to be determined (Hayashida et al., 2003a). PL-D2 contains two chitin-binding domains and has been compared to lectin C. It was found that three aromatic residues in the carbohydrate binding sites of PL-C correspond to and mimic the residues that interact with the trisaccharide in the PL-D2 complex (Hayashida et al., 2003b). Phytolacain G has also been isolated from the roots as well (Susner et al., 2004). It is a cysteine protease with lectin-like activities that include mitogenic effects to lymphocytes and haemagglutination.

PAP as Immunotoxins

One method that is used to introduce *P. americana* toxins to tumor cells is through its binding to a hormone or other protein in the form of an immunotoxin (Schlick et al., 2000). For example, a construct of a bacterial plasmid encoding PAP with gonadotropin-releasing hormone (GnRH) has been formulated, since GnRH has receptor

sites on several tumor lines. This complex was shown to inhibit the growth of Ishikawa cell line by inhibiting protein synthesis in a dose-dependent manner. The purified PAP inhibited protein synthesis less than the complex, while either part of the complex alone had no effect on tumor growth.

A similar study was performed using the GnRH-PAP conjugate to determine if inhibition results in various cancer cell lines (Yang et al., 2003). The cell lines included Chinese hamster ovary cells, mice with a gonadotroph tumor cell line, as well as human breast, prostate, and endometrial cell lines. There was a dose-dependent toxicity that varied according to the number of receptors as well as the time of exposure. Therefore, the Chinese hamster ovary cells without receptors showed no toxicity. The toxicity was inhibited by introducing analogs of GnRH, suggesting another method of introducing the toxin to the receptors.

It has been suggested that PAP may be a better candidate for the synthesis as an immunotoxin than ricin A, which, like PAP, has been shown to inhibit eukaryotic ribosomal activity (Ramakrishnan and Houston, 1984). Ricin A was linked to an antibody by noncleavable *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester cross-link and was found to be nontoxic to leukemia cells. PAP coupled to the same antibody was toxic. However, both toxins were cytotoxic when linked to a cleavable disulfide bond. The suggested use of PAP over ricin A as an immunotoxin was also made based on the improved stability of PAP over ricin A and the fact that it is more easily purified.

PAP Inhibits Human Immunodeficiency Virus (HIV)

PAP has been found to inhibit HIV when attached to monoclonal antibodies to CD4 cells (Zarling et al., 1990). The process occurs by the inhibition of protein synthesis of HIV-1, which leads to decreased production of the virus. It has also been suggested that the inhibition of HIV by the three isoforms of pokeweed antiviral protein, PAPI, PAPII, and PAPIII, results in part by depurination of viral RNA in a concentration-dependent manner (Rajamohan et al., 1999). The isoforms inhibited the replication of HIV in human peripheral blood mononuclear cells, whereas ricin does not, although ricin does inhibit protein synthesis as well as does PAP alone in cell-free translation assays. It was predicted and shown that PAPIII is a stronger inhibitor of HIV, due to its distribution of charge and its surface structure, as well as a stronger depurinator of HIV-1 RNA (Kurinov and Uckun, 2003).

The immunotoxin, TXU(Anti-CD7)-PAP, has been shown to inhibit HIV in cynomolgus monkeys and in severe combined immunodeficient (SCID) mice (Uckun et al., 1998). The activity of this immunotoxin was compared to activities of zidovudine, 2',3'-didehydro-2',3'-dideoxythymidine, unconjugated PAP, and B53-PAP and was shown to be the favored choice. Importantly, the use of the immunotoxin resulted in no side effects in the mice at doses that were well-tolerated by the monkeys.

PAP activity against HIV led to a study to determine whether or not PAP can be used in spermicidal gels to help prevent the spread of HIV (D'Cruz et al., 2004). A specialized PAP-containing spermicidal gel was developed for a study involving mouse vaginas with the evidence that there are no side effects from the toxin that reduced

reproductive capacity, the survival of neonates, or the development of offspring. For example, there was no significant change in organ weights among fetuses from mothers given the spermicide, blood parameters of liver and kidney functions were not negatively altered, and there was no inflammation of reproductive parts of mothers given the treatment.

Research on *P. americana* for Cancer Treatment

B43-PAP is a pan-B immunotoxin made by linking a human B-cell specific monoclonal antibody, known as B43 (anti-CD19), to PAP. B43-PAP has been found to inactivate the 60S ribosomal subunit and has been used to treat human t(4,11) Acute Lymphoblastic Leukemia (ALL) in mice (Jansen et al., 1992). This is paramount, because this specific leukemia is the most common form of leukemia in human infants, and methods currently used for therapy have not exhibited promising results. When mice were injected with this leukemia cell line, severe immunodeficiency and death resulted. However, with the introduction of B43-PAP, long-term survival of 60% of mice resulted.

Another study supports the finding that patients with B-lineage ALL may benefit from the B43-PAP immunotoxin (Uckun et al., 1986). B43-PAP inhibited blasts in all 10 patients involved in this study. Protein synthesis of B-lineage ALL cells was variably decreased among patients with increasing concentrations of PAP. There was a maximum kill of >99.96% of leukemic cells after treatment. Further, there was relatively little toxicity, less than 40%, to progenitor cells not of the ALL lineage. The authors argue that this toxin should be used as a standard protocol for the removal of residual

clonogenic leukemia cells when patients with B-cell derived ALL opt to do autologous bone marrow transplants. They add that current methods do not ensure that leukemia cells and their remnants are completely removed.

A similar study used a combination of B43-PAP with cytosine arabinoside, a common chemotherapeutic agent, to treat SCID mice (Messinger et al., 1996). The results were that the combination leads to long-term event-free survival of the disease. Other chemotherapeutic drugs used in combination with B43-PAP were not as powerful in producing long-term, event-free survival as using B43-PAP alone.

The same toxin was later used to determine whether mice with SCID could be defended against human pre-B cell ALL (Uckun et al., 1992). The results showed a substantial improvement of event-free survival (EFS) of mice when treated with B43-PAP. Mice injected with human pre-B cell ALL exhibited disseminated and fatal leukemia with 15 of 27 mice having paraplegia. All ALL-injected SCID mice died after 31 days. A 3 day treatment regimen of the immunotoxin resulted in only 16 of 44 developing leukemia after 74 days. The chance of long-term survival was around 60% for mice treated with the immunotoxin compared with control samples.

The effects of a toxin similar to B43-PAP, TXU-PAP, on T-lineage ALL cells have been determined (Waurzyniak et al., 1997). The study was performed using murine and primate models. BALB/c mice exhibited cardiac toxicity after exposure to the TXU-PAP but SCID mice with human T-lineage ALL had an increase in leukemia-free survival. Controls in the study consisted of mice treated with phosphate buffered saline (PBS), mice treated with unconjugated TXU antibody, and mice treated with B43-PAP.

All of the control mice died from leukemia within 80 days, whereas around 80% of mice treated with a cumulative dose over 3 days of 15µg TXU-PAP and 100% of mice treated with a cumulative dose over 3 days of 30µg TXU-PAP were leukemia-free after 120 days. Cynomolgus monkeys, which were used in the study to attest half-life of the immunotoxin and side effects of the therapy, were found to tolerate the therapy well. The toxin was found to have a half-life of around 8 hours.

Similarly, an immunotoxin was formed from PAP and TP3 (anti-p80) that works against the p80 antigen of osteosarcoma cells (Ek et al., 1998). TP3-PAP shrunk the tumors in the cheeks of 3 Golden Syrian hamsters 57± 19% three days after treatment. However, twelve mice receiving doses of 4 or 5 mg/kg all died within 15 days. The authors suggest that nontoxic levels of this treatment may be an option for treating osteosarcomas.

It was suggested that the esculentoside M fraction from the roots of *P. americana* could be useful in inhibiting multiple drug resistant human ovarian cancer 2780 AD cells. Several novel triterpene saponins were isolated from the roots, and the calcein accumulation test was performed with this compound scoring the highest. However, the cytotoxicity was weak toward the cell lines tested. It should be noted that other esculentosides isolated from *Phytolacca* species have been found to have anti-inflammatory activities such as inhibiting interleukin-1, interleukin-6, and tumor necrosis factor alpha (TNFα) (Wang et al., 1996).

Mechanisms of Action

Research concerning the effect of PAP and other extract constituents of *P. americana* on molecular mechanisms is limited. However, in the human embryonic kidney cell line 293T apoptosis did not occur upon exposure of PAP (Chan Tunga et al., 2008). It was found that although PAP depurinated the α-sarcin/ricin loop of 28s rRNA and caused damage to the ribosome, all of this occurred without an inhibition of overall translation. Alternatively, PAP induced JNK activation without apoptosis. The conclusion of this isolated study was that at low concentrations PAP inhibits cell proliferation without causing apoptosis. Research on the changes in gene expression after exposing cancer cells to *P. americana* extracts using various array techniques is scarce. Although studies of PAP and other extracts of *P. americana* on molecular mechanisms of cancer cells are limited, there are studies concerning the effects of other herbs on molecular mechanisms.

Herbs Causing Apoptosis

Abrin, a natural poison from seeds of the rosary pea, is a member of the type II family of RIPs and was found to inhibit protein synthesis and then induce apoptosis in a caspase 3-dependent manner (Narayanan et al., 2004). The process was independent of caspase 8 but resulted in mitochondrial membrane damage and reactive oxygen species production.

Studies have been performed on other types of herbs as well. It has been shown that hepatoma cell growth can be inhibited by *P. radix* by the induction of the p53-

independent apoptosis pathway (Lee et al., 2002). By an alternative pathway, it has been shown that a selection of herbs inhibits cell proliferation and induces apoptotic pathways in hepatic stellate cells. The study was performed using the herbs *Angelica sinensis, Carthamus tinctorius, Ligusticum chuanxiong, Salvia miltiorrhiza, and Stephania tetrandra.* The latter two herbs exhibited the strongest apoptotic changes with 52% and 45% of cells showing apoptotic changes. The evidence supported that the method of apoptosis involved the FAS and BCL2-associated X protein (BAX) genes, which were upregulated, and the B-cell lymphoma-extra large (BCL-XL) gene, which was down-regulated.

Other studies support the idea that different herbs that cause apoptosis of cancer cells work in different ways. An herbal extract known as Magnolol, from *Magnolia officinalis*, has been shown to inhibit the synthesis of DNA and activate apoptosis in liver and colon cancer cells from the cell line Colo-205 (Lin et al., 2002). There was an increase in apoptosis and an accompanying increase in the protein p21. More importantly, it did not have these effects in cell lines such as keratinocytes, fibroblasts, or human umbilical vein endothelial cells.

Other mechanisms of apoptosis by other herbs have also been reported. A Chinese medication called Inchin-ko-to that contains the herbal extract, *Artemisiae capillaris spica*, exhibits apoptotic effects of liver cancer cells in rats (Yamamoto et al., 1996). The effects occur by transforming the growth factor β-1 (TGF-β1) pathway in a rat hepatoma cell line referred to as McA-RH8994. Another experiment involving Chinese medicinal herbs was shown to inhibit the proliferation of HL-60 cells, a human

leukemia cell line (Dong et al., 1997). The specific ingredients, tetrandine and berbamine, were found to induce apoptosis with evidence of cell shrinkage, apoptotic bodies, and DNA fragmentation, while *Coriolus versicolor* did not. *Solanum incanum* is another Chinese herb from which solamargine is purified. This portion of the herb has been reported to cause human hepatocytes and skin fibroblasts to express the tumor necrosis factor receptor I (TNFR1), which has been known to lead to apoptosis (Hsu et al., 1996).

Scutellaria barbata is a Chinese herbal medicine that has been shown to have antiproliferative activity toward lung, breast, and other cancers (Yin et al., 2004). Research has determined that lung cancer A549 is inhibited by ethanol extracts of this plant. cDNA microarray has been performed to indicate that the major pathways by which the extracts inhibit growth of A549 cells is through apoptosis with genes that are involved in the cell cycle, nucleic acid binding, and protein phosphorylation.

It is generally agreed that some, but not all, of the RIPs work by way of apoptosis (Nielsen and Boston, 2001). DNA fragmentation, nuclei fragmentation, cytoplasmic densification, and changes in mitochondrial membrane potential have all been documented after Types I and II exposures to plant and animal cells. However, not all Type I RIPs induce apoptosis. Whether or not apoptosis occurs, the activity seems to depend on the variation in uptake of the RIP. While one study supported the idea that PAP specifically does not cause cell death through apoptosis, more studies are needed to determine whether PAP causes apoptosis to a variety of cell lines (Chan Tunga et al., 2008).

Caspases

Caspases are the cysteine aspartate-specific protease machinery of apoptosis (Kuida et al., 1998; Yuan and Horvitz, 2004; Danial and Korsmeyer, 2004). Caspases 2, 8, 9, and 10 are considered initiator caspases while caspases 3, 6, and 7 are considered effector caspases. Cytochrome c release from the mitochondria into the cytosol is one mechanism that begins apoptosis, whereby cytochrome c binds to apoptotic protease activating factor 1 (APAF-1), which triggers caspase 9 activation (Slee et al., 1999; Zou et al., 1997). Some studies suggest that caspase 9 is necessary to activate caspases 2, 3, 6, 7, 8, and 10, while caspase 3 is necessary for the activations of caspases 2, 6, 8, and 10. One study showed that deletions of caspase 9 prevent caspase 3 activation in embryonic mice brains (Kuida et al., 1998).

It was shown in nematodes that APAF-1 promotes the processing of caspase 9 *in vivo* (Hu et al., 1998). It was also found that in addition to cytochrome c activating caspase 9 by being bound to APAF-1, that procaspase 9 can activate procaspase 3 to the complex of APAF-1 and procaspase 9 (Hu et al., 1999; Jiang and Wang, 2000). Alternatively, it was found that without cytochrome c, dATP binds poorly to APAF-1, since an apoptosome is formed with APAF-1 and cytochrome c through the activation of dATP binding to APAF-1. Further, in an apparently synergistic manner, procaspase 9 promotes the further cytochrome c-dependent binding of dATP to APAF-1. Another note is that BCL-XL inhibits the association of APAF-1 with caspase 9 in mammalian cells (Hu et al., 1998).

Cell death initiators such as apoptosis regulator proteins derived from B-cell lymphoma 2 (BCL2) and BCL2 interacting domain (BID) are involved in the regulation of apoptosis through the release of cytochrome c from the mitochondria (Kluck et al., 1997; Yang et al., 1997; Luo et al., 1998). BCL2 was shown to act in-situ and within intact cells to prevent the release of cytochrome c whereas ZVAD-FMK, a caspase inhibitor, did not block its release into the cytosol (Kluck et al., 1997). On the other hand, BID is cleaved by caspase 8, itself activated by TNF and TNFR superfamily member 6 (FAS), and then moves to the mitochondria to cause the release of cytochrome c (Luo et al., 1998). Thus, BCL2 and BID work antagonistically to one another.

It has been shown that caspase 2 is activated by cytotoxic stress and it then causes permeability of the mitochondria. The permeability then causes the amplification of the release of caspases which then cause the cell to disassemble (Lassus et al., 2002). Upon opening of the permeability transition pore, procaspases 2 and 9 are released from the mitochondria into the cytosol (Susin et al., 1999). Caspase 2 has not only been shown to release cytochrome c but also apoptosis-inducing factor (AIF) and second mitochondria-derived activator of caspases protein (SMAC) (Guo et al., 2002).

It has further been shown that during exposure to the chemotherapeutic drug etoposide that caspase 2 similarly acts upstream of the mitochondria (Robertson et al., 2002). When caspase 2 activity is blocked, procaspase 3 and 9 activities are weakened, and phosphatidylserine presentation on the membrane of the mitochondria and DNA fragmentation are also reduced.

The importance of caspase 3 in the role of apoptosis has been well established. Pathways that lead to the activation may be dependent or independent of the release of cytochrome c (Porter and Janicke, 1999). Likewise, its activation may be dependent or independent of caspase 9 activation.

Caspase 3 has been found to affect various substrates and, thus, impact cell death in several ways. One such effect is to activate the DNA Fragmentation Factor (DFF) (Liu et al., 1997). The presence of this protein leads to DNA fragmentation with the presence of caspase 3, which was unable to induce this fragmentation alone in HeLa cells. After purification of DFF and as increasing volumes of DFF were added to the reactions, DNA fragmentation increased.

To further verify the importance of caspase 3 in DNA fragmentation, Janicke et al. (1998) investigated the impact on MCF-7 cells, which have no caspase 3 activity due to a deletion in the caspase 3 gene. The result was the absence of DNA fragmentation. Morphological features of apoptosis, such as cell blebbing and cell shrinkage were also not present. When MCF-7 cells have caspase 3 activity restored, they again sensitized to the apoptotic effects of the chemotherapeutic agents doxorubicin and etoposide (Yang et al., 2001)

Kothakota et al. (1997) showed that gelsolin is one important substrate cleaved by caspase 3 in FAS-stimulated cells that is responsible for apoptotic morphological changes. In response to gelsolin cleavage, cells detach *in vitro*, become rounder, and cause nuclear fragmentation. Without the presence of gelsolin, it was discovered that cells have delayed blebbing and nuclear fragmentation.

Caspase 6 activity has been shown to be triggered by p53, an important gene in apoptosis caused by chemotherapy (MacLachlan and El-Deiry, 2002). Another target of p53 in triggering apoptosis has been shown to be APAF-1 (Robles et al., 2001). It was further shown that caspase 3 and 7, other effector caspases, were not activated in response to p53.

It has been shown that one of the effects of caspase 6 is on the cleavage of lamin A, a fibrous structure of the nuclear envelope. For example, resveratrol (RSV) is a phytoalexin that is produced from plants with the purpose of fighting fungi or bacteria. It has been shown to elicit apoptosis in HCT-116 cells by caspase 6 activation and the cleavage of lamin A (Lee et al., 2006). Blocking caspase 6 activity with inhibitors caused cleavage of lamin A and apoptosis to cease. Partially blocking caspase 6 activity caused a reduction in lamin A cleavage and apoptosis. It was further shown that the cleavage of lamin A by caspase 6 is necessary for the production of nuclear condensation, shrinking, and the formation of apoptotic bodies in HeLa cells (Ruchaud et al., 2002; Broers et al., 2002).

Some work has been done to determine what blocks and activates caspase 8. For example, the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) blocks the activation of caspase 8 (Wang, 1998). Caspase 8 is activated by FAS, also known as CD95, which recruits FAS-associated protein with death domain (FADD), procaspase 8, and Caspase 8 (FLICE)-inhibitory proteins (C-FLIP) (Krueger et al., 2001). All of these make up the death-inducing signaling complex, DISC. After procaspase 8 is activated to the DISC, it goes through several steps of proteolytic

cleavage and activation to form mature caspase 8, leading to cell death. C-$FLIP_L$ and C-$FLIP_s$ are 2 alternative types of C-FLIP that may either initiate or block caspase 8 cleavage steps that lead to apoptosis (Chang et al., 2002).

It was found that select anticancer drugs allow the hastened activation of procaspase 8 by tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) in human colon cancer cell lines (Lacour et al., 2001). This then allows caspase 8 activation and the activation of BID of the BCL2 family and the subsequent activation of effector caspases.

Herbs Inhibiting Cancer Cell Proliferation Through Caspase Activity

Although research has not been performed to show whether *P. americana* extracts increase caspase activity, there are reports regarding the change in caspase activity caused by other herbs. One such plant extract is the mistletoe lectin I (ML-I) from the plant *Viscum album* (Bantel et al., 1999). While this extract was found to have cytotoxic effects such as cell shrinking and chromatin condensation on T- and B- cell leukemia cell lines, treatment with a caspase inhibitor prevented apoptotic effects. In addition, caspase 8, 9, and 3 activities were measurable.

A hexane extract has been produced from the resin Chios mastic gum, which is produced from the *Pistacia lentiscus* tree. This extract has been shown to cause anoikis to HCT-116 colon cancer cells (Balan et al., 2005). This form of apoptosis had also been attributed to the activity of caspases 3, 8, and 9 but with the cell detachment not

dependent upon caspase activity. In a similar study, an extract of *Radix Sophorae Tonkinensis* was shown to elevate caspase 3 activity in HepG2 cells (Chui, 2005).

Members of the *Brassica* genus of plants include Brussel sprouts, cabbage, radishes, and cauliflower. These plants produce indole-3-carbinol that has been shown to reduce the proliferation of breast, colon, and endometrial cancer cells through apoptosis (Aggarwal and Ichikawa, 2005). Antiapoptotic genes such as BCL2 were shown to be downregulated, while caspase 3 and 9 activities were increased along with a coinciding cytochrome c release.

Hypericum perforatum L., commonly known as St. John's wort, contains an active component, hyperforin. Hyperforin was shown to increase caspase 9 and 3 activities in leukemia U937 cells and to increase caspase 8 and 3 activities in K562 human histiocytic lymphoma cells (Hostanska et al., 2003). As with the other herbs mentioned, this caspase inhibition makes hyperforin of St. John's wort a potential candidate for cancer treatment.

Chlorophyllin is derived from chlorophyll and may be useful as a chemotherapeutic agent. Research has recently been performed to determine the molecular pathway through which it works (Diaz et al., 2003). It was found that upon exposure to HCT-116 colon cancer cells, apoptosis-inducing factor is released into the cytosol from the mitochondria, carried to the nucleus, and then nuclear lamins are cleaved. Caspases 8, 6, BID, and BCL2 homologous antagonist/killer (BAK) are involved in the process.

From these studies, it is clear that there are herbs and herbal extracts that increase various caspase activities. Some of the caspase activations lead directly to apoptosis. Other herbs, however, cause apoptosis through other mechanisms or in addition to caspase involvement. Therefore, it is important to understand gene upregulations and downregulations that underlie antiproliferative properties of herbs. This becomes complex, because many affected genes are interconnected through various pathways.

Relevant Genes Involved in Cancer Cell Cycle, Apoptosis, and Signal Transduction

The WNT/β-catenin pathway has been found to be altered in most colon cancers, since it drives tumorigenesis. It has recently been shown that CDK8 is associated with cell proliferation of colon cancer tumors and is found to be present in a region of recurrent copy number gain in many colon cancers. HCT-116 cells are known to be dependent upon β-catenin for proliferation, and compounds such as diclofenac® alter the signaling in this pathway by NF-κB activation (Cho et al., 2005a). It was found that Kirsten rat sarcoma viral oncogene homolog (KRAS) and myelocytomatosis viral oncogene homolog (MYC) genes were also necessary for the proliferation of this cell line along with CDK8 (Firestein et al., 2008). Notably, it was suggested that CDK8 may be a good target for therapeutic interventions because suppression of CDK8 reduced expression of other genes involved in colon cancers such as MYC, axis inhibition protein 2 (AXIN2), and lymphoid enhancer-binding factor 1 (LEF1).

It is known that the activation of NF-κB blocks the activation of caspase 8 (Wang et al., 1998). I Kappa B alpha is the product of the nuclear factor kappa light polypeptide

gene enhancer in B cells inhibitor alpha (NFKBIA) gene that binds to NF-κB to prevent its activation, which ultimately serves to block apoptosis. Polymorphisms of the NFKBIA gene have been associated with a predisposition to cancers such as multiple myeloma (Spink et al., 2007). The gene itself was found to be upregulated in metastatic colon cancer stem cells and, thus, was working to block the function of NF-κB (Botchkina et al., 2009).

In a colon cancer study involving 15 males and 20 females, all positively expressed mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*) (MLH1) and mutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*) (MSH2), both proteins essential to DNA repair, while 60% and 40% expressed β-catenin membranous and β-catenin nuclear proteins, respectively (Kapiteijn et al., 2001). Further, 18% expressed BCL2 and 29% expressed p53. In this study, there were variations in genetic expression within the sample and compared to samples of rectal cancers, supporting the idea that different individual's cancers have different gene expressions.

In another study of the colonic adenocarcinomas, adenomatous polyposis coli (APC), KRAS, and p53 mutations were found in only 11% of samples, negating the previous thought that these mutations usually occur together in colon cancers (Samowitz et al., 2007). Among the colon cancers tested, 59% had frameshift mutations in the APC gene. In other colon cancer cell lines, such as SW480 cells, KRAS oncogene is often the most clinically important (Tokunaga et al., 2000). Alternatively, feline sarcoma oncogene (C-FES) has been associated with colorectal cancers as a tumor suppressor rather than an oncogene (Delfino et al., 2006). C-FES protein levels were found to either

not be present at all or to be present in low numbers in HCT-116 cells. When this protein was introduced into the cells, cell growth was suppressed.

Modifier of Min 1 (MOM1) is a gene that influences polyp size and multiplicity in multiple intestinal neoplasia (MIN) mice. The Modifier of Min 2 (MOM2) mutation is the result of a spontaneous mutation and resistant MOM2 alleles can suppress the polyps in APC mutated mice in a dominant fashion (Silverman et al., 2002). It is widely regarded that the APC gene inactivation is often the initiating step in human colorectal cancer transformation. Identifying modifiers of various loci of APC can be important to studying adenoma incidence (Baran et al., 2004).

Cell survival is promoted by growth factors such as phosphatidylinositol 3-kinase (PI3K) that phosphorylate the serine/threonine kinase murine thymoma viral oncogene (AKT), which then causes the phosphorylation of BCL2-associated death promoter (BAD), a BCL2 member, and the prevention of apoptosis (Datta et al., 1997). AKT itself can also be activated by interleukin-3 in a PI3K-dependent manner (De Peso et al., 1997). Regulation of BAD has been shown to occur through its phosphorylation at serine-112, activated by retrovirus associated sequence oncogene (RAS) and murine leukemia viral oncogene homolog 1 (RAF), and its phosphorylation at serine-136, mediated by AKT (Zha et al., 1996; Fang et al., 1999). BAD then binds with 14-3-3 protein and is transported to the cytoplasm, causing BAD not to heterodimerize with survival proteins BCL-XL and BCL2. Alternatively, BAD can also be phosphorylated by C-JUN N-terminal kinase (JNK) at serine-128 (Donovan et al., 2002). This antagonizes the ability of growth factors to inhibit BAD-influenced apoptosis. Sulindac sulfide, a metabolite of

the nonsteroidal anti-inflammatory drug, sulindac®, has been shown to inhibit phosphorylation of BAD in colon cancer cells (Rice et al., 2003).

BAX is a BCL2 related gene that heterodimerizes with BCL2 (Korsmeyer et al., 1993). The ratio of the two proteins is important in determining whether or not apoptosis will continue. In patients with colorectal cancer, low BAX levels have been correlated with a poor prognosis in patients with liver metastasis (Sturm et al., 1999). Best survivals are seen in patients with a working p53/BAX pathway. Low BAX protein expressions also indicate a poor prognosis in Union for International Cancer Control (UICC) stage III primary colorectal cancer (Schelwies et al., 2002). One study found that a reduction in heterodimerization of BAX with BCL2 may be a contributing factor to colorectal cancer development, while the suppressed heterodimerization of BAD with BCL2 may contribute to advanced tumor development (Hattori et al., 2000).

BCL2 has long been considered a mitochondrial membrane protein that functions to prevent the release of cytochrome c from the mitochondria and block cell death (Hockenberry et al., 1990; Yang et al., 1997). Further, it has been shown that BCL2 suppresses p53 activity in HCT-116 cells (Jiang and Milner, 2003). When BCL2 was silenced by RNA interference (RNAi), the result was an increased rate of apoptosis through p53 activation. Thus, one strategy in the treatment of colorectal cancers may be to target BCL2 activity.

TNF receptors are death receptors that facilitate the activation of caspase 8, leading to apoptosis. TNF-related apoptosis-inducing ligand (TRAIL) is expressed in a variety of cell types but induces apoptosis in mostly neoplastic cell types. Inhibiting

PI3K has been shown to induce activation of TRAIL, leading to cleavage and activation of caspase 8 (Rychahou et al., 2005). Similarly, there is evidence that PI3KCA, a polymorphism of the PI3KR1 gene that has been linked as a colon cancer risk (Li et al., 2008), is an oncogene in ovarian and breast cancer (Campbell et al., 2004). Therefore, inhibitors of PI3K/AKT signaling have been proposed to be potential therapeutic agents for cancer.

Members of the TNF receptor superfamily span the cell membrane and are involved in inflammatory and immune functions (Gaur et al., 2003). Patients with mutations of the TNFR superfamily 1A (TNFRSF1A) are more resistant to TNF-induced apoptosis (D'Osualdo et al., 2006). Mutations of this receptor are a characteristic of patients with TNF receptor-associated periodic syndrome (TRAPS) who commonly experience problems with inflammation (Aksentijevich et al., 2001). Patients with mutations of the TNFSF10B are associated with type 2 diabetes (Philippe et al., 2008). TNFSF10B, also proapoptotic, was found to be upregulated in type 1 diabetes patients due to cytokine deprivation (Jailwala et al., 2009). A separate receptor of TNF is TNFRSF25 (DR3), which is expressed on immune cells and osteoblasts, and mutations are associated with rheumatoid arthritis (Borysenko et al., 2005). The DR3 extracellular domain is made up of 4 homologous cysteine-rich domains, and this part of the receptor is necessary for local t-cell accumulation and cytokine production (Meylan et al., 2008).

It has been shown that HIV-1 Tat interactive protein 2, 30kDa (HTATIP2) is a gene involved in anti-invasion of cancer cells (Diaz et al., 2007). This gene was downregulated in response to the upregulation of the ovarian cancer invasion gene, mucin

16 (MUC16), along with the upregulation of cadherin-1, type 1, E-cadherin (epitheilial) (CDH1), fibronectin 1 (FN1), interleukin 1 beta (IL1B), and matrix metalloproteinase-7 (MMP7) and matrix metalloproteinase-9 (MMP9). Similarly, it was shown that when protocadherin 10 (PCDH10), whose methylation is associated with poor prognosis in gastric cancer patients, has restored expression, the HTATIP2 anti-invasion gene is restored along with genes such as FAS, caspase 8, JUN, and CDKN1A (p21) apoptotic genes (Yu et al., 2009).

One of the genes that C-MYC activates is telomerase reverse transcriptase (TERT), on which telomerase complex activity is dependent (Wu et al., 1999). TERT is upregulated in most immortal cell lines to stabilize telomeres throughout cell cycling due to the upregulation by C-MYC by way of its E-box (Greenberg et al., 1999). In colon cancer tissue, there has been a correlation between overexpression of survivin, an inhibitor of apoptosis, and telomerase activity through the activation by TERT (Endoh et al., 2005).

In some cases of hereditary non-polyposis colorectal cancer (HNPCC), there is a polymorphism of ataxia telangiectasia (ATM) present, known as D1853N (Maillet et al., 2000). For those who carry the ATM polymorphism and are carriers of either the MLH1 or MSH2 mutation, both of which are important to DNA repair, there is an 8 times greater risk of colorectal cancer. The authors suggested that the ATM polymorphism affects the number of MLH1 and MSH2 mutations. Further, it has been shown that ATM regulates the phosphorylation of p53 at serine 46 after ionizing radiation, and the protein kinase that is likely responsible for this activity is homeodomain-interacting protein kinase 2

(Dauth et al., 2007). After UV exposure, it is p38 that phosphorylates p53 at serine 46 (Takekawa et al., 2000).

It has been shown that cyclins D1, D3, and E1 are often overexpressed in breast cancer (Malumbres et al., 2001). Mitogenic growth factors stimulate breast cancer cells and activate cyclin E in the late G1 phase with a coinciding activation of CDK complexes and a move to the S phase (Musgrove et al., 1993; Aleem et al., 2005). It has been suggested that G1/S-specific cyclin E1 (CCNE1) and G1/S-specific cyclin E2 (CCNE2) qualify as prognostic markers for lymph node negative breast cancer patients (Sieuwerts et al., 2006).

CDK2 is a protein kinase that has been known to phosphorylate and regulate the retinoblastoma (RB) gene (Meyerson et al., 1992), itself a tumor suppressor found in increased expression in colorectal cancers (Lothe et al., 1992). CDK2 was found to be increased in colorectal cancers and may prevent the retinoblastoma protein from breaking the cell cycle (Yamamoto et al., 1995). The cyclin D1/CDK4 complex also phosphorylates and inactivates RB (Meyerson et al., 1992). Cyclin-dependent kinase 4 (CDK4) proteins were found to be decreased in human colonic adenocarcinoma cell line Caco-2 upon exposure to resveratrol, a polyphenol present in grapes, peanuts, and red wine, when compared to Caco-2 and HCT-116 cells not exposed (Wolter et al., 2001).

The cell division cycle 25 (CDC25) family is known to activate the cyclin dependent kinases, and CDC25A functions early in the cell cycle (Jinno et al., 1994). The nuclear protein phosphatase CDC25A is considered a protooncogene with increases in expression in cancerous colon epithelial tissue (Dixon et al., 1998). In fact, all CDC25

phosphatases have been shown to function as potential oncogenes (Galaktionov et al., 1995) and are differentially regulated in human colorectal carcinomas (Hernandez et al., 2001). In fibroblasts of mice, CDC25A may interact with activated HA-RAS or RB1 loss to play a role in transformation (Galaktionov et al., 1995).

p21 (CDKN1A/CIP1/WAF1) is a p53-regulated cyclin-dependent kinase inhibitor (Ogino et al., 2006). In colorectal cancer, p53 mutations cause down-regulation of p21. It has been shown that cell cycle arrest of p53 ceases in p21 deficient cells, indicating that p21 is necessary for the cell cycle arrest of p53 (Waldman et al., 1995). In fact, herbal extracts may alter the gene expressions of p21 and p53 in various cancer cells. For example, *P. quinquefolius* was shown to exert anti-proliferative effects on HCT-116 colon cancer cells through changes in the expression of apoptotic proteins (King and Murphy, 2009). After exposure to this extract, the expressions of p21 and p53 were upregulated with phospho-MEK levels decreased and the cell cycle phase arrested at G0/G1. Furthermore, cells deficient in p21 had reduced cell viability and more dead cells with a coinciding increase in BAX as well as the cleavage of caspase 3. In a separate study, gene expression changes were determined after exposure to ginseng and ginsenoside RG3 (Luo et al., 2008). There were 76 significant genes changes after treatment, some influencing apoptosis.

High percentages of aberrant methylations of p16, encoding the cyclin-dependent kinase inhibitor 2A (CDKN2A), have been associated with colon and colorectal polyps (Petko et al., 2005). Similarly, mutations in this gene are associated with cutaneous malignant melanomas (Foulkes et al., 1997). It follows that aberrations of p16, as well as

KRAS, are predictors of poor prognosis in human colorectal cancers (Esteller et al., 2001).

Checkpoint homolog (CHEK2) has been referred to as a multiorgan cancer susceptibility gene (Cybulski et al., 2004). This is because the CHEK2 protein is important in the DNA damage response. Thus, mutations in this gene cause problems with repair of DNA damage. One specific CHEK2 mutation, 1100delC, has been found to correlate with a hereditary breast and colorectal cancer, which is known as the HBCC phenotype (Meijers-Heijboer et al., 2003). In addition, the variant CHEK I157T has been associated with both familial and sporadic colorectal cancers (Kilpivaara et al., 2006).

The E2F1 transcription factor has been found to play a role in gastric, breast, and colon cancers (Petrocca et al., 2008; Louie et al., 2004; Kasahara et al., 2000). Overexpression of the E2F1 gene has been found to correlate with thymidylate synthase expression in colon cancer (Kasahara et al., 2000). This enzyme ultimately becomes thymidine triphosphate, which is used in DNA synthesis and repair. E2F transcription is regulated by the RB protein, the entire pathway which regulates DNA replication and is disrupted in many cancers (Nevins, 2001).

Both ETS1 and ETS2 of the E-twenty six (ETS) family of transcription factors have been implicated as targets in the RAS signaling pathways, in addition to the activator protein-1 (AP-1)/ATP-dependent proteolysis factor (APF) family (Yang et al., 1996). This is done through the phosphorylation of conserved threonine residues. The ETS factor then regulates the FBJ murine osteosarcoma viral oncogene homology (C-FOS) promoter. It has additionally been noted that the ETS2 transcription factor is

important to the sustained transformation of prostate cancer cells (Sementchenko et al., 1998).

C-FOS has been found to be expressed in an increased proportion in aberrant colonic crypts, preneoplastic lesions of colon cancer (Stopera et al., 1992). FOS and JUN families make up the AP-1 complex. The signal is carried from RAS to AP-1 through the V-RAF-1 murine leukemia viral oncogene (RAF)/mitogen activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) mitogen activated protein (MAP) kinase pathway. The ERK/MAP kinase pathway has been implicated in cell growth regulation. In a model that is conserved from yeast to humans, RAF phosphorylates and activates MEK1 (MAP2K1), which phosphorylates and activates ERK1 (Widmann et al., 1999). It was shown in colon cancer cell lines that C-JUN and FOS-related antigen 1 (FRA-1) activities depend on ERK activities such that elevated ERK-MAP kinase activity leads to protection of FRA-1 against proteosomal degradation in colon cancer cells (Vial et al., 2003). It is important to note that mutations of RAF itself have been shown to be involved in 15% of human colon cancers (Davies et al., 2002; Mercer and Pritchard, 2003). Thus the entire pathway has been targeted for cancer treatment (Roberts and Der, 2007). In addition, in a study involving the effect of circumin on colon cancer cells, there was a decrease in the gene expression of MAP2K2, while another study showed decreases in the expression of MAP2K1 (van Erk et al., 2004; Kim et al, 2002). This suggests that circumin could be useful in the treatment of colon cancers.

The S100 genes produce a family of calcium binding proteins that regulate cell proliferation and extracellular signal transduction. S100A4 has been studied in regard to

clinical and metastatic effects of non-small cell lung, breast, and gastric cancers (Kimura et al., 2000; Rudland et al., 2000; Yonemura et al., 2000). The metastatic effects are derived from its ability to activate nonmuscle myosin (Yonemura et al., 2000), and it is highly correlated with patient demise (Rudland et al., 2000). It was also concluded that S100A4 gene is a strong prognostic marker in human colorectal cancer (Gongoll et al., 2002; Cho et al., 2005b). Associations were made with increased expression of the gene, and it was found to be statistically correlated most with tumor stage pT3/3, secondary metastasis, RB, p16, and women (Gongoll et al., 2002).

The V-ERB-B2 erythroblastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian) (ERBB2) type I receptor tyrosine kinase has been implicated in the progression of human colon cancer malignant expression (Porebska et al., 2002). It was shown that the flavonoid quercetin can decrease the expression of both ERBB2 and ERBB3 in HT29 colon cancer cells (Kim et al., 2005). This was done through inhibition of cell growth and induction of apoptosis as seen through reduced levels of BCL2.

Methylation of the synuclein-γ (SNCG) gene, which activates the MAPK signal transduction pathway, has been found to be expressed in a high percentage in various cancers, such as uterine, breast, and colon (Morgan et al., 2009; Jia et al., 1999) There has also been a strong correlation between this gene and metastasis in various cancer types (Liu et al., 2005). The expression of SNCG has been found to be greater in patients with colorectal cancer hepatic metastasis (Hu et al., 2009).

It has been suggested that AKT, a serine/threonine protein kinase known as protein kinase B, plays a role in the prevention of apoptotic activity of human cancers such as colorectal cancer (Kandel et al., 2002). Its activation may lead to genetic instability by controlling the G2/M cell cycle progression, and it can overcome p53 checkpoints, equating p53 inactivation. This was recently shown in a study using HCT-116 cell lines. It has also been shown that mitochondrial respiration problems in some cancer cells can cause AKT activation (Pelicano et al., 2006). This is thought to lead to an increase in ATP production through glycolysis, increases in NADH, and inactivation of phophatase and tensin homolog (PTEN), a tumor suppressor gene. This is also thought to give cancer cells a survival and drug-resistance advantage.

Apoptosis is induced in colon cancer cells by the hydrophobic bile acid, deoxycholic acid, while the chemopreventative agent, ursodeoxycholic acid (UDCA), protects HCT-116 cells from this apoptosis (Yui et al., 2005). It has been shown that hydrophobic bile acids induce apoptosis in these cells by releasing cytochrome c from the mitochondria although the mechanism is unknown. UDCA protects the cells by acting downstream of the release of cytochrome c, since its presence was not inhibited, although caspase 8 and caspase 9 activations were inhibited. UDCA instead arrests the cell growth of colon-derived tumor cells. Similarly, the HCT-116 cancer cell line's resistance to sodium deoxycholate (NaDOC) has been shown to involve increases in GRP78 promoter, BCL2, and NF-κB (Crowley-Weber et al., 2002). Each of these genes protects cells against apoptosis. Furthermore, it has also been found that HRFI gene overexpression

inhibits the apoptosis caused by chemotherapeutic agents with upregulations of the BCL2 gene (Sasaki et al., 2006).

It has been shown through microarray studies that the alteration of gene expression of extracellular matrix components may be responsible for the multidrug resistance of a line of MCF-7 breast cancer cells (Iseri et al., 2009). Cells known to be resistant to paclitaxel®, docetaxel®, vincristin®, and doxorubicin® displayed upregulations in the gene expressions of collagen, fibronectic, syndecan, integrin receptor subunits alpha 5 and beta 1, and metalloproteinases, among others when compared with drug sensitive MCF-7 cells. However, the authors stressed that the association between drug resistance and invasiveness remains to be resolved. From this information, it is apparent that upregulations of genes associated with colon cancer may be responsible for drug resistance as well.

It should be noted that there are other causes besides mutations and upregulations and downregulations of gene activities that can lead to increased chances of colorectal cancers. Microsatellite instability (MSI) can occur as can chromosomal instability (CIN) or translocations (Grady et al., 2004). Therefore, testing gene expression alone does not provide a full picture of an individual's risk of cancer or what is occurring physiologically. In as many as 15% of colorectal cancers, there is MSI in the form of mismatch repair (MMR) gene mutations or hypermethylation of the MLH1 promoter. Microsatellite repeats may also result from MSI, namely transforming growth factor beta receptor 2 (TGFBR2) and BAX. In other cases, there are mutations or methylations in budding uninhibited by benzimidazoles 1 (BUB1), budding uninhibited by

benzimidazoles 1 homolog beta (yeast) BUBR1, mitotic checkpoint regulators, or Aurora kinase A (STK15) amplifications. Therefore, it is necessary to understand the mechanistic activity of plant extracts against cancer cells through studying the molecular pathways that are affected.

Although work has been performed to determine the effects of *P. americana,* mostly in the form of PAP, on a limited number of leukemia cell lines, no work has been performed to determine the effects on colon or breast cancer cells. There has also been very limited research reporting the effect of *P. americana* extracts or their fractions on cancer cells at the molecular level. Preliminary studies performed in our lab comparing the antiproliferative effects of extracts derived from leaves, berries, and roots of *P. americana* indicated that roots exhibit the strongest antiproliferative activity against select cancer cells *in vitro*.

Therefore, this study was carried out to: 1) investigate the inhibitory activity of *Phytolacca americana* root extracts against the proliferation of HCT-116 and MCF-7 cancer cells *in vitro*; 2) test the antiproliferative effect of the fractions isolated from the most active extract of *P. americana* root; 3) compare the inhibitory activity of the most active *P. americana* root extract against HCT-116 cells to that of pure protein antiviral protein (PAP); 4) conduct apoptosis assays to understand the mechanism of action of the most potent *P. americana* root extract and its most active fraction against HCT-116 cells; and 5) determine changes that occur at the level of gene expression after HCT-116 cells are exposed to the most potent *P. americana* root extract and its most active fraction.

The hypotheses that were tested in this study are as follows: 1) *P. americana* ethanol extract inhibits the proliferation of cancer cells through the mitochondrial apoptotic pathway as exemplified through increased activity of one or more of caspases 2, 3, 6, 8, and 9. 2) The ethanol extract of *P. americana* causes increases in expression of genes involved in the mitochondrial apoptotic pathway such as BAD, BCL2, caspase 8, and APAF-1.

CHAPTER 3

MATERIALS AND METHODOLOGY

Sample Collection and Preparation

Roots of *P. americana* were harvested from the NC Agricultural and Technical State University farm in Greensboro, North Carolina. The roots were washed, cut, and kept frozen at -80ºC. The samples were freeze-dried using a Labconco free-zone freeze dryer (Kansas City, Missouri) for 72 hours. Following freeze drying, the samples were powdered using a grinder (Reustch 1640, Germany). The powdered materials were individually soaked in 100% deionized water or 80% ethanol (1:5 w/v) or 80% methanol overnight with continuous stirring. Solid material was removed by centrifugation at 7000 x*g* for 20 minutes at 4ºC. The liquid supernatant was collected in a flask and the final residue was discarded. The water, ethanol, and/or methanol in the supernatant were evaporated under reduced pressure using a Rotovapor (Buchi, Germany). The yields of the extracts were collected as crude water (PRW), ethanol (PRE), or methanol (PRM) extracts of *P. americana* roots.

Cell Culture

Two lines of cancer cells, (1) human colorectal adenocarcinoma (HCT-116) cultured in 89% McCoy's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin; and (2) human breast adenocarcinoma (MCF-7) cultured in 89% Roswell Park Memorial Institute (RPMI-1640) medium with 0.1% HEPES buffer

solution, 10% fetal bovine serum, and 1% penicillin/streptomycin, were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cancer cells were maintained in a 5% CO_2 incubator at 37ºC with fresh media added every 2 to 3 days for the duration of the study to allow normal cell growth and proliferation.

Antiproliferative Activity Assay

The antiproliferative activity of the crude extracts (PRW, PRE, and PRM) derived from the roots of *P. americana* was evaluated at concentrations of 0, 400, 800, 1600, and 3200 µg/ml using the 3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrazolium bromide dye (MTT) assay (Mossman, 1983). This is a colorimetric assay that measures the activity of enzymes that reduce MTT to formazan dyes, resulting in a purple color. Extracts were dissolved in 0.5% dimethyl sulfoxide (DMSO). The dissolved extracts were filter sterilized using a 0.22 µm syringe filter. The cancer cells' viability was confirmed using Trypan Blue Exclusion Test (ATCC, Manassas, VA). Forty microliters of viable HCT-116 or MCF-7 cells at concentrations of 6.25 x 10^6 cells/ml were seeded in a 96-well microplate and incubated for 2 hours to allow for cell attachment, after which 20 µl of the various concentrations of dissolved crude extracts was added. Phosphatidylinositol 3 kinase inhibitor (PI3KI) (Cell Signaling Technology, Danvers, MA) was used as a positive control at 200 µg/ml. The microplates were then incubated for 24, 48, and 72 hours at 37ºC in a 5% CO_2 atmosphere. After each incubation time period, aliquots of MTT (Sigma-Aldrich, St. Louis, MO) were added to each well at 4:15 ratio and incubated for an additional 3 hours, then washed gently with 0.01 N HCl in isoproponal

to dissolve the tetrazolium crystals. Absorbance was read at 490 nm using an Elx808 Ultra Microplate Reader (BioTek, Vienna, VA). All concentrations and time intervals were repeated six times.

Fractionation of the Ethanol Extract

A total of 5.68 g of the remaining ethanol extract was further fractionated using 110 ml each of hexane and water, followed by ethyl acetate, and then butanol. Each fraction was separately obtained by removing the liquid portion via rotavapor evaporation for the following yields: 0 mg hexane, 22.04 mg ethyl actetate, 354.44 mg butanol, and 4734.08 mg water fraction. Each fraction, excluding hexane, was tested for its antiproliferative activity following the same method as described above except at concentrations of 25, 50, 100, and 200 μg/ml and for 48 and 72 hours. Pokeweed antiviral protein (PAP) from spring leaves was obtained from Dr. Friedland at the City University of New York (CUNY) and tested at concentrations of 2.5, 5, 10, 20, and 40 μg/ml for 48 hours.

Caspase Analysis

After determining the most active extract and/or fraction derived from pokeweed against both cell lines, caspase analysis was carried out to study the apoptotic effect of the crude ethanol extract of *P. americana* (PRE) and its water fraction (PREW) on HCT-116 cells. The ApoTarget™ Colorimetric Protease Assay Sampler Kit from Invitrogen (Carlsbad, CA) that is capable of determining the activity of caspases 2, 3, 6, 8, and 9 was

used. This kit detects caspase levels by measuring free *p*-nitroaniline at an absorbance of 405 nm. Free *p*-nitroaniline increases as caspases cleave the *p*-nitroaniline-labeled peptides in cells. PRE was tested at 1600 and 3200 µg/ml and its corresponding water fraction, PREW, was tested at 400, 800, and 1600 µg/ml. A negative control was also used that consisted of HCT-116 cells exposed only to 0.5% DMSO, whereas all extracts or fractions were dissolved in 0.5% DMSO. After 48 hour incubation at 37ºC in a 5% CO_2 atmosphere, the cells were resuspended in chilled Cell Lysis Buffer and incubated on ice for 10 minutes. The microplates were then centrifuged for 1 minute at 2196 x *g* for 5 minutes. The supernatant was transferred to a fresh microplate and placed on ice. Next, 50 µL of 2x Reaction Buffer, which contains 10 mM DTT, and 5 µL of 4 mM caspase substrate were added to each sample, followed by a 1.5 hour incubation. The plates were then read at 405 nm using an Elx808 Ultra Microplate Reader (BioTek, Vienna, VA). All concentrations and time intervals were repeated at least six times.

Human Cancer Pathway Finder Realtime PCR Profiler

The Human Cancer Pathway Finder Realtime PCR (HCPFRPCR) Profiler (S.A. Biosciences, Frederick, MD) was used to determine which of 84 genes, including those of cell cycle control, DNA damage repair, signal transduction, transcription factors, apoptosis, adhesion, angiogenesis, invasion, and metastasis, are activated or inactivated in HCT-116 cells upon exposure to PRE and PREW (Das et al., 2009). The specific genes recognized by the profiler are listed in Table 1, along with their descriptions.

Table 1. Genes studied using the HCPFRPCR Profiler

Symbol	Description	Symbol	Description
AKT1	V-akt murine thymoma viral oncogene homolog 1	MCAM	Melanoma cell adhesion molecule
ANGPT1	Angiopoietin 1	MDM2	Mdm2 p53 binding protein homolog (mouse)
ANGPT2	Angiopoietin 2	MET	Met proto-oncogene (hepatocyte growth factor receptor)
APAF1	Apoptotic peptidase activating factor 1	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)
ATM	Ataxia telangiectasia mutated	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
BAD	BCL2-associated agonist of cell death	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
BAX	BCL2-associated X protein	MTA1	Metastasis associated 1
BCL2	B-cell CLL/lymphoma 2	MTA2	Metastasis associated 1 family, member 2
BCL2L1	BCL2-like 1	MTSS1	Metastasis suppressor 1
BRCA1	Breast cancer 1, early onset	MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
CASP8	Caspase 8, apoptosis-related cysteine peptidase	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
CCNE1	Cyclin E1	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
CDC25A	Cell division cycle 25 homolog A (*S. pombe*)	NME1	Non-metastatic cells 1, protein (NM23A)
CDK2	Cyclin-dependent kinase 2	NME4	Non-metastatic cells 4, protein
CDK4	Cyclin-dependent kinase 4	PDGFA	Platelet-derived growth factor alpha polypeptide
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
CFLAR	CASP8 and FADD-like apoptosis regulator	PLAU	Plasminogen activator, urokinase
CHEK2	CHK2 checkpoint homolog (*S. pombe*)	PLAUR	Plasminogen activator, urokinase receptor
COL18A1	Collagen, type XVIII, alpha 1	PNN	Pinin, desmosome associated protein
E2F1	E2F transcription factor 1	RAF1	V-raf-1 murine leukemia viral oncogene homolog 1
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	RB1	Retinoblastoma 1

Table 1. Genes studied using the HCPFRPCR Profiler Cont.

Symbol	Description	Symbol	Description
ETS2	V-Ets erythroblastosis virus E26 oncogene homolog 2 (avian)	S100A4	S100 calcium binding protein A4
FAS	Fas (TNF receptor superfamily, member 6)	SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin), member 5
FGFR2	Fibroblast growth factor receptor 2	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	SNCG	Synuclein, gamma (breast cancer-specific protein 1)
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	SYK	Spleen tyrosine kinase
HTATIP2	HIV-1 Tat interactive protein 2, 30kDa	TEK	TEK tyrosine kinase, endothelial
IFNA1	Interferon, alpha 1	TERT	Telomerase reverse transcriptase
IFNB1	Interferon, beta 1, fibroblast	TGFB1	Transforming growth factor, beta 1
IGF1	Insulin-like growth factor 1 (somatomedin C)	TGFBR1	Transforming growth factor, beta receptor 1
IL8	Interleukin 8	THBS1	Thrombospondin 1
ITGA1	Integrin, alpha 1	TIMP1	TIMP metallopeptidase inhibitor 1
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	TIMP3	TIMP metallopeptidase inhibitor 3
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	TNF	Tumor necrosis factor (TNF superfamily, member 2)
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	TP53	Tumor protein p53
ITGB5	Integrin, beta 5	TWIST1	Twist homolog 1 (Drosophila)
JUN	Jun oncogene	EPDR1	Ependymin related protein 1 (zebrafish)
MAP2K1	Mitogen-activated protein kinase kinase 1	VEGFA	Vascular endothelial growth factor A

To produce cDNA required to perform this analysis, RNEasy kit (Quiagen, Valencia, CA) was used to extract RNA from unexposed HCT-116 cells, HCT-116 cells exposed to PRE at concentrations of 1600 µg/ml and 3200 µg/ml, and HCT-116 cells exposed to PREW at concentrations of 800 µg/ml and 1600 µg/ml. All extracts and fractions were dissolved in 0.5% DMSO and the unexposed cells were exposed only to 0.5% DMSO (control). HCT-116 cells were incubated in the 96 well microplate along with the specified concentration of extract or fraction in the same manner as they were in the antiproliferative assay. The extra step that involves DNase exposure was included in the RNA extraction process to ensure that any remaining DNA was destroyed. After RNA extraction, verification of RNA purity was determined by use of NanoDrop Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington, DE), and cDNA was created using the RT^2 First Strand Kit (S.A. Biosciences).

Fold changes comparing the experimental groups and the control (unexposed) were determined for each gene tested. Averages and standard deviations of at least 2 replicates per extract or fraction were determined. An average was also obtained for housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB) that are included in each exposure. The average threshold value of the five housekeeping genes was determined and this average was subtracted from each gene average, which was obtained from the 2 replicates to determine the threshold cycle of each gene. This calculation was performed for the extracts and fractions tested as well as for the control groups, which were derived from unexposed cells. Next, to consider the inverse proportional relationship between the threshold cycle and the original expression level of the gene, the

negative result of that difference was taken to the second power, to consider the doubling of the amount of product per cycle. The fold difference was then determined by dividing the result of the experimental sample by the control sample. The fold up or down change was determined by dividing the fold difference by 1 if greater than 1 or dividing it into -1 if the fold change was less than 1. A fold up or down regulation of ±2 was considered significant (SA Biosciences, Frederick, MD).

Statistical Analysis

For the antiproliferative and caspase studies, data was analyzed by Analysis of Variance (ANOVA) using Statistical Analysis Software (SAS, 2000) with mean treatments at each concentration tested separately by comparing the percent reduction. Least significant means was run as a post ANOVA test to determine significance at $P \leq 0.05$. For the Cancer Profiler, an upregulation or downregulation of ±2 was considered significant using the formula developed by Pfaffl (2001).

CHAPTER 4

RESULTS AND DISCUSSION

Antiproliferative Effects of *P. americana* Extracts on HCT-116 Colon and MCF-7 Breast Cancer Cells

The ethanol extract of *P. americana* root (PRE) inhibited the proliferation of HCT-116 cells to a greater degree than the methanol (PRM) and water (PRW) extracts at the same concentrations (Figures 1-3). The average inhibition rate of the crude ethanol extract against HCT-116 cells was 20% at 3200 µg/ml after 72 hours (Figure 3). There was no significant inhibitory activity ($P \geq 0.05$) of any of the *P. americana* crude extracts against MCF-7 cells *in vitro* (Figures 4-6).

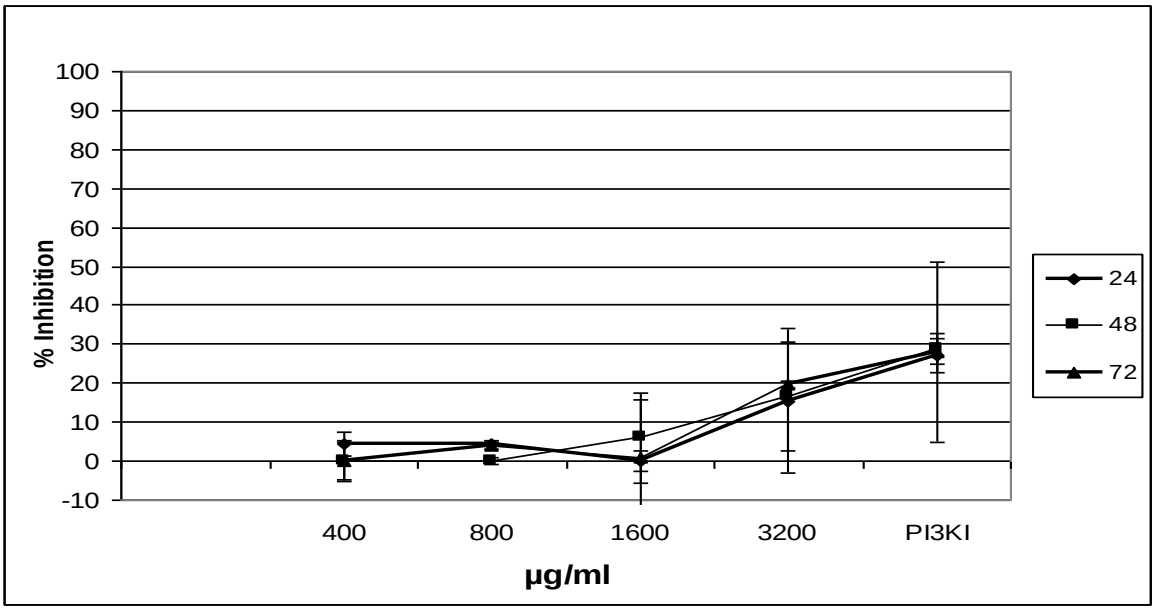

Figure 1. The percent inhibitory activity of ethanol extract (PRE) of *P. americana* against HCT-116 cancer cells at 24, 48, and 72 hours ($P \leq 0.05$)
PI3KI=Phosphatidlyinositol 3 kinase inhibitor (used as a positive control) at 200 µg/ml

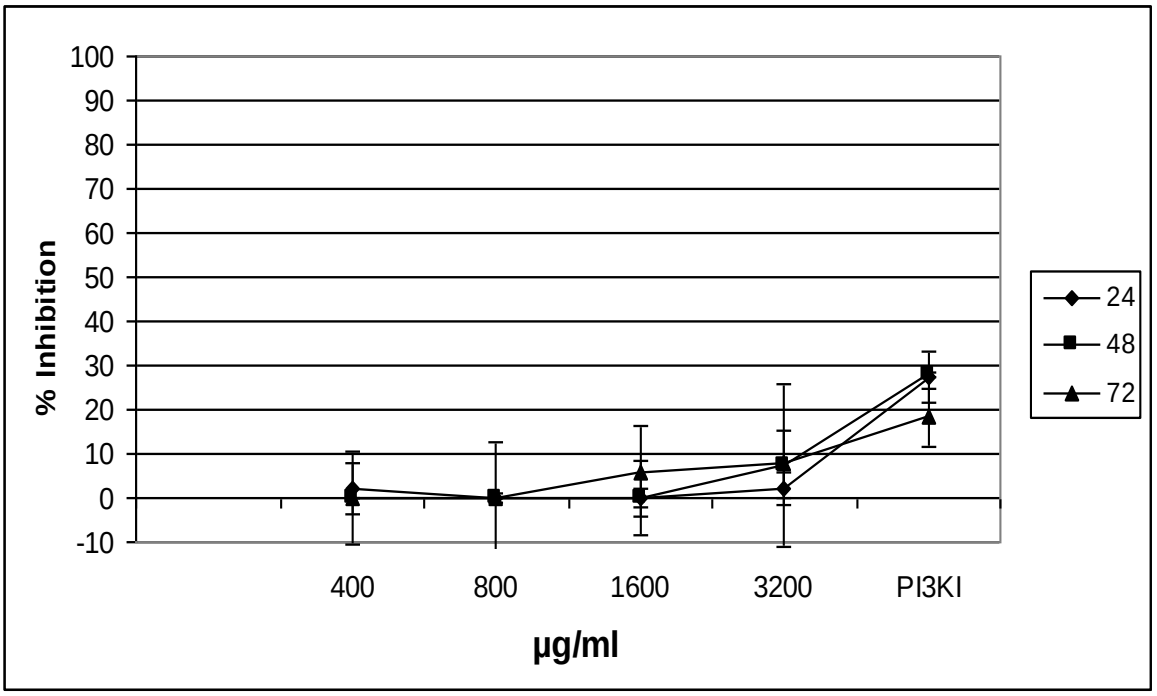

Figure 2. The percent inhibitory activity of methanol extract (PRM) of *P. americana* against HCT-116 cancer cells at 24, 48, and 72 hours (P≤0.05)

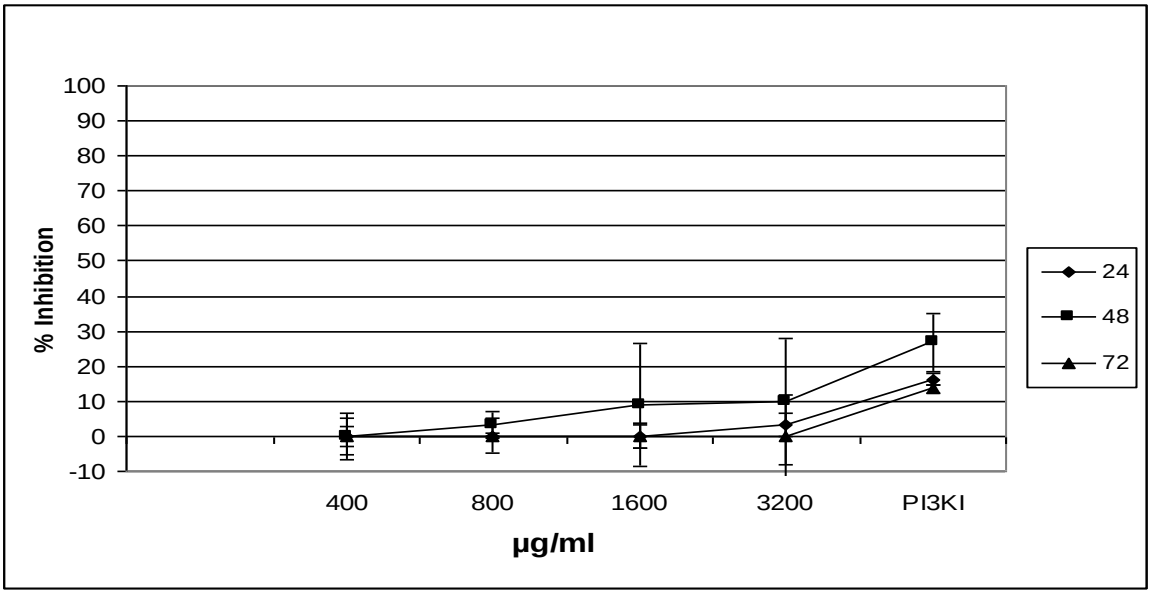

Figure 3. The percent inhibitory activity of water extract (PRW) of *P. americana* against HCT-116 cancer cells at 24, 48, and 72 hours (P≤0.05)

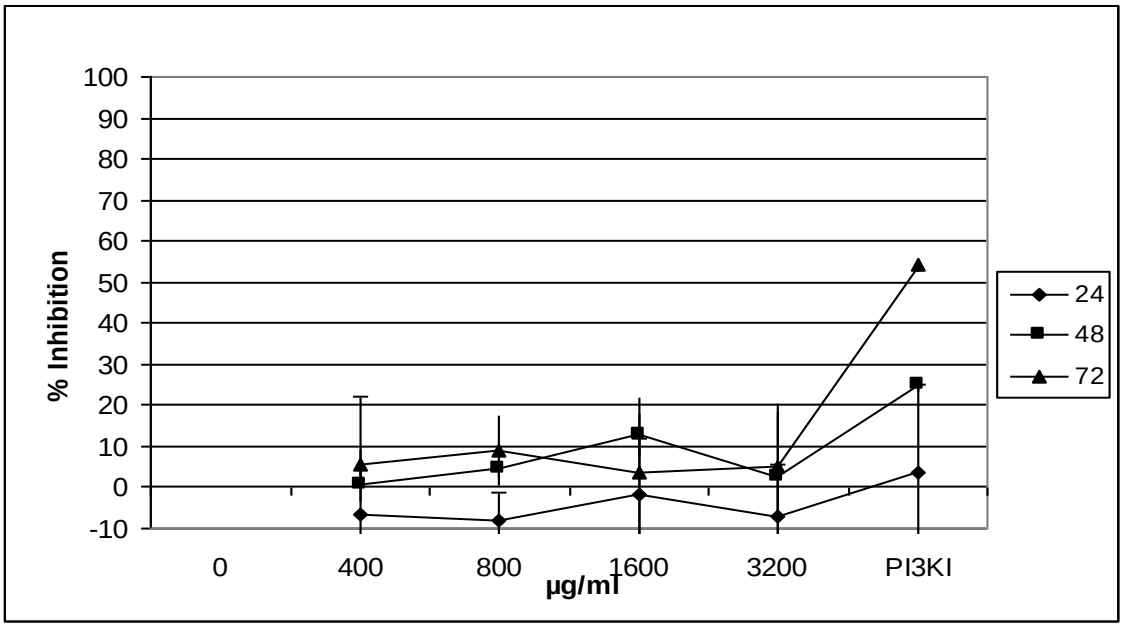

Figure 4. The percent inhibitory activity of ethanol extract (PRE) of *P. americana* against MCF-7 cancer cells at 24, 48, and 72 hours (P≤0.05)

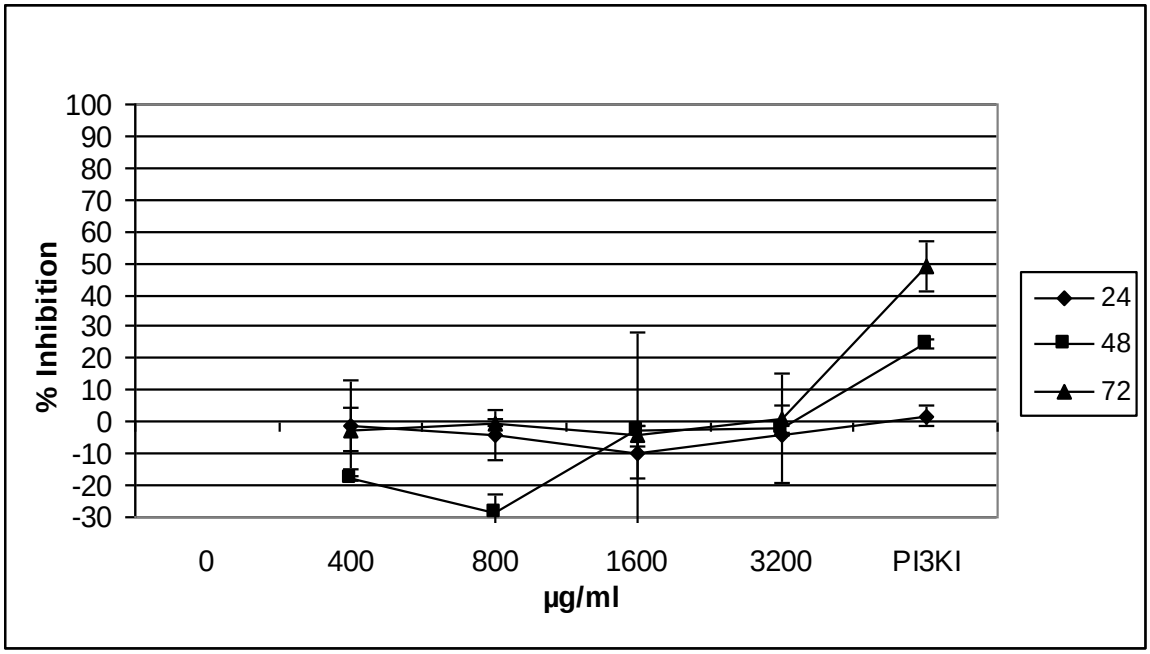

Figure 5. The percent inhibitory activity of methanol extract (PRM) of *P. americana* against MCF-7 cancer cells at 24, 48, and 72 hours (P≤0.05)

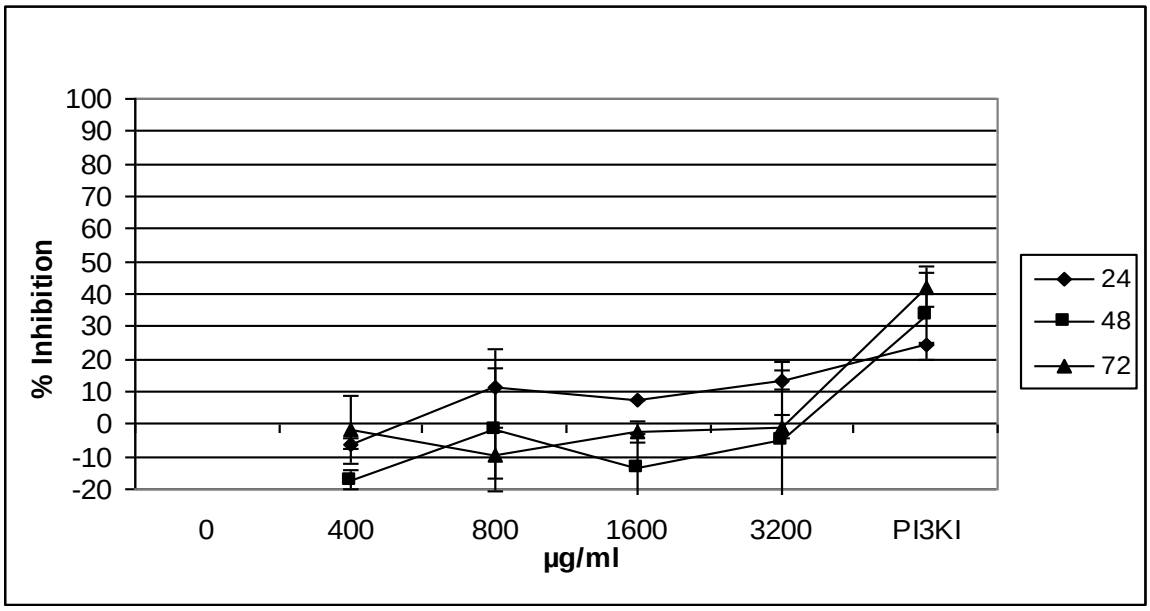

Figure 6. The percent inhibitory activity of water extract (PRW) of *P. americana* against MCF-7 cancer cells at 24, 48, and 72 hours (P≤0.05)

It is important to recognize that the compounds that result from the extraction process often vary depending on the extraction methods. For example, using water for extraction will yield water-soluble extracts, while extraction with alkali solutions results in water-insoluble extracts (Mizuno, 1996). Furthermore, chemically fractionating the crude extracts after processing often results in a variety of groups that have different functions from one another (Lindholm, 2002). For example, methanol was used to make an extract from the leaves of the *Crataegus pinnatifida* tree and then this extract was further fractionated with dicholormethane and water (Min et al., 2000). Uvaol and ursolic acid were two cytotoxic triterpenes that resulted from this *C. pinnatifida* fractionation. 3-Oxo-ursolic acid was then synthesized from ursolic acid. Although the first 2 fractions exhibited moderate anticancer activities against murine L1210 and human

cell lines, they exhibited weak activities against A549, XF498, and HCT15, among others. However, 3-Oxo-ursolic acid exhibited strong activities against all cell types tested.

Similarly, the mushroom *Hypsizygus marmoreus* has been shown to produce 1,3-α-Glucan and 1,3-β-Glucan from its fruiting bodies (Motoi, 2003). Only the purified 1,3-β-Glucan polysaccharide exhibited antitumor activity against Sarcoma 180 tumor in mice, while 1,3-α-Glucan did not. In another study, five polysaccharides were derived from the Basidiomycete, *Grifola frondosa*, which had diverse molecular masses with different biological activities (Lee, 2003). A few of the fractions resulted in strong free radical and scavenging activity, while other fractions increased the proliferation of fibroblasts.

These studies highlight the idea that it may be more precise to use fractionated groups of mushroom extracts when studying their effects, since the raw extracts likely contain diverse groups with functions that vary. However, some of the individual fractions may work synergistically and, thus, result in stronger anti-carcinogenic activity than chemically separated compounds.

In this study, the ethanol extract inhibited the HCT-116 cells more than the methanol and water extracts. Ethanol extracts are known to yield hydrophobic compounds, such as benzo-dioxanes and phenolic acids, and possibly saponins and glycosides (Mizuno, 1996; Takahashi et al., 2003). These are the types of bioactive compounds that are likely responsible for the increases in inhibitory activity by the ethanol extract found in this study.

The next step in this study was to fractionate the *P. americana* ethanol extract to determine whether one fraction contained most of the inhibitory activity or if the activity on the HCT-116 cells was synergistic. The fractions that resulted were ethyl acetate, butanol, and water (PREW). At a concentration of 200 µg/ml, the ethyl acetate fraction inhibited the proliferation of HCT-116 cells by 8% (Figure 7), the butanol fraction inhibited the growth of HCT-116 cells by up to 7% (Figure 8), and the water fraction showed the greatest inhibitory activity of up to 18% (Figure 9).

These results, at first, suggest that the inhibitory activity of the ethanol extract may be synergistic and that fractionating may reduce its antiproliferative effect, requiring that the intact extract may be necessary to exhibit pronounced effects. However, lower concentrations were used in performing antiproliferative studies for each fraction than those used in studies to test the antiproliferative activity of PRE.

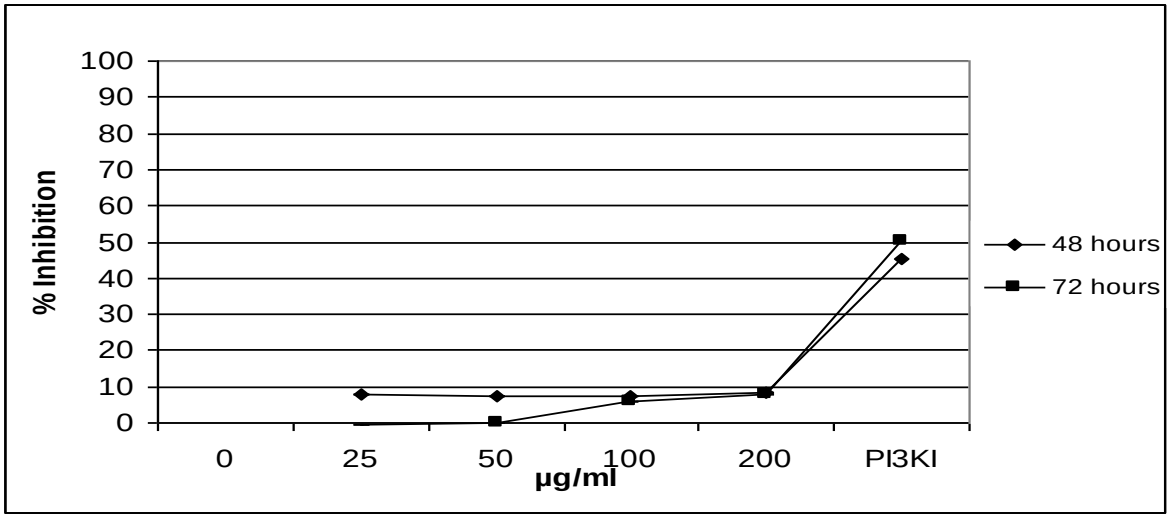

Figure 7. The percent inhibitory activity of ethyl acetate fraction derived from PRE against HCT-116 cancer cells at 48 and 72 hours (P≤0.05)

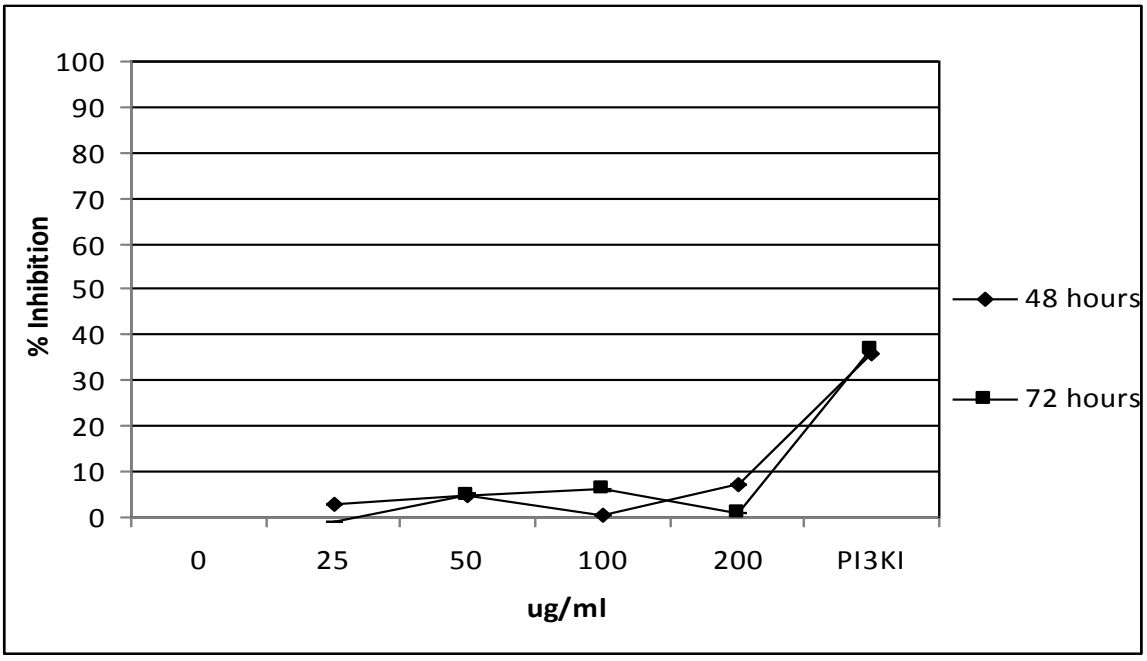

Figure 8. The percent inhibitory activity of butanol fraction derived from PRE against HCT-116 cancer cells at 48 and 72 hours (P≤0.05)

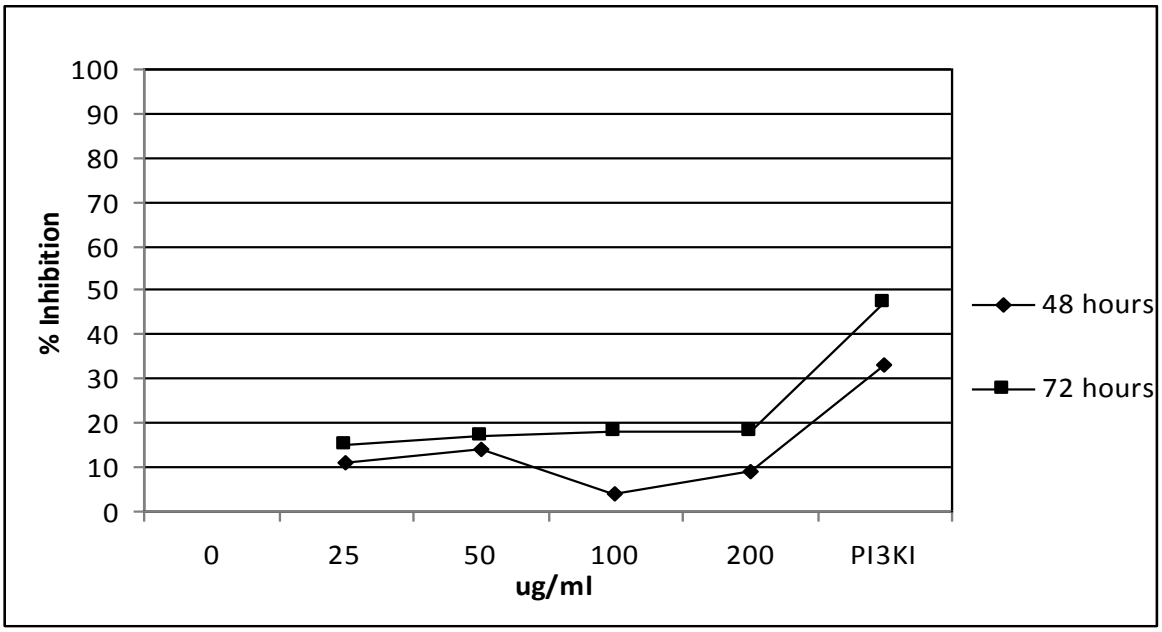

Figure 9. The percent inhibitory activity of water fraction (PREW) derived from PRE against HCT-116 cancer cells at 48 and 72 hours (P≤0.05)

The fraction yield of PREW (4734.08 mg) was 83.35% of the starting material of ethanol extract used in the fractionation process. At this percent yield, to correlate with the ethanol extract of 3200 µg/ml that was tested, 2667 µg/ml of PREW would have been needed. This would likely have resulted in greater inhibitions of HCT-116 cells exposed to PREW.

The comparison study included pure pokeweed antiviral protein (PAP) isolated from *P. americana* and the crude ethanol extract of pokeweed (PRE). The inhibitory activity of PAP against HCT-116 cells at 5 µg/ml correlated closely (19% inhibition, Figure 10) with the inhibitory rate of PRE tested at 3200 µg/ml (18%) after 48 hours of exposure.

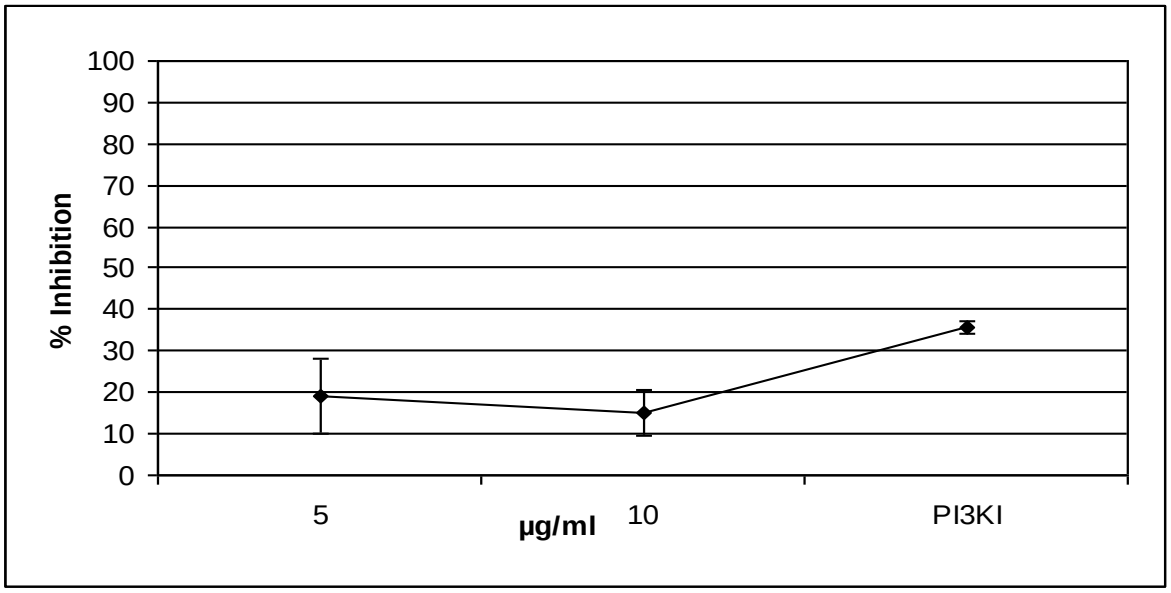

Figure 10. The percent inhibitory activity of PAP against HCT-116 cancer cells after 48 hours of exposure (1st trial) (P≤0.05)

However, the growth inhibitory activity of concentrations greater than 5 µg/ml varied in their activities as exemplified in Figure 11. It would be best to repeat the experiments using a wide range of concentrations. However, repeated studies with PAP are very difficult at this time due to the scarcity of this pure protein.

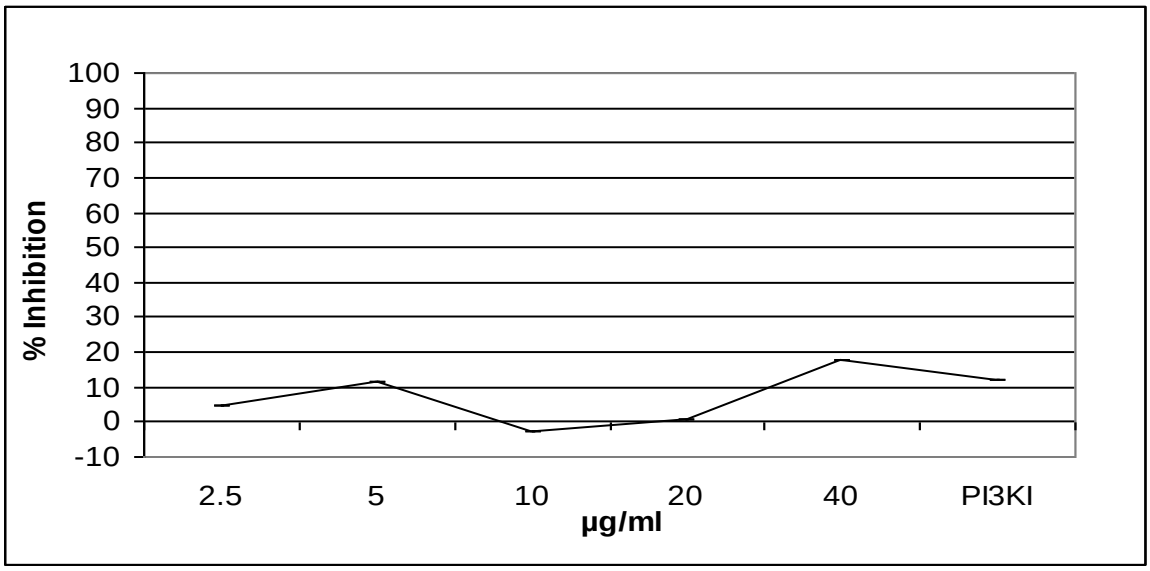

Figure 11. The percent inhibitory activity of PAP against HCT-116 cancer cells after 48 hours of exposure (2nd trial) (P≤0.05)

Caspase Activity of HCT-116 Colon Cancer Cells Exposed to *P. americana* Ethanol Extract and its Water Fraction

For the ethanol extract of *P. americana* (PRE), caspase 2 had a 43% decrease in activity when HCT-116 cells were exposed to PRE at 1600 µg/ml. A slight change in activity, a 6% increase was observed in HCT-116 cells after 48 hours of exposure to PRE at the 3200 µg/ml (Table 2). Caspase 3 activity increased by 12% at 1600 µg/ml but

decreased by 23% at 3200 µg/ml. Caspase 6 had the most significant change in activity with an increase of 340% when using a PRE concentration of 1600 µg/ml and an increase of 887% when using a concentration of 3200 µg/ml. Caspase 8 activity in HCT-116 cells increased by 12% with a PRE concentration of 1600 µg/ml and decreased by 9% after exposure of the cells to 3200 µg/ml of PRE. Caspase 9 activity showed a significant ($P \leq 0.05$) increase in activity of 216% at 1600 µg/ml of PRE and an increase in activity of 12% after exposure to 3200 µg/ml of PRE.

Table 2. Measured absorption (405 nm) and change in caspase activity after HCT-116 cells were exposed to PRE ($P \leq 0.05$)

Caspases	Concentrations (µg/ml)			
	0	**16**	**32**	**PI3KI**
2	0.04±0.0004	0.02±0.0002 (-43)	0.04±0.0008 (6)	0.10±0.0040 (148)
3	0.05±0.0020	0.05±0.0015 (12)	0.04±0.0012 (-23)	0.09±0.0063 (91)
6	0.00±0.0000	0.02±0.0004 (340)	0.06±0.0240 (887)	0.05±0.0025 (681)
8	0.03±0.0003	0.03±0.0012 (12)	0.03±0.0003 (-9)	0.10±0.0065 (343)
9	0.02±0.0008	0.06±0.0030 (216)	0.02±0.0008 (12)	0.04±0.0020 (150)

Numbers in parentheses indicate % change in caspase activity; PI3KI tested at 200 µg/ml

It is apparent from Figure 12 that the activity of caspase 6, an effector caspase, was significantly ($P \leq 0.05$) upregulated at both concentrations of PRE derived from the roots of *P. americana.* Similar results were confirmed in a similar study in which chlorophyllin was shown to work as a chemotherapeutic agent using a mechanism involving caspase 6 as well as caspase 8, BID, and BAK, ultimately causing the cleavage

of nuclear lamins (Diaz et al., 2003). It is also apparent from Figure 12 that caspase 9 was upregulated when the cells were exposed to 1600 µg/ml of PRE. However, the activity was found to be relatively weak compared to the other caspases due to activity not increasing with increasing concentration.

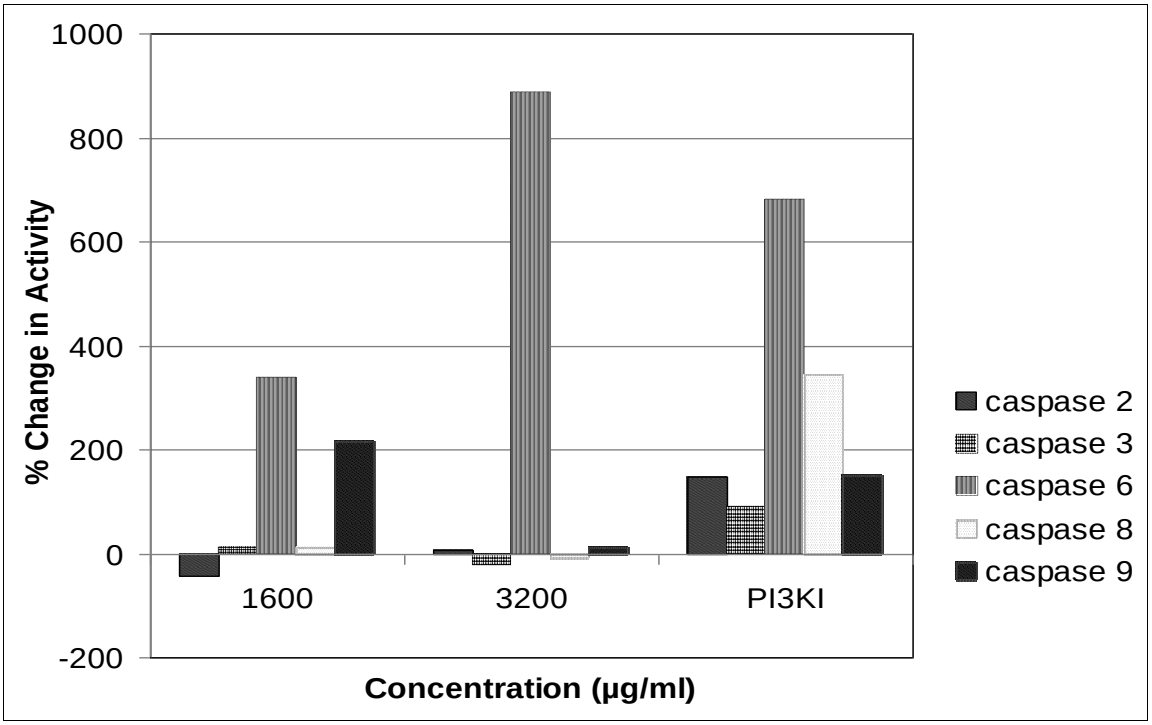

Figure 12. Change in caspase 2, 3, 6, 8, and 9 activities induced in HCT-116 cells after incubation with PRE at 1600 and 3200 µg/ml for 48 hours

Caspase 9 is an initiator caspase necessary to activate caspase 6 and other effector caspases (Slee et al., 1999; Zou, 1997). Other herbs, such as *Viscum album*, Chios mastic gum, members of the *Brassica* genus of plants, and *Hypericum perforatum* L. were shown to initiate caspase 9 activity along with other enzymes involved in the apoptotic

process. The increases in caspases 9 and 6 caused by PRE correlate well in this study, suggesting that the mitochondrial apoptotic pathway was affected by this extract.

Following exposure of the water fraction (PREW) derived from PRE, the mitochondrial apoptotic pathway was also affected. Caspase 2 showed decreases in activity at all concentrations tested in this study (Table 3). Caspase 3 activity was increased by 69% after HCT-116 cells were exposed to 400 µg/ml of PREW and increased by 24% at 1600 µg/ml. However, at a concentration of 800 µg/ml, there was a decrease of 18%. For caspase 6 activity, there was an 80% decrease at 400 µg/ml, a 49% decrease at 800 µg/ml, and a coinciding decrease of 51% at 1600 µg/ml. For caspase 8, there was no change in activity at 400 µg/ml, an increase in activity of 137% at the concentration of 800 µg/ml, and an increase of 24% at the concentration of 1600 µg/ml. Caspase 9 had no change in activity at 400 µg/ml, a 12% increase in activity at 800 µg/ml, and an increase of 39% at 1600 µg/ml.

Table 3. Measured absorption (405 nm) and change in caspase activity after HCT-116 cells were exposed to PREW (P≤0.05)

Caspases	Concentrations (µg/ml)				
	0	**4**	**8**	**16**	**PI3KI**
2	0.02±0.0006	0.02±0.0008 (-2)	0.02±0.0006 (-23)	0.02±0.0006 (-26)	0.07±0.0035 (191)
3	0.03±0.0015	0.05±0.0015 (69)	0.02±0.0008 (-18)	0.04±0.0008 (24)	0.10±0.0010 (227)
6	0.06±0.0030	0.01±0.0003 (-80)	0.03±0.0012 (-49)	0.03±0.0015 (-51)	0.04±0.0000 (-21)
8	0.01±0.0002	0.00±0.0000 (0)	0.03±0.0015 (137)	0.02±0.0006 (24)	0.03±0.0009 (134)
9	0.02±0.0010	0.00±0.0000 (0)	0.03±0.0012 (12)	0.03±0.0012 (39)	0.07±0.0042 (193)

Numbers in parentheses indicate % change in caspase activity; PI3KI tested at 200 µg/ml

It is apparent from Figure 13 that caspase 3 activity in HCT-116 cells exposed to PREW at 400 µg/ml is upregulated. It is slightly upregulated after exposure to 1600 µg/ml of the same fraction. This upregulation, however, was not significant ($P \geq 0.05$), because the increase in activity did not continue with subsequent increases in concentration. That is, the increase in activity of caspase 3 was apparent at the lowest concentration and slightly apparent at the highest concentration tested with a slight downregulation after exposure to 800 µg/ml of PREW. The activity did not rise with increasing concentrations. It may be that since there are many compounds associated with this fraction, some of those compounds act antagonistically depending on the concentration used.

Caspase 8 activity of HCT-116 cells was upregulated significantly ($P \leq 0.05$) only after exposure to 800 µg/ml of PREW with a slight upregulation at 1600 µg/ml. In addition, caspase 9 activity in HCT-116 cells was upregulated slightly after exposure to 800 µg/ml of PREW, and slightly more upregulated after the cells were exposed to 1600 µg/ml of the same fraction. This was a significant increase in activity ($P \leq 0.05$).

Caspases 8 and 9 are initiator caspases and caspase 3 is an effector caspase. As previously stated, various pathways that lead to the activation of caspase 3 may be dependent or independent of the release of cytochrome c (Porter et al., 1999). Caspase 3 affects a number of substrates and, thus, impact cell death in several ways, including the activation of gelsolin and DFF, leading to both nuclear and DNA fragmentation (Kothakota et al., 1997; Liu et al., 1997). Caspase 8 is usually activated by the FAS gene (Krueger et al., 2001). This is interesting because exposing HCT-116 cells to 800 µg/ml

of water fraction resulted in a 2.2-fold upregulation of FAS. An upregulation of FAS did not occur with the exposure to the water fraction at 1600 µg/ml.

Similar to the results displayed in Table 3, Chios mastic gum was able to activate caspases 3, 8, and 9 in HCT-116 cells (Balan, 2005). In addition, *Viscum album* exposure resulted in measurable caspase 8, 9, and 3 activities in T- and B- cell leukemia cell lines (Bantel et al., 1999). *Hypericum perforatum* L., commonly known as St. John's wort, contains an active component, hyperforin, which has been shown to increase caspase 8 and 3 activities in K562 human histiocytic lymphoma cells (Hostanska, 2003).

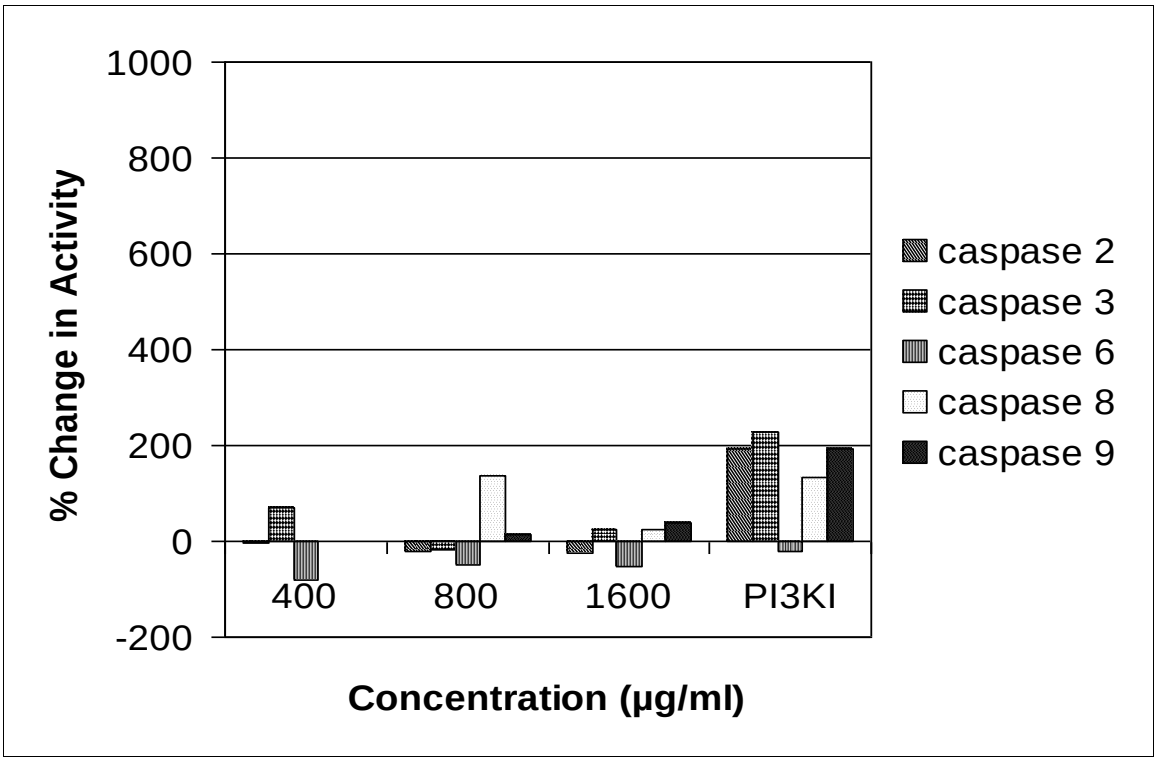

Figure 13. Change in caspase 2, 3, 6, 8, and 9 activities induced in HCT-116 cells after incubation with PREW at 400, 800, and 1600 µg/ml for 48 hours

From the above results, the water fraction appears to slightly downregulate caspase 2, an initiator caspase, and 6, an effector caspase. This makes the use of the water fraction in activating apoptosis questionable. While this fraction resulted in activation of one branch of the apoptotic chain, that involving caspases 3, 8, and 9, it resulted in the inactivation of another, the branch involving caspases 2 and 6.

It has been reported that there are mechanisms that lead to apoptosis other than caspase mediation (Broker et al., 2005). Other models include autophagy, mitotic catastrophe, and paraptosis. Autophagy is a catabolic process in which the cell degrades its own constituents. Although there are several varieties of autophagic processes, all degrade intracellular components via the lysosome. Paraptosis is activated by TNF receptor family members TAJ/TROY (TNFRSF19) and an insulin-like growth factor I receptor (ILGFR1), characterized by increased cytoplasmic vacuolization and increased cell electron density (Wang et al., 2004; Sperandio et al., 2004). Defective cycle checkpoints cause mitotic catastrophe, and it is still debated as to whether or not this is completely p53 and caspase independent (Castedo et al., 2004).

Another possibility is that the antiproliferative effects that result from HCT exposure to PRE and its corresponding water fraction, PREW, are not derived from apoptosis at all. The mechanism could be similar to that seen in the human embryonic kidney cell line 293T after exposure to PAP, whereby PAP induced JNK activation without apoptosis (Chan Tunga et al., 2008). Alternatively, other genes could be activated to slow or stop the cell cycle without causing apoptosis.

Human Cancer Pathway Finder Realtime PCR Profiler

Table 4 lists the genes with the corresponding extracts that resulted in greater than or equal to ±2-fold change when compared to HCT-116 cells that were unexposed, indicating that the upregulation or downregulation in gene activity is significant. Whether or not the change in gene transcription is considered desirable to the treatment of colon cancer was determined by understanding the role that the particular gene has on colon or other types of cancer. For example, if a known oncogene was downregulated more than 2-fold in HCT-116 cells after exposure to one of the extracts of fractions when compared to unexposed HCT-116 cells, then that was considered to be a desirable gene change.

The expression of several genes has been found to be associated with HCT-116 cell proliferation. It has been shown that HCT-116 cells are dependent upon the β-catenin pathway for proliferation (Cho et al., 2005; Kapiteijn et al., 2001). K-RAS and MYC have also been shown to be necessary for their proliferation (Firestein et al., 2008). Additionally, BCL2 was found to suppress p53 activity in HCT-116 cells (Jiang and Milner, 2003).

Although some genes specifically associated with HCT-116 cells are present on the Cancer Pathway Finder RT-PCR Profiler, others are not since this test was designed for use in the study of a variety of cancer types. The MYC, p53, and BCL2 genes are present on this array while K-RAS is not. However, genes associated with K-RAS are present, such as RAF and FOS.

Table 4. Genes that resulted in up and down regulations of greater than or equal to ±2 when compared to the unexposed control

Extract	Gene	Fold change	Extract	Gene	Fold Change
W800	FAS	2.2, D	E1600 (cont.)	IGF1	2.9, U
	IGF1	2.6, U		ITGA3	2.6, U
	ITGA4	2.6, D		ITGA4	2.9, D
	MMP2	2.3, U		MCAM	2.1, U
	MYC	25.5, U		MMP2	2.6, U
	TERT	2.6, U		MTA1	2.6, U
W1600	CDK4	2.0, U		MTA2	3.1, U
	MYC	34.8, U		MYC	-7.1, D
	NFKBIA	-2.1, U		PIK3R1	2.1, U
	NME4	-2.1, D		PLAUR	2.2, U
	SYK	-3.2, U		TERT	2.9, U
	TEK	-2.1, D		TGFB1	2.9, U
	THBS1	-2.2, D		TIMP1	3.8, U
	VEGFA	2.1, U		TNFRSF10B	2.1, D
E1600	AKT1	2.6, U		TNFRSF25	3.2, D
	BCL2L1	3.2, U		VEGFA	3.2, U
	CDC25A	2.5, U	E3200	IFNA1	-2.9, U
	E2F1	3.5, U		MYC	-19.7, D
	ERBB2	2.3, U		PLAU	-2.1, D
	FOS	2.1, U		TEK	-2.4, D

(W800= water fraction 800 µg/ml, W1600= water fraction 1600 µg/ml, E1600= ethanol extract 1600 µg/ml, E3200= ethanol extract 3200 µg/ml; D= desirable gene change, U= undesirable gene change)

In this study, the gene with the greatest fold up or downregulation of transcription in all extracts and fractions tested on HCT-116 cells was MYC. For the duplicate runs of all genes tested, the standard deviation was <0.05. The same holds for the duplicates of the control runs with the exception of the MYC gene which resulted in a standard deviation of 6.0. According to Table 4, the MYC gene was upregulated 25.5-fold after exposure to PREW at a concentration of 800 µg/ml (W800) and 34.8-fold when exposed to PREW at a concentration of 1600 µg/ml (W1600 extract). However, the MYC gene was down-regulated -7.1-fold after exposure to PRE at a concentration of 1600 µg/ml

(E1600) and -19.7-fold after exposure to PRE at a concentration of 3200 µg/ml (W3200). The changes in regulations are consistent with strengthening extract or fraction concentration. The MYC gene is a homolog of the V-MYC myelocytomatosis avian viral oncogene. This gene has been found to be necessary for the proliferation of the HCT-116 colon cancer cells and influences the β-catenin pathway (Firestein et al., 2008; Cho et al., 2005). With further testing, use of the ethanol extract of poke root could prove to inhibit the function of this gene, whereas the water fraction increased the transcription of this gene.

With the W800 fraction, the FAS gene was upregulated by 2.2-fold. This gene is a member of the TNF receptor superfamily. TNF is involved with inducing inflammation and apoptosis. Therefore, the upregulation of this gene in cancer cells is desirable. FAS can activate caspase 3 and 8 (Kothakota et al., 1997; Luo et al., 1998) and is involved in the death-inducing signaling complex, DISC (Krueger et al., 2001). As mentioned previously, caspase 8 activity was increased by 137% after the HCT-116 cells were exposed to PREW at 800 µg/ml. This is a good correlation because caspase 8 is activated by the FAS gene.

Insulin-like growth factor 1 (IGF1), also known as somatomedin C, was upregulated 2.6-fold by exposure to the W800 fraction. IGF1 is an activator of the AKT signaling pathway that stimulates cell growth and proliferation, inhibits apoptosis, and is involved in angiogenesis. This would be a detrimental upregulation in cancer cells. Likewise the upregulation of matrix metallopeptidase 2 (MMP2) of 2.3-fold is unwanted because it is a gelatinase A involved in extracellular matrix degradation, invasion, and

metastasis. It was shown that activations of MMP2 are associated with Dukes A and C stages of colorectal cancer (Liabakk et al., 1996). Dukes A means the cancer is only affecting the innermost colon lining or just slightly growing into the layer of muscle while Dukes C indicates that the cancer has spread to at least one lymph node. In addition, the upregulation of TERT, telomerase reverse transcriptase, of 2.6-fold could be invaluable because this gene is involved in stabilizing cell lines during cell cycling. It was shown that TERT activation occurs early during tumorigenesis *in vivo* in human breast and colon tissues (Kolquist et al., 1998). In fact, MYC is known to activate TERT (Wu et al., 1999). Therefore, it is a good correlation in this study that both genes were found to be upregulated.

The upregulation of integrin alpha 4 (ITGA4) of 2.6-fold could be seen as desirable since this gene is an integrin, which is involved in cell adhesion. Many metastatic cells lose their ability to adhere, but in this case the adherence is increased. ITGA4 was found to be commonly methylated, or silenced, in colorectal cancers and the methylated form could be a promising marker for early detection of colon neoplasms (Ausch et al., 2009). Integrins, however, are generally complex in their roles with cancer cells. Various integrins may be activated or inactivated in different cancer cell lines. ITGA4 was actually found to be upregulated in H1650 lung cancer cells (Fan et al., 2009).

According to the genes that are upregulated by exposure of HCT-116 cells to W800 fraction, it does not appear to be advisable to use this fraction on colon cancer cells. Four of the six genes that were found to be upregulated are genes that would preferably be downregulated. Only two of the upregulated genes, FAS and ITGA4, would be

desirable to be upregulated in cancer cells because one contributes to apoptosis and the other sustains cellular adhesion. However, according to the antiproliferative assay results of the water fraction as well as the fact that caspase 8 activity was increased by 137% in HCT-116 cells exposed to W800 fraction, there still may be a cause for further research. It could also be that there are other genes involved in the process that are not included on the Human Cancer Pathway array. It should be noted that the activity of caspase 8 on the array was upregulated 1.2-fold. This does correlate with the result that FAS was upregulated 2.2-fold, since they work together in the mitochondrial apoptotic pathway.

With the W1600 fraction, entirely different genes were up or down regulated when compared to the W800 fraction. CDK4, MYC, and vascular endothelial growth factor A (VEGFA) were upregulated while NFKBIA, non-metastatic cells 4 (NME4), spleen tyrosine kinase (SYK), tyrosine endothelial kinase (TEK), and thrombospondin 1 (THBS1) were downregulated. As with the W800 fraction, understanding the functions of these genes is important in determining whether or not the changes in gene function are beneficial.

CDK4 is cylin-dependent kinase 4 and is involved in cell cycle control and DNA damage repair. It has been shown to phosphorylate and inactivate the tumor suppressing RB gene (Meyerson et al., 1992), which could cause cancer to increase. Cyclin-dependent kinase 4 (CDK4) proteins decreased upon exposure to resveratrol, a polyphenol present in grapes, peanuts, and red wine, in Caco-2 and HCT-116 cells when compared to control groups (Wolter et al., 2001). However, CDK4 was upregulated 2-

fold when HCT-116 cells were exposed to the W1600 fraction in this study, indicating that this exposure could cause the cancer cells to further proliferate.

The VEGFA gene was upregulated 2.1-fold in our study after exposure of HCT-116 colon cancer cells to the W1600 fraction. This gene is involved in angiogenesis and appears to be regulated by matrix metalloproteinases to determine vascular patterns (Lee et al., 2005). Thus, the upregulation of this gene would be harmful in the treatment of cancer. Similarly, the downregulation of SYK of -3.2-fold is undesirable. This gene is known to be involved in adhesion and was downregulated in metastatic breast cancers and is suggestive of a poor prognosis, although the exact role of SYK in breast cancer is questionable (Toyama et al., 2003). When treating colon cancer, it would be desirable to have an increase in SYK transcription to maintain cell adhesion.

NFKBIA was downregulated 2.1-fold in HCT-116 cells exposed to the W1600 fraction. This gene product binds to NF- κB to prevent its activation, which serves to block apoptosis induction (Wang et al., 1998). Therefore, a downregulation of this gene caused by W1600 is not ideal, because it is not present to prevent NF-κB from blocking apoptosis. As a result, apoptosis is blocked by NF-κB and the cancer cells continue to proliferate.

The downregulation of NME4 of -2.1-fold is helpful because this gene is involved in invasion and metastasis. It has been shown that the NME4, as well as other markers, are associated with poor prognosis and disease progression of myelodysplastic syndrome (Kracmarova et al., 2008). The downregulations of TEK of -2.1-fold and THBS1 of -2.2-fold in HCT-116 cells exposed to the W1600 fraction are promising because these genes

are involved in angiogenesis (Dales et al., 2004; Park et al., 2000). In fact, THBS1 was proposed to be the primary gene involved in colorectal carcinogenesis (Park et al., 2000), suggesting that the downregulation of this gene is especially valuable in the early prevention or treatment of colon cancer.

A few changes in gene activity after exposure of HCT-116 cells to W1600 are promising. Several of the genes that have undesirable activity were downregulated, such as NME4, TEK, and THBS1. These downregulations of undesirable gene activity could lead to improved prognosis. There were, however, a few genes with undesirable activity that were upregulated, such as CDK4, MYC, and VEGFA. There were also the SYK and NFKBIA genes that were undesirably downregulated. Since W1600 was promising in its antiproliferative activity of HCT-116 cells and had a mixture of desirable and undesirable changes in gene activity, further testing including *in vivo* animal bioassays would be necessary to determine whether this fraction is a good candidate for treatment of colon cancer.

The E1600 exposure activated more genes than any of the other extracts and most of the genes that were activated were genes that were not significantly (±2-fold) affected by the other extracts. Genes that were upregulated included AKT1, BCL2-like 1 (BCL2L1), CDC25A, E2F1, ERBB2, FOS, IGF1, integrin alpha 3 (ITGA3), integrin alpha 4 (ITGA4), melanoma cell adhesion molecule (MCAM), MMP2, metastasis associated 1 (MTA1), metastasis associated 1 family member 2 (MTA2), MYC, PIK3R1, plasminogen activator urokinase receptor (PLAUR), TERT, transforming growth factor beta receptor 1 (TGFB1), TIMP metallopeptidase inhibitor 3 (TIMP1), TNFRSF10B,

TNFRSF25, and VEGFA. The only downregulation determined was of MYC, which was previously discussed. Of these genes, IGF1, ITGA4, MMP2, and TERT were upregulated by similar amounts as the W800 fraction. The transcription of VEGFA was upregulated after W1600 exposure as well.

As was discussed with W800, IGF1 activates the AKT signaling pathway to stimulate cell growth and proliferation and inhibits apoptosis. It is also involved in angiogenesis. Therefore, the upregulation that occurred after exposure to E1600 of the IGF1 gene is not preferred in the treatment of colon cancer. Also upregulated after HCT-116 cells were exposed to W800 was ITGA4, which is an integrin gene involved in cell adhesion. This is a desirable increase in gene activity because metastatic cells lose cell adhesion rather than gain that ability. As previously mentioned, ITGA4 has been found to be methylated in colorectal cancers although its activity was increased in H1650 lung cancer cells (Ausch et al., 2009). MMP2 is a gelatinase A gene involved in invasion and metastasis and was upregulated with E1600 as well as with W800. These upregulations of MMP2 are undesirable. In addition, TERT is involved in stabilizing cell lines during cell cycling and its activation occurs early in tumorigenesis of breast and colon tissues (Kolquist et al., 1998). TERT was critically upregulated after exposure to W800 and E1600. As discussed with W1600, VEGFA is involved in angiogenesis. Collectively, the upregulations of the VEGFA gene that resulted from W1600 and E1600 exposure are detrimental in the treatment of colon cancer.

Most of the genes that were upregulated due to exposure of E1600 were not significantly (±2-fold) affected by the other extracts at different concentrations. ITGA3

is an integrin that was upregulated by this extract concentration only. Although involved in adhesion, the upregulation of this gene has been associated with enhanced cellular communication in tumor tissue (Sultmann et al., 2005), making the upregulation of this gene undesirable.

AKT1 is involved in the phosphorylation of BAD and so prevents apoptosis (Datta et al., 1997). It has also been suggested that AKT, a protein kinase B serine/threonine kinase, plays a role in the prevention of apoptotic activity of human cancers such as colorectal cancer (Kandel et al., 2002). Using HCT-116 cell lines, Kandel et al. (2002) suggested that the activation of AKT may lead to genetic instability by controlling the G2/M cell cycle progression so that it can overcome p53 checkpoints, leading to p53 inactivation. It has also been shown that cancer cells can gain a survival and drug-resistance advantage by a process that begins with mitochondrial respiration problems in some cancer cells that can cause AKT activation (Pelicano et al., 2006). This is then thought to lead to an increase in ATP production through glycolysis, increases in NADH, and inactivation of phophatase and tensin homolog (PTEN) resulting in advantageous activity. It is clear that the upregulation of AKT activity of 2.6-fold found in our study is not advantageous.

BCL2L1 upregulations are associated with the prevention of apoptosis and drug resistances. For example, HCT-116 cancer cell lines resistant to sodium deoxycholate (NaDOC) have been shown to increase the expression of BCL-2, among other genes (Crowley-Weber et al., 2002). The upregulation of 3.2-fold exemplified by exposure to E1600 is not a preferable characteristic for colon cancer treatment.

Likewise, CDC25A is considered a protooncogene with increases in expression in cancerous colon epithelial tissue (Dixon et al., 1998). Studies in fibroblasts of mice show that CDC25A may interact with activated HA-RAS or RB1 loss to play a role in transformation (Galaktionov et al., 1995). The 2.5-fold upregulation of CDC25A that resulted from exposure to E1600 in this study is not desirable.

E2F1 transcription factor has been found to play a role in colon and other cancers (Petrocca et al., 2008; Louie et al., 2004; Kasahara et al., 2000). Furthermore, overexpression of the E2F1 gene has been found to correlate with thymidylate synthase expression in colon cancer, which correlates with poor prognosis (Kasahara et al., 2000). In addition, E2F transcription is regulated by the retinoblastoma tumor suppressor protein (RB), the entire pathway of which regulates DNA replication and is disrupted in many cancers (Nevins et al., 2001). Upon exposure to E1600, the transcription of this gene was upregulated 3.5-fold in HCT-116 cells.

The ERBB2 type I receptor tyrosine kinase has been implicated in the progression of human colon cancer malignant expression (Porebska et al., 2002). The gene encodes a member of the epidermal growth factor receptor family (EGF) of receptor tyrosine kinases. Although it was shown that quercetin can decrease the expression of ERBB2 and ERBB3 in HT-29 colon cancer cells (Kim et al., 2005), the level of expression was upregulated in this study when HCT-116 cells were exposed to E1600.

FOS and JUN families make up the AP-1 (activator protein-1) complex and are involved in signal transduction. FOS has been found to be expressed in an increased proportion in aberrant colonic crypts, preneoplastic lesions of colon cancer (Stopera et al.,

1992). In this study, FOS was found to increase 2.1-fold after exposure to E1600 in HCT-116 cells. Since FOS is associated with colonic crypts and lesions, this increase is considered detrimental.

MCAM is involved with cell adhesion and is associated with metastasis of malignant melanoma (Bar-Eli, 1997; Xie et al., 1997). It has been shown to play a role in tumor thickness and metastatic potential in nude mice. This gene was upregulated 2.1-fold in this study after HCT-116 cells were exposed to E1600. Furthermore, MTA1 is associated with an increased invasion and metastasis and has been seen in gastrointestinal, colon, and lung cancers (Toh et al., 1997; Sasaki et al., 2002). This gene was upregulated 2.6-fold after exposure to E1600 in this study. Likewise, MTA2 is also involved in invasion and metastasis and has been associated with more aggressive forms of epithelial ovarian cancer (Yuxin et al., 2006). MTA2 was upregulated 3.1-fold in HCT-116 cells exposed to E1600. PLAUR is also involved in invasion and metastasis and the expression of PLAUR was enhanced 2.2-fold after exposure to this same extract. Each of these upregulations is detrimental to the treatment of colon cancer.

TIMP1 is a metallopeptidase inhibitor, and it has been found that TIMP1 levels are higher in pancreatic cancer tissues, since it is associated with increased cell proliferation and anti-apoptosis (Gong et al., 2000). TIMP1 expression was also found to be higher in malignant breast cancer than in nonmalignant breast cancer (Yoshiji et al., 1996; McCarthy et al., 1999). TIMP1 was also upregulated more than five-fold in early stages of colorectal carcinomas (Nosho et al., 2005). However, TIMP1 was shown to bring about less invasiveness in pancreatic cancers (Rigg and Lemoine, 2001). In

addition, it has been shown that introduction of TIMP1 into a Kaposi's Sarcoma cell line resulted in reduced tumor growth in nude mice, suggesting an antiangiogenic impact (Zacchigna et al., 2004). The associations of TIMP1 to various cancers, although conflicting, suggest that the upregulation of TIMP1 that resulted after exposure of HCT-116 cells to E1600 in this study are likely undesirable, especially judging from the effect on colon cancer cells.

TGFB1 encourages angiogenesis, although it has been demonstrated that the protein can suppress early events in the development of colon cancer (Engle et al., 1999). The problem is that human colon tumor cell lines are often resistant to the growth-inhibitory effects of TGFB1 leading to increased invasiveness. Although TGFB1 inhibits epithelial cell growth *in vitro*, it promotes extracellular matrix remodeling. TGFB1 levels increased 2.9-fold in this study. The interpretation of this value is questionable because it appears that at first TGFB1 can inhibit cell proliferation but overall, its levels could contribute to carcinogenesis. It is considered a transforming growth factor, so for the purposes of this study, the upregulation is considered harmful.

PI3KR1 is involved in signal transduction. In fact, inhibiting PI3K was shown to induce activation of TRAIL, leading to cleavage and activation of caspase-8 (Rychahou et al., 2005). Further, PI3KCA is a suspected oncogene in ovarian and breast cancer (Campbell et al., 2004). Therefore, inhibitors of PI3K/AKT signaling have been proposed to be potential therapeutic agents for cancer. However, PI3KR1 was actually upregulated after HCT-116 cells were exposed to E1600 in this study, a detrimental gene change.

Tumor necrosis factor receptor superfamily members 10B and 25 were upregulated in HCT-116 cells exposed to E1600. These receptors are proapoptotic (Jailwala et al., 2009; Borysenko et al., 2005). Involved in this process is BID, which is cleaved by caspase-8, itself activated by TNF and FAS. BID then moves to the mitochondria to cause the release of cytochrome c (Luo, 1998). TNFRSF10B and TNFRSF25 were upregulated 2.1 and 3.2-fold, respectively, in HCT-116 cells exposed to E1600. These upregulations are desirable in the treatment of colon cancer since the receptors are involved in the mitochondrial apoptotic pathway.

Of the up and downregulations caused by exposing HCT-116 colon cancer cells to E1600, the vast majority of the changes were detrimental. The exceptions were the changes associated with MYC, TNFRSF10B, and TNFRSF25. It would be easy to make the conclusion that E1600 may not be an ideal treatment for colon cancer cells according to this data. However, as has been previously stated, exposure of HCT-116 cells to E1600 resulted in increased antiproliferative activity and also upregulated caspase 6 activity by 340%, thereby suggesting that it may still be promising to treat colon cancer cells with this extract. Furthermore, it is likely that there are other genes not represented on this assay that may have desirably altered expressions.

Only four genes were significantly (±2-fold) upregulated or downregulated by exposing HCT-116 colon cancer cells to E3200. These were IFNA1, MYC, plasminogen activator urokinase (PLAU), and TEK. Three out of four of these changes in gene activity are desirable changes.

Interferon alpha 1 (IFNA1) is involved in angiogenesis. Introduction of an IFNA1 into a highly angiogenic Kaposi's sarcoma cell line reduced tumor growth in mice (Albini et al., 2000). It was suggested that class I interferons can be used to treat vascular tumors. In this study, IFNA1 was downregulated -2.9-fold. It would, however, be desirable to see this gene upregulated, since its activation can reduce tumor growth.

PLAU is a plasminogen activator involved in invasion and metastasis. It is involved in the degradation of the extracellular matrix and tumor cell migration and proliferation (Ploug et al., 2002). Strategies that help control the activities of this gene and its protein would potentially fight against invasive cancers. In this study, PLAU was downregulated -2.1-fold, suggesting that E3200 could be an antagonist to the extracellular matrix degrading and invasive activities of colon cancer cells.

TEK was downregulated in HCT-116 cells by exposure to E3200 in this study, just as it was after exposure to W1600. The TEK gene encodes the angiopoietin-1 receptor gene. TEK system activation has been implicated in shorter survival in breast cancer patients because the activation contributes to neoangiogenesis (Dales et al., 2004). In this study, the greater than 2-fold downregulations seen after W1600 and E3200 exposure suggests that these extracts could be helpful in the treatment of metastatic cancers, such as colon cancer.

As previously discussed, the MYC gene has been found to be necessary for the proliferation of the HCT-116 colon cancer cell line and influences the β-catenin pathway (Cho et al., 2005). E3200 inhibited the expression of this gene -19.7-fold, suggesting that it may be an option in the treatment of colon cancer cells, since it may inhibit the

proliferation of HCT-116 cells. Thus, in this study, E3200 downregulated MYC, PLAU, and TEK, all of which are critical downregulations in the treatment of colon cancer. Only IFNA1 was a downregulation in an undesirable direction since its expression may reduce tumor growth. Overall, the exposure of HCT-116 cells to E3200 seems to work well in inactivating undesirable genes and yet not activating genes that are undesirable. Of course, there may be other genes that are upregulated or downregulated that are not present on the array used in this study that may impact the inhibition of the growth of the HCT-116 cells.

Several genes known to have an influence specifically on colon cancer cell lines that were present in this cancer array were not significantly (±2-fold) upregulated or downregulated in this study. Those genes were RB1, BCL2, and p53. The RB/E2F1 pathway is critical in regulating DNA replication, and this pathway is dysfunctional in most human cancers (Nevins, 2001). As previously mentioned, in one study of 15 males and 20 females with colon cancer, 18% expressed BCL2 and 29% expressed p53. There were variations in genetic expression within the sample, supporting the idea that different individual cancers have different gene expressions. Not specific to colon cancer cell lines, CHEK2 has been referred to as a multiorgan cancer susceptibility gene, because the CHEK2 protein is important in the DNA damage response (Cybulski et al., 2004). In this study, there was no up or downregulation in CHEK2 activity.

It is very important to understand that there are other genes involved in the progression of colon cancer that are absent in this array. Examples of such genes are those from the WNT/β-catenin pathway, such as CDK8, APC, AXIN2, and LEF, as well

as K-RAS, MLH1, MSH2, C-FES, MOM2, p21, MEK, CHEK I157T, and p16. Further studies may need to focus on whether or not these genes are altered in HCT-116 cells after exposure to the extracts and fractions used in this study.

Not included on this cancer pathway profiler, the WNT/β-catenin pathway is important, because it is altered in most colon cancers, since it drives tumorigenesis (Cho et al., 2005). One study showed that CDK8 is associated with cell proliferation of colon cancer tumors by its presence in a region of recurrent copy number gain and that CDK8 kinase activity plays a role in β-catenin-driven transformation (Firestein et al., 2008). Further, it was found that CDK8 and K-RAS are both necessary for the proliferation of colorectal cancer. The authors went on to suggest that CDK8 may be a good target for therapeutic interventions, because its suppression resulted in reduced expression of other genes involved in colon cancers such as MYC, AXIN2, and LEF1. Aberrant activity of the WNT pathway leads to inappropriate activation of LEF1, which is not normally activated in intestinal epithelium (Lau et al., 2001).

AXIN2 mutations have been found to contribute to colorectal cancer through the activation of β-catenin/T-cell factor signaling (Liu et al., 2000). In one study, for 11 of 45 colorectal cancer cases AXIN2 was mutated, resulting in defective DNA mismatch repair, thereby linking the cases to the APC pathway.

In another study of the colonic adenocarcinomas, K-RAS, p53, and adenomatous polyposis coli (APC), mutations were found in only 11% of samples (Samowitz et al., 2007). This finding negated the previous thought that these mutations usually occur together in colon cancers. Interestingly, among the colon cancers tested, 59% had

frameshift mutations in the APC gene. K-RAS and APC are genes that are not present on the gene profiler used in this study.

It is widely regarded that the APC gene inactivation is often the initiating step in human colorectal cancer transformation. Identifying modifiers of various loci of APC can be important to studying adenoma incidence (Baran et al., 2004). The MOM2 mutation is a spontaneous mutation and resistant MOM2 alleles can suppress the polyps in APC mutated mice (Silverman et al., 2002). Thus, the MOM2 gene could also serve as a marker in tracking influence of various extracts on the proliferation of colon cancer cells.

In the same study previously mentioned involving 15 males and 20 females with colon cancer, all positively expressed MLH1 and MSH2, both proteins essential to DNA repair. Moreover, 60% and 40% expressed β-catenin membranous and β-catenin nuclear proteins, respectively (Kapiteijn et al., 2001). Discovering the effects of extracts and their fractions on upregulations and downregulations of MLH1, MSH2, and β-catenin membranous and nuclear proteins would be valuable in understanding inhibitions in proliferation of colon cancer cells.

Another gene that may potentially be an important marker to understanding antiproliferative activity that results from the exposures of various extracts to colon cancer cells is C-FES. Although known to have transforming capabilities, this gene has been associated with tumor suppression among various colorectal cancers (Delfino et al., 2006). Levels of this protein were found to either not be present or present in low

numbers in HCT-116 cells. When this protein was introduced into the cells, cell growth was suppressed.

p21 is a p53-regulated cyclin-dependent kinase inhibitor (Ogino et al., 2006). In colorectal cancer, p53 mutations cause down-regulation of p21. It has been shown that cell cycle arrest of p53 ceases in p21 deficient cells, indicating that p21 is necessary for the cell cycle arrest of p53 (Waldman et al., 1995). After exposure to *P. quinquefolius*, the expressions of p21 and p53 were upregulated, while phospho-MEK levels decreased, and the cell cycle phase arrested at G0/G1 (King and Murphy, 2009). Furthermore, cells deficient in p21 had reduced cell viability and more dead cells with a coinciding increase in BAX and caspase 3 protein cleavage.

In addition to monitoring changes in p21, p16 may also be important. High percentages of aberrant methylations of p16, which encodes the cell cycle inhibitor CDKN2A, have been associated with colon and colorectal polyps, since it normally acts as a tumor suppressor (Petko et al., 2005). Similarly, mutations in this gene are associated with cutaneous malignant melanomas (Foulkes et al., 1997). It follows that aberrations of p16, as well as K-RAS, are predictors of poor prognosis in human colorectal cancers (Esteller et al., 2001). Similarly, CHEK I157T has been associated with both familial and sporadic colorectal cancers (Kilpivaara et al., 2006). Therefore, determining whether or not the activities of these genes are up or downregulated after exposure of colon cancer cells to PRE and PREW would be valuable information.

Judging from the contribution of quite a few other genes to colon cancer that are not on this cancer pathway profiler, one can understand how antiproliferative activity was

determined in this study and yet some of the extracts did not upregulate or downregulate genes where they may have been expected to do so. It is highly likely that the activities of any of the other genes listed above were altered in response to exposure of HCT-116 cells to the extracts and fractions used in this study. These gene changes would likely contribute to the antiproliferative qualities that the ethanol extract of *P. americana* and its water fraction were shown to exhibit.

CHAPTER 5

CONCLUSION

In this study, there was no significant ($P \geq 0.05$) antiproliferative effect of the ethanol (PRE), methanol, and water extracts of *P. americana* toward MCF-7 breast cancer cells. PRE exhibited stronger antiproliferative activity against HCT-116 colon cancer cells than the methanol and water extracts tested at the same concentrations. After fractionating PRE, the water fraction (PREW) was found to have a greater antiproliferative effect than the ethyl acetate and butanol fractions. Preliminary results of the antiproliferative effects of PAP against HCT-116 cells showed much promise and correlated well with the results of PRE.

The activity of caspase 6 increased in HCT-116 cells exposed to both concentrations of PRE used in the study. Caspase 9 activity showed an upregulation after exposure to 1600 µg/ml of PRE. This is a good correlation since caspase 9 is an initiator caspase and caspase 6 is an effector caspase. Caspase 8 activity in HCT-116 cells was significantly ($P \leq 0.05$) upregulated after exposure to 800 µg/ml of PREW while caspase 9 was slightly upregulated after exposure to 800 µg/ml and 1600 µg/ml of PREW. Caspase 3 was upregulated in HCT-116 cells after exposure to 400 µg/ml and 1600 µg/ml of PREW. Activations of both caspases 3 and 9 are a good correlation since caspase 9 is required to activate caspase 3.

Some genes from the Human Cancer Pathway were upregulated or downregulated in a manner that is desirable in the treatment of colon cancer, while other gene activities

changed in a manner that is undesirable in colon cancer treatment. These changes indicate that the extracts and fractions impact transcription. The most desirable gene changes appear to be from W1600 and E3200 exposures of HCT-116 cells *in vitro*. Of the genes significantly affected by exposure to W1600, 3 out of 8 changes could potentially be beneficial in the treatment of colon cancer. Those genes were NME4, TEK, and THBS1 while the undesirable changes involved NFKBIA, CDK4, MYC, VEGFA, and SYK. Of the genes significantly affected by exposure to E3200, 75% were changes that could benefit the treatment of colon cancer. The desirable changes in gene activity were MYC, PLAU, and TEK while the change in gene activity that was undesirable was IFNA1. The W800 and E1600 exposures resulted in more undesirable changes than desirable changes in gene expression.

In summary, PREW at 200 µg/ml inhibited HCT-116 cell proliferation by 18% after 72 hours of exposure. The greatest increase in caspase activity by PREW was caspase 8, which was increased by 137% after exposure of HCT-116 cells to 800 µg/ml of this fraction for 48 hours. Exposure of HCT-116 cells to PREW at 1600 µg/ml for 48 hours resulted in desirable gene changes with the downregulations of NME4, TEK, and THBS1. The former is involved in invasion and metastasis, while the latter two are involved in angiogenesis.

PRE at 3200 µg/ml inhibited the proliferation of HCT-116 cells 20% after 72 hours of exposure. At the same concentration, caspase 6 activity was increased by 887%. Also at that concentration, PRE caused downregulations of MYC, PLAU, and TEK. MYC is an oncogene known to be necessary for HCT-116 proliferation. PLAU is a

plasminogen activator that is involved in invasion and metastasis, while TEK encodes angiopoietin-1, which results in an increase in the number of blood vessels. Each of these downregulations is desirable in the treatment of colon cancer.

The hypothesis of this study was that antiproliferative activity would be caused by members of the apoptotic pathway, such as the caspases that were tested and apoptosis-related genes on the Cancer Pathway. This study demonstrated that caspase activities of 6 and 9; and 3, 8 and 9 were increased in HCT-116 cells exposed to PRE and PREW, respectively. The Cancer Pathway studies indicated that only a few gene changes that are desirable in the treatment of colon cancer were related to apoptosis. FAS, which directly increases caspase 8 activity, was upregulated by PREW at 800 µg/ml. TNFRSF10B and TNFRSF25 both activate caspase 8 and were upregulated after exposure to PRE at 1600 µg/ml. The other gene changes that were recognized as being desirable in the treatment of colon cancer were oncogenes or related to invasion and metastasis, angiogenesis, and adhesion.

To understand how these beneficial changes would truly aid in the treatment of colon cancer, further testing is necessary. There are other genes that are involved in colon cancer, and understanding how exposures affect those genes would be helpful. Also, animal testing would ultimately be necessary to determine whether or not the exposures of PRE or PREW reduce tumor size as well as to understand what physiological side effects may occur on animals due to PRE and PREW exposures tested in this study.

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