COMPOUNDS OF *VACCINIUM MACROCARPON* INHIBIT DU145 HUMAN PROSTATE CANCER CELLS

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Abstract

Prostate cancer is the second most prevalent cancer in North American males. A series of *in vitro* studies were conducted to examine the effect of compounds from the American cranberry (*Vaccinium macrocarpon*) on the behavior of DU145 human prostate cancer cells. These compounds (Proanthocyanidin [Pacs], Flavonol [Flavs], Ursolic acid [UA]) were hypothesized to be the principle contributors to previous research where *V. macrocarpon* treatment of DU145 cells was found to induce apoptosis and changes in matrix metalloproteinase (MMP) activity. Pacs treatment of DU145 cells induced apoptosis by up-regulating the protein expression of intrinsic pro-apoptotic proteins culminating in induction of the final stages of apoptosis, poly ADP-ribose protein (PARP) cleavage and nucleosome formation. Flav treatment of DU145 human prostate cells also induces apoptosis through the intrinsic pathway. These inductions led to cytochrome c induction leading to DNA cleavage, but without the PARP cleavage normally present in apoptosis induction. UA, a compound found in the skin of cranberries, was studied for its ability to induce changes in the activity of MMPs released from DU145 cells. UA inhibited the activity of MMP-9, Pro-MMP-2 and MMP-2. UA treatment of DU145 cells induces the expression of tissue inhibitor of matrix metalloproteinases -1/-2 (TIMP-1/-2). Extracellular matrix metalloproteinase protein inducer (EMMPRIN) and reversion inducing cysteine rich protein with kazal motifs (RECK), two regulators of MMP activity, were both inhibited by UA treatment of DU145 cells. Cell signaling pathways associated with the regulation of MMP activity by UA were also evaluated. It was observed that the effect of UA was mediated through a MAPK and NF-κB dependent manner. These results suggest that *V. macrocarpon* is a viable and important source of anti-cancer compounds.

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Finally and most of all, I would like to thank my family and particularly my girlfriend, Kristen, who not only supported me and kept me sane during the last few stressful months, and was always there to help and offer encouragement. This journey has been sincerely enjoyable and I look forward to new paths.

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LIST OF ABBREVIATIONS

α MEM	α-Minimum Essential Media
AB	Alamar Blue assay
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
APAF-1	Apoptotic Peptidase Activating Factor
ATP	Adenosine-5'-Triphosphate
Bcl-2	B-cell lymphoma-2
BSA	Bovine Serum Albumin
CARD	Caspase Activation and Recruitment Domain
Caspase	cysteine aspartases
Da	Dalton
DM	Defined Media
DMSO	Dimethyl sulfoxide
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-3-gallate
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FADD	Fas-Associated Death Domain
FasL	Fas Ligand

FasR	Fas Receptor
FBS	Fetal Bovine Serum
Flavs	Flavonols
DISC	Death Inducing Signalling Complex
DNA	Deoxyribonucleic Acid
DR	Death Receptor
GSE	Grape Seed Extracted
HCl	Hydrochloric Acid
IAP	Inhibitors of Apoptosis
JNK	c-Jun N-terminal Kinases
LNCap	Lymph Node Carcinoma of the Prostate
MAPK	Mitogen Activated Protein kinase
MMP	Matrix Metalloproteinase
MT-MMP	Membrane Type Matrix Metalloproteinases
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide Assay
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
Pacs	Proanthocyanidins
PAGE	Polyacrylamide Gel Electrophoresis
Par-4	Prostate Apoptosis Response-4
PARP	Poly ADP-Ribose Protein
PBS	Phosphate Buffered Solution
PI3k	Phosphoinositide-3-kinase

PMSF	Phenylmethylsulphonylfluoride
PKC	Protein Kinase C
PTP	Mitochondrial Permeability Transition Pore
RECK	Reversion Inducing Cysteine Rich Protein with Kazal Motifs
SDS	Sodium Dodecyl Sulfate
Smac/Diablo	Second Mitochondria-derived Activated of Caspases/Direct Inhibitor of Apoptosis Binding Protein with Low pl
Strep-HRP	Streptavidin-Horseradish Peroxidase
tBid	Truncated Bid
TBS	Trizma Buffered Solution
TBS-T	TBS supplemented with 0.05% Tween-20
TIMP-1	Tissue Inhibitors of Matrix Metalloproteinases-1
TIMP-2	Tissue Inhibitors of Matrix Metalloproteinases-2
TNF	Tumour Necrosis Factor
TNFα	Tumour Necrosis Factor α
TNFβ	Tumour Necrosis Factor β
TNFR	Tumour Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UA	Ursolic acid
UTI	Urinary tract infections
UV	Ultra-violet
VDAC	Voltage Dependent Anion Channel
VEGF-A	Vascular Endothelial Growth Factor

Chapter One: Introduction

1.1 Introduction

Despite increased education and research into the study of cancer, incidences of some forms of cancer have been steadily increasing over the past 10 years (Kucuk 2002). In Asian countries, cancer rates have also raised particularly cancers that predominate in Western society. These changes in cancer rates have been attributed in part to the Westernization of the Asian diet (Tsugane and Sasazuki 2007). This phenomenon has prompted many researchers to explore changes in diet as a means to modify cancer risks. Diets high in fruits and vegetables appears to lower cancer risk (Lee 2009; Tsugane and Sasazuki 2007). Therefore, it is suggested that the lowered risk of developing some types of cancer may be attributed to specific compounds found within plants, particularly polyphenolic compounds, which are found in large quantities (Chung et al. 2009; Morin et al. 2008; Neuwirt et al. 2008). These observations have led to an increase in research involving compounds such as proanthocyanidins (Pacs), flavonols (Flavs) and ursolic acid (UA) due to their anti-cancer properties attributed to them (Luceri et al. 2002).

1.1.1 Rationale

Previous work has demonstrated the potential for whole cranberry to modulate anti-cancer behaviours in prostate cancer cells (MacLean 2009). Specifically, treatment of DU145 human prostate cancer cells with whole cranberry extract inhibits the activity of proteins linked to tissue invasion and induces apoptosis through the intrinsic pathway (MacLean 2009). DU145 human prostate cancer cells were chosen for this study as the cell line is a well studied prostate cancer cell line that is hormone refractory and is an

example of a malignant prostate cancer. Data further suggests that whole cranberry extract mediates its effects through the induction of internal stresses within the cell culminating in apoptosis rather than through the interaction with receptors found in the extracellular membrane.

The ability for tumour cells to invade and subsequently migrate to secondary sites is one of the chief behaviours which distinguish them from normal cells. Blueberries and cranberries have been shown to inhibit matrix metalloproteinases (MMP) activity through the modulation of inhibitors and inducers of MMP expression rather than through direct interaction (Maclean 2009; Matchett 2005). Previously, whole cranberry extract was observed to inhibit the secretion of MMPs in DU145 via the induction of protein expression of TIMP-1/-2 and RECK along with the suppression of EMMPRIN (MacLean 2009). The pathways that govern the effects seen in previous work have not fully been elucidated.

1.1.2 Hypotheses

The investigations presented in this thesis test the general hypothesis that **cranberry phytochemicals affect the behaviour of prostate cancer cells *in vitro*.** More specifically, it was hypothesized that Pacs and Flavs induce apoptosis in DU145 cancer cells through the intrinsic or extrinsic apoptotic pathways or both. Furthermore, it was hypothesized UA would inhibit the activity of MMPs in DU145 human prostate cancer cells by modulating the expression of MMP regulators.

1.1.3 Objectives

I. Whether Pacs induce apoptosis in DU145 cancer cells through either the intrinsic or extrinsic apoptotic pathways or both.

II. Whether Flavs induce apoptosis in DU145 cancer cells through either the intrinsic or extrinsic apoptotic pathways or both.

III. Whether UA inhibits the activity of MMPs in DU145 human prostate cancer cells by modulating the expression of MMP regulators.

Chapter Two: Literature Review

2.1 Literature Review

2.1.1 The American Cranberry (*Vaccinium macrocapon*)

V. macrocapon, also known as the North American cranberry is a shrub native to the north-eastern portion of North America and the Pacific northwest. Cranberries can be cultivated commercially and are a major crop for commercial operations in the north-eastern United States, eastern Canada and Quebec. Cranberries are harvested and sold either as unprocessed whole berries or can be processed into gel or juice. Cranberries have been found to be a good source of vitamin C, fibre, and other nutrients. The nutrient complement of *V. macrocarpon* contains many antioxidants, compounds with a strong ability to quench free radicals when compared to other food sources or fruit sources (Boivin et al., 2007; Vinson et al. 2001; Wu et al. 2004). Recent research into the potential bioactive effects of cranberry phytochemicals has led to an increase in demand for nutraceutical extracts derived from cranberry to combat everything from cancer to urinary tract infections.

Many of the health benefits attributed to cranberries are due to the cranberry's high content of phenolic compounds. Phenolics are chemical compounds that contain an aromatic group along with a hydroxyl group. Anthocyanin, one of the common phenolic compounds found in cranberry, has been shown to have strong reactive oxygen species quenching properties which can protect against many oxidating events (Vinson et al. 1995). While this effect is important for cellular health, there are other effects seen with cranberry treatment that have not been associated with anthocyanins (Neto et al. 2006;

Seeram 2006; Vinson et al. 1995). Other important phenolics of interest include flavonols, proanthocyanidins, and triterpenoids (Figure 2.1) (Neto et al. 2008). These three phenolic subfamilies represent the majority of the phenolics in cranberry. Neto et al (2008) found that one variety of American Cranberry, Early Black Cultivar, was particularly rich in phenolics such as proanthocyanidins.

2.1.2 The Health Benefits of the American Cranberry

V. macrocarpon is viewed as an important source of anti-cancer compounds. The observation of anti-cancer properties were first discovered as the result of research into the effect of some components of the *V. macrocarpon* and their ability to modulate the diseases involving the stomach or urogential system. It has been observed that consuming a processed form of cranberry resulted in a reduction of urinary tract infections (UTIs) and plaque formations on urogential epithelial cells (Foo et al. 2000; Howell et al. 1998). Further research has shown that one of the bioactives found in cranberry, proanthocyanidins, competes for receptor/anchor points with bacteria that cause UTIs. After the initial observations of proanthocyanidins competing for anchor locations with bacteria, it was also found that proanthocyanidins also compete for anchor locations with *Heliobacter pylori*, the bacteria that cause gastric ulcers. *H. pylori* have been found to have the potential to induce to the formation of gastric cancer through the generation of the gastric ulcers. This important finding offers a potential mechanism for Pacs to reduce the risk of ulcers and prevent gastric cancer. The linking of cranberry consumption to the relief of various ailments resulted in the expansion of research into other studies including cancer. Finding potential anti-cancer qualities in a fraction of

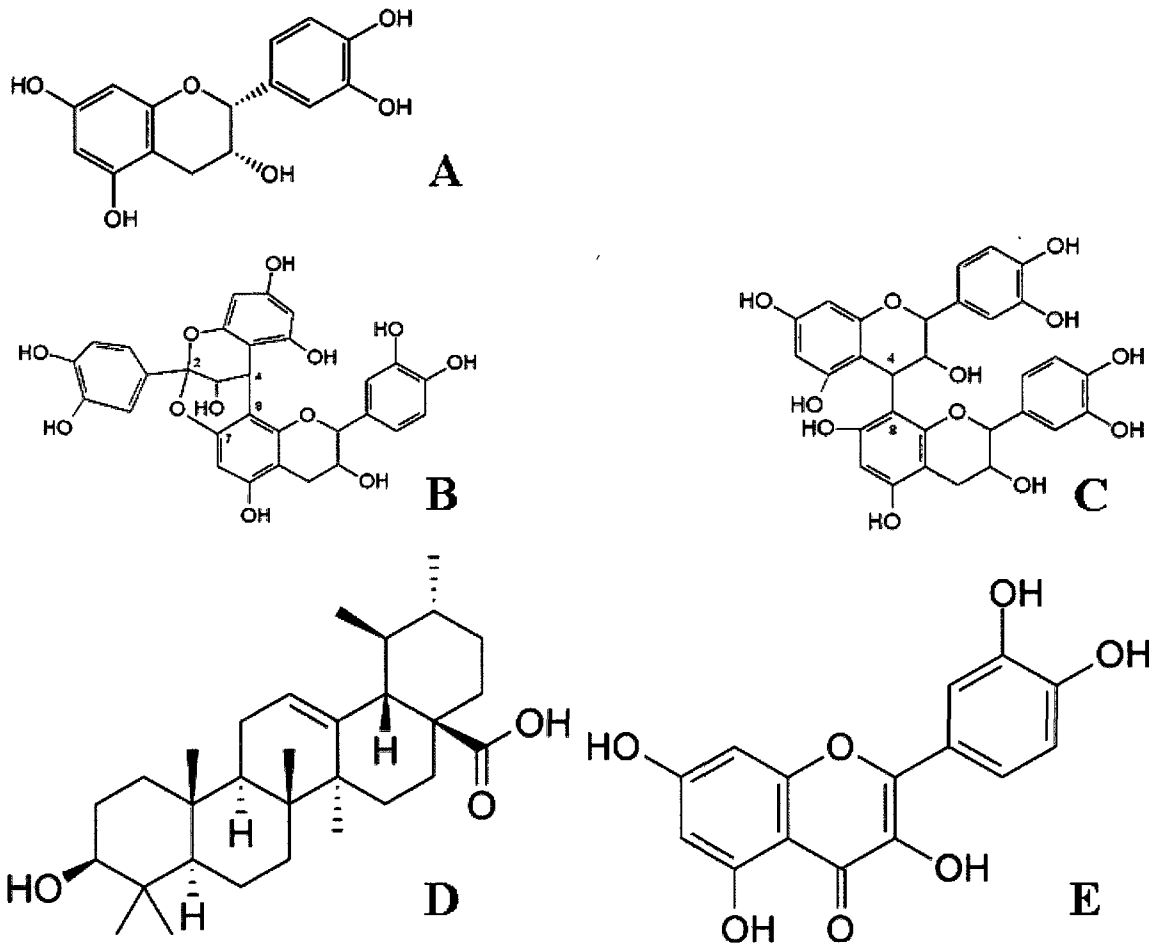

Figure 2.1 Representative structures of the various fractions used in this study. The structures are a representative monomer of oligmeric Pacs (A), Oligmeric Pacs in an α-linked conformation (B), Oligmeric Pas in a β-linked conformation (C), structure of UA (D), and the structure of Quercetin, the principal compound in the Flav fraction (E).

cranberry during the same time as interesting novel research into the effects of phytochemicals derived from other food sources has led to a further interest in the phenolic compounds found in the American Cranberry.

Early work with *V. macrocapon* suggested that it may have other anticancer properties (Neto et al. 2008). Research with cranberries found that an extract prepared from whole cranberries could inhibit the proliferation of several cell lines which represent several forms of prostate cancer. These cell lines include LNCaP, PC3, DU145, RWPE-1, RWPE-2, and 22Rv1 (Biovin et al., 2007; Ferguson et al., 2004; Murphy et al., 2003; Seeram et al., 2004). Whole cranberry extract inhibits proliferation in human prostate cancer cells is via several mechanisms including: cell cycle arrest and apoptosis (Sun et al., 2006; Yan et al., 2002). The anticancer effects of *V. macrocarpon* have been attributed to several different sub-fractions of compounds found naturally in the fruit.

2.1.2.1 Proanthocyanidins

Pacs in the American cranberry are found naturally as dimers, trimers and larger conglomerate oligomers of epicatechin or poly-flavan-3-ols. Pacs polymers are commonly found linked with the more common B-type linkage between the structural units. Interestingly, cranberry Pacs contain the more rare, A-type linkage. Some investigators hypothesized that this A-type linkage may lead to an enhancement of the normal cytotoxicity associated with Pacs treatment (Neto et al., 2008). *In vitro* studies into the cytotoxicity of cranberry derived Pacs have shown that this phenolic can induce cytotoxicity in a number of cell lines, including prostate cancer cells (Ferguson et al., 2004; Seeram et al., 2004; Liberty et al., 2007; Neto et al., 2006). Grape seed extracted

(GSE) Pacs have been found to inhibit the growth of several cancer lines, including prostate cancer. (Agarwal et al., 2000; Kim et al., 2003; Mantena et al., 2006; Tyagi et al., 2003; Ye et al. 1999). Previous studies of compounds structurally related to Pacs such as: epigallocatechin-3-gallate (EGCG) or proanthocyanidin gallates have been observed to induce the extrinsic pathway of apoptosis in AA549 lung cancer cells, MCF-7 breast cancer cells and LNCaP prostate cells (Kuo et al. 2004; Kuo et al. 2005; Siddiqui et al. 2007). The extrinsic pathway is induced through ligand-receptor activation eventually leading to cellular death induction. The activation or initialization of the two main apoptotic pathways differs as unlike the extrinsic pathway which is induced through a ligand-receptor binding; the intrinsic pathway is activated through internal cellular stresses to induce apoptosis. GSE Pacs and structurally related compounds have also been observed to induce the intrinsic pathway through induction of protein expression of key proteins such as Bax and B-cell lymphoma-2 (Bcl-2) (Du and Lou, 2008). GSE Pacs have been observed to inhibit the expression of both Bcl-2 and Bax in H9C2 cardiomyocytes (Du and Lou, 2008). Bid, a protein with ramifications for cytochrome c release and overall apoptosis initialization, has been observed to be induced in a long list of cancer cell lines by EGCG (Nishikawa et al. 2006). In contrast, GSE and cranberry derived Pacs have not been reported to modulate the cleavage or expression of Bid. Cytochrome c, a small mitochondrial segregated protein with importance for propagation of an intrinsic apoptotic signal has been observed to be released in larger concentrations when DU145 human prostate cancer cells are treated with GSE Pacs (Agarwal et al. 2002). Pacs derived from either cranberry or grape seed have both been reported to induce cysteine aspartases (caspases) -2, -3 and -8 (Chatelain et al.2008; Nomoto et al.

2004). Cleavage of the caspase, caspase-3, is a central feature of both intrinsic and extrinsic pathways and leads to irreversible induction of the final stages of apoptosis.

2.1.2.2 Flavonols

Flavonols are a large family of metabolites that have been found to have wide ranging effects on cells. Initially, Flavs were studied for their contribution to the ability of fruit to inhibit or quench reactive oxygen species. Treatment with enriched Flav fractions from olive leaves and cranberries was found to have benefits in scavenging free radicals and protecting against lipid oxidation (Lee et al. 2009; Kathirvel et al. 2009; Kathirvel and Richards 2009). Further research has found that treatment with Flavs induces apoptosis and cytotoxicity in a variety of cells including breast, oesophageal, and colon cancer (Li et al., 2009; Zhang et al. 2008). Poly ADP-ribose protein (PARP), described in detail below, is an anti-apoptotic protein involved in DNA repair. Flavonolic compound treatments of LNCaP and PC3 prostate cancer cells have been observed to induce PARP cleavage (Haddad, 2008; Lee et al. 2008).

Quercetin, a widely studied Flav, has been reported to induce the extrinsic pathway by induction of Fas and TNF-related apoptosis-inducing ligand (TRAIL), two key receptors of the extrinsic pathway (Chen et al. 2007; Russo et al. 1999). Quercetin has also been observed to induce the intrinsic pathway in a wide range of cell lines including Lymph Node Carcinoma of the Prostate (LNCaP) prostate cancer cells (Chien et al. 2009; Lee et al. 2008; Xu et al. 2008; Psahoulia et al. 2007; Ramos and Aller. 2008). Quercetin and kaempferol both induce Bax and inhibit Bcl-2 protein expression in several cell lines including LNCaP human prostate cancer cells (Chien et al. 2009; Lee et

al. 2008; Xu et al. 2008). Treatment of colon cancer and leukemia cells with quercetin induces the cleavage of Bid, an important protein that induces apoptosis (Psahoulia et al. 2007; Ramos and Aller. 2008). Bid cleavage into truncated Bid (tBid) leads to the release of cytochrome c from the mitochondria. HL-60 leukemia cells treated with apigenin and related flavonoids have been shown to induce cytochrome c release, and activate caspase-3/-9 (Wang et al. 1999). This observation is important as cytochrome c release leads to the formation of a conglomerate of proteins which leads to activation of caspase-9 which then cleaves and activates caspase-3 (Wang et al. 1999). Caspase-3 activation leads to the last irreversible stages of apoptosis. In summary, the Flav fraction, like the Pacs fraction have constituents capable of inducing apoptosis through either the extrinsic and/or the intrinsic pathway of apoptosis.

2.1.2.3 Ursolic Acid

While the majority of the phenolics found in cranberries exist within the fruit itself, the highest concentrations of UA are found within the peel of the fruiting body. UA has three structural conformations, an aglycone, or as either a cis or trans *p*-hydroxycinnamate ester form. Treatment with UA has been observed to inhibit proliferation in several cancers cell-lines including those of prostate and breast cancer (Murphy et al., 2003; Novotny et al., 2001). The DU145 prostate cancer cell line has been observed to be inhibited by UA treatment (Zhang et al. 2009 [2]). UA has been found to induce apoptosis in a number of cell lines through several mechanisms, including the induction of intracellular calcium signalling, cytochrome c release linked with caspase-3 activation, induction of JNK kinase leading to Bcl-2 phosphorylation to

activate caspase 3, and direct activation of caspases -3, -8 and -9 (Andersson et al. 2003; Baek et al. 1997; Kim et al. 2000; Zhang et al. 2009 [1]). UA has also been observed to induce apoptosis through a non intrinsic method without involving Bax, Bcl-2 or mitochondrial permeabilization (Choi et al. 2000).

Of importance for this investigation, UA has been found to inhibit MMP activity in a variety of cell lines (Pathak et al. 2007; Shan et al. 2009; Xavier et al. 2009). UA treatment inhibited MMP-9 activity in HT1080, Jurkat, 293, KBM-5, H1299 and U937 cancer cells (Hee-Jae et al. 1998; Hee-Jae et al. 1996; Shishodia et al. 2003). UA treatments of HT1080 and HaCaT have been observed to either have no effect (Cha et al. 1996) or induce the expression of MMP-2 activity (Lee et al. 2003). The changes in MMP activity may be due to the ability of UA to interact with several modulators of MMPs. UA has been observed to inhibit TIMP-1/-2 expression in fibrosarcoma cells (Hee-Jae et al. 1996). EMMPRIN and RECK, two other modulators described in this thesis have not been studied in relation to UA treatment. These two proteins are studied to observe whether the inhibition of MMP activity is due to modulation of key regulators rather then through direct interaction between the compound (UA) and the MMPs of interest. There are several key signal transduction pathways through which MMP activity is commonly modulated. These include the mitogen activated protein kinase (MAPK), protein kinase C (PKC), phosphoinositide-3-kinase (PI3K) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signal transduction pathways (Achiwa et al. 2007; Liu and Jiang 2007; Pathak et al. 2007; Shan et al. 2009; Xavier et al. 2009; Manu and Kuttan, 2008; Shishodia et al. 2003).

2.1.3 Properties that Contribute to Cancer

Cancer is a diverse disease that includes any ailment characterized by excessive cell growth culminating in the formation and development of tumours. These neoplastic (tumourgenic) cells arise by the accumulation of genetic mutations that result in a cell with selective advantages over non-transformed cells. These selective advantages can be described as analogous to the process of natural selection. While a cell line population differs from a natural population due to a lack of genetic richness and therefore do not undergo natural selection as a organism population may, a general example using the inheriting of characteristics can be used for clarity. As mutations arise, a cell population can receive a set of genetic benefits that allows cells to ignore replication constraints or instead produce their own signals for growth. This accumulation of genetic benefits can act additively or synergistically to produce a cell that can out-compete normal cells. It is possible for multiple mutations to act interdependently in complex ways which accounts for the vast continuum of cancer behaviours. Cancers differ not only in their progenitor cells, but also which genetic mutations they contain. Many of these mutations allow them to circumvent the normal cellular restrictions that allow for millions of cells to form a functioning conglomerate such as tissue.

Cells in the human body are under constant pressure to maintain homeostasis. Therefore, cells are regulated in an effort to organize their growth or death so that they can form complex tissues and organs. Normal cells excrete or break down the surrounding matrix to release chemical factors that allow for intercellular communication and to allow them to modulate the general growth rates of surrounding cells (O'Hayre et al. 2008). In some cancers, these extrinsic factors can act as a positive feedback loop

(Legewie 2009). This feedback loop allows the cells to speed up their own rate of growth without relying on the production of growth factors from other cells (Legewie, 2009).

Proper tissue regeneration and growth requires cellular proliferation to be tightly regulated. Cell tissue homeostasis requires that select cells undergo apoptosis in response to the extrinsic signals. Kerr et al. (1972) first described apoptosis as a possible mechanism for cancer cell inhibition. In their study, hormone-dependent neoplasms were deprived of hormones which resulted in the systemic apoptosis of the cells. Further research into apoptosis induction revealed that cancer cells commonly show defects in the normal cell apoptotic pathways (Li et al. 2009). The vast majority of cancers have been found to have mutations in the p53 protein (Laurie et al. 2006). p53 is a very important tumour suppressor protein that is normally involved in DNA repair when genetic material is damaged and if the damage is too extensive, induces apoptosis. The loss of proper p53 function allows the cell to accumulate mutations without the normal checks and balances in place to ensure cellular health and the continuity or cohesiveness of tissues (Demidenko et al. 2005; Li et al. 2009). The DU145 human prostate cancer cell line expresses this mutated p53 gene (Demidenko et al. 2005; Hickman et al. 2002). The relevance of the DU145 human prostate cancer cell's p53 mutation is that the mutation allows the cells to evade some of the normal cellular repair mechanisms which would be activated to induce apoptosis.

Cancer growth involves the formation of neoplasms from normal cells as mutations such as inactive p53 accumulate, which eventually leads to the formation of a primary tumour. As cancer develops, the primary tumour tends to invade surrounding tissue, eventually shedding cells into vascular vessels at which point the cells then

migrate to secondary locations throughout the body. Each of these secondary sites has the potential to form an independent tumour which can then give rise to tertiary sites.

Cellular invasion can be facilitated through a variety of signalling pathways and proteases such as a family of extracellular matrix (ECM) degrading proteins, matrix metalloproteinases (MMPs). The MMP family of proteins degrade the ECM and allow cells to access compounds that can be derived from this degraded ECM. This degradation also allows cells access to the margins of the tissue around the tumour in order to invade the surrounding area. MMPs are important mediators in the interaction between cancer cells and their microenvironment. Dysfunction in the normal regulation of MMPs can lead to induction of cellular proliferation, increased tissue invasion and induction of vascular vessel formation, three important events in tumourgenesis (Chang and Werb. 2001).

2.1.3.1 Prostate cancer

DU145 human prostate cancer cells are of particular interest as the cancer line represents a hormone-independent malignant cell line. Therefore, DU145 human prostate cancer cells are an example of an advanced malignant prostate cancer that is independent of hormonal control. In a clinical setting, prostate cancer develops or acquires mutations in much the same way as many other cancers. Initially, prostate cancer cells form growths within the prostate tissue, eventually giving rise to a neoplasm after many separate mutations occur (Feldman and Feldman, 2001). The change from a benign neoplasm into a metastatic prostate tumour can often take years or decades (Klotz, 2005). The slow rate of growth for prostate cancer is an important factor, as this property allows

the use of tissue specific treatments in an attempt to cure a patient before the cancer spreads to secondary sites (Klotz, 2005).

Prostate cancer treatment often takes the form of targeted irradiation or prostatectomy, both of which results in a better patient prognosis. Late stage prostate cancer patients often present with secondary metastases to the bone. Once prostate cancer metastasises to a secondary location, the difficulty for treatment increases (Feldman and Feldman, 2001; Navarro et al., 2002). Often the only treatment option is to drastically reduce the amount of testosterone in the body as prostate cells are dependent on this hormone to survive. To take advantage of the dependence on testosterone requires either physical (removal of testes) or chemical (testosterone antagonists) castration. The disadvantage of these treatments is that the side effects tend to be extreme. Loss of nerve function or erectile dysfunction along with loss of libido, loss of weight/appetite, problems urinating and general feminization have all been included as possible side effects (American Cancer Society. 2009). While chemical or physical castration can increase survival, the benefits are often short-term for the patient with regression ultimately averaging 24 months (Scholz et al. 2005).

New cancer regrowth is common as the prostate normally contains both hormone-sensitive and -insensitive cells therefore; treatment generally fails to eliminate both types of cells. Androgen ablation by physical or chemical castration leads to the depletion or death of hormone-sensitive cells, but allows the hormone-insensitive cells to multiply. Once a tumour changes from predominately hormone-sensitive to hormonal-insensitive, treatment options decrease. The median survival time for hormone refractory prostate cancer patients averages 8-12 months (Feldman and Feldman, 2001; Navarro et al. 2002).

The aggressiveness of hormone refractory cancer along with the lack of sustainable treatment options has stimulated research into novel therapies.

The use of *in vitro* cell studies can lay the groundwork for later *in vivo* and human studies interested in examining novel compounds that can be used to treat prostate cancer. Many cell lines have been developed to mimic different stages of prostate cancer. These stages range from benign neoplasms, hormone sensitive tumours, and highly metastatic hormone-independent metastases. The DU145 human prostate cancer cell line is an example of a metastatic hormone-independent cancer cell line. The DU145 cell line over-expresses growth factors and receptors independently of any external growth signals. This leads DU145 cells to become autonomous with respect to growth signals and inhibitors. The pathways associated with the propagation of growth factors are arranged in positive feedback loops that, when mutated, allows these cells to provide all the growth signals required for their own propagation (Manes et al. 1999; Russell et al. 1998). DU145 cell proliferation is induced in normal serum conditions due in part to the situation mentioned above.

p53 is an important tumour suppressor protein that can be used as a prognostic indicator of the malignancy of a cancer (Li et al., 2009; Stackhouse et al. 1999). This protein, when mutated, can give a cancer a selective advantage over normal cells, as the mutated cell can no longer repair the novel mutations that arise as part of normal attrition. DU145 cells have been found to contain a selectively mutated p53 gene that allows the cell line to evade apoptosis and gain an advantage over normal cells (Demidenko et al. 2005; Hickman et al. 2002). The p53 protein in DU145 prostate cancer cells has two separate mutations in its amino acid structure (Demidenko et al. 2005; Isaacs et al.,

1991). These mutations increase the stability of the p53 protein, which means less p53 is normally degraded and the protein accumulates in the cytoplasm (Tang et al., 1998). While the protein is more stable, the mutation also changes the protein's binding affinity for DNA. Functional p53 binds readily to damaged DNA and either repairs the damage, or if the DNA damage is extensive, induces apoptosis (Hickman et al. 2002). If p53 is mutated, transcription of proteins involved in apoptosis does not readily occur (Li et al. 2009). This allows the cell to survive even in the presence of apoptotic stimuli and to accumulate a series of mutations without normal cellular machinery inducing apoptosis. The presence of a mutated p53 has also been suggested to be the chief reason why some cancer cells can evade the induction of apoptosis that would normally occur when serum or growth factor deprivation occurs (Hickman et al. 2002; Hui et al. 2006; Tang et al. 1998).

2.1.3.2 Risks Associated with Cancer Development

While internal factors such as hormones, the immune system, and inherited mutations can play a role in the development of cancer, it has been theorized that many external factors also play a role in cancer development. In some situations an almost limitless number of external factors can be the key contributor to cancer generation. Some of these factors include exposure to radiation, carcinogenic chemicals, infectious organisms, poor diet, and lifestyle choices (American Cancer Society, 2009; Wiseman, 2008). Recent studies suggest that a diet rich in fruit and vegetables and low in animal fats contributes to a reduction in the rates of some cancers (Shirai et al. 2002; Willett, 2000). Willett (1995) suggested that as many as one third of all cancer cases could be

avoided if people made modifications to their diet and lifestyle. As information concerning the role of diet in cancer formation increases, new targets for possible treatments are being studied (Mazhar and Waxman, 2004; Nguyen et al., 2006).

The link between diet and cancer can be demonstrated by using observations of changing cancer rates in Asian populations. The Westernization of the Asian diet correlates with increased cancer rates of cancers, including prostate cancer, prevalent in Western societies (Cook et al., 1999; Neuwirt et al. 2008; Schulman et al. 2001; Wiseman, 2008). In particular, increases in prostate cancer rates in westernized Asian populations suggest that this cancer has a strong dietary component. Studies correlating dietary changes to increases in prognosis suggest that personal changes in diet could potentially be used to help prevent or manage cancer (Chung et al. 2009; Morin et al. 2008; Neuwirt et al. 2008). The potential for dietary compounds to affect prostate cancer is being studied to determine if there are potential complementary alternatives to current prostate cancer therapies.

2.1.3.3 Methodologies of Phytochemical Research

The link between diet and cancer has been the focus of a number of epidemiological studies performed by researchers from a diverse range of disciplines. The disciplines that focus on health research may have complementary goals, but approaches in achieving these goals often differ. The holistic approach relies on studying the effect of whole foods in a diet to observe changes in cancer and other pathologies. The more reductionist view observes the effects of compounds derived from these foods.

Research methodologies into the interaction between diet and cancer can be broken into two general categories. Nutritional research, which evaluates the effects of whole foods and their health related properties, often on the whole organism. Often nutritional research focuses on the general benefits that allow the prevention of cancer rather than on the treatment and curing of an already developed malignancy. The second category, called pharmacognosy, is an approach wherein compounds derived from natural sources, such as plants, are studied to determine the effects these compounds can have on disease. This category focuses on the production of novel compounds that may be used to prevent or treat a malady at the whole organism level. Pharmacognosy is not restricted to physiological relevant quantities for treatment, as research in this category often attempts to determine the mechanism by which these novel compounds operate. The development of plant-based drugs to treat cancer is often the objective of research for those working with this methodology. A wide variety of bioactives with potential health benefits have been derived from fruits and vegetables. Some of these compounds have been found to have anti-cancer potentials, but the mechanisms of these phytochemicals have not been fully elucidated and a full understanding of their potential is lacking. Many of the compounds that are derived from plants are often secondary metabolites with diverse roles in plant defence. Many of these secondary metabolites can have a wide ranging effect on physiologies of organisms that consume the plant. Of interest for this investigation are the effects that select secondary metabolites have on human cancer cells.

Prostate cancer is a relatively slow growing cancer in comparison to other cancers (Klotz, 2005). This is of particular importance as it allows for a long time period for

intervention with various anti-cancer treatments. One of the more important strategies when considering prostate cancer treatment and prevention is the modification of diet. The benefits of dietary intervention compared to medical treatment with chemical compounds revolve around the low risk of toxicity involved with the consummation of food over the use of purified drugs. Dietary intervention is of interest for prostate cancer due to the long development time before the cancer becomes malignant and metastasizes to secondary locations. Prostate cancer also presents as a heterogeneous tumour composed of both hormone dependent and independent cells. It is often uncertain whether a benign prostate tumour will ever truly become malignant enough to threaten a patient's life. Lack of predictability taken in concert with some of the adverse side-effects of common invasive treatments of prostate cancer explains why many doctors often suggest a 'watchful waiting' approach at an initial diagnosis of early stage prostate cancer. This causes many prostate cancer patients to look for alternatives for treatment, possibly including dietary intervention (Bemis et al., 2006; Kao and Devine, 2000; Lippert et al. 1999).

While human studies examining the effects of dietary bioactives on cancer may be the most relevant to human health and translate directly to treatments, the challenges associated with such studies lead to considerable obstacles. Some of these challenges include reproducibility, expense of clinical trials, ethical guidelines/observations, and even the quality and safety of compounds. Due to some of these reasons, *in vivo* and *in vitro* models are often used to determine the effects of plant bioactives on cells and animals, which permits researchers to hypothesise and extrapolate the effects of these bioactives on humans. The *in vitro* method for research has several key benefits,

including the ability to screen a large number of bioactives for potential in treatment of any malady that can be modeled in a cell line. The *in vitro* method of research allows for the underlying mechanisms to be studied at a cellular level and to understand changes within the cellular environment that can be modulated by a bioactive compound. Though there are benefits to the *in vitro* method of research, there are also disadvantages. These disadvantages include difficulty extrapolating whether the compound would interact the same in an organism, the uncertainty that the dosages assayed would be available in physiological quantities within a living organism, and whether the original compound would undergo possible modifications when introduced into the body of the organism. Generally, the disadvantage of *in vitro* work is that the findings cannot be directly transferred to a multicellular living system. While the results may not be directly transferable, *in vitro* studies are important as a foundation for later experiments, including clinical and animal studies (Jeffery and Keck, 2008). Despite the method of research, studies into cancer can focus on many separate aspects or properties of a malignant cell, including the ability of a cell to invade and migrate to secondary locations.

2.1.4 Introduction: Tissue Remodelling

Many tumours remain benign as long as they remain at their primary site. Surgical intervention is especially successful in treatment of prostate cancer that has not moved beyond the margins of the prostate. When a cancer tumour invades surrounding tissue and gains access to vascular tissue there are fewer treatment options. Once a tumour gains access to a lymphatic or blood vessel, tumour cells can spread to secondary

sites where they begin to form new tumours. With prostate cancer, secondary metastasises most commonly develop in bone tissue.

Invading the cohesive tissue around a tumour is difficult for cancerous cells. Therefore, tumour cells use a complex series of proteins to degrade the extracellular matrix. For a tumour to invade secondary sites it must first degrade the extracellular environment allowing a free margin for growth. To cleave the extracellular environment, both non-transformed and cancerous cells use a family of specialized proteases, the MMPs (Chang and Werb, 2001).

2.1.4.1 Extracellular Matrix

The interaction between the tumour and its microenvironment has begun to garner interest since the tumour is no longer viewed as a self sustaining unit but rather as a complex tissue that interacts intimately with the surrounding matrix. The ECM is a complex amalgamation of fibroblasts, immune cells, vascular tissue, indigenous growth factors, collagen, elastin, laminin, fibronectin and mitogenic proteins such as cytokines; that together interact to offer structural and mechanical support to cells and tissues (Pupa et al. 2002). While the principle responsibility of the ECM is structural support, the ECM can affect other processes such as cellular proliferation, differentiation, adhesion, migration, and tissue morphogenesis (Assoian and Marcantonio 1996; Ingber and Folkman 1989, Juliano and Haskill. 1993). A central process of tumour progression is the remodelling of the ECM via proteolysis to allow the activation and liberation of chemicals found contained within the ECM (Pupa et al. 2002). These liberated factors

can act as inducers or inhibitors of growth, angiogenesis or even as a source of energy to further support the intensive growth of the cancer cells (Pupa et al. 2002).

For metastasis to occur, neoplastic cells must cleave and degrade the surrounding ECM to allow migration and growth through this barrier. These processes are accomplished through the use of various proteases such as: cysteine proteases (cathepsin B), aspartate proteases (cathepsin D), serine proteases (elastase), and MMPs (Andreasen et al. 1997; Heck et al. 1990).

2.1.4.2 The Matrix Metalloproteinases

A common cellular mediator of ECM degradation is a multigene family of proteases, the MMPs. MMPs have roles in important physiological events such as wound repair, tissue homeostasis, organogenesis and vasculogenesis (Chang and Werb, 2001). Mutations of the pathways that regulate MMP expression and activity allows cancer cells to take advantage of normal MMP function and modulate it to benefit invasion, metastasis and angiogenesis (Yu and Stamenkovic, 2000; Sternlicht and Werb, 2001). The link between cancer and MMPs was first suggested when the potential for MMPs to degrade important ECM structural constituents (collagen, fibronectin, laminin, proteoglycans) was observed to be exploited by cancer cells (Matrisian, 1990). The MMP family consists of 25 zinc-dependent proteases that, while individually selective, as a whole can readily degrade all the principal components of the ECM.

Each of the individual members of the MMP family target specific subunits of the ECM, yet still show some functional overlap (Hyun-Jeong and Parks, 2007; Sternlicht and Werb, 2001). The difference in specificity between MMP family members is due to

differences in structure and domain complement. This leads to differences in substrate specificity, matrix binding, cellular localization and inhibitor binding (Powell and Matrisian, 1996). The collagenases within the MMP family (MMP-1, -8, -13, and -18) directly interact with the triple-helical fibrillar collagens in the ECM. Stromelysins (MMP-3, -10, and -11) are another subgroup of MMPs which target proteins found imbedded within the ECM. Some MMPs remain attached to the plasma membrane and are named membrane-type MMPs (MT-MMPs; MT-MMP-14, -15, -16, -17, -24 and -25). MT-MMPs targets vary widely, including gelatin, fibronectin, and laminin. The gelatinases (MMP-2 and -9) also digest gelatin like the MT-MMPs, but are more similar to the collagenases and as such typically target a specific collagen (type IV) and gelatin (denatured collagen). The gelatinases are important to study as their roles in ECM degradation are the most commonly observed in relation to cancer metastasis, in particular, prostate cancer (Brehmer et al., 2003; Upadhyay et al., 1999).

2.1.4.3 Tissue Remodelling: Modulators of MMP Activity

With the extensive research that has focused on tissue invasion, new hypotheses have been proposed concerning the tumour-microenvironment relationship (Rakisky et al. 2001). While cancer cells may propagate their own growth signals and ignore normal mitogenic inhibitors, cleavage of the ECM can lead to liberation of novel modulators of cellular growth. One of the interactions that cancer cells mediate is contact with the ECM.

The ECM can act as a barrier that can inhibit cellular growth and help keep a tumour segregated from a blood supply. The cleavage of the ECM can therefore lead to

an induction of cancer cell growth and potentially to the induction of angiogenesis. The induction of angiogenesis relies on the liberation of pro-angiogenic factors from the degradation of the ECM. Some of the important factors that are liberated by MMP mediated degradation of the ECM are vascular endothelial growth factor A (VEGF-A) and select angiopoietins (Bergers et al., 2000). A highly malignant cancer cell that ignores all growth inhibitory signals and reproduces at a greater than normal rate has a comparably higher rate of metabolism. This rate of growth requires a large amount of resources. The generation of a blood supply is a very important step for a tumour as it allows access to oxygen and resources directly to the source of the cancer. Cancer cells can use these newly formed blood vessels as a mode of dissemination for migration of metastatic cells allowing 'colonization' of other areas elsewhere in the body (Folkman, 1972).

The cleavage of the ECM also leads to the induction of angiogenesis. The induction of angiogenesis relies on the liberation of pro-angiogenic factors from the degradation of the ECM. Some of the important factors that are liberated by MMP mediated degradation of the ECM are vascular endothelial growth factor A (VEGF-A) and select angiopoietins (Bergers et al., 2000). A highly malignant cancer cell that ignores all growth inhibitory signals and reproduces at a greater than normal rate has a comparably higher rate of metabolism. This rate of growth requires a large amount of resources. The generation of a blood supply is a very important step for a tumour as it allows access to oxygen and resources directly to the source of the cancer. Cancer cells can use these newly formed blood vessels as a mode of dissemination for migration for

metastatic cells allowing 'colonization' of other areas elsewhere in the body (Folkman, 1972).

Along with the effect of MMPs on the ECM, they are also responsible for the induction of many membrane bound proteins found on the outside of the cell membrane. These proteins often have diverse roles throughout the cell and some have direct effects on proliferation, such as fibroblast growth factor receptor (Cauwe et al., 2007; Chang and Werb, 2001). In addition, MMPs have a variety of important roles that allow cancer cells that take advantage of these proteases to gain a selective advantage over unmutated cells in the body.

2.1.4.4 Gelatinases

The gelatinases (MMP-2 and -9) are involved in the degradation of gelatin and collagen which are two of the most common compounds found in the ECM (Matrisian, 1990). MMP-2 and -9 have the potential to be exploited to benefit a cancer cell as described previously. The roles in angiogenesis, removal of the ECM as a barrier, liberation of ECM growth inducers, activation of growth factors/proteins, and their degradation of two of the most ubiquitous compounds in the ECM mean that MMP-2 and -9 are important targets for cancer research. Urine and blood levels of MMP-2 and -9 can be correlated to the degree of malignancy of cancer, particularly prostate cancer (Kuniyasu et al., 2003; Zhang et al., 2004). However, studies have not conclusively determined whether MMP-9 alone could be used as an indicator of the presence or malignancy of cancer (Turpeenniemi-Hujanen, 2005). Due to the potential damage these

two MMP can cause when they are utilized by cancer cells, they are potentially important targets for prostate cancer treatment and prevention

2.1.4.5 Modulation of MMP Activity: Methods of Activation

The potential for MMPs to be used as a target to help reduce the spread of a cancer is due in part to the multiple methods in which this family of proteins can be inhibited. The regulation of MMPs can occur in several ways, three key regulation events are: 1) zymogen cleavage, 2) inhibition of the active enzyme, and 3) modulation of gene expression.

The gene expression of MMPs is complex. This complexity mirrors the importance and diversity of gelatinases' roles in normal ECM remodelling and cellular processes. In almost all cancer cells, baseline MMP activity is elevated when compared to normal cells, leading to increases in all facades of MMP interaction with the ECM (Brehmer et al., 2003; Cauwe et al., 2007; Yan and Boyd, 2007).

The pathways which regulate the cellular translocation of MMPs are vulnerable to mutations or inductions via cellular pathways commonly up-regulated in cancers. Cancer cells normally produce their own MMPs, but have also shown an ability to induce surrounding untransformed cells to release increased concentrations of MMPs by secreting factors either through cell-cell channels or into the ECM. EMMPRIN is a trans-membrane protein that is often over-expressed on the cellular membrane of cancer cells. Normally, EMMPRIN induces the production of MMP-1, -2 and -3 (Caudroy et al., 2002; Gabison et al., 2005, Sun and Hemler, 2001). Therefore, in situations where

EMMPRIN release is induced, the protein can attenuate the production of these MMPs in nearby cells (Caudroy et al., 2002; Gabison et al., 2005, Sun and Hemler, 2001).

2.1.4.6 Modulation of MMP Activity: Zymogen Cleavage

MMPs are produced and secreted as inactive zymogens or pro-MMPs. A zymogen commonly needs some form of activation, often in the form of cleavage of an inhibitory domain before the protein becomes functional. MMPs are zinc-dependent pepsidases that require a Zn^{2+} ion in the catalytic site in order for the activated protein to function. The inhibitory or prodomain of the zymogen contains a cysteine residue (cysteine^{73}) which acts to form a bond between a thiol group and the Zn^{2+} cofactor. This interaction stabilizes the zymogen by incubating the Zn^{2+} catalytic site which results in a protein with no function. Often the zymogen is cleaved and this bond is broken through a method called the 'cysteine switch' (Hyun-Jeong and Parks, 2007).

2.1.4.7 Modulation of MMP Activity: Inhibition of the Active Enzymes

Generally, the regulation of activated MMPs takes place outside the cell through binding with the TIMPs (Westermarck and Kahari, 1999). The TIMP-MMP inhibition occurs as a reversible binding and takes place in a 1:1 ratio. The N-terminal portion of the TIMPs interacts with the catalytic site of the MMP which effectively segregates the Zn^{2+}, resulting in the same function of the Thiol-Zn^{2+} bond in the zymogen isoform (Blavier et al., 1999; Westermarck and Kahari, 1999). The general baseline MMP activity is dependent on TIMP levels; therefore increased MMP activity requires the balance to shift towards MMP concentration before extensive ECM remodelling occurs.

Cancer cells often have down-regulated TIMP levels leading to an induction of invasive potential.

There are currently four known TIMPs (TIMP1-4) that have been discovered. Each TIMP has specific MMP targets with some overlap with one or two of the other members. This study examines two of these, TIMP-1 and TIMP-2 which are commonly associated with the gelatinases. TIMP-1 binds preferentially to MMP-9 whereas TIMP-2 binds and associates with MMP-2 (Cauwe et al., 2007, Westermarck and Kahari, 1999).

2.1.4.8 Modulation of MMP Activity: Modulation of Gene Expression

The signal transduction pathways leading to increases in gene expression and overall MMP activation are complex. There are several common signal transduction pathways that can modulate MMPs and have been found to be activated when the baseline level of MMPs is elevated (Kajanne et al., 2007; Gordon et al., 2009, Zhang et al., 2009 [2]). These pathways are: nuclear factor kappa light chain enhancer of activated B cells. (NF-κB), p38, jun N-terminal kinase (JNK), mitogen activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K) and protein kinase C (PKC) pathways (Kajanne et al., 2007; Gordon et al., 2009, Zhang et al., 2009 [2]). The pathways p38 and JNK can be categorized as sub-pathways within the MAPK super-pathway as they rely heavily on signals propagated by members of the MAPK family. Some of these pathways, such as MAPK, can be induced by the binding of extracellular factors to receptors found on the cell membrane such as epithelial growth factor receptor and vascular endothelial growth factor receptor (Dempke and Heinemann, 2009; Kajanne et al., 2007). The cleavage and liberation of mitogenic factors from the ECM can lead to

activation of these receptors which suggests a possible feed forward mechanism for MMP activation via the cleavage mediated by MMPs. Studies assessing these receptors as potential novel targets in reducing the overall malignancy of a tumour focus on eliminating the signal before it activates specific signal transduction pathways in cancer cells (Dempke and Heinemann, 2009).

2.1.5 Introduction: Cellular Death

Cell division is closely regulated to maintain normal development and homeostatic conditions at the level of the organism (Hickman et al. 2002). This process requires a balance between cell growth and death; the deregulation of cell division results in uncontrolled cell growth which eventually leads to tumour formation (Guo and Hay 1999). Evasion of the signalling mechanisms or signal transduction pathways is common in neoplastic cells as these proliferation/survival pathways are often altered (Guo and Hay 1999, Hengartner 2000, Hickman et al. 2002). The ability of cancer cells to circumvent the normal cellular machinery to control cell proliferation has been studied for many years (Hickman et al. 2002, Gaur and Aggarwal 2003). MacLean (2009) found that phenolic compounds extracted from *V. macrocarpon* could induce cellular death through induction of caspase-8 and -9. Whole cranberry extract was observed to induce apoptosis through the modulation of various pro-apoptotic proteins such as Bid/tBid, Bax, prostate apoptosis response-4 (Par-4) and cytochrome c release (MacLean 2009).

2.1.5.1 Cell Death Pathways

In order for the tissues to remain functional, the body relies on the constant renewal and death of cells. Accidental cellular death often occurs as the result of damage to the cell due to physical trauma, heat, or radiation. This unregulated cell death is known as necrotic death or necrosis. Necrotic death is characterized by uncontrolled cellular swelling, chromatin degradation, and enzymatic digestion of the cellular organelles followed by rupture of the plasma membrane. This process leads to the uncontrolled purge of the cell cytoplasm into the extracellular matrix. Necrotic cell death is often followed by an inflammatory response as the body reacts to the excess cellular waste by cleaning and sterilizing the tissue.

Cellular death can also occur in a tightly regulated manner that avoids the wholesale release of the cytoplasmic matter of the cell to the extracellular space. Regulated cell death relies on the use of ATP to control and systematically close down cellular processes that lead to cell death. This programmed cell death can be induced in several different ways, but the most common incarnation is apoptosis. Apoptosis is a tightly regulated mode of cell death that is characterized by a defined set of morphological changes. When a cell undergoes apoptosis the cell loses both the normal symmetry and the anchorage of the cell to surrounding cells/tissues. The cell starts to shrink, partly due to cellular blebbing (budding of vesicles containing degraded portions of the cytoplasm), followed by the systematic degradation of the nuclear membrane and cleavage of the chromosomal material. The systematic degradation of a cell due to apoptosis is closely regulated by a family of proteolytic proteins called the cysteine-aspartic acid proteases (caspases).

The caspases do not induce apoptosis independently but rely on a large number of proteins that are linked together to create a series of signal transduction pathways. Activation of the effector caspases causes induction of signal transduction proteins which eventually leads to the cleavage of the zymogens of caspase-3 and -7 (Slee et al. 1999; Van de Craen et al., 1999). These signal transduction proteins eventually lead to the cleavage and activation of another subsection of caspases (-2, -6, -8 and -10) (Slee et al. 1999; Van de Craen et al., 1999). Caspase-3, known as the "gate-keeper", is of particular interest, as the signal transduction pathways always end with the cleavage of caspase-3. This cleavage is a central event in apoptosis which leads to the irreversible propagation of apoptotic signals. Caspase-3 activation leads to the cleavage of downstream targets such as poly-ADP-ribose-polymerase (PARP), an important protein in DNA repair. Cleavage of PARP leads to the inactivation of this protein which prevents the protein from binding to DNA and repairing damage (Oliver et al. 1998). Once caspase-3 deactivates PARP, caspase-3 is free to target the chromatin within the nucleus and cleave the DNA into mono- and oligonucleosome fragments. This chromatin cleavage is an important final stage of apoptosis and is often described as one of the key events of this process (Yuste et al. 2005).

2.1.5.2 Key Pathways of Apoptosis – Extrinsic Pathway

As mentioned previously, caspase-3 zymogen cleavage is a central event in apoptosis. Cleavage of pro-caspase-3, the zymogen of caspase-3, is induced through two pathways, the extrinsic, which is involved in propagation of external apoptotic signals through intramembrane receptor proteins known as death receptors, and the intrinsic,

which is involved in the induction of apoptosis with an emphasis on the mitochondria-mediation of apoptosis (Figure 2.2). The intrinsic pathway is induced through internal stressors such as cytoskeletal stress, organelle loss of integrity, DNA damage, loss of anchorage, and growth factor withdrawal. (Von Ahsen et al., 2000). The extrinsic pathway is activated by the binding of external ligands to and subsequent activation of death receptors.

Each death receptor has specific ligands that activate them. Tumour necrosis factors alpha and beta (TNFα/TNFβ) are two apoptotic cytokines that induce apoptosis through interaction with TNF receptors (TNF-R1, TNF-R2) found within the plasma membrane. TNF-related apoptosis-inducing ligand (TRAIL) is another apoptosis ligand, but differs from independent ligands TNFα/TNFβ in that TRAIL is an intramembrane protein that will ligate with its target death ligands, which include death receptors 4 and 5 (DR4, DR5). Another common ligand is the Fas ligand (Fas), which is also an intermembrane protein that is a part of the TNF family but does not interact with the TNFRs.

Instead, Fas interacts with its own distinct death receptor (FasR) to form a trimer. This trimer then activates adaptor proteins distinct to the receptor, Fas associated death domain (FADD). FADD, once induced, moves and forms part of a large conglomerate of protein. This complex consists of FADD, pro-caspase-8, and pro-caspase-10. The complex, called the death inducing signalling complex (DISC), activates the caspase-8 and -10 zymogens leading to propagation of the death signal resulting in the cleavage of caspase-3 and -7 (Lawen, 2003). Once caspase-3 is activated the apoptotic signal is no longer reversible and cell deconstruction is induced. In Type I cells, DISC formation and caspase-3 cleavage is directly responsible as described above, but Type II cells differ in

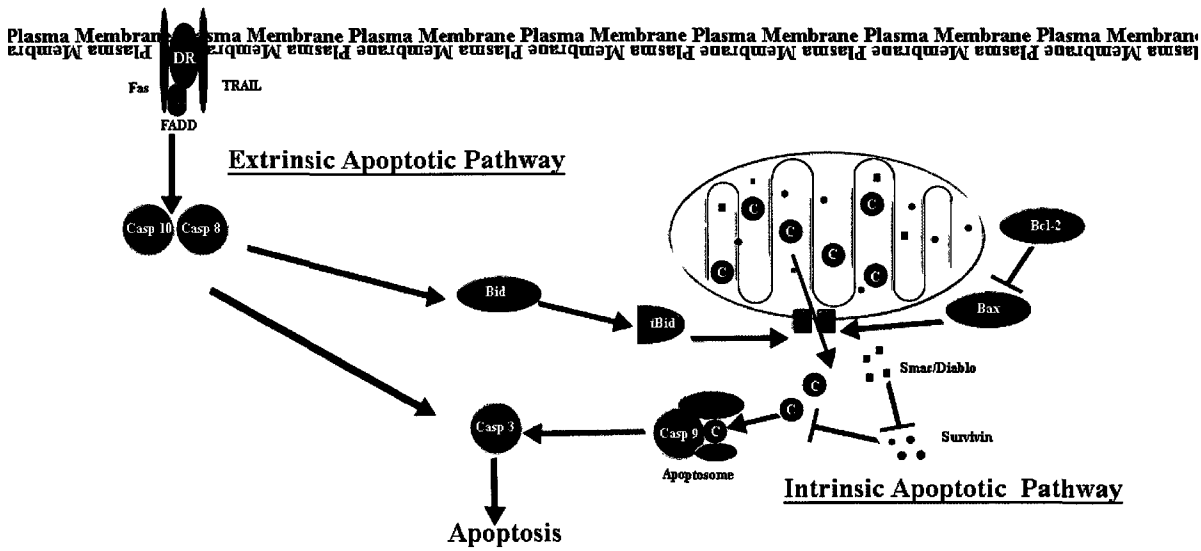

Figure 2.2 The interaction of key proteins involved in the intrinsic and extrinsic apoptotic pathways

their mode of propagation of the apoptotic signal. Type II cells will be induced through ligands via death receptors, like Type I cells, but rather than interacting directly with caspase-3 using DISC, the signal is instead shunted to the intrinsic pathway where the signal is used to induce apoptosis (Scaffidi et al. 1998). The intrinsic pathway differs from the extrinsic pathway by the fact that the intrinsic pathway takes its cues from internal stressors and relies on an organelle for mediation of the apoptotic signal.

2.1.5.3 Key Pathways of Apoptosis – Intrinsic Pathway

The second pathway that leads to the cleavage of pro-caspase-3 is the intrinsic pathway. As mentioned previously this pathway relies heavily on a single organelle, the mitochondrion. Normally the membranes of mitochondria are stable, permitting this organelle to perform many functions including segregating select proteins from the cytosol. To change the permeability of the mitochondrial membrane in an apoptotic episode, the cell relies on a family of proteins known as the Bcl-2 family. This large family of proteins contains both pro- and anti-apoptotic proteins that often work in opposition to each other. Bax and Bak, two of the pro-apoptotic proteins, will dimerize together in the outer mitochondrial membrane to generate a pore thereby liberating segregated proteins. Bcl-2, the most common anti-apoptotic protein in the Bcl-2 family, antagonizes the formation of this Bax/Bak pore. The formation of the Bax/Bak pore allows the release of many proteins in the inter-membrane space of the mitochondrial, such as cytochrome c. Once released, cytochrome c will form a part of a conglomerate protein, not unlike the DISC in the extrinsic pathway, combining with apoptotic peptidase

activating factor (APAF-1) and pro-caspase-9 which together are called the apoptosome. The apoptosome uses energy donated by adenosine-5'-triphosphate (ATP) to activate caspase-9. Caspase-9, once activated, dimerizes and the proteins show remarkable affinity for pro-caspase-3. Once these proteins are associated together, they irreversibly cleave and active caspase-3. When caspase-3 is activated, the final stages of apoptosis are induced and cell death is guaranteed.

2.1.5.4 Key Pathways of Apoptosis – Intrinsic Pathway and the Mitochondrion

Mitochondria have a role in healthy cells as the centre for ATP synthesis and can acts as a repository for Ca^{2+} ions. But when the cell is subjected to apoptotic stimuli the mitochondria is also involved in apoptotic signalling. The outer mitochondrial membrane is a semi-permeable lipid bilayer that allows molecules under 5000 Da to cross due to the presence of porins. The inner mitochondrial membrane is highly convoluted to increase its surface area, but lacks pores and is therefore much more difficult to permeate. The inner membrane space along with the inner mitochondria membrane is used extensively to produce energy for the cell via the electron transport chain (ETC). The space between the outer and inner mitochondria membranes, known as the intermembrane space, is the key repository for mitochondrial segregated Ca^{2+} involved in intracellular signalling. Ca^{2+} is normally actively transported across the outer membrane of the mitochondria where is stored in the intramembrane space until 1) it reaches a threshold concentration, or 2) the cell undergoes an induction of Ca^{2+} release resulting in a change in the outer mitochondrial membrane via the mitochondrial permeability transition pore (PTP). The

formation of the PTP and the release of the Ca^{2+} from the mitochondria may induce an apoptotic event (Chipuk et al., 2006).

Research into the effect of the mitochondria in apoptosis was initially focused on the effect of select members of the bcl-2 family of proteins. Bcl-2, an inhibitor of apoptosis was observed to localize to the outer mitochondrial membrane when a cell undergoes an apoptotic signal (de Jong et al., 1994; Hockenbery et al., 1991; Krajewski et al., 1993; Monaghan et al., 1992). Follow-up studies on apoptosis found that two select factors were needed for apoptosis to occur, ATP and cytochrome c. (Liu et al., 1996). Cytochrome c is a small protein found in the intermembrane space and in the inner mitochondrial membrane where it is used within the ETC to shunt electrons between complexes. After it was determined that cytochrome c was also an integral contributor to apoptosis, research focused on this protein to elucidate its full role in apoptosis. It was discovered that following apoptotic induction, the mitochondria underwent an event that allowed the rapid release of nearly all the mitochondrial sequestered cytochrome c, allowing for the propagation of the apoptotic signal. It was also found that Bcl-2 localization to the mitochondria during apoptotic events actually inhibited the mitochondrial release of cytochrome c and therefore acted as an anti-apoptotic protein (Kluck et al., 1997; Yang et al., 1997).

Cytochrome c, once released from the mitochondria binds, with the help of ATP, to APAF-1. The oligomerization of cytochrome c, ATP and APAF-1 forms the apoptosome which is involved in caspase cleavage. The formation of the apoptosome allows a conformational change in APAF-1 opening the protein's caspase activation and recruitment domains (CARD) to interact with pro-caspase-9. APAF-1's CARD binds

two caspase-9 zymogens to dimerize just prior to mutual activation of each other (Acehan et al., 2002; Boatright et al., 2003; Li et al., 1997; Pop et al., 2006). Once caspase-9 is activated it remains attached to the apoptosome and forms a caspase-9 holoenzyme which binds readily to pro-capase-3 and -7 (Chao et al., 2005; Jiang and Wang, 2000; Rodriguez and Lazebnik, 1999; Yin et al., 2006). Once the apoptosome binds with pro-caspase-3 or -7 it cleaves the inhibitory portion and activates the caspases which leads to further propagation of apoptosis.

Cytochrome c is not the sole apoptotic inducing/regulating protein segregated in the mitochondria; there are several others of note. These include second mitochondria-derived activated of caspases (Smac/Diablo) and survivin. These two proteins actively compete with each other. As part of a large family of inhibitors, survivin's effect on the induction of apoptosis in neoplastic cells has been studied extensively (Salvesen and Ducklet 2002). Importantly, survivin shows differential expression depending whether it is expressed in cancer versus non-cancer cells. Survivin directly inhibits caspase activation leading to inhibition of any apoptotic stimuli. Smac/Diablo acts to inhibit activation of members of the inhibitors of apoptosis (IAP) family. Therefore, since Smac/Diablo inhibits inhibitors of apoptosis it is a pro-apoptotic protein.

The release of apoptotic proteins from the mitochondria is a central event in the intrinsic apoptosis pathway. Cytochrome c was found to be of primary importance in apoptotic induction, and has been studied extensively. There are two general methods for the release of cytochrome c from the mitochondria into the cytoplasm. The first involves the PTP, which can be used to release proteins segregated in the mitochondria. The

second is through direct pore formation of the outer mitochondrial membrane by pro-apoptotic bcl-2 proteins, including Bax or Bid.

2.1.5.5 Mitochondrial Permeability Transition Pore (PTP)

Many different processes rely on a change in the permeability of mitochondria. The intrinsic pathway relies partially on the change in membrane potential of the mitochondria to allow an apoptotic signal to be propagated to caspase-3. The formation of the PTP, once induced, allows the transfer of small cytoplasmic molecules or mitochondrial segregated proteins into and out of the mitochondria. This change in permeability affects both the outer and inner mitochondrial membranes. This is therefore one of the only methods that allows proteins segregated in the inner membrane space to pass through the mitochondrial membranes. This change in permeability results in osmotic deregulation which causes the mitochondria to swell and the outer membrane to rupture. This rupture allows pro-apoptotic proteins normally segregated in the mitochondria to enter the cytoplasm (Basso et al., 2005). The formation of mitochondrial PTP has been observed to be induced by endoplasmic reticulum (ER) stress (Breckenridge et al. 2003; Rizzuto et al. 1998). This may be associated with another possible apoptotic pathway that is not very well understood, ER mediated apoptosis. The ER acts as a Ca^{2+} repository similar to the mitochondria. When induced, the ER will release sequestered Ca^{2+} which leads to increases in calcium ion levels around the mitochondria. This induces the mitochondria to create PTP openings by an association between cyclophilin D and cytosolic Ca^{2+} (Chipuk et al., 2006). PTP induction does not

occur at normal cytosolic levels of Ca^{2+}, but rather once the calcium level reaches a 'critical point' (Chipuk et al., 2006).

Despite the potential for PTP formation to be one of the primary routes of cytochrome c release, there are some important distinctions suggesting that PTP formation may not be a common method of cytochrome c release during the induction of an apoptotic signal. One of the events associated with PTP formation is the general swelling of the mitochondria which leads to wholesale rupture of the outer mitochondrial membrane. This event causes irreversible damage to the mitochondrion, destroying all its membrane potential. Once the membrane potential is disrupted the proton gradient on which the ETC relies is destroyed. Therefore, PTP formation will disrupt the potential for the cell to generate ATP which is integral for certain events further down the intrinsic pathway. As noted above, ATP is required for the cleavage of caspase-9 and caspase-3 by the apoptosome (Acehan et al., 2002; Genini et al., 2000; Jiang and Wang, 2000). It has been observed that the loss of the critical ATP inhibits the creation of the PTP in the mitochondrial outer membrane (Crompton 1999; Eguchi et al. 1997; Leist et al., 1997; Nicotera et al., 1998; Nicotera et al., 2000). The lack of ATP as energy source changes cellular death from an apoptotic event into caspase independent cell death. This caspase independent programmed cell death resembles necrosis rather than the final stages of apoptosis (Crompton 1999; Eguchi et al. 1997; Kass et al., 1996; Leist et al., 1997; Nicotera et al., 1998; Nicotera et al., 2000). For PTP to be a common method for apoptotic stimuli, induced cytochrome c release from the outer membrane would have to allow the membrane to remain functional to allow the membrane potential to stay intact.

There is a possibility that the PTP can still induce apoptosis if only a subset of mitochondria undergo cellular swelling and rupture, leaving a population of mitochondria functional and productive. Furthermore, Huser et al. (1998) and Petronilli et al. (1999) described a transient PTP opening that resulted without the expected cellular swelling and loss of outer membrane integrity while still allowing cytochrome c to be released.

2.1.5.6 Outer Membrane Permeabilization by bcl-2 Family Pore Formation

Rather than PTP which results in the general rupture of mitochondria, there is a second method for permeabilization which relies on proteins to form a pore in the outer mitochondrial membrane. Several studies have found that cytochrome c release occurred in the absence of mitochondrial swelling and also allowed the mitochondrial membrane potential to remain intact (Bossy-Wertzel et al., 1998; Kluck et al., 1997; Yang et al., 1997). Later studies found that this method of apoptosis could still be induced in protein knockout cell lines (cell lines missing important proteins involved in PTP formation) and mice lacking integral components of PTP formation (Kokoszka et al., 2004; Nakagawa et al., 2005). This exciting finding suggested that there was a second method for cytochrome c release that was independent of PTP formation. This method relies on a group of proteins, the bcl-2 family, to form pores in the outer mitochondrial membrane to release sequestered proteins.

The bcl-2 family is a large group of related proteins, many of which either induce or inhibit apoptosis. While the range of effects may differ across the family, they all share structural similarities. All bcl-2 members contain at least one of four distinct bcl-2 homology domains (BH1, BH2, BH3, and BH4). The absence or presence of these

homology domains can be used to further subdivide the family into three groups. Commonly localized to either the ER or the mitochondrion, the anti-apoptotic proteins of the bcl-2 family (Bcl-2, Bcl-xL, Bcl-w, etc.) are characterized by having all four homologies (BH1-4) (Krajewski et al., 1993; Susin et al., 1996). These anti-apoptotic proteins inhibit apoptosis by ligating with the pro-apoptotic bcl-2 proteins and sequestering them (Adams and Cory 2001; Gross et al. 1999).

The pro-apoptotic bcl-2 proteins are characterized by only having the first three homologies (BH1-3). The pro-apoptotic bcl-2 proteins (Bax, Bak, Bok, etc.) all have roles in the permeabilization of outer mitochondrial membranes (Marzo et al., 1998). These proteins are normal constituents of healthy cells, but are found in an inactive form to ensure apoptosis is not randomly induced. The third class of bcl-2 proteins are involved in the activation of the pro-apoptotic proteins. This last group of proteins (Bik, Bid, Bim, Bad, Bmf, Noxa, Puma, Blk, etc.) only contain the third BH domain, BH3. BH3 proteins interact to activate the BH1-3 proteins either directly or indirectly. The BH3 proteins Bid and Bim directly interact with the BH1-3 proteins to induce the formation of homo-dimers. This conformation is required for these proteins to form pores. Bad, Bik, Bmf, Noxa act indirectly to induce apoptosis by inhibiting the BH1-4 proteins. These proteins bind to the active site of the BH1-4 proteins leading to either the displacement of proteins already present or to competition with BH1-3 proteins for open active sites (Chipuk et al., 2006).

The BH1-3 proteins have been shown to induce PTP formation in certain situations, but of more significance is the ability of these proteins to localize in the outer mitochondrial membrane and form pores (Kuwana et al., 2002; Letai et al., 2002; Marzo

et al., 1998; Narita et al., 1998; Shimizu et al., 1999). Kuwana et al. (2002) showed that Bax and Bak could induce cytochrome c release without inducing PTP formation, but used simplified liposomes as models of the outer mitochondrial membrane. While these proteins have been observed releasing cytochrome c in isolation of other factors, it has been hypothesised that other factors may be required to act synergistically with Bax and Bak to release enough cytochrome c in a timely fashion for apoptosis to be induced. These secondary factors (such as cardiolipin) may be required, but passive since they have no direct role in regulating whether the outer mitochondrial membrane is permeabilitized (Chipuk et al., 2006).

The heterodimerization of BH1-4 proteins with the pro-apoptotic BH1-3 proteins inhibits the ability of BH1-3 to form pores. Of critical importance is the cellular 'balance' of these proteins as the direction the ratio becomes skewed directly determines whether the cell remains in a quiescent state, or whether the cell starts to undergo apoptosis. BH3 proteins, while important as mediators for the BH1-4 and BH1-3 proteins are also involved in apoptosis induction.

Bid, a BH3 protein, interacts with BH1-3 proteins, but has low affinity for these proteins and therefore is a poor inducer of apoptosis. Bid's contribution to apoptosis differs in comparison to other BH3 proteins. Rather than solely interacting with the Bcl-2 members, Bid is cleaved into tBid which then interacts with the mitochondrial membrane to form pores not unlike the pro-apoptotic BH1-3 proteins. Bid in its native form is not as potent as the truncated form but is unique in that it has locations perfect for binding to caspases. These sites allow caspase-8 to bind to and cleave the inhibitory N-terminal domain from Bid, creating tBid. The caspase-8 mediated cleavage of Bid allows access

to its pro-apoptotic BH3 domain. This access allows tBid to interact with a co-factor, cardiolipin, which is ubiquitous in the inner mitochondrial membrane (Kim et al., 2004; Li et al., 1998). As noted previously, caspase-8 is a normal member of the extrinsic pathway and Bid's preference for this caspase suggests a possible mode of 'cross-talk' between the extrinsic and intrinsic pathways.

BH1-3 induced permeabilization of the mitochondrial layer permits the exodus of cytochrome c without the normal disadvantages of PTP such as disruption of the ETC resulting in the generation of ATP that is integral in later stages of the intrinsic pathway. Permeabilization of the outer mitochondrial membrane alone is not enough to induce apoptosis. Cytochrome c is normally sequestered in the mitochondria and the majority of it is localized to the inner mitochondrial membrane where is it associated or attached to a four tailed diphosphatidylglycerol lipid found only in the mitochondria, cardiolipin. This protein when found in the inner mitochondrial membrane acts to stabilize proteins involved in the ETC. These facts are important as the induction of apoptosis requires the release of nearly 100% of a mitochondria's complement of cytochrome c (Goldstein et al., 2000). Outer membrane permeabilization only releases an estimated 20% of the mitochondrial complement. However, it has been observed that tBid can release 100% of the mitochondrial cytochrome c at a fraction of the concentration needed for Bax to release 20% (Jurgenmeiser et al., 1998; Scorrano et al., 2002). This difference may be explained by the observation that tBid can bind to cardiolipin. tBid induces the release of cytochrome c from the cardiolipin allowing cytochrome c to leave the inner mitochondrial membrane and enter the intermembrane space. This interaction between tBid and cardiolipin causes dramatic changes in the cristae structure of the inner

mitochondrial membrane (Jurgenmeiser et al., 1998). The tBid interaction with cardiolipin is independent of the BH3 domain on which the tBid mediated oligmization of Bax/Bak relies (Scorrano et al., 2002). It has been hypothesised that the induction of apoptosis by tBid may be completely independent of its ability to interaction with Bax/Bak.

To induce the changes observed with Bid requires the protein be cleaved into tBid. tBid then localizes to the outer mitochondrial membrane and forms homo-trimers that insert themselves into the membrane forming a pore to allow the exodus of cytochrome c from the mitochondria (Grinberg et al., 2002). The late stages of the interaction between tBid and the cardiolipin found within the inner mitochondrial membrane have not been fully elucidated. Regardless, the observations of tBid's effects on the mitochondria suggest that tBid may be able to act independently of other factors allowing for apoptosis induction even in the presence of defences to stop apoptosis such as inhibition of Bax or induction of Bcl-2.

2.1.5.7 Non-mitochondrial Proteins Involved in Apoptosis Induction

The vast majority of apoptotic machinery is conserved across all cell types, with the exception of a small list of proteins that are unique to certain cells. Prostate antigen response-4 (Par-4) is one example that was first discovered by Sells et al. (1994) when androgen independent prostate cancer cells were induced to undergo apoptosis. Par-4 mediated apoptotic induction may be a possible means of inducing apoptosis even in the presence of anti-apoptotic defences engineered by cancer cells. Par-4 readily induces

apoptosis in hormone independent cancer cells which is important as the apoptotic machinery normally present in these cells is highly mutated.

Mutated apoptotic pathways make it difficult to induce apoptosis in cancer cells which is one of the chief reason cancer cells out-compete normal cells so readily (Chakraborty et al., 2002). Par-4 sensitizes cancer cells by inducing not one, but rather both the extrinsic and intrinsic apoptotic pathways (Bergmann et al., 2004; Boehrer et al., 2002). Par-4 induces apoptosis via the extrinsic pathway by inducing the transfer of death receptors from the Golgi apparatus where excess receptors are stored. The receptors (Fas-FasR) are transferred from the Golgi apparatus to the plasma membrane where they are inserted and begin by heightening any external death signal. Signal amplification leads to a higher induction of FADD, the adaptor protein associated with Fas. FADD then indirectly induces the cleavage of pro-caspase-8 via induction of DISC formation (Chakraborty et al. 2001). Par-4's affect on the intrinsic pathway differs from the method it uses to induce the extrinsic pathway.

Rather than inducing the transfer of a protein like in the extrinsic pathway, Par-4 inhibits an anti-apoptotic protein involved in the intrinsic pathway, Bcl-2 (Qui et al. 1999). Since Par-4 inhibits an inhibitor of apoptosis, it acts to change the critical Bax/Bcl-2 ratio in favour of Bax, leading to apoptosis induction. Par-4 may provide an important target for apoptosis induction in hormone independent prostate carcinoma which is highly resistant to apoptotic stimuli.

2.1.6 Summary

Cranberries have been found to be a source of phytochemicals capable of affecting many forms of cancer, including prostate cancer. To test the effects of specific phytochemicals on prostate cancer, DU145 human prostate cancer cells were used to represent a malignant, hormone independent form of prostate cancer. The ability of a cancer cell to ignore death signals or apoptosis is a key mode through which cancer cells become malignant. In this work two apoptotic pathways were studied to understand the effect through which cranberry phytochemicals may induce apoptosis in DU145 human prostate cancer cells. These pathways, the intrinsic and extrinsic pathways, differ by their mode of induction and their constituent proteins. To understand what effects phytochemicals have on these apoptotic pathways, key proteins of either pathway were studied to examine potential changes in expression. The hypothesis of this study was that Pacs will induce apoptosis in DU145 human prostate cancer cells through both or either, the intrinsic and extrinsic pathways.

Also studied in this body of work, was the ability of cancerous cells to express certain proteins involved in the degradation of the ECM. The ability of a cell to degrade the ECM involves the MMP family of proteins and their protein modulators. Changes in the modulators of MMPs indicate a potential effect on the expression of MMPs within the cell. It is hypothesised in this study that UA will inhibit MMP expression of DU145 human prostate cancer cells by modulating MMP regulators.

The previously described topics within this chapter discuss in detail some of the individual members and effectors of cancer focusing on the induction of apoptosis and on MMP expression and regulation. Changes in these processes and proteins would support

the general hypothesis: Cranberry phytochemicals from *V. macrocarpon* will affect the behaviour of human prostate cancer cell *in vitro*.

Chapter Three: Proanthocyanidins from *Vaccinium macrocarpon* Induce Programmed Cell Death in DU145 Human Prostate Cancer Cells via the Intrinsic Pathway of Apoptosis

3.1 Introduction

Prostate cancer is the most diagnosed cancer, and is the second-most lethal cancer for men (American Cancer Society 2009; Canadian Cancer Society 2008). Treatment options for prostate cancer involve surgical intervention (prostatectomy) or hormone deprivation. These treatment options tend to have severe side effects which, taken in concert with the median age of prostate cancer onset of 65 years, often leads health advocates to suggest 'watchful waiting' (American Cancer Society 2009). This passive approach is often unsatisfactory and consequently leads many patients to search for new treatment options, such as dietary intervention.

The use of plants or herbal supplements to treat illnesses has been popular in Asian cultures for thousands of years. Current research has focused on the health benefits of various plants, such as the American cranberry. Extracts from *V. macrocarpon* have been found to affect prostate cancer cells by inducing apoptosis (MacLean 2009). MacLean (2009) also observed that several different fractions were responsible for the induction of apoptosis seen with the whole cranberry extract. One of these is the proanthocyanidins (Pacs) fraction (Ferguson et al., 2004; Seeram et al., 2004; Liberty et al., 2007).

Cellular apoptosis generally occurs through two apoptotic pathways, the intrinsic and extrinsic pathways (Reuter et al. 2008). The extrinsic pathway is dependent on the induction of apoptosis through a ligand-receptor mediated model (Reuter et al. 2008).

The intrinsic pathway of apoptosis is induced through internal cellular stresses which modulate the permeability of the mitochondria (Reuter et al., 2008). The work presented in this chapter elucidates the mechanism whereby the Pacs fraction of *V. macrocarpon* induces apoptosis in DU145 human prostate cancer cells. It was hypothesized that Pacs treatment of DU145 human prostate cancer cells will induce apoptosis through modulation of the intrinsic or extrinsic apoptotic pathways or both.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (Oakville, ON). Unless otherwise noted, antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA).

3.2.2 Preparation of Proanthocyanidin Extracts

The proanthocyanidin fraction from American cranberry (*Vaccinium macrocarpon*) was prepared and characterized in the laboratory of Dr. Catherine Neto, Department of Chemistry and Biochemistry at University of Massachusetts-Dartmouth as described in the Appendix.

3.2.3 Cell Culture

Human prostate adenocarcinoma cancer cells (DU145 cells) (ATCC, Manassas, VA) were cultured in α-minimum essential media (αMEM; Gibco, Burlington, ON)

supplemented with 1% antibiotic-antimycotic (Gibco) and 10% Fetal Clone III fetal bovine serum (FBS; Hyclone/VWR Canlab, Mississauga, ON). The DU145 cells were grown on 100 mm plastic tissue culture plates (Falcon, Mississauga, ON) at a temperature of 37 °C in the presence 5% CO_2. The FBS supplemented αMEM was removed and replaced with defined media (DM) consisting of αMEM supplemented with 5 μg/mL transferrin and 2.5 μg/mL insulin 24 hours prior to treatment. Stock cell cultures were prepared by freezing cells in a solution of 5% dimethyl sulfoxide (DMSO) and 95% αMEM culture medium. This cell pellet was later sonicated as described below.

3.2.4 Solubilization of Proanthocyanidin Extracts for Cell Treatments

The Pacs fraction was dissolved immediately before treatment in a 1:9 ratio of DMSO dissolved in DM (vol:vol) supplemented as described above. DMSO did not exceed a maximum of 1% concentration on the treatment plate.

3.2.5 Bradford Protein Assay

To assay the protein concentration of samples used for Western blots or for various assays the Bradford method was used to determine protein concentration. Bradford assay protein dye (Biorad Mississauga, ON) was diluted in 2 μL of protein sample and 1598 μL of distilled water per microcuvette tube.

Bradford reagent (400 μL) was added to each microcuvette which was then inverted to ensure proper mixing. Samples were incubated for 7 minutes at room temperature. Post-incubation, the protein sample was read at a wavelength of 595 nm. A series of

dilutions of bovine serum albumin (BSA) of known protein concentration was used to generate a standard curve. The protein samples of unknown concentrations were compared against the standard curve.

3.2.6 Sonication

Stored cell pellets were removed from the -80 °C freezer and stored on ice until resuspension in 50 mM Tris-HCL [pH 7.4] at a volume of 50-150 µL proportional to the pellet size. Phenylmethylsulphonylfluoride (PMSF) and HALT protease inhibitors cocktail (Fisher Scientific, Canada) were added to inhibit the action of proteases released from the cell once the cellular membrane was lyzed. The sample was sonicated using setting 2 on the Sonic Dismembrateor model 100 (Fisher Scientific, Ottawa, ON) for 10 seconds, and then replaced on ice. The samples were sonicated three times before they were subjected to a Bradford Protein assay as detailed above.

3.2.7 Alamar Blue Assay

The Alamar Blue assay (AB; Invitrogen, Burlington, ON) measures cell viability using a redox indicator that is metabolized by cells. This indicator, resazurin, is exposed to cells on plates and is reduced intracellularly by the viable cells on the plate, yielding a pink fluorescent product, resorufin (O'Brien et al. 2000). The conversion of resazurin to resorufin serves as an indicator to measure cellular metabolic activity, and therefore viability and is quantified by fluorescence spectroscopy. The active reagent of the AB assay is a nontoxic alternative to the more common MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay.

Cells were suspended in αMEM supplemented with FBS and then subcultured onto 96 well plates at a density of 3500 cells/well and allowed to incubate for 36 hours. Twenty-four hours prior to treatment with Pacs, the cell media was removed and replaced with serum free αMEM, also known as DM. Immediately prior to treatment the serum-free DM was removed and replaced with 5 ml of fresh DM. Cultures were then treated with varying concentrations of the Pacs fraction derived from the American cranberry. Treatment concentrations ranged from 0-25 μg/mL for of Pacs. Cells were treated for 6 hours and the AB reagent was placed on the plate 3 hours into the treatment at a volume of 10% of the culture medium (20 μL AB reagent added to 200 μL cell culture medium). At the end of the treatment, the plate was removed from the incubator and cooled to room temperature before reading fluorescence at 595 nm. Cell viability data were then graphed and cell treatments were normalized to control.

3.2.8 Immunoblot Analysis

Cells were subcultured at a density of $3.5x10^5$ on 100 mm plastic culture plates and incubated at 37°C at 5% CO_2. Cells were allowed to grow for 36 hours in αMEM, at which point, if the confluency was ~60%, then αMEM was removed and replaced with DM for 18 hours prior to treatment with an enriched fraction from cranberry. Cells were treated with Pacs (0, 10, and 25 μg/mL) for 6 hours. The media was then removed and discarded and the cells were washed three times by chilled PBS to ensure the removal of all fractions and media traces. The cells were then trypsinized using a 10% (vol/vol) solution of Trypsin-EDTA (diluted in PBS and warmed to ~37°C; Sigma-Aldrich, Oakville, ON). After 5 minutes of trypsin incubation, αMEM supplemented with FBS

was used to deactivate the trypsin. Cells were gently liberated from the plate with tapping and washing the cell monolayer with 5 mL αMEM from a glass pipette. The cells, now suspended in αMEM, were removed and placed into 15 mL Falcon tubes which were then centrifugated at 1000 x *g* for 5 minutes. The supernatant was removed, and the pellet was resuspended in PBS then centrifuged at 1000 x *g* for another 5 minutes. The supernatant was removed and the pellet resuspended in fresh phosphate buffered solution (PBS) before being moved from the original 15 mL tube and placed in a fresh, chilled microcentrifuge tube and centrifuged at 1000 x *g* for 5 minutes. The supernatant was then very carefully removed and the pellet was stored at – 80°C.

Prior to use, the cell sample was sonicated as outlined above, then the extracted lysate was subjected to a Bradford protein assay. Once the protein concentration of the sample was determined the lysates were diluted in a 3:1 ratio of Laemmli buffer (50 mM Tris-HCL [pH 6.8], 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol, and 100 mM β-mercaptoethanol). The diluted sample was then boiled for 3 minutes before being cooled at -20°C for a minimum of 5 minutes. The sample was then centrifugated briefly at 1000 x *g* to combine the sample and the condensation coating the inside of the microcentrifuge tube. Equal amounts of protein were then loaded into each of the various wells, and the samples were electrophoresed using 7-12% polyacrylamide gels resolved at an amperture of 35 mA. During electrophoresis, the gels were bathed in 1x running buffer (200 mM glycine, 25 mM trizma-base, and 4 mM SDS) in a volume of 1 L of dH_2O). While the samples of proteins were resolved through the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel, the nitrocellulose membranes were

bathed in Tris transfer buffer (50 mM Trizma-base, 40 mM glycine, in a volume of 0.2 L methanol diluted in 0.8 L dH_2O).

After the sample is electrophoresed through the gel, the gel was removed from the running apparatus and washed briefly with transfer buffer before being loaded on the pre-bathed nitrocellulose membranes and transferred from the gel onto the membrane. The proteins were transferred from the gel to the nitrocellulose membrane at a current of 65 V for 75 minutes. After the transfer of the proteins to the nitrocellulose membrane was complete, the membranes were washed in blocking solution (5% BSA in 1X Trizma buffered solution [TBS] supplemented with 0.05% Tween-20) for 1.5 hours. The membrane was then cut horizontally to allow multiple antigenic targets to be assayed from the same SDS-PAGE gel. The membranes were stored on a shaker in primary antibody diluted in 2.5% BSA solution for 30 minutes.

The membranes were then washed three times with TBS supplemented with 0.05% Tween-20 (TBS-T) for 30 minutes with 10 minutes washes in the solution prior to removal. After washing, the membranes were stored on a shaker with specific secondary antibodies (diluted 1:1000 in PBS) for 1.5 hours. The secondary antibody was removed, and the membranes washed briefly with TBS-T while being agitated for 10 minutes. This washing was done in triplicate. The TBS-T was removed after the third incubation and SigmaFast BCIP-NBT tablets used to develop the antibodies to visualize the proteins found within the membrane. Computer analysis was performed using the software Bio1D in conjunction with the Bio-Print gel documentation system (Montreal Biotechnology, Montreal, QC) to quantify the bands and to provide a densitometric determination of the protein expression.

3.2.9 Mitochondrial Isolation

The mitochondrion can act as a reservoir for some of the proteins involved in apoptosis (Lopaczynski and Zeisel 2001). To assay the expression of the mitochondrial segregated proteins required the removal of the mitochondria from the sample. One of the mitochondrial sequestered proteins, Smac/Diablo, is commonly released when the cell undergoes apoptosis. A Mitochondria Isolation kit (Pierce Biotechnology, Nepean, ON) was used to accurately partition the mitochondria from the cytosolic fraction.

After treatment, cells were trypsinized as described previously before being counted. Cells ($3x10^7$) were centrifuged at 800 x *g* for 10 minutes in a microcentrifuge tube. The supernatant was carefully removed and discarded, and the pellet was resuspended in 800 µL of Mitochondria Isolation Reagent A supplied by the kit. The pellet was vortexed for 5 seconds and allowed to incubate on ice for 2 minutes. A volume of 20 µL of Mitochondria Isolation Reagent B was then added to the tube and the microcentrifuge tube was then vortexed and stored for 10 minutes on ice. During the 10 minutes on ice, each sample was vortexed every 2 minutes. Samples (10 µL) from each tube were pipetted onto a hemocytometer, and morphological changes (rupture of the cellular membrane, or cell lysis) were noted.

Mitochondria isolation reagent C (800 µL) was then introduced to the cell lysates which were then inverted twice before being stored on ice for 15 minutes. The sample was centrifuged at 4°C and 700 x *g* for 10 minutes to remove insoluble cell material. The supernatant was removed and placed into fresh microcentrifuge tubes that had been pre-chilled on ice. This sample was then centrifuged at 12000 x *g* for 5 minutes to pellet any organelles, such as mitochondria. The supernatant was carefully removed and placed in

chilled microcentrifuged, and both the supernatant (cytoplasmic portion of the cell) and the mitochondrial pellet were stored at -80°C. Prior to analysis, the cytoplasmic sample protein levels were evaluated with the Bradford assay as described previously.

3.2.10 Nucleosome Enrichment

Human DNA, like all eukaryotic DNA, is organized into subunits of structure consisting of DNA wrapped around histone proteins (H2A, H2B, H3, and H4). Theoretically, this structure is akin to a long string of yarn periodically wrapped around multiple balls evenly spaced apart along the DNA chain. Due to the close proximity of the histones to the DNA, endonucleases are inhibited from cleaving the DNA at that site, and therefore the cleavage happens between the histones in the internucleosomal linker regions (Burgoyne et al., 1974). This cleavage results in the production of mono- and oligonucleosome units which are now small enough to exit the nucleus and enter the cytoplasm. The formation of these oligonucleosomes in cranberry fraction-treated DU145 cells was assayed using the Roche Cell Death ELISA Plus kit (Laval, QC). The Roche cell death kit is based on a quantitative sandwich-enzyme immunoassay principle using monoclonal antibodies directed against DNA and histones. Initially, cell lysates were loaded into a 96-well streptavidin-coated microplate. The lysates were then incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. The histone-biotin antibody binds to the histone component of any nucleosomes present in the sample and also binds to the streptavidin plate via the antibody's biotinylation during incubation. Following the removal of unbound antibodies by washing, the amount of nucleosomes present is determined by evaluating peroxidase-mediated cleavage of 2,2'-azino-bis(3-

ethylbenzthiazoline-6-sulphonic acid (ABTS) which is quantified based upon the change in colour. This colour change is proportional to the concentration of nucleosomes present.

The DU145 human prostate cancer cells were subcultured into 96 well plates at a density of 3500 cells/well and allowed to grow in αMEM for 36 hours at 37°C and 5% CO_2 before the αMEM was removed and replaced with DM, 18 hours prior to treatment. The DU145 cells were then treated with Pacs dissolved into a 1:9 DMSO:DM (vol:vol) solution. The Pacs-enriched DM sample was diluted in the media to a concentration of 0, 10 and 25 μg/mL. Post-treatment, the media was carefully removed using a pipette, and the cells were bathed in 200 μL of lysis buffer and incubated for 30 minutes at room temperature with periodic agitation. The agitation was used to help liberate the cells from the plate and to ensure that no cells were protected from the buffer by being sheltered by other cells or the cell fragments of a lyzed cell. The remaining cells were removed from the wells using a pipette to triturate the liquid after 30 minutes of the lysis buffer. The lysis buffer containing the lysed cells from the wells were transferred into microcentrifuge tubes and centrifuged at 200 x *g* for 10 minutes. After the centrifugation was complete, 20 μL of the supernatant (which contained the cytoplasmic fraction) was placed into the streptavidin-coated multiwall plate supplied as part of the nucleosome enrichment assay.

The supernatant samples in the streptavidin-coated plate were then diluted using 80 μL of immunoreagent (1 anti-histone-biotin: 1 anti-DNA-peroxidase: 18 incubation buffer [vol:vol:vol]). The plate was covered with an adhesive cover foil and incubated at room temperature with gentle agitation for 3 hours. After incubation, the plate was

washed three times (300 μL per well per wash), followed each time with gently tapping the plate on paper towels to remove the excess supernatant/immunoreagent from the wells. This wash was important to remove any excess nucleic material that remained unbound to the plate. Following the washes, 100 μL of ABTS solution was pipetted into each well and the plate was incubated at room temperature for 20 minutes while covered in aluminum foil to protect the plate and solution from any possible degradation by ultra-violet (UV) light. The reaction of the ABTS solution was ceased using the ABTS stop solution supplied by the assay kit. Absorbance was read at 405 nm. Triplicate absorbance readings for each treatment were averaged, and the value of a blank well containing incubation buffer, ABTS solution and ABTS stop solution, but devoid of DU145 cells, was subtracted from these averages.

The enrichment of the wells was calculated using the formula:

$$\text{Enrichment factor} = \frac{\text{mU of the cranberry-treated DU145 cells}}{\text{mU of the corresponding vehicle-treated cells}}$$

$$\text{mU} = \text{absorbance } [10^{-3}]$$

This enrichment factor was indicative of the mono- and oligonucleosome enrichment of the various concentrations assayed. The treatments were run in triplicate for this protocol for nucleosome enrichment.

3.2.11 Measurement of PARP activity

Poly ADP-ribose protein (PARP) is cleaved by members of the ICE family of proteins (e.g. caspase-3) into an 85 kDa protein as part of the normal degradation observed during apoptosis. Once cleaved, PARP becomes deactivated, but can be used as an important marker for apoptosis. Functional PARP is an important mediator of DNA repair in healthy and cancerous cells (Huerta et al. 2007). PARP cleavage induced by PACs treatment was assayed using the Universal Colorimetric Assay Kit (R&D Systems, Cedarlane Laboratories, Burlington, ON). The basis of this assay involves a histone-coated multiwell plate which binds any biotinylated PARP present in samples.

DU145 cells were grown on 100 mm plates under αMEM for 36 hours after which the αMEM was removed and replaced with DM for 24 hours. Immediately prior to treatment, the DM was replaced with fresh DM which was then supplemented with varying concentrations of Pacs (0, 10, and 25 μg/mL). The cells were then removed from the treatment plates using a 10% Trypsin/PBS solution. The trypsin was deactivated by using αMEM which contains FBS. FBS can be used as an inhibitor for trypsin. Cells were collected after trypsinization and placed in pre-chilled 15 mL falcon tubes. The cell suspension was then centrifuged at 400 x *g* for 10 minutes at 3°C. After centrifugation, the supernatant was removed and discarded, and the pellet was resuspended using 1 mL PBS 1X. Aliquots containing 10^7 cells were removed from the 15 mL tube and placed into pre-chilled 1 mL centrifuge tubes before being centrifuged again at 10000 x *g* for 20 seconds at 6°C. The supernatant was discarded and the cell pellet was resuspended in 15 cell volumes of lysis buffer consisting of 1x PARP Buffer, 0.4 mM PMSF (phenylmethylsulphonylfluoride), 1X Halt Protease inhibitors (Pierce Biotechnology, Nepean, ON), 0.4 mM NaCl and 1 % Triton. Suspensions were incubated on ice for 30

minutes, with vortex mixing every 3 minutes. The suspension was then centrifuged at 10000 x *g* for 10 minutes at 4°C to remove any insoluble material such as cellular membranes. Post-centrifugation, the supernatant was removed and placed in a new pre-chilled 1 mL micro-centrifuge tube, and a Bradford protein assay was performed.

Based on the protein assay results, the PARP plate wells were loaded with samples of equal protein concentrations in triplicate for each treatment concentration. The cell lysates were incubated for 15 minutes at room temperature before the plate was washed with 1x PBS supplemented with triton (1%), and then tamped lightly on paper towels to remove the wash solution. After washing the plate, 50 µL of Strep-HRP was placed in each well and incubated for 1 hour at room temperature. The plate was then washed 4 times with PBS-Trition (1%), and again tamped lightly on paper towels to remove the excess wash buffer. TACS-Sapphire, the primary reagent of the Roche Kit, was added at a volume of 50 µL to each well, and the plate was incubated for 30 minutes at room temperature in the dark before the first and second fluorescence readings were taken at 495 nm. At 35 minutes, 0.2 N HCl was added to all wells to deactivate the TACS-Sapphire reagent, and the plate was then reread at 595 nm.

3.2.12 Image Capture and Gel Quantification

Image capture of the developed gels was performed using the Bio-Print gel documentation system purchased from Montreal Biotech (Montreal, QC). The quantification of the various blots was conducted using the Bio 1D program provided as part of the gel documentation system. The protein expression of actin was used as a loading control for all Western blot analysis performed for this thesis. Blocked

membranes were cut horizontally to allow multiple proteins to be assayed, providing that the protein size of the primary and secondary/tertiary protein targets sizes were distinct. This method is of particular benefit as it allows direct correlation of the loading control with the protein of interest assayed.

3.2.13 Statistics

Unless specified elsewhere all statistical analysis consisted of an one-way analysis of variance (ANOVA) with Tukey post-hoc test. To ensure data fit a gaussian or normal distribution, a Kolmogorov-Smirnov (with Dallal-Wilkinson-Lilliel P value) analysis was performed. P-value of < 0.05 was considered significant. All analysis was preformed using the Graphpad Prism suite of programs (Graphpad Software Inc., La Jolla, CA).

3.3 Results

3.3.1 Evaluating DU145 Cell Viability in Response to Pacs Treatment

The inhibition of cell viability was significant at the higher concentrations 10, 25, and 50 μg/mL Pacs, but not at the lowest dosage assayed, 1 μg/mL Pacs (Figure 3.1). DU145 human prostate cells show reduced viability when treated with 10, 25 and 50 μg/mL Pacs for 6 hours (Figure 3.1). The Pacs-mediated decrease in viability of DU145 human prostate cancer cells at concentrations 10 and 25 μg/mL was statistically significant ($P < 0.01$), and also at 50 μg/mL of Pacs ($P < 0.001$).

3.3.2 Evaluating Markers of Late Stage Apoptosis

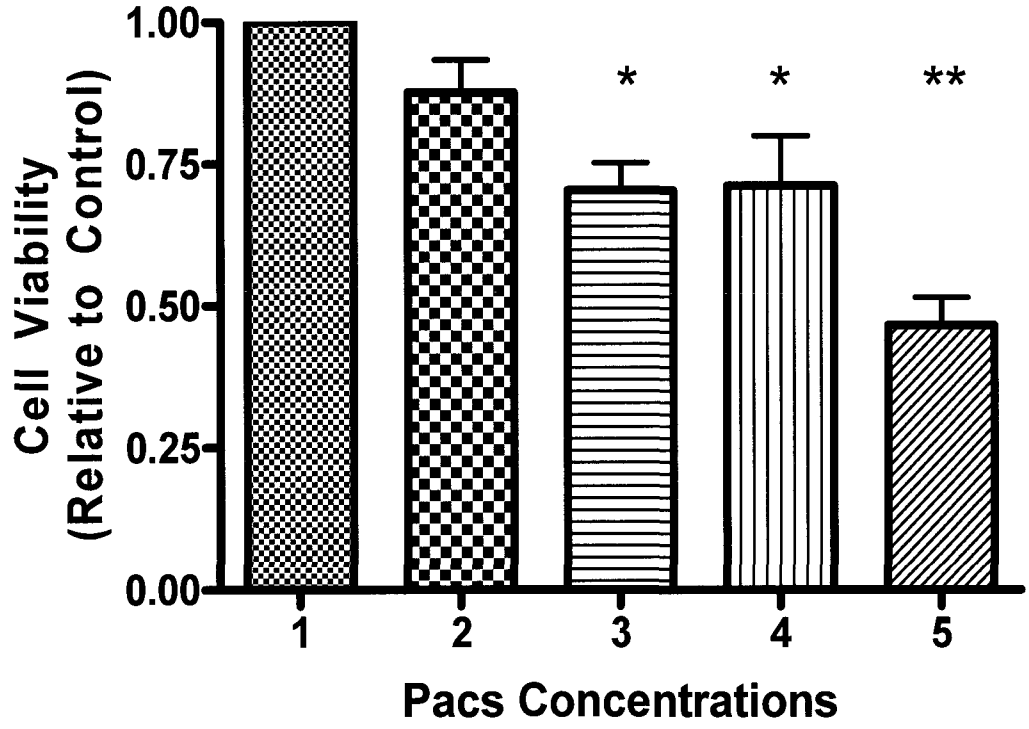

Figure 3.1: DU145 human prostate cancer cells treated with Pacs significantly inhibits cell viability at 10, 25 and 50 μg/mL concentrations. Cell viability of DU145 human prostate cells in the absence of Pacs (1), in the presence of 1 (2), 10 (3), 25 (4), and 50 (5) μg/mL Pacs over 6 hours. Data was analysized with ANOVA with a tukey post-hoc test. An asterisk indicates $P < 0.01$, a double asterisk represents $P < 0.001$. The graph is representative of results obtained from eight independent experiments. Each independent experiment was supported with 2 assay replicates.

Nucleosome formation is an important apoptotic event that can be used to indicate programmed cell death. This event constitutes one of the final steps of cellular apoptosis. DU145 human prostate cancer cells were assayed for the induction of nucleosomes. DU145 human prostate cancer cells treated with 10 and 25 μg/mL of Pacs resulted in an induction of nucleosome formation (Figure 3.2). The induction of nucleosome formation was statistically significant with a P value of < 0.05 and < 0.01 at the concentrations of 10 and 25 μg/mL, respectively.

3.3.3 Western Blot Analysis of Proteins Involved in the Extrinsic Apoptotic Pathway

Apoptotic cell death can be mediated through several different pathways. The two most important are: the intrinsic and extrinsic cell death pathways. The extrinsic pathway is activated by extracellular signals propagated using ligands, and relies heavily on a subset of cellular receptors to transmit the induction of apoptosis from outside the cell to the proteins found in the cytoplasm. Fas and TRAIL are two such receptors.

DU145 human prostate cancer cells treated with Pacs (25 μg/mL) for 6 hours showed an inhibition of Fas (Figure 3.3) and TRAIL (Figure 3.4) protein expression. Fas relies on other proteins such as FADD to propagate any extrinsic signal from the receptor to the other members of the extrinsic pathway of apoptosis. Treatment of DU145 human prostate cancer cells with Pacs (10 and 25 μg/mL) for 6 hours resulted in inhibition of FADD protein expression (Figure 3.5).

3.3.4 Western Blot Analysis of Protein Involved in the Intrinsic Pathway

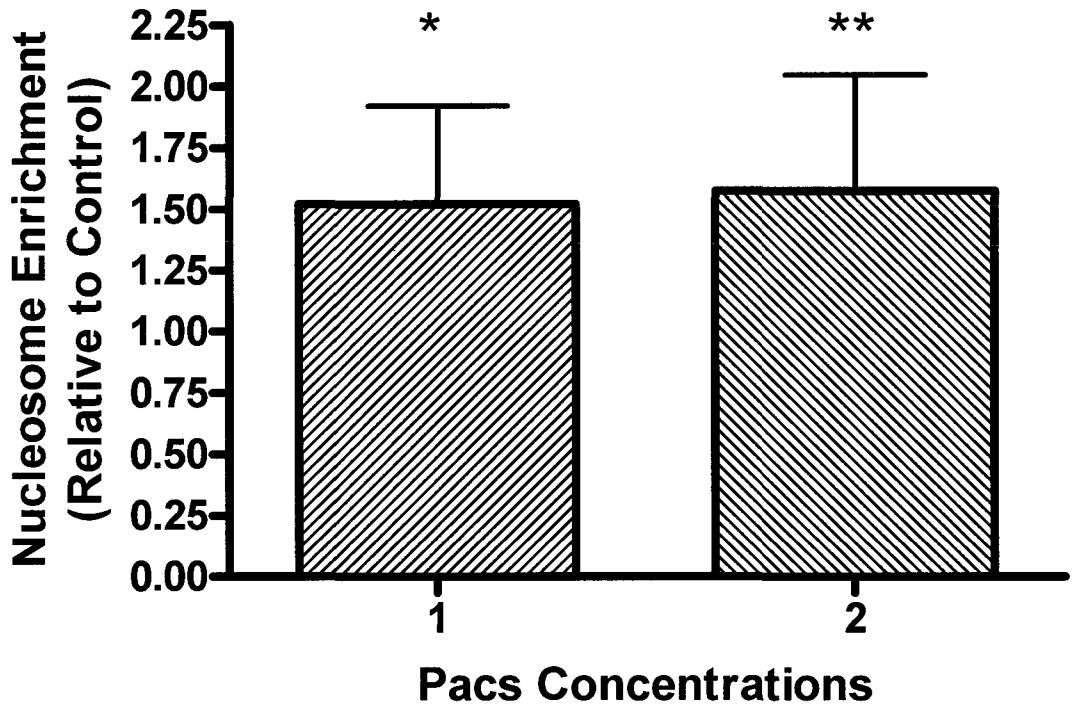

Figure 3.2: Pacs treatment of DU145 human prostate cancer cells induce oligonucleosome formation. Induction of nucleosome formation in cells in the presence of 10 (1), or 25 (2) μg/mL Pacs for 6 hours. Nucleosome enrichment at 0.0 is indicative of no enrichment. Data was analysized with ANOVA with a tukey post-hoc test. An asterisk denotes a signficance with $P < 0.05$ and double asterisk represent $P < 0.01$. The graph is representative of results obtained from three independent experiments. Each independent experiment was supported with two assay replicates.

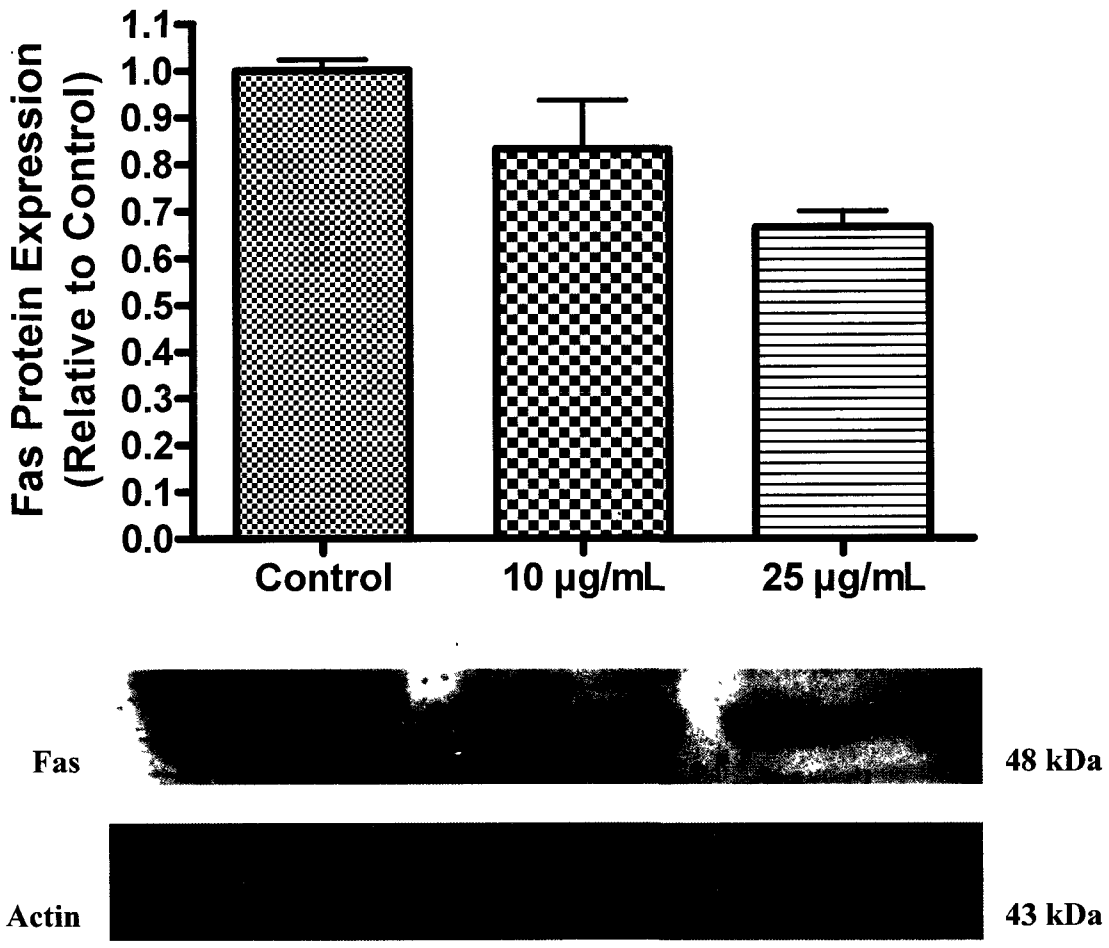

Figure 3.3 Pacs treatment of DU145 human prostate cancer cells inhibits the expression of Fas. Protein expression of Fas in the absence of Pacs (Vehicle present; Negative Control) and protein expression in the presence 10 and 25 µg/mL Pacs for 6 hours. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with two assay replicates.

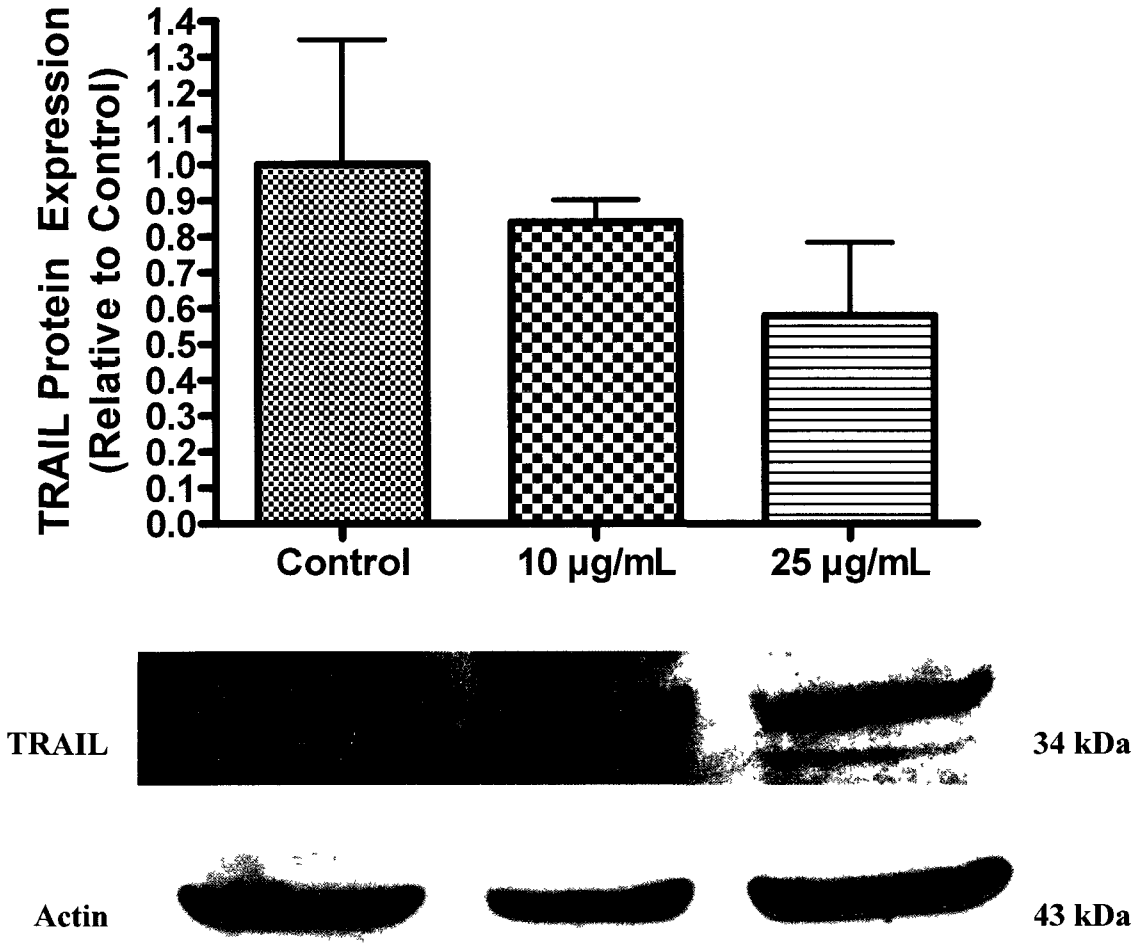

Figure 3.4 Pacs treatment of DU145 human prostate cancer cells inhibits the expression of TRAIL. Protein expression of TRAIL in the absence of Pacs (Vehicle present; Negative Control) and protein expression in the presence 10 and 25 µg/mL Pacs for 6 hours. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with two assay replicates.

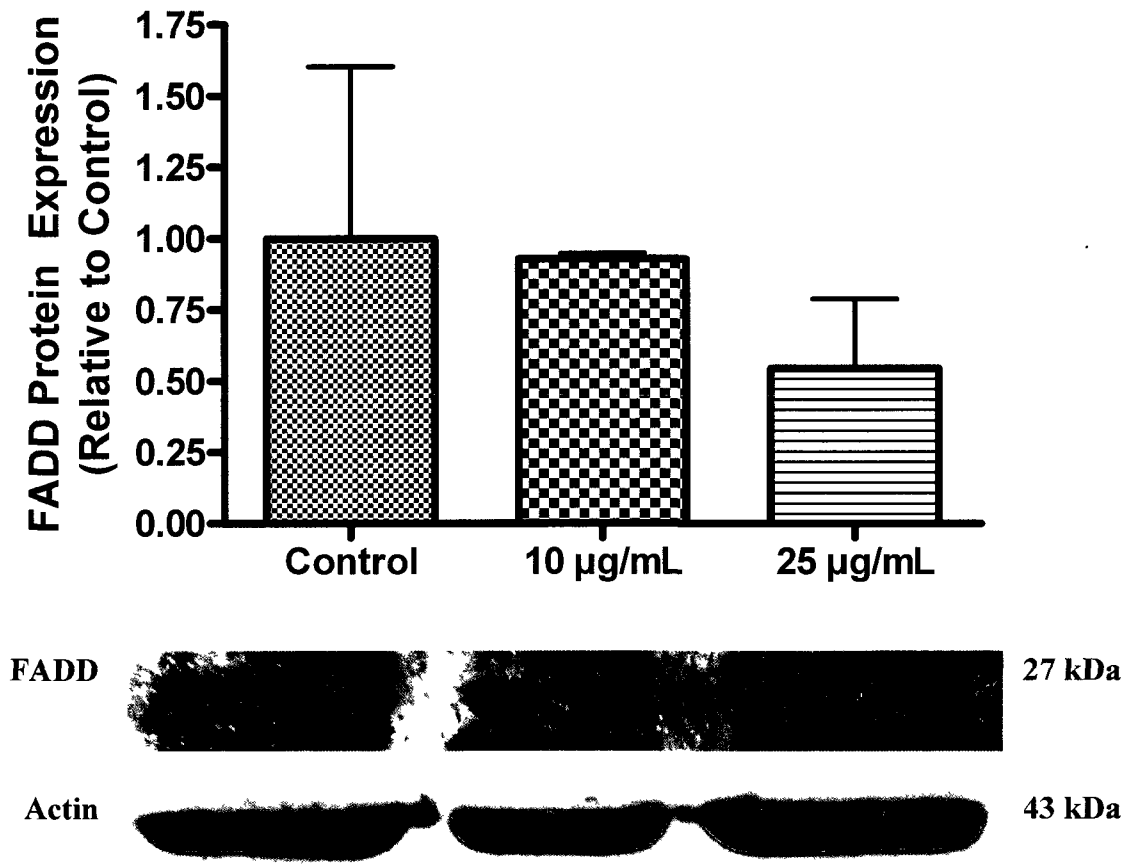

Figure 3.5 Pacs treatment of DU145 human prostate carcer cells inhibits the protein expression of the adapator protein, FADD. Protein expression of FADD in the absence of Pacs (Vehicle present; Negative Control) and in the presence 10 and 25 µg/mL Pacs for 6 hours. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with assay replicates.

Pacs treatment of DU145 human prostate cancer cells at 10 and 25 μg/mL for 6 hours had an inhibitory effect on Bcl-2 protein expression (Figure 3.7). Bax protein expression was inhibited by Pacs treatment at 25 μg/mL for 6 hours (Figure 3.6). Bid, another Bcl-2 protein, was inhibited at 10 and 25 μg/mLof Pac treatment for 6 hours (Figure 3.8). Inhibition of Bid protein expression was statistically significant at 25 μg/mL of Pacs treatment ($P < 0.01$). The activation of Bid requires the cleavage of the protein into tBid. Pacs treatment of DU145 human prostate cells resulted in an induction of tBid protein expression (Figure 3.9).

3.3.5 Western Blot Analysis of Proteins Segregated to the Mitochondria

The expression levels of two other mitochondrial segregated proteins, Smac/DIABLO and Survivin, were also assayed. The protein VDAC, which is found only in the mitochondria, was used as a control to measure the relative purity of the sample. Treatment with Pacs (10 and 25 μg/mL) for 6 hours induced cytochrome c protein expression in DU145 cells (Figure 3.10). Pacs treatment of DU145 human prostate cancer cells resulted in an inhibition of survivin (Figure 3.11) and Smac/DIABLO (Figure 3.12) protein expression. The induction of protein expression of both cytochrome c and Smac/DIABLO was statistically significant at 25 μg/mL, $P < 0.01$ and $P < 0.05$, respectively.

3.3.6 Non-mitochondrial proteins involved in the apoptotic pathways

Pacs treatment resulted in an induction in Par-4 protein expression in DU145 cells (Figure 3.13). Treating DU145 human prostate cancer cells with Pacs (10 and 25 μg/mL) induced PARP cleavage in DU145 cancer cells (Figure 3.14).

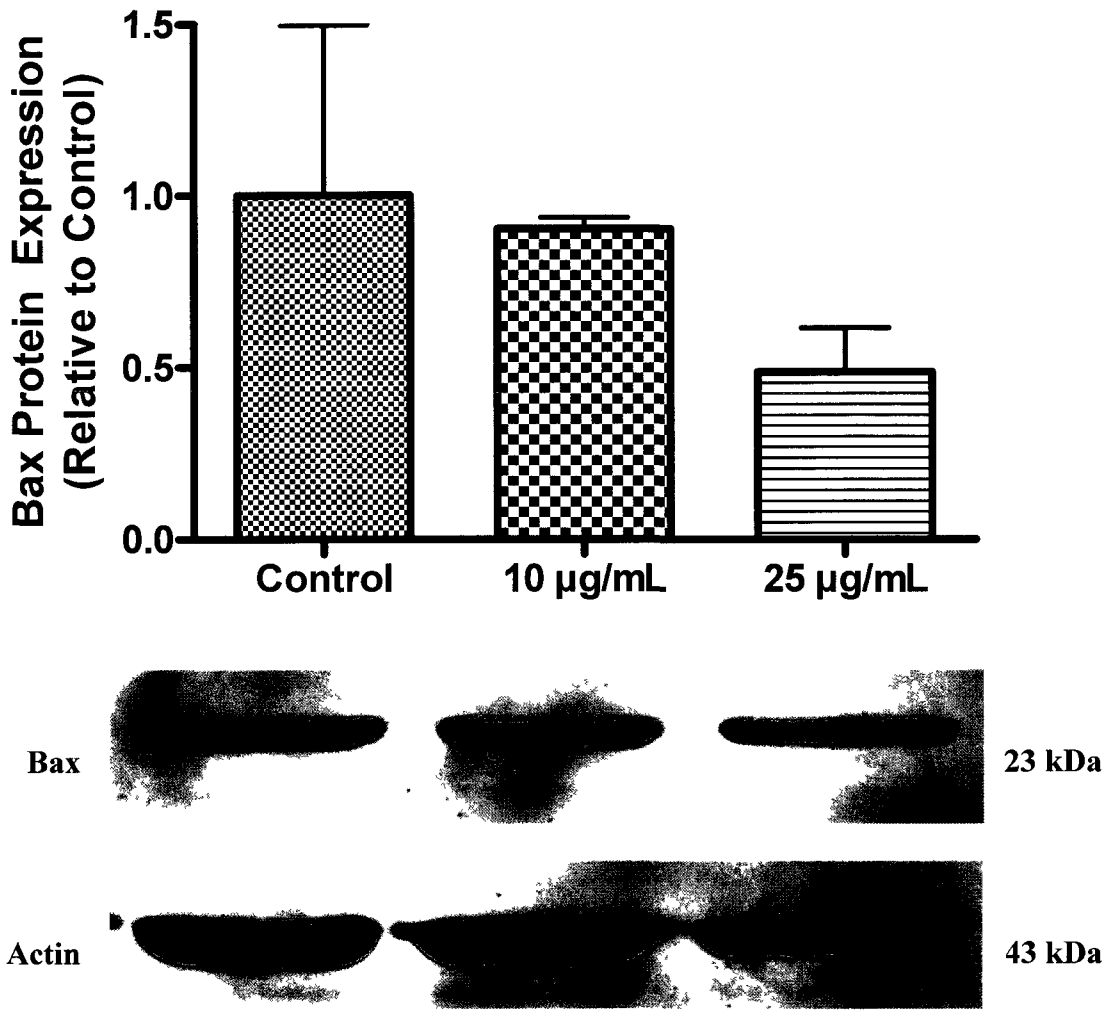

Figure 3.6 Pacs treatment of DU145 human prostate cancer cells inhibits the protein expression of Bax. Protein expression of Bax in the absence of Pacs (Vehicle present; Negative Control) and in the presence 10 and 25 µg/mL Pacs over the treatment time of 6 hours. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with a single assay replicate.

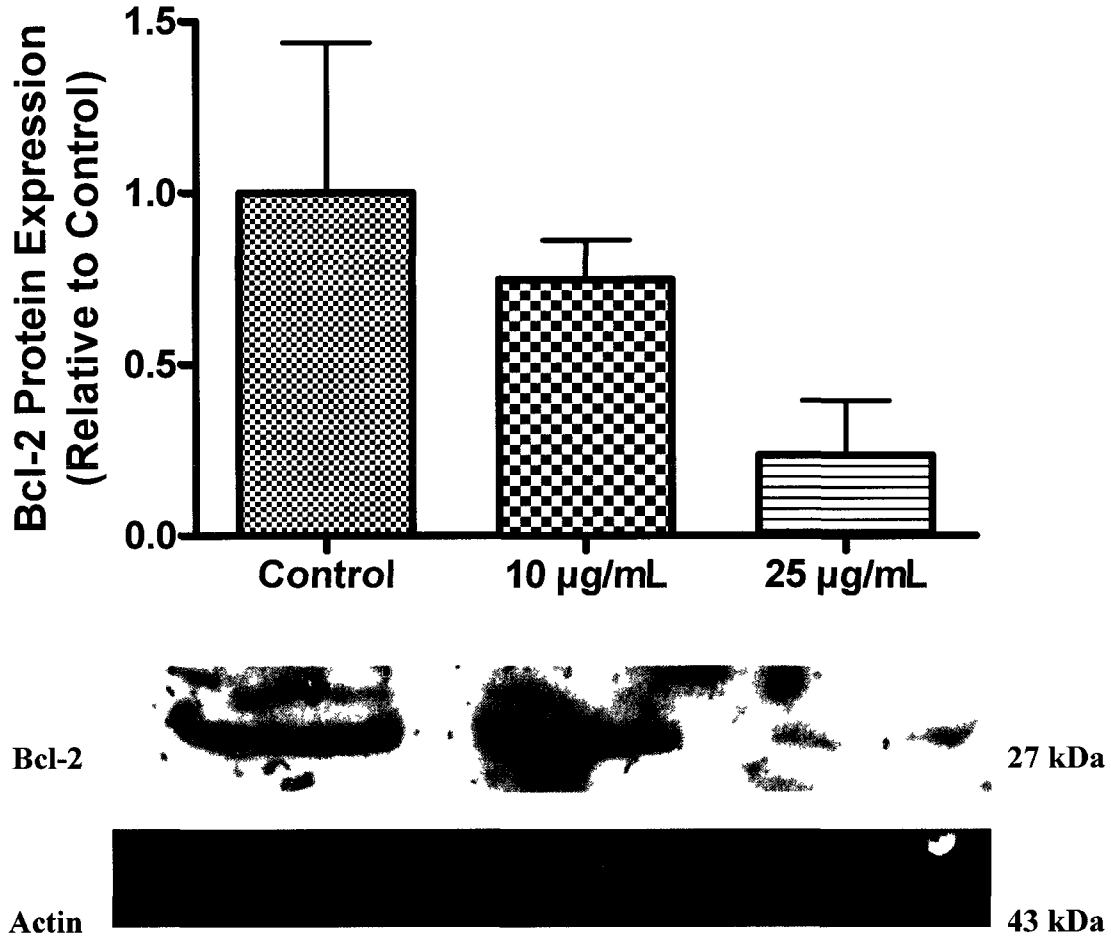

Figure 3.7 Pacs treatment of DU145 human prostate cancer cells inhibits the protein expression of Bcl-2. Protein expression of Bcl-2 in the absence of Pacs (Vehicle present; Negative Control) and in the presence 10 and 25 µg/mL Pacs over the treatment time of 6 hours. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with a single assay replicate.

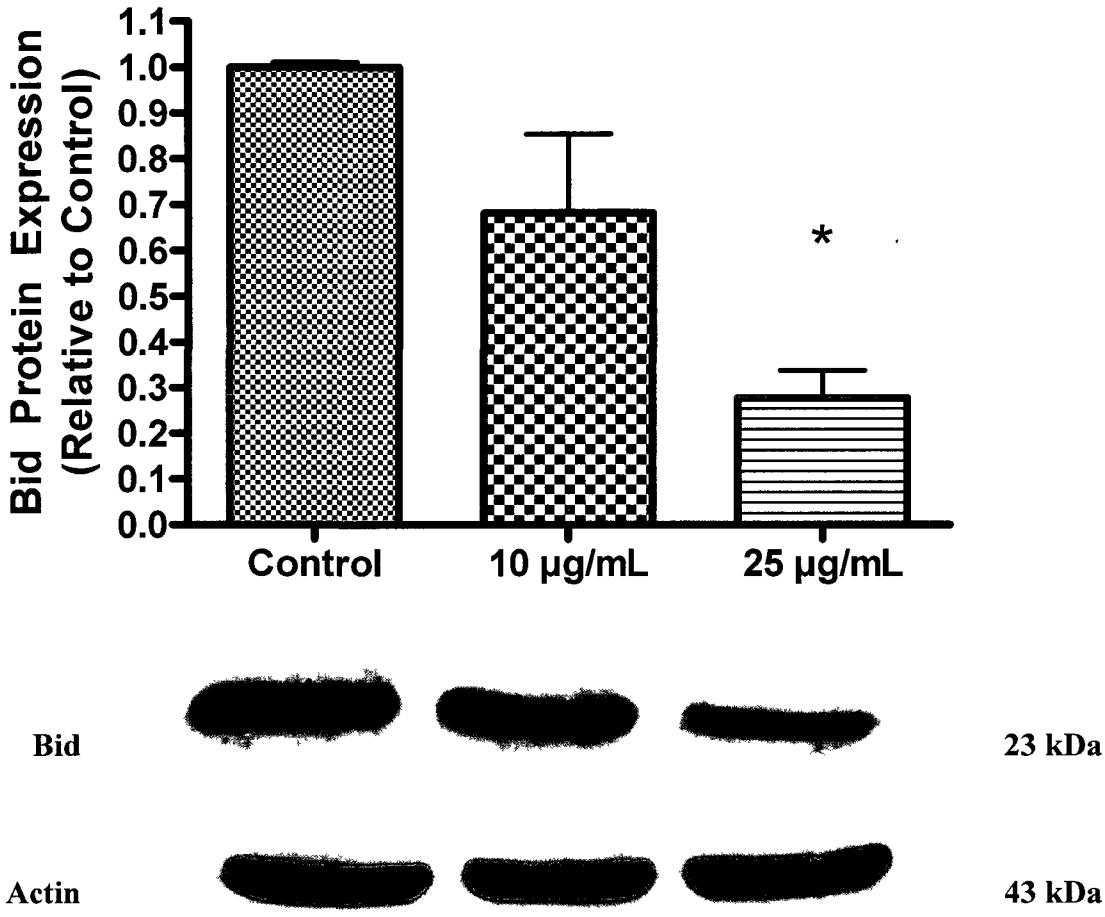

Figure 3.8 Pacs treatment of DU145 human prostate cancer cells induces the cleavage of Bid. Protein expression of Bid in the absence of Pacs (Vehicle present; Negative Control) and in the presence 10 and 25 µg/mL Pacs for 6 hours. Actin protein levels are indicated as a loading control. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk denotes a statistical significance of $P < 0.01$. The data are representative of results obtained from five independent experiments. Each independent experiment was supported with one assay replicate.

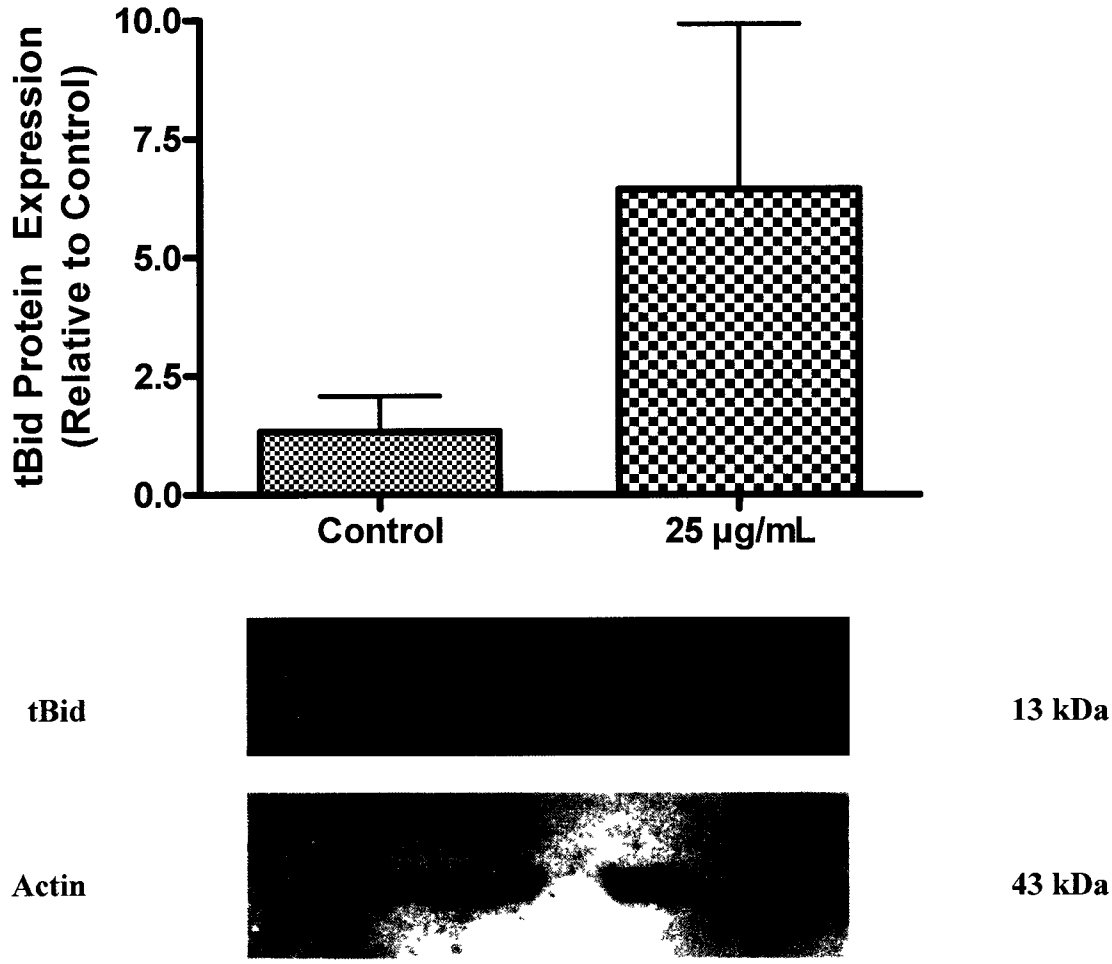

Figure 3.9 Pacs treatment of DU145 human prostate cancer cells cells induces the activation of tBid. Protein expression of tBid in the absence of Pacs (Vehicle present; Negative Control) and in the presence of 25 µg/mL Pacs for 6 hours. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with one assay replicate.

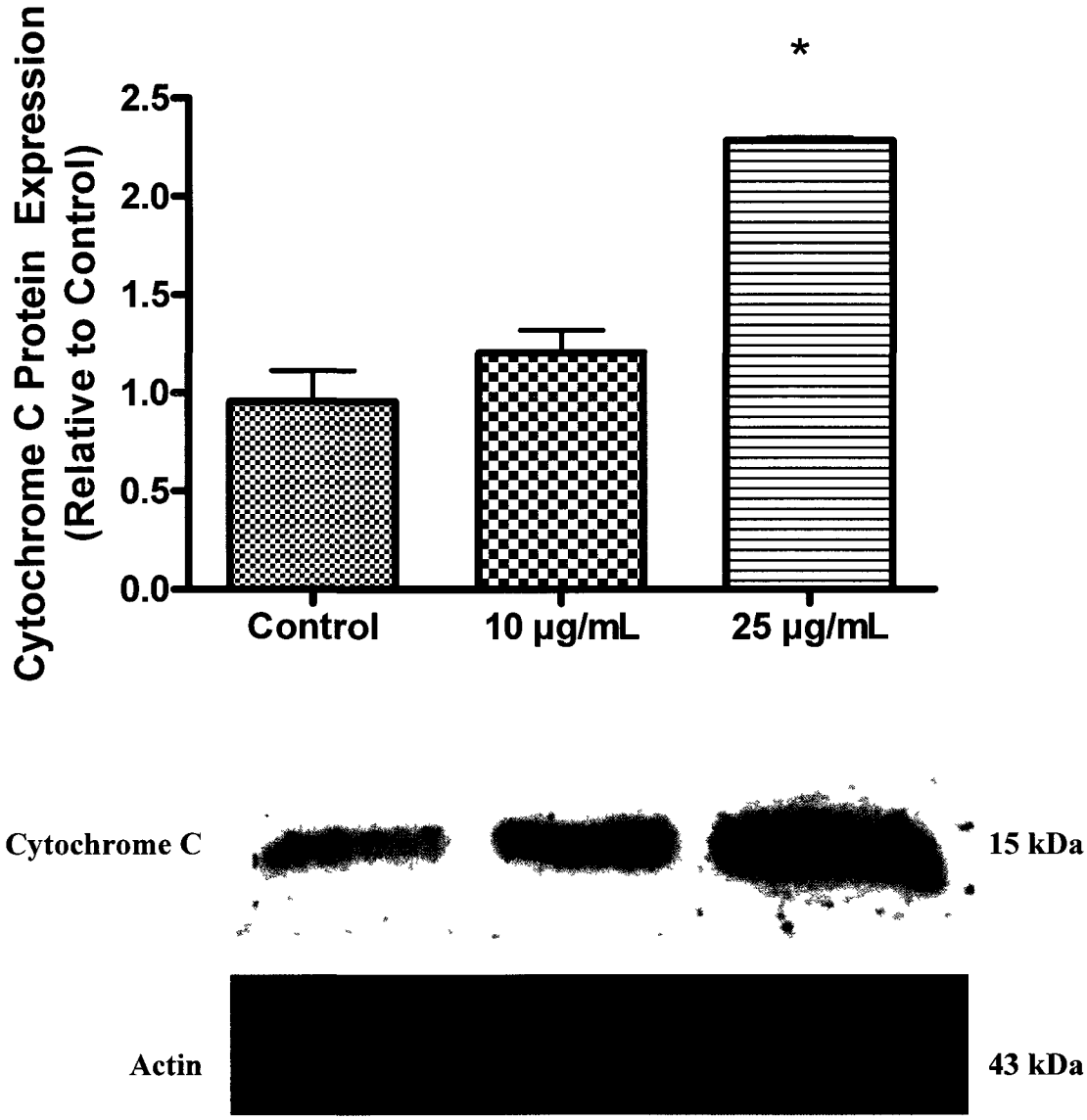

Figure 3.10 Pacs treatment of DU145 human prostate cancer cells induces the protein expression of cytochrome c. Protein expression of cytochrome c in the absence of Pacs (Vehicle present; Negative Control) and in the presence 10 and 25 µg/mL Pacs for 6 hours. Data was analysized with ANOVA with a tukey post-hoc test. Single Star denotes statisical signficance of $P < 0.01$. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment was supported with one assay replicate.

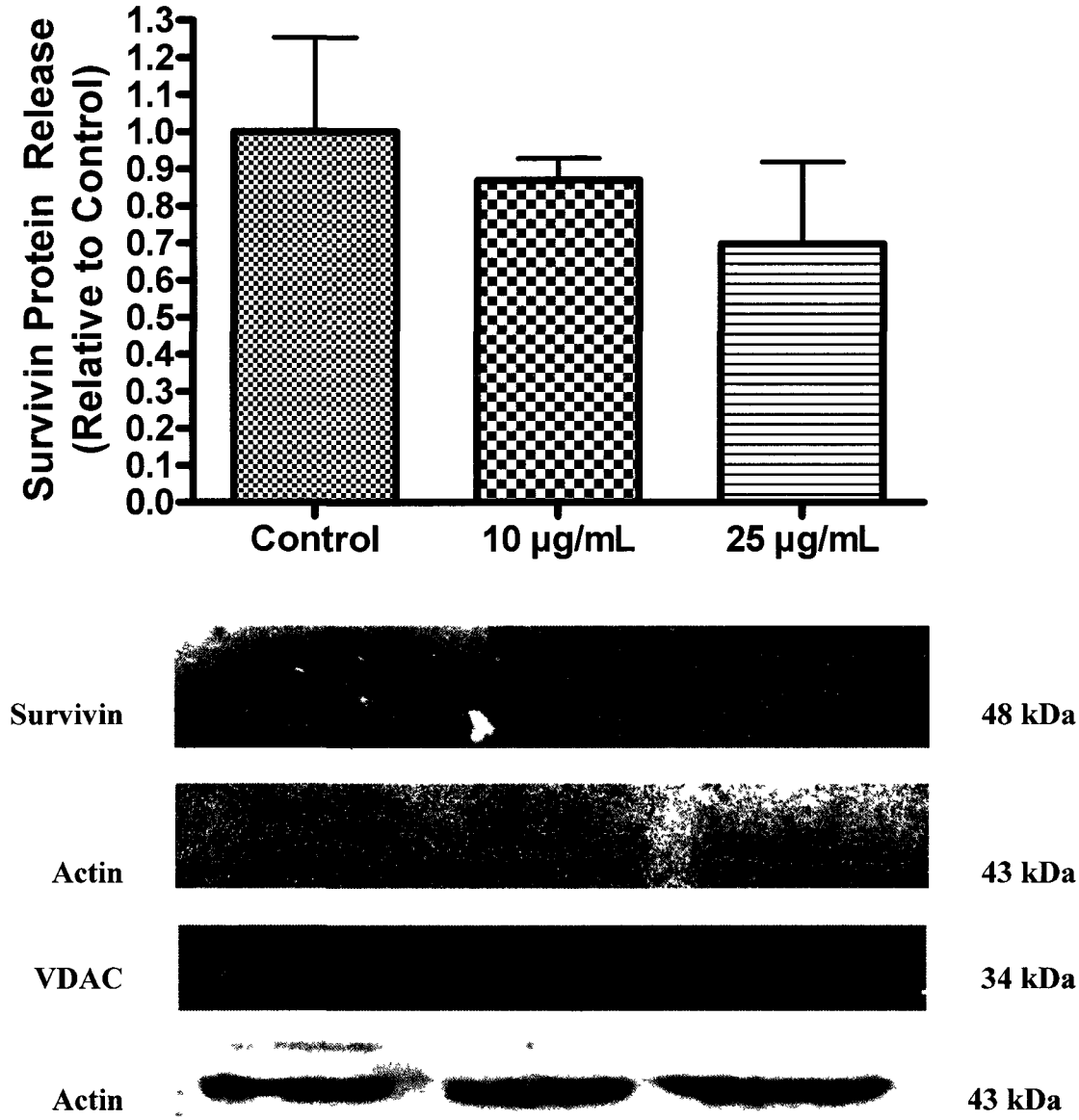

Figure 3.11 Pacs treatment of DU145 human prostate cancer cells inhibits the mitochondrial release of Survivin. Protein expression of survivin in the absence of Pacs (Vehicle present; Negative Control) and protein expression of survivin in the presence 10 and 25 µg/mL Pacs for 6 hours. To ascertain the presence of mitochondria within the cytoplasmic sample VDAC, a mitochondrial segregated protein, was assayed. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment was supported with one assay replicate

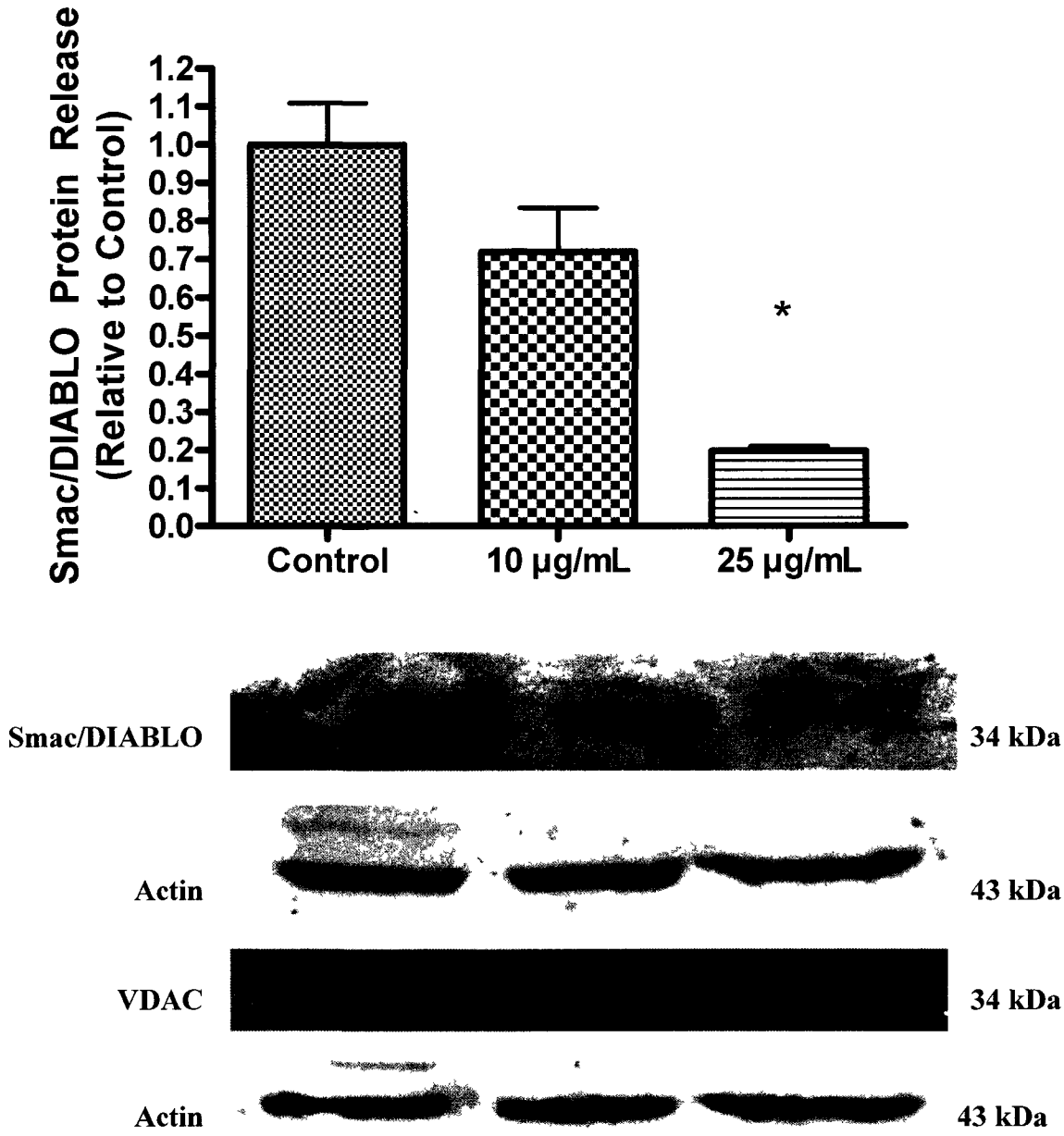

Figure 3.12 Pacs treatment of DU145 human prostate cancer cells inhibits the mitochondrial release of Smac/DIABLO. Protein expression of Smac/DIABLO in the absence of Pacs (Vehicle present; Negative Control) and protein expression of Smac/DIABLO in the presence 10 and 25 µg/mL Pacs for 6 hours. To ascertain the presence of mitochondria within the cytoplasmic sample VDAC, a mitochondrial segregated protein, was assayed. Data was analysized with ANOVA with a tukey post-hoc test. Singleasterisk denotes statisical significance of $P < 0.05$. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments with one assay replicate

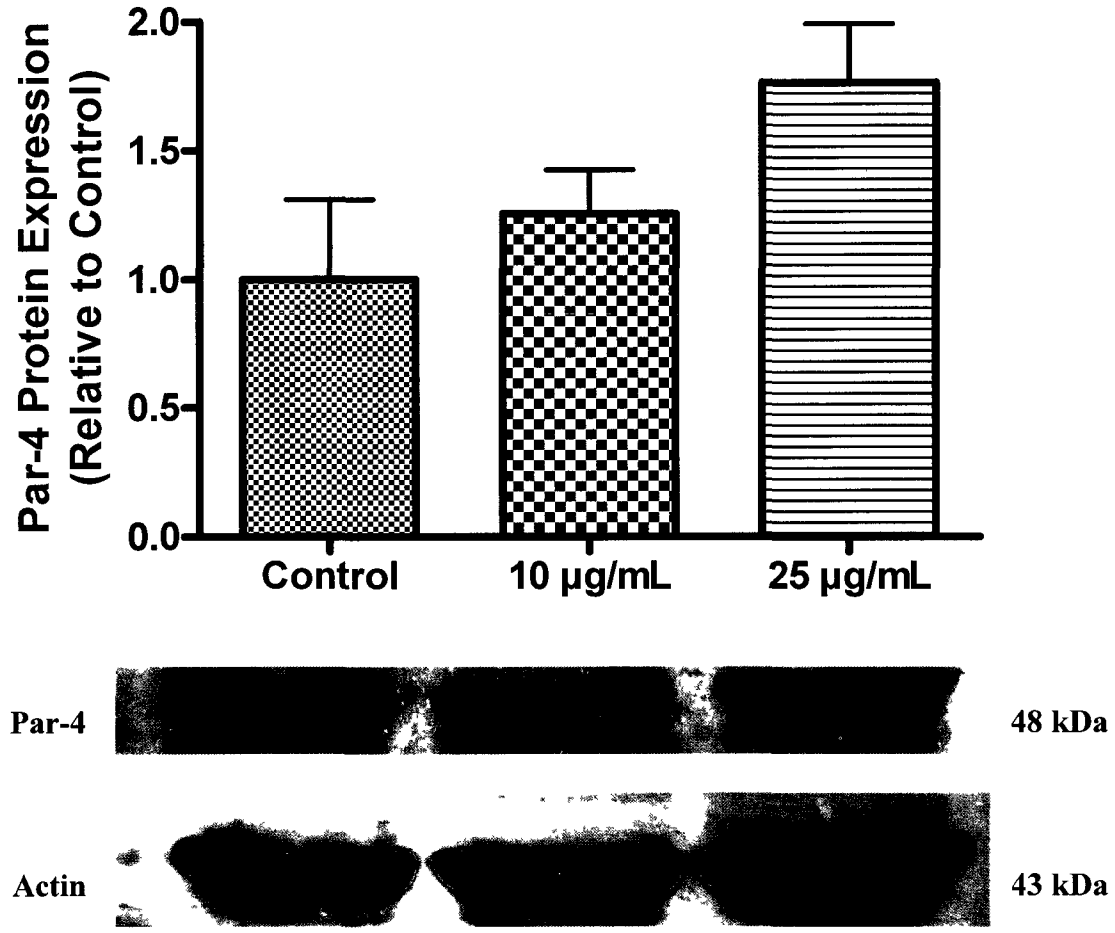

Figure 3.13 Treatment of DU145 human prostate cancer cells with Pacs induces the expression of Par-4. Protein expression of Par-4 in the absence of Pacs (Vehicle present; Negative Control) and in the presence 10 and 25 µg/mL Pacs following 6 hour exposure. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment was supported with one assay replicate.

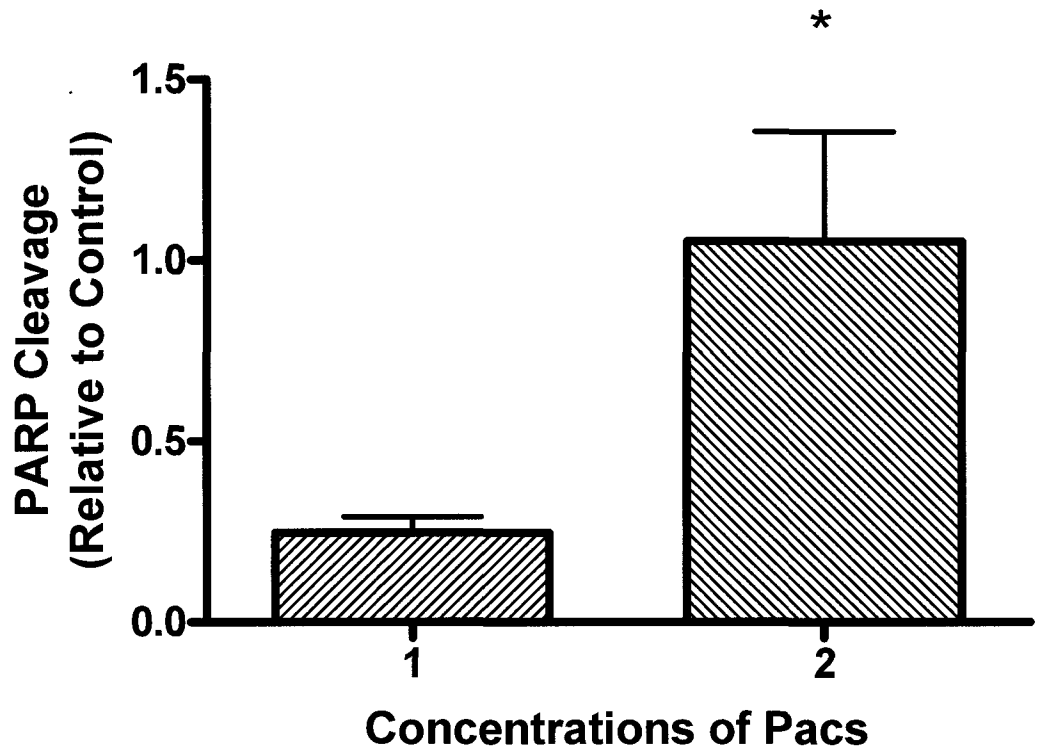

Figure 3.14 Pacs treatment of DU145 prostate cancer cells induces the caspase-3 cleavage of PARP at 25 μg/mL. PARP cleavage in the presence of 10 (1) and 25 (2) μg/mL Pacs following 6 hours of treatment. PARP cleavage of 0.0 is indicative of no cleavage. Data was analysized with ANOVA with a tukey post-hoc test. The single star represents significance with a P value of < 0.05. The data is representative of results obtained from three independent experiments. Each independent experiment was supported with two assay replicates.

3.4 Discussion

The research presented in this chapter examined the mechanisms through which Pacs, a fraction of *V. macrocarpon*, induced apoptosis in DU145 human prostate cancer cells. It was hypothesized that Pacs treatment of DU145 human prostate cancer cells would induce apoptosis through the induction of proteins involved with either the intrinsic or extrinsic pathways or both.

To substantiate the induction of programmed cell death, two key events of apoptosis were studied: formation of nucleosomes and cleavage of PARP. The induction of apoptosis involves the formation of nucleosomes which is considered a defining event (Kuo et al. 2005). Nucleosome formation is the generation of short individual chains of DNA associated with solitary histones, and is the general result of caspase-3 cleavage of cellular DNA (Michelin et al. 2003). The treatment of DU145 cells with 10 and 25 μg/mL of Pacs induced the formation of nucleosomes statistically with $P < 0.05$ and < 0.01, respectively. The second defining event of apoptosis assayed as part of this chapter's work is the cleavage of PARP, a protein involved in DNA repair (Agarwal et al. 2002; Li et al. 2009). PARP cleavage is generally the preceding event prior to nucleosome formation. Activated caspase-3 will bind to and cleave PARP which allows caspase-3 to then cleave DNA without the interference of the PARP repair mechanism (Huerta et al. 2007). Once caspase-3 has eliminated the possibility for DNA repair by cleaving PARP, caspase-3 binds to the genetic material of the cell and cleaves the chromatin into oligonucleosomes (Agarwal, et al. 2002; Huerta et al. 2007; Li et al. 2009). Consequently, the cleavage of PARP is a key event of apoptosis, and is indicative of the activation of caspase-3 and the irreversible induction of apoptosis (Hsu et al. 2009;

Li et al. 2009). Treatment of DU145 human prostate cancer cells with Pacs at a concentration of 25 μg/mL for 6 hours induced the cleavage of PARP ($P < 0.05$), but at 10 μg/mL there was no statistically significant change. Together, the inductions of PARP cleavage and nucleosome formation indicate the activation of caspase-3 (Hsu et al. 2009; Michelin et al. 2003).

The induction of apoptosis in DU145 human prostate cancer cells treated with Pacs is similar to that reported in other cancer cell-lines (Engelbrecht et al. 2007, Hu and Qin, 2006). Pacs obtained from grape seed extract (GSE) induced apoptosis in CaCo2 colon cancer cells and 14.3D10 human acute myeloid leukemia cells in doses consistent with those reported here (Engelbrecht et al. 2007, Hu and Qin, 2006). As far as can be observed, this is the first study detailing the modulation of PARP by Pacs treatment in DU145 human prostate cancer cells. Previous work with whole cranberry extract treatment of this cell line reported an induction of PARP cleavage consisted with the finding presented in this chapter (MacLean. 2009).

The extrinsic pathway is initially induced through the interaction between ligands and their specific death receptors. The ligands and receptors described in this chapter include Fas, TRAIL, and FADD. Pacs treatment of DU145 cells resulted in the inhibition of Fas, TRAIL, and FADD. This inhibition of death receptors and adaptor proteins suggests that Pacs treatment leads to a general inhibition of the extrinsic pathway. However, a gallate derived from *Myrica rubra* with structural similarities to Pacs monomers has been observed to induce Fas protein expression in a number of cell lines including A549 lung cancer cells and MCF-7 breast cancer cells (Kuo et al. 2004; Kuo et al. 2005). The discrepancy of some non-cranberry derived Pacs inducing the extrinsic pathway in the

literature contrasts with the inhibition with Pacs on DU145 human prostate cancer cells observed in this study. The difference may be due to cell line specific characteristics. Another possible reason could be a variance in structure between proanthocyanidins and the proanthocyanidin gallates or potentially the combination of compounds in the extract used in this study. Epigallocatechin-3-gallate (EGCG), a flavanol compound structurally similar to Pacs, has been reported to sensitize LNCaP human prostate cancer cells to TRAIL-mediated apoptosis (Siddiqui et al. 2007). This observation is interesting, as work in this chapter describes down-regulation of TRAIL in general, rather than sensitization of the cell to TRAIL-mediated apoptosis.

Intrinsic pathway mediated apoptosis requires the involvement of the mitochondrion and the Bcl-2 family of proteins (Neuwirt et al. 2008). Two common Bcl-2 proteins involved in apoptotic regulation that were studied were Bcl-2 and Bax (Mackey et al. 1998). Bcl-2 acts antagonistically with Bax; Bax induces apoptosis through pore formation whereas Bcl-2 inhibits the pore formation by Bax (Mackey et al. 1998). These proteins are so important to the intrinsic pathway induction that they are generally considered to be overriding factors (Mackey et al. 1998). In a viable cell, Bcl-2 proteins outnumber Bax proteins, leading to an inhibition of apoptosis. When the number of Bax outnumbers Bcl-2 proteins, apoptosis is induced. Although Pacs treatment inhibited the expression levels of both Bax and Bcl-2, the rate of inhibition of these proteins could be further studied. If there was a difference, it is possible that there would be less Bcl-2 protein present to inhibit Bax pore formation, which would lead to the induction of apoptosis through the release of cytochrome c from the mitochondria. The change from a Bcl-2 to a Bax dominant ratio has been observed in several prostate cell lines (including

DU145 cells) treated with Pacs derived from GSE (Neuwirt et al. 2008). The inhibition of both Bcl-2 and Bax protein expression in H9C2 cardiomyocytes cells treated with GSE Pacs were also observed (Du and Lou, 2008).

The formation of pores via Bax may not release all the cytochrome c necessary for the propagation of an apoptotic signal. Bid, another Bcl-2 family member was observed to induce the release of cytochrome c from the mitochondrion (Yan et al. 2003). Bid is a BH3 protein, and as such, interacts with the BH1-4 and BH1-3 subgroups of the Bcl-2 family to activate or inhibit their function; but Bid has also been found to directly interact with the mitochondria and release of cytochrome c (Yan et al. 2003). This induction of cytochrome c release requires Bid to be cleaved into tBid, a 15 kDa truncated form (Yan et al. 2003). Treatment of HLE, HepG2, HuH-7 and PLC/PRF/5 lung cancer cell-lines with EGCG was observed to induce the protein expression of tBid (Nishikawa et al. 2006). tBid can interact with the outer mitochondrial membrane and homotrimerize to form pores through interactions with cardiolipin (Yan et al. 2003). Cardiolipin is a protein found within the inner mitochondria membrane that commonly binds to mitochondrially segregated cytochrome c to stabilize the protein for interaction with the electron transport chain (ETC) (Iverson and Orrenius, 2003). When tBid interacts with cardiolipin, the protein breaks its bonds with cytochrome c (Iverson and Orrenius 2003). This event leads to the near total release of cytochrome c from mitochondria following tBid formation. DU145 human prostate cancer cell treatment with Pacs at 10 and 25 μg/mL resulted in the down-regulation of cleavage of Bid; 25 ug/mL Pacs showed an induction of tBid expression. Therefore, it is probable that Pacs treatment leads to

cleavage of Bid into tBid potentially culminating in the release of cytochrome c from the mitochondrion.

Cytochrome c is a small protein involved in shunting electrons between components of the ETC. Eventually, it was discovered that cytochrome c played multiple roles within the cell (Liu et al., 1996). This small protein, once released from the mitochondria, is an integral component of the induction of apoptosis. To propagate the apoptotic signal mitochondrial released cytochrome c interacts with Apaf-1, along with ATP, to form the apoptosome (Wright et al. 2007). This heteromer binds with the zymogen of caspase-9 and enacts the proper conformation and proximity between two pro-caspase-9 proteins, which induces the dual cleavage and activation of these proteins (Wright et al. 2007). The activation of caspase-9 leads directly to the caspase-9 mediated activation of caspase-3, the executioner caspase. Pacs treatment of DU145 human prostate cancer cells at 10 and 25 μg/mL induces cytochrome c protein expression. This induction of protein expression appears to be novel. Current research with GSE has found that GSE Pacs can induce changes in Pacs release from the mitochondria, but did not study the effect of GSE Pacs on cytochrome c protein expression (Agarwal et al. 2002).

Cytochrome c is not the sole apoptotic influencing protein that is segregated in the mitochondria. Smac/Diablo and survivin are two apoptotic modulating proteins that are also released by changes in the permeability of the outer mitochondrial membrane. Survivin, a member of the inhibitor of apoptosis (IAP) family of proteins is inhibited in DU145 cells treated with 10 and 25 μg/mL Pacs for 6 hours. Smac/Diablo has been shown to inhibit anti-apoptotic proteins, including survivin and other members of the IAP family (Martinez-Ruiz et al., 2008). Pac treatment (10 and 25 μg/mL) of DU145 cells for

6 hours dose-dependently inhibits the release of Smac/Diablo from the mitochondria. The inhibition of survivin allows a cell to induce apoptosis, as the inhibition of an inhibitor equates to induction (Martinez-Ruiz et al., 2008). The inhibition of Smac/Diablo may be a compensatory measure of the cell or mitochondria in an effort to regain homeostasis in the face of a general induction of apoptosis and dysregulation of the cell and mitochondria.

The proteins discussed previously are proteins found in all cell types, but there are also important organ-specific cellular proteins that affect apoptosis. For example, Par-4 is a pro-apoptotic protein initially discovered in prostate cells (Mundle, 2006). Par-4 has been suggested to induce apoptosis in the absence of any other apoptotic modulators (Mundle, 2006). Pacs treatment (10 and 25 μg/mL) of DU145 human prostate cells induces the protein expression of Par-4. This observation is the first example of Par-4 interaction with Pacs treatment. Par-4 is an important protein to study in relation to prostate cancer as it can induce apoptosis independently from the activation of the extrinsic or intrinsic pathways.

Importantly, activation of Par-4 leads to the cleavage of pro-caspase-8. Caspase-8 cleaves Bid into tBid, and therefore the induction of Par-4 may be the mechanism through which the induction of Bid cleavage occurs. The observation of Par-4 induction when cells are treated with Pacs supports previous reports where whole cranberry treatment of DU145 human prostate cancer cells induced Par-4 protein expression (MacLean 2009).

The induction of apoptosis by Pacs treatment has several mechanisms of activation through which the fractions may mediate their effects. Pacs treatment led to the inhibition of receptors of the extrinsic pathway, thereby ensuring that this fraction induces apoptosis exclusively through the intrinsic pathway. The intrinsic pathway is induced by deregulating the Bax:Bcl-2 ratio, and by inducing cytochrome c expression which would lead to propagation of the apoptotic stimulus leading to caspase-3 activation (Lee et al. 2008). Induction of Bid cleavage was also observed and could be an independent event or may be linked to the induction of Par-4 protein expression in DU145 cancer cells treated with Pacs. Par-4 is independently activated outside the extrinsic and intrinsic apoptotic pathways, but when active, interacts and activates caspase-8 leading to tBid formation. tBid induction would lead to the liberation of some—if not all—of the cytochrome c found in the mitochondria, leading to apoptosome formation and eventual caspase-3 activation.

The work outlined in this research suggests that Pacs may, at least be partially responsible for the induction of apoptosis observed with treatment of whole cranberry extract. Perhaps more importantly, Pacs may comprise a novel set of pharmacological compounds that could be studied in respect to prostate cancer, or cancer treatment in general. Whereas the consumption of cranberries may lead to overall health benefits via apoptotic induction in cancer cells, these effects may be limited in comparison to the possible benefits of development of a novel drug for direct treatment of cancer.

Chapter Four: Flavonols from *Vaccinium macrocarpon* Induce Programmed Cell Death in DU145 Human Prostate Cancer Cells

4.1 Introduction

Cancer is a complex disease characterized by the accumulation of cellular mutations. These mutated cells rapidly divide, providing them with a selective advantage over normal cells. This selective advantage relies on these cells gaining several properties that enable the population of cancer cells to out-compete non-cancerous cells, and include the ability to disregard inhibitors, evade apoptosis and produce their own inducers for cellular growth (Hanahan and Weinberg, 2000).

One of these properties, the ability of a cell to evade apoptosis can be correlated to an increase in malignancy (Hanahan and Weinberg, 2000). Consequently, cell death pathways are commonly mutated in cancer cells. Despite these apparent changes, some compounds are able to induce death in cancer cells and are often targeted for study. The secondary metabolites from plants, in particular, are known to contain many apoptosis-inducing molecules (Haddad 2008; Neto et al. 2008).

The induction of apoptosis through two key pathways, the intrinsic and extrinsic was studied in this portion of work. These two pathways differ in the mechanisms of their induction, but eventually they both activate a protein, caspases-3, which once activated leads to irreversible cellular death. To establish which molecule(s) may be responsible for this observation, whole cranberry extract can be further fractionated to derive specific enriched fractions of various components. One of these fractions, flavonols (Flavs), forms the basis of this study. Flavs have been found to have a diverse range of properties

including the ability to induce apoptosis and quench reactive oxygen species (Lee et al. 2009; Kathirvel et al. 2009; Kathirvel and Richards 2009; Zhang et al. 2008). More recently, the Flav fraction has also been shown to be one of the principle fractions of the whole cranberry extract to induce apoptosis in DU145 human prostate cancer cells (MacLean 2009).

This work outlined here details the effect of Flav treatment on the mechanism(s) that induce apoptosis in DU145 human prostate cells. It is hypothesized that Flavs will induce cellular death in DU145 human prostate cancer cells through one of the two major apoptotic pathways inherent to cells or both. Both the intrinsic and the extrinsic pathway will be explored.

4.2 Materials and Methods

4.2.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (Oakville, ON). Unless otherwise noted, antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA).

4.2.2 Preparation of Flavonol Extracts

The Flav fraction from American cranberry (*Vaccinium macrocarpon*) was prepared and characterized in the laboratory of Dr. Catherine Neto, Department of

Chemistry and Biochemistry at University of Massachusetts-Dartmouth as described in the Appendix.

4.2.3 Cell Culture

Please refer to Chapter 3 (section 3.2.3) for an overview of the cell culture techniques used with DU145 human prostate cancer cells.

4.2.4 Solubilization of Flavonol Extracts for Cell Treatments

The Flavs fraction was dissolved immediately before treatment in a 1:9 ratio of DMSO dissolved in DM (v/v) supplemented as described previously. DMSO did not exceed a maximum of 1% concentration on the treatment plate.

4.2.5 Bradford Protein Assay

Please refer to Chapter 3, section 3.2.5 (Bradford Protein Assay) for a detailed overview of the Bradford Protein Assay.

4.2.6 Sonication

Please refer to Chapter 3, section 3.2.6 (Sonication) for a detailed overview of the sonication protocol.

4.2.7 Alamar Blue Assay

Please refer to Chapter 3, section 3.2.7 (Alamar Blue Assay) for a detailed overview of the Alamar Blue Assay.

4.2.8 Mitochondrial Isolation

Please refer to Chapter 3, section 3.2.8 (Mitochondrial Isolation) for a detailed overview of the Mitochondrial Isolation procedure.

4.2.9 Nucleosome Enrichment

Please refer to Chapter 3, section 3.2.9 (Nucleosome Enrichment) for a detailed overview of the Nucleosome Enrichment Assay.

4.2.10 Measurement of PARP Activity

Please refer to Chapter 3, section 3.2.10 (Measurement of PARP Activity) for a detailed overview of the PARP Assay.

4.2.11 Immunoblot Analysis

Please refer to Chapter 3, section 3.2.11 (Immunoblot Analysis) for a detailed overview of the Western blotting analysis.

4.2.12 Image Capture and Gel Quantification

Please refer to Chapter 3, section 3.2.12 (Image Capture and Gel Quantification) for a detailed overview of the gel documentation system and programs.

4.2.13 Statistics

Please refer to Chapter 3, section 3.2.13 (Statistics) for an overview of the statistics used to analyze the data. Cytotoxicity of DU145 human prostate cancer cells treated with

Flavs was analyzed with the Kruskal-Wallis test with post-hoc Dunn's Multiple Comparison test as the data did not fit a normal distribution.

4.3 Results

4.3.1 Evaluating DU145 Cell Viability in Response to Flavonol Treatment

Flav treatment of DU145 cells revealed a decrease in cell viability of 5, 8, 9, and 15% relative to the control for the concentrations of 1, 10, 25 and 50 μg/mL Flavs, respectively (Figure 4.1). The observed inhibition of cell viability of DU145 human prostate cancer cells was only significant at the highest dose assayed, 50 μg/mL Flavs, $P < 0.01$.

4.3.2 Evaluating Markers of Late-Stage Apoptosis in Flavonol Treated DU145 Human Prostate Cancer Cells

The common factor between both the intrinsic and extrinsic apoptotic pathways is the protein caspase 3. Once caspase-3 is activated by the apoptotic pathways of a cell, the protein migrates to the nucleus where it cleaves the genetic material of the cell. This cleavage results in the formation of nucleosomes. The formation of nucleosomes is a defining event of apoptosis. Therefore, this event can be used as an indicator of the induction of apoptosis (Yuste et al. 2005). Treatment of DU145 human prostate cancer cells with 25 and 50 μg/ml Flavs resulted in an induction in nucleosome formation which was significant at the highest concentration assayed (50 μg/ml; $P < 0.01$) (Figure 4.2).

4.3.3 Western Blot Analysis of Proteins Involved in the Extrinsic Apoptotic Pathway

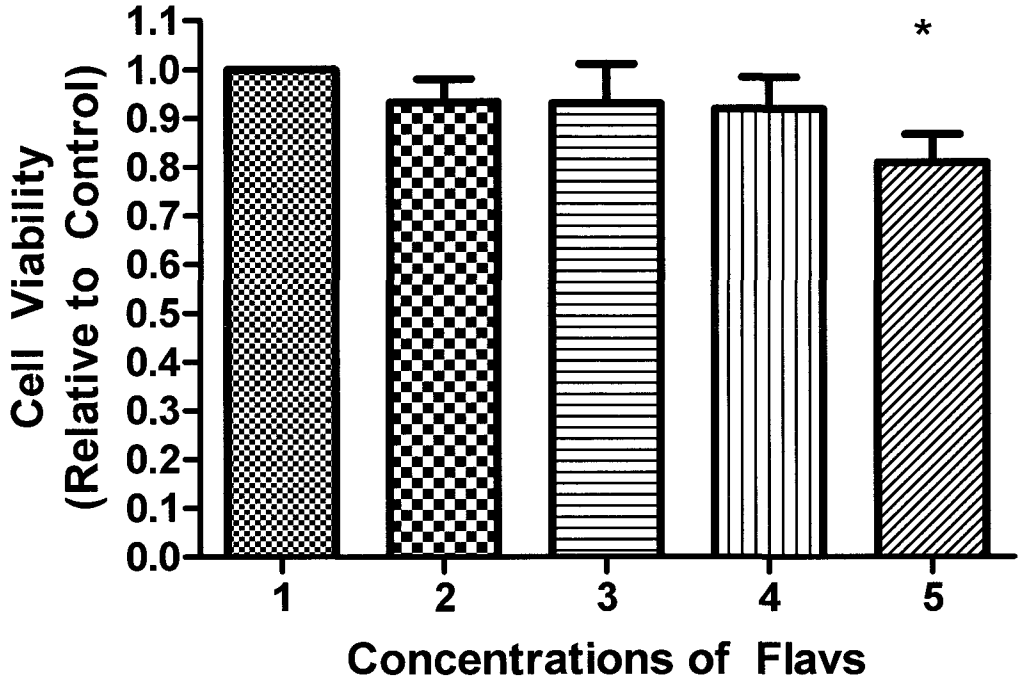

Figure 4.1 Flav treatment of DU145 human prostate cancer cells inhibited cell viability. Viability of cells in the absence of Flavs (1), in the presence of 1 (2), 10 (3), 25 (4), and 50 (5) μg/mL Flavs for 6 hours. Data was analyzed with the Kruskal-Wallis test with post-hoc Dunn's Multiple Comparison test as the data did not fit a normal distribution. Single asterisk is representative of $P < 0.01$. The graph is representative of results obtained from five independent experiments. Each independent experiment consisted of three assay replicates.

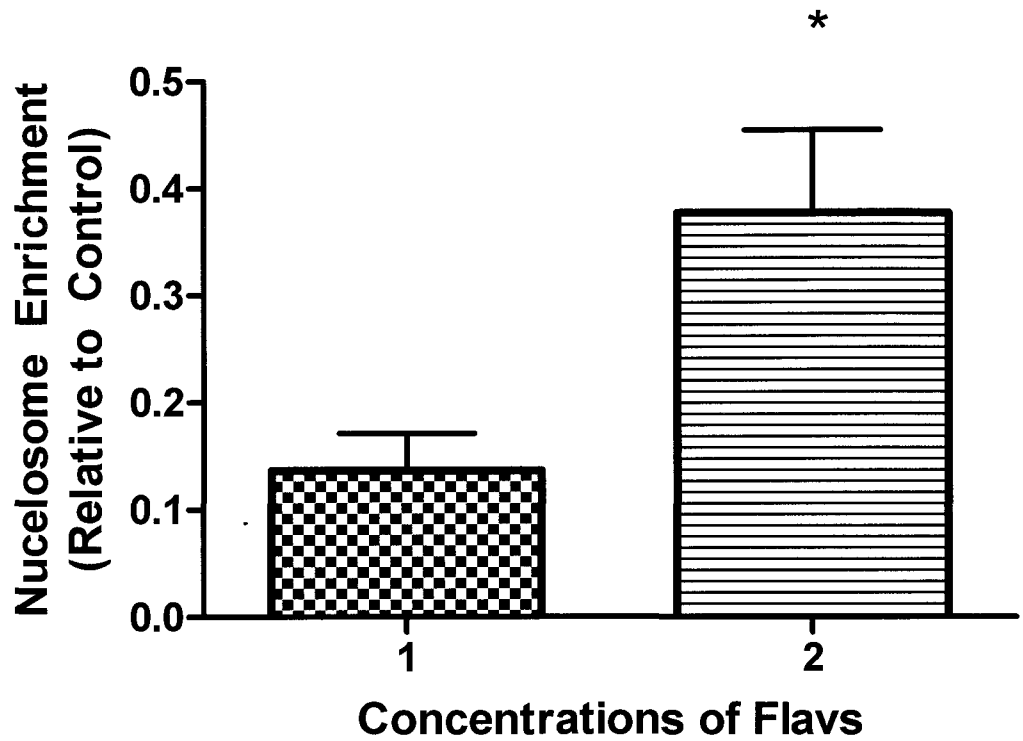

Figure 4.2 Flav treatment of DU145 human prostate cancer cells induce the formation of oligo-nucleosomes. Nucleosome enrichment of cells treated: in the presence of 25 (1), and 50 (2) μg/mL Flavs for 6 hours. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk indicates P value of < 0.01. Nucleosome Enrichment of 0.0 is indicative of no cleavage (Negative control). The graph is representative of results obtained from four independent experiments. Each independent experiment consisted of three assay replicates.

The extrinsic apoptotic pathway involves a series of death receptors and the coupling ligands that induce their activation. TNF-related apoptosis inducing ligand (TRAIL) and Fas were selected due to their presence on the plasma membranes of both transformed and non-transformed cells. These proteins induce the signal in the receptors to proteins involved in the extrinsic pathway.

Flav treatment at 25 and 50 μg/mL for 6 hours inhibited FADD protein expression (Figure 4.5), but Western blotting analysis of the protein Fas showed that Flav treatment (25 and 50 μg/mL) had no apparent affect on its expression (Figure 4.3). Similar to FADD, the expression of TRAIL was inhibited when DU145 cells were treated with Flavs (25 and 50 μg/mL) for 6 hours (Figure 4.4).

4.3.4 Western Blot Analysis of Proteins Involved in the Intrinsic Apoptotic Pathway

A cell has multiple pathways through which it can mediate cellular apoptosis. The intrinsic pathway relies on activation by internal stresses, and involves a series of proteins that interact with the mitochondria to regulate and propagate apoptosis. The protein expressions of three key members of the Bcl-2 family were studied: Bax, Bcl-2 and Bid.

Western blot analysis on DU145 cells treated with Flavs (25 and 50 μg/ml) for 6 hours showed an induction of Bax expression (Figure 4.6). In contrast, Bcl-2 expression was inhibited in these cells (Figure 4.7). Bcl-2 inhibition at 50 μg/ml of Flav treatment was statiscally significant at $P < 0.01$. Treatment of DU145 cells with the Flav fraction at

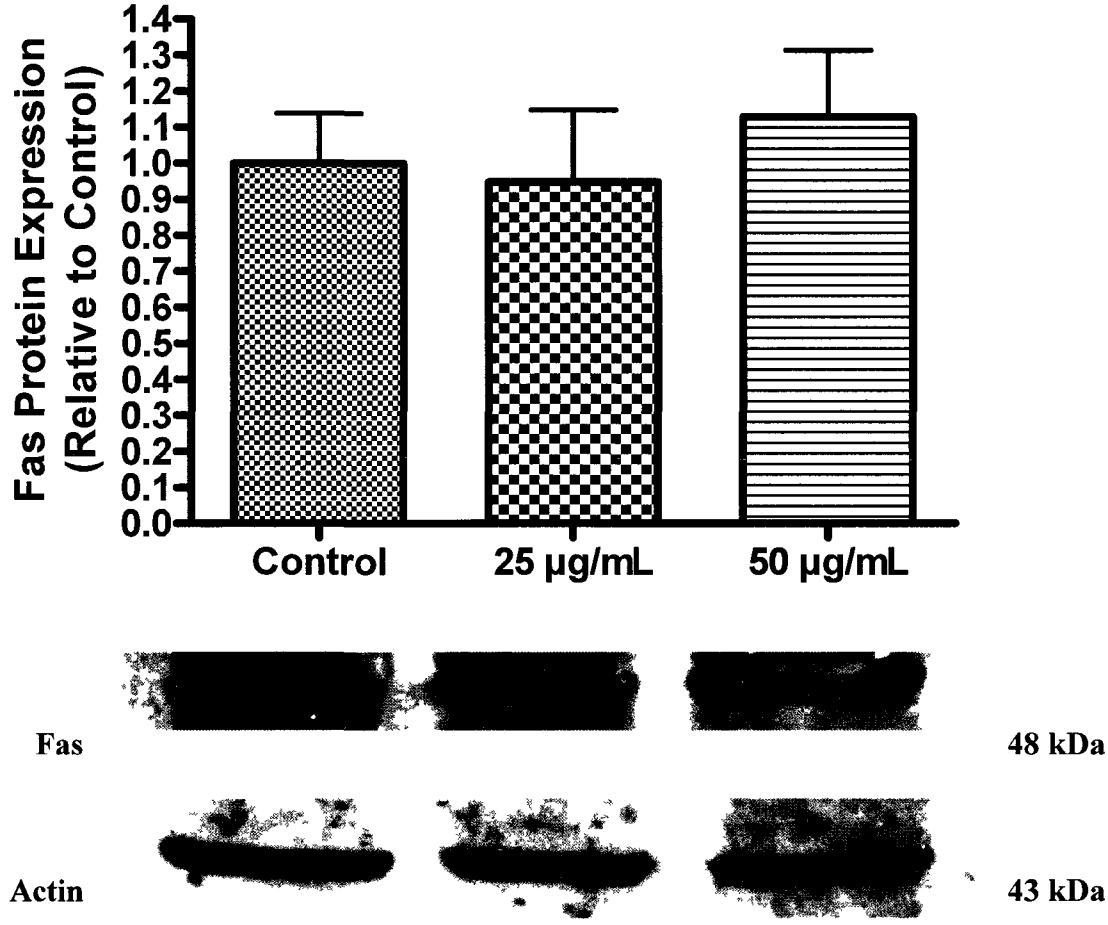

Figure 4.3 Flav treatment of DU145 human prostate cancer cells had no apparent effect on Fas protein expression. Western blotting analysis was performed using cellular extracts from cells following 6 hours of Flav treatment. Protein expression of Fas in the absence of Flavs (Vehicle present; Negative Control) and protein expression in the presence 25 and 50 µg/mL Flavs following 6 hour treatment. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

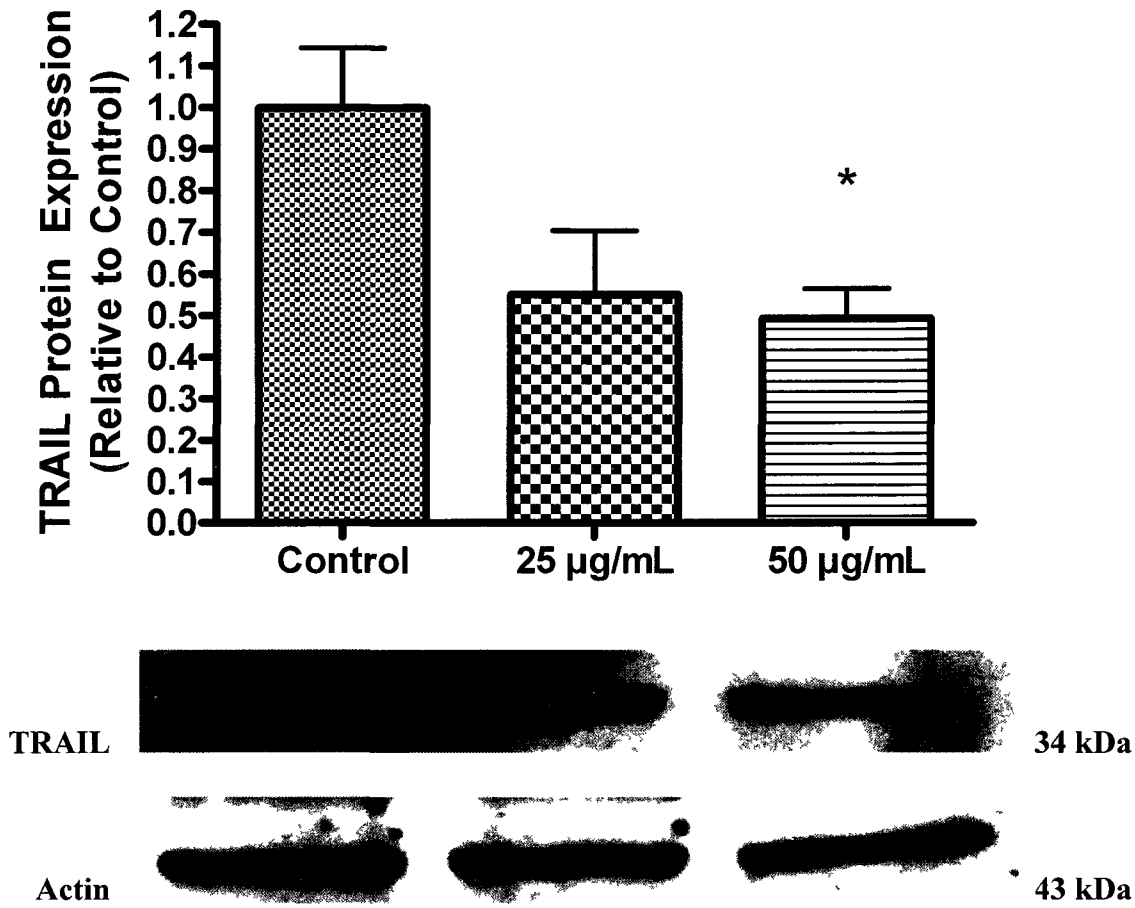

Figure 4.4 Flav treatment of DU145 human prostate cancer cells inhibited the protein expression of TRAIL. Protein expression of TRAIL in the absence of Flavs (Vehicle present; Negative Control) and protein expression in the presence 25 and 50 µg/mL Flavs following 6 hour treatment, respectively. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk denotes statisical significance of $P < 0.05$. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

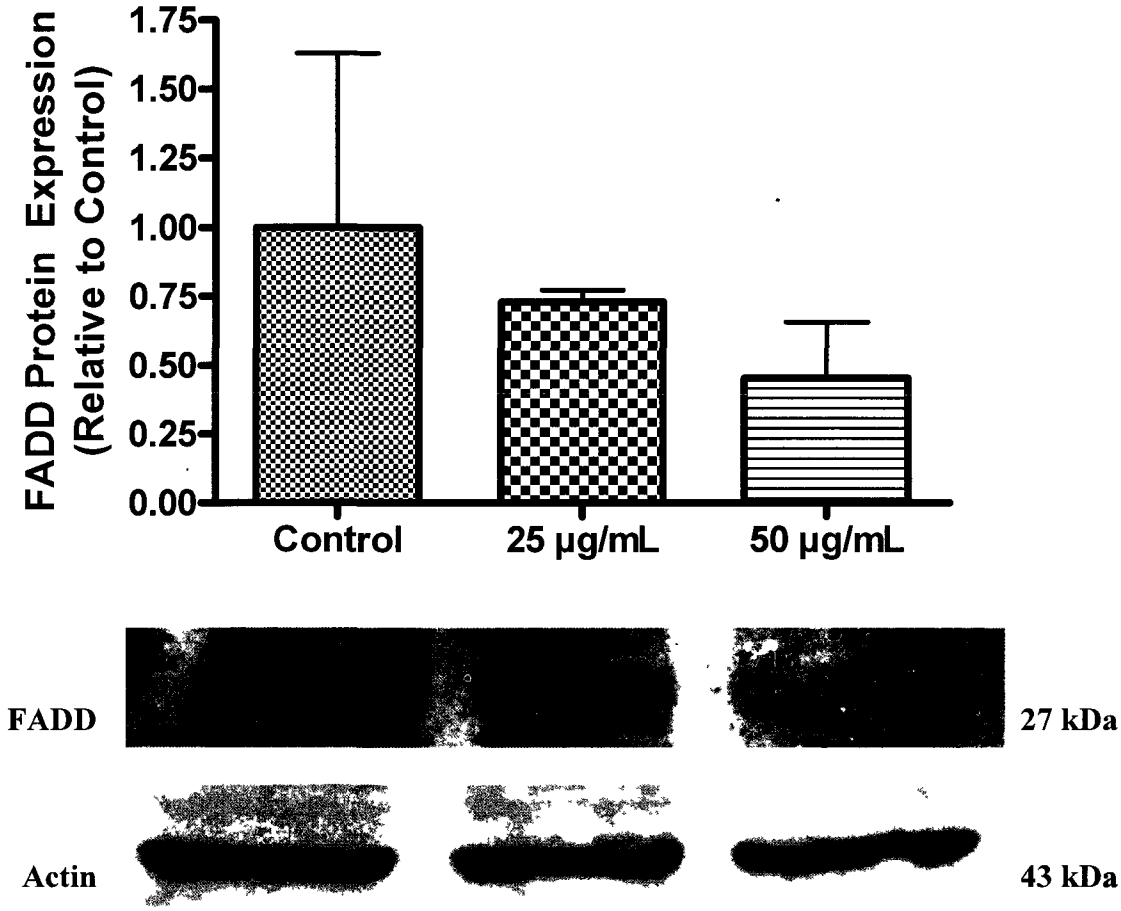

Figure 4.5 Flav treatment of DU145 human prostate cancer cells inhibited the protein expression of FADD. Protein expression of FADD in the absence of Flavs (Vehicle present; Negative Control) and protein expression in the presence 25 and 50 µg/mL Flavs following 6 hour treatment, respectively. Single asterisk denotes statisical significance of $P < 0.05$. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

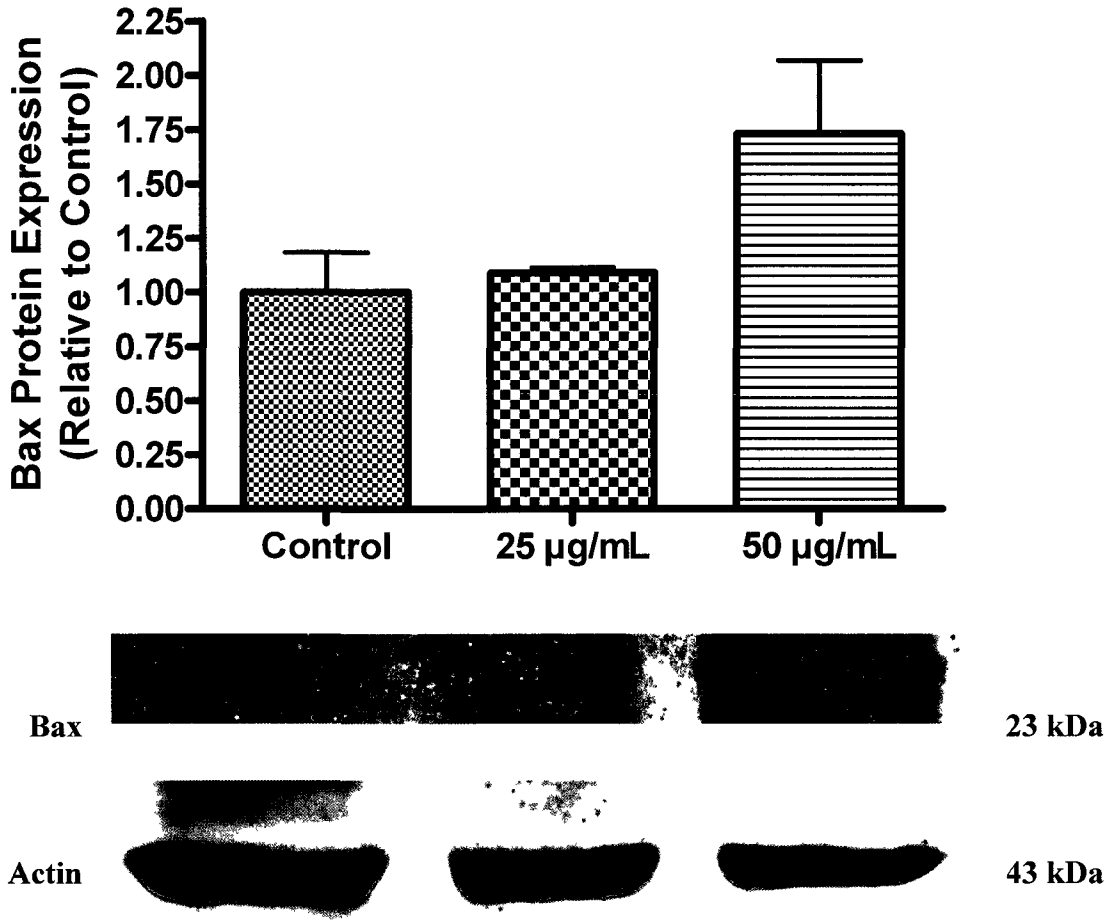

Figure 4.6 Flav treatment of DU145 human prostate cancer cells induces the protein expression of Bax. Protein expression of Bax in the absence of Flavs (Vehicle present; Negative Control) and in the presence 25 and 50 µg/mL Flavs following 6 hour treatment. Actin protein levels are indicated as a loading control. The data is representative of results obtained from four independent experiments. Each independent experiment consisted of two assay replicates.

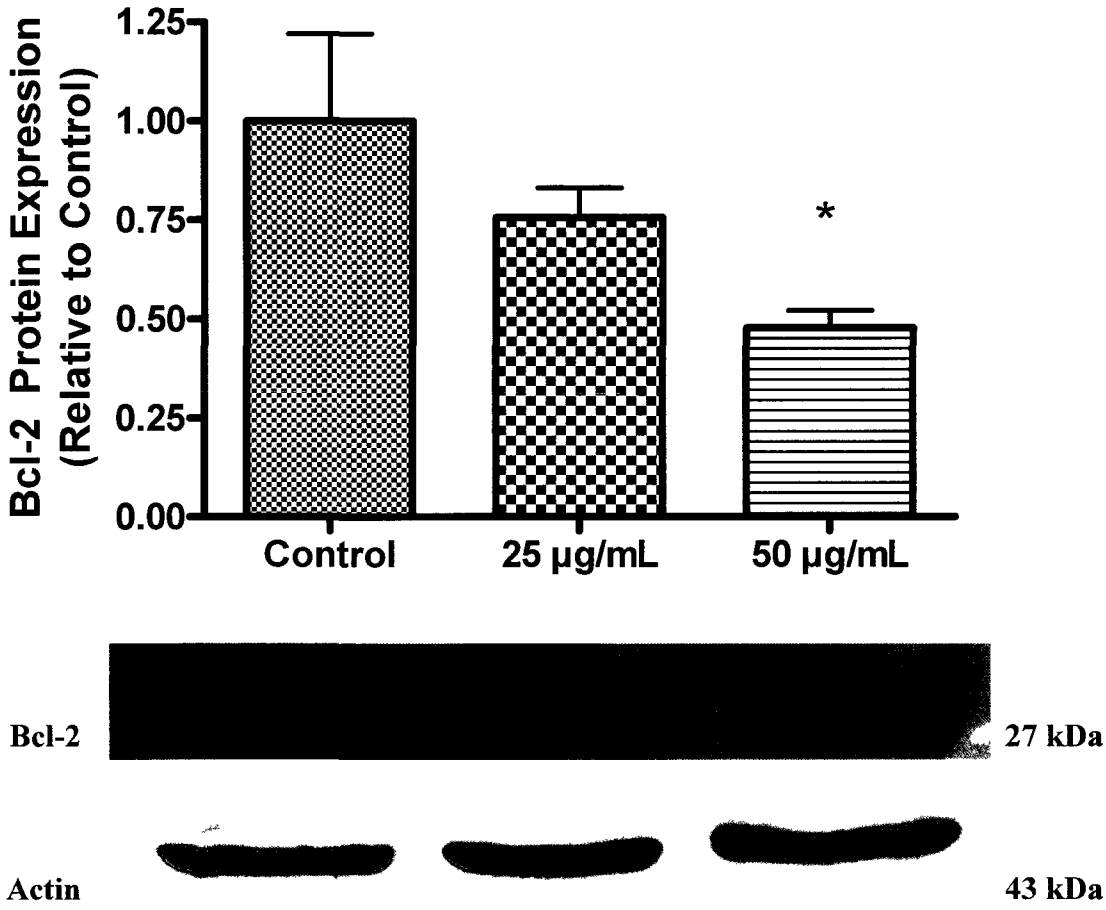

Figure 4.7 Flav treatment of DU145 human prostate cancer cells inhibits the protein expression of Bcl-2. Protein expression of Bcl-2 in the absence of Flavs (Vehicle present; Negative Control) and in the presence 25 and 50 µg/mL Flavs following 6 hour treatment. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk denotes a statisical signficance of $P < 0.05$. Actin protein levels are indicated as a loading control. The data is representative of results obtained from four independent experiments. Each independent experiment consisted of two assay replicates.

25 and 50 μg/mL for 6 hours induced the statistically significant cleavage of Bid, $P < 0.01$ and $P < 0.001$ respectively (Figure 4.8). Treatment of DU145 human prostate cancer cells with the Flav fraction (25 and 50 μg/ml) resulted in an inhibition of Bid and an induction of tBid (Figure 4.9).

4.3.5 Western Blotting Analysis of Mitochondrial Derived Modulators of the Intrinsic Pathway.

Whole cell lysates of DU145 cells treated with Flavs show a dose-dependent induction in cytochrome c expression (Figure 4.10). The formation of pores within the outer membrane of the mitochondria does not liberate cytochrome c solely, but can liberate other mitochondrial segregated proteins (Martinez-Ruiz et al., 2008). Smac/Diablo protein expression was found to be induced by Flav treatment of DU145 human prostate cancer cells at 25 and 50 μg/mL for 6 hours (Figure 4.11). Smac/Diablo was statistically significantly induced at 50 μg/mL ($P < 0.01$). Since Smac/Diablo is a mitochondrial protein, it was critical to remove all of the mitochondria from the cell samples. To ensure that the removal of the mitochondria had occurred, the expression of a protein excessively found in the mitochondrion was assayed. The voltage dependent anion channel (VDAC) was chosen as an indicator due to its exclusivity to the mitochondria. No VDAC protein expression was observed in the samples, indicating that the cytoplasmic fraction was devoid of mitochondria (Figure 4.11). This result ensured that the protein expression observed is a measure of the release of Smac/Diablo protein from the mitochondria.

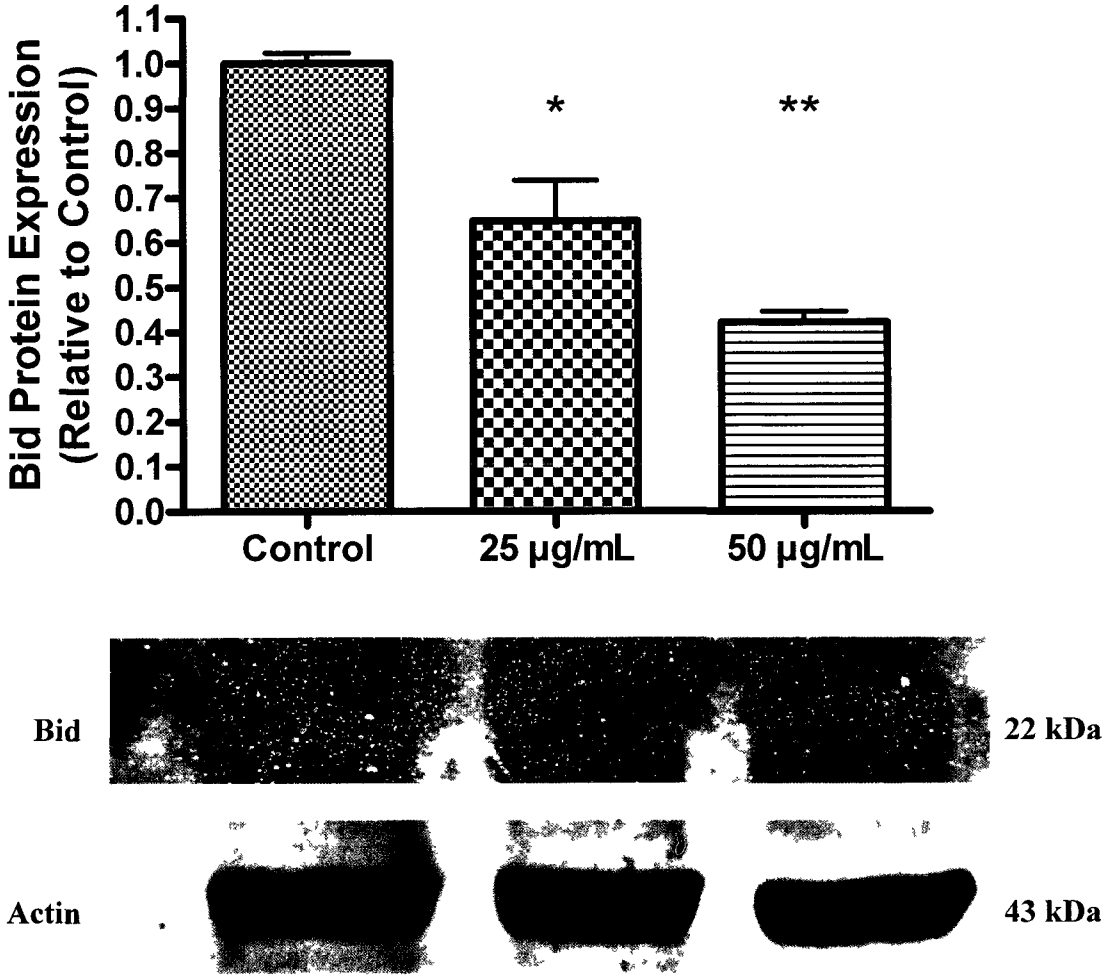

Figure 4.8 Flav treatment of DU145 human prostate cancer cells induces the cleavage of Bid. Protein expression of Bid and tBid in the absence of Flavs (Vehicle present; Negative Control) and in the presence 25 and 50 µg/mL Flavs for 6 hours, repectively. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk denotes statistical signficance of $P < 0.01$ and two asterisks denotes signficiance of $P < 0.001$. Actin protein levels are indicated as a loading control. The data is representative of results obtained from three independent experiments. Each independent experiment consisted of two assay replicates.

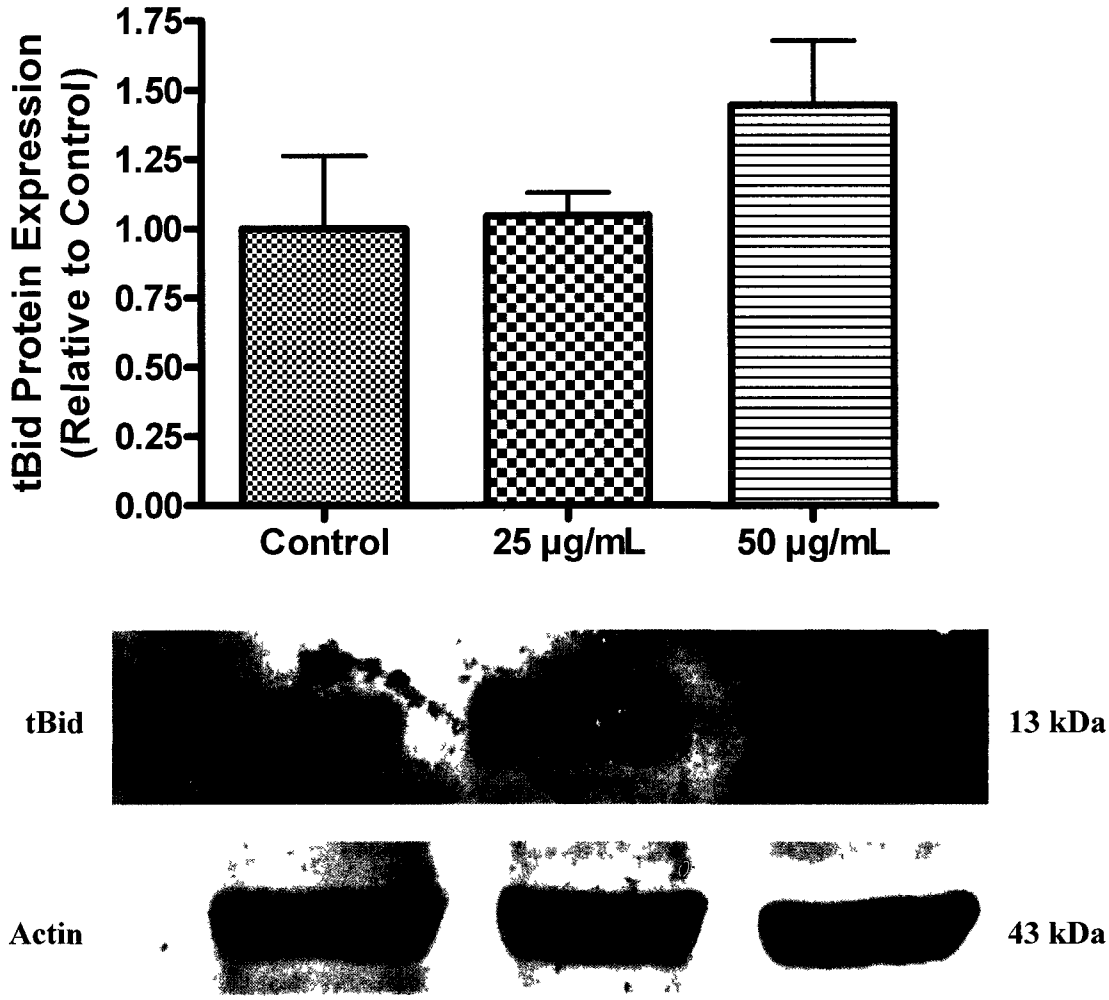

Figure 4.9 Flav treatment of DU145 human prostate cancer cells induces the activation of tBid. Protein expression of Bid and tBid in the absence of Flavs (Vehicle present; Negative Control) and in the presence 25 and 50 µg/mL Flavs for 6 hours, repectively. Actin protein levels are indicated as a loading control. The data is representative of results obtained from three independent experiments. Each independent experiment consisted of two assay replicates.

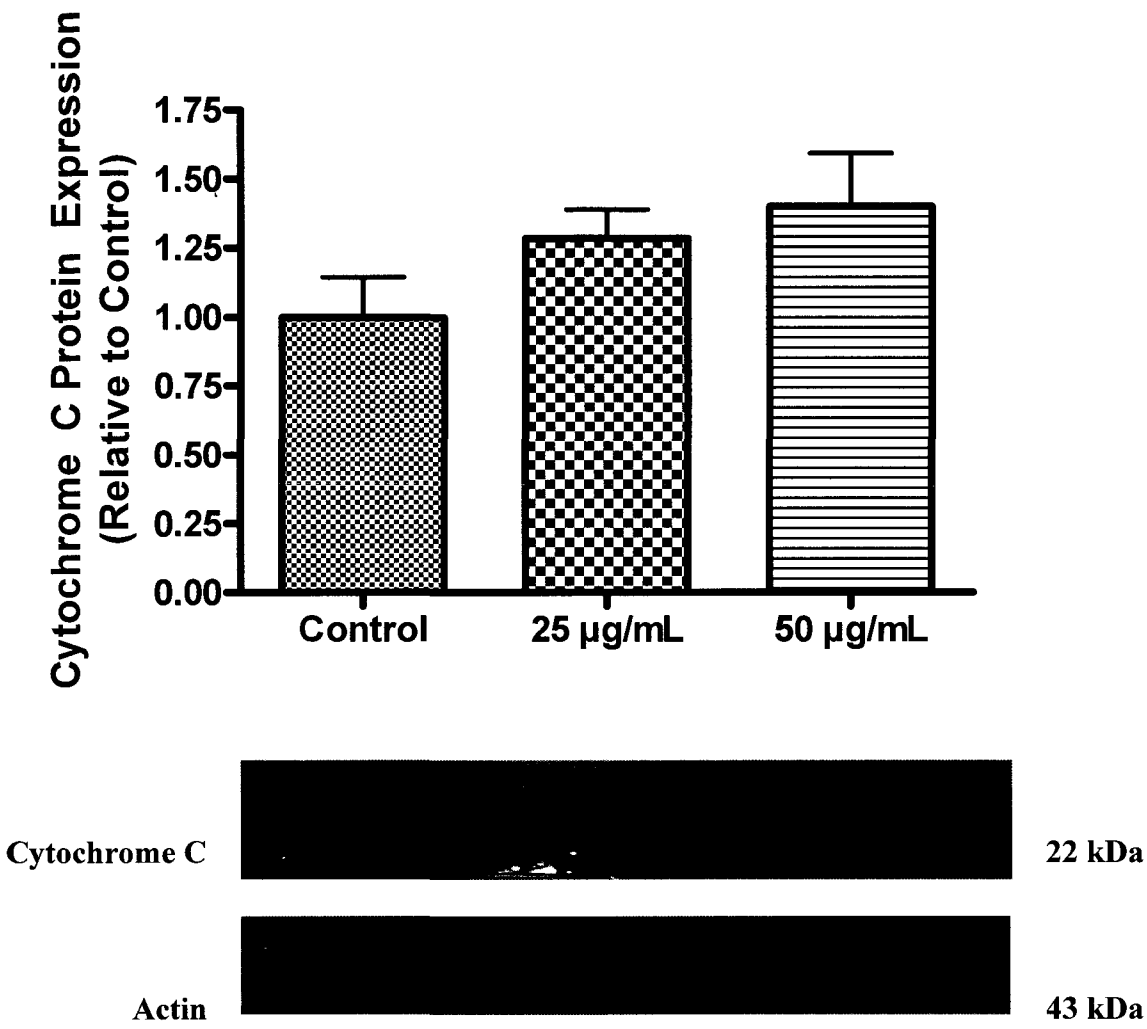

Figure 4.10 Flav treatment of DU145 human prostate cancer cells induces the expression of cytochrome c. Protein expression the cytochrome c in the absence of Flavs (Control) and protein expression of in the presence 25 and 50 µg/mL Flavs following 6 hour treatment. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

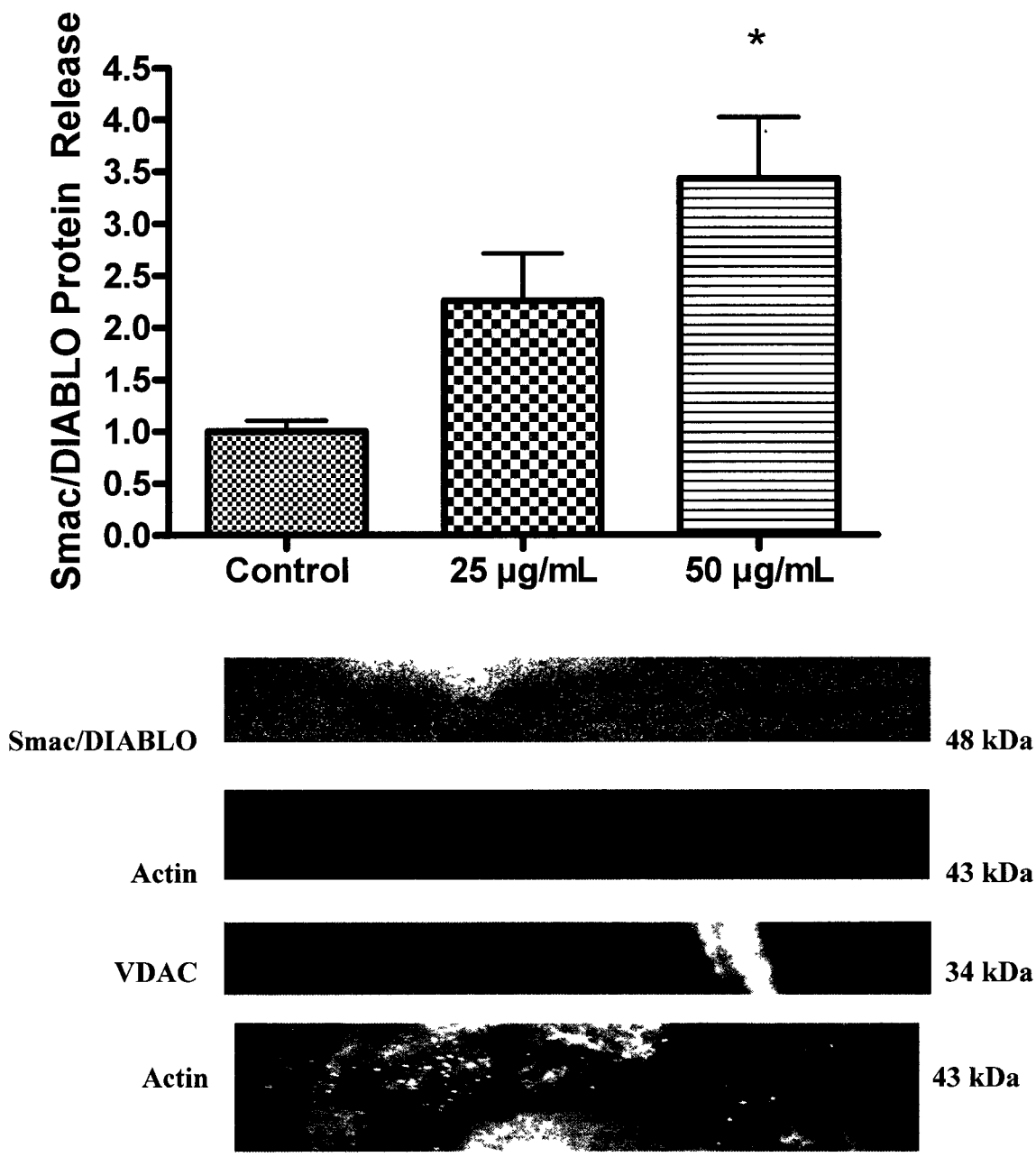

Figure 4.11 Flav treatment of DU145 human prostate cancer cells induces the expression of Smac/DIABLO. Protein expression the Smac/DIABLO in the absence of Flavs (Control) and protein expression of in the presence 25 and 50 µg/mL Flavs following 6 hour treatment. Data was analysized with ANOVA with a tukey post-hoc test. Single Asterisk denotes a statisical signficance of $P < 0.01$. To ascertain the presence of mitochondria within the cytoplasmic sample VDAC, a mitochondrial segregated protein, was assayed. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

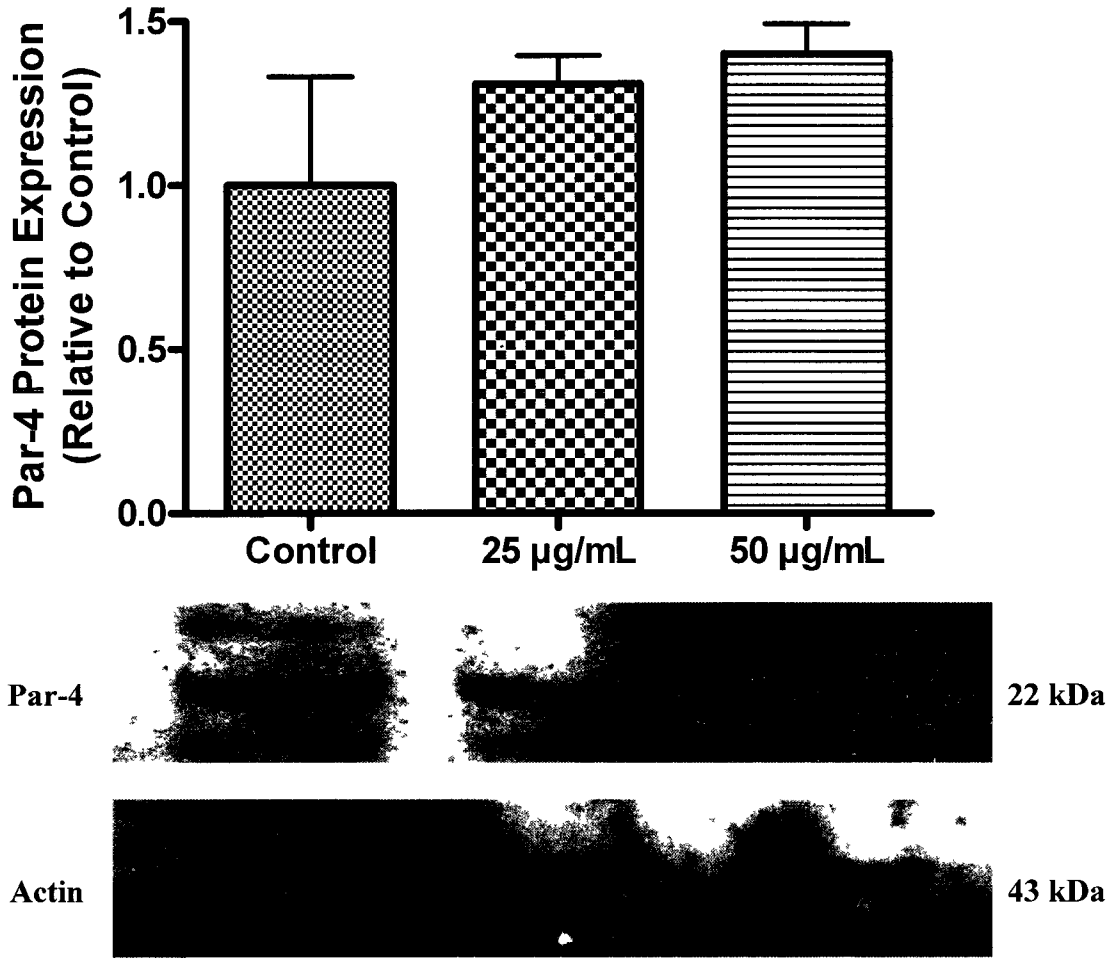

Figure 4.12 Flav treatment of DU145 human prostate cancer cells induces the expression of Par-4. Protein expression of Par-4 in the absence of Flavs (Control) and protein expression in the presence 25 and 50 µg/mL Flavs following 6 hour treatment, respectively. Actin protein levels are indicated as a loading control. The data are representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

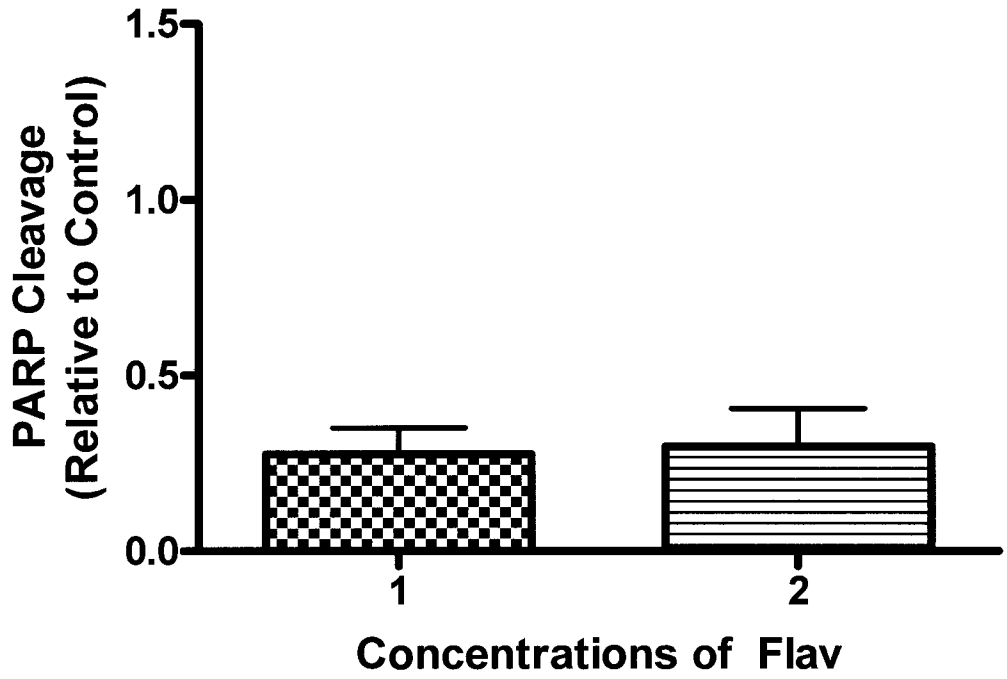

Figure 4.13 Flav treatment of DU145 human prostate cancer cells had no apparent effect on the cleavage of PARP. PARP cleavage in the presence 25 (1) and 50 (2) μg/mL Flavs following 6 hour treatment, respectively. PARP cleavage of 0.0 is indicative of no cleavage. The graph is representative of results obtained from three independent experiments. Each independent experiment consisted of two assay replicates.

4.3.6 Non-mitochondrial Proteins Involved in the Apoptotic Pathways.

In normal cells, Par-4 appears to have a minor affect on managing apoptosis, but in cancer cells, elevated levels of Par-4 may be enough to induce full apoptotic death without any other cellular mediators (Berikhanov et al. 2009). Flav treatment induced the protein expression of Par-4 in DU145 cells after 6 hours of treatment (Figure 4.12). Cleavage of another protein, PARP, is often an important indicator for apoptosis. This inactivation of PARP allows caspase-3 to systematically digest the chromatin within the cell. Using an assay to test for levels of cleaved PARP, it was observed that there was no apparent change in the cleavage of PARP when DU145 human prostate cancer cells were subjected to Flavs (25 and 50 μg/mL) for 6 hours (Figure 4.13).

4.4 Discussion

This study demonstrated that cranberry-derived Flavs have the ability to induce oligonucleosome formation and various proteins involved in apoptosis. These findings support previous research into the induction of apoptosis in cancer cells by Flav treatment through modulation of the protein expression of the Bcl-2 family of proteins, and cytochrome c (Li et al. 2009; Zhang et al. 2008). The Flav induction of apoptosis in DU145 cells induced and inhibited many of the same proteins as the apoptosis induction mediated by the Pacs fraction described in Chapter 3. Taken together, the evidence suggests that both Flavs and Pacs induce apoptosis in DU145 human prostate cancer cells through the intrinsic pathway while inhibiting the extrinsic pathway.

Flav treatment of DU145 human prostate cancer cells leads to a reduction of viability at higher doses; but only at the highest dose assayed (50 μg/mL) was there

significant inhibition of viability ($P < 0.01$). The induction of nucleosome formation is significant at 50 μg/mL ($P < 0.01$). There are several key end events that are often observed during apoptosis induction including nucleosome induction and PARP cleavage (Huerta et al. 2007).

While Flav treatment of DU145 human prostate cancer cells induced nucleosome formation there was no apparent change in PARP cleavage with treatment of Flavs at 25 and 50 μg/mL for 6 hours. Functional PARP repairs any damage to DNA including the potential cleavage of DNA by caspase-3 (Herceg and Wang, 2001). The significant induction of nucleosome formation, while not inducing PARP cleavage, is an interesting finding as the cleavage of PARP by caspase-3 is beneficial for caspase-3 to then digest the nucleic material of the cell (formation of nucleosomes) without PARP interfering and repairing the damage (Herceg and Wang 2001). Therefore, PARP is still present in its functional form and working to repair damage to the DNA.

The induction of apoptosis and formation of nucleosomes in the presence of functional PARP could be due to several reasons. First, in order for Flav treatment of DU145 human prostate cancer cells to induce PARP cleavage, cells may require treatment longer than 6 hours before PARP cleavage is induced (MacLean. 2009). Secondly, a constituent of the Flav fraction may be ineracting with PARP inhibiting its function while also blocking the caspases-3 induced cleavage. Thirdly, another unknown factor not found within the Flav fraction may be required, or perhaps there is a competing factor in the Flav fraction that interferes with the induction of PARP cleavage. Three Flavs, quercetin, fisetin and 2-2'dihydroxychalcone, have been observed to induce PARP cleavage (Haddad, 2008; Lee et al. 2008). The Flav fraction used within this study is

predominately quercetin. Therefore the fraction should induce apoptosis similar to literature but the lack of PARP cleavage suggest that there could be even be compounds present that may be able to inhibit or modulate the effect of the quercetin within the fraction. A final possibility is that the Flav fraction acts on DU145 human prostate cancer cells to induce apoptosis, independent of the cleavage of PARP. Morin, a Flav derived from *Maclura pomifera*, was able to induce apoptosis in oral squamous cells independent of the cleavage of PARP (Brown et al. 2003). While the induction of apoptosis events in DU145 human prostate cancer cells by Flav treatment is not as pronounced as Pacs (induced both PARP cleavage and nucleosome formation), there is still a significant enrichment of nucleosomes at the highest concentration assayed to understand this enriched fractions affects on DU145 human prostate cells.

To further understand the induction of apoptosis by Flavs, expressions of proteins commonly involved in apoptotic pathways were studied. The receptors and adaptor proteins that form the primary source of induction for the extrinsic pathway were assayed using Western blotting analysis. Flav treatment of DU145 human prostate cells at concentrations 25 and 50 μg/mL had an inhibitory affect on the expression of TRAIL and FADD protein expression. Fas unlike the other proteins involved in the extrinsic pathway, had no apparent change in protein expression during treatment. The Flav fraction down regulated the protein expression of receptors and adaptor proteins involved in the induction of apoptosis through the extrinsic pathway. This finding contrasts with the previous results regarding quercetin, a widely studied Flav. Quercetin has been observed to sensitize a number of cancer cell lines to the induction of apoptosis through TRAIL (Chen et al. 2007). Quercetin has also been observed to induce Fas-mediated

apoptosis in HBP-ALL human leukemia cells (Russo et al. 1999). The observed apoptotic effects of quercetin through the extrinsic pathway contrasts with the observations presented in this thesis that show a Flav-mediated inhibition of the extrinsic pathway. The Flav fraction inhibited some members of the extrinsic pathway while having no apparent affect on others. This discrepancy may be due to other components of the Flav fraction masking or out-competing any quercetin present. The inhibition of extrinsic receptor proteins could even be a compensatory measure of the cell to regain homeostasis, rather than due to any direct interaction by the Flavs (Knott et al. 2003). Despite the hypotheses presented above to explain the diversion between literature and the observed results the most likely explaination would be that the observed results are due to a cell line specific response..

The Bcl-2 family of proteins was assayed for their involvement in the induction of apoptosis by Flav treatment. Flav treatment of DU145 human prostate cells appeared to induce the cleavage of Bid into its active form, tBid. Quercetin has been observed to induce the cleavage of Bid into tBid in both colon cancer cells and leukemia cells (Psahoulia et al. 2007; Ramos and Aller. 2008). This observation of quercetin-mediated cleavage of Bid is consistent with the observed cleavage of Bid due to Flav treatment of DU145 human prostate cancer cells described in this chapter. The induction of Bid cleavage can be caused by proteins outside the normal apoptotic process. Par-4 is a prostate-specific protein involved in the induction of apoptosis. Flav treatment of DU145 human prostate cells resulted in an induction of protein expression of Par-4. This induction of Par-4 is a novel finding, as Par-4 modulation by Flav has not been previously reported. Par-4 induction may relate to the induction of Bid cleavage

mentioned previously, as Par-4 commonly activates caspase-8, which in turn cleaves Bid into tBid (Li et al. 1998). The induction of Bid cleavage could contribute to the formation of pores in the outer mitochondrial membrane, leading to induction of apoptosis through the intrinsic pathway. Other Bcl-2 family members were also assayed for their potential to be modulated by Flav treatments of DU145 cells. Flav treatment induced the expression of Bax, and inhibited Bcl-2 protein expression, and current literature supports this finding (Chien et al. 2009; Lee et al. 2008; Xu et al. 2008). For example, quercetin and kaempferol both induced Bax and inhibited Bcl-2 protein expression in a number of cell lines including MDA-MB-231 human breast cancer cells, HeLa cervical carcinoma cells, LNCaP human prostate cancer cells and L-02 embryonic liver cells (Chien et al. 2009; Lee et al. 2008; Xu et al. 2008).

The induction of Bax and inhibition of Bcl-2 protein expression suggest that Flav treatment could redistribute the Bax/Bcl-2 ratio in favor of Bax. This redistribution is important as it could create a pro-apoptotic environment within the cell, leading to the release of cytochrome c and the induction of apoptosis. The inhibition of Bcl-2 and induction of Bax protein expression suggest that there could be a change in the important Bax/Bcl-2 ratio, in addition to the Flav-mediated induction of tBid formation. These inductions could both lead to the liberation of cytochrome c and other apoptotic modulating proteins from the mitochondria leading to propagation or induction of apoptosis (Grinberg et al. 2002; Kuwana et al. 2002).

Cytochrome c is an important protein that forms the part of the apoptosome which activates caspase-9, leading to irreversible apoptotic induction via caspase-3 activation (Michelin et al. 2003). Flav treatment of DU145 prostate cancer cells induced protein

expression of cytochrome c. The induction of cytochrome c may have implications for the release of cytochrome c from the mitochondria via Bax-mediated pores. Normally, cardiolipin is found within the mitochondria and is saturated with cytochrome c; only about 20% of cytochrome c proteins are loose within the intermembrane space (Jurgensmeier et al., 1998; Scorrano et al. 2002). The induction of cytochrome c could be directly attributed to the population of unbound cytochrome c suggesting that the Bax-mediated pores could potentially liberate enough cytochrome c alone to propagate the apoptotic stimuli without contributions from other proteins. Smac/Diablo is a pro-apoptotic protein that is also segregated within the intermembrane space of the mitochondria. Unlike Pacs treatment, which inhibited this protein, Flav treatment induced the release of Smac/Diablo. Although Flavs, including quercetin, have not been studied with respect to Smac/Diablo release, previous findings reported an induction of Smac/Diablo release after treatment with polyphenols derived from bran (Kong et al. 2009). The induction of Smac/Diablo release is important as this protein acts to inhibit a diverse range of inhibitors, including the family of inhibitors of apoptosis (IAPs) (Dai et al. 2009). This induction would allow for the inhibition of many factors that would contribute to the inhibition of apoptosis.

In conclusion, Flav treatment of DU145 cells induced apoptosis as indicated by nucleosome enrichment through induction of the intrinsic pathway. This finding was shown in multiple ways: 1) by the potential change of the Bax/Bcl-2 ratio; 2) by the induction of Bid cleavage; 3) by the induction of Smac/Diablo release; and 4) by increased cytochrome c protein expression. These findings suggest that the Flav fraction is a source of compounds that should be studied further as a potential source of novel

pharmacological compounds to treat prostate cancer. The results presented in this chapter suggest that the Flav fraction is at least partially responsible for the induction of apoptosis observed in DU145 cells treated with whole cranberry extract.

Chapter Five: Ursolic acid, a Constituent of *Vaccinium macrocarpon* Inhibits MMP Activity in DU145 Human Prostate Cancer Cells.

5.1 Introduction

According to the Canadian Cancer Society (2008), approximately 40-45% of the population can be expected to develop at least one form of cancer throughout their lifetime. By the year 2020, it is expected that cancer rates will increase to roughly 50% (World Health Organization 2003). Prostate cancer is the third most prevalent cancer in North American men, and is just slightly less prevalent than colorectal cancer (Canadian Cancer Society 2008). Although the numbers of new and more comprehensive treatments and screening methods have increased, epidemiological data suggests that prostate cancer rates are still rising and are expected to continue to rise (Canadian Cancer Society 2008). Prostate cancer is a relatively slow growing cancer, and as such, alternative therapies in combination with conventional approaches are an increasing likelihood for the treatment and management of this condition (or disease) (American Cancer Society. 2008).

Prostate cancer patients often turn to lifestyle and dietary changes in an effort to combat their disease. Many of the plants and fruits that are commonly consumed as part of a normal diet contain chemicals that are capable of modulating cellular function. The theory is that some of these secondary metabolites can alter the function of a cancerous cell. Chemical extracts from fruits have been observed to have diverse effects on cancer and other maladies (Aggarwal and Shishodia 2006). Ursolic acid (UA) is a triterpene found in many fruits including the North American cranberry (*V. macrocarpon*). Current

research suggests that this pentacyclic acid possesses a number of beneficial properties, including both anti-inflammatory and anti-cancer effects (Ikeda et al. 2008).

A tumour can be viewed as a functional tissue within its own microenvironment. The way a tumour interacts with the surrounding tissue or matrix is an important determinant of its behaviours (Radisky et al. 2001). The extracellular interaction between the cell and the matrix is partially due to the activity of matrix metalloproteinases (MMPs) and their subsequent degradation of the extracellular matrix (ECM) (Pupa et al. 2002). MMPs are an important group of proteases that are often over-expressed in many forms of cancer (Davidson et al. 2002). These zinc-dependent endopeptidases are also important in the interactions between 'normal' (i.e. non-transformed) cells and their immediate environment. MMPs are especially critical to the way that cancerous cells interact with and modulate the ECM. The ability to degrade the extracellular matrix (ECM) arises through two mechanisms: the post-transcriptional induction of activity or modulation of regulators of MMPs (Davidson et al. 2002). It is hypothesised that UA treatment of DU145 human prostate cells will inhibit the activity of MMPs via modulation of their regulators. The work presented in this chapter demonstrates the effect of UA on MMP activity in DU145 cells and elucidates aspects of the mechanisms involved.

5.2 Materials and Methods

5.2.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (Oakville, ON). Unless otherwise noted, antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA).

5.2.2 Cell Culture

Please refer to Chapter 3 (section 3.2.2) for an overview of the cell culture used with DU145 human prostate carcinoma cells.

5.2.3 Solubilization of UA for Cell Treatments

Ursolic acid (Sigma) was dissolved immediately before treatment in a 1:9 ratio of DMSO dissolved in DM (v/v) supplemented as described previously. DMSO did not exceed a maximum of 1% concentration on the treatment plate. Ursolic acid treatments were for 6 hour durations at concentrations 0 μM, 10 μM, and 25 μM.

5.2.4 Bradford Protein Assay

Please refer to Chapter 3, section 3.2.4 (Bradford Protein Assay) for a detailed overview of the Bradford Protein Assay.

5.2.5 Sonication

Please refer to Chapter 3, section 3.2.5 (Sonication) for a detailed overview of the sonication protocol.

5.2.6 Alamar Blue Assay

Please refer to Chapter 3, section 3.2.6 (Alamar Blue Assay) for a detailed overview of the Alamar Blue Assay.

5.2.7 Immunoblot Analysis

Please refer to Chapter 3, section 3.2.7 (Immunoblot Analysis) for a detailed overview of the Western blotting analysis.

5.2.8 Gelatin Gel Electrophoresis

Gelatin gel electrophoresis, also known as zymography, is a common technique used to evaluate MMP activity. Zymography uses SDS-PAGE to separate MMPs based on size and relies on the ability of MMPs to degrade denatured collagen (gelatin). These areas of degradation can then be visualized through the addition of dyes to the gel; bands of clearing are indicative of activity. Briefly, Triton X-100, a detergent, is used to remove the SDS. The proteins are then allowed to re-form structurally and become catalytically active. The gels are then bathed in a buffer that is complementary to MMP function, thereby allowing the MMPs to degrade the collagen that is found within the poly-acrylamide gel. Undigested collagen is dyed using a simple protein dye, in this case Coomassie Blue R-250. This allows for the detection of areas of digested collagen that is indicative of MMP activity.

Cells were subcultured on 100 mm plates at a density of 3.5×10^5 cells/plate and allowed to incubate for 36 hours at 37°C in a 5% CO_2 incubator. Following this growth period, the culture medium was removed and replaced with defined media (DM) for 24 hours. DM was then removed and replaced with fresh DM before UA was added to the culture plate. The DU145 human prostate cancer cells were then treated with UA for 6 hours. Cells were treated with 10 μM and 25 μM of UA. Control cells received vehicle alone. Following this treatment, cell culture medium was carefully removed and placed

in 15 mL Falcon tubes, and these tubes were centrifuged at 250 x *g* for 2 minutes to pellet particulates and cell debris. The supernatant was then removed and stored at -80°C in 15 mL Falcon tubes.

For analysis, the cell media was diluted in a 3:1 ratio with Laemmli buffer (500 mM Tris-HCL [pH 6.8], 1 mM SDS, 10 nM bromophenol blue and 10 mM glycerol) and was incubated for 20 minutes at 37°C. The samples were then electrophoresed through a stacking gel and a resolving gel at 40 mA. The gel was washed in zymography buffer 1 (50 mM Tris-HCl [pH 7.4] with 2.5% Triton X-100) for 1 hour at room temperature. This gel was then bathed with zymography buffer 2 (50 mM Tris-HCl [pH 7.4]) for 30 minutes at room temperature. Following this bathing period, the gel was further incubated at 37°C in the presence of zymography buffer 3 (50 mM Tris-HCl [pH 7.4] with 2.5% Triton X-100, and 5 mM $CaCl_2$) for 48 hours.

After zymograph buffer 3, the gel was then washed with dH_2O for 10 min. The gel was stained using a protein dye (0.25% Coomassie Blue R-250 in a solution of 45:50:5 [v/v/v] of water, methanol and acetic acid, respectively) while being gently agitated for 1.5 hours. The gel was then destained with a solution of water, methanol and acetic acid (85:10:5 [v/v/v]) for 1 hour. The location (molecular weight), and the zone of clarity which resulted, were indicative of the identity of the MMP and its activity, respectively.

5.2.9 Image Capture and Gel Quantification

Please refer to Chapter 3, section 3.2.11 (Image capture and gel quantification) for a detailed overview of the gel documentation system and programs.

5.2.10 Statistics

Please refer to Chapter 3, section 3.2.12 (Statistics) for an overview of the gel documentation system and programs. Cytotoxicity of DU145 human prostate cancer cells treated with UA was analyzed with the Kruskal-Wallis test with post-hoc Dunn's Multiple Comparison Test as cytotoxic data did not fit normal distribution.

5.3 Results

5.3.1 UA-mediated Cytotoxicity in DU145 Human Prostate Cancer Cells

An Alamar Blue assay was performed to assess the effect of UA on the viability of DU145 human prostate cancer cells. UA treatment of DU145 cells resulted in a decrease in cell viability. As shown in Figure 5.1, UA (at 10, 25 and 50 μM) significantly inhibited the viability of DU145 cells ($P < 0.001$).

5.3.2 UA Inhibits MMP Activity in DU145 Human Prostate Cancer Cells

Neto et al. (2006) reported that cranberry treatment could inhibit the MMP-2 and -9 activities in DU145 cells; MacLean et al. (2007) confirmed this observation. UA has been observed to have an inhibitory effect on MMPs in some cell lines (Pathak et al. 2007; Shan et al 2009; Xavier et al. 2009). DU145 cells were treated with UA (0 μM, 10 μM, and 25 μM) for 6 hours. The UA treatment of DU145 cells at 10 and 25 μM resulted in an inhibition of MMP-9 (Figure 5.2), MMP-2 (Figure 5.4) and Pro-MMP-2 (Figure 5.3) activity. The inhibition of MMP-9 and Pro-MMP-2 were significant at 25 μM, $P < 0.05$ and $P < 0.05$ respectively.

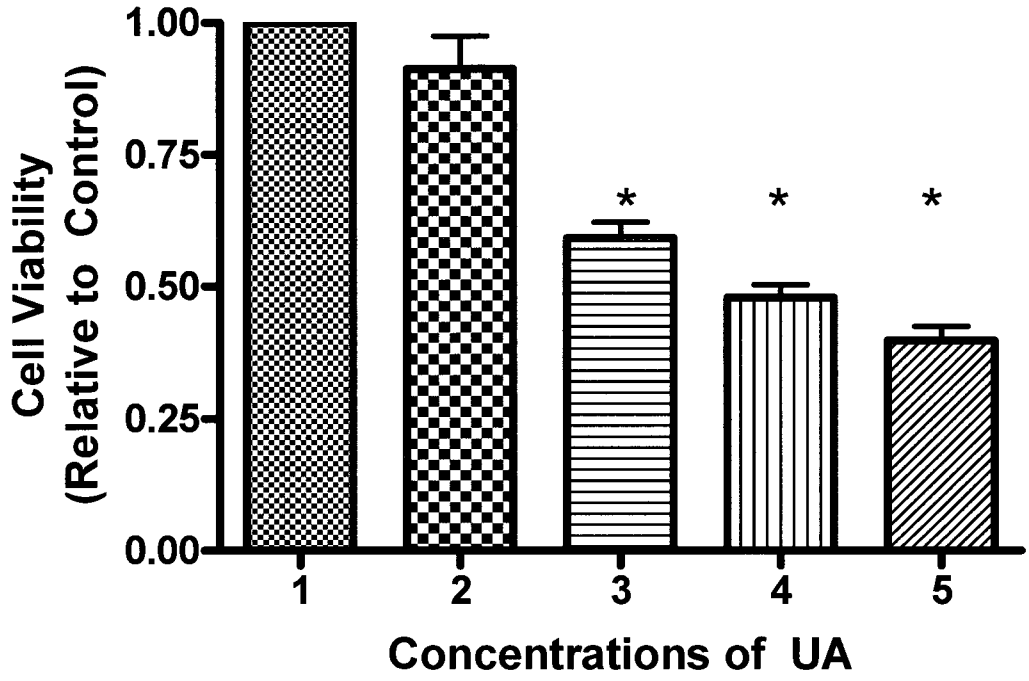

Figure 5.1 UA treatment of DU145 human prostate cells inhibits cellular viability. Cytotoxicity was assessed using an Alamar blue assay. Cell viability in control cells(no UA, vehicle alone) (1), and in cells treated with 1 μM of UA(2), 10 μM of UA (3), 25 μM of UA (4) and 50 μM of UA (5) for 6 hours respectively. Data was analyzed with the Kruskal-Wallis test with post-hoc Dunn's Multiple Comparison test as the data did not fit a normal distribution. Single stars are representative of a significance with $P < 0.001$. The graph is representative of results obtained from four independent experiments, each with 15 assay replicates.

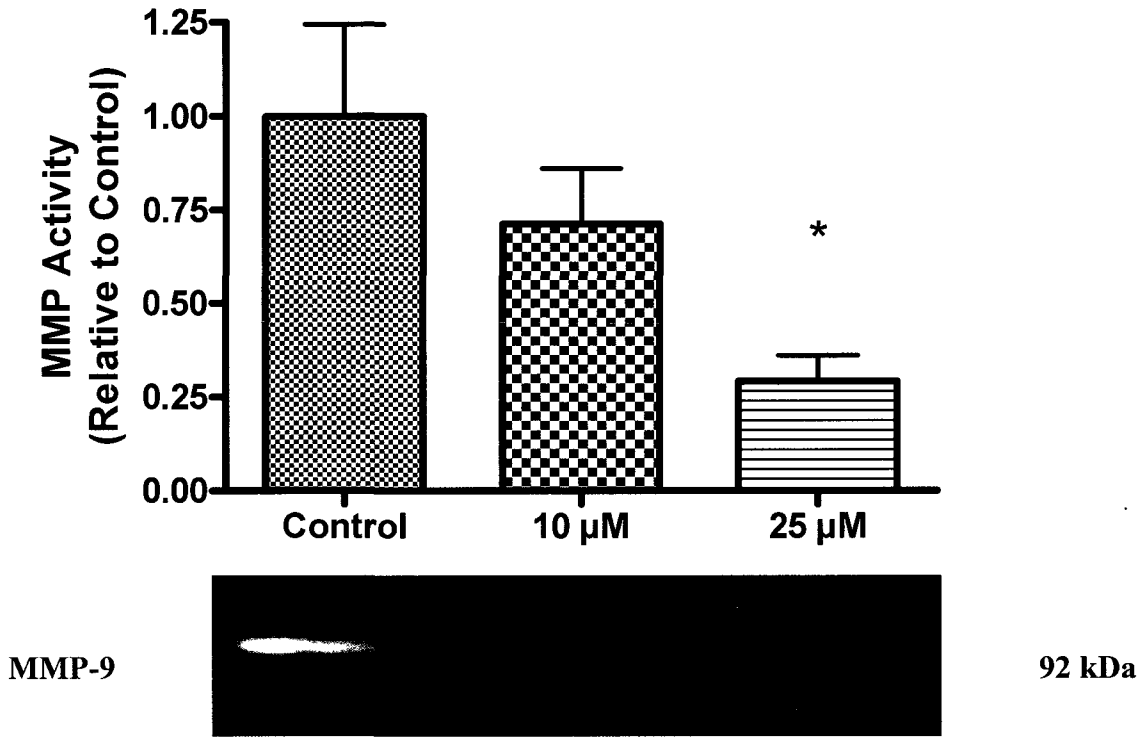

Figure 5.2 UA treatment of DU145 human prostate cancer cells inhibits the activity of MMP-9. Gelatin gel electrophoresis (zymography) was performed using media from cells following 6 hours of UA treatment. Gelatinolytic activity in cells in the presence of velicle absence of UA are shown as Control and the gelatinolytic activity of cells following 6 hour treatment with UA 10 µM are labeled accordingly. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk denotes statisical signficance of $P < 0.05$. The data are representative of results obtained from at least two indipendent experiments with two assay replicates.

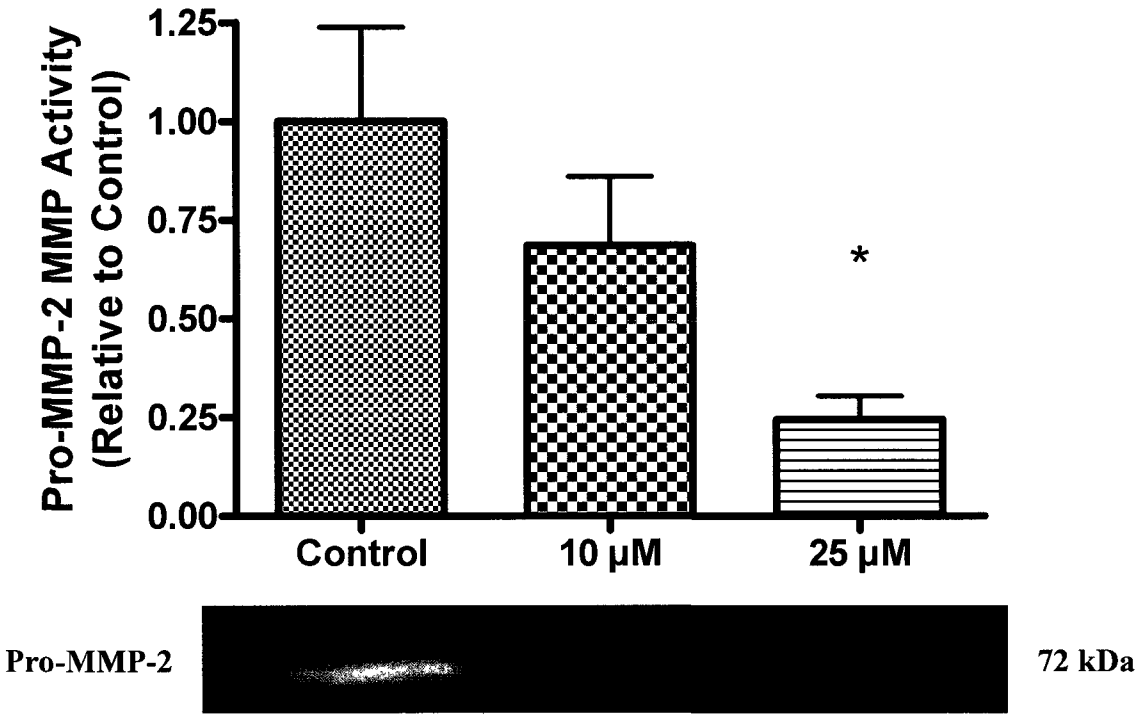

Figure 5.3 UA treatment of DU145 human prostate cancer cells inhibits the activity of Pro-MMP-2. Gelatin gel electrophoresis (zymography) was performed using media from cells following 6 hours of UA treatment. Gelatinolytic activity in cells in the presence of velicle absence of UA are shown as Control and the gelatinolytic activity of cells following 6 hour treatment with UA 10 µM are labeled accordingly. Data was analysized with ANOVA with a tukey post-hoc test. Single asterisk denotes statisical signficance of $P < 0.05$. The data are representative of results obtained from at least two indipendent experiments with two assay replicates.

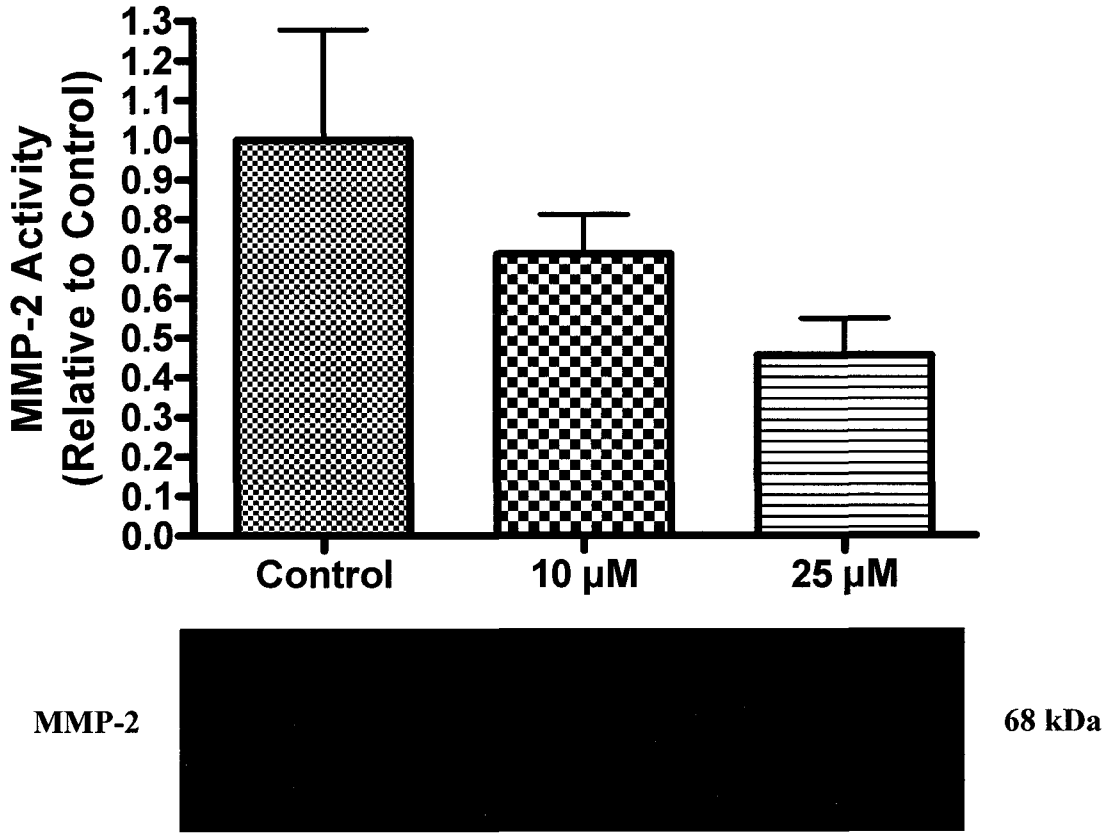

Figure 5.4 UA treatment of DU145 human prostate cancer cells inhibits the activity of MMP-2. Gelatin gel electrophoresis (zymography) was performed using media from cells following 6 hours of UA treatment. Gelatinolytic activity in cells in the presence of velicle absence of UA are shown as Control and the gelatinolytic activity of cells following 6 hour treatment with UA 10 µM are labeled accordingly. The data are representative of results obtained from at least two indipendent experiments with two assay replicates.

5.3.3 **UA Effects on Protein Regulators of MMP Expression in DU145 Human Prostate Cancer Cells.**

Treatment of DU145 cells with whole cranberry extract was observed to modulate the expression of proteins associated with the regulation of MMPs (MacLean et al. 2007). This inhibition of MMP activity may be through interaction of UA with the regulators of MMPs. DU145 cells were treated with concentrations of 10 μM, and 25 μM UA for 6 hours.

Treatment of DU145 cells with UA resulted in an increase in the expression of two inhibitors of MMPs: tissue inhibitors of matrix metalloproteinases, TIMP-1 (Figure 5.5) and TIMP-2 (Figure 5.6). Extracellular matrix metalloproteinase inducer (EMMPRIN), an inducer of MMP activity was inhibited by UA treatment (Figure 5.7). Reversion inducing cysteine rich protein with kazal motifs (RECK) was inhibited by treatment with UA (Figure 5.8).

5.4 **Gel Gelatin Electrophoresis Analysis of Signal Transduction Pathways**

There are several pathways common to regulation of cellular behaviour including MMP governance. These pathways are: mitogen activated protein kinase (MAPK), the phosphoinositide-3-kinase (PI3K), and protein kinase C (PKC). The study the mechanisms behind the effect of UA on DU45 cells required the use of the inhibitors: PD98059, LY294002, and Calphostin C, which affect the MAPK, PI3K, and PKC pathways, respectively. As shown in Figure 5.9, the UA mediated inhibition of MMP-9, Pro-MMP-2 and MMP-2 was relieved in the presence of the inhibitor PD98059.

Treatment with LY294002 and Calphostin C had no apparent effects on the observed inhibition of MMP activity by UA.

Another key pathway that can affect MMPs is the NF-κB mediated pathway. To investigate the involvement of the NF-κB pathway, the inhibitors JSH-23 and SM-7368 were used. The inhibitions of MMP-9, MMP-2, and Pro-MMP-2 by UA treatment were relieved by treatment with the inhibitor JSH-23 (Figure 5.10). Treatment with SM-7368 had no effect on the UA-mediated inhibition. The involvement of the various signal transduction pathways involved in the induction of UA-mediated inhibition of MMPs are summarized in Figures 5.11, 5.12 and 5.13.

5.5 Discussion

Many phytochemicals have been found to have wide-ranging effects on biological systems. UA is no different; to date; as many as 120 different "bioactivities" have been ascribed to this molecule (Jaki et al. 2008). As discussed previously, DU145 human prostate cancer cells show decreased cell viability when treated with UA. This inhibition of cellular viability is consistent with several reports which illustrate UA's cytotoxicity in a number of cell lines derived from prostate, epithelial and cervical cells, such as HL-60, BGC, Bel-7402, B16F-10, LNCaP, PC-3, DU145 and Hela cancer cells (Kassi et al. 2007; Ma et al. 2005; Manu and Kuttan 2008; Yim et al. 2006; Zhang et al. 2009 [1]). The reduction of cellular viability for 10, 25 and 50 μM treatments of UA were statistically significant ($P < 0.001$). UA treatment of DU145 cancer cells at the lowest concentration used in this study, 1 μM over 6 hours, inhibited cell viability, but not

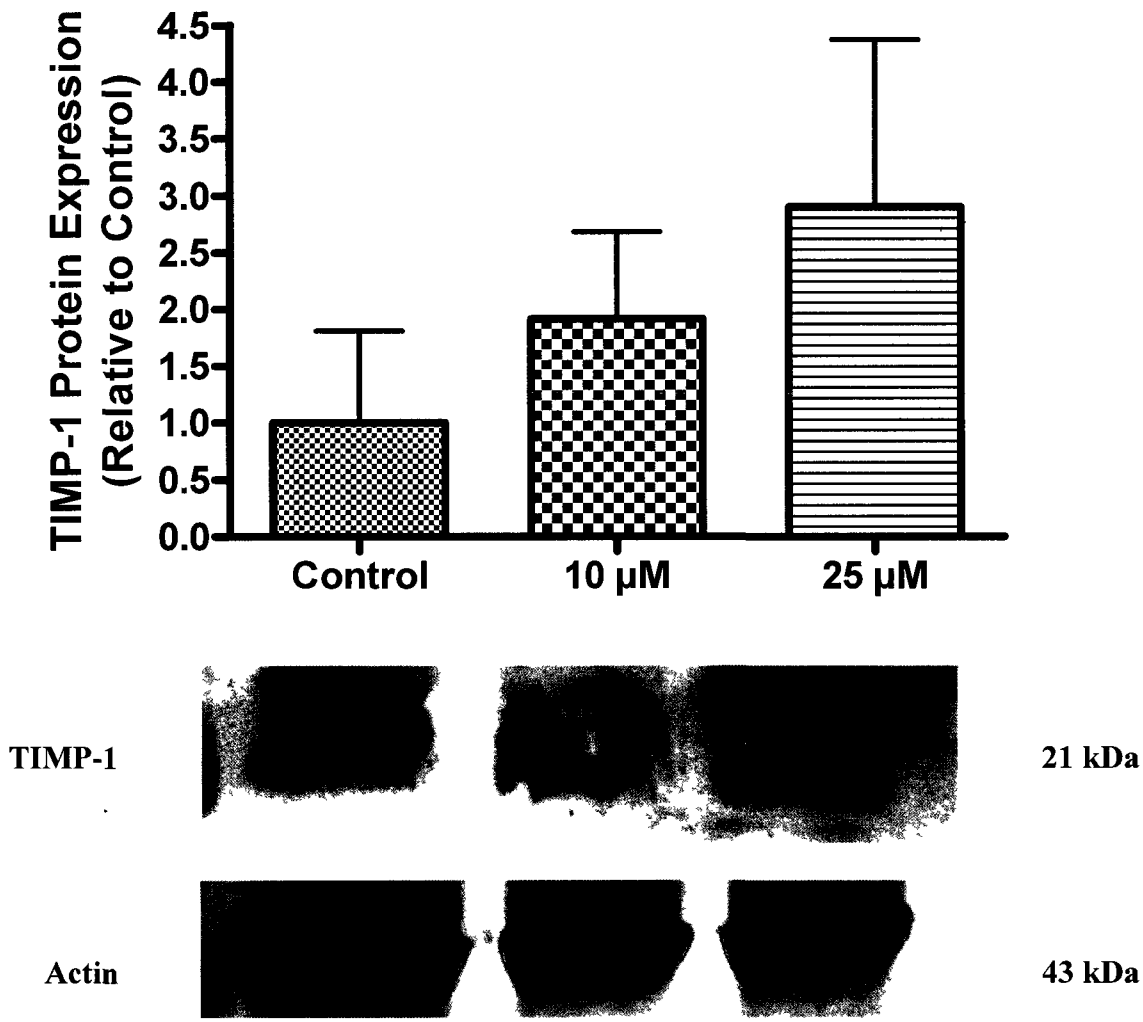

Figure 5.5 UA treatment of DU145 human cancer cells induces in a dose dependent manner the protein expression of TIMP1. Western blotting analysis was performed using extracts from cells following 6 hours of UA treatment. TIMP-1 protein expression in prostate cells not treated with UA(Control) and in the presence of UA treatment at 10 µM and 25 µM of UA, respectively. Actin protein levels are indicated as a loading control. The data is representatative of results atained from two independent experiments. Each independent experiment consisted of two assay replicates.

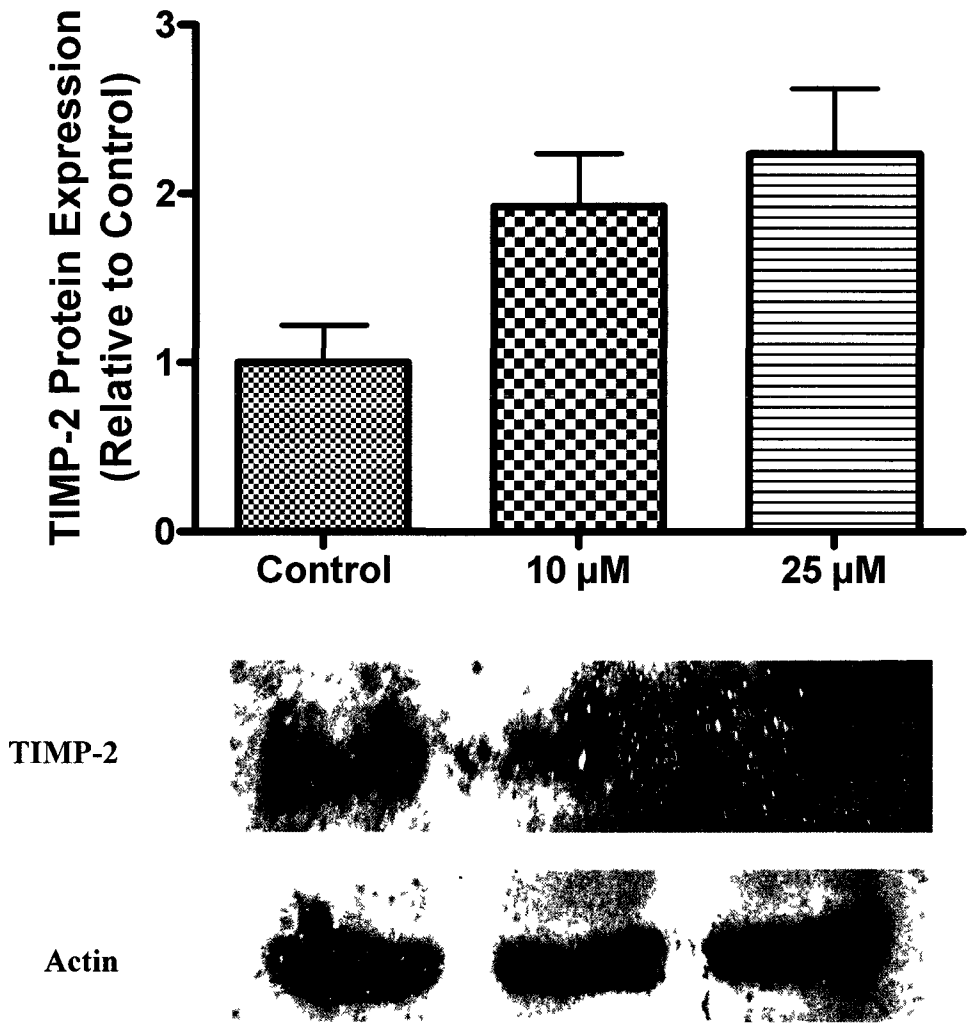

Figure 5.6 UA treatment of DU145 human cancer cells induces in a dose dependent manner the protein expression of TIMP-2. Western blotting analysis was performed using extracts from cells following 6 hours of UA treatment. TIMP-2 protein expression in prostate cells not treated with UA(Control) and in the presence of UA treatment at 10 µM and 25 µM of UA, respectively. Actin protein levels are indicated as a loading control. The data is representatative of results atained from two independent experiments. Each independent experiment consisted of two assay replicates.

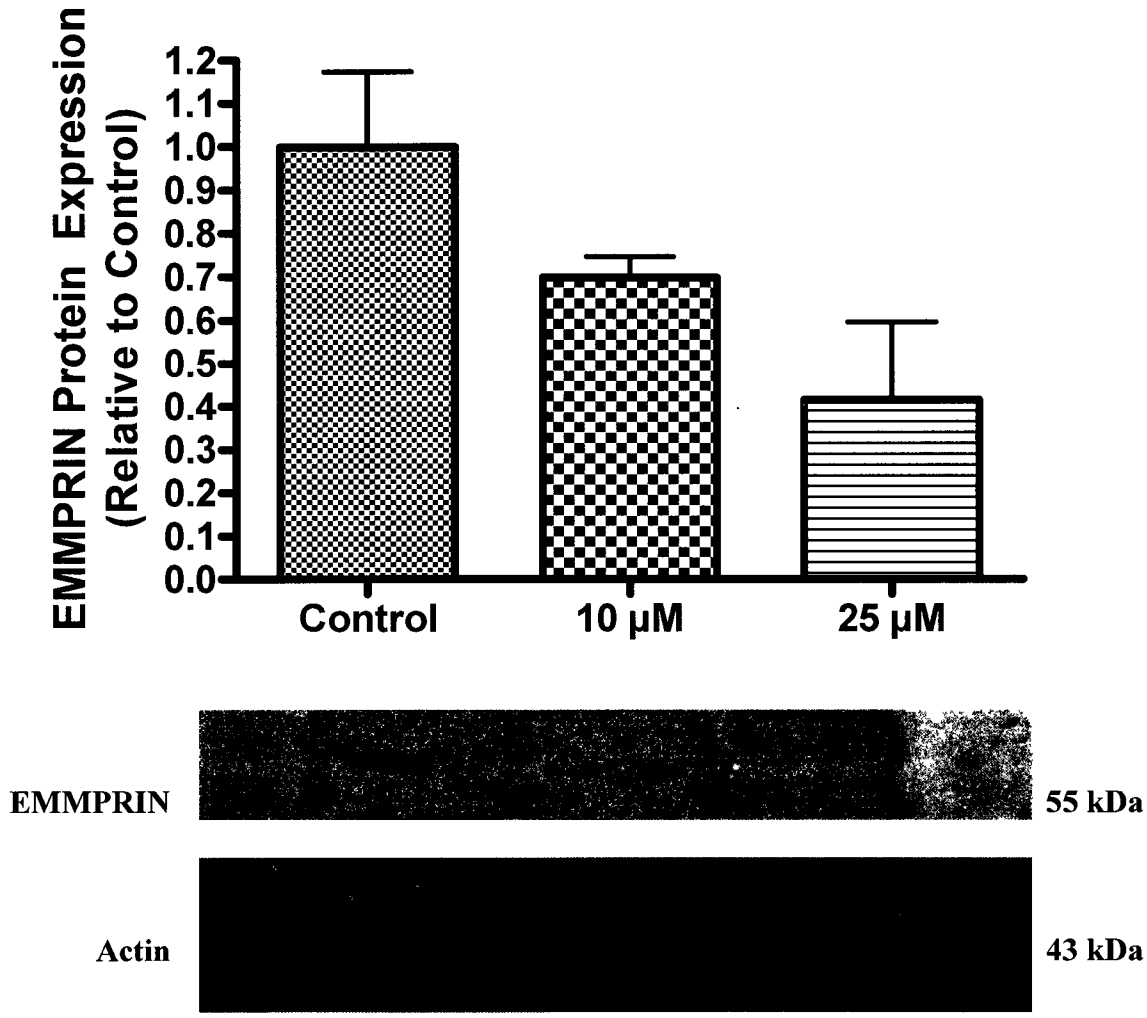

Figure 5.7 UA treatment of DU145 human prostate cancer inhibits the expression of EMMPRIN. Western blotting analysis was performed using extracts from cells following 6 hours of UA treatment. EMMPRIN protein expression in the absence of UA (Control) and protein expression levels of EMMPRIN in cells in the presence of 10 μM and 25 μM UA, respectively. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

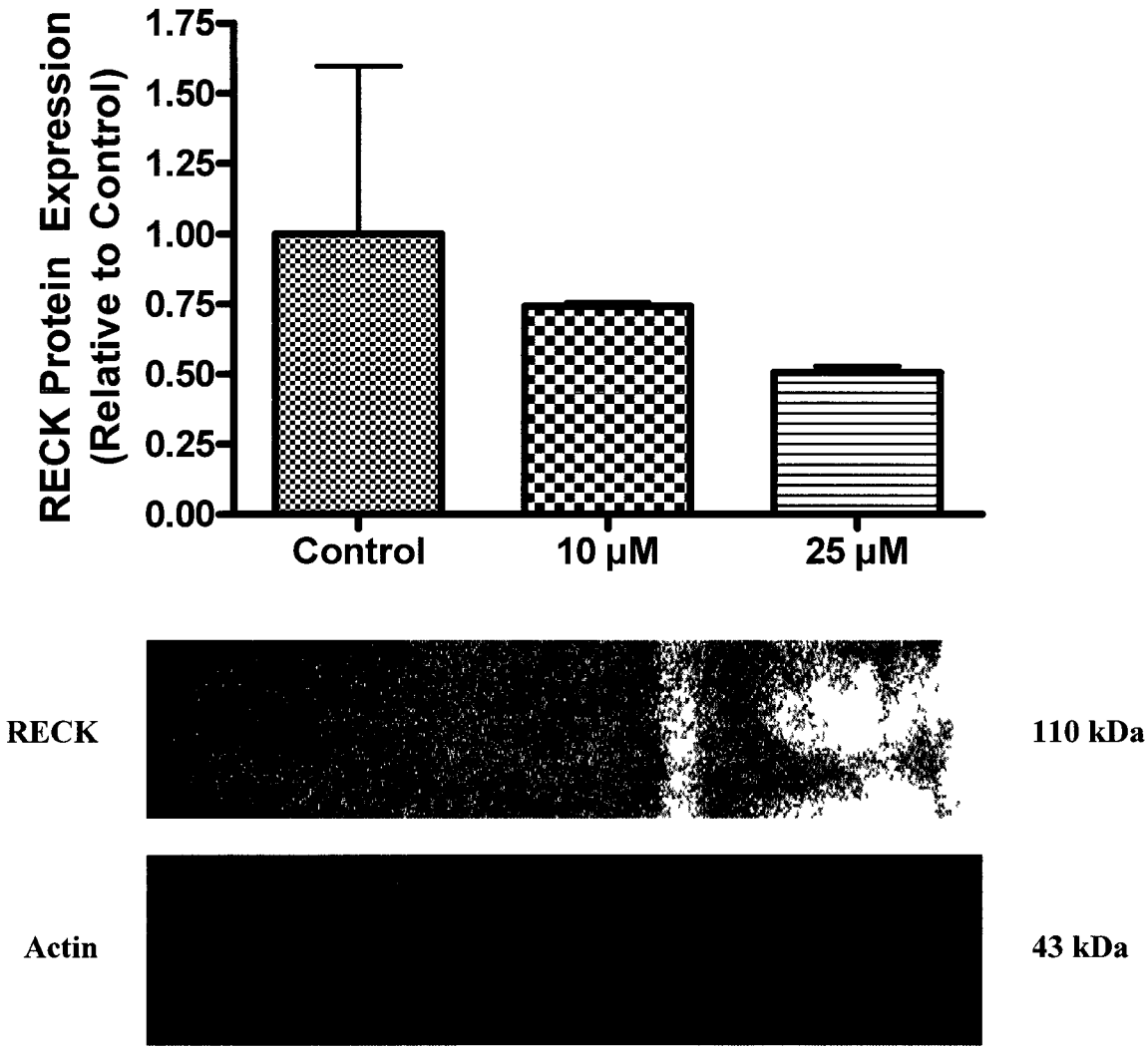

Figure 5.8 UA treatment of DU145 human prostate cancer inhibits the expression of RECK. Western blotting analysis was performed using extracts from cells following 6 hours of UA treatment. RECK protein expression in the absence of UA (Control) and protein expression levels of RECK in cells in the presence of 10 μM and 25 μM UA, respectively. Actin protein levels are indicated as a loading control. The data is representative of results obtained from two independent experiments. Each independent experiment consisted of two assay replicates.

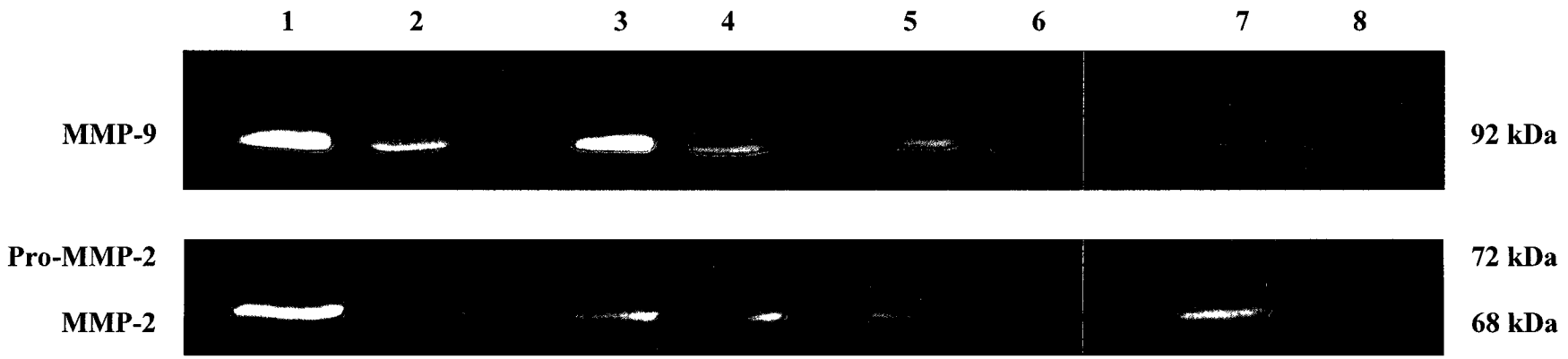

Figure 5.9 Partial relief of the UA mediated inhibition of Pro-MMP-2, MMP-2, and MMP-9 occurs in the presence of PD98059(an inhibitor of the MAPK pathway). Gel gelatin electrophoresis was performed on aliquots of cell media after 6 hours of UA treatment in the presence or absence of specific inhibitors. Gelatinolytic activity in DU145 cells as follows: DU145 cells in the absence of UA(1), in the presence of UA (25 μM)(6 hrs)(2), in the presence of PD98059 alone (3), pre-treated with PD98059 for 1 hour followed by 6 hour exposure to UA (25 μM)(4); in the absence of UA and the presence of LY294002(5), pre-treated with LY294002 for 1 hour followed by 6 hour exposure to UA (25 μM)(6); treated with Calphostin C alone(7) and pre-treated with Calphostin C for 1 hour followed by 6 hour exposure to UA (25 μM)(8). PD98059, LY294002, Calphostin C were used at the concentrations 10 μM, 50 μM and 500 nM, respectively. The data presented is representative of MMP-9, Pro-MMP-2 and MMP-2 results from at least two independent experiments with duplicate assay replicates.

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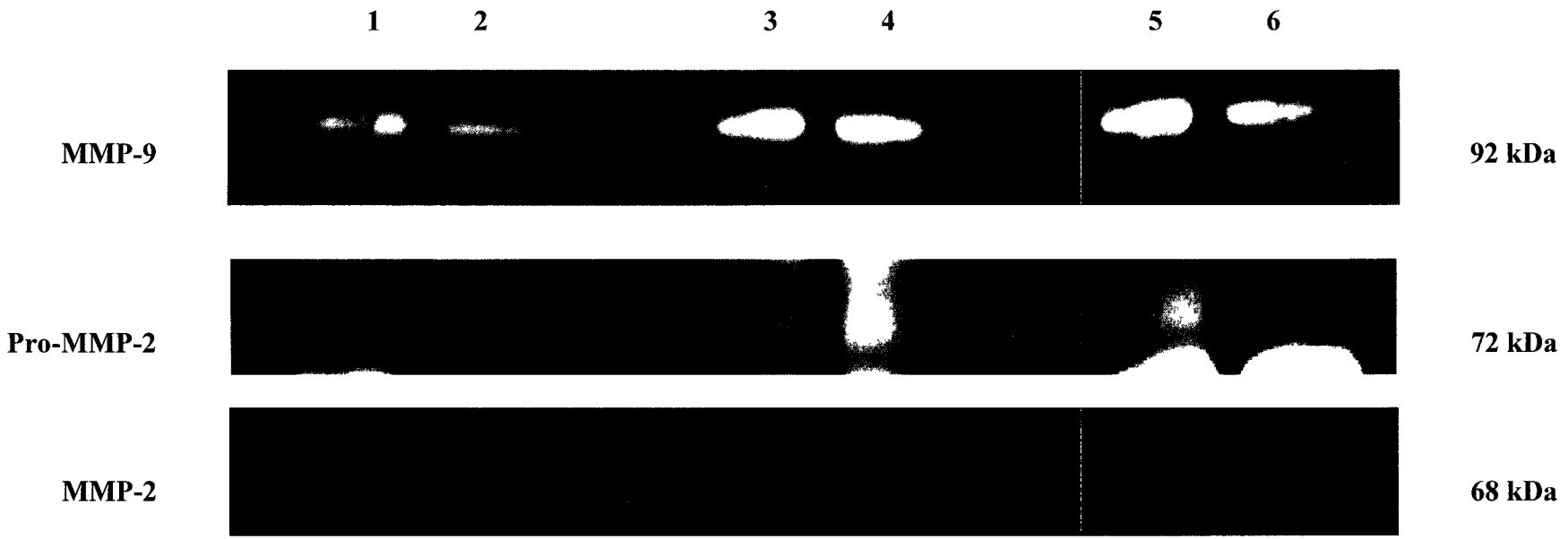

Figure 5.10 Relief of the UA mediated inhibition of MMP-2, Pro-MMP-2 and MMP-9 occurs in the presence of JSH-23 (an inhibitor of the NF-κB pathway). Gelatinolytic activity of DU145 cells in the absence of UA(1), in the presence of UA (25 μM duration 6 hrs)(2), in the presence of JSH-23 alone (3), pre-treated with JSH-23 for 1 hour followed by 6 hour exposure to UA (25 μM)(4); in the absence of UA and the presence of SM-7368(5), pre-treated with SM-7368 for 1 hour followed by 6 hour exposure to UA (25 μM)(6). JSH-23 and SM-7368 were used at the concentrations of 50 μM and 10 μM, respectively. The data presented is representative of MMP-9, Pro-MMP-2 and MMP-2 results from at least three independent experiments with duplicate assay replicates.

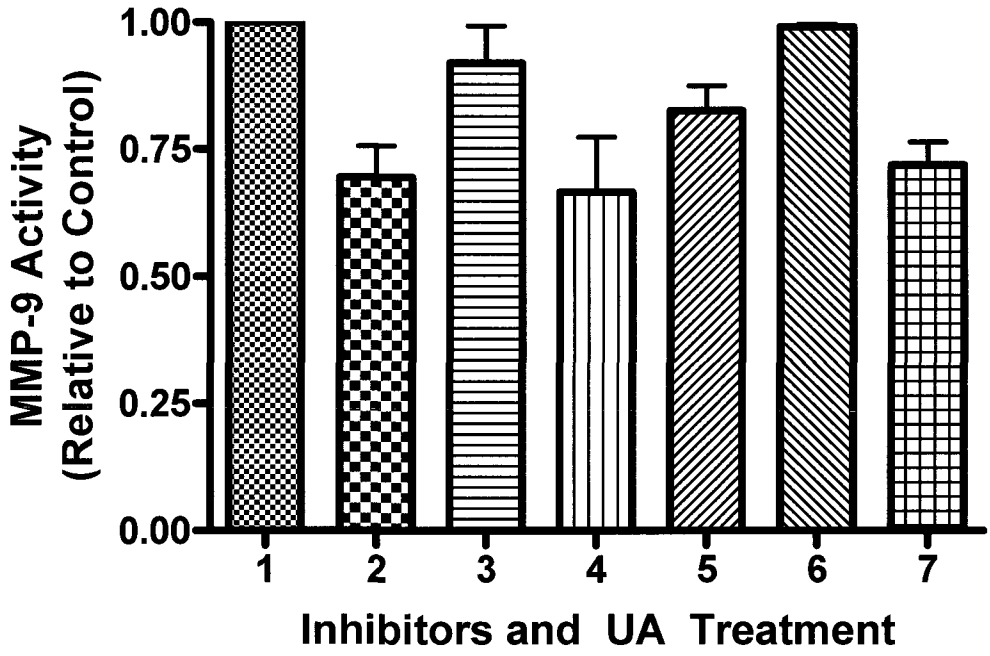

Figure 5.11 The inhibitors of the MAPK and NF-κB mediated signal transduction pathways abolished the UA mediated inhibition of MMP-9 activity. Gel gelatin electrophoresis was performed on aliquots of cell media after 6 hours of UA treatment in the presence or absence of specific inhibitors. Gelatinolytic activity is shown as follows: DU145 cells in the absence of UA(1), in the presence of UA (25 μM)(6 hrs)(2), pre-treated with PD98059 for 1 hour followed by 6 hour exposure to UA (25 μM)(3); pre-treated with LY294002 for 1 hour followed by 6 hour exposure to UA (25 μM)(4); pre-treated with Calphostin C for 1 hour followed by 6 hour exposure to UA (25 μM)(5); pre-treated with JSH-23 for 1 hour followed by 6 hour exposure to UA (25 μM)(6); pre-treated with SM-7368 for 1 hour followed by 6 hour exposure to UA (25 μM)(7). PD98059, LY294002, Calphostin C, JSH-23, and SM-7368 were used at the concentrations 10 μM, 50 μM, 500 nM, 50 μM, and 10 μM, respectively. Single asterisk denotes statistical significance of $P < 0.05$. The graph is representative of results obtained from at least three independent experiments. Each independent experiment consisted of duplicate assay replicates.

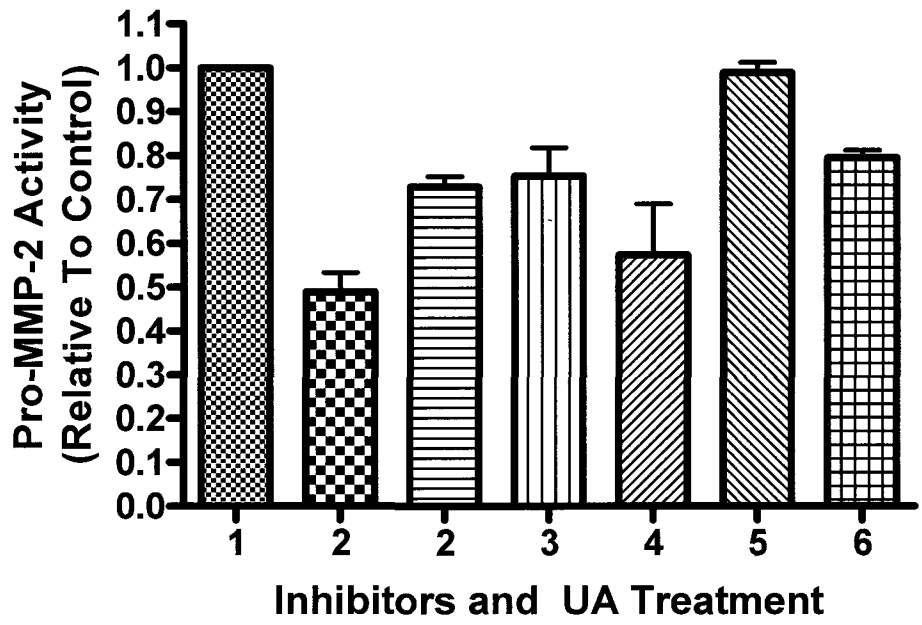

Figure 5.12 The inhibitors of the NF-κB mediated signal tranduction pathway abolished the UA mediated inhibition of Pro-MMP-2 activity . Gel gelatin electrophoresis was performed on aliquots of cell media after 6 hours of UA treatment in the presence or absence of specific inhibitors. Gelatinolytic activity is shown as follows: DU145 cells in the absence of UA(1), in the presence of UA (25 μM)(6 hrs)(2), pre-treated with PD98059 for 1 hour followed by 6 hour exposure to UA (25 μM)(3); pre-treated with LY294002 for 1 hour followed by 6 hour exposure to UA (25 μM)(4); pre-treated with Calphostin C for 1 hour followed by 6 hour exposure to UA (25 μM)(5); pre-treated with JSH-23 for 1 hour followed by 6 hour exposure to UA (25 μM)(6); pre-treated with SM-7368 for 1 hour followed by 6 hour exposure to UA (25 μM)(7). PD98059, LY294002, Calphostin C, JSH-23, and SM-7368 were used at the concentrations 10 μM, 50 μM, 500 nM, 50 μM, and 10 μM, respectively. Single asterisk denotes statistical significance of $P < 0.05$. Two asterisks denotes signficance of $P < 0.01$ The graph is representative of results obtained from at least three independent experiments. Each independent experiment consisted of duplicate assay replicates.

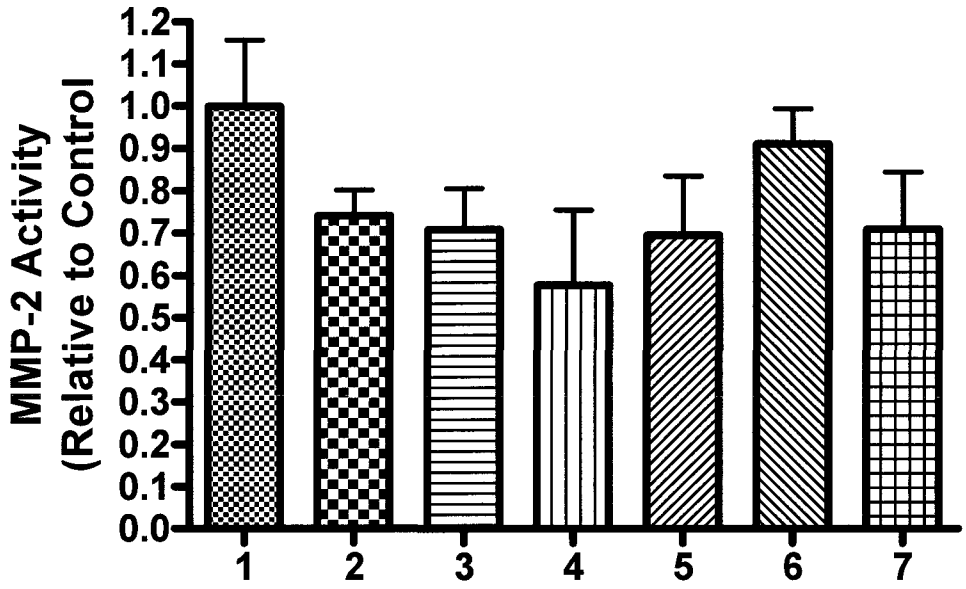

Figure 5.13 The inhibitors of the NF-κB mediated signal tranduction pathway ly abolished the UA mediated inhibition of MMP-2 activity. Gel gelatin electrophoresis was performed on aliquots of cell media after 6 hours of UA treatment in the presence or absence of specific inhibitors. Gelatinolytic activity is shown as follows: DU145 cells in the absence of UA(1), in the presence of UA (25 μM)(6 hrs)(2), pre-treated with PD98059 for 1 hour followed by 6 hour exposure to UA (25 μM)(3); pre-treated with LY294002 for 1 hour followed by 6 hour exposure to UA (25 μM)(4); pre-treated with Calphostin C for 1 hour followed by 6 hour exposure to UA (25 μM)(5); pre-treated with JSH-23 for 1 hour followed by 6 hour exposure to UA (25 μM)(6); pre-treated with SM-7368 for 1 hour followed by 6 hour exposure to UA (25 μM)(7). PD98059, LY294002, Calphostin C, JSH-23, and SM-7368 were used at the concentrations 10 μM, 50 μM, 500 nM, 50 μM, and 10 μM, respectively. The graph is representative of results obtained from at least three independent experiments. Each independent experiment consisted of duplicate assay replicates.

significantly. The observation of UA mediated cytotoxicity coincides with the published literature on UA (Aparecida et al. 2006; Hollosy et al. 2000; Ikeda et al. 2008; Kassi et al. 2007; Ma et al. 2005; Manu and Kuttan 2008; Meng et al. 2009 Yim et al. 2006; Zhang et al. 2009 [1]).

Treatment of DU145 human prostate cancer cells with UA (10 and 25 μM) for 6 hours inhibited the activity of MMP-9 and Pro-MMP-2, and MMP-2 activity. These findings correlate with the inhibition observed with UA treatment of cell lines other then DU145 human prostate cancer cells, such as HT1080, Jurkat, 293, KBM-5, H1299 and U937 (Hee-Jae et al. 1998; Hee-Jae et al. 1996; Shishodia et al. 2003). These observations also support previous work from our laboratory in which inhibition of MMP activity was observed when treating DU145 cells with whole cranberry extract (MacLean 2009). The effects of UA on apoptosis via the inhibition of MMP expression may be linked through glucocorticoid receptor localization (Lai et al. 2007). Lai and colleagues' suggested that there is a connection between UA-induced apoptosis and an increase in MMP gene induction. Lai's work was interesting, as they also observed that UA had a cytotoxic effect on DU145 cells, however, rather than an induction of MMP activity, they showed an inhibition of MMP activity is presented. Taken together, these data suggest that cells utilize different proteins to control and modulate MMPs regulation both directly and indirection by modulation of the mechanisms behind their production.

Two important modulators of MMP activity are TIMP-1 and TIMP-2 (Visse and Nagase 2003). TIMP-1 and TIMP-2 are inhibitors of MMP activity and bind directly to the active forms of MMPs in a 1:1 ratio (Visse and Nagase 2003). UA treatment (10 and 25 μM) of DU145 human prostate cancer cells resulted in an induction of TIMP-1/-2 protein expression. The

induction of TIMP-1/-2 protein expression in response to UA treatment observed in this study is similar to observations of UA affects resulting in an induction of both TIMP-1 and TIMP-2 in fibrosarcoma cells in which treatment of UA inhibited MMP activity and expression (Hee-Jae et al. 1996).

There are two other temporal modulators of MMP activity which were examined in this chapter, EMMPRIN and RECK. UA treatment (10 and 25 μM) of DU145 human prostate cancer cells was observed to inhibit both EMMPRIN and RECK protein expression. The inhibition of EMMPRIN correlates well with the UA-mediated inhibition of MMP-9, MMP-2 and Pro-MMP-2 protein expression, as EMMPRIN is an inducer of MMP activity. RECK is an inhibitor of MMPs, which would be expected to be induced by UA treatment similar to the TIMP induction. Interestingly, RECK protein expression was counter to the expected result, and was shown to be inhibited by treatment of UA (10 and 25 μM). Changes in RECK expression could be tied to the induction of gene expression observed in the literature with UA treatment (Chang et al. 2006; Shan et al. 2009). RECK gene expression is strongly related to the methylation status of the gene promoter: specifically, hypermethylation of the RECK gene has been correlated with a decrease in cancer prognosis (Chang et al. 2006; Khanh et al. 2008). UA has been observed to interact with an important signal transduction pathway, MAPK, the modulation of this pathway may lead to changes in gene expression of RECK, leading to the inhibition observed (Chang et al. 2006). Alternatively, the inhibition of RECK could also be a cell line specific effect or potentially be a compensatory measure by a cell induced to undergo apoptosis, but still trying to regain homeostasis. After an examination of the literature, it appears that no observations on the effect of UA or any other terpenoids on EMMPRIN or RECK have been reported. Therefore, this present inhibition of both EMMPRIN and RECK in DU145 human prostate cancer cells

treated with UA may be the first demonstration of UA-mediated alterations in EMMPRIN and RECK expression.

After observing the effects of UA on MMP activity and MMP modulators, detailed inhibitor studies were performed in order to elucidate the possible signalling pathways utilized by UA to modulate the MMP activity of DU145 human prostate cancer cells. Four key signal transduction pathways were chosen through which UA may be mediating its effects: MAPK, PI3K, PKC and NF-κB. A partial relief of UA-mediated inhibition of MMP-9 activity was observed when treating with inhibitor PD98059, which specifically inhibits the MAPK pathway. Indeed, UA has been observed to induce much of its cytotoxic effects through the MAPK pathway (Achiwa et al. 2007; Liu and Jiang 2007; Pathak et al. 2007; Shan et al. 2009; Xavier et al. 2009).

UA has been shown to induce apoptosis through the inhibition of the NF-κB pathway (Achiwa et al. 2007; Manu and Kuttan 2008; Shishodia et al. 2003). To date, the correlation between UA-mediated inhibition of MMP activity in DU145 human prostate cancer cells and the modulation of the NF-κB signal transduction pathway has not been fully investigated. Following the initial inhibitor experiment, a pair of NF-kB inhibitors, JSH-23 and SM-7368 were used to inhibit UA mediated MMP inhibition of DU145 cells. A relief of the inhibition of MMP activity by UA on MMP-9, MMP-2 and Pro-MMP-2 was observed when treated with JSH-23, but not with SM-7368. JSH-23 targets the p65 subunit of NF-kB and inhibits the translocation of activated p65 from the cytoplasm to the nucleus where it induces gene regulation. Therefore, as the treatment with JSH-23 completely inhibits the effect of UA on MMP-9, MMP-2 and Pro-MMP-2 activity, this suggests that UA relies, in part, on the translocation of the p65 subunit of NF-κB to exert its effect.

To summarize, treatment of DU145 human prostate cancer cells with UA inhibits MMP-9, MMP-2 and Pro-MMP-2 activity. This inhibition of activity is due in part to the concomitant induction of TIMP-1/-2 and inhibition of EMMPRIN. The UA-mediated inhibition of the gelatinases is potentially due to the compound's ability to interact with MAPK and NF-κB signal transduction pathways. To the best of our knowledge, this work describes the first demonstration of UA-mediated inhibition of MMP activity through a combination of MMP modulators and signal transduction pathways in DU145 human prostate cancer cells.

Chapter six: General Discussion

6.1 Conclusions

This thesis presents the results from studies examining the effects of treatment by specific concentrated fractions derived from the American cranberry (*V. macrocarpon)* on DU145 human prostate cancer cells. Previous studies have found that whole cranberry extract induces apoptosis and modulates MMP activity in DU145 cells (MacLean 2009; Matchett 2005). The present study builds on this work to study several enriched fractions from cranberries and their effect on DU145 human prostate cancer cells. Specifically, Proanthocyanidins (Pacs) and Flavonols (Flavs), two enriched flavonoid fractions, were studied to further understand their influence on the observed induction of apoptosis by treatment with whole cranberry extract. Ursolic acid (UA), the third compound of interest, was studied to elucidate the mechanisms behind the compound's ability to modulate MMP expression.

In this study, Pacs and Flavs induced apoptosis *in vitro* via modulation of proteins in the pathways of apoptosis. Pacs treatments induced the intrinsic apoptotic pathway of apoptosis while inhibiting the extrinsic pathway. Flavs treatments differed slightly; while the fraction inducted apoptosis through the intrinsic pathway, the extrinsic pathway was not inhibited as comprehensively as Pacs treatment of DU145 human prostate cells.

Initially nucleosome formation and Poly ADP ribose polymerase (PARP) cleavage, two events used as indicators of apoptosis (Huerta et al. 2007; Kuo et al. 2005) were assayed. Pacs treatment at concentrations of 10 and 25 μg/mL resulted in a significant induction of nucleosome formation ($P < 0.05$ and $P < 0.01$, respectively) and induced PARP cleavage at 25 μg/mL ($P <$

0.05). PARP cleavage was not statistically significant at 10 μg/mL. Flavs treatments of DU145 cells also induced nucleosome formation, but only at 50 μg/mL ($P < 0.05$) and did not statistically significantly induce PARP cleavage even at the highest dose tested 50 μg/mL. Consequently, this shows that there is a differential effect on the induction of apoptosis between the two fractions assayed. Of interest is the observation of the induction of cytotoxicity and nucleosome formation at 50 μg/mL of Flav treatment, while at the same concentration there was no induction of PARP cleavage. It is possible that there was an induction of apoptosis without the presence of PARP cleavage, though the caspase-3 mediated induction of apoptosis generally results in both the cleavage of PARP followed by the induction of nucleosome formation. This indicates that the inhibition of PARP cleavage is not due to a direct and complete inhibition of caspase-3, as nucleosome formation is induced. Rather it is possible that the induction of apoptosis without PARP cleavage is due to either transient inhibition of caspse-3, which is unlikely as this would also inhibit nucleosome formation, or possibly the constituents are reacting in complex methods to induce apoptosis while inhibiting PARP cleavage. Potentially, the compounds found in the enriched Flav fraction may be acting on PARP to inhibit the binding of caspases-3 or to counter the caspase-3 mediated cleavage of PARP. Further work into the Flavs induction of apoptosis in the absence of PARP cleavage would be required to substantiate any hypotheses.

To further substantiate the apoptotic affect observed with treatment of DU145 cells with the enriched fractions, key proteins of both the intrinsic and extrinsic pathway were assayed to understand what proteins the fractions may be using to induce apoptosis (Figure 6.1). One of the principal defining features of the extrinsic pathway is that the pathway is induced through activation of death receptors (DR) in the extracellular membrane. Ligands secreted to the

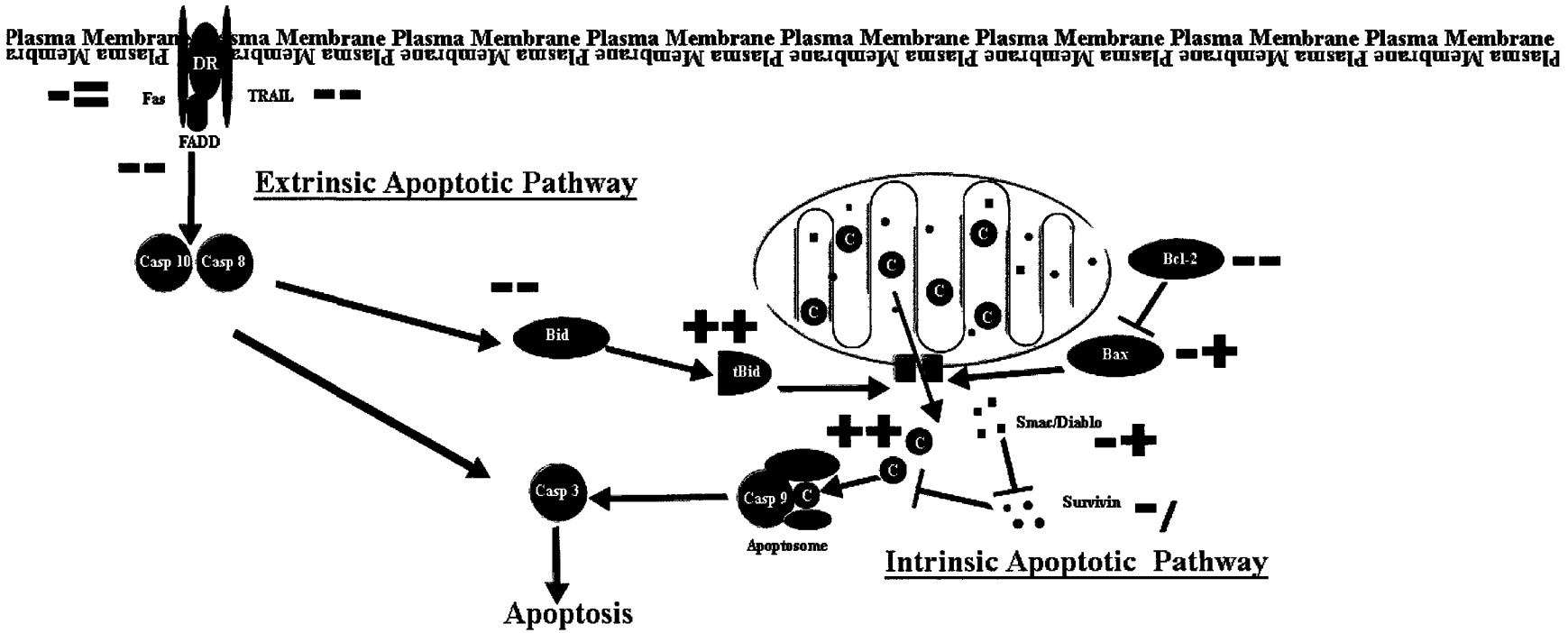

Figure 6.1 The interaction of key proteins involved in the extrinsic and intrinsic pathways. The symbol on the left denotes the effect on the protein when DU145 cells are treated with Pacs. The symbol on the right denotes the effect on the protein when DU145 cells are treated with Flavs. – is indicative of inhibition, + is indicative of induction, = is indicative of no change, and / represents that Survivin was not studied with respect to Flav treatment of DU145 cells

extracellular membrane may induce apoptosis, though other external ligands can also induce apoptosis through these DRs. The key ligands Fas and TRAIL were studied to understand whether the Flavs or Pacs enriched fractions affected the extrinsic pathway. Treatment with both Pacs and Flavs resulted in an inhibition of Fas and TRAIL protein expression indicating that these fractions may be interacting with the extrinsic pathway. This effect does not appear to be restricted to the cellular ligands. FADD, an adaptor protein associated with the Fas receptor, was also inhibited by treatment of both Pacs (10 and 25 μg/mL) and Flavs (25 and 50 μg/mL). The induction of nucleosome enrichment by both Pacs and Flavs in concert with the induction of PARP cleavage at 25 μg/mL of Pacs indicates apoptosis is occurring. The assay of key proteins involved in the extrinsic pathway suggests that there are multiple proteins within this pathway that are inhibited by Pacs and Flavs treatment. Despite Pacs and Flavs modulating the extrinsic pathway, this effect is not related to the induction of apoptosis, therefore the apoptosis already observed must be through other means.

The other common apoptotic pathway, the intrinsic apoptotic pathway, was an obvious choice for further study. Select bcl-2 family proteins were assayed to determine whether there was any effect by treatment of DU145 cells by either Pacs or Flavs on this pathway. Bax a pro-apoptotic protein and Bcl-2 its antagonist were both inhibited in DU145 human prostate cancer cells by treatment of 10 and 25 μg/mL of Pacs. Flav treatment at 25 and 50 μg/mL inhibited Bcl-2 and induced Bax protein expression. The inhibition of both Bax and Bcl-2 by Pacs treatment complicates the conclusions that can be generated from a change in these two bcl-2 proteins. The relative difference between the two proteins can be used as an indicator as to whether the membrane of the

mitochondrion will change permability and release sequestered proteins leading to the induction of apoptosis. The Flav mediated inhibition of Bcl-2 and induction of Bax strongly suggest that there would be a change towards a cell with little inhibition of the pore formation by Bax potentially leading to an induction of apoptosis. Pacs treatment of DU145 cells leading to an inhibition of both Bax and Bcl-2 is inconclusive. Potentially if the inhibition is skewed towards a stronger inhibition of Bcl-2, an environment to induce apoptosis may still occur, but current data in this study is not comprehensive. To substantiate the change in permeability of the mitochondrion and the induction of apoptosis, Bid, another bcl-2 family protein capable of inducing pores was studied.

Both Pacs (10 and 25 μg/mL) and Flavs (25 and 50 μg/mL) inhibited the protein expression of Bid. Activation of Bid requires the cleavage of the protein into a 15 kDa truncated form called tBid. To examine whether the inhibition of Bid protein expression was due to changes in protein amounts within the cell, or due to the cleavage of Bid into the truncated form, the protein expression of tBid was assayed. Both Pacs (25 μg/mL) and Flavs (25 and 50 μg/mL) induced the formation of tBid suggesting that Bid is being activated potentially leading to apoptosis induction. Therefore, the observed inhibition of Bid is due to the cleavage or activation of Bid, rather than a reduction in protein expression. While the proteins upstream in the mitochondrion appear to be induced and therefore set up an environment that would liberate proteins from the mitochondrion, mainly cytochrome c, further work on the downstream proteins are required to understand whether an apoptotic signal will result in apoptosis.

Protein expression of cytochrome c was induced by both Pacs (10 and 25 μg/mL) and Flavs (25 and 50 μg/mL) in DU145 cells. This finding suggests that a change in the

permeability of the mitochondrion would lead to a larger induction of apoptosome formation leading to a larger apoptotic signal. The increase in protein expression of cytochrome c, along with the induction of proteins that form pores that increase the permeability, suggests that both Pacs and Flav fractions induce apoptosis through the intrinsic pathway. Several mitochondrion-segregated proteins associated with apoptosis proteins were also studied. Survivin, a member of the inhibitor of apoptosis family (IAP), was inhibited by Pacs treatment at 10 and 25 μg/mL. Survivin was not studied with Flav treatment in lieu of studying another protein associated with the extrinsic pathway. Smac/DIABLO, an inhibitor of the IAP family, was inhibited by Pacs treatment at 10 and 25 μg/mL, but was induced by Flav treatment at 25 and 50 μg/mL. Pacs inhibition of Smac/Diablo could potentially allow other members of IAP to inhibit Pacs mediated apoptosis, but the inhibition of Survivin, one of the most well studied IAPs, would suggest that the inhibition of Smac/Diablo not be an overriding factor for the initialization of apoptosis by Pacs. Counter to the inhibition of Smac/Diablo, Flav induced Smac/Diablo release suggesting that this fraction would further exasperate the cell towards an apoptotic induction.

Of interest to this study was the understanding of whether the effects of Pacs and Flavs treatment were segregated to the apoptotic proteins involved in apoptosis pathways directly or whether the fractions also induce effects on apoptosis by interacting with proteins outside the normal apoptotic pathways. Par-4, a potent inducer of apoptosis is an example of a tissue specific protein that is not normally included as part of the apoptotic pathways (Sells et al., 1994). Both Pacs (10 and 25 μg/mL) and Flavs (25 and 50 μg/mL) treatment of DU145 human prostate cells induced Par-4 protein expression. This

suggests that these enriched fractions may induce key proteins in the intrinsic pathway, and have the potential to induce proteins outside the common apoptotic pathways to modulate or induce apoptosis.

In general, these findings support the hypotheses that Pacs and Flavs induce apoptosis in DU145 prostate cancer cells through either the intrinsic or extrinsic apoptotic pathways or both. Induction of apoptosis by both Pacs and Flavs treatments occur through the intrinsic pathway and both fractions show potential to down regulate the extrinsic pathway. UA a compound derived from cranberry was also studied. The UA portion of this study did not focus on the ability of this compound to induce apoptosis, rather focused on the ability of this compound to modulate the MMP activity of DU145 human prostate cells.

The results of the UA portion of this study are described in detail in the discussion of Chapter 5. To summarize briefly, UA inhibits MMP activity in DU145 cells by modulating the expression of key regulators of MMPs. This regulation involved two signal transduction pathways, NF-kB and MAPK. As such, the hypothesis that UA inhibits the activity of MMPs in DU145 human prostate cancer cells by modulating the expression of MMP regulators was supported.

This study supports the hypotheses that "Pacs and Flavs induce apoptosis in DU145 cancer cells through either the intrinsic or extrinsic apoptotic pathways or both" and "UA inhibits the activity of MMPs in DU145 human prostate cancer cells by modulating the expression of MMP regulators". These findings further support the general hypothesis that "**Cranberry phytochemicals affect the behavior of prostate cancer cells *in vitro***"

The results presented here are generally supportive of current published research. Despite this there are several interesting deviations from the current literature. Research on the effects of Pacs has not looked extensively at the extrinsic pathway though polyphenols have been studied with respect to the extrinsic pathway, and have been reported to induce extrinsic pathway (D'Archivio 2008). Fas protein expression was observed to be induced by extracts from tea and coffee along with proanthocyanidin-gallates derived from *Myrica rubra* (D'Archivio 2008; Kuo et al. 2004; Kuo et al. 2005). The effect of EGCG, a compound similar in structure to the Pacs monomers, is observed to induce apoptosis at least partially through a TRAIL-mediated path (Siddiqui et al. 2007). This suggests that while the study reported here showed inhibition of the extrinsic pathway; similar compounds induce or act through the extrinsic pathway. The inhibition of the extrinsic pathway may be a cell line specific effect in the presence of Pacs or Flav treatment. The inhibition could also be a compensatory measure by the cell to regain normal function in the presence of an apoptotic induction through the intrinsic pathway. The difference may also be due to difference in the compounds found in the Pacs fraction or perhaps this difference may be a cell line dependent effect.

The effect of EGCG on the intrinsic pathway has been observed to induce Bax and inhibit Bcl-2 protein expression (Hsu et al. 2004; Qanungo et al. 2005; Roy et al. 2005). Grape seed extracted (GSE) Pacs have been reported to induce Bax and inhibit Bcl-2 in DU145 cells (Neuwirt et al. 2008). Interestingly, not all GSE Pacs studies have consistent results. The inhibition of both Bax and Bcl-2 by Pacs treatment reported in this study correlates with GSE Pacs treatment of cardiomyocytes which resulted in the inhibition of both proteins (Du and Lou, 2008). Treatment of EGCG induced the

cleavage of Bid, another bcl-2 family member, into tBid in a number of cancer lines, not including prostate cancer (Nishikawa et al. 2006). The observation of Pacs mediated modulation of the Bcl-2 family members' correlates well with current literature.

The results obtained with the enriched Flavs fraction are also consistent with the current literature. Dihydroxyflavonol treatment of cardiac cells has been observed to inhibit Fas and Bax protein expression (Wang et al. 2009). Dihydoxyflavonol has also been reported in the previous study to inhibit the release of cytochrome c from the mitochondria (Wang et al. 2009). Dihydroflavonol extracted from *Blumea balsamifera* has been observed to inhibit FADD protein expression and induce the cleavage of Bid into tBid (Hasegawa et al. 2006). Interestingly, Hasegawa et al. (2006) did not report any change in the protein expression of Bax and Bcl-2.

UA mediated inhibition of MMP activity correlates and expands on the current literature. UA inhibition of MMP activity has not been observed in prostate cancer cells, but several other cancer cell types have been inhibited by UA treatment (Hee-Jae et al. 1998; Hee-Jae et al. 1996; Shishodia et al. 2003). In this study, UA induced the protein expression of both TIMP-1 and TIMP-2 which is supported by current literature on UA treatment of fibrosarcoma cells where similar inductions have been observed (Hee-Jae et al. 1996). UA also inhibited the protein expression of EMMPRIN and RECK, two modulators of MMP activity. This appears to be the first use of EMMPRIN and RECK to study the effects of UA treatments and therefore are novel.

UA has been shown to decrease MMP activity, though the mechanism by which this inhibition occurs is unknown. In contrast, cytotoxic and apoptotic effects of UA have

been comparably well studied. UA mediated cytotoxicity and apoptosis have been associated with both MAPK and NF-kB pathways (Achiwa et al. 2007; Liu and Jiang 2007; Manu and Kuttan 2008; Pathak et al. 2007; Shan et al. 2009; Shishodia et al. 2003; Xavier et al. 2009). This study has found that UA inhibition of MMP activity occurs through either MAPK or NFkB pathways. The observations that UA can interact with these pathways support the literature describing the affects of this compound.

6.2 Limitations

The results presented are generally supported in current literature. However, a number of differences have been noted. These are likely due to cell specific effects and/or differences in the constituents making up the enriched fractions. Further reasons are described in detail below.

There are several limitations that are inherent to this *in vitro* study and to *in vitro* studies in general. Firstly, concentrations used may be higher than could be found available within tissues when used in organisms. Also the ingestion and transit through the digestive and vascular systems may alter the structure of compounds and therefore potentially change their effects observed *in vitro*. Secondly, it is difficult to extrapolate the effects observed *in vitro* to human studies or even *in vivo* animal studies. Thirdly, some of the compounds used such as: Pacs and Flavs were enriched fractions not pure samples. Therefore it is difficult to understand what effect a purified fraction would have on a system. Potentially the effects may be similar, but to build hypotheses of what purified fractions would do based on the study of enriched fractions is difficult. Finally, the generation of the fractions themselves may differ. While every effort was made to

ensure the same fraction sample was used in each portion of this study, it was not possible to only use a single enriched fraction sample. As such, there could be confounding variables in the study due to the common deviations in the preparation of the individual batches of the enriched fractions leading to slightly different effects between them.

There are some other limitations that are more specific to this study. While the Pacs and Flavs studies assay key proteins of both the intrinsic and extrinsic pathways, there are many proteins that affect apoptosis that are not studied. Elaboration of this study to include a wider subset of proteins would be beneficial to understand the full effect of the Pacs and Flav fractions. Particular proteins of interest, such as: APAF-1, Bak and Bcl-xL are all required for the normal induction of apoptosis. APAF-1 is involved in the formation of the apoptosome which is required for caspases-3 activation. While cytochrome c is also required, without APAF-1 no apoptosome would be formed. Bax is an important pro-apoptotic protein, but requires a heterodimerization with Bak to form the pores to change the permeability of the mitochondrion. Bcl-xL is an important anti-apoptotic protein that has important implications for inhibiting both Bax and tBid pore formation. The expansion of this study to encompass other proteins would strengthen this study and any hypotheses generated from it.

Furthermore, there are some difficulties that limit the research presented in this study. Chief among these difficulties is the induction of cytotoxicity by UA. The cytotoxicity of Pacs and Flavs does not limit their study as studying apoptosis requires the initiation of cellular death. The study of UA mediated MMP inhibition is different. As UA was observed to induce statistically significant cell death in DU145 cells, it

becomes difficult to ensure that the results generated from the UA study, namely the inhibition of MMP activity, is due to the compounds ability to modulate key proteins and regulators and not due to the reduction in viable cells producing MMPs. The observation of changes in protein expression of both activators and inhibitors of MMP activity show that the UA mediated MMP inhibition is due, at least in part, to the compound's ability to interact with the regulators, but this result is clouded by the induction of cellular death by UA.

Another limitation of this work is the choice of a single cell line of study. While DU145 human prostate cancer cells are a well studied prostate cell line, the use of other prostate cell lines, perhaps a hormone sensitive line such as LNCaPs would provide a more structured view on the effects of these fractions on prostate cancer cells. The lack of an alternative "normal" prostate cell line also poses problems as the effects on the DU145 cancer cells may also affect the behaviour of normal untransformed cells. Without the expansion of this work to inclusion of a "normal" prostate cell line it is difficult to suggest the direct benefit of UA. These limitations do not directly contradict the results presented in this investigation, but rather highlight potential methods or targets that would be beneficial to explore. Further work to answer many of the limitations would increase our understanding of the effects of the enriched fractions from cranberries

6.3 Future Directions

Previously, MacLean (2009) found similar results where the induction of apoptosis and inhibition of MMP expression was observed by treatment of DU145 human prostate cancer cells with whole cranberry extract. The analysis of the Pacs/Flavs/UA mediated

effects elucidates and identifies some of the mechanisms through which constituents of *V. macrocarpon* may influence specific behaviors of prostate cancer cells *in vitro*. The inhibition of MMP activity by UA and the induction of apoptosis by Pacs and Flavs fractions on DU145 cells provide further evidence in support of the potential health benefits associated with cranberries. Therefore, these results offer further support for the inclusion of cranberries as part of a "cancer protection" healthy diet.

While the inclusion of cranberries in a "cancer protection" diet may be supported by this study, future work is warranted before the introduction of concentrated or enriched fractions are used in human studies. As listed previously within the limitations, the concentrations used pose some problems. While the concentrations used induce potential therapeutic effects, it is unknown if these concentrations can be reached, or that the compounds of interest will reach the desired target-of-interest unmodified. Therefore, human studies with Pacs/Flav enriched fractions and UA are a distance from use in human studies. Further research into the *in vivo* effects of these compounds would be an obvious choice. Along with preliminary *in vivo* studies, the elaboration of *in vitro* studies would allow for a more comprehensive understanding of the induction of behavior in DU145 human prostate cancer cells.

While UA was purchased as a purified compound, Pacs and Flavs fractions were enriched fractions that potentially contain many different compounds that could be of interest for future work. The targeting of specific compounds within the various fractions would be beneficial to discover the principal effectors. This could eventually allow targeted work on purified compounds that could lead to the development of pharmacological products.

6.4 Summation

In summary, **cranberry phytochemicals can affect the behavior of prostate cancer cells *in vitro*.** *V. macrocarpon* is a source of potential anti-cancer compounds that have the ability to modulate the behavior of prostate cancer cells. As such, cranberries may provide potential chemoprevention and/or chemoprotective properties against prostate cancer and possibly other cancers too. Much more investigation is warranted. Research to clarify and identify the exact nature of the compounds or combinations of compounds responsible for the response noted in this study is required. Further *in vitro* studies could extend the investigations described in this thesis. The work described in this thesis also lays the foundation for future *in* vivo studies and for clinical trials to validate the potential for cranberries to be a valuable source of anti-cancer compounds.

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Appendix

Prearation and Chromatographic Analysis of Cranberry Extracts by the Laboratory of Dr. Catherine Neto

Solvents

Solvents used in extraction and analysis were HPLC-grade and were purchased from Pharmco (Brookfield, CT, USA). Sephadex LH-20 was purchased from GE Healthcare (Westborough, MA, USA). Diaion HP-20 was purchased from Supelco, Inc. (St. Louis, MO, USA). Commercial standards used in the HPLC analysis were cyanidin-3-galactoside and quercetin-3-galactoside (Chromadex; Irvine, CA), p-hydroxycinnamic acid (Sigma-Aldrich; St. Louis, MO, USA) and procyanidin A2 (Indofine; Hillsborough, NJ). All other materials, unless stated otherwise, were purchased from Sigma-Aldrich Canada (Oakville, ON).

Preparation of cranberry extracts/fractions

Cranberry fruit (*Vaccinium macrocarpon;* Early Black variety) was harvested in November 2006 at the State Bog in Wareham, Massachusetts, USA. The fruit was flash-frozen in liquid nitrogen and stored at -20^0 C until use. For preparation of whole cranberry extract polyphenolic extract, 1.06 kg of fruit was extracted several times with 300 mL aliquots of 40/40/19/1 methanol/acetone/water/formic acid at room temperature, each time pulsing with a Waring blender for 5 minutes, filtering after 30 minutes, and collecting the filtrate. This was repeated with the pulp until most of the color was gone.

The combined filtrates were concentrated *in vacuo* and then freeze-dried. Free sugars were removed to produce a concentrated whole crude extract (WCE) using chromatography on a Diaion HP-20 column (4.5 X 30 cm). The sample was then applied to the column, allowed to adsorb, then washed with several column volumes of distilled water to remove free sugars. The column was then eluted with methanol until no pink color remained and then rinsed with acetone. The methanol and acetone extracts were combined, concentrated and freeze-dried to produce 10.5 g of WCE.

Fractions were prepared from WCE to investigate activities of the flavonoids predominant in cranberries, including flavonols, and proanthocyanidins. These were separated using Sephadex LH-20 chromatography (3.0 X 22 cm column) as follows. WCE (500 mg) was dissolved in a minimum amount of distilled water and applied to the column. Fractions were collected and analyzed for composition by HPLC. The column was firstly eluted with 70/30 methanol/water yielding a fraction containing predominantly phenolic acids (yellowish in color), followed by a second fraction containing primarily flavonol glycosides (reddish-yellow). Finally, the column was eluted with 70/30 acetone/water to produce a fraction containing mainly proanthocyanidins (pinkish-tan in color). Separation of 500 mg of crude extract yielded approximately 35 mg of flavonol-enriched fraction (Flav) and 195 mg of proanthocyanidin-enriched fraction (Pac).

Characterizations of Extracts

Extracts and fractions were analyzed with a Waters HPLC chromatography system equipped with two pumps, an ultraviolet-visible photodiode array detector (PDA) and

Millennium software. The crude extract (WCE) was analyzed using a Waters Symmetry C18 reversed phase column (4.6 X 250 mm) and gradient elution program employing solvent A (4% aqueous acetic acid) and solvent B (4% acetic acid in methanol). Samples were dissolved in 100% solvent A at a concentration of 20 mg/mL for crude extract and 5 mg/mL for enriched fractions. Linear gradient elution at a flow rate of 0.80 mL/min began at 0 minutes with 99% solvent A with a gradient to 80% solvent A over 30 minutes, a gradient of 70% solvent A at 70 minutes and finally a gradient of 100% solvent B at 90 minutes. Flavonol glycosides are detected at 355 nm and proanthocyanidin at 280 nm. Content of the fractions was quantified by the standard curve method employing a commercial standard of cyanidin-3-galactoside and using the above program with peak absorbances measured at 520 nm. The Flav was analyzed with diode-array detection on a Waters Symmetry C18 reverse- phase column (3.0 X 150 mm). The HPLC program used gradient elution employing solvent A (2% aqueous acetic acid) and solvent B (2% acetic acid in methanol). Samples were prepared with 50/50 solvent A and B at 3 mg/mL concentration and analyzed using a linear gradient beginning at 0 minutes with 95% solvent A, a gradient to 75% solvent A over 5 minutes, a gradient to 65% solvent A at 25 minutes, a gradient to 60% solvent A at 35 minutes, and a gradient to 5% solvent A at 45 minutes. Flavonol glycosides were quantified as quercetin-3-galactoside by the standard curve method, measuring peak absorbance at 355 nm. The proanthocyanidin-enriched fraction (PAC) was analyzed by HPLC using a Waters Xterra C8 column (4.6 x 250 mm) to verify the presence of proanthocyanidins and the absence of contaminants. The program used gradient elution with solvent A (2% aqueous acetic acid) and solvent B (methanol). Samples were prepared in 50/50 solvent

A and B at 10 mg/mL concentration and analyzed using a gradient with a flow rate of 0.8 mL/min, beginning at 0 minutes with 100% solvent A, with a gradient to 100% solvent A at 5 minutes, then a linear gradient to 100% solvent B at 60 minutes, holding until 80 minutes. Proanthocyanidins were detected by characteristic peak elution patterns at 280 nm (which is the absorbance maximum for epicatechin-based proanthocyanidins). Procyanidin A2 was identified in the extracts based on comparison to a commercial standard. The composition of the proanthocyanidin fraction was verified by MALDI-TOF MS analysis by Dr. Christian Krueger at the University of Wisconsin using methods previously described (Neto et al. 2006).

Analysis of Fractions

The HPLC chromatogram for the Flavs is shown in Figure 1. The presence of flavonol glycosides was verified by detection of peaks with absorbance maxima close to 355 nm. Peaks characteristic of anthocyanins were not present. The major flavonol glycosides detected at 355 nm were identified based on comparison to published data (Yan et al. 2002; Vvedenskaya et al. 2004). These are identified in Figure 3. Total flavonol content in the crude extract was 6.4% and in the flavonol-enriched fraction 53%. The Flavs did not contain a significant quantity of anthocyanins based on HPLC analysis. The MS profile shown in Figure 2 indicates that the sample contains proanthocyanidin oligomers ranging in size from two to eight epicatechin units with at least one A-type. Based on peak intensity, the major oligomers appear to be dimers, trimers and tetramers with lesser amounts of the larger oligomers present; similar to the composition reported previously (Neto et al. 2006). The presence of procyanidin A2 in the crude extract and in the PAC fraction was confirmed by HPLC in comparison to the commercial standard.

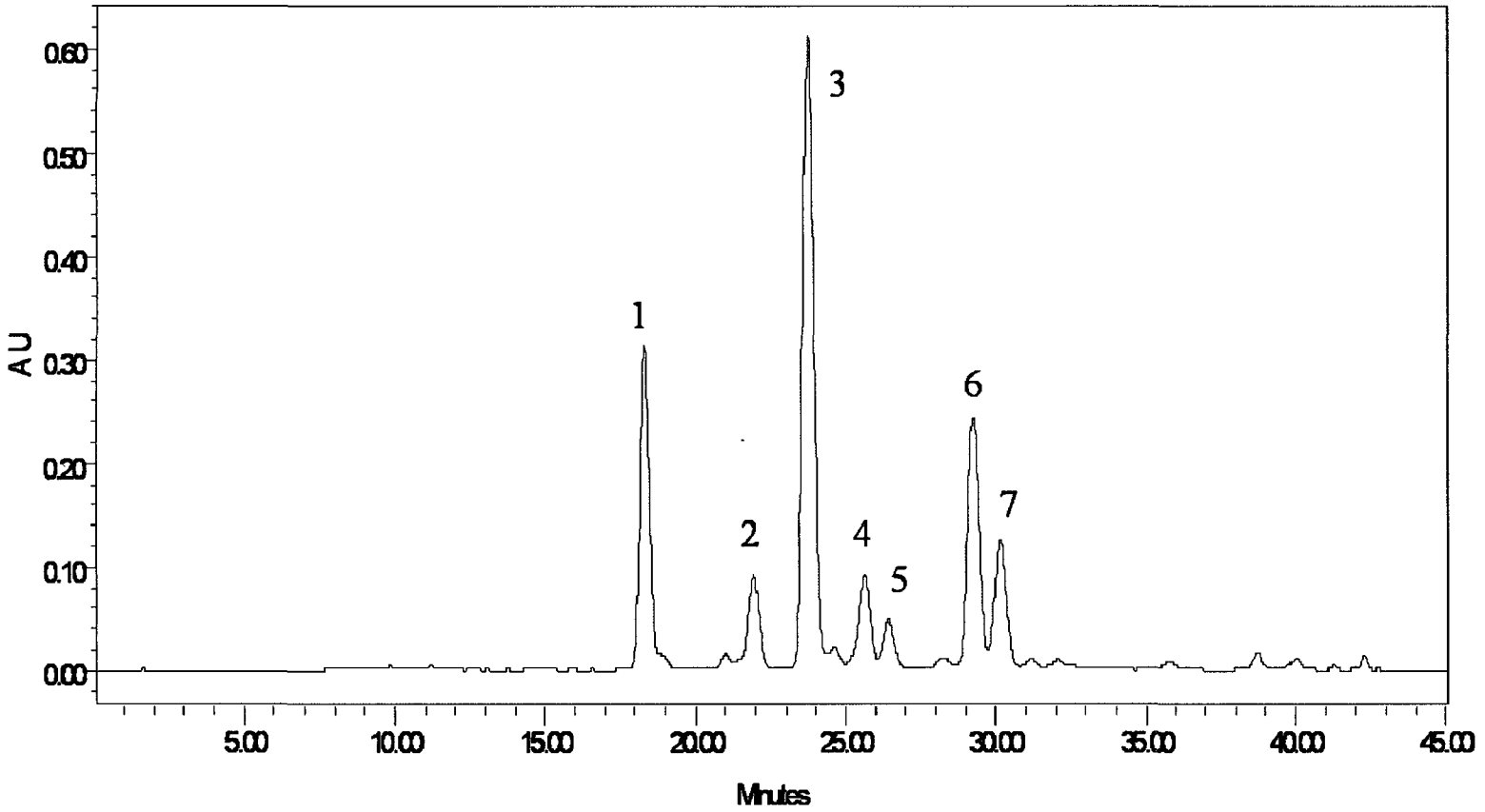

Figure A.1: HPLC profile of flavonol-enriched cranberry fraction (FLAV). Flavonol glycosides were detected at 355 nm and identified by comparison to published data as (1) myricetin-3-galactoside, (2) myricetin-3-arabinoside, (3) quercetin-3-galactoside, (4) quercetin-3-xyloside, (5) quercetin-3-arabinofuranoside , (6) quercetin-3-arabinopyranoside and (7) quercetin-3-rhamnoside.

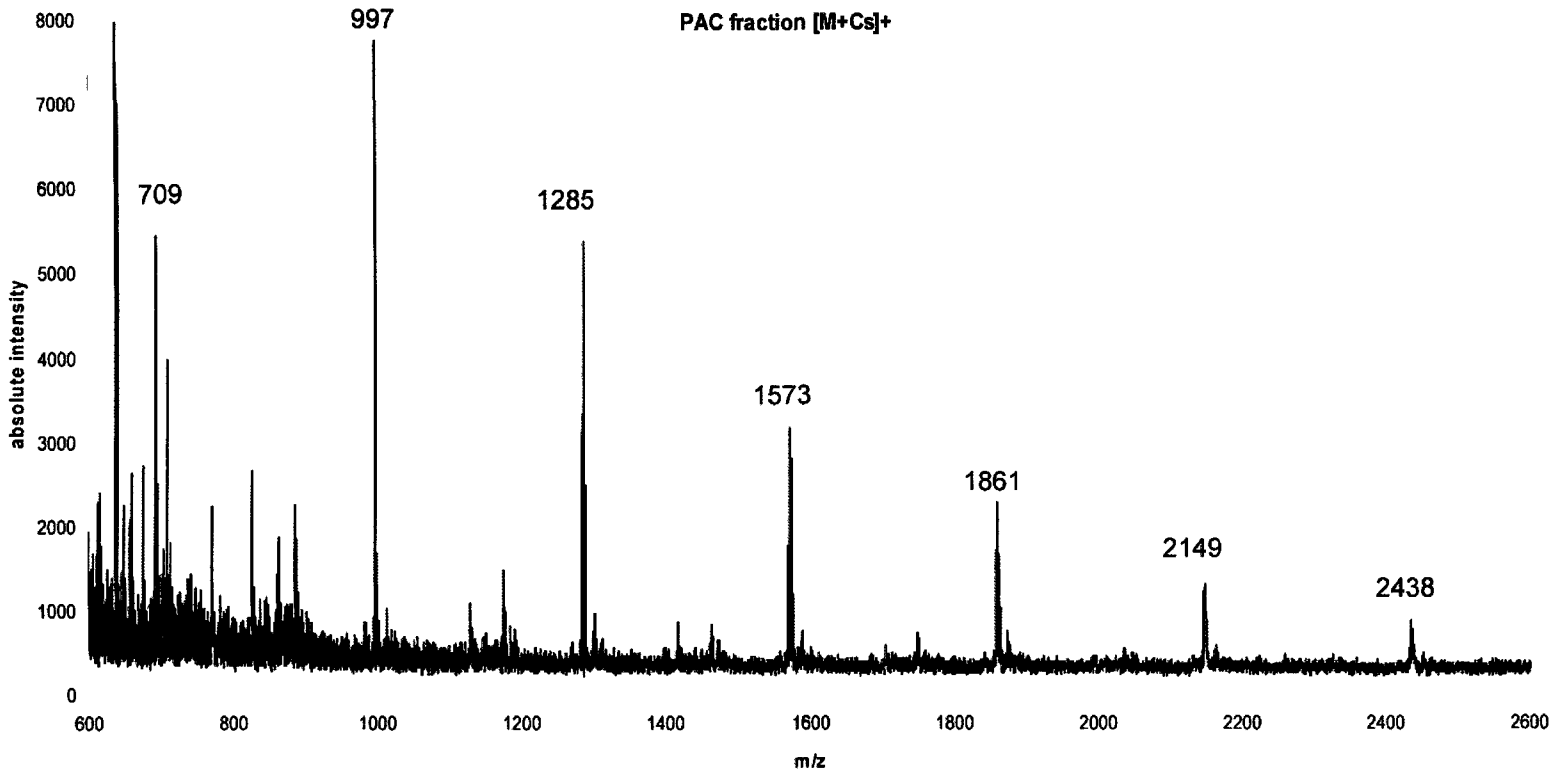

Figure A.2: MALDI-TOF mass spectrum of proanthocyanidin-enriched cranberry fraction (PAC) showing the presence of A-linked proanthocyanidins. Masses detected represent epicatechin dimer (M+Cs = 709 amu), trimer (M+Cs = 997 amu) tetramer (M+Cs = 1285 amu) and larger oligomers.