ANTI-CANCER ACTIVITIES OF *VACCINIUM MACROCARPON*: APOPTOSIS INDUCTION AND MATRIX METALLOPROTEINASE INHIBITION

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ABSTRACT

Increasingly, dietary intervention is being used as a means of preventing the onset or delaying the development of cancer. Targets of such intervention often include cell death pathways and the mechanisms responsible for cancer invasion and metastasis. Previously, research has identified the North American Cranberry's (*Vaccinium macrocarpon*) apoptosis-inducing and invasion-limiting activities in a variety of tumour cell lines, though little work has been conducted to determine the mechanisms responsible for these anticancer activities. The work presented in this thesis serves to elucidate some of those mechanisms by evaluating the effects of cranberry phytochemicals on DU145 human prostate adenocarcinoma and MDA-MB-231 human breast carcinoma cells. Briefly, it was found that treating DU145 cells with cranberry extracts induces apoptotic cell death through caspase-8-mediated cleavage of Bid to truncated Bid, resulting in the release of apoptogenic factors from the mitochondria and the subsequent activation of caspase-9 – ultimately resulting in PARP inhibition and an increase in cytoplasmic nucleosome enrichment (hallmarks of apoptosis). Furthermore, cranberry has also been observed to directly inhibit the gelatinolytic activity of matrix metalloproteinases as well as impairing their secretion from DU145 and MDA-MB-231 cells. This study has thus elucidated some of the mechanisms responsible for cranberry's induction of programmed cell death and inhibition of MMP activity, and has thus established the potential value of cranberry phytochemicals in the development of novel chemopreventative strategies.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$\Delta\psi m$: Mitochondrial transmembrane potential

αMEM: Alpha minimum essential medium

AB: Alamar Blue

ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

AP: Alkaline phosphatase

ANTHO: Anthocyanin

BAX: Bcl-2 associated X protein

BCIP: 5-Bromo-4-chloro-3-indolyl phosphate

BCL-2: B-cell lymphoma-2 protein

BH: Bcl-2 homology

BSA: Bovine serum albumin

CARD: Caspase activation and recruitment domain

cytC: Cytochrome C

DISC: Death inducing signaling complex

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunoSorbent assay

EMMPRIN: Extracellular matrix metalloproteinase inducer

ER: Endoplasmic reticulum

ETC: Electron transport chain

FADD: Fas-associated death domain

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FLAV: Flavonol

GPSE: Grape seed extract

HPLC: High pressure liquid chromatography

HRP: Horseradish peroxidase

IAP: Inhibitor of apoptosis proteins

IGF: Insulin-like growth factor

IGFBP-3: IGF binding protein-3

INT: 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride

JNK: c-jun NH(2)-terminal kinases

LEHD: Ac-Leu-Glu-His-Asp-pNA

LETD: Ac-Leu-Glu-Thr-Asp-pNA

LDH: Lactate dehydrogenase

MALDI-TOF MS: Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

MAP-K: Mitogen activated protein kinase

MMP: Matrix metalloproteinase

MOMP: mitochondrial outer membrane permeabilization

MT1-MMP: Membrane type 1 matrix metalloproteinase

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NAD^+/H: Nicotinamide adenine dinucleotide

NBT: nitro blue tetrazolium chloride

NF- κB: nuclear factor kappa-B

PAC: Proanthocyanidin

PAGE: Polyacrylamide gel electrophoresis

PAR-4: Prostate apoptosis response-4

PARP: poly-ADP-ribose-polymerase

PAWT: Pineapple weed tea

PBS: Phosphate buffered saline

PCD: Programmed cell death

PMSF: Phenylmethanesulphonylfluoride

PTP: Permeability transition pore

RIPA: Radio immunoprecipitation assay buffer

RNA: Ribonucleic acid

SDS: Sodium dodecyl sulfate

Smac: Second mitochondria-derived activator of caspases

STS: Staurosporine

TBE: Tris/Borate/EDTA

TIMP: Tissue inhibitor of matrix metalloproteinase

TNF-α/β: Tumour necrosis factor alpha/beta

TRAIL: TNF-related apoptosis-inducing ligand

tBID: truncated BID

VEGF: Vascular endothelial growth factor

VDAC: Voltage-dependent anion channel

CHAPTER ONE

Introduction and Literature Review

1.1 Introduction

As people around the world adopt a Western lifestyle and diet, cancer is set to become the leading cause of death globally. The worldwide incidence of cancer has doubled from 1975 to 2000, and is expected to double again by 2020 (IARC, 2008). The burden of this growth may prove too heavy for health care providers to bear, despite increased understanding of cancer-related processes and the development of therapies to target those processes. Chemopreventative strategies need to be developed so that the onset of cancer can be avoided. As diet and nutrition have been implicated in the development of many cancers, attention is increasingly focused on the potential anticancer benefits of dietary and lifestyle changes. Individual foods, and even chemical constituents of those foods, are being evaluated to determine how they may impede the development and progression of a variety of cancers (Donaldson, 2004). The work presented in this thesis evaluates the potential anticancer activities of cranberry polyphenols in *in vitro* prostate and breast models of cancer, with the goal of determining the mechanisms responsible for previously reported anticancer activities.

1.1.1 Research objectives and general hypotheses

The overall purpose of this thesis was to elucidate the mechanisms responsible for cranberry's previously observed anticancer activities, namely its cytotoxicity and its matrix metalloproteinase (MMP)-inhibiting activity. This work thus provides a detailed overview of cranberry's mechanistic effects, data that is of value when designing new *in vitro* or *in vivo* research projects into cranberry's anticancer activities. The rationale behind the hypotheses addressed is found in Chapter 1, section 1.2.3, which summarizes the previously documented anticancer activities possessed by *Vaccinium macrocarpon*. Detailed explorations of the mechanisms and pathways responsible for apoptosis induction and matrix metalloproteinase inhibition are reviewed in section 1.2.4 and 1.2.5, respectively. This background information is necessary to put into context the specific objectives of the research presented herein.

Although previous studies had identified the apoptogenic potential of cranberry phytochemicals, few attempts had been made to explore the pathways through which cranberry exerts its cytotoxic effects - the first major objective of this thesis, addressed in chapter 2. **It was hypothesized** that cranberry inhibits DU145 cell proliferation via induction of the intrinsic pathway of apoptosis. Specific objectives were designed to evaluate this hypothesis: determine the effect of cranberry treatment on DU145 cell viability, evaluate markers of apoptosis and necrosis to determine the mechanism of cell death and evaluate caspase activation to further elucidate the pathways involved in cranberry-mediated cell death.

Furthermore, extensive immunoblot analysis was employed in order to evaluate the effect(s) of cranberry treatment on the protein expressions of key enzymes involved in both the intrinsic and extrinsic apoptotic pathways.

Cranberry has also been documented to inhibit matrix metalloproteinase (MMP) secretion, though the mechanisms whereby cranberry inhibits the secretion of MMPs 2 and 9 have not yet been fully elucidated (Neto *et al.*, 2006). Therefore, the second major objective of this thesis is to evaluate the effect(s) of cranberry treatment on regulators of MMP expression and activity in both prostate and breast carcinoma cells (addressed in chapter 3). Based on previous work with blueberry phytochemicals, **it was hypothesized** that cranberry's inhibition of MMP secretion occurs in response to decreased expression of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) and MT1-MMP (Membrane Type 1 – Matrix Metalloproteinase) and increased production of the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) 1 and 2. To evaluate this hypothesis, zymography was employed to determine the effect of cranberry treatment on MMP activity and secretion from MDA-MB-231 and DU145 cells. Western immunoblot analysis and real-time RT-PCR was employed to address whether cranberry treatment alters the expression of MMP-9, MT1-MMP, EMMPRIN, or the TIMPs.

1.2 Literature Review

1.2.1 Cancer: an accumulation of acquired characteristics

The last 20+ years of cancer research has revealed cancer to be a disease involving dynamic genomic changes. The transformation of normal human cells into malignant cancer cells is a complex process involving numerous sequential genetic mutations that may occur over a considerable period of time, as evidenced by the age-dependent incidence of most cancers (Renan, 1993). These mutations confer selective advantages upon the cancer cell against its normal competitor. Thus, through a process analogous to Darwinian evolution, the cancer cell population retains modifications that increase their ability to proliferate. Transformed cells thus possess a seemingly limitless number of morphological features, molecular pathways and cellular behaviours that differentiate them from normal cells. Hanahan and Weinberg (2002) have proposed that this array of differences can be distilled down to six essential alterations in cell physiology characteristic of all cancers; self-sufficiency in growth signals, insensitivity to growth-inhibition signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis, and metastasis and tissue invasion. Each of these features allows a cancer cell to bypass our innate anticancer defense mechanisms and wreck havoc in the body.

Although each of these acquired capabilities is vital to the overall development and progression of cancer, only three capabilities will be discussed herein, those that are of particular relevance to the work conducted. The first discovered acquired capability of cancer was self-sufficiency in growth signaling. In

normal cells, growth and proliferation is a coordinated process involving extensive intercellular communication through soluble regulatory growth molecules. These mitogenic growth signals are transmitted into the cell by transmembrane receptors specific for particular signaling ligands. Neoplastic cells meanwhile, do not depend upon exogenous growth stimulation. This autonomous growth results, in part, from constitutive expression of both growth factors and their receptors, which operate in self-contained autocrine loops (Fedi *et al.*, 1997).

Independence from external growth signaling is one of the mechanisms whereby transformed cells evade apoptosis, as growth factor withdrawal is an apoptotic stimulus in normal cells. Apoptosis is a form of programmed cell death in which a cell responds to pro-death signals by committing to a highly regulated cellular suicide. Tissue homeostasis depends upon apoptosis to maintain the balance between cell replication and cell death (discussed in more detail, Chapter 2). The dramatic growth characteristic of tumours results not only from increased cell proliferation but also from decreased cell attrition through evasion of apoptosis. Apoptosis was first identified as a barrier to cancer in 1972, when it was observed that rapidly growing, hormone-dependent tumours undergo massive apoptosis in response to hormone withdrawal (Kerr *et al.*, 1972). Further research revealed that many tumours possess mutations that cripple the apoptotic machinery intrinsic to all cells. Mutations of the p53 tumour suppressor gene, for example, result in the loss-of-function of its protein product, thereby removing a key component of apoptosis machinery responsible for the detection of DNA damage and other

abnormalities such as oncogene hyperexpression and hypoxia (Harris, 1996; Levine, 1997).

During the development of most cancers, the primary tumour mass sheds cells that invade adjacent tissues, eventually traveling to distant sites where they may form secondary cancer settlements (metastases). A sequence of events is believed to occur in all cases of metastasis: escape of cells from the primary tumour, invasion of cells into adjacent tissues, entry of cells into the lymphatic or blood circulatory systems (intravasation), survival and transport of cancer cells through the circulatory systems, escape of tumour cells from the circulatory system (extravasation) and, finally, attachment of cells at the metastatic site and growth resulting in the formation of a secondary tumor. This incredibly complex process arises following a barrage of mutations that significantly alter the cell's phenotype, and is thus one of the characteristics of transformed cells. Invasion and metastasis both rely on extensive interactions between the tumour cell and its immediate environment, the extracellular matrix (ECM). These interactions are mediated by a variety of signaling pathways, though this thesis focuses exclusively on the role of a particular family of ECM-degrading enzymes, the matrix metalloproteinases (MMPs). As will be discussed in chapter 3, MMPs are important arbiters of the tumour-ECM relationship, and their aberrant regulation in tumour cells is required for tissue invasion, intra- and extravasation, amongst other activities (Chang and Werb, 2001; Egeblad and Web, 2002).

1.2.1.1 Prostate cancer

The development and progression of prostate cancer illustrates many of the acquired characteristics just described. As prostate cells accumulate mutations, they undergo transformation and their proliferation accelerates, resulting in the formation of tiny, undetectable neoplasms. As transformed cells continue to proliferate, they spread into surrounding prostate tissue, eventually forming a tumour (Feldman and Feldman, 2001). This process may take many decades, and thus many tumours are never detected, except during autopsy. It has been revealed by autopsy that 30-40% of men over fifty, who died due to causes unrelated to cancer, had undetected prostate cancer (American Cancer Society, 2008). This slow development provides ample opportunity to treat organ confined prostate cancers once detected, primarily with targeted irradiation and/or prostatectomy, both of which have generally good outcomes resulting in improved patient prognosis.

Unfortunately, prognosis becomes considerably worse once the cancer spreads beyond the margins of the prostate and metastasizes to distant secondary sites (Feldman and Feldman, 2001; Navarro *et al.*, 2002). As prostate cancer is often undetectable while confined, up to 60% of patients present with metastases, most to the bone. In this situation, the only treatment option is an endocrine therapy called androgen ablation, in which androgens are suppressed, leading to the apoptotic regression of the androgen-dependent prostate cells. This therapy results in extended regressions (median of ~24 months), but is not curative as androgen ablation inevitably selects for those prostate cancer cells whose growth is androgen-

independent, ultimately causing the development of extremely aggressive and metastatic hormone-refractory prostate cancer. There is no treatment for this type of cancer and the median survival time decreases to somewhere between 8 and 12 months (Feldman and Feldman, 2001; Navarro *et al.*, 2002). The inherent inability for current treatments to effectively deal with prostate cancers has stimulated intense research into novel chemopreventative and chemotherapeutic strategies for prostate cancer.

Researchers employ a variety of models to investigate prostate cancer, including *in vitro* cell culture systems. A range of cell lines have been developed to model different stages of prostate cancer, from normal prostate cells, to benign neoplasia cells, androgen responsive cells and androgen refractory cells. The studies presented in this thesis employed DU145 prostate adenocarcinoma cells that had metastasized to the brain. The DU145 cell line is one of the "classical" androgen refractory prostate cancer cell lines and is used to model the final stages of prostate cancer. Reflecting the growth factor autonomy of advanced prostate cancers (Lorenzo *et al.*, 2003), DU145 cells constitutively express growth factors and their receptors, and thus lose their reliance on extrinsic growth factors. DU145 cells have been found to express all the components of the insulin-like growth factor (IGF), the epidermal growth factor (EGF), and the fibroblast growth factor (FGF) signaling pathways, which are arranged into a self-contained autocrine loops that provide all the growth promoting signaling required for continued proliferation (Manes *et al.*, 1999; Russell *et al.*, 1998). These modifications increase the survivability of DU145

cells in the absence of trophic factors. It fact, DU145 cell proliferation *increases* upon serum starvation (Tang *et al.*, 1998).

DU145 cells, like many other "successful" cancer cells, harbour mutations of the p53 gene that confer upon the cancer a selective advantage by providing the cells with a means of evading apoptosis. DU145 cells contain two p53 mutations (Isaacs *et al.*, 1991) that dramatically increase the stability of the encoded p53 protein, leading to an accumulation of the protein in the cell's cytosol (Tang *et al.*, 1998). This p53 product, however, cannot function as a transcription factor as the mutation prevents it from being able to bind to DNA. As functional p53 is required for the transcription of some apoptotic machinery, the overall result of these mutations is the suppressed ability to respond to apoptotic stimuli (Isaacs *et al.*, 1991). The aforementioned resilience of DU145 cells to growth factor withdrawal has been attributed, in part, to p53 mutations (Tang *et al.*, 1998).

1.2.1.2 Breast cancer

As with other forms of cancer, breast cancer is the final outcome of multiple interacting hereditary and environmental factors resulting in uncontrolled breast cell proliferation, facilitated by abnormal growth signaling. Though risk factors have been identified, the cause of any individual breast cancer is usually unknown. Most breast tumours are identified by self-examination, and if the cancer is detected while localized to the breast, a number of treatment options are available. These include surgical removal (mastectomy) of the tumour, often accompanied by chemotherapy

and/or radiotherapy. Suppression of female sex hormones estrogen/progesterone can lead to apoptotic regression of the tumour cells, but only if these cells express either the estrogen receptor or progesterone receptor. Unfortunately, breast cancer is a metastatic disease, thus complicating treatment considerably. Extensive lymphatic networks in the breast provide an escape route for cancer cells to metastasize first to nearby lymph nodes (usually under the arm) and from there to distant secondary sites throughout the body, including the lungs, bones and brain (Lacroix, 2006). Breast cancer development is common in women, even if the disease doesn't always present itself: 30-50% of women aged 40 to 50 years who die from causes unrelated to cancer have been found to harbour premalignant, microscopic breast tumours (Black and Welch, 1993). The prevalence of breast cancer has made it the focus of considerable research efforts, with particular efforts to prevent or delay breast cancer metastasis, which increases mortality considerably.

A variety of cell lines have been developed to model breast cancer in an *in vitro* setting, including the MCF-10A and the MDA-MB-231 cell lines (used in this research). The MCF-10A cell line was derived from the mastectomy tissue of a woman with fibrocystic disease. The cells from this tissue spontaneously gave origin to the immortalized MCF-10A cell line. Analyses have concluded that this cell line has the characteristics of luminal ductal cells, is non tumorigenic, and exhibits minimal genetic alterations (Soule, *et al.*, 1990; Tait *et al.*, 1990). These cells thus represent "normal" cells and are useful for studying the earliest stages of breast neoplasia. The MDA-MB-231 cell line was isolated from the pleural effusion of a

woman with breast cancer metastasized to the lungs (Cailleau *et al.*, 1978), and has since become one of the most common breast cancer cell lines used in research. Recent analyses of MDA-MB-231 gene expression profiles, cell morphology, and behaviour have reconfirmed this cell line's usefulness as a model of advanced stage breast cancer. Like DU145 cells, MDA-MB-231 cells are invasive and metastatic to bone and brain (Lacroix and Leclercq, 2004) and harbour p53 mutations that encode a stabilized p53 protein product distinct from wild-type p53. This p53 protein suppresses MDA-MB-231 cell apoptosis that would otherwise occur in response to serum withdrawal (Hui *et al.*, 2006).

1.2.2 Nutrition and Cancer

1.2.2.1 A Wealth of Epidemiological Evidence

Since the 1950's, epidemiological studies have been highlighting many of the risk factors associated with the development and progression of cancer. While internal factors (inherited mutations, hormones, and immune conditions) play a role in this process, it is well established that a seemingly infinite number of external risk factors contribute significantly to the risk of developing cancer. These risk factors include carcinogenic/mutagenic chemicals, radiation (including solar), infectious organisms, poor diet and a sedentary lifestyle, amongst many others (Wiseman *et al.*, 2008). In particular, the relationship between diet, lifestyle and cancer onset is becoming increasingly clear. Epidemiological studies indicate that consumption of a fruit and vegetable-rich diet contributes to reducing the risk of certain cancers

(Steinmetz and Potter, 1991), and that a third of all cancers could be avoided by making appropriate modifications to one's diet (Willett *et al.*, 1995). Both essential and non-essential nutrient components of fruits, vegetables and whole grains contribute to this anticancer activity.

Accumulating evidence suggests that diet exerts some influence on the development and progression of prostate cancer (Barnes, 2001; Mazhar and Waxman, 2004; Nguyen *et al.*, 2006). Prostate cancer is more prevalent in Asian men who have recently immigrated to the United States than it is in their counterparts in the East. This increased cancer incidence has been attributed to dietary changes, including decreased consumption of fruits and vegetables (Cook *et al.*, 1999; Schulman *et al.*, 2001; Wiseman, 2008). In response to this epidemiological data, ongoing studies seek to determine how diet can be used to manage prostate cancer. The East-West dichotomy in cancer incidence also extends to breast cancer: Asians have a 10-fold lower incidence of breast cancer than do Westerners (Wiseman, 2008) and Americans have the highest incidence of breast cancer in the world (American Cancer Society, 2008). However, it doesn't appear that these regional differences are related to diet, as studies have revealed that the link between nutrition and breast cancer is less evident than that found in other cancers. An extensive review of prospective observational studies revealed few significant correlations between diet and breast cancer (van Gils *et al.*, 2005) with the exception that increased alcohol consumption and weight gain contribute to disease onset (Michels *et al.*, 2007). *In vitro* and *in vivo* studies continue to explore

the potential effects of dietary compounds on breast and prostate cancer development.

1.2.2.2 Nutritional Research and Pharmacognosy

Given the wealth of epidemiological evidence demonstrating a vital link between some forms of cancer and diet, it should come as no surprise that a huge number of researchers from a range of disciplines are dedicating themselves to fully exploring this relationship. The objectives of these research endeavours vary widely, as do the approaches used to investigate the hypotheses generated from epidemiological data. More holistic approaches investigate the multiple interactions between different foods within a diet on mammalian physiology or on particular pathologies. Some researchers focus on whole foods while others, adhering to a decidedly more reductionist worldview, investigate individual dietary compounds for particular cancer-related activities of interest.

In the broadest sense, research into the potential health promoting and anticancer properties of foods (particularly fruits, vegetables, and whole grains) can be divided into two categories, depending upon the objectives of the research. Nutrition-based research evaluates the health-related effects of consuming whole foods (or mixtures of foods in a diet). As such, nutrition research focuses on the potential of physiologically relevant food quantities to prevent, rather than treat, cancer. The second category of research, pharmacognosy, evaluates the pharmacological potential of compounds isolated from natural sources, most often

plants. Pharmacognosists focus on the search for novel drugs from natural sources and the physical, chemical and biochemical properties of those drugs. As this type of research isn't limited to physiological doses of food-derived compounds, its purview extends beyond cancer chemoprevention into the development of drugs designed for cancer treatment.

These fields of research have revealed that many of the anticancer benefits imparted by consumption of fruit and vegetables can be attributed to their phytochemicals (plant produced chemicals). Over five thousand individual phytochemicals have been identified in vegetables, fruits, grains, though the structure and functions of the majority of these compounds are still unknown. Phytochemicals are the products of secondary metabolism in plants, and act as defense mechanisms against various pathogens, parasites and predators or, alternatively, as chemical attractants for pollinators (Fraenkel, 1959). Researchers continue to explore the physiological effects of phytochemical consumption on mammalian physiology.

As carcinogenesis is a slow process often taking many decades, there is ample opportunity to intervene with chemopreventative strategies. Diet-derived compounds are of particular interest in chemoprevention, as it is expected that consumption of dietary phytochemicals over long periods of time may have less adverse toxic effects than intervention with synthetic compounds (as it easier to attain toxic doses of purified compounds). Prostate cancer is an ideal target for dietary chemoprevention, as detected tumours display remarkable heterogeneity

with respect to stage and grade, and it is extremely difficult to determine whether the cancer will progress to become a metastatic killer, and thus whether treatment is required. This fact, plus the reality that treatment is not always curative (but *is* accompanied by terrible side effects) leads many physicians to advocate a "watchful waiting" approach in which treatments are administered only after symptoms appear. Many patients in this situation actively seek the means to manage their disease through complementary alternative therapies and lifestyle/diet changes (Lippert *et al.*, 1999; Kao and Devine, 2000; Bemis *et al.*, 2006).

The most relevant model for the evaluation of the anticancer activity of dietary phytochemicals is the human subject. Unfortunately, epidemiological data by itself is not sufficient for the design of human clinical trials due to the inherent variability and irreproducibility of such data. Furthermore, clinical trials are extremely expensive, are subject to considerable ethical scrutiny, and need to be very carefully planned (safety/efficacy dosing needs to be at least partially established prior to conducting the trial). These facts provide an important role for both *in vitro* and *in vivo* models to determine the potential effects of dietary components in humans. *In vitro* studies of dietary phytochemicals are used to screen for bioactivities and to determine in detail the mechanisms responsible for such activities. While useful for mechanistic studies, there are obvious limitations to the use of *in vitro* models for evaluating the efficacy of dietary phytochemicals, as they cannot take into account the bioavailability of these compounds, nor their *in vivo* distribution throughout the body's tissues. Furthermore, the *in vivo* active agent may be quite different from that found in the fruit/vegetable as a result of the

modifications that often occur during digestion and absorption. For these reasons, results from *in vitro* studies cannot be directly extrapolated to dietary effects, though such results are essential for the development of effective *in vivo* and human clinical trials (Jeffery and Keck, 2008).

This brief survey of the research landscape describes some of the many approaches taken to study the relationship between diet and cancer development/progression. While these approaches and the models they employ may differ considerably from one another, each is required to produce the mosaic of research needed to understand the chemoprevention of cancer by components of the diet. The research presented in this thesis focuses primarily on the effects of whole cranberry extracts on *in vitro* models of cancer and was required to determine the detailed mechanisms and pathways by which cranberry exerts its anticancer activities at the cellular level (specifically with regards to apoptosis induction and matrix metalloproteinase inhibition). While these results cannot be directly extrapolated to dietary effects, this research may be of value when considering the design of future animal feeding studies. Furthermore, as these *in vitro* models represent late stage metastatic prostate and breast cancers, this work may be applicable to the field of pharmacognosy and the evaluation of cranberry phytochemicals as pharmacological agents.

1.2.3 *Vaccinium macrocarpon*, the North American Cranberry

The North American Cranberry (*Vaccinium macrocarpon*) is a low, creeping shrub of the Ericaceae family native to Northeastern America and the Pacific Northwest. Cranberry is cultivated as a major commercial crop in a number of the Northeastern states (primarily Wisconsin and New Jersey) and in Québec, Nova Scotia and Prince Edward Island (CCCGA, 2008). The small red berries of the cranberry bush are either sold fresh, or are processed into a number of products including juice, sauce, sweetened dried cranberries, and neutraceutical extracts or pills. In recent years, there has been a growing recognition of cranberries for their nutrient content, phytochemical composition and antioxidant qualities (Vinson *et al.*, 2001). Cranberries contain moderate amounts of vitamin C and fibre, and a high Oxygen Radical Absorbance Capacity (ORAC) rank compared to other commonly consumed foods (Wu *et al.*, 2004; Boivin *et al.*, 2007). This antioxidant status has been attributed to cranberry's high content of phenolics, chemical compounds containing an aromatic ring bearing a hydroxyl group. Many of these phenolics, particularly the anthocyanins, are strong antioxidants that have the potential to prevent oxidative damage caused by reactive oxygen species (Vinson *et al.*, 1995; Pietta, 2000; Wang and Jiao, 2000; Wang and Stretch, 2001; Yan *et al.*, 2002). Cranberry's redox status, however, is but one aspect of this berry's health-promoting effects.

Of 20 fruits analyzed by Vinson *et al.* (2001), cranberries contained the highest content of total phenolics per serving by weight. Many of cranberry's biological activities have been attributed to these compounds, which include

anthocyanins, flavonols (usually as glycosides), flavan-3-ols (catechins), proanthocyanidins (condensed tannins), stilbenoids (resveratrol), phenolic acids, hydrolysable tannins (ellagitannins, gallotannins) and triterpenoids like ursolic acid (Seeram, 2006; Neto *et al.*, 2006; Rimando *et al.*, 2004). Our collaborators have identified the Early Black cultivar of cranberry as being particularly rich in anthocyanins and proanthocyanidins (Neto *et al.*, 2008). An overview of cranberry's flavonoid and flavan-3-ol content is provided in Table 1.1.

1.2.3.1 Early Observations of *V. macrocarpon* activity

Some of the first observed health promoting effects of cranberry [juice] consumption include the prevention of urinary tract infections and plaque formation (by preventing *Escherichia coli* and *Streptococcus mutans* adherence, respectively) (Howell *et al.*, 1998; Foo *et al.*, 2000). The phytochemicals found to be responsible for this activity (cranberry proanthocyanidins) were found to also prevent the adhesion of *Heliobacter pylori* to the gastric mucus, thereby providing a mechanism whereby cranberry may prevent gastric ulcers and cancer (Burger *et al.*, 2000; Zhang *et al.*, 2005). These findings, coupled with research into the polyphenolic compounds found in other functional foods, stimulated a great deal of interest into other potential properties of *V macrocarpon*, including possible anticancer activities.

The earliest evidence of cranberry's potential anticancer activity came from a study of *Vaccinium* species that showed cranberry's ability to downregulate the expression of ornithine decarboxylase, a key enzyme involved in the promotion of

Table 1.1. Flavonoid and flavan-3-ols content in cranberry fruit (by class).

Phytochemical compound	**mg/100 g (Mean ±SD)**
Anthocyanins[1]	91.88
Cyanidin[2]	41.81 ± 2.86
Peonidin[2]	42.10 ± 3.64
Delphinidin[2]	7.66 ± 1.93
Malvidin[2]	0.31 ± 0.22
Flavonols[1]	21.96
Quercetin[2]	15.09 ± 1.06
Myricetin[2]	6.78 ± 1.67
Kaempferol[2]	0.09 ± 0.03
Flavan-3-ols monomers[1]	7.26
(-)-Epicatechin[2]	4.37 ± 0.93
(-)-Epigallocatechin[2]	0.74 ± 0.28
(-)-Epigallocatechin 3-gallate[2]	0.97 ± 0.48
(+)-Catechin[2]	0.39 ± 0.16
Proanthocyanidins[1]	411.5
Dimers[3]	25.93 ± 6.12
Trimers[3]	18.93 ± 3.39
4-6 unit oligomers[3]	70.27 ± 13.07
7-10 unit oligomers[3]	62.90 ± 14.71
Polymers[3]	233.48 ± 49.08

1. Sum of individual components in each class

2. USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. January 2007. http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/Flav02-1.pdf

3. USDA Database for the PAC Content of Selected Foods, August 2004. http://www.nal.usda.gov/fnic/foodcomp/Data/PA/PA.pdf

tumour proliferation (Bomser *et al.*, 1996; Kandil *et al.*, 2002). Whole polyphenolic extracts of cranberry have been shown to inhibit the proliferation of a number of different cell lines, including those representative of prostate (LNCaP, PC3, DU145, RWPE-1, RWPE-2, 22Rv1) and breast carcinomas (MDA-MB-435, MCF-7). The mechanisms responsible for this inhibition include cell cycle arrest and induction of apoptosis (Yan *et al.*, 2002; Murphy *et al.*, 2003; Ferguson *et al.*, 2004; Seeram *et al.*, 2004; Sun *et al.*, 2006; Boivin *et al.*, 2007). These effects have been attributed to a variety of cranberry phytochemicals, as will be currently reviewed.

1.2.3.2 Anticancer Activities of Cranberry Phytochemicals

Antiproliferative and Cytotoxic Effects

Based on weight, cranberries are one of the leading fruit sources of quercetin. Quercetin glycosides comprise approximately 75% of the total flavonol content of cranberries (the rest primarily being myricetin). Quercetin has been studied extensively due to its prevalence among fruits and vegetables, and has been shown to be a potent anticancer agent in *in vitro* models. Quercetin's abundance and activities are such that it is likely to be a primary source of cranberry's anticancer activities (Neto, 2007). This flavonol is able to prevent the proliferation of a number of different cancer cell lines, including breast, colon, pancreatic and leukemic cancers through cell cycle arrest and the induction of apoptosis (Murphy *et al.*, 2003; Lee *et al.*, 2002; Choi *et al.*, 2001; Seeram *et al.*, 2004; Ramos *et al.*, 2005; Richter *et*

al., 1999). Quercetin's induction of apoptosis in a rat colon cancer model involved the mitochondrial pathway through modulation of Bax and Bcl-2 protein expression (Volate, *et al.*, 2005).

The proanthocyanidins (PAC) contained within *V. macrocarpon* consist primarily of dimers, trimers and larger oligomers of epicatechin or poly-flavan-3-ols. Cranberry PACS are unique in that their constituent monomers are joined by two types of linkages, the common B-type linkage (found in grape seed and other PAC extracts) and the less common A-type linkage (rare, except in *Vaccinium* species). The presence of A-type linkages may enhance PAC-mediated cytotoxicity (Neto *et al.*, 2008) and *V. macrocarpon* PACs have shown *in vitro* antiproliferative activities in a variety of cell lines, including prostate carcinoma cells (Ferguson *et al.*, 2004; Seeram *et al.*, 2004; Liberty *et al.*, 2007; Neto *et al.*, 2006). Grape seed extract (GPSE) PACs have been widely studied, and have been reported to inhibit the growth of tumour cell lines (including breast and prostate) through mechanisms that include the induction of apoptosis (Ye *et al.*, 1999; Agarwal *et al.*, 2000; Agarwal *et al.*, 2000; Tyagi *et al.*, 2003; Kim *et al.*, 2004; Mantena *et al.*, 2006). Studies in oral squamous cell carcinomas have shown that the antiproliferative effects of grape seed extract and cranberry extract are similar, with both extracts inducing apoptosis by activation of caspase-2 and -8 (Chatelain *et al.*, 2008). However, caution should be taken when comparing PACs from different plant species, as the structural variability inherent in PACs likely affects their biological functions.

Resveratrol is a stilbene found in whole polyphenolic extracts of cranberry (Rimando *et al.*, 2004) that has been shown capable of inhibiting proliferation via the promotion of apoptosis in a number of cell lines, including the DU145 cell line (Lin *et al.*, 2002; Joe *et al.*, 2002; Kim *et al.*, 2003; Shih *et al.*, 2004). *Vaccinium* fruits are also abundant in anthocyanins, compared with other foods, with the primary anthocyanins being galactosides of cyanidin and peonidin (Neto *et al.*, 2006). Anthocyanins are particularly powerful antioxidants, though they have little growth-inhibitory or antiproliferative properties (Seeram *et al.*, 2004; Murphy *et al.*, 2003).

The most abundant of cranberry's triterpeneoids (all of which are located in the peel) is ursolic acid, which is found in either the aglycone form or the *cis* or *trans* *p*-hydroxycinnamate ester form. Cranberry-isolated ursolic acid has been shown to inhibit the growth of several types of tumour cell lines, including MCF-7 breast cells and DU145 prostate cells (Murphy *et al.*, 2003, Novotny *et al.*, 2001). Ursolic acid induced apoptosis in human leukemia cells through alterations to intracellular calcium signaling (Baek *et al.*, 1997), in human hepatoblastoma cells through cytochrome c release and caspase-3 activation (Kim *et al.*, 2000) and in HT-29 colon cells via the activation of caspases 3, 8 and 9 (Andersson *et al.*, 2003). Of even more relevance to the work presented in this thesis, ursolic acid has also been shown to induce apoptosis in prostate epithelial cells through caspase activation (via a mechanism not involving Bcl-2 or Bax expression regulation, nor mitochondrial dysfunction) (Choi *et al.*, 2000).

Anti-invasive and Anti-metastatic Effects

While the majority of studies evaluating cranberry's anticancer effects have focused on its inhibition of proliferation, there is a growing body of evidence that supports the berry's ability to inhibit processes related to invasion and metastasis. A whole cranberry polyphenolic extract has been reported to inhibit the expression of MMPs 2 and 9 in DU145 cells in a dose-dependent manner. The PAC extract also inhibits MMP expression, though is less capable than the whole polyphenolic extract (Neto *et al.*, 2006). This anticancer effect has also been observed in response to treatment with highbush blueberry (*Vaccinium angustifolium*) (Matchett *et al.*, 2006), a close relative of the North American cranberry. The blueberry phenolics of particular importance with respect to this activity appear to be the PACs and purified ursolic acid and its hydroxycinnamate esters (Kondo *et al.*, 2004). Some of the phytochemicals responsible for this activity have been evaluated in other studies. It's been found that ursolic acid decreases MMP-9 expression in human fibrosarcoma cells (Cha *et al.*, 1996), an activity mediated by nuclear factor kappa-B (NF- κB) inhibition (Shishodia *et al.*, 2003). GPSE PACs have been reported to inhibit MMP-2 and –9 expressions in a dose-dependent manner in prostate cancer cell lines through the inhibition of NF- κB and mitogen-activated protein kinase (MAP-K) activation (Vayalil *et al.*, 2004).

Taken together, previous research into cranberry's anticancer effects provide the rationale for the hypotheses addressed in this thesis.

1.2.4 Cell Death and Apoptosis

The induction of apoptotic death in malignant cells is the goal of many chemopreventative and chemotherapeutic strategies. As many anticancer agents have been discovered in natural plant sources, the search for novel phytochemicals displaying anticancer activities continues at a tremendous rate. Previous work conducted in the laboratory of Dr. Robert Hurta has indicated that polyphenolic extracts of *V. macrocarpon* induce DU145 cell death through activation of caspase-3 (24 hour treatment, 1000 μg/ml) (Matchett, unpublished results). The focus of this research project was to determine the signaling mechanisms whereby cranberry treatment results in caspase-3 activation, with the hypothesis that apoptosis occurs through regulation of the Bcl-2 family of proteins resulting in the release of mitochondria-sequestered cytochrome c. An overview of the pathways and mechanisms whereby apoptotic stimuli induce apoptosis follows.

1.2.4.1 An overview of cell death mechanisms

In all multicellular organisms there is a constant process of death and renewal as that organism's cells die and are supplanted by newly divided replacements. Cells often die in response to unanticipated and accidental damage arising from physical trauma, heat, radiation, and oxygen deprivation, which lead to wild perturbations in cellular homeostasis, ultimately resulting in a form of cell death characterized as necrosis. Necrotic cells undergo swelling, chromatin digestion, and enzymatic digestion of cellular organelles followed by a complete and

irreversible loss of cell function and plasma membrane integrity, ultimately resulting in the release of cellular contents into the surrounding environment eliciting an inflammatory response.

Cell death that occurs according to an intricately regulated, ATP-dependent intracellular program is called programmed cell death (PCD). Of the many forms of PCD, apoptosis is the most common and is required to preserve the balance between cell division and attrition, thereby maintaining organism and tissue homeostasis. If cell apoptosis outstrips cell proliferation, ravaging diseases such as AIDS or neurodegenerative disorders will result. If apoptosis is inhibited and cells divide at a greater rate than they die, a malignant tumour may develop. Apoptosis is coordinated by a series of tightly regulated biochemical events that lead to characteristic morphological changes including loss of cellular asymmetry and attachment, membrane blebbing (budding of cytosol -containing apoptotic vesicles from the cell), cell shrinkage, nuclear fragmentation and chromosomal DNA fragmentation. The orderly destruction of an apoptotic cell is carried out by a family of proteolytic enzymes called the cysteine-aspartic acid proteases (caspases), often referred to as the central executioners of apoptosis.

Activation of the executioner (effector) caspases is intricately regulated by a vast network of signaling pathways that integrate a variety of apoptotic stimuli. Regardless of the stimulus, all pathways ultimately converge to activate procaspase-3 and -7 (via cleavage) and initiate an irreversible, self-amplifying proteolytic cascade (involving caspases-2, -6, -8 and -10) (Slee *et al.*, 1999; Van de Craen *et al.*,

1999). The activated effector caspases then cleave downstream cell targets including poly-ADP-ribose-polymerase (PARP), an enzyme that maintains genomic integrity by repairing single strand DNA breaks. Caspase-3's cleavage and inhibition of PARP renders the enzyme unable to repair the DNA damage caused by apoptotic stimuli and is a marker of apoptosis (Oliver *et al.*, 1998). Another critical target of caspase activity is the chromatin contained within the nucleus, which is degraded into characteristic mono- and oligonucleosome fragments in the final stages of apoptosis.

1.2.4.2 Regulation of apoptosis: extrinsic apoptotic pathway

Caspase-3 is activated by one of two major apoptotic pathways – the intrinsic, mitochondria-mediated pathway, and extrinsic, death receptor-mediated pathway. The intrinsic pathway responds to apoptotic signals from a variety of stressors, including cytoskeleton damage, endoplasmic reticulum (ER) stress, DNA damage, loss of adhesion, and growth factor withdrawal (amongst others) (Von Ahsen *et al.*, 2000). The extrinsic pathway of apoptosis is activated by the binding of extracellular death-ligands (apoptogenic cytokines) to their receptors on the outer surface of the cell membrane. Tumor necrosis factors alpha and beta (TNF-α, TNF-β) cytokines are apoptotic stimuli that act through ligation to the TNF receptors (TNF-R1, TNF-R2) located on the cell membrane. Likewise, TNF-related apoptosis-inducing ligand (TRAIL) is a transmembrane protein that binds to death receptors 4 and 5 (DR4, DR5) and the fas ligand (FasL) is a transmembrane protein belonging to

the TNF family that binds to its receptor (FasR) to trigger its trimerization. Upon activation of these membrane "death receptors" by their respective ligands, associated adaptor proteins (such as the Fas-associated death domain [FADD]) are recruited to the activated receptor, as are procaspases-8 and -10. Together, these molecules form the death inducing signaling complex (DISC) that is responsible for the activation of caspase-8 and -10. These activated "initiator" caspases then proteolytically cleave and activate caspases-3 and -7, thereby stimulating the final, irreversible stages of apoptosis (Lawen, 2003). In some cells (Type I), DISC assembly and the subsequent activation of caspase-8 are sufficient for the complete induction of apoptosis (via caspase-3 activation). However, in many cells (Type II), apoptosis initated by death receptor activation is carried out via the intrinsic apoptotic pathway (Scaffidi *et al.*, 1998), a pathway focused on one organelle of primary importance – the mitochondrion. Our attention now turns to that vitally important arbiter of both cell life and cell death.

1.2.4.3 Regulation of apoptosis: the mitochondrion

The mitochondrion's structure is crucial to the organelle's participation in ATP synthesis and the regulation of calcium (Ca^{2+}) and apoptosis signaling. The outer phospholipid bilayer membrane is perforated with transmembrane porins that allow for the diffusion of small (<5000 Da) molecules across its surface. Within the mitochondrion, there is a second highly convoluted membrane, separated from the outer membrane by the intermembrane space. The inner membrane is

completely impermeable to ions and contains the components of the electron transport chain (ETC). In addition to serving as the powerhouse of the cell, the mitochondrion is a central mediator of intracellular Ca^{2+} signaling. Cytosolic calcium enters the intermembrane space though outer membrane porins, and from there it is pumped into the mitochondria matrix via a calcium uniporter. Should the matrix calcium concentration reach a critical level, calcium is released back into the cytosol via the mitochondrial permeability transition pore (PTP) through a mechanism that may result in the activation of apoptosis signaling (Chipuk *et al.*, 2006). This is but one mechanism whereby the mitochondria's role as the cell's powerhouse is superseded by that as the coordinator of cell suicide.

Early evidence of the mitochondria's potential involvement in apoptosis signaling came with the observation that the B-cell lymphoma-2 (Bcl-2) protein, a known inhibitor of apoptosis (Hockenbery *et al.*, 1991), was localized to the outer mitochondrial membrane (Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; de Jong *et al.*, 1994). Later studies revealed that two factors were required for the activation of caspase-3 in isolated cytosols -- dATP and a 15 kDa protein that was purified, characterized, and found to be cytochrome c (Liu *et al.*, 1996). Cytochrome c (cytC) is a small heme protein found within the intermembrane space and the inner mitochondrial membrane where it shuttles electrons between complexes III and IV of the ETC. This observation triggered a period of frenetic research that established a framework for the mitochondria's role in apoptosis. Following exposure to apoptotic stimuli, mitochondria-sequestered cytC is rapidly released into the cytosol, where it participates in apoptosis signaling. These investigations also

revealed that Bcl-2's anti-apoptotic activity stems from the protein's ability to block mitochondrial release of cytC (Kluck *et al.*, 1997; Yang *et al.*, 1997). The pathways surrounding cytC release and apoptosis signaling are summarized in Figure 2.1.

Upon release into the cytosol, cytC and dATP bind to the apoptotic protease-activating factor 1 (Apaf-1) protein causing a conformational change that exposes Apaf-1's caspase activation and recruitment domains (CARD). This results in the oligomerization of apaf-1/cytC/dATP protein complexes to form the apoptosome, a large oligomeric protein complex responsible for procaspase-9 recruitment and activation. When two caspase-9 monomers are pulled into close proximity by the oligomerized CARD domains of the apoptosome, they dimerize and undergo mutual activation (Acehan *et al.*, 2002; Boatright *et al.*, 2003; Li *et al.*, 1997; Pop *et al.*, 2006; Zhou *et al.*, 1997). Activated caspase-9 remains bound to the apoptosome, forming the caspase-9 holoenzyme, which displays remarkable affinity and specificity for caspase-3/-7 cleavage and activation (Rodriguez and Lazebnik, 1999; Jiang and Wang, 2000; Chao *et al.*, 2005; Yin *et al.*, 2006).

CytC is not the sole apoptogenic weapon in the mitochondrion's arsenal, as a number of other apoptosis-regulating proteins are sequestered within the intermembrane space. These include the second mitochondria-derived activator of caspases (Smac; Smac/DIABLO), which indirectly facilitates caspase activation through its inhibition of the inhibitors of apoptosis proteins (IAP), a family of endogenous apoptosis inhibitors including x-linked IAP (XIAP), c-IAP1, c-IAP2, Op-IAP and survivin (Gray *et al.*, 2000; Du *et al.*, 2000; Verhagen *et al.*, 2000). IAPs halt

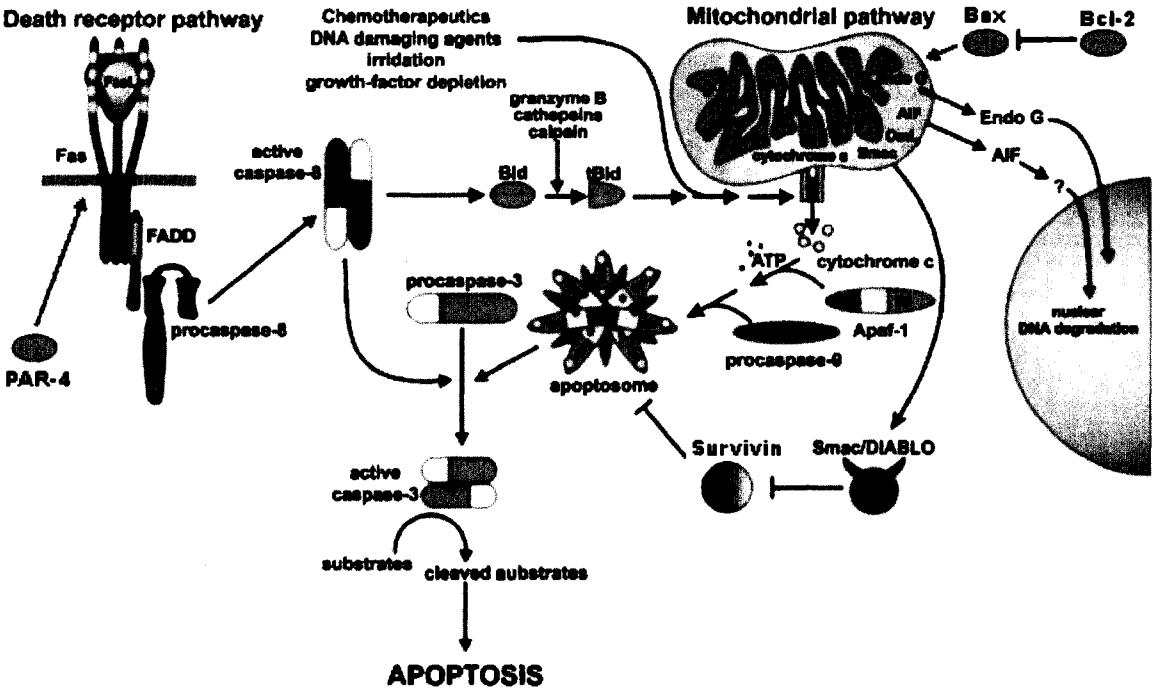

Figure 2.1: An overview of apoptotic pathways focused on the mitochondrion (modified from van Loo *et al.*, 2002).

the apoptotic cascade by directly interfering with the activation of caspases (Bratton *et al.*, 2001), hence Smac/DIABLO's role in perpetuating apoptotic signals.

The release of cytC and other apoptotic stimuli from the mitochondria into the cytosol is a central coordinating event in apoptosis signaling and is thus subject to intricate regulation. There are two primary mechanisms whereby cytC may be released from the mitochondrion (Figure 2.2): the first involves the mitochondrial permeability transition pore (PTP), and the second involves direct permeabilization of the outer mitochondrial membrane by proapoptotic Bcl-2 proteins.

1.2.4.4 Opening of the mitochondrial permeability transition pore

Opening of the mitochondrial PTP allows small cytosolic molecules and ions to pass from the cytosol through the otherwise impermeable inner mitochondrial membrane into the mitochondrial matrix, resulting in an osmotic imbalance. The matrix volume swells and the outer membrane ruptures, releasing sequestered apoptogenic proteins into the cytosol (Basso *et al.*, 2005). Mitochondrial PTP opening is implicated in apoptosis that occurs in response to ER stress, stress that causes a sustained increase in the Ca^{2+} levels in cytosol immediately surrounding a mitochondrion. When this occurs, matrix Ca^{2+} increases until a critical point is reached ("calcium overload") in which calcium interacts with cyclophilin D (matrix component of the PTP) to induce PTP opening (Chipuk *et al.*, 2006).

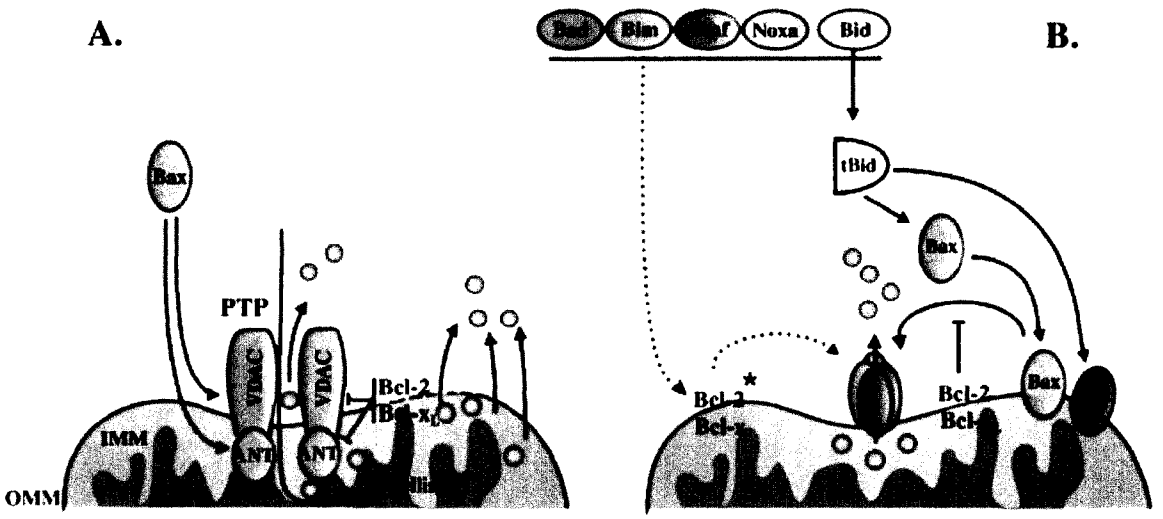

Figure 2.2: Cytochrome c may be released from the mitochondria by either one of two mechanisms. (A) Proapoptotic Bcl-2 proteins may trigger opening of the mitochondrial permeability transition pore, leading to matrix swelling, mitochondria rupture and cytochrome c release. (B) Alternatively, members of the Bcl-2 family of proteins may form transmembrane pores in the outer mitochondrial membrane, resulting in cytochrome c release from the intermembrane space into the cytosol (van Loo *et al.*, 2002).

There are limitations to the PTP model that call into question its usefulness as a general mechanism for cytochrome c release. First, PTP opening and outer membrane rupture leads to the disruption of the mitochondrial transmembrane potential ($\Delta\psi m$), thus dissipating the proton gradient required for ATP synthesis (Zamzami *et al.*, 1995; Kroemer *et al.*, 1995). The execution phase of apoptosis, however, depends upon ATP at a number of critical junctures including the activation of the apoptosome (Jiang and Wang, 2000; Genini *et al.*, 2000; Acehan *et al.*, 2002). Indeed, should an apoptotic stimulus disrupt ATP synthesis, cell death that was initiated as apoptosis switches to a caspase-independent form of PCD that resembles necrosis (Kass *et al.*, 1996; Leist *et al.*, 1997; Eguchi *et al.*, Nicotera *et al.*, 1998; Nicotera *et al.*, 2000). For PTP opening to be implicated in apoptosis, the overall bioenergetics of the cell must remain intact. This would be possible if the PTPs only opened in a subset of the cell's mitochondria, as occurs during localized Ca^{2+} release from proximal ER. Transient PTP opening (called flickering) has been observed in response to some apoptotic stimuli, and allows for cytC release without resulting osmotic disequilibrium and outer membrane rupture (Huser *et al.*, 1998; Petronilli *et al.*, 1999).

1.2.4.5 Permeabilization of the outer mitochondrial membrane by Bcl-2 proteins

It has been reported that cytC release is not always accompanied by $\Delta\psi m$ dissipation or matrix swelling (Kluck *et al.*, 1997; Yang *et al.*, 1997; Bossy-Wertzel *et al.*, 1998), and that knockout cells and mice lacking key components of the mitochondrial PTP can still undergo apoptosis in response to a range of stimuli (Kokoszka *et al.*, 2004; Nakagawa *et al.*, 2005). Therefore in addition to PTP-dependent apoptosis, there must be a second mechanism of cytC release that is independent of PTP opening. This model relies on the Bcl-2 family of proteins to induce mitochondrial outer membrane permeabilization (MOMP), thus releasing sequestered cytochrome c.

The Bcl-2 family of proteins contains members that are both pro- and antiapoptotic. Though they vary considerably in their activities, all family members share at least one of four characteristic Bcl-2 homology (BH) domains (BH1, BH2, BH3, BH4). The presence or absence of these homology domains is used to broadly divide this protein family into three groups. The anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL, Bcl-w) possess all four BH domains (BH1-4) and are localized to mitochondria and ER membranes (Krajewski *et al.*, 1993; Susin *et al.*, 1996). These BH1-4 proteins inhibit apoptosis by binding and sequestering the proapoptotic Bcl-2 proteins(Gross *et al.*, 1999; Adams *et al.*, 2001; Puthalakath *et al.*, 2002).

All pro-apoptotic members of the Bcl-2 family lack the BH4 domain. Multidomain proapoptotic Bcl-2 proteins (Bax, Bak, Bok) have BH domains 1-3, and are absolutely required for the permeabilization of the mitochondrial outer membrane. That stated, BH1-3 proteins are unable to induce apoptosis on their own. This is evident as BH1-3 proteins are constitutively expressed even in non-apoptotic cells. Indeed, these proteins are dormant until activated by the other group of proapoptotic Bcl-2 proteins – those that possess only the BH3 domain (Bik, Bid, Bim, Bad, Bmf, Noxa, Puma, Blk). BH3-only proteins can either directly or indirectly activate the BH1-3 proteins Bax and Bak. The direct activators (Bid, Bim) transiently interact with the BH1-3 proteins to catalyze their homo-oligomerization, which is required for activation. The indirect activators (Bad, Bim, Bmf, Noxa) perpetuate apoptosis signaling by binding to the active site of the BH1-4 proteins, thereby displacing the proapoptotic proteins that would otherwise be sequestered (either BH1-3 multidomain proteins or BH3-only direct activators) (Chipuk *et al.*, 2006).

Upon activation, BH1-3 proteins are able to promote PTP opening (Marzo *et al.*, 1998; Narita *et al.*, 1998; Shimizu *et al.*, 1999) in certain apoptotic pathways, but more importantly, they are also able to insert themselves into the outer mitochondrial membrane forming pores that allow for the release of intermembrane space proteins (Letai *et al.*, 2002; Kuwana *et al.*, 2002). The ability of Bax and Bak to induce cytC release in the absence of mitochondrial PTP has been shown using defined liposomes as models of "simplified" mitochondria (Kuwana *et al.*, 2002). A number of studies have suggested that certain mitochondrial membrane

components (like cardiolipin) may be required for Bax/Bak-mediated membrane permeabilization. However, these potential partners are but passive bystanders in the process and have no role in regulating whether pemeabilization occurs – that "decision" is made exclusively by the Bcl-2 proteins (Chipuk *et al.*, 2006).

The heterodimerization of functionally opposing Bcl-2 family members allows antiapoptotic proteins to inhibit their proapoptotic homologues, and vice versa. Therefore, the balance of pro- to antiapoptotic Bcl-2 members determines whether apoptosis will occur (Gross *et al.*, 1999; Adams *et al.*, 2001; Puthalakath *et al.*, 2002). The multiple interactions between Bcl-2 family members provides a series of cytosolic checks and balances whereby activation of proapoptotic multidomain Bcl-2 proteins requires not only direct activators (Bid, Bim), but also the rest of the BH3-only proteins that serve to offset the inhibitory effects of antiapoptotic Bcl-2 proteins.

Bid (a BH3-only direct activator) normally has a low affinity for BH1-3 proteins, and is not a potent inducer of apoptosis. Bid, however, is unique amongst BH3 proteins in that it contains two sites that are ideally suited as caspase-8 substrates. Activated caspase-8 cleaves off Bid's inhibitory N-terminal domain, yielding the truncated form of the protein (tBid). Cleavage also exposes tBid's pro-apoptotic BH3 domain, allowing tBid to interact with its mitochondria-anchored receptor, cardiolipin (Luo *et al.*, 1998; Li *et al.*, 1998; Kim *et al.*, 2004). Caspase-8's role in the activation of Bid thus illustrates a mechanism whereby activation of the

extrinsic apoptotic pathway may result in cross-talk with the intrinsic pathway of apoptosis.

Bax/Bak-mediated mitochondrial outer membrane permeabilization allows for the release of cytC without disrupting ATP synthesis, thus allowing apoptosis to proceed. However, outer membrane permeabilization is by itself insufficient to catalyze the release of all sequestered cytC: 85% of cytC is attached to the inner mitochondrial membrane bound to cardiolipin, a four-tailed phospholipid unique to the mitochondria that stabilizes ETC proteins like cytC. As the induction of apoptosis results in rapid, nearly complete release of cytC from the mitochondria (Goldstein *et al.*, 2000), any proposed mechanism for cytC release must account for all of the sequestered protein. While activated Bax can only trigger the release of 20% of the total mitochondrial pool of cytC, tBid is able to release nearly 100% of mitochondrial cytC at a 500 fold lower concentration (~0.2 nM) (Jurgensmeier *et al.*, 1998; Scorrano *et al.*, 2002). Upon binding to outer membrane cardiolipin, tBid catalyzes the redistribution of cytC from the inner membrane to the intermembrane space through a mechanism involving dramatic remodeling of cristae structure. This activity occurs independently of the BH3 domain required for Bax/Bak oligomerization (Scorrano *et al.*, 2002).

It has also been suggested that tBid is able to trigger apoptosis without involvement of either Bax or Bak. Once activated, a small proportion of the tBid population forms trimers that insert themselves into the outer mitochondrial membrane, forming a pore large enough for cytC release (Grinberg *et al.*, 2002). It is

not yet known the extent to which this activity contributes to the overall permeabilization of the mitochondrion in physiological settings, but does indicate a mechanism whereby the truncation of Bid may be able to circumvent the antiapoptotic defenses of tumour cells (such as Bax repression and Bcl-2 overexpression).

1.2.4.6 Prostate apoptosis response-4 (Par-4): A key mediator of apoptotic responses in hormone-refractory cancers

Although apoptosis-regulating mechanisms are well conserved amongst different cell types, some apoptotic machinery, like Par-4, is cell type specific. Par-4 was first identified when androgen independent prostate cancer cells were induced to undergo apoptosis (Sells et al., 1994) and has been since characterized as a means of circumventing the anti-apoptotic defenses of prostate cancer cells (Gurumurthy and Rangnekar, 2004). Par-4 selectively mediates apoptosis in hormone-refractory cancers (Chakraborty et al., 2001), which is intriguing, as these cancers tend to have defective apoptotic machinery that typically makes the induction of apoptosis more difficult to achieve. The Par-4 gene product is known to sensitize cells to apoptotic stimuli by affecting both the extrinsic and intrinsic apoptotic pathways (Bergmann et al., 2004; Boehrer et al., 2002). Par-4 promotes apoptosis through the intrinsic apoptotic pathway by down-regulating bcl-2 (Qiu et al., 1999), thus preparing the cell for release of apoptogenic factors from the mitochondria. Furthermore, Par-4 activates the extrinsic pathway of apoptosis by catalyzing the golgi-dependent translocation of both Fas and FasL to the cell membrane. This Fas-FasL trafficking

causes FADD activation and DISC assembly, ultimately resulting in caspase-8 activation (Chakraborty *et al.*, 2001).

1.2.5 Cancer cell invasion and matrix metalloproteinase activity

Many types of cancer are readily treated in the early stages of development and progression, so long as the tumour cells remain localized and don't metastasize to secondary sites. This is particularly true of prostate and breast carcinomas. If caught early enough, complete surgical removal of the tumour is often possible via either prostatectomy or mastectomy (often accompanied by chemo- or radiotherapy). This window of opportunity is closed, however, once the cancer becomes metastatic, entering either the circulatory or lymphatic systems to spread to distant parts of the body – skeletal metastases being common secondary sites in both breast and prostate cancer.

Tumor metastasis is a complicated process that involves extensive interactions between invading cancer cells and the extracellular environment. The growing tumour is constantly interacting with the extracellular matrix (ECM) through a variety of enzyme- and cytokine-mediated signaling events that enhance growth-signaling pathways and promote angiogenesis, tissue invasion and metastasis. Physical barriers to carcinoma cells must be overcome for invasion into nearby tissue to occur, and degradation of the vasculature basement membrane is a necessary precondition for metastasis. Interactions between the tumour cell and the

ECM are mediated by a variety specialized proteolytic enzymes produced by both cancer cells and surrounding stromal cells (Chang and Werb, 2001).

1.2.5.1 The matrix metalloproteinases

Of the many proteolytic enzymes responsible for mediating the relationship between the tumour and its environment, perhaps none are more critical to the processes of invasion and metastasis than the matrix metalloproteinases (MMPs). The MMP family contains at least 24 zinc-dependent proteolytic enzymes that are collectively capable of digesting most ECM structural components, with each member having a distinct, yet overlapping substrate specificity (Hyun-Jeong and Parks, 2007). Family members differ from one another in the presence or absence of domains that contribute to their substrate specificity, matrix binding, cellular localization and inhibitor binding (Powell and Matrisian, 1996). The collagenases (including MMPs 1, 8, 13 and 18) selectively degrade triple-helical fibrillar collagens, the major components of bone and cartilage while the stromelysins target a range of ECM proteins and include MMPs 3, 10 and 11. The membrane-type MMPs (MT-MMPs; includes MMPs 14-17, 24, 25) are characterized by their attachment to the cell membrane and can degrade a variety of ECM components, including gelatin, fibronectin, and laminin. The gelatinases (MMPs 2 and 9) are selective for type IV collagen and gelatin (denatured collagen), and are the focus of this research as their activities are essential for the ECM remodeling that accompanies invasion and metastasis.

1.2.5.2 MMP activities in neoplastic tissues

MMPs are involved in many normal physiological processes such as wound repair, tissue and bone remodeling, vasculogenesis, and organogenesis and are also responsible for mediating a range of extracellular signaling pathways vital to the maintenance of tissue homeostasis (Chang and Werb, 2001). Abnormal regulation of MMP expression results in enhanced tumour survival and growth, angiogenesis, invasion and metastasis (Stamenkovic, 2000; Sternlicht and Werb, 2001). MMPs were first implicated in the progression of cancer because of their ability to degrade key ECM and connective tissue components, namely the collagens, fibronectins, laminins, and proteoglycans (Matrisian, 1990). These ECM structures are the physical barriers that impede cell proliferation and invasion into new tissues. As such, the breakdown of these structures constitutes the rate-limiting step of tissue invasion and metastasis (Sternlich and Werb, 2001). MMPs 2 and 9 are primarily responsible for the degradation of gelatins and collagens (Matrisian, 1990), two of the more ubiquitous ECM structures, and vital components of vasculature basement membrane.

MMPs do far more than just remodel ECM structure. The proteolysis of the ECM by active MMPs releases sequestered growth factors that are subsequently activated by proteolytic cleavage by MMPs. MMPs also promote the cleavage and activation of over 40 different cell surface-bound proteins, many of which (like the fibroblast growth factor receptor) have roles in growth regulation (Chang and Werb, 2001; Parks *et al.*, 2004; Cauwe *et al.*, 2007). MMPs further encourage cancer

development through the promotion of angiogenesis (new vasculature formation) by releasing and activating ECM-sequestered pro-angiogenic factors such as vascular endothelial growth factor A (VEGF-A) and the angiopoietins (Bergers *et al.*, 2000). Formation of tumour vasculature grants the tumour access to oxygen and nutrients and provides the cancer a route whereby it can metastasize to distant tissues (Folkman, 1972). MMPs therefore, are not just degraders of ECM components, but instead are extracellular processing enzymes involved in regulating a wide variety of cell-cell and cell-matrix signaling events.

Given the wide range of cancer-promoting activities of MMPs 2 and 9, it should not be a surprise that their expressions correlate with cancer progression and poor patient prognosis. Increased plasma and urine levels of MMPs 2 and 9 have been correlated with the degree of metastasis in prostate cancer patients (Moses *et al.*, 1998; Gohji *et al.*, 1998). Biopsies of prostate tumors have likewise found that MMP-2 and -9 mRNA expression and enzymatic activity can predict advanced stage prostate cancer (Kuniyasu *et al.*, 2003; Zhang *et al.*, 2004). The prognostic value of MMP-2 has also been established in breast cancers. A number of studies have shown that increased MMP-2 expression is linked to an aggressive clinical course in breast carcinomas, and that patients with MMP-2 negative tumours have more favourable disease outcomes (Talvensaari-Mattila *et al.*, 2003; Sivula *et al.*, 2005). Studies investigating the potential prognostic value of MMP-9 have proven to be inconclusive (Turpeenniemi-Hujanen, 2005). Combined with extensive *in vitro* and *in vivo* experimental studies, this clinical data provides rationale for the development of therapies designed to inhibit the activities of MMPs 2 and 9.

1.2.5.3 Regulation of MMP expression and activity

MMPs are intriguing targets for therapeutic intervention not only because of their relevance to invasion and metastasis, but also because there are so many avenues through which their expression and activity can be regulated. Catalytic activity of MMPs is regulated at three distinct points – gene expression, proenzyme/zymogen activation, and enzyme inactivation. The regulation of MMP gene expression is extraordinarily complex, as would be expected given their crucial importance in organism development and in the maintenance of tissue homeostasis. MMP gene expression is controlled by promoters that are responsive to a wide range of cytokines, hormones and growth factors including the interleukins, interferons, platelet derived growth factor, epidermal growth factor, vascular endothelial growth factor, tumour necrosis factor alpha, transforming growth factor beta, amongst many others. Transcriptional regulation of MMPs is also responsive to oncogenic transformation, accounting for the observations of increased MMP expression in most all cancers (Cauwe *et al.*, 2007; Yan and Boyd, 2007).

The production and secretion of MMPs is also responsive to a variety of cancer related signaling pathways. While cancer cells themselves are capable of producing and secreting MMPs into the ECM, they are also capable of co-opting the MMP-producing machinery of nearby stromal cells through a variety of cell-bound or soluble signaling factors. These factors include the extracellular matrix metalloproteinase inducer (EMMPRIN), a 58 kDa transmembrane protein located on the surface of tumour cells that has been shown to induce the production of MMPs 1

2 and 3 in stromal cells (Gabison *et al.*, 2005). A growing body of evidence is also unveiling EMMPRIN's autocrine function, as it is capable of stimulating MMP production in tumour cells as well as in stromal cells (Sun and Hemler, 2001; Caudroy *et al.*, 2002).

1.2.5.4 Regulation of MMP activity: pro-MMP activation

The majority of MMPs are secreted in an inactive (latent) form called either a zymogen or a pro-MMP, which requires an activation step to gain its proteolytic activity. MMP activity requires an unencumbered Zn^{2+} ion present in the catalytic site. The prodomain of MMP zymogens contains a cysteine residue (Cys^{73}) that stabilizes the pro-MMP in its inactive form by forming a bond between its thiol group and the Zn^{2+} in the catalytic domain. The intact Cys^{73} thiol-Zn^{2+} bond sequesters the Zn^{2+} from the catalytic site, thereby rendering the MMP inactive. MMP activation thus requires disruption of this bond through a mechanism called the "cysteine switch" (Hyun-Jeong and Parks, 2007).

There are three distinct mechanisms whereby the thiol-Zn^{2+} interaction can be broken, yielding a catalytically active MMP: 1) direct cleavage of the zymogen prodomain by other proteinases, 2) oxidation of the free thiol group by oxidants and 3) allosteric interference within the zymogen that forces apart the prodomain and the catalytic site. All mechanisms render the MMP catalytically active, but in mechanisms that do not involve proteolytic cleavage (i.e. 2 and 3), the MMP is still considered a zymogen until the prodomain is lost. However, these mechanisms also

render the MMP susceptible to inter- or intramolecular autolytic cleavage (Springman *et al.*, 1990; van Wart and Birkedal-Hansen, 1990; Hyun-Jeong and Parks, 2007). Loss of the prodomain renders the MMP completely functional, and reduces the enzyme's total mass by 8-10 kDa.

In many cases, active MMPs are responsible for the proteolytic cleavage of their zymogen counterparts, suggesting a possible feed-forward cascade of MMP activation in response to MMP-activating stimuli. MMP-9, for example, is activated by MMPs 2, 3, 7, 10, and 13. Given the heterogeneity of the MMP family, this mechanism could also be responsible for fine tuned control of MMP activation *in vivo* by other MMPs (Nagase, 1997). Activation of proMMP-2 requires cooperative action between MT1-MMP and the tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). TIMP-2's C-terminal end interacts with both proMMP-2 and MT1-MMP to bring them into close proximity so that MT1-MMP can cleave off proMMP-2's prodomain (Cao *et al.*, 1996; Hyun-Jeong and Parks, 2007).

MMP activation can also be controlled by oxidants produced by cells and secreted into the extracellular environment. Oxidation of the cysteine^{72} prodomain thiol breaks the thiol-Zn^{2+} bond (flipping the cysteine switch), an event that is subsequently followed by autolytic cleavage and loss of the zymogen prodomain. There is considerable evidence that in *in vitro* settings, reactive oxygen species (ROS) are able to activate a number of proMMPs through autolytic cleavage – hypochlorous acid, for example, activates (amongst others) proMMP-9 (Weiss *et al.*,

1985; Peppin and Weiss, 1986), while peroxynitrate can activate proMMPs including proMMP-2 (Okamato *et al.*, 1997).

1.2.5.5 Regulation of MMP activity: inhibition of activated MMPs

Activated MMPs are regulated through reversible binding to the tissue inhibitors of matrix metalloproteinases (TIMPs) (Wojtowicz-Praga *et al.*, 1997). The TIMPs are a family of secreted proteins that associate with MMPs in a 1:1 ratio to inhibit their activity. The N-terminal domain possessed by TIMPs contains residues that interact with the Zn^{2+}-binding region of the MMP's catalytic domain, thereby impairing the MMP's ability to cleave substrates (Blavier *et al.*, 1999). Net MMP activity thus results from the balance of MMPs and TIMPs, and this balance is largely responsible for the regulation of ECM remodeling. Imbalances in the MMP/TIMP ratios also play a major role in the development of an invasive phenotype in neoplastic diseases. To date, four members of the TIMP family have been discovered: TIMPS 1-4. This study focused on the protein expressions of TIMP-1 and TIMP-2, as these two TIMPs are most directly involved in the regulation of MMPs 2 and 9. TIMP-1 and TIMP-2 both bind to a variety of MMPs, though it has been shown TIMP-1 preferentially binds to MMP-9, whereas TIMP-2 preferentially binds to MMP-2, and is not an effective inhibitor of MMP-9 (Murphy and Willenbrock, 1995; Gomez *et al.*, 1997; Wojitowicz-Praga *et al.*, 1997; Cauwe *et al.*, 2007).

CHAPTER 2

Vaccinium macrocarpon Phytochemicals Induce Programmed Cell Death in DU145 Human Prostate Carcinoma Cells.

2.1 Introduction

The goal of many chemotherapeutic and chemopreventative strategies involves halting the seemingly limitless expansion of tumour cell populations, either through cell cycle arrest or the induction of apoptotic pathways. Whole cranberry extracts have been shown to inhibit the proliferation of tumour cell lines, including those representative of prostate (LNCaP, PC3, DU145, RWPE-1, RWPE-2, 22Rv1) and breast (MDA-MB-435, MCF-7) carcinomas through mechanisms that include cell cycle arrest and apoptosis (Yan *et al.*, 2002; Murphy *et al.*, 2003; Ferguson *et al.*, 2004; Seeram *et al.*, 2004; Sun *et al.*, 2006; Boivin *et al.*, 2007). These effects can be attributed to a variety of cranberry phytochemicals, including the flavonols (particularly quercetin), the proanthocyanidins, stilbenes (resveratrol) and the triterpeneoids (particularly ursolic acid) (Neto *et al.*, 2006). The work presented in this chapter elucidates the mechanisms whereby cranberry triggers apoptotic death in DU145 prostate cancer cells, and it was hypothesized that these mechanisms focus primarily on the intrinsic pathway of apoptosis.

2.2 Materials and Methods

All materials, unless otherwise indicated, were attained from Sigma-Aldrich (Oakville, ON).

2.2.1 Preparation of cranberry extracts

The whole cranberry, the anthocyanin (ANTHO), flavonol (FLAV), and proanthocyanidin (PAC) extracts were prepared and characterized by the laboratory of Dr. Cathy Neto as described in Appendix A.

2.2.2 Cell culture

Human DU145 prostate adenocarcinoma cells (ATCC, Manassas, VA) were cultured on 100mm plastic tissue-culture dishes (Falcon, Mississauga, ON) in αMEM (Gibco, Burlington, ON) supplemented with 1% (vol/vol) antibiotic-antimycotic (Gibco) and 10 % (vol/vol) Fetal Clone III fetal bovine serum replacement (FBS; Hyclone/VWR Canlab, Mississauga, ON) at 37 °C and 5% CO_2. In order to prevent potential genetic drift arising from continuous culture, cells were only cultured for a maximum of 30 passages, at which point cells were discarded and replaced with cells frozen at -80 °C in a solution of 5% DMSO and 95% cell culture medium.

2.2.3 Solubilization of cranberry extracts for cell treatments

Immediately prior to use, cranberry extract (or the extract of cranberry fractions) was solubilized in methanol at a concentration of 20 μg/μL, as per laboratory practice. This cranberry solution was then diluted to the concentrations required for experiments in cell culture medium either with or without FBS (10% vol/vol). Initial work with the Alamar blue viability assay revealed no appreciable toxicity in response to methanol treatment (up to concentrations as high as 2.5%). Cells not treated with cranberry or its extracts (negative control cells) were treated with medium containing methanol at a concentration equal to that found in the highest tested concentration of cranberry in that experiment (usually either 1% or 2.5% methanol).

2.2.4 Measurements of cell viability

MTT assay

The MTT assay is amongst the most commonly employed assays used for the determination of either cytotoxicity or cellular proliferation. In this quantitative colorimetric assay, the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is reduced by living cells to water-insoluble purple formazan crystals, which are then solubilized in iso-proponaol (Mossman, T., 1983). It was originally believed that the tetrazolium salt is reduced exclusively by mitochondrial dehydrogenases on the respiratory chain of viable cells (Slater *et al.*,

1963), though it is now known that microsomal and cytosolic fractions are also involved in MTT reduction (Berridge and Tan, 1993; Gonzalez and Tarloff, 2001).

DU145 cells were plated into a 48 well plate at $2x10^5$ cells/well. This cell number was chosen following optimization of the MTT assay for the intrinsic metabolic activity of DU145 cells to ensure that the assay was capable of detecting minor changes in the overall population viability. Cells were incubated for 24 hours prior to treatment, allowing the cells to become attached to the plate and enter the logarithmic phase of growth. Cell culture medium was removed and replaced with a solution of cranberry extract (0-250 μg/ml) diluted in cell culture medium (either with or without 10% FBS). Cells were treated for either 6 or 24 hours.

Following treatment, cell culture medium was carefully removed and replaced with fresh medium (to prevent treatment interference with the assay). MTT reagent, reconstituted in 1X Hanks Balanced Salt Solution, was added to the cell culture medium in an amount equal to 10% of the culture medium volume. Cells were then returned to the incubator for three hours, during which time purple formazan crystals were formed. Following the incubation period, formazan crystals were dissolved by adding an amount of MTT Solubilization Solution equal to that of the original culture medium volume (200 μL). Plates were mixed on a gyratory shaker for half an hour prior to trituration (pipetting up and down) of the formazan crystal suspension. Plates were again briefly agitated on the shaker prior to spectrophotometrically measuring the absorbance at 570 nm. Triplicate assay

replicates were averaged, the background absorbance subtracted and the absorbance of treated cells compared to that of the vehicle treated controls.

Alamar Blue assay

The Alamar Blue (AB) assay (Invitrogen, Burlington, ON) serves to measure cellular metabolic activity with the goal of determining the survival and proliferation of mammalian cells. This is a one step assay in which a redox indicator (resazurin) is added to growing cells in culture. This blue, non-fluorescent, redox indicator is then reduced intracellularly by viable cells, yielding a pink fluorescent product, resorufin (O'Brien *et al.*, 2000). The extent of this conversion serves as a reflection of cell viability, and can be quantified by fluorescence spectroscopy. The AB reagent and MTT reagent differ in their redox potentials and the location where the reagents interact with the electron transport chain (ETC). AB reagent (resazurin) displaces O_2 as the final electron acceptor of the ETC. MTT, meanwhile, intervenes in the electron transport process at an earlier stage – it accepts electrons prior to their being passed to the cytochromes, thereby shutting down the ETC (Gonzales and Tarloff, 2001; Hamid *et al.*, 2004). AB reagent is thus non-toxic, unlike the MTT reagent.

Optimization of the AB assay for the DU145 cell line was conducted to determine the ideal assay conditions such as optimal plating density (5000 DU145 cells/well) and duration of incubation with AB reagent (three hours). DU145 cells, diluted to a concentration of $5x10^4$ cell/ml in cell culture medium supplemented

with 10% FBS, were seeded at a density of 5000 cells /well in 96 well cell culture plates. Cells were allowed to incubate for twenty-four hours prior to treatment and were then treated with either the whole cranberry extract, the PAC extract, FLAV extract, or the ANTHO extract at concentrations ranging from 0 μg/ml (vehicle-treated control) to 500 μg/ml. To determine the effects of fetal bovine serum on cranberry cytotoxicity, these extracts were diluted in either serum-free medium, medium supplemented with 1% FBS, or medium supplemented with 10% FBS. Cells were treated for either six or twenty four hours. Following treatment, AB reagent was added to each well at a volume 10% that of the cell culture medium in the well (i.e. 10 μL AB added to 100 μL cell culture medium). Cells were returned to the incubator for three hours, at which point the plate was removed from the incubator, allowed to cool to room temperature (ensuring accurate fluorescence readings), and fluorescence was read at 595nm. The reduction of AB reagent by treated cells was compared to that of vehicle-treated controls. Viability of treated cells was expressed as a fraction of that of vehicle-treated cells.

2.2.5 Measurement of necrosis: LDH release assay

Cranberry's potential to induce necrotic cell death in DU145 cells was determined by evaluating the release of lactate dehydrogenase (LDH) from cranberry-treated cells into the supernatant. The release of LDH, a ubiquitous cytoplasmic protein, into cell culture medium is indicative of the decreased plasma membrane integrity that occurs in response to necrosis. LDH release was evaluated

using the Cytotoxicity Detection Kit (LDH) from Roche Applied Science (Roche Diagnostics, Laval, QC). This assay determines LDH activity in an enzymatic test in which NAD^+ is reduced to NADH by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, the diaphorase catalyst transfers 2 H^+ from NADH to the tetrazolium salt INT, which is then reduced to formazan. The amount of formazan produced is directly proportional to the enzymatic activity of the LDH enzyme, which is proportional to the number of cells with damaged plasma membranes (Decker and Lohmann-Matthes, 1988).

To test the cranberry's ability to induce necrosis in DU145 cells, cells were seeded at 5000 cells/well (100 μL of a $5x10^4$ cells/ml suspension) in a 96 well plate. This seeding density was determined following experiments to optimize the assay to take into account the LDH content of DU145 cells. Cells were then incubated for 24 hours, at which point cells were treated with cranberry extracted diluted in cell culture medium supplemented with 1% FBS (maximum allowable concentration of FBS, as FBS contains LDH). Cells were treated with 200 μL cranberry over a range of concentrations (0 μg/ml to 250 μg/ml) for either 6 or 24 hours (triplicate wells used for assay replicates). Some cells were also treated with 2% triton X-100 for one hour as a positive control (as triton causes complete cell lysis and release of all cytoplasmic LDH). Following treatment, 100 μl of cell culture supernatant was carefully removed from each well and transferred into corresponding wells of a 96 well assay microplate. 100 μl of freshly prepared reaction mixture (catalyst + dye solution) was added to the cell culture medium and allowed to incubate at room temperature for 30 minutes. Sample absorbance was read at 490 nm (595 nm used

as a reference wavelength). The average absorbance values of the assay triplicates were calculated and subtracted from each value was the absorbance readings from the background control. Cranberry-mediated cytotoxicity was evaluated by comparing the effects of cranberry treatment to the effects of triton treatment according to the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{abs. cranberry treated cells - abs. vehicle treated cells}}{\text{abs. triton-treated cells - abs. vehicle treated cells}} \times 100$$

2.2.6 Measurement of cytoplasmic nucleosome enrichment

One of the hallmarks of late stage apoptosis is endonuclease-mediated cleavage of DNA. Eukaryotic DNA is organized into nucleosomes, which are structures consisting of DNA complexed with core histone proteins (H2A, H2B, H3 and H4). Histone proteins prevent endonucleases from being able to cleave nucleosomal DNA, so endonucleases instead target double stranded DNA at the most accessible internucleosomal linker regions (Burgoyne *et al.*, 1974). This selective targeting of DNA by endonucleases results in the production of mono- and oligonucleosome fragments, which then escape the nuclear envelope and enter the cytoplasm. Mono- and oligonucleosome formation in cranberry-treated DU145 cells was evaluated using the Roche Cell Death ELISA Plus kit (Laval, QC). This assay is based on a quantitative sandwich-enzyme immunoassay principle using monoclonal antibodies directed against DNA and histones. Cell lysates are loaded into a 96 well streptavidin-coated microplate. Incubated with the cell lysate is a mixture of anti-histone-biotin and anti-DNA-peroxidase. During the incubation period, the anti-

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. Following the removal of unbound antibodies by washing, the amount of nucleosomes present is determined by evaluating peroxidase-mediated cleavage of ABTS (which yields a colorimetric signal proportional to the amount of nucleosomes present).

DU145 cells were plated into each well of a 96 well plate (5000 cells/well), and were allowed to incubate for 24 hours at 37 °C (5% CO_2). Cells were then treated for 24 hours with either the proanthocyanidin fraction or flavonol fraction of cranberry, diluted in serum-free cell culture medium at the following concentrations: 0 μg/ml, 10 μg/ml, 25 μg/ml and 50 μg/ml. Cells were also treated with 5 μM staurosporine as a positive control for the induction of apoptosis (Th'ng *et al.*, 1994; Zhang *et al.*, 1996). Following treatment, the cell culture supernatant was carefully removed, and the cell pellet was resuspended in 200 μL of lysis buffer. Following a 30-minute incubation at room temperature, the microplate was agitated on a gyratory shaker for 30 minutes to facilitate cell detachment. Tituration was also necessary to ensure complete removal of cells from the microplate. Cell lysates were then transferred into eppendorfs and centrifuged at 200 x g for 10 minutes. 20 μl of supernatant was then transferred (in triplicate) into the streptavidin-coated microplate for analysis, as was 80 μl of immunoreagent (1 anti-histone-biotin: 1 anti-DNA-peroxidase: 18 incubation buffer [vol:vol:vol]). Following a 3-hour incubation at room temperature with gentle shaking on a gyratory shaker, each well

was washed 3 times with incubation buffer prior (250 µl/well). Once wells were dry after the final washing, 100 µl ABTS solution was pipetted into each well. After ~20 minutes on a gyratory shaker, the reaction was stopped using 100 µl ABTS stop solution. Absorbance was read at 405 nm (490 nm measured as a reference). The triplicate absorbance readings for each sample were averaged, and the background value (Incubation buffer, ABTS solution, ABTS Stop solution) was subtracted from each of those averaged values. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated using the following formula:

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

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

2.2.7 Measurement of PARP activity

Upon activation, caspase-3 cleaves PARP into an 85 kDa fragment which lacks activity. Thus, PARP activity can be evaluated as a marker of apoptosis. Cranberry-treated DU145 cell lysates were evaluated for PARP activity using the PARP Universal Colorimetric Assay Kit (R&D Systems, Cedarlane Laboratories, Burlington, ON). This assay measures the incorporation of biotinylated (ADP-ribose) onto histone proteins in a 96-well plate. DU145 cells were seeded at $5x10^5$ cells/dish in 100mm cell culture dishes. 24 hours later, cells were treated with cranberry at concentrations ranging from 10 µg/ml to 100 µg/ml for 6 and 24 hours. Following treatment, cells were washed three times in ice-cold 1x PBS and were removed from

the cell culture dish via exposure to a 10% (vol/vol) solution of trypsin-EDTA in 1x PBS at 37°C for 2 minutes. Cells were washed with 5 mL cell culture medium (supplemented with 10% FBS as a trypsin inhibitor) and were then collected in 15 ml tubes. $1x10^7$ cells were centrifuged at 400 x g for 10 minutes at 4 °C and the supernatant was carefully removed and discarded. The pellet was then resuspended in 1x PBS, centrifuged at 10,000 x g (12 seconds) and, after removal of the supernatant, resuspended again in 5 - 10 pellet volumes of cold cell lysis buffer (1X PARP buffer, 0.4 mM PMSF, 0.4 M NaCl, 1x Halt Protease Inhibitor cocktail [Pierce Biotechnology, Nepean, ON] and 1% Triton X-100). Cells were incubated in the buffer on ice for 30 minutes with periodic vortexing. Insoluble material was removed from the cell suspension by centrifugation at 10,000 x g for 20 minutes. Cell lysate supernatant was aliquoted into fresh chilled eppendorfs and stored at -80°C.

The protein concentration of the supernatant was determined using Bio-rad's Bradford assay (Mississauga, ON). 2 μL of protein suspension was diluted in 1598 μL of distilled water in a microcuvette. 400 μL of Bio-rad's Bradford assay reagent was added to each cuvette, mixed by inverting, and allowed to incubate for seven minutes, at which time absorbance was read at 595 nm. A serial dilution of bovine serum albumin (BSA) of known concentrations was used to prepare a standard curve. Using the line of best fit from that curve, absorbance readings were converted to protein concentrations (μg/ml).

30 μg of protein from each treatment was loaded, in triplicate, into the wells of a histone-coated plate, as was 25 μl of 1X PARP cocktail. The plate was incubated at room temperature for one and a half hours. Following this incubation period, the plate was washed 5 times with 1x PBS (200 μL/well), ensuring that all liquid was removed between each washing. Wells were then incubated with 50 μL Strep-HRP for 20 minutes at room temperature, followed by another 5 washes with 1x PBS (200 μL/well). Post washing, the plate was incubated 50 μL TACS-Sapphire in the dark for 30 minutes. TACS-Sapphire is an HRP substrate that generates a soluble blue color. Following this incubation, the reaction was terminated with 0.2 M HCl, and absorbance at 450 nm was measured. A serial dilution of supplied PARP-HSA enzyme was prepared for use as a standard curve (1 unit/25 μL, 0.5 units/25 μL, 0.1 units/25 μL, 0.05 units/25 μL, and 0.01 units/25 μL). Absorbance readings of cranberry-treated cell lysates were fit into the standard curve to determine the standard PARP units.

2.2.8 Measurement of Caspase-8 and -9 activities

Caspase activation remains *the* hallmark of apoptotic pathways. As such, bioluminescent caspase assays (Caspase-Glo, Promega) were employed to evaluate the activation of caspases-8 and -9 in cranberry-treated DU145 cells. The Caspase-Glo provides a luminogenic caspase substrate in a buffer system optimized for caspase activity, luciferase activity and cell lysis. The luminogenic substrates employed in the caspase-8 and -9 assays contain tetrapeptide LETD and LEHD

sequences, respectively. Each substrate is specific for the activity of the caspase under evaluation. Cleavage of the caspase substrate releases aminoluciferin, a substrate for luciferase. The Ultra-Glo Recombinant Luciferase then acts upon the aminoluciferin to generate a stable luminescent signal. As the luminogenic substrates in these assays are not substrates for the luciferase until cleaved to release luciferin, the signal to noise ratio (background) is inherently quite low. For this reason, this assay is incredibly sensitive, and can detect caspase activation in as few as 1500 cells (Niles *et al.*, 2004; Meisenheimer *et al.*, 2008).

To evaluate the effect of cranberry treatment on the activities of caspases 8 and 9 in DU145 cells, cells were seeded at a density of 5000 cells/well in 96 well tissue culture plates (plating density determined following assay optimization for DU145 cells to ensure maximum sensitivity for caspase detection). Clear cell culture plates were used to evaluate cell growth and confluence while white walled plates (Nalge NUNC, VWR, Misissauga, ON) were used to measure the luminescent output of the assay. Cells were incubated for 24 hours, and were then treated with either a whole cranberry extract, a proanthocyanidin-enriched extract, or a flavonol-enriched extract. Concentrations of these extracts ranged from 0 μg/ml to 50 μg/ml, and treatment durations from 3 to 24 hours were evaluated. Upon completion of the treatment, cell culture supernatant was carefully removed from each well and was replaced with room temperature 1x PBS (50 μL/well). 50 μL of freshly reconstituted Caspase-Glo reagent was added to each well, and the plate was mixed on a plate shaker for 10 minutes, ensuring complete cell lysis. Plates were then incubated at room temperature for 3 hours prior to reading the luminescence in a

plate-reading luminometer. Caspase activity of treated DU145 cells was expressed as a fold difference relative to vehicle-treated control cells.

2.2.8 Immuoblot analysis

$5x10^5$ DU145 cells were seeded in 100mm plastic tissue-culture dishes, and incubated at 37°C for 24 hours. After 24 hours, serum-containing medium was removed and replaced with serum free medium for a few hours. Cells were then treated with a whole cranberry extract (10 μg cranberry/ml or 100 μg cranberry/ml for either 6 or 24 hours). Following the treatment, cells were washed three times in ice-cold 1x PBS to remove all traces of cell culture medium and treatment. To remove adherent cells from the cell culture dish, cells were exposed to a 10% (vol/vol) solution of Trypsin-EDTA in PBS at 37°C for 2 minutes. Cells were washed with 5 mL cell culture medium (supplemented with 10% FBS as a trypsin inhibitor) and were then collected in 15 ml tubes. Cells were pelleted by centrifugation at 500xg for 10 minutes. Cell culture medium was carefully removed from the cell pellet, which was then reconstituted in 1X PBS. Cell suspension was then transferred to an eppendorf tube and cells were again pelleted at 500xg for 10 minutes. Upon removal of the supernatant (1X PBS), the cell pellet was then stored at -80°C.

Prior to use in immunoblot analysis, the cell pellet was reconstituted in 100-150 μl RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM PMSF, 1 mM EDTA,

1% Brij-40, 1% Sodium deoxycholate, 0.1% Sodium orthovanadate 0.1% SDS and 1X Halt protease inhibitor cocktail [Pierce Biotechnology, Nepean, ON]). Cells were maintained on ice for 20 minutes, with periodic vortexing every 5 minutes. Following this period of incubation, cells were briefly lysed (3 seconds) by sonication. The cell lysates were centrifuged at 10,000 rpm for 20 minutes at 4 °C to pellet cellular debris. The supernatant, containing cytosolic proteins, was carefully removed from the pellet. Cell lysate supernatant was then aliquoted, and stored at -80 °C.

For the immunoblot analysis of mitochondria-sequestered cytC, it was necessary to prepare a mitochondria-free cytosolic fraction. This procedure required extensive troubleshooting, as it is quite difficult to selectively permeabilize the cellular membrane without affecting the integrity of the mitochondrial outer membrane. To isolate mitochondria from cranberry-treated DU145 cells, the Pierce Mitochondria Isolation Kit was employed (Pierce Biotechnology, Nepean, ON), according to the provided protocol (with a number of alterations). Briefly, cells were harvested by trypsinization as previously described, and $2x10^7$ cells were centrifuged in an eppendorf tube at 800 x g for 10 minutes. The supernatant was carefully removed and discarded. 800 μL of Mitochondria Isolation Reagent A was added to the pellet, followed by vortexing (5 seconds) and a 2-minute incubation on ice. Following this incubation, 40 μL of Mitochondria Isolation Reagent B was added. The eppendorf was then vortexed and incubated on ice for ten minutes (vortexing every minute). During this step, cell morphology was periodically evaluated under light microscopy, until cells appeared swollen (which occurred after approximately

ten minutes). 800 μL of Mitochondria Isolation Reagent C was then added to the cell suspension, the tube was inverted several times to mix, and was then incubated on ice. Cell morphology was evaluated until cells became swollen and burst (after approximately 15 minutes). The suspension was then centrifuged at 700 x g for 10 minutes at 4°C (to remove cell membrane debris). The supernatant was then transferred to a new eppendorf tube, and was centrifuged again, this time at 12,000 x g for 5 minutes. The supernatant (cytosolic proteins without mitochondria) was removed and stored at – 80 °C, as was the pellet (isolated mitochondria).

The total protein concentration of each sample was determined using Bio-rad's Bradford assay. 2 μL of protein suspension was diluted in 1598 μL of distilled water in a microcuvette and mixed with 400 μL of Bio-rad's Bradford assay reagent, and allowed to incubate for seven minutes. At this time, absorbance was read at 595 nm. A serial dilution of bovine serum albumin (BSA) of known concentrations was used to prepare a standard curve. Using the line of best fit from that curve, absorbance readings were converted to protein concentrations (μg/ml).

Protein extracts were mixed in a 3:1 ratio with laemmli buffer (50 mM Tris-HCL [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM β-mercaptoethanol) and heated in a thermocycler at 95 °C for 3 minutes. Equal amounts of protein (50 μg) were resolved on 10-15% SDS-PAGE mini-gels at 10 mA/gel through the stacking gel, and 20 mA/gel through the resolving gel. Following electrophoresis in 1X running buffer (3.0 g tris-base, 14.4 g glycine, and 1.0 g sodium dodecyl sulfate [SDS] in 1 L H_2O, pH 8.3), gels were gently shaken in

transfer buffer (5.6 g tris-base, 3.0 g glycine in 0.8 L H_2O and 0.2 L MeOH) for thirty minutes prior to transferring the protein unto nitrocellulose membrane (Bio-rad) for 1 hour and 45 minutes at 65 volts. Membranes were incubated in blocking solution (5% bovine serum albumin in 1x TBS 0.05% Tween-20) overnight at 4°C, and then incubated in primary antibodies (1:200 dilution in 1% BSA in 1X PBS 0.05% Tween-20) also overnight at 4°C. The membranes were then washed in 1x TBS 0.05% Tween-20 for 30 minutes (3 10 minute washes) and then incubated in alkaline phosphatase (AP)-conjugated secondary antibodies (1:1000 dilution) for two hours. Following a second round of washes, membranes were developed using SigmaFast BCIP-NBT tablets to provide colorimetric determination of protein expression.

2.2.9 Image capture and gel quantification

Image capture of developed Western blots was performed with the AlphaImager gel documentation hardware (Alpha Innotech, San Leandro, CA) and quantified with Bio-Rad's Quantity One software package (Mississauga, ON). Actin protein expression was used as a loading control for all Western blot analysis performed. Blocked membranes were sliced horizontally into strips to allow for the probing of multiple antigen targets from the same SDS-PAGE gel, which is particularly important when evaluating actin as a loading control.

2.3 Results

2.3.1 Evaluating DU145 cell viability in response to cranberry treatment

Treating DU145 human prostate carcinoma cells with a whole extract of cranberry resulted in a dose-dependent decrease in cell viability, as measured by the MTT assay (Figure 2.3). The cytotoxic effects of cranberry depended not only on treatment dose and duration, but also on the presence of FBS in the medium, which impeded cranberry-mediated cytotoxicity.

Anomalous results from early MTT assays of cranberry-treated DU145 cells suggested that cranberry treatment caused a dramatic *increase* in cell viability, despite the observation of cellular morphology indicative of apoptotic death. A cell-free experiment was carried out in which cranberry conditioned medium was incubated with the MTT reagent for three hours. Surprisingly, this incubation resulted in a dose-dependent formation of formazan crystals, as observed by microscopy and absorbance at 595nm (Figure 2.4A). This MTT-reducing activity was also evaluated in three subfractions of cranberry (PAC, ANTHO and FLAV) and it was found that the ANTHO and FLAV fractions were the more potent MTT reducers (Figure 2.4B). The presence/absence of serum has no effect on cranberry's reduction of MTT to formazan, so results were pooled to provide the data in Figure 2.4A/B. To prevent cranberry from interfering with the MTT assay, cranberry-conditioned medium was carefully removed from treated DU145 cells and replaced with fresh cell culture medium prior to running the assay (yielding the data presented in Figure 2.3).

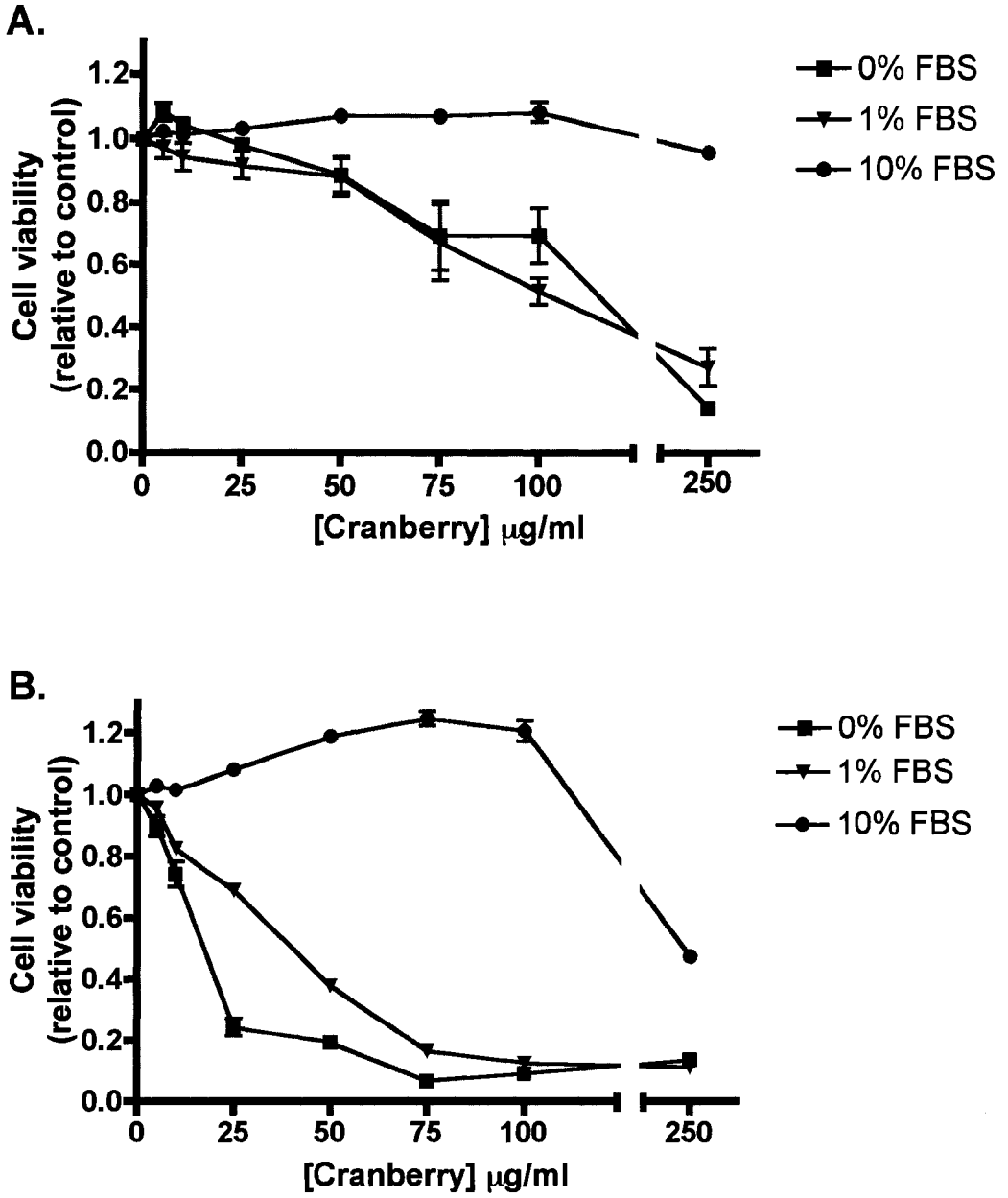

Figure 2.3: An evaluation of cranberry-mediated cytotoxicity in DU145 cells. DU145 cell viability was evaluated using the MTT assay following treatment with 5, 10, 25, 50, 75, 100, or 250 µg/ml whole cranberry extract for either 6 hours (A) or 24 hours (B). Cranberry conditioned cell culture medium contained either 0% FBS, 1% FBS, or 10% FBS (vol/vol). Values attained from three independent experiments (n=3) with three assay replicates. The reduction of MTT in cranberry-treated cells was compared to that of the vehicle-treated controls, so the values indicated are arbitrary units (relative to control). Data points where no error bars are visible indicate negligible variation between biological replicates.

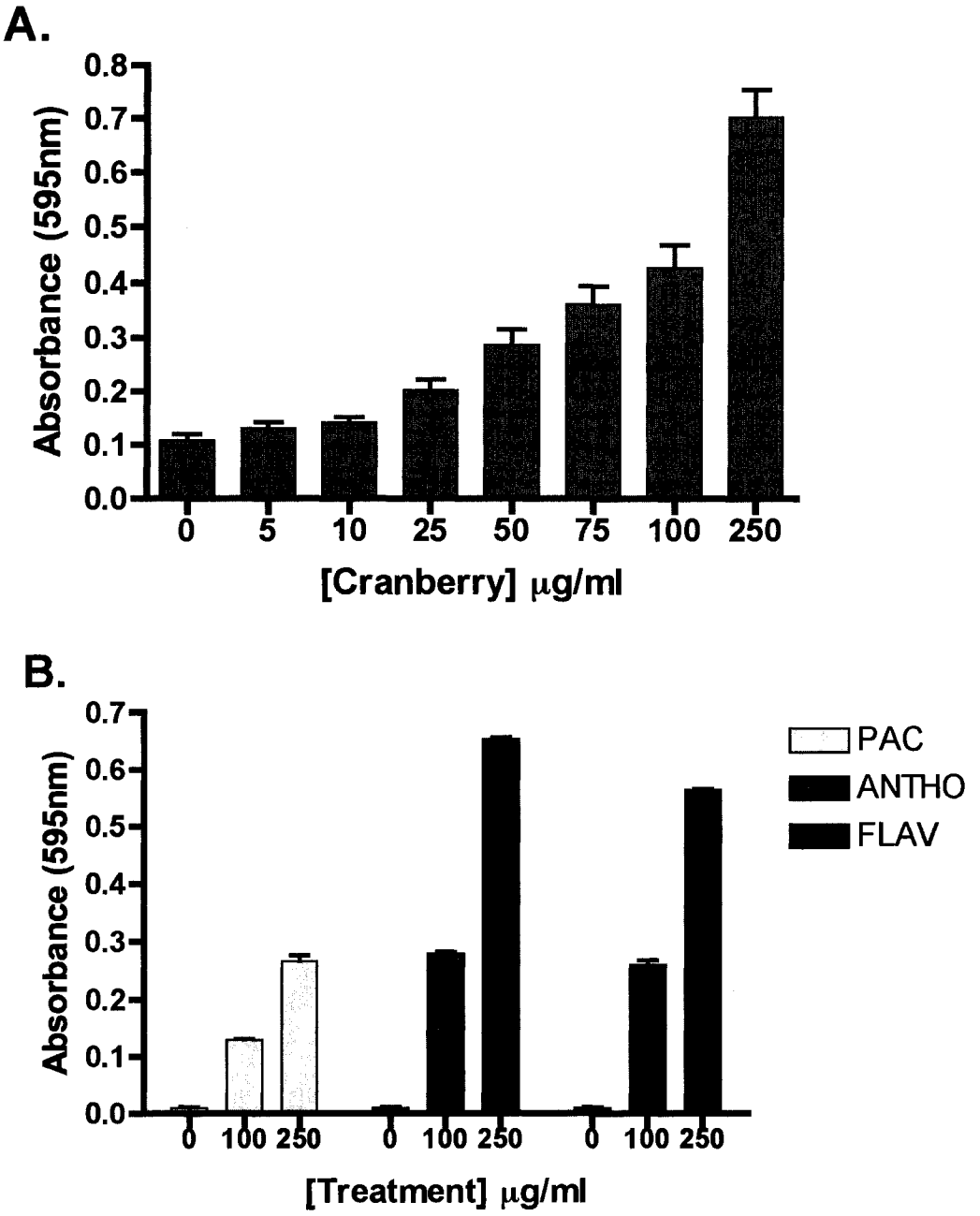

Figure 2.4: An evaluation of cranberry's MTT reducing activity. The ability of the whole cranberry extract (A) and the three subfractions of cranberry (B) to reduce MTT to formazan was evaluated in a cell free system. Results attained from two independent experiments (n=2) with three assay replicates.

For the reasons just presented, there was considerable concern as to whether the MTT assay could provide an accurate assessment of cranberry-mediated viability. Removing treatment medium prior to running the assay was necessary to prevent cranberry from reducing the MTT reagent, but medium renewal may have also resulted in the loss of viable, but loosely attached, DU145 cells. Furthermore, it couldn't be determined with any certainty that all cranberry was removed from the wells following medium renewal. It was thus decided to employ the Alamar Blue (AB) assay as a second means of evaluating DU145 cell viability, as cranberry-conditioned medium is unable to reduce the AB agent. The AB assay revealed that treating DU145 cells with a whole extract of cranberry resulted in a dose- and time-dependent decrease in cell viability (Figure 2.5). It was also found that the phytochemicals responsible for this cytotoxicity were concentrated in the PAC and FLAV fractions, as the ANTHO fraction showed little cytotoxic effects (Figure 2.6). The lack of ANTHO-induced cytotoxicity can also be taken as further evidence that the vehicle used to solubilize cranberry, methanol, itself exerts negligible cytotoxicity. As was observed with the MTT assay, FBS inhibits cranberry-mediated cytotoxicity.

An attempt was made to elucidate the mechanism whereby the presence of FBS in cell culture medium inhibited cranberry-mediated cytotoxicity. As previous work with the DU145 cell line had shown that these cells aren't adversely affected by growth factor (or FBS) withdrawal (Tang *et al.*, 1998), it was hypothesized that the proteins present in FBS bind and sequester cranberry phytochemicals, thereby inhibiting their cytotoxic effects. This hypothesis was generated, in part, from work

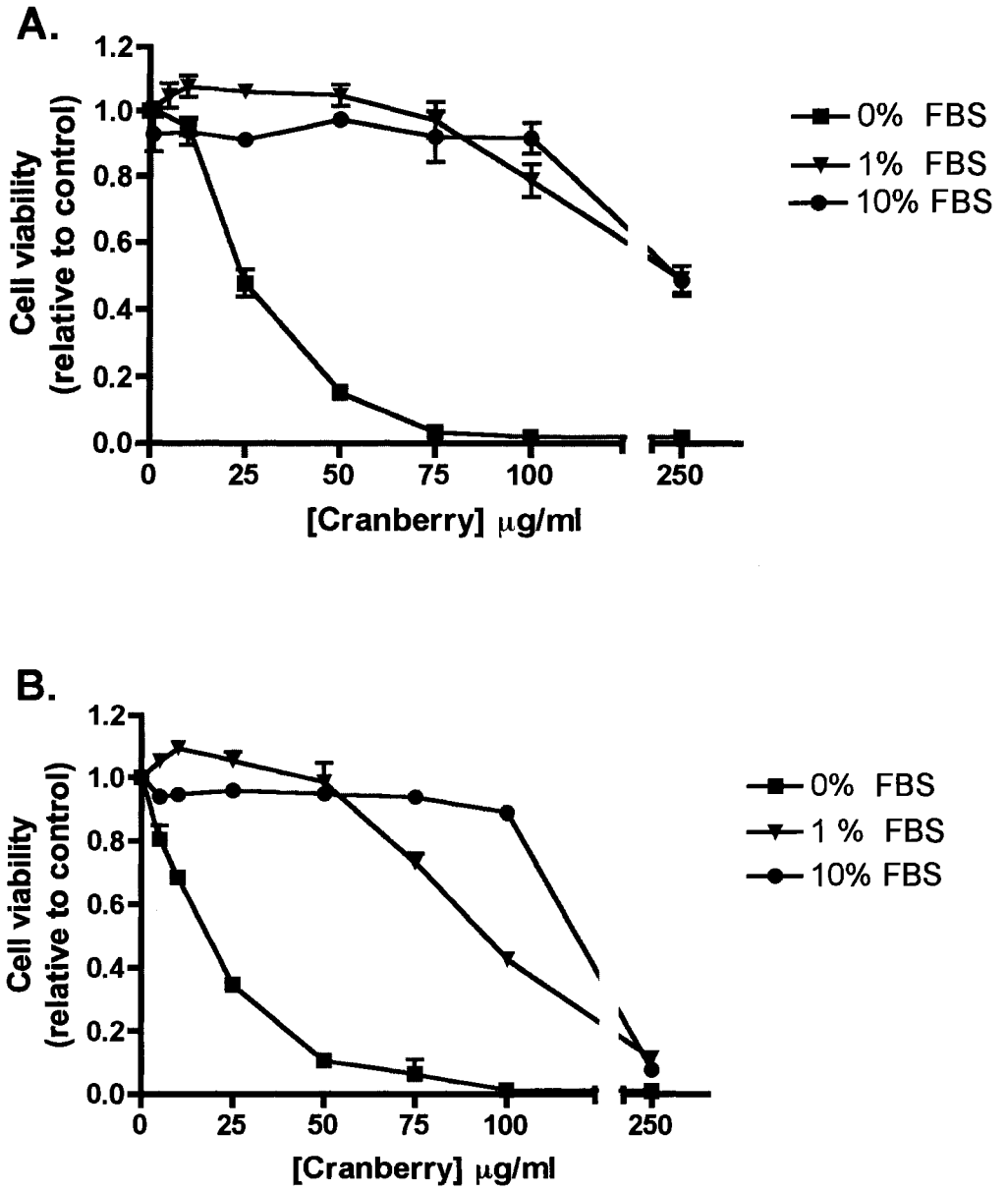

Figure 2.5: An evaluation of cranberry-mediated cytotoxicity in DU145 cells. DU145 cell viability was evaluated using the Alamar Blue assay following treatment with 5, 10, 25, 50, 75, 100, or 250 μg/ml whole cranberry extract for either 6 hours (A) or 24 hours (B). Cranberry conditioned cell culture medium contained either 0% FBS, 1% FBS, or 10% FBS (vol/vol). Values attained from four independent experiments (n=4) with triplicate assay replicates. The reduction of AB reagent in cranberry-treated cells was compared to that of the vehicle-treated controls, so the values indicated are arbitrary units (relative to control). Data points where no error bars are visible indicate negligible variation between biological replicates.

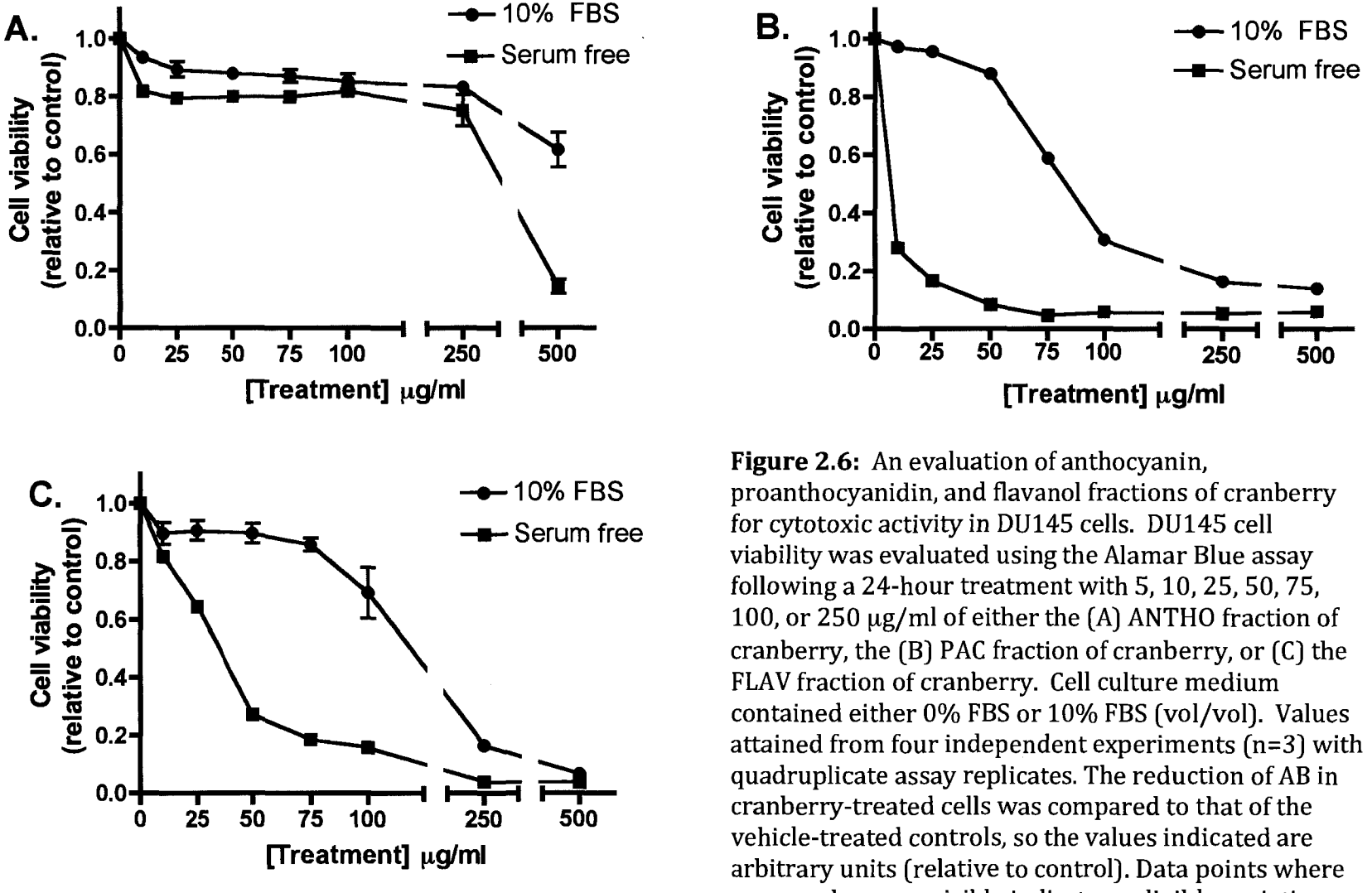

Figure 2.6: An evaluation of anthocyanin, proanthocyanidin, and flavanol fractions of cranberry for cytotoxic activity in DU145 cells. DU145 cell viability was evaluated using the Alamar Blue assay following a 24-hour treatment with 5, 10, 25, 50, 75, 100, or 250 µg/ml of either the (A) ANTHO fraction of cranberry, the (B) PAC fraction of cranberry, or (C) the FLAV fraction of cranberry. Cell culture medium contained either 0% FBS or 10% FBS (vol/vol). Values attained from four independent experiments (n=3) with quadruplicate assay replicates. The reduction of AB in cranberry-treated cells was compared to that of the vehicle-treated controls, so the values indicated are arbitrary units (relative to control). Data points where no error bars are visible indicate negligible variation between biological replicates.

that had shown that FBS protects Jurkat T cells from lysophosphatidylcholine-induced cytotoxicity and that the observed FBS-mediated cytoprotection was attributed to bovine serum albumin (BSA) present in the serum (BSA directly binds to lysophosphatidylcholine in Jurkat T cells) (Kim *et al.*, 2007). As bovine serum albumin (BSA) is the primary constituent protein in FBS, this hypothesis was tested by preparing a BSA solution (in serum free alpha MEM) of a concentration equal to that of the protein concentration of FBS (32.5 mg/ml) (concentration provided by HyClone Laboratories, Certificate of Analysis). Cranberry was diluted to either 100 μg/ml or 250 μg/ml in cell culture medium that was either serum-free, or supplemented with 10% FBS or 10% BSA solution. 2 μM staurosporine (STS), likewise diluted in one of three cell culture solutions, was used as a positive control. Cell viability after 24 hours was evaluated using the AB assay. This experiment thus attempted to determine whether FBS's cytoprotective effects are conferred by protein-cranberry interactions. BSA did not confer the same cytoprotective effect as FBS against cranberry-induced cytotoxicity. However, it should be noted that STS-mediated cytotoxicity was unaffected by the cell culture medium in which it was diluted (Figure 2.7). The observation that the presence of serum doesn't attenuate STS-cytotoxicity suggests that the cytoprotective effects of FBS may be specific to cranberry (as opposed to general cytotoxic insult).

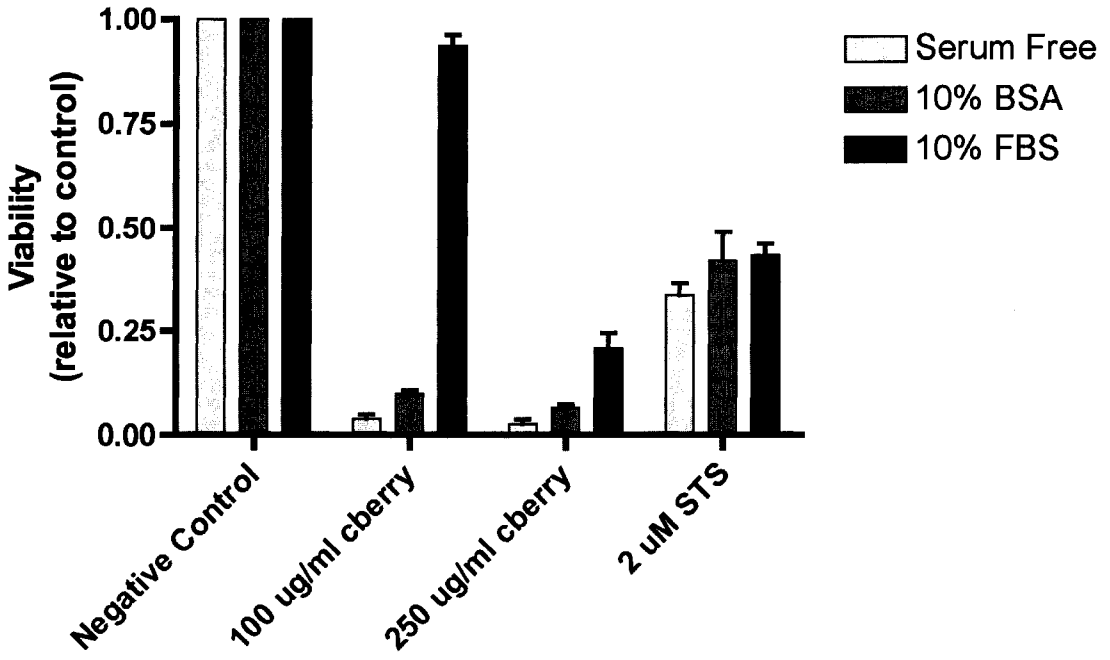

Figure 2.7: A comparison of the effects of BSA and FBS on cranberry-mediated cytotoxicity in DU145 cells. DU145 cell viability was evaluated using the Alamar Blue assay following a 24-hour treatment with either 100 μg/ml or 250 μg/ml cranberry diluted in cell culture medium containing either 10% FBS, 10% BSA solution, or no FBS/BSA. Values attained from three independent experiments (n=3) with triplicate assay replicates. The reduction of AB in cranberry-treated cells was compared to that of the vehicle-treated controls, so the values indicated are arbitrary units (relative to control).

2.3.2 Evaluating markers of late-stage apoptosis and necrosis in cranberry treated DU145 cells

Cell culture supernatant from cranberry-treated DU145 cells was evaluated for LDH activity, the presence of which is indicative of decreased plasma membrane integrity in response to necrosis. It was found that cranberry treatment results in a dose- and time-dependent increase in cell culture LDH activity (Figure 2.8A). However, this result was overshadowed by observations from a cell-free experiment that revealed cranberry's inherent "LDH activity" (Figure 2.8B). This assay measures LDH's conversion of lactate to pyruvate indirectly by recording the concomitant reduction of INT (a tetrazolium salt) to formazan. Thus, cranberry's interference with the LDH assay occurs according to the same mechanism whereby it interferes with the MTT assay.

The cleavage of DNA into mono- and oligonucleosomes is one of the final events that occur in PCD. The presence of nucleosomes in cell lysates is thus indicative of apoptosis, while nucleosomes in cell culture medium indicates loss of plasma membrane integrity and necrosis. DU145 cell lysates and cell culture supernatants were evaluated for nucleosome enrichment following a 24-hour treatment with either the PAC or FLAV enriched fractions of cranberry (Figure 2.9). This assay revealed that PAC treatment (25 μg/ml) resulted in a ~25-fold enrichment in nucleosomes over vehicle-treated controls. Upon treatment with 50 μg/ml PAC, nucleosomes were no longer present in the cell lysates, but were instead found in the cell culture supernatant, suggesting necrotic cell death. FLAV-treated

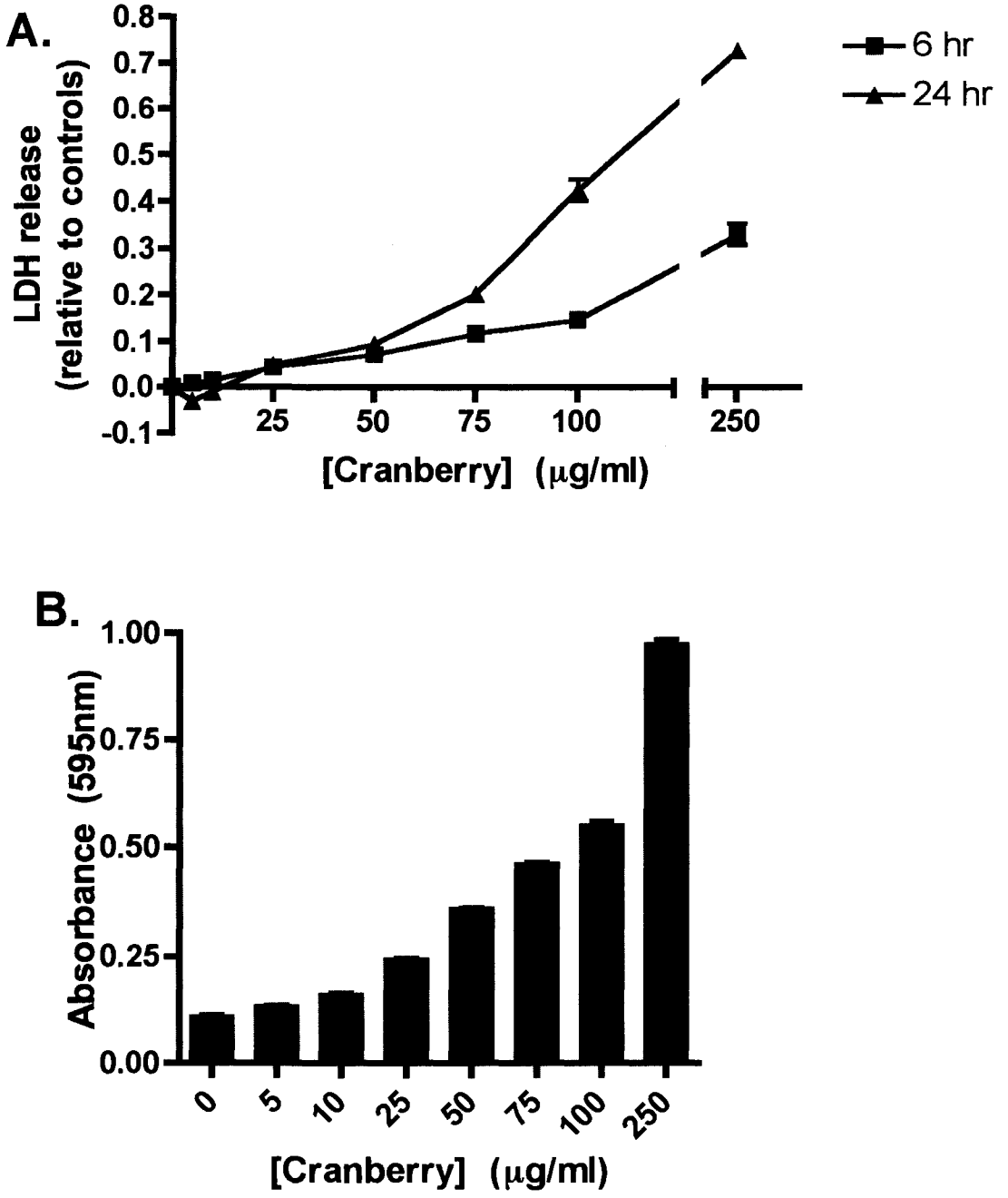

Figure 2.8: An evaluation of cranberry-mediated LDH release in DU145 cells. (A) An evaluation of LDH activity in the cell culture supernatants of cranberry-treated DU145 cells (following 6 and 24 hour treatments in cranberry-conditioned cell culture medium supplemented with 1% FBS). Results from two independent experiments (assay duplicates). (B) The effect of cranberry-conditioned medium on the reduction of the INT (tetrazolium salt) reagent in a cell free system. Results from two independent experiments (assay duplicates).

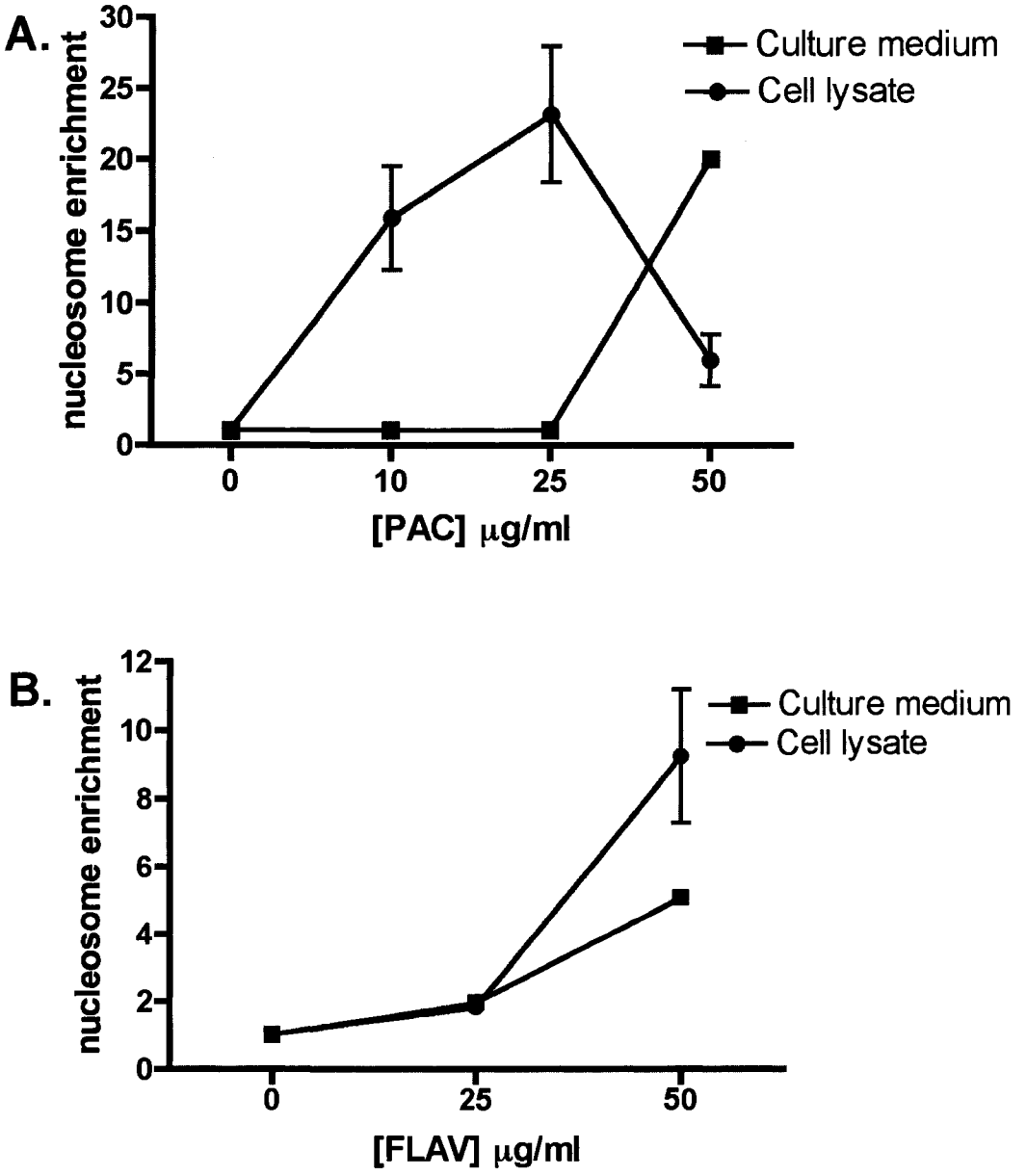

Figure 2.9: Analysis of nucleosome enrichment and localization in PAC (A) and FLAV (B) treated DU145 cells. Results are those attained from two independent experiments (assay duplicates) for the evaluation of cytosolic nucleosome enrichment, or a single experiment for the evaluation of nucleosome enrichment in cell culture supernatants.

DU145 cells meanwhile, exhibited a modest increase in cytoplasmic nucleosome enrichment at 25 μg/ml, and a more notable increase (9-fold) at 50 μg/ml. FLAV treatment also resulted in a modest (~5 fold) enrichment in cell culture nucleosomes. However, analysis of necrosis is preliminary (n=1, assay duplicates).

Treating DU145 cells with a whole extract of cranberry resulted in a slight decrease in PARP activity, particularly after 24 hours, suggesting that the enzyme is being cleaved by caspase-3, thereby decreasing its activity (Figure 2.10).

2.3.3 Evaluating the activation of caspases-8 and -9 in DU145 cells in response to cranberry treatment

Caspase-8 and -9 activities were evaluated in cranberry-treated DU145 cells to determine the signaling mechanisms whereby apoptosis is induced. The effect a whole extract of cranberry on DU145 cells was evaluated over a range of concentrations (5 μg/ml to 250 μg/ml) and over a range of treatment durations (1.5 hrs, 3 hrs, 4.5 hrs, 6 hrs, 7.5 hrs, 9 hrs, 12 hrs, 24 hrs and 48 hrs). Under the assay conditions tested, the whole cranberry extract was unable to activate either caspases-8 or -9 (data not shown). Consultation with Promega revealed that their luciferase enzyme is inhibited by resveratrol (Bakhtiarova *et al.*, 2006), one of the phytochemical components of cranberry (Rimando *et al.*, 2004). As it was thought that the resveratrol present in the whole extract may have been inhibiting the luciferase, further studies focused on the PAC and FLAV fractions rather than the whole cranberry extract, as these fractions do not contain stilbenes.

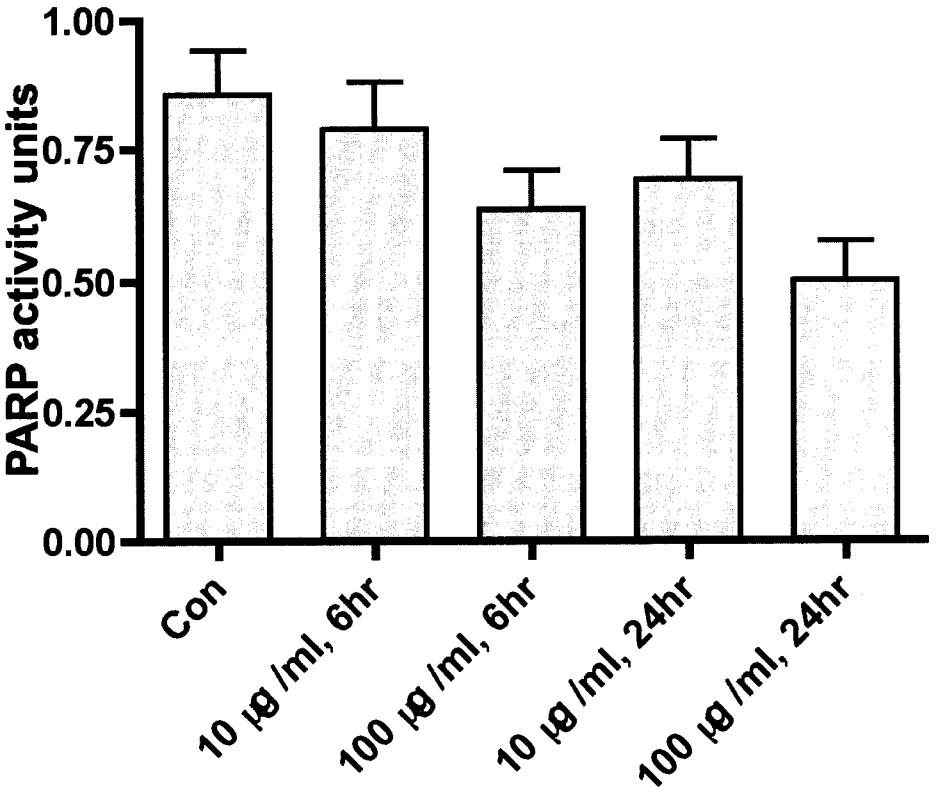

Figure 2.10: PARP activity in DU145 cells in response to cranberry treatment. Results attained from three independent experiments, with three assay replicates for each experiment.

Treating DU145 cells with the 25 μg/ml PAC fraction of cranberry (diluted in serum free medium) resulted in a ~3 fold increase in the activity of caspase-8 after 6 hours and a ~3 fold increase in the activity of caspase-9 after 12 hours (Figure 2.11) (n=4). At the highest tested dose of the PAC fraction, 50 μg/ml, there was no observed increase in the activities of either caspase-8 or -9. This data suggests that lower concentrations of PACs (up to 25 μg/ml) induce apoptosis through a mechanism reliant primarily upon caspase-8 activation, with the apoptotic signals being amplified by the activation of caspase-9 at a later timepoint. The highest tested dose of PAC (50 μg/ml) yields no caspase activation, corroborating the earlier suggestions that this dose exerts necrotic effects (Figure 2.9A). Treating DU145 cells with 25 μg/ml and 50 μg/ml FLAV cranberry extract resulted in a ~3 fold increase in caspase-8 and -9 activity after 12 hours treatment (Figure 2.12) (n=4).

2.3.4 Immunoblot analysis of key proteins involved in the regulation of apoptosis

The expressions of key proteins involved in the extrinsic and intrinsic pathways of apoptosis were evaluated by Western immunoblot analysis. The observed activation of caspase-8 suggests potential involvement of the extrinsic pathway of apoptosis in mediating cranberry cytotoxicity. Recall that this pathway is activated by ligation of proapoptotic ligands to their cell membrane death receptors, to catalyze DISC and caspase-8 activation. The possibility that *V. macrocarpon* polyphenols regulate the protein expression of TNF-related apoptosis-

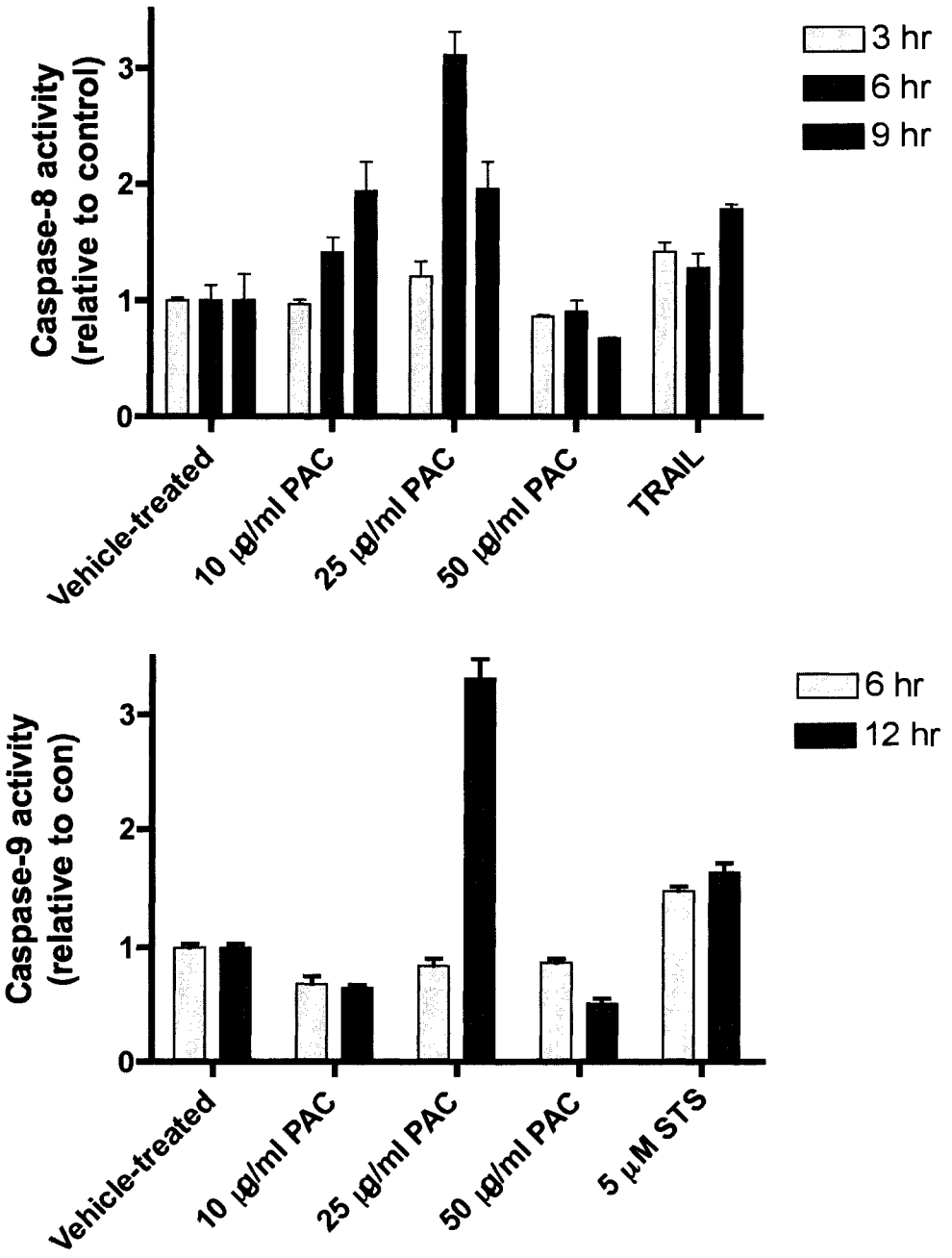

Figure 2.11: The effect of PAC treatment on the activities of caspase-8 and -9 in DU145 cells. DU145 cells were treated with the PAC-enriched fraction of cranberry (10 µg/ml, 25 µg/ml, 50 µg/ml) for the times indicated. Values are those attained from three independent experiments (three assay replicates). Caspase activity in cranberry-treated cells was compared to that of the vehicle-treated controls, so the values indicated are arbitrary units (relative to control).

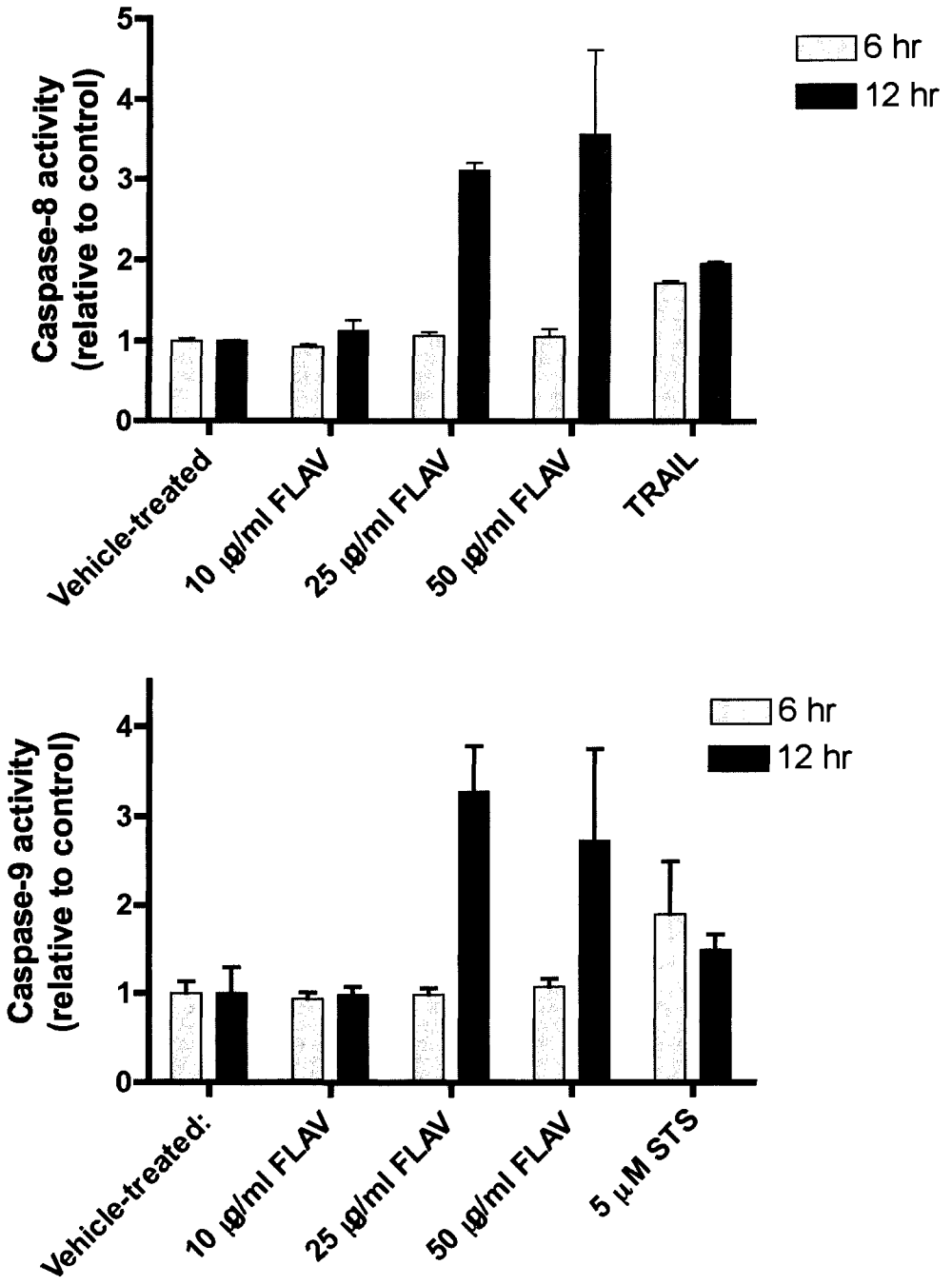

Figure 2.12: The effect of FLAV treatment on the activities of caspase-8 and -9 in DU145 cells. DU145 cells were treated with the FLAV-enriched fraction of cranberry (10 µg/ml, 25 µg/ml, 50 µg/ml) for the times indicated. Values are those attained from three independent experiments (three assay replicates). Caspase activity in cranberry-treated cells was compared to that of the vehicle-treated controls, so the values indicated are arbitrary units (relative to control).

inducing ligand (TRAIL) was studied, as this ligand's apoptogenic effects are specific to a number of tumour cell lines and is non-toxic to normal cells. Likewise, potential changes to the protein expression of the Fas-associated death domain (FADD) were also evaluated in response to cranberry treatment. FADD is an adaptor molecule that bridges the activation of death receptors to the activation of caspase-8 through its contribution to the formation of the death inducing signaling complex (DISC). Western blot analysis revealed that cranberry treatment of DU145 cells had no effect on the protein expressions of either TRAIL (n=2) or FADD (n=2) (Figure 2.13). Analyses of Fas and FasL protein expressions in response to cranberry treatment were inconclusive.

Caspase-9 activation occurs following the release of cytochrome C (cytC) from the mitochondria into the cytosol, where it participates in the assembly of the caspase-9-activating apoptosome. To evaluate cytC release, mitochondria were isolated and removed from cranberry-treated DU145 cells, leaving only the cytosolic fraction. Immunoblot analysis of this cytosolic fraction revealed a dose- and time-dependent increase in the protein concentration of cytC, indicative of the protein's release from the mitochondria (n=4) (Figure 2.14). Protein expression of the voltage-dependent anion channel (VDAC), an ubiquitous protein found on the surface of the outer mitochondrial membrane, was used as a control to ensure that there was no mitochondrial contamination of the cytosol fraction. The protein expression of survivin, a mitochondria-sequestered antiapoptotic protein, and Smac/DIABLO, a mitochondria-sequestered proapoptotic protein, were likewise evaluated in response to cranberry treatment (Figure 2.15). An increase in survivin

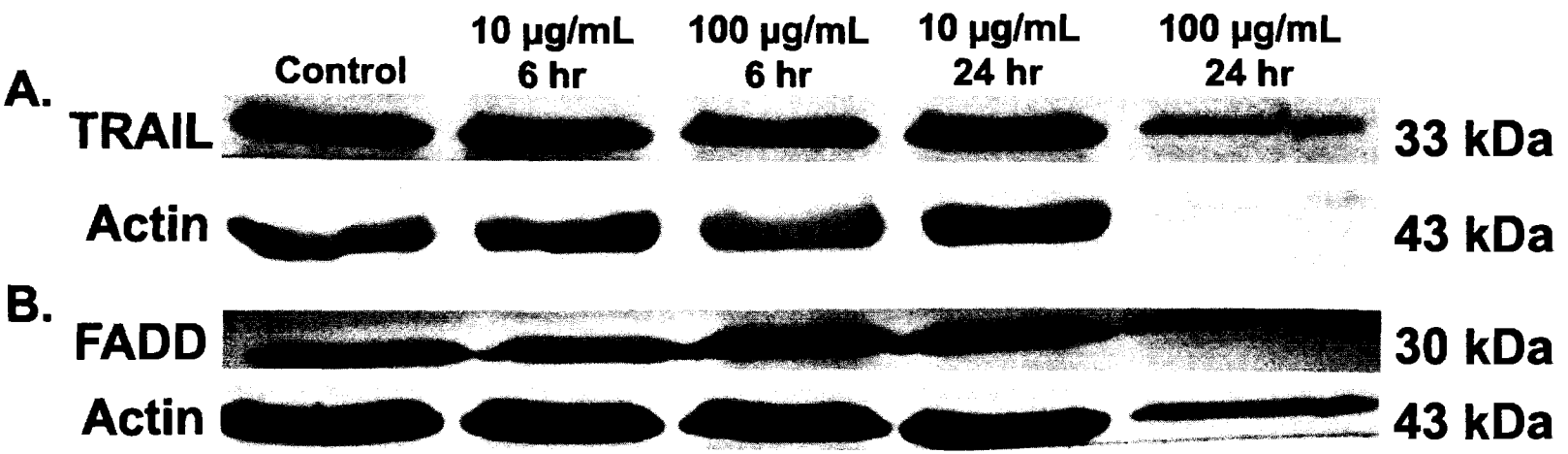

Figure 2.13: The effect of cranberry treatment on the protein expressions of TRAIL (A) and FADD (B) in DU145 cells. The data shown are representative of data attained from two independent experiments (each with 3-4 assay replicates).

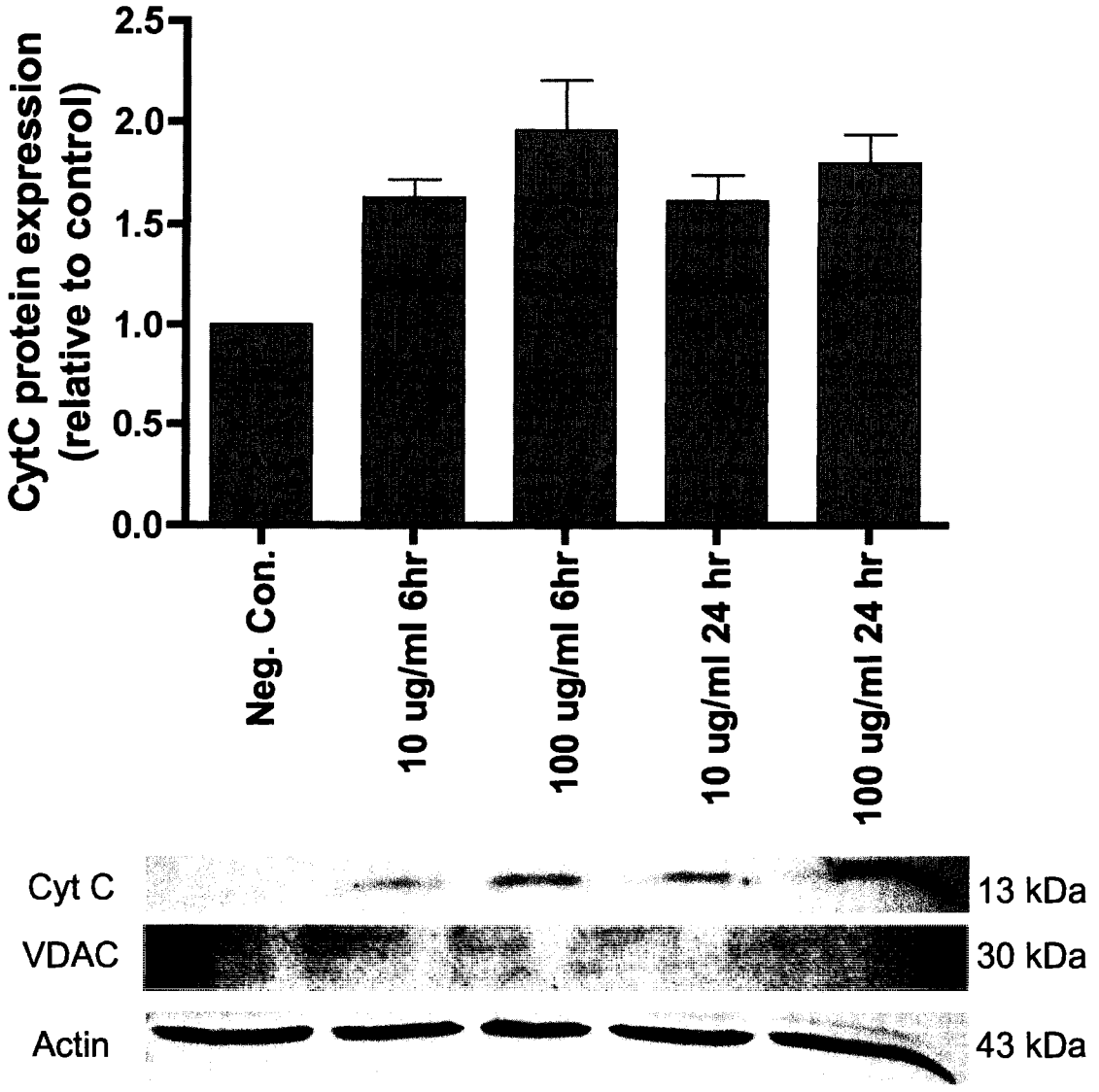

Figure 2.14: The effect of cranberry treatment of DU145 cells on cytosolic cytochrome c expression. Evident is a dose- and time-dependent increase in cytosolic cytochrome c expression. VDAC was employed as a control for mitochondrial contamination of the cytosolic fraction. The data shown are representative of data attained from four independent experiments (each with 3-4 assay replicates).

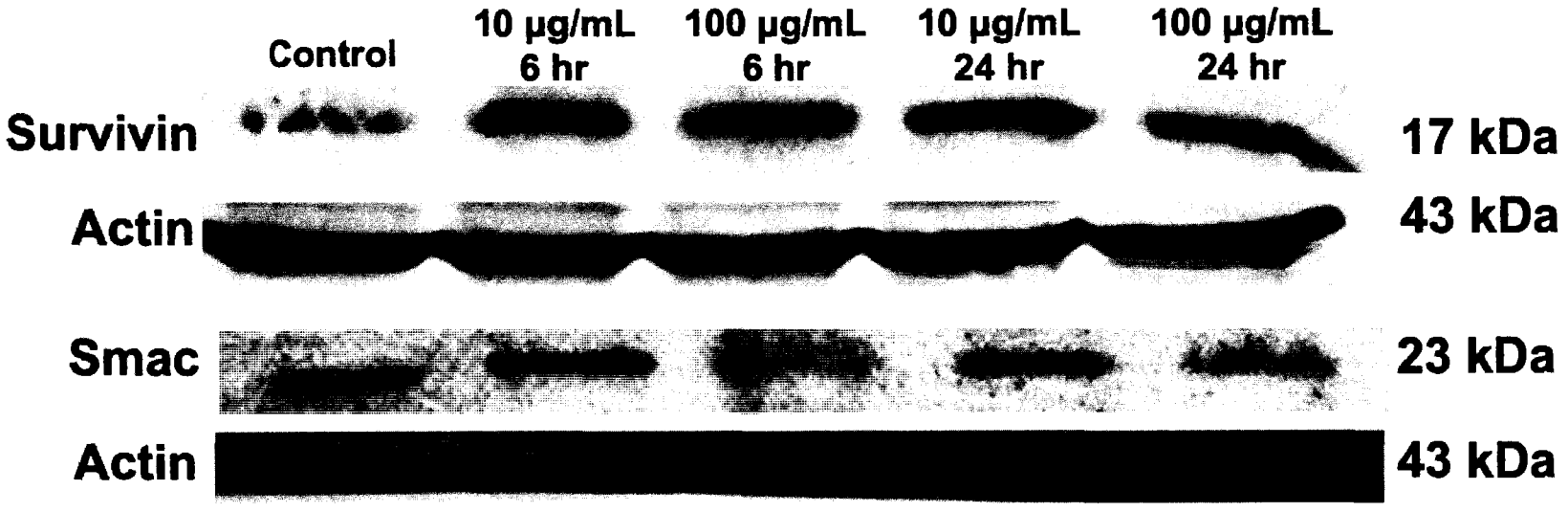

Figure 2.15: The effect of cranberry treatment of DU145 cells on the protein expressions of survivin and Smac/DIABLO. Western blots are representative of observations recorded in two independent experiments (survivin) or one independent experiment (smac/DIABLO), each with 2-3 assay replicates.

protein expression (n=2) and Smac/DIABLO protein expression (n=1) suggest permeabilization of the outer mitochondrial membrane and release of apoptosis-related intermembrane space proteins.

Release of cytC and other apoptogenic factors from the intermembrane space is regulated by the activities of the Bcl-2 family of proteins. Traditionally, the ratio of Bax to Bcl-2 is used as the key factor to determine whether apoptosis will occur. Western blot analysis of Bax and Bcl-2 protein expressions revealed that cranberry treatment causes an increase in Bax protein expression (n=4), while it had no effect on the protein expression of Bax's antiapoptotic homologue, Bcl-2 (n=3) (Figure 2.16), suggesting that the overall balance of these proteins may favour apoptosis. Another Bcl-2 family protein, Bid, was also studied as a potential link between the extrinsic and intrinsic apoptotic pathways. Caspase-8's cleavage of Bid to truncated Bid (tBid) dramatically increases the apoptogenic activity of the protein. Western blot analysis has revealed a time-dependent increase in the protein expression of truncated BID in response to cranberry treatment (n=3) (Figure 2.17).

Par-4 is a protein known to sensitize androgen-refractory prostate cancer cells to apoptotic stimuli through both the intrinsic and extrinsic pathways. Treating DU145 cells with 25 μg/ml cranberry for 6 hours resulted in an increase in Par-4 protein (n=2) (Figure 2.18). Par-4 upregulation may be responsible for the activation of caspase-8, as active Par-4 catalyzes the transfer of the Fas ligand and the Fas receptor to the cell membrane, where it participates in the DISC activation.

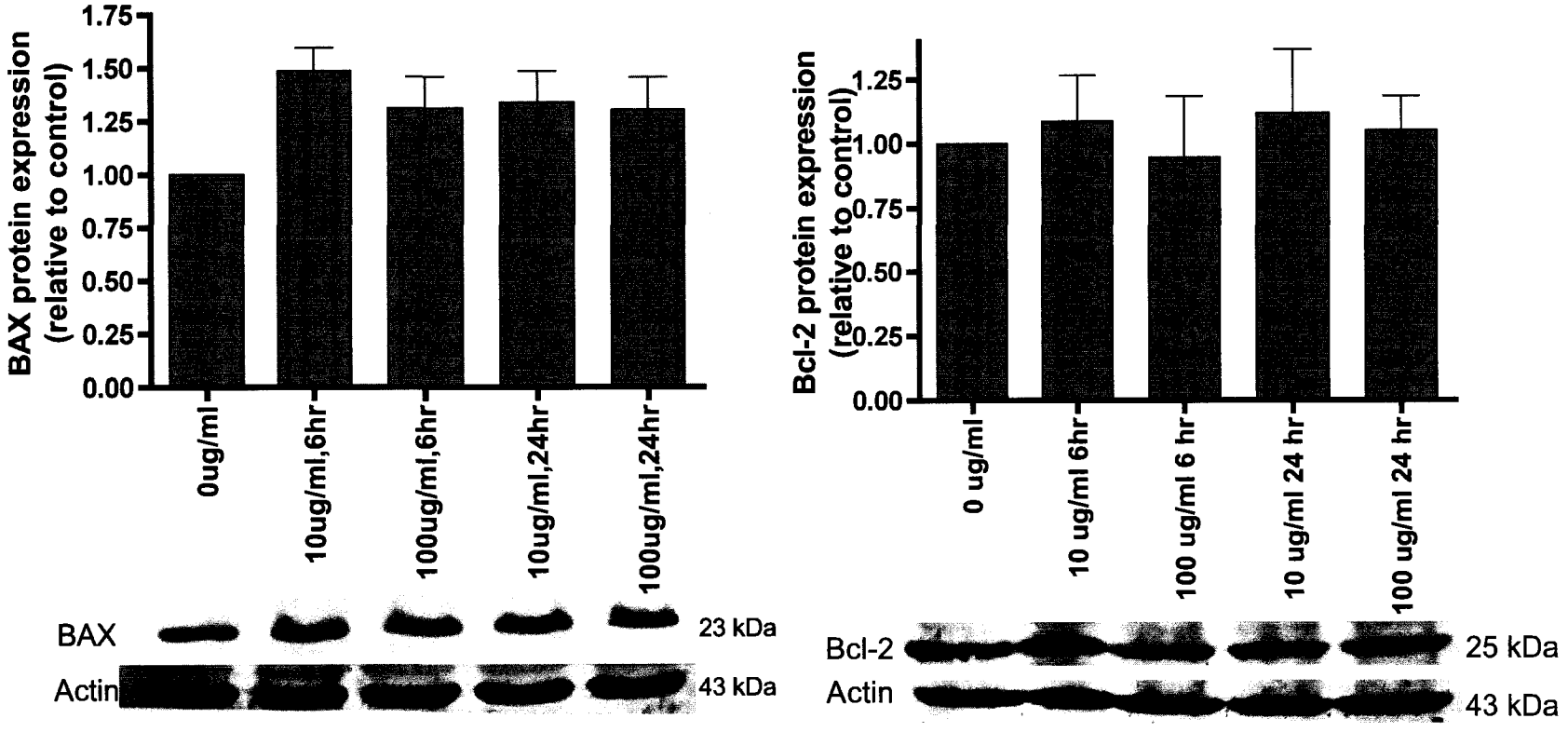

Figure 2.16: The effect of cranberry treatment of DU145 cells on the protein expressions of Bax and Bcl-2. Western blots are representative of those seen in four independent experiments for Bax, and three independent experiments for Bcl-2 (each with 3 assay replicates).

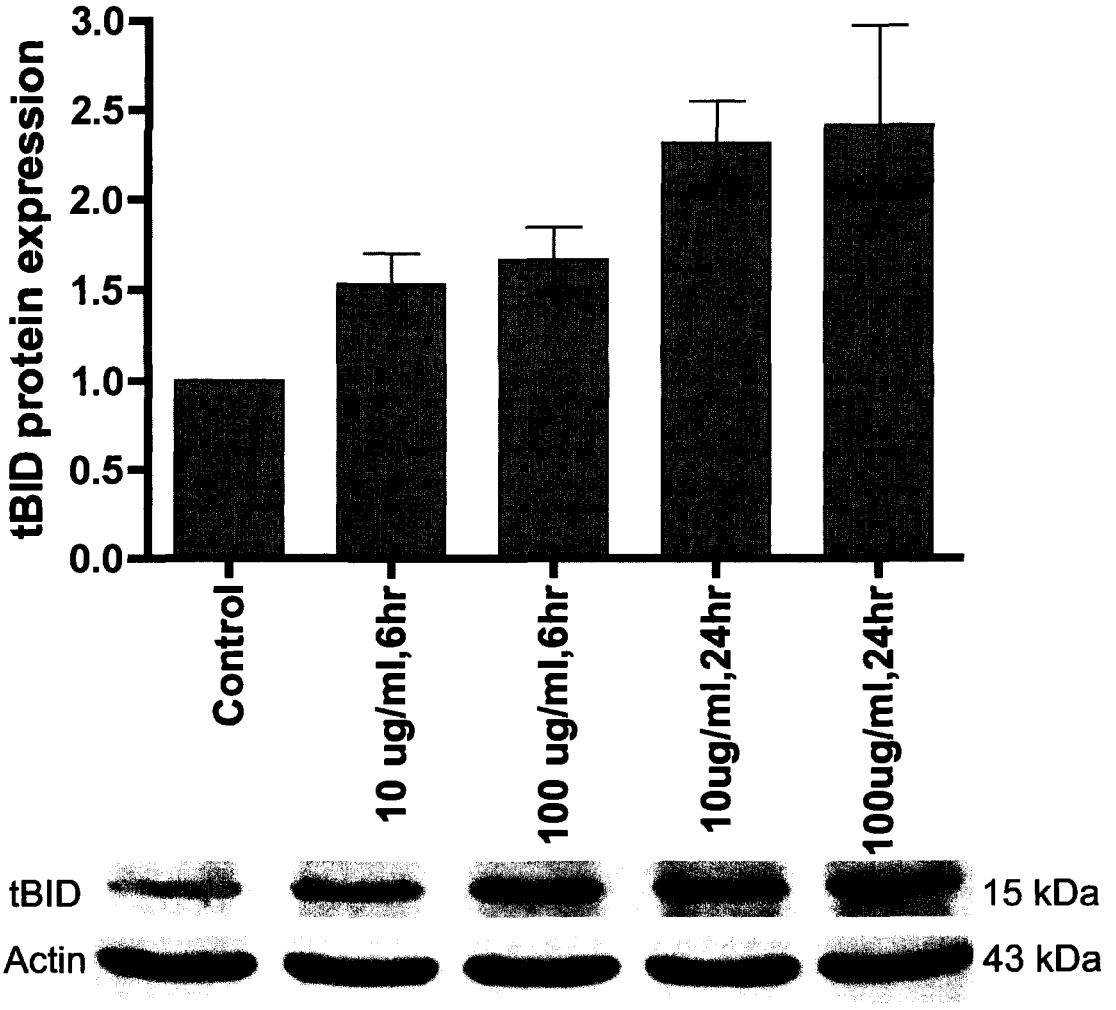

Figure 2.17: The effect of cranberry treatment on the protein expression of truncated Bid (tBid) in DU145 cells. The data shown are representative of data attained from three independent experiments (each with 3-4 assay replicates).

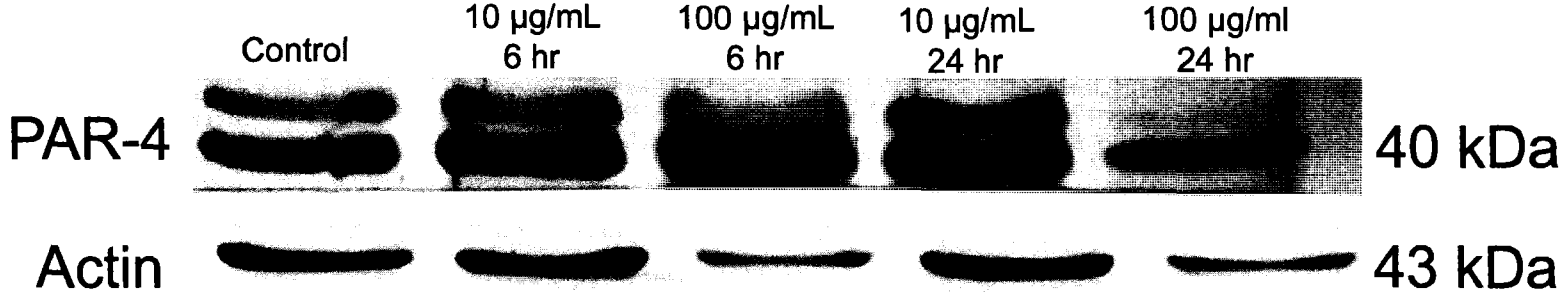

Figure 2.18: The effect of cranberry treatment on the protein expression of Par-4 in DU145 cells. The data shown are representative of data attained from two independent experiments (each with 3-4 assay replicates).

Actin is commonly employed as a loading control in immunoblot analysis, as it is constitutively and abundantly expressed in cells. As seen in the some of the above immunoblots (Figures 2.13, 2.15 and 2.18) treating DU145 cells with high doses of cranberry caused a marked decrease in actin expression. A similar observation is noted in other cell systems as high doses of gallic acid (50 μg/ml) reduced the total cellular protein yield and dramatically decreased actin expression. It was proposed that decreased actin expression occurs in response to aggressive and massive cell death (Veluri *et al.*, 2006), which appears to also be occurring in DU145 cells in response to cranberry treatment. As these observations may call into question the validity of actin as a loading control, band densitometry is not shown in those situations where actin expression appears to be regulated (as it cannot be determined with certainty that actin is representative of total protein loading). Note that the observed actin inhibition occurred in a small subset of the total immunoblot analyses conducted. It is proposed that this discrepancy arises from the non-standarized nature of the polyphenol extracts. It is possible that lot to lot variation may be responsible for variability in the "strength" of cranberry's cytotoxic effects.

2.4 Discussion

This investigation has explored and elucidated many of the apoptosis-linked events that occur within cranberry-treated DU145 cells. The proposed mechanism that follows is based on the observations recorded during this study and an

extensive review of cell death pathways in prostate carcinoma cells, and is summarized in Figure 2.19.

2.4.1 Cranberry induces programmed cell death by triggering release of mitochondrial-sequestered cytochrome c.

The central coordinating event in cranberry-mediated programmed cell death appears to be the release of mitochondria-sequestered cytochrome c into the cytosol, an event that occurs as early as 6 hours after treatment (the earliest tested time point). Cytosolic cytochrome c is required for apoptosome assembly and caspase-9 activation. Increased protein expressions of survivin and smac/DIABLO, two other mitochondria-sequestered proteins, provide further support for the mitochondria's role in cranberry-mediated cytotoxicity. These proteins compete with one another to regulate the activation of caspase-9, an event that is first observed 12 hours after treatment with either the FLAV fraction of cranberry (25 μg/ml, 50 μg/ml) or the PAC fraction of cranberry (25 μg/ml). Caspase-9, the initiator caspase of the intrinsic apoptotic pathway, catalyzes the activation of caspase-3, the central executioner caspase. The expected downstream effects of caspase-3 activation are observed in cranberry-treated DU145 cells, namely inhibition of PARP activity and cleavage of DNA into mono- and oligonucleosomes.

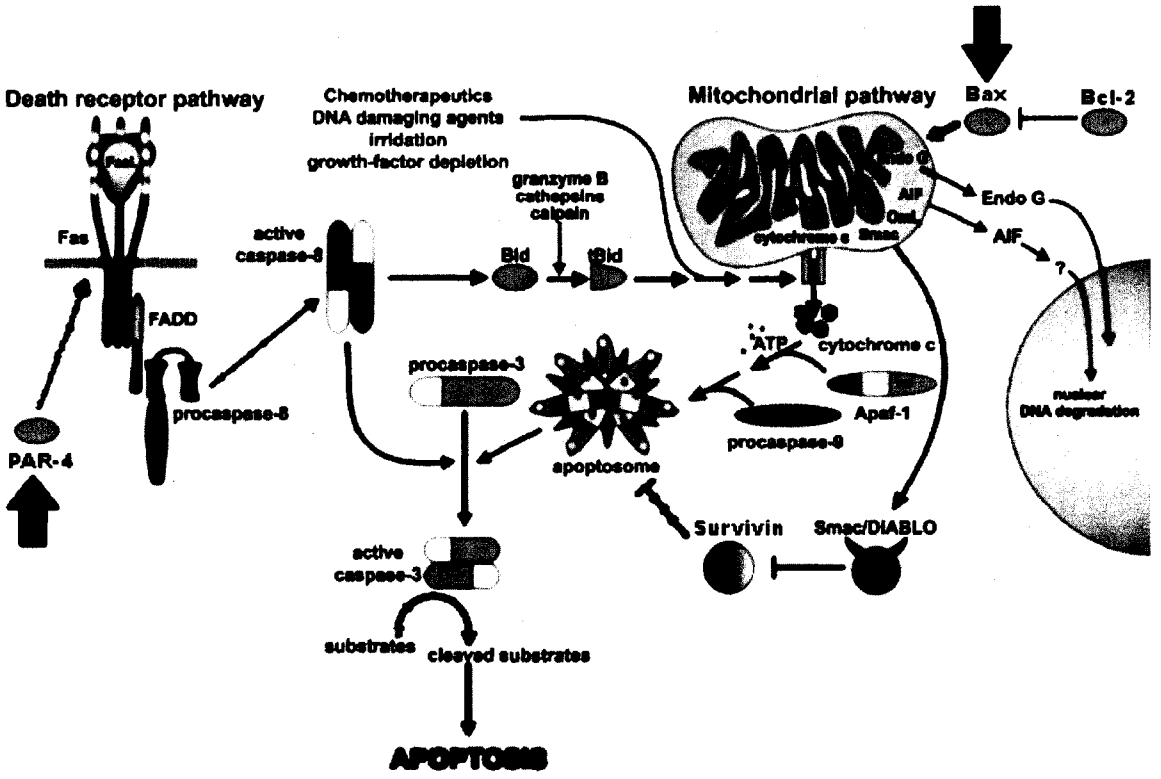

Figure 2.19: An overview of the pathways whereby cranberry exerts its pro-apoptotic effects. Cranberry-coloured arrows highlight the avenues through which cranberry treatment of DU145 cells results in apoptosis induction (modified from van Loo *et al.*, 2002).

2.4.2 Cytochrome C release is mediated by regulation of Bcl-2 proteins, through a mechanism likely involving Caspase-8 and Par-4.

Upon determining cranberry's ability to induce apoptosis through the release of cytC from the mitochondrion, attention turned to the possible mechanisms responsible for triggering this critical event. The Bcl-2 proteins are the primary players in this set of regulatory pathways and the balance of pro- and anti-apoptotic family members often determines whether apoptosis will occur. It was hypothesized that treating DU145 cells with cranberry would result in a notable increase in the ratio of Bax protein expression to Bcl-2 protein expression. Cranberry treatment had no discernable effect on the protein expression of Bcl-2 and increased that of Bax. It is not clear whether the resulting shift in the Bax:Bcl-2 ratio would be sufficient to induce apoptosis.

Bax and Bcl-2 are but two members of a large family of apoptosis-regulating proteins. Though the Bax:Bcl-2 ratio is still considered a valid indicator of whether apoptosis will occur, the concept is somewhat simplistic given the complex network of post-translational mechanisms responsible for apoptosis regulation. Recall that Bax and its proapoptotic homologue Bak are constitutively expressed in many non-apoptotic cells, indicating that expression alone is not sufficient for apoptosis. Indeed, cytosolic monomeric Bax is not apoptogenic until it is triggered to oligomerize and translocate to the mitochondrion. This activation only occurs following transient interaction with the BH3-only proteins (either Bim or Bid). Bid is secreted as a protein with little activity, and only becomes an apoptotic stimulus upon cleavage into its active fragment, truncated Bid (tBid), and this study has

shown that cranberry treatment of DU145 cells increases the protein expression of the 15-kDa fragment of Bid.

It is proposed that the observed cleavage of Bid in response to cranberry treatment is mediated by the activation of caspase-8, an event that occurs following either a 6-hour treatment with the PAC fraction of cranberry, or a 12-hour treatment with the FLAV fraction of cranberry. Increased expression of tBid is seen after 6 hours treatment with a whole cranberry extract, as is the release of cytochrome c from the mitochondrion. That caspase-9 activation is only seen after 12 hours treatment (with either PAC or FLAV) suggests that the activation of this initiator caspase occurs downstream of caspase-8 activation, Bid cleavage, and cytochrome c release.

Caspase-8 is typically activated following the ligation of proapoptotic ligands to their membrane bound death receptor, though activation of this initiator caspase has been observed to occur without ligand-receptor interactions upon treatment with anticancer drugs (Wesselborg *et al.*, 1999), and other mechanisms continue to be explored. Given the data presented in this thesis, it is suggested that the observed upregulation of Par-4 protein expression may be one of the potential mechanisms responsible for caspase-8 activation. Par-4 upregulation leads to the golgi-dependent translocation of Fas and FasL from the cytosol to the plasma membrane, resulting in FADD recruitment and DISC assembly (Chakraborty *et al.*, 2001). As would be expected, increased Par-4 expression has been shown to cause

caspase-8 activation, Bid cleavage and mitochondrial release of cytC (Boehrer *et al.*, 2002).

2.4.3 Hypotheses generated and future directions

2.4.3.1 Investigation of potential Par-4 activities

The observed upregulation of Par-4 may have effects beyond the activation of caspase-8 and may also explain some of the other events observed in cranberry-treated DU145 cells. As such, Par-4 is worthy of further investigation. The first task in any such investigation needs to be an evaluation of this protein's phosphorylation state, as Par-4's activity is subject to post-translational modifications. Phosphorylation of Par-4's T155 residue by protein kinase A positively regulates the protein's apoptotic activity (Gurumurthy *et al.*, 2005), while phosphorylation of the S249 residue by Akt (V-akt murine thymoma viral oncogene homolog 1; protein kinase B) prevents Par-4's nuclear translocation, promoting cell survival (Goswami *et al.*, 2005). It is thus recommended that immunoblot analyses of Par-4 be conducted using antibodies capable of discerning between the protein's phosphorylation states.

It is hypothesized that the increase in Bax protein expression observed in response to cranberry treatment is a result of Par-4 upregulation. Recall that DU145 cells harbour p53 mutations that impair the transcription of proapoptotic Bcl-2 proteins like Bax (Miyashita and Reed, 1995). Active Par-4 circumvents this endogenous defense against apoptosis by translocating to the nucleus where it

inhibits the transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor highly expressed in DU145 cells (Chakraborty *et al.*, 2001). In DU145 cells, inhibition of NF-κB increases the activity of c-jun NH(2)-terminal kinases (JNKs), which then phosphorylate the p53 mutant at the serine 15 residue, thereby restoring the wild-type function of the mutated protein (Zerbini *et al.*, 2005), leading to increased Bax expression.

A review of the literature reveals support for this hypothesis. It has been reported that cranberry juice inhibits NF-κB activation in cell lines including PC-3 prostate carcinoma cells (Boivin *et al.*, 2007), and that quercetin (a major constituent of *V. macrocarpon*) likewise inhibits NF-κB (Kim *et al.*, 2005). GPSE PACs induce apoptosis in DU145 cells through inhibition of NF-κB (Dhanalakshmi *et al.*, 2003; Vayalil *et al.*, 2004), through it remains to be seen if cranberry PACs have the same effect. The proposed phosphorylation of mutant p53 protein in response to cranberry treatment also finds support in the literature. Resveratrol, one of cranberry's component phytochemicals (Rimando *et al.*, 2004), induces p53 phosphorylation (Lin *et al.*, 2002; Kim *et al.*, 2003) through a pathway requiring JNK (She *et al.*, 2002). Treating LNCaP cells with GPSE PACs causes p53 phosphorylation (Kaur *et al.*, 2006), and in DU145 cells, these PACs activate JNKs thereby inducing apoptosis (Agarwal *et al.*, 2000; Tyagi *et al.*, 2003). It is proposed that cranberry phytochemicals operate through a similar pathway. This hypothesis should be first evaluated by determining NF-κB activity in cranberry treated DU145 cells, followed by immunoblot analyses of the phosphorylated form of the p53 protein.

2.4.3.2 Investigation of growth signaling pathways

The data presented in this thesis indicates that cranberry's induction of apoptosis is far more dramatic in serum-free cell culture medium than in medium supplemented with 10% FBS, an effect that cannot be explained by serum protein binding to cranberry phtyochemicals (Figure 2.7). It thus appears that growth factor withdrawal and cranberry treatment work together to induce apoptosis in DU145 cells. The proposed phosphorylation of p53 via Par-4 upregulation provides a potential explanation for this observation. Recall that DU145 cells, due to their p53 mutations, do not undergo apoptosis in response to growth factor withdrawal (serum starvation), as apoptosis induced by trophic support withdrawal requires functional p53 (Tang *et al.*, 1998). Restoration of wild-type p53 function by phosphorylation is thus hypothesized to render these cells susceptible to apoptosis by growth factor withdrawal.

Another possible explanation for cranberry's enhanced cytotoxicity in the absence of serum focuses on the autocrine growth factor signaling loops inherent to DU145 cells. As reviewed in the introduction, DU145 cells are completely self-sufficient with respect to exogenous growth factors (explaining their proliferation in the absence of serum). This self-sufficiency however, appears to be abrogated via cranberry treatment. It is thus hypothesized that cranberry interferes with one or more components of these autocrine growth factor loops. Quercetin has been reported to induce apoptosis in colorectal and pancreatic tumour cells by blocking the EGFR tyrosine kinase activity (Richter *et al.*, 1999; Lee *et al.*, 2002), and may be

able to do so in DU145 cells as well. Furthermore, treating PC-3 prostate cancer cells with quercetin also decreases the culture medium levels of insulin-like growth factors (IGFs) through increased secretion of the IGF binding protein-3 (IGFBP-3), resulting in decreased proliferation and increased apoptosis (Vijayababu *et al.*, 2006). This particular autocrine loop is discussed in more detail in Chapter 4. It is recommended that an extensive investigation be conducted to determine what effect, if any, cranberry treatment has on the autocrine signaling loops in DU145 cells. DU145 cells should be treated with a whole cranberry extract in serum-free medium supplemented with individual growth factors, to better elucidate which autocrine feedback loops are affected by cranberry treatment.

2.4.4.3 Evaluation of necrotic-like programmed cell death.

The data presented in this thesis indicates that cytochrome c and other mitochondria-sequestered apoptogenic factors are released into the cytosol following permeabilization of the outer mitochondrial membrane following tBid-dependent homo-oligomerization of Bax and Bak. While this mechanism accounts for the majority of recorded observations, it does not adequately explain a few key findings that seem to indicate the induction of necrosis in cranberry-treated DU145 cells. Recall that FLAV treatment not only increases cytosolic nucleosome enrichment, but also increases the enrichment of nucleosomes in the cell culture medium, albeit to a lesser extent (Figure 2.9B). It is possible that the observed release of nucleosomes into the cell culture supernatant is a result of secondary

necrosis – an artifact of *in vitro* systems. Normally, apoptotic bodies are engulfed by phagocytes, preventing the release of cytosolic contents into the extracellular environment. However, in an *in vitro* setting, there are no phagocytes, so apoptotic bodies eventually lyse, releasing their contents (secondary necrosis). A more interesting trend is noted when evaluating the effects of PAC treatment on DU145 cells: The cytoplasmic nucleosome enrichment observed following a 24 hour treatment with 25 μg/ml PAC is abruptly replaced with an enrichment in extracellular nucleosomes at 50 μg/ml PAC (Figure 2.9A), indicating a switch from apoptotic cell death to necrotic cell death. This data is supported by observations of caspase-8 and -9 activities: at 25 ug/ml both caspases are activated by PAC treatment, but at 50 μg/ml, no such activation is apparent (Figure 2.11).

Based on the data presented, it is hypothesized that higher doses of cranberry may have the ability to trigger a caspase-independent (i.e. non-apoptotic) form of programmed cell death (PCD) resembling necrosis. Indeed, a growing body of research is beginning to dispense with the traditional dichotomy between PCD and necrosis, as accumulating evidence is showing that some forms of necrosis occur in a regulated manner and may simply represent an alternative execution phase of PCD (Proskuryakov *et al.*, 2003; Boujrad *et al.*, 2007). It is thus suggested that higher concentrations of cranberry may exert mitotoxic effects, causing mitochondrial rupture and abrogation of ATP synthesis pathways. As such, it is recommended that the potential effect of cranberry treatment on mitochondrial transmembrane potential ($\Delta\psi m$) be evaluated. $\Delta\psi m$, a marker of mitochondrial integrity, can readily be tested through use of the fluorescent dye JC-1. Futhermore, the extent to which

cranberry's cytotoxicity is mediated through caspase activation should be evaluated (easily accomplished through use of the broad spectrum caspase inhibitor, Z-VAD-FMK).

2.4.6 Conclusions

This study has described the cytotoxic effects of *V. macrocarpon* phytochemicals on DU145 prostate cancer cells, and has highlighted some of the mechanisms proposed to be responsible for this cytotoxicity. Cranberry treatment activates the apoptotic cascade by triggering the release of cytochrome c from the mitochondria though a mechanism involving the proteolytic cleavage of Bid to truncated Bid, most likely by activated caspase-8. It is hypothesized that at least some of the cytotoxicity exerted by *V. macrocarpon* is related to the upregulation of Par-4 protein expression, though more work is required to evaluate this hypothesis.

The work presented in this chapter highlights the potential value of *V. macrocarpon* as a source of novel phytochemicals that may be useful in the development of chemopreventative and chemotherapeutic strategies. This study also provides proof-of-concept for the potential benefits of cranberry consumption, and argues for further evaluation of this concept in an *in vivo* setting.

CHAPTER 3

Vaccinium macrocarpon-Mediated Regulation of Matrix Metalloproteinase Activity in Prostate and Breast Carcinoma cells

3.1 Introduction

Matrix metalloproteinases (MMPs) are a family of endopeptidases that digest extracellular matrix (ECM) proteins. Unregulated MMP activity can play a major role in cancer expansion, invasion and metastasis, and MMP inhibition has thus emerged as a new target for cancer chemoprevention. Previous investigations have identified cranberry's ability to inhibit the secretion of MMPs 2 and 9 from DU145 prostate cancer cells (Neto *et al.*, 2006). This study, based on that previous work, attempts to elucidate the mechanisms responsible for cranberry-mediated MMP inhibition in DU145 cells, and to determine whether the same effects are seen in breast cancer cells. The hypothesis that cranberry treatment would alter MMP secretion from DU145 and MDA-MB-231 cells by controlling the expression of key MMP-regulating enzymes was evaluated using gelatin-gel electrophoresis, immunoblot analysis and real time PCR.

3.2 Materials and Methods

All materials, unless otherwise indicated, were attained from Sigma-Aldrich (Oakville, ON).

3.2.1 Preparation of cranberry extracts

The whole cranberry, the anthocyanin (ANTHO), flavonol (FLAV), and proanthocyanidin (PAC) extracts were prepared and characterized by the laboratory of Dr. Cathy Neto as described in Appendix A.

3.2.2 Cell culture

All cell lines (ATCC, Manassas, VA) were cultured on 100mm plastic tissue-culture dishes (Falcon, Mississauga, ON) at 37 °C and 5% CO_2. Human DU145 prostate adenocarcinoma cells were cultured in αMEM (Gibco, Burlington, ON) supplemented with 1% (vol/vol) antibiotic-antimycotic (Ab-Am; Gibco) and 10 % (vol/vol) Fetal Clone III fetal bovine serum replacement (FBS; Hyclone/VWR Canlab, Mississauga, ON). MDA-MB-231 breast carcinoma cells were cultured in Dulbecco's MEM (Gibco) supplemented with 1% Ab-Am and 10% FBS. MCF-10A breast epithelial cells were cultured in Minimum Essential Basal Medium (MEBM) supplemented with bovine pituitary hormone (52 μg/ml), hydrocortisone (0.5 μg/ml), human epidermal growth factor (0.01 μg/ml), insulin (5 μg/ml), 1% Ab-Am and 5% FBS (as per the recommendations of ATCC). These supplemental growth factors are required as the MCF-10A cell line, though transformed, is not cancerous

and still depends upon the presence of exogenous growth factors for continued proliferation.

3.2.3 Solubilization of cranberry extracts for cell treatments

Please refer to Chapter 2 (section 2.2.3) for an overview of methanolic solubilization of cranberry fractions.

3.2.3 Alamar Blue assay

Please refer to Chapter 2 (section 2.2.4) for an overview of the Alamar blue assay protocol.

3.2.4 Gelatin-gel electrophoresis (zymography)

One of the more commonly employed techniques for the evaluation of MMP secretion is substrate zymography, which evaluates MMPs by their molecular weight, the degradation of their preferred substrate, and the extent of that degradation. In zymography, proteins are separated by electrophoresis under denaturing, nonreducing conditions on a polyacrylamide gel containing a specific substrate co-polymerized with acrylamide. Following the removal of SDS by Triton X-100 (required to render MMPs catalytically active), gels are incubated in a MMP-developing buffer and MMPs within the gel digest the polymerized substrate. Undigested substrate is then detected with a general protein stain, such as

Coomassie Blue R250. The activity of latent, physiologically inactive, MMP zymogens can also be seen in zymography, as SDS causes the dissociation of Cys^{73} from the zinc (the "cysteine switch"), rendering the zymogens active (Fernandez-Resa *et al.*, 1995; Woessner, 1995; Leber and Balkwill, 1997; Snoek-van Beurden and Von den Hoff, 2005). As this study is focusing on the effect of cranberry treatment on the expression of MMPs 2 and 9, gelatin zymography was employed.

DU145 cells, MDA-MB-231 cells, or MCF-10A cells were plated in 100mm plastic tissue-culture dishes ($5x10^5$ cells/dish), and incubated at 37°C for 24 hours. Following this incubation period, cell culture medium was removed and replaced with serum-free medium supplemented with a whole extract of cranberry or an anthocyanin-enriched fraction of cranberry. Treatments lasted for 24 hours and ranged from 0 μg/ml to 250 μg/ml. Following the treatment, cell culture medium was carefully removed and centrifuged at 250 xg to pellet any cellular debris. Cleared cell culture medium was aliquoted and stored at -80°C until required.

Cell culture medium was mixed in a 3:1 ratio with non-reducing 4x laemmli buffer (50 mM Tris-HCL [pH 6.8], 10% SDS, 0.1% bromophenol blue and 10% glycerol) and incubated for 20 minutes at 37°C. 20 μL of the medium-laemmli mixture were resolved on 10% SDS-PAGE mini-gels supplemented with 1 mg/ml gelatin. Samples were resolved at 15 mA/gel through the stacking gel, and 30 mA/gel through the resolving gel in 1x running buffer (3.0 g tris-base, 14.4 g glycine, and 1.0 g sodium dodecyl sulfate [SDS] in 1 L H_2O, pH 8.3). Gels were then washed in 50 mM Tris-HCl with 2.5% triton X-100 (pH 7.4) at room temperature (3 1 hour

washes). Gels were then incubated at 37°C in 50 mM Tris-HCl pH 7.4, 5 mM $CaCl_2$ and 50 μM $ZnCl_2$ for 24-48 hours. Gels were then rinsed with ddH_2O and stained with 0.25% Coomassie Blue R250 in a solution of water, methanol and acetic acid (45:50:5 vol/vol/vol) for thirty minutes then destained in a solution of water, methanol and acetic acid (85:10:5 vol/vol/vol) until bands were well defined (approximately thirty minutes, with repeated destaining steps). The size of the zones of clearing at 92 kDa and 62-72 kDa indicate the gelatinolytic activity of MMPs 9 and 2 respectively.

3.2.5 Real-time reverse transcription polymerase chain reaction

The effect of treating MDA-MB-231 breast carcinoma cells with cranberry on the expression of MT1-MMP and MMP-9 mRNA was evaluated using real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Real-time RT-PCR is the most sensitive and reliable method for the analysis of gene expression. PCR is a reaction that allows one to monitor the amplification of a DNA fragment by the development of a fluorescent signal. By monitoring fluorescence during this amplification process, one can determine the amount of DNA fragment originally in the sample, thus allowing a researcher to determine the fold change in gene expression of treated cells, relative to vehicle-treated controls (Bustin *et al.*, 2005; Wong and Medrano, 2005).

A real-time RT-PCR set up involves several components. High quality mRNA from cranberry-treated MDA-MB-231 cells is converted into a cDNA template (via a

reverse transcription reaction). Primers are short nucleotide sequences designed to be complementary to a nucleotide sequence unique to the gene of interest (in this case, either MT1-MMP, MMP-9, or Actin). DNA polymerase is a heat sensitive enzyme that adds free nucleotides present in the buffered reaction mixture to the template-bound primers in a sequence that is complementary to the original template DNA (which is, itself, complementary to the original mRNA). During this amplification process, molecules of SYBR green become incorporated to the lengthening double stranded DNA. Upon incorporation, the SYBR green will produce a fluorescent signal. It is the detection of this signal that is used to determine the relative amount of template DNA present in the test sample (Bustin *et al.*, 2005; Wong and Medrano, 2005).

The process whereby template DNA is amplified occurs in a series of 40 repeated temperature changes called cycles, with each cycle consisting of two discrete temperature steps, all controlled by the thermocycler. The process begins with the initialization step, in which the thermocycler heats the reaction mixture to 95 °C for 10 minutes in order to activate the DNA polymerase. The 40 cycles which follow consist of two steps: a 15 second step at 95°C and a 1 minute step at 60°C. The 95°C step allows the DNA template and primers to dissociate (by disrupting hydrogen bonds), yielding single strands of DNA. The second step (60°C) lowers the temperature such that primers can anneal (bind) to the single-stranded DNA template, and DNA polymerase can catalyze the addition of nucleosides to the template cDNA (beginning with the primer). During this stage, the SYBR green fluorescence is measured in order to determine the quantity of template present. In

PCR, there exists a threshold at which the thermocycler is first able to distinguish SYBR green fluorescence arising from DNA amplification from background fluorescence. The cycle number at which this threshold detection occurs is used to determine the amount of template DNA originally contained within the sample (abundant genes are detected earlier than less prevalent genes) (Bustin *et al.*, 2005; Wong and Medrano, 2005).

MDA-MB-231 cells were plated into 100mm plastic tissue-culture dishes (seeded at $5x10^5$ cells/dish), and incubated at 37°C for 24 hours. Following this incubation period, cell were treated with a whole cranberry extract (diluted in cell culture medium supplemented with 10% FBS) at 0 μg/ml (vehicle-treated control), 50 μg/ml, 100 μg/ml and 250 μg/ml. Following a 24-hour treatment, cells were rinsed three times in ice-cold 1x PBS and were removed from the cell culture dish by exposure to a 10% (vol/vol) solution of Trypsin-EDTA in PBS at 37°C for 2 minutes. Cells were washed with 5 mL cell culture medium (supplemented with 10% FBS as a trypsin inhibitor) and were then collected in 15 ml tubes. Cells were pelleted by centrifugation at 500xg for 10 minutes. Cell culture medium was carefully removed from the cell pellet, which was then reconstituted in 1X PBS. Cells were then transferred to an RNase-free eppendorf tube, centrifuged, and the supernatant removed.

Total RNA was extracted from the cell pellet using the Qiagen RNeasy Mini Kit (Mississauga, ON) according to the manufacturers instructions. The resulting RNA was stored in RNase-free water at -80°C until required. The total concentration of

RNA was determined by spectroscopy. RNA was diluted 50 fold in 10 mM Tris-HCl pH 7.0 buffer, was subsequently loaded into an RNase-free glass microcuvette, and the absorbance of the solution read at 260 nm to determine the RNA concentration (1 optical density unit = 44 μg/ml, such that the concentration of the RNA sample = 44 μg/ml x Absorbance at 260 nm x dilution factor [50]). The potential presence contaminants in the RNA solution was determined by diluting the RNA 50 fold in 10 mM Tris-HCl pH 7.5 buffer, and reading the solution absorbance at 260 nm and 280 nm. The Absorbance 260 nm / Absorbance 280 nm ratio provides an estimate of RNA purity, with pure RNA having a ratio between 1.9-2.1. Only RNA deemed "pure" (i.e. free of contaminants such as buffers, salts, etc.) was used for subsequent analyses. The integrity and size distribution of the RNA was evaluated by denaturing agarose gel electrophoresis and ethidium bromide staining. A 1% agarose gel was prepared by gently heating and stirring 50 mL of 0.5x TBE Buffer (Tris/Borate/EDTA: 44.5 mM Tris base, 44.5 mM Boric acid, and 1mM EDTA), 1 μL of ethidium bromide and 0.5 g agarose. Once the cast gel had polymerized, it was buffered in 0.5x TBE buffer, and samples were prepared: 2 μL of RNA sample was mixed with 5 μL of RNase-free water and 1 μL of loading dye (Track-It; Invitrogen). Samples were loaded into the gel, a current was applied, and following electrophoresis bands were visualized under ultraviolet light. RNA was deemed to have sufficient integrity only if the ribosomal bands were well defined, and the apparent ratio of 28S RNA to 18S RNA was approximately 2:1. If these characteristics weren't apparent, it was determined that the sample suffered degradation during RNA purification.

Once the concentration, purity and integrity of the RNA had been determined, 1 μg of RNA was transcribed into cDNA using the Qiagen QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions. The kit employs a two-step procedure in which any potential genomic DNA contamination is eliminated followed by cDNA synthesis by reverse transcription. Real-time RT-PCR gene expression analysis of the cDNA template was carried using SuperArray RT^2 PCR primers for MMP-9, MT1-MMP and Actin (SABiosciences; Cedarlane Canada; Burlington, ON) and Qiagen QuantiFast SYBR Green PCR Master Mix, according to manufacturers' instructions, using the Stratagene MxP3000 QRT-PCR thermocycler. Actin expression was analyzed as a housekeeping gene control.

3.2.6 Immuoblot analysis

Please refer to Chapter 2, section 2.2.8 (Immunoblot analysis) for a detailed overview of the Western immunoblot analysis protocol.

3.2.7 Image capture and gel quantification

Please refer to Chapter 2, section 2.2.9 (Image capture and gel quantification) for a detailed overview of these methods.

3.3 Results

3.3.1 Evaluating cranberry-mediated cytotoxicity in DU145, MDA-MB-231 and MCF-10A cells

As shown in chapter 2, cranberry exerts dose and time dependent cytotoxicity against DU145 prostate adenocarcinoma cells. Treating MDA-MB-231 cells and MCF-10A cells with a whole extract of cranberry (diluted in serum-free medium) likewise resulted in a dose dependent decrease in cell viability (Figure 3.1), as measured by the Alamar Blue assay. It should be noted that both MDA-MB-231 cells and MCF-10A cells are more resistant to cranberry-mediated cytotoxicity than the DU145 cells. Unfortunately, cranberry-mediated cytotoxicity is not selective for the tumourigenic breast cell line, as the response of MDA-MB-231 cells and MCF-10A cells to cranberry treatment is nearly identical. Treating the breast cell lines with less concentrated doses of cranberry appears to increase the proliferation of cells, rather than exerting a cytotoxic effect.

3.3.2 Cranberry inhibits MMP expression in cell and in cell-free systems

The previously reported inhibition of MMP-2 and -9 secretion in response to cranberry treatment of DU145 cells (Neto *et al.*, 2006) was re-evaluated. This was deemed necessary as the previously employed concentrations of cranberry ranged from 100 μg/ml to 1000 μg/ml, concentrations that, in my work, resulted in nearly complete loss of cell viability. To take into account the potential effect of cranberry

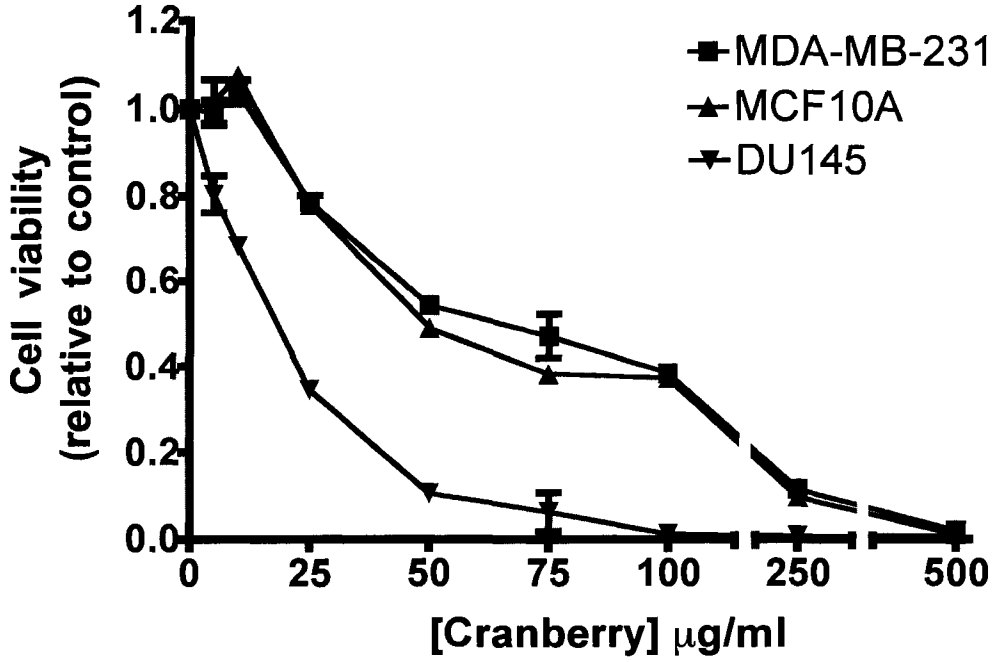

Figure 3.1: An evaluation of cranberry's cytotoxic effects in MDA-MB-231, MCF-10A and DU145 cells. Cells were treated with a whole cranberry extract at concentrations from 0 μg/ml to 500 μg/ml for 24 hours. Reduction of the Alamar Blue reagent in cranberry-treated cells was compared to that of vehicle-treated control cells, so viability is relative to control. Results are from four independent experiments, with three assay replicates per experiment. Data points where no error bars are visible indicate negligible variation between biological replicates.

-mediated cytotoxicity on the apparent inhibition of MMPs 2 and 9, DU145 cells were treated with the same doses of cranberry that were used in the evaluation of cytotoxicity. Treating DU145 cells with a whole extract of cranberry (24 hours) resulted in a dose-dependent decrease in the secretion of MMP-9, proMMP-2 and MMP-2, as measured by the gelatinolytic activity of the enzymes via substrate zymography (Figure 3.2). Three independent experiments were conducted, with six assay replicates for each biological replicate. Expression of MMP-9, MMP-2, and proMMP-2 decreases following treatment with 50 μg/ml cranberry and this decreased expression is maintained or enhanced at all higher concentrations of cranberry.

To determine whether the previously described inhibition of MMP expression in response to cranberry treatment is unique to prostate cancer cell lines, two breast cell lines (MCF-10A, MDA-MB-231) were treated with increasing doses of cranberry for 24 hours. Cell culture medium was then analyzed by gelatin zymography. No gelatinolytic activity was observed in MCF-10A cells (data not shown) though MDA-MB-231 cells produce ample quantities of both MMPs 2 and 9, and this production of MMPs appears to be inhibited by cranberry treatment (Figure 3.3). Secretion of both MMP 9 and MMP 2 decreases in response to cranberry treatment in a dose-dependent manner, even at the lowest concentrations (5 μg/ml, 10 μg/ml) – concentrations that do not exert cytotoxic effects against the MDA-MB-231 cell line.

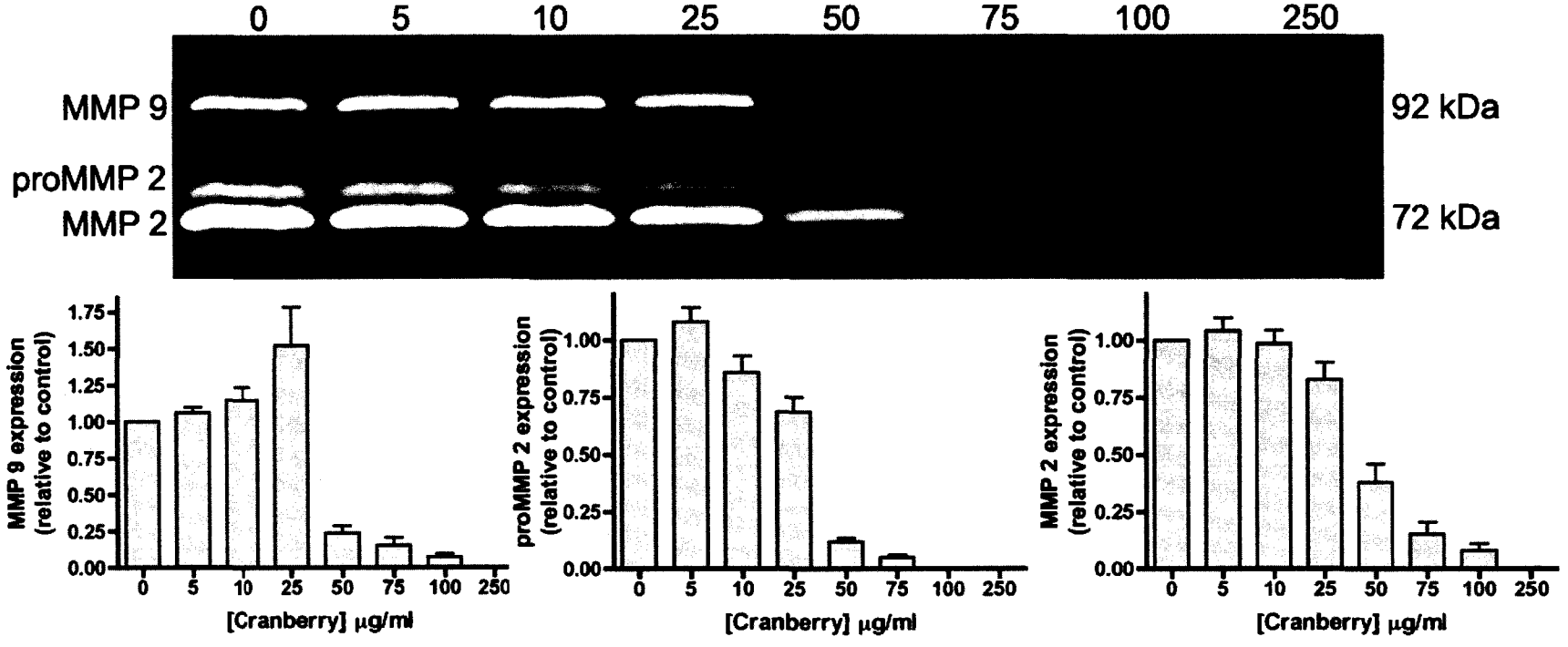

Figure 3.2: The effect of cranberry treatment on the expressions of MMP-9, proMMP-2 and MMP-2 secreted from DU145 cells. Cells were treated with increasing concentrations of whole cranberry extract (0 µg/ml to 250 µg/ml) for 24 hours. The zymogram shown is representative of three independent experiments. The histograms summarize the results attained from three independent experiments (six assay replicates for each biological replicate), and show the MMP expression relative to the vehicle treated control.

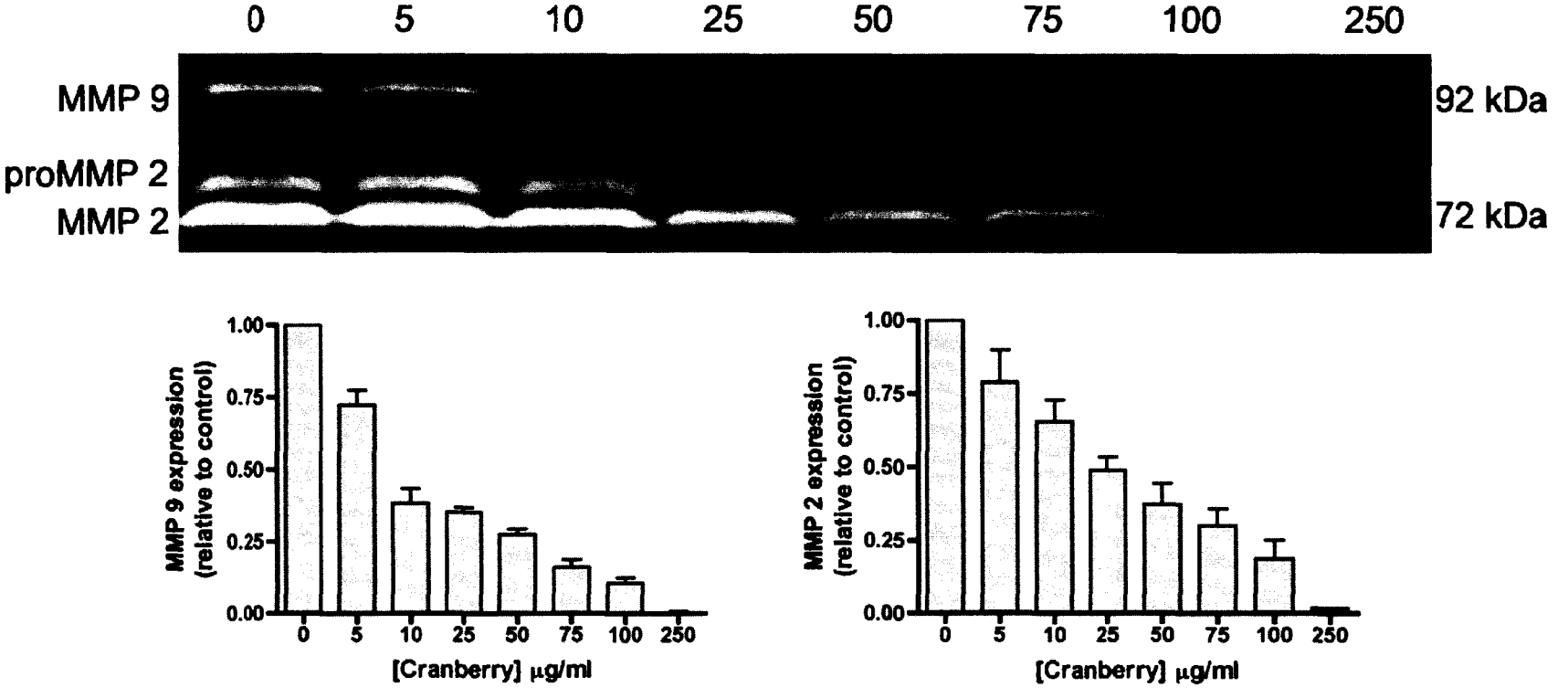

Figure 3.3: The effect of cranberry treatment on the expressions of MMP-9 and MMP-2 secreted from MDA-MB-231 cells. Cells were treated with increasing concentrations of whole cranberry extract (0 µg/ml to 250 µg/ml) for 24 hours. The zymogram shown is representative of three independent experiments. The histograms summarize the results attained from three independent experiments (six assay replicates for each biological replicate), and show the MMP expression relative to the vehicle treated control.

To further explore the observed inhibition of MMP secretion in response to cranberry treatment, an experiment was conducted to determine whether cranberry could directly inhibit the gelatinolytic activity of already secreted MMPs. MDA-MB-231 and DU145 cells were cultured in serum-free medium for 24 hours. Cell culture medium was then collected and aliquoted into 1 mL portions. Cranberry (diluted in MeOH to a concentration of 20 μg/ml) was then added to each aliquot of cell-conditioned medium (containing secreted MMPs), such that the final concentration of cranberry in each aliquot ranged from 5 μg/ml to 250 μg/ml. Cell culture medium (with added cranberry) was then incubated for 3 hours at 37 °C, followed by gelatin zymography. Surprisingly, it was revealed that cranberry treatment inhibited the "expression" of MMPs 2 and 9 secreted by both DU145 cells and MDA-MB-231 cells. As cranberry exerted the same effect on MMPs regardless of the cell line from which they were secreted, results were pooled (Figure 3.4). The results presented in figure 3.4 thus represent four independent experiments, two assay replicates each.

An experiment was also conducted to determine whether the anthocyanin (ANTHO) fraction of cranberry possesses the ability to inhibit MMP expression in DU145 cells. This particular fraction of cranberry was selected, as it had been shown in chapter 2 to exert little cytotoxicity against DU145 cells (Figure 2.6). Therefore, by evaluating the ANTHO fraction, we are able to evaluate MMP inhibition without concern about cytotoxicity. Figure 3.5 shows that the ANTHO fraction of cranberry did inhibit MMP 2 and 9 expressions, though the inhibition is muted compared to that which results from treatment with the whole extract. These experiments were not conducted in the MDA-MB-231 cell line.

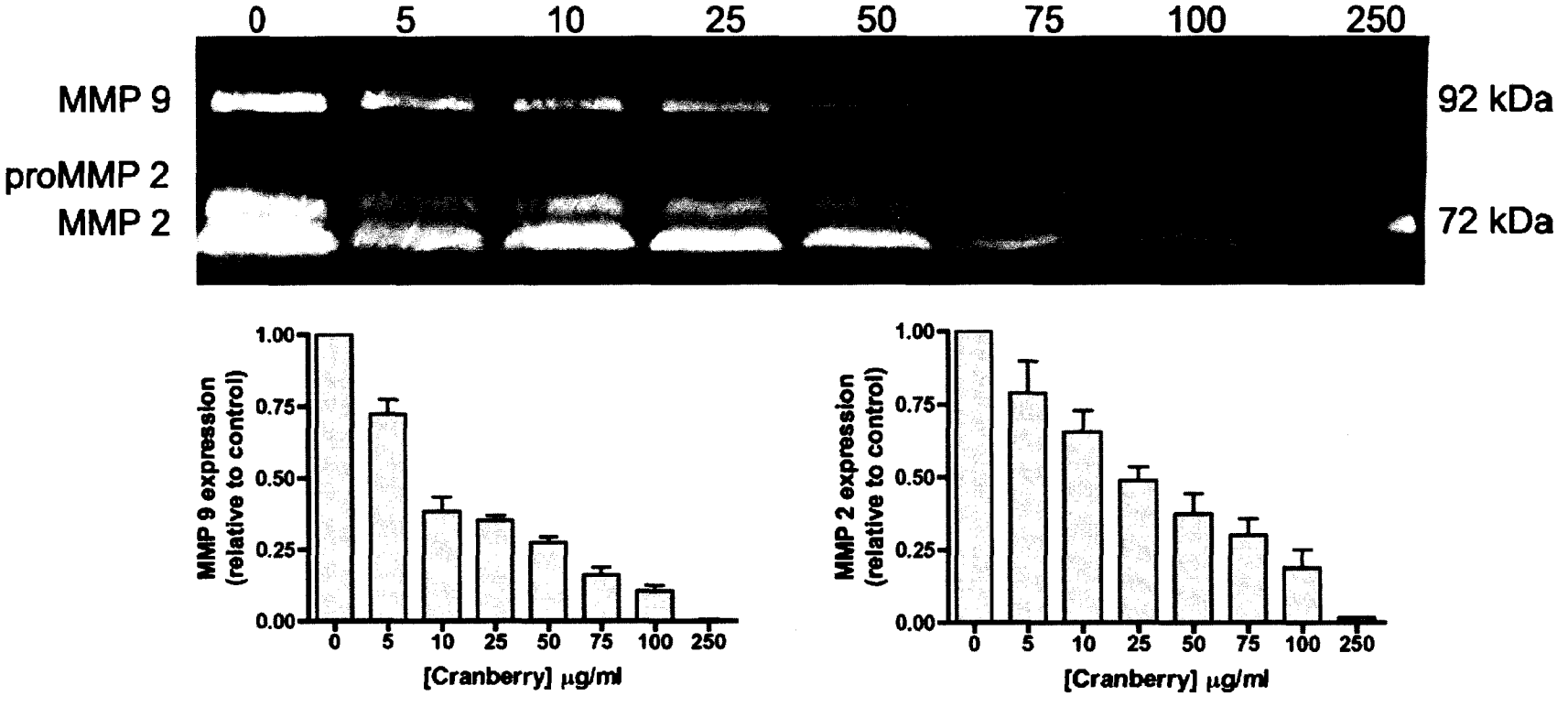

Figure 3.4: Adding cranberry to cell conditioned medium (containing secreted MMPs) causes a dose dependent decrease in the gelatinolytic activity of MMPs. The histograms summarize the results attained from four independent experiments (two assay replicates for each biological replicate), and show the MMP expression relative to cell-conditioned medium without added cranberry extract.

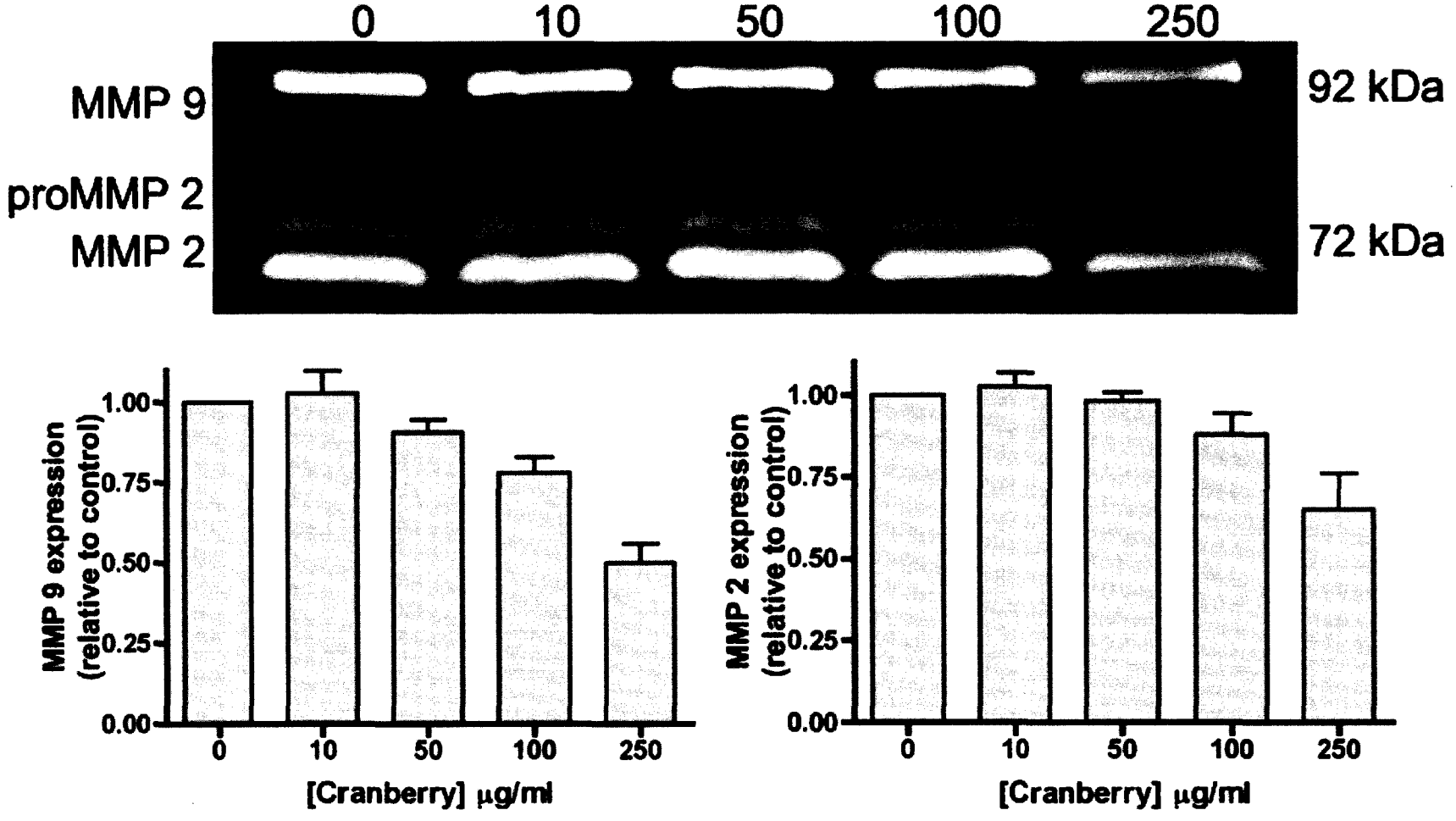

Figure 3.5: The effect of ANTHO treatment on the expressions of MMP-9 and MMP-2 secreted from DU145 cells. Cells were treated with 10, 50, 100, or 250 μg/ml ANTHOs for 24 hours, and the zymogram shown is representative of three independent experiments. The histograms summarize the results attained from those three experiments (four assay replicates for each), and show the MMP expression relative to the vehicle treated control.

3.3.3 Immunoblot analysis of cranberry-treated DU145 cells reveals changes in the protein expressions of MMP regulators.

Cranberry's ability to alter the protein expression of key MMP-regulating proteins was evaluated in DU145 cells (though not MDA-MB-231 cells), to evaluate a potential mechanism of action in an *in vivo* environment. Treating DU145 cells with 100 μg/ml whole cranberry extract (for 2, 6 and 24 hours) revealed a slight time-dependent increase in the protein expressions of both TIMPs 1 and 2 (Figure 3.6) and a time-dependent decrease in EMMPRIN protein expression (Figure 3.7).

3.3.4 QRT-PCR analysis of MMP-9 and MT1-MMP

Cranberry's potential ability to regulate the expression of MMP-9 and MT1-MMP mRNA was evaluated in MDA-MB-231 cells, as it had been hypothesized that inhibition of MMP-9 mRNA transcription could be responsible for the observed decrease in MMP-9 secretion in response to cranberry treatment. Likewise, inhibition of MT1-MMP would account for any observed decreases in MMP-2 activity. Figure 3.8 shows that treating MDA-MB-231 cells with a whole extract of cranberry (diluted in serum-containing medium) causes a dose- and time-dependent *increase* in the levels of MMP 9 and MT1-MMP mRNA expression (as revealed by QRT-PCR). Results are those attained from three independent trials, with three assay replicates per condition. This evaluation was not conducted for the DU145 cell line.

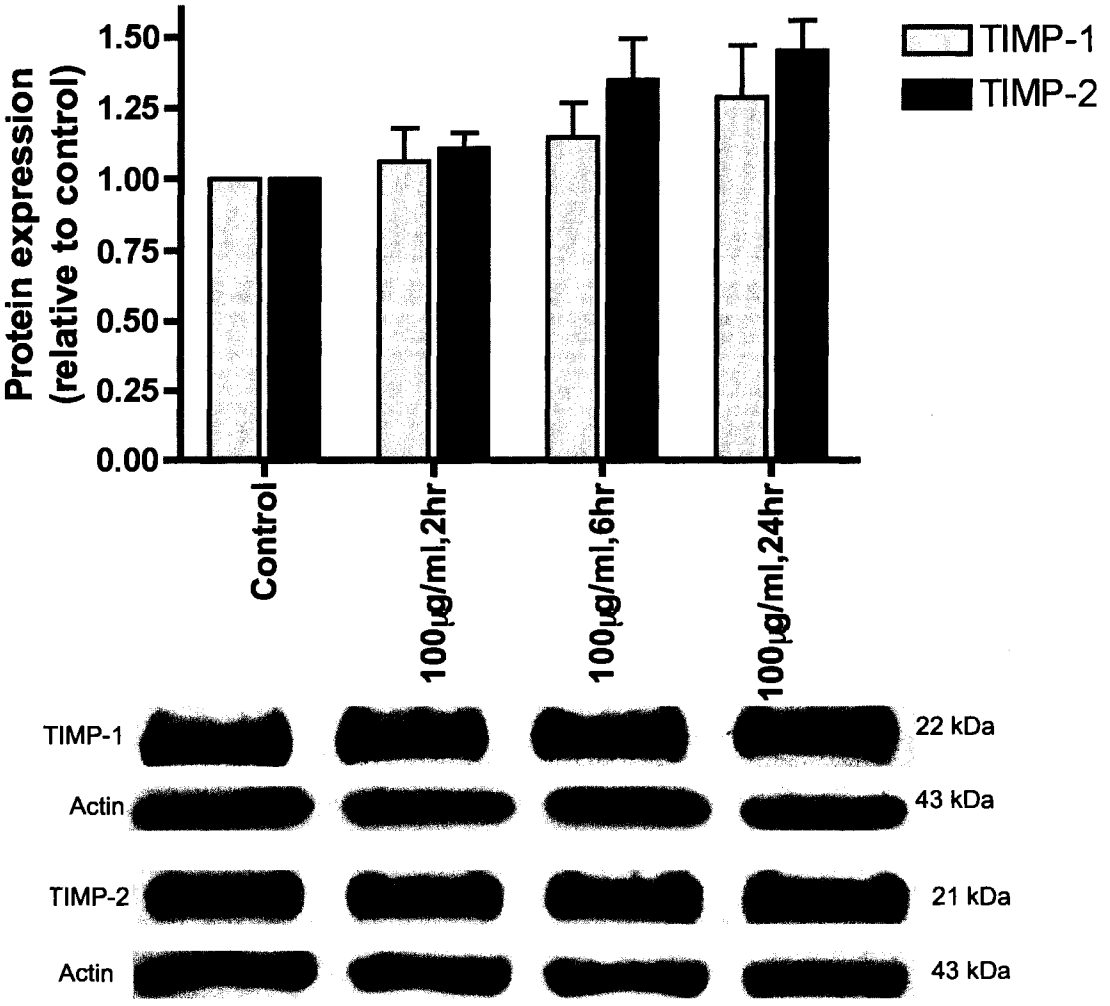

Figure 3.6: Treating DU145 cells with a whole cranberry extract (100 µg/ml) for increasing durations (2, 6 and 24 hours) increases the protein expressions of TIMPs 1 and 2. Immunoblots shown are representative of those observed from two independent experiments. The histograms summarize all results attained from two independent experiments, each with four assay replicates. TIMP protein expression was adjusted for that of the actin loading control, and those values were compared to the vehicle treated control (arbitrarily set at 1).

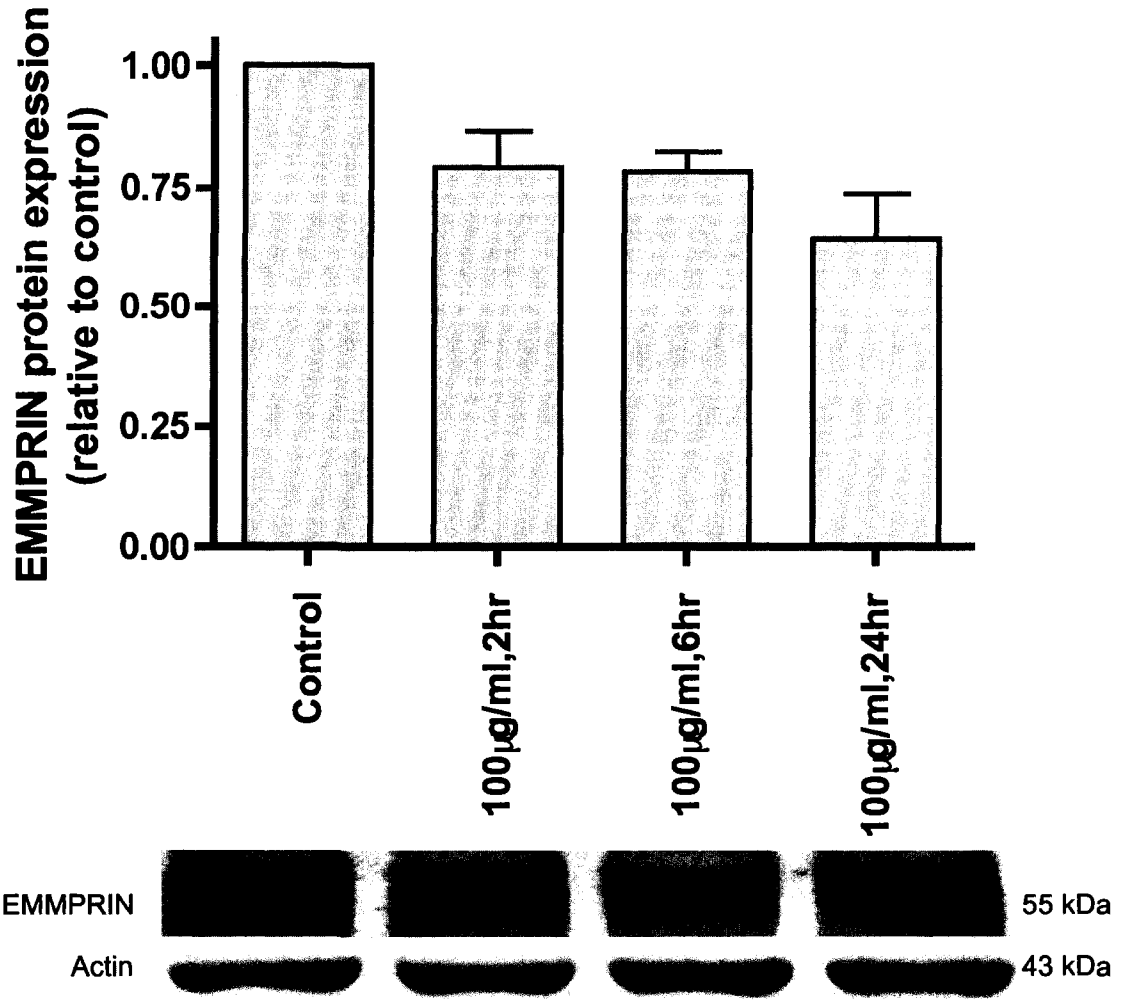

Figure 3.7: Treating DU145 cells with a whole cranberry extract (100 µg/ml) for increasing durations (2, 6, 24 hours) decreases the protein expression of EMMPRIN. The above immunoblot is representative of those observed from two independent experiments. The histograms summarize all results attained from two independent experiments, each with four assay replicates. EMMPRIN protein expression was adjusted for that of the actin loading control, and those values were compared to the vehicle treated control (arbitrarily set at 1).

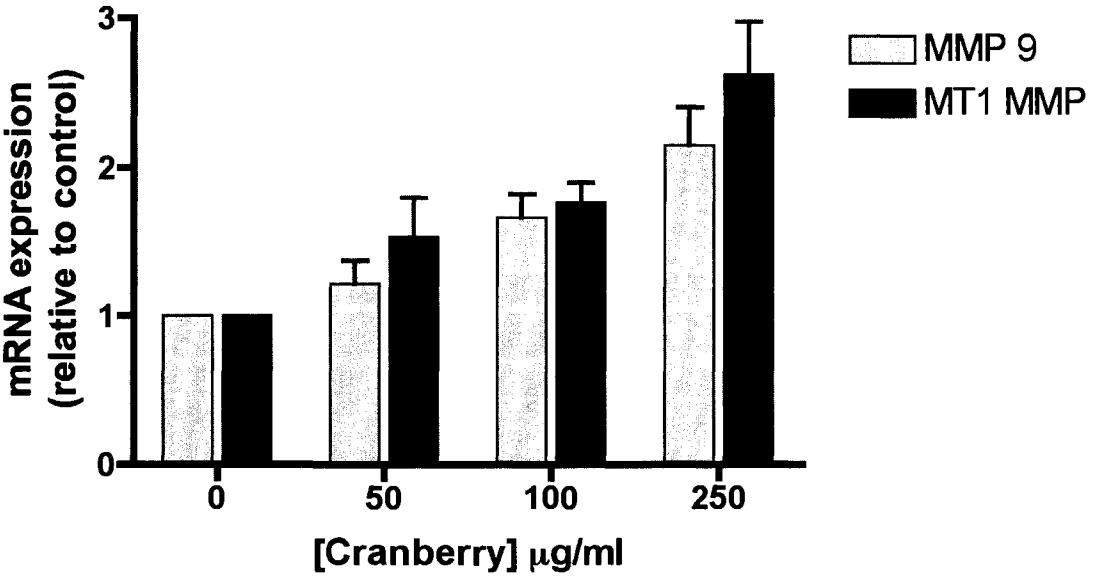

Figure 3.8: Treating MDA-MB-231 cells with a whole extract of cranberry for 24 hours (50 μg/ml, 100 μg/ml, and 250 μg/ml) results in a dose-dependent increase in the levels of MMP-9 and MT1-MMP mRNA transcripts. Results from three independent experiments, three assay replicates for each biological replicate.

3.4 Discussion

The purpose of the work presented in this chapter was to explore the previously reported inhibition of MMPs 2 and 9 in cranberry-treated DU145 cells (Neto *et al.*, 2006) and to evaluate potential mechanisms responsible for this inhibition, and also to evaluate MMP inhibition in breast cells.

3.4.1 The effect of cranberry cytotoxicity on the observed inhibition of MMPs

The observation that cranberry's cytotoxicity is enhanced in serum-free medium has implications for the analysis of cranberry-mediated MMP inhibition. Zymography can only be employed to evaluate the gelatinolytic activity of serum-free cell conditioned medium, as FBS has an inherent gelatinolytic activity that interferes with zymography (Garbisa *et al.*, 2001). Treating DU145 cells with whole cranberry extract for 24 hours causes a dose-dependent decrease in viability at concentrations as low as 5 and 10 μg/ml cranberry, with almost no viable cells remaining at 100 μg/ml. Obviously, there was some concern that the previously observed inhibition of MMPs 2 and 9 in response to cranberry treatment may have been overstated, given the cytotoxic effects of cranberry in this cell line. Indeed, comparing cranberry-mediated cytotoxicity with the observed inhibition of MMP expression revealed similar trends at doses greater than 25 μg/ml.

To further evaluate the relationship between cytotoxicity and inhibition of MMP expression, DU145 cells were treated with the anthocyanin fraction of cranberry, which has only slight cytotoxic effects at 250 μg/ml (Figure 2.6). Gelatin-

gel electrophoresis revealed that the inhibition of MMP 2 and 9 by the anthocyanin fraction is considerably less pronounced than that seen when cells are treated with the whole cranberry extract. That stated, the inhibition of MMP expression at 250 μg/ml is greater than the cytotoxicity at that concentration, suggesting that the anthocyanin fraction of cranberry may have the ability to inhibit MMP 2 and 9 secretion apart from its cytotoxic effects.

The effect of cranberry treatment on the expression of MMP-2 and -9 was also evaluated in MDA-MB-231 cells to determine whether cranberry's effects were cell type specific. It was found that increasing doses of cranberry treatment resulted in decreased expression of both MMP-2 and -9. As was the case in the DU145 cell line, this decrease in MMP expression appears to correlate with cranberry's cytotoxic effects in the breast carcinoma cells. However, MMP inhibition and cranberry cytotoxicity don't always correlate, as there is a decrease in MMP-2 and -9 expressions at 5 μg/ml and 10 μg/ml cranberry, doses that are *not* cytotoxic in this cell line. This suggests that cranberry-mediated inhibition of these MMPs may, at lower doses, occur independently of cytotoxic effects. At these lower doses of cranberry, the proliferation of both cell types appears to increase. This may be attributable to a hormetic effect (a favourable biological response to a toxic substance), which is often seen in tumour cell lines treated with low doses of antiproliferative agents, including flavonoids (Calabrese and Baldwin, 2003). The observation that cranberry phenolics exert the same antiproliferative effect against MCF-10A and MDA-MB-231 cells seems to contradict work conducted by our collaborators, who have shown that a whole cranberry extract selectively induces

apoptosis in tumorigenic MCF-7 cells, while not significantly affecting apoptosis in the "normal" MCF-10A breast cells (Griffin *et al.*, 2005; Neto *et al.*, 2006).

3.4.2 Cranberry appears to directly inhibit MMP activity in cell free systems.

Cytotoxicity is not the only complicating factor interfering with the analysis of cranberry's inhibition of MMP expression. As described in figure 3.4, treating cell conditioned medium (containing secreted MMPs 2 and 9) with increasing doses of cranberry results in a dose-dependent decrease in the "expression" of both MMP-2 and -9. However, in this experimental setup, it is not possible that cranberry can be affecting the *abundance* of these proteins, as the MMPs had already been secreted prior to introducing cranberry as a variable. Therefore, cranberry must be affecting the activity of secreted, active MMPs (as measured by gelatin zymography). Before discussing in detail such possibilities, the limitations of substrate zymography need be considered.

Gelatin zymography detects the presence and abundance (expression) of MMPs 2 and 9 in cell culture medium by measuring their gelatinolytic activity. However, there are a number of reasons why the MMP "activity" seen in zymography should not be confused with *in situ* MMP activity (in cell culture medium). First, SDS causes the dissociation of MMP-TIMP complexes during electrophoresis, such that the activity measured by zymography does not take into account the inhibitory effects of any TIMPs that may be present in the cell culture medium. A second limitation of zymography is that it cannot be used to accurately

assess the cysteine switch mechanism responsible for the activation of MMP zymogens. This limitation stems from the fact that the SDS in the gel disrupts the Cys^{72}-Zn^{2+} bond, thereby rendering the MMP zymogens active in the zymogram gel, regardless of whether or not they were catalytically active *in situ*. Zymography is thus unable to detect the difference between latent and active MMP zymogens, and is only able to detect the difference between MMP zymogens that have not undergone proteolytic cleavage and those MMPs that have. Proteolytic cleavage is "seen" in zymography due to the loss of the 8 kDa prodomain (Snoek-van Beurden and Von den Hoff, 2005). Therefore, when considering cranberry's direct effect on MMP activity, one must consider both what may occur *in situ*, and what would be evident via zymography (as the two may not be the same).

The observed direct inhibition of MMP activity by phytochemicals is not without precedent. Extracts of raspberry, blackberry, and two grape varieties were shown to inhibit both MMP-2 and -9, as measured by three different MMP activity assays (including zymography), though no suggestions were made as to their mechanism of action (Tate *et al.*, 2004). Zinc ion chelation by flavonoids has been suggested to be a potential mechanism responsible for MMP inhibition. Quercetin, for example, is able to bind divalent metal cations, including Fe^{2+} and Zn^{2+} (Guo *et al.*, 2007). Furthermore, dietary flavonoids (including quercetin and epigallocatechin 3-gallate) have been shown to interact with trace metals in cell culture medium (Kuo *et al.*, 1998; Chen *et al.*, 2007). However, preliminary investigations suggest that cranberry's inhibition of MMP activity is operating through a mechanism distinct from Zn^{2+} chelation, as this possibility was evaluated by incubating zymogram gels

in developing buffer containing high concentrations of $ZnCl_2$ (up to 250 μM). Cranberry's inhibition of MMPs' gelatinolytic activities was unaltered by the addition of the $ZnCl_2$ to the developing buffer (results are included in the zymograms used to produce Figure 3.4). Zn^{2+} chelation, however, is not the sole possible mechanism for the observed inhibition, as inhibition of zinc metallopeptidases (enzymes related to MMPs) by flavonoids has also been observed to occur through a mechanism independent of zinc chelation (Parellada *et al.*, 1998). This direct inhibition arises from specific structural interactions that may be applicable to the observations of MMP inhibition, by cranberry.

Another proposed mechanism that could explain cranberry's ability to inhibit the gelatinolytic activity of MMPs focuses on cranberry's redox activity. Cranberry phytochemicals are known to exert considerable antioxidant effects. Indeed, the research presented in this thesis has confirmed cranberry's role as a strong reducing agent, as it was seen that cranberry is able to catalyze the reduction of tetrazolium salts MTT and INT to formazan in cell culture medium (Figure 2.4, 2.8). How might cranberry's promotion of a reduced cellular environment inhibit the gelatinolytic activity of MMPs? Recall that oxidants molecules promote proMMP activation through the oxidation of the cysteine^{72} thiol group, thereby breaking the thiol-Zn^{2+} bond that maintains the zymogen in a latent form. This ultimately leads to autolytic cleavage and activation of the MMP (Hyun-Jeong and Parks, 2007). The extracellular environment is typically maintained in a more oxidized state than the intracellular environment, a balance maintained by a variety of membrane bound redox-modulating proteins and the constant diffusion of reactive oxygen species (ROS)

from the cell into the extracellular matrix (Moriatry-Craige and Jones, 2004; Chaiswing *et al.*, 2008). Cell culture medium is thus expected to contain a ready supply of ROS capable of MMP activation. However, cranberry's well-documented ROS scavenging activities would neutralize these oxidants, thereby resulting in decreased thiol oxidation and inhibition of proMMP activation.

Support for this redox hypothesis can be found in the literature. Reducing the extracellular environment (via overexpression of extracellular superoxide dismutase) was found to inhibit the invasive capacity of DU145 cells through the inhibition of MMP-2 activity (Chaiswing *et al.*, 2008). Treating transformed fibroblast cells with antioxidants likewise inhibited MMP activity through a mechanism thought to have involved blocking the active site residues through the cysteine switch mechanism (Tyagi *et al.*, 1996).

Future work to evaluate cranberry's inhibition of MMP-2 and -9 must take into account the two confounding variables highlighted in this chapter: 1) cranberry-mediated cytotoxicity in serum-free medium and 2) cranberry's direct inhibition of the gelatinolytic activity of MMPs, as revealed by zymography. Therefore, it is recommended that DU145 and MDA-MB-231 cells be treated with cranberry diluted in cell culture medium containing 10% FBS. Under these conditions, both cell lines can be treated with up to 75 μg/ml cranberry without concern about cytotoxic effects (as shown in Chapter 2). The FBS in the medium will also serve as a source of growth factors to stimulate MMP production in DU145 and MDA-MB-231 cells. Following a 24- or 48- hour treatment, the cranberry-

conditioned, serum-containing medium would then be replaced with serum and cranberry-free defined medium for 24 hours (allowing for the secretion of MMPs into the medium). Analysis of this medium via zymography would provide information as to how cranberry treatment affects the MMP production in these cell lines. Alternatively, gelatinase-free FBS can be prepared for use in experiments measuring gelatinase activity by zymography as described by Garbisa *et al.* (2001).

3.4.3 Evaluating other MMP-related effects of cranberry treatment in MDA-MB-231 and DU145 cells

It was hypothesized that cranberry-mediated inhibition of EMMPRIN protein expression in DU145 cells could account for the previously reported decrease in MMP-9 expression that occurs in response to cranberry treatment. EMMPRIN not only regulates the production of MMP-9 from stromal cells, but also has an autocrine function, as it is able to regulate MMP-9 production from tumour cells. The cranberry-mediated decrease in EMMPRIN protein expression might be responsible, to an extent, for the decrease in MMP-9 expression seen in response to cranberry treatment. However, as already discussed, there are a number of other factors that make interpreting this result somewhat difficult. Based on previous work conducted evaluating the effect of blueberry treatment on the activities of TIMP-1 and -2 (Matchett *et al.*, 2006), it was hypothesized that cranberry treatment would increase the protein expressions of these MMP inhibitors. Treating DU145 cells with whole cranberry increased TIMP-1 and -2 expressions. Contrary to what was

predicted, treating MDA-MB-231 cells with cranberry resulted in a dose-dependent increase in the mRNA expressions of MMP-9 and MT1-MMP.

3.4.4 Evaluating the effect(s) of other phytochemicals/natural products on MMP secretion and activity

The research presented herein has explored the inhibition of MMP secretion and activity by *Vaccinium macrocarpon* phytochemicals, and has elucidated some of the mechanisms responsible for this inhibition. Cranberry, however, is not unique amongst plants in its ability to inhibit MMP activity, as a great deal of research has been conducted to identify other potential MMP inhibitors from natural sources. This work has revealed an incredibly variety of plant extracts capable of inhibiting MMP activity (Garbisa *et al.*, 2001; Mannello, 2006; Rose *et al.*, 2005; Tate *et al.*, 2004). In order to evaluate just how common these MMP-regulating activities might be amongst plant phytochemicals, a series of experiments were conducted to evaluate how aqueous extracts of *Matricaria matricariodies* (pineapple weed) might be able to inhibit MMP secretion and activity. This work is presented in Appendix B, and serves to illustrate the seemingly ubiquitous nature of MMP regulation by plant phytochemicals, even though the mechanisms responsible for such inhibition may vary considerably.

3.4.5 Future directions

MMP expression and activity correlates with the development and progression of advanced metastatic cancers, particularly cancers of the breast and the prostate. Many pharmaceutical companies turned their attention and resources to the development of MMP-inhibiting agents with the hope that MMP inhibition would prevent basement membrane breakdown and ECM remodeling, thus constraining cancer that would otherwise be metastatic. Several drugs that directly inhibit the catalytic activity of MMPs were tested in phase III clinical trials, though none were effective (Coussens *et al.*, 2002). The problem with all of these drugs is that they indiscriminately target a range of metalloenzymes. In the complex environment of a tumour, many MMPs affect multiple and sometimes opposing processes at the same time, some of which restrain disease and promote healthy tissue (Coussens *et al.*, 2002; Parks *et al.*, 2004). Therefore, for any MMP-inhibiting agent to be useful, it must be able to selectively inhibit those MMPs required specifically for tissue invasion and metastasis (such as MMP-2 and -9) whilst sparing the other metalloenzymes that have beneficial functions. The observation that cranberry is able to directly inhibit the activity of MMPs 2 and 9 is certainly intriguing, though it is, as of yet, unknown if cranberry can *selectively* inhibit those MMPs that promote cancer. Future investigations should thus focus on determining the extent to which cranberry inhibits other metalloenzymes, and the mechanisms whereby cranberry inhibits the activities of MMPs 2 and 9 in cell free systems.

CHAPTER 4

Conclusions, General Discussion and Future Directions

4.1 Conclusions and General Discussion

The investigations presented in this thesis have explored and elucidated some of the mechanisms responsible for *Vaccinium macrocarpon*'s previously described anticancer activities. While cranberry's antiproliferative effects on tumour cell lines had previously been reported to involve the induction of apoptosis, little work had been done to identify the apoptotic machinery involved in this process. The work presented in this thesis identifies the release of mitochondria-sequestered cytochrome c into the cytsol and the subsequent activation of caspase-9 as major events in cranberry-mediated apoptosis, events that ultimately lead to PARP cleavage and inhibition, DNA cleavage into nucleosomes and apoptotic cell death. This work has also identified upregulation of Bax protein expression and caspase-8-mediated cleavage of Bid to truncated Bid as the likely causes of cytochrome c release. While other mechanisms have yet to be explored, it appears that upregulation of Par-4 protein expression in response to cranberry treatment may be the trigger for caspase-8 activation.

It was hypothesized that cranberry's cytotoxic effects were mediated solely through the intrinsic pathway of apoptosis, though the observations of caspase-8 activation and tBid cleavage clearly indicate that elements of the extrinsic pathway are engaged in response to cranberry treatment. While the effector machinery of cranberry-mediated apoptotic death involves the mitochondrial components of the intrinsic pathway, the upstream signaling pathways haven't been sufficiently elucidated by this study. The upregulation of Par-4 expression provides a number of targets for further investigation, many of which have been highlighted in the Discussion of Chapter 2.

The cytotoxic effects of three cranberry fractions were evaluated in DU145 cells, and it was determined that the PAC and FLAV fractions of cranberry were primarily responsible for the observed cytotoxicity of the whole polyphenol extract, as the ANTHO fraction had little effect. This study has also identified the inhibition of cranberry-mediated cytotoxicity by fetal bovine serum. It was originally believed that the presence of FBS inhibited cranberry cytotoxicity through direct protein binding and sequestration of cranberry phytochemicals, though work with bovine serum albumin ruled out this possibility. It is thus hypothesized that growth factor withdrawal and cranberry treatment synergize to induce apoptotic cell death, somewhat surprising given the extensive documentation of the growth factor signaling autonomy of DU145 cells. Perhaps cranberry treatment interferes with the autocrine loops that grant DU145 cells this autonomy. To further discuss this possibility, our attention turns to the objectives addressed in Chapter 3 of this thesis, those that concern the matrix metalloproteinases.

It was hypothesized that the previously reported inhibition of MMP-2 and -9 by cranberry treatment of DU145 cells was effected through increased TIMP-1 and -2 protein expressions and decreased EMMPRIN expression. While these effects *were* observed in response to cranberry treatment, they may be rendered somewhat less noteworthy with the observation that cranberry is able to directly inhibit the gelatinolytic activities of secreted MMP-2 and -9. This is a novel finding that is worthy of and needing further study, as the mechanism whereby cranberry inhibits MMP activity is currently unknown.

Cranberry's direct inhibition of MMP-9 activity is hypothesized to play a role in *V. macrocarpon*-induced cytotoxicity in DU145 cells, particularly in the absence of serum. This thesis does not provide the reason for the enhanced cytotoxicity of cranberry in serum free medium, though it has been suggested (Chapter 2 Discussion) that the increased expression of Par-4 may result in phosphorylation of the mutant p53 protein product, thereby rendering the cell susceptible to growth factor withdrawal-induced apoptosis. However, another hypothesis is that cranberry treatment may abrogate the autocrine growth factor signaling loops that provide DU145 cells with their self-sufficiency in growth signaling. One of the mechanisms whereby cranberry could effect such a change has already been described - its inhibition of MMP-9 activity.

The insulin-like growth factor (IGF) autocrine signaling loop, of which DU145 cells express all the components, is at least partly responsible for the proliferation of DU145 cells in serum-free medium. This loop is regulated by extracellular IGF-

binding proteins (IGFBP) that bind and sequester IGF peptides from their receptors, thereby interrupting growth signaling. The IGF-1 binding activity of IGFBP-3 is regulated by MMP-9, which cleaves the binding protein to inhibit its activity. Inhibition of MMP-9 in DU145 cells via the addition of exogenous TIMP-2 or antisense MMP-9 cDNA is accompanied by decreased proteolysis of IGFBP-3, which then accumulates in the cell culture medium and sequesters IGF-1, causing an 80% inhibition of DU145 cell proliferation. This effect is reversed by adding exogenous IGF-1 (Manes *et al.*, 1999). MMP-9 inhibition could thus explain decreased viability of DU145 cells in response to cranberry treatment, though only in serum free medium. Further support for this potential mechanism of cranberry-mediated inhibition of viability is provided by the observation that treating PC-3 prostate cancer cells with quercetin decreases the culture medium levels of insulin-like growth factors (IGFs) through increasing serum concentration of IGF binding protein-3 (IGFBP-3), ultimately resulting in decreased proliferation and increased apoptosis (Vijayababu *et al.*, 2006). Furthermore, it is suggested that this pathway may be one of the means by which cranberry induces Par-4 expression. It has been reported that the inhibition of apoptosis by exogenous IGF-1 is associated with decreased Par-4 expression (Chung and Park, 2007). It remains to be seen if MMP-9 inhibition can play a role in the activation of Par-4 by decreasing the availability of IGF-1.

The mechanism just describe could also be responsible for some of cranberry's effects in the MDA-MB-231 cell line. Studies have shown that these cells are also reliant upon autocrine IGF loops, and inhibition of IGFR has been shown to

slow the growth of MDA-MB-231 cells in serum-free conditions (Arteaga, 1992). This autocrine loop is less well defined in MDA-MB-231 cells as compared to DU145 cells, and it is not yet clear if its abrogation could induce apoptosis. It is recommended that further investigation of cranberry-mediated apoptosis focus on these autocrine pathways, as it appears possible that *V. macrocarpon* phytochemicals may be able to render advanced stage prostate and breast carcinoma cells more sensitive to the withdrawal of trophic support. This would of considerable value in a chemopreventative adjuvant therapy, as independence in growth signaling is one of the means by which carcinoma cells evade typical therapeutic intervention.

It is tempting to arrange the pathways and mechanisms (both observed and hypothesized) proposed to be responsible for cranberry-mediated apoptosis into convenient cause and effect cascades. However, one must realize that multiple independent cell death pathways may be simultaneously induced by cranberry treatment, due to the wealth of phytochemical constituents contained within the whole extract. Therefore, it would be unwise to assign cranberry *a* method of cell death induction, when in reality it is far more likely that multiple mechanisms act in concert. This consideration adds a level of complexity to the evaluation of mechanisms responsible for cranberry-mediated cytotoxicity.

4.2 Future directions and considerations

In addition to suggesting specific experiments to address the hypotheses generated in this thesis, I would also like to suggest a re-evaluation of the *in vitro*

models employed to study cranberry's anticancer activities. The work presented in this thesis provides proof of concept that cranberries contain potentially valuable phytochemicals for the development of novel chemo-preventative and -therapeutic strategies. However, this model only evaluates cranberry compounds in a pharmacological setting. Advanced stage prostate and breast carcinoma cells were treated with relatively high "doses" of cranberry extracts for durations shorter than 24 hours. This work therefore yields data of more use to drug-synthesizing pharmacognosists than researchers evaluating the effects of dietary cranberry consumption or those who are designing *in vivo* feeding studies.

If the goal of this research is to provide valuable information about the potential benefits of cranberry consumption, or to assist in the development of animal feeding studies, it is recommended that a broader range of cell models be employed. While oncologists and geneticists have long acknowledged the morphological and physiological continuum along which cells develop from "normal" to neoplastic (Sogn *et al.*, 2005), the vast majority of *in vitro* researchers continue to rely exclusively upon tumour cells in the evaluation of mechanisms and processes relevant to cancer progression. However, developing an *in vitro* model of cancer that is representative of early, middle, and late stages of tumorigenesis is particularly important when evaluating the potential of dietary components to impact upon the progression of cancer. Determining the effects of these dietary components on normal and preneoplastic cells is particularly important when one is evaluating whether the compounds can prevent normal cells from becoming

preneoplastic and/or slow the progression of preneoplastic cells to the complete cancer phenotype.

As such, it is recommended that the use of immortalized normal human prostate epithelial cells (Clonetics) be considered for future evaluations of cranberry's potential anticancer activities. Although somewhat more difficult to maintain, if used efficiently these cells can provide considerable information about the potential effects of cranberry phytochemicals on preneoplastic cells. Likewise, use of androgen-responsive prostate cancer cells (like the LNCaP cell line) could suggest potential benefits of cranberry consumption during the earlier stages of prostate cancer development, prior to the onset of untreatable metastatic disease. Previously conducted studies indicate that the cytotoxicity of *Vaccinium* phytochemicals may be enhanced in the LNCaP cell line, compared to DU145 cells (Ferguson *et al.*, 2004; Schmidt *et al.*, 2006). With respect to breast cancer, expanded use of the MCF-10A cell line could provide information more relevant to the prevention of breast cancer by cranberry phytochemicals. The other advantage of using "normal" cells is that they grow more slowly than their cancerous counterparts, such that the effects of cranberry can be studied at lower concentrations for longer periods of time (up to a week).

The vast majority of the work conducted throughout the course of this investigation has focused primarily upon the anticancer activities of the whole cranberry extract, with subfractions of cranberry being used in assays only when the whole extract was not appropriate. As discussed earlier, working with whole

extracts has particular benefits when attempting to evaluate the effects of consuming whole foods, though as already highlighted, the design of the experiments presented in this thesis tend to represent pharmacological intervention. However, there are distinct drawbacks to the use of a whole, non-standarized extract of plant phytochemicals, apart from the variability inherent to such a treatment. Using complex mixtures of phytochemicals can make it difficult to clearly identify cause and effect relationships, as a variety of phytochemicals may be affecting different targets along cell death- and MMP-regulating pathways. For example, the PAC and FLAV extracts are able to activate caspase-8 and -9 in DU145 cells, while the whole cranberry extract cannot. While it was hypothesized that this discrepancy resulted from resveratrol's inhibition of luciferase, it is also possible that in DU145 cells, non-FLAV and non-PAC polyphenols may be inducing PCD through a caspase-independent mechanism, thus killing cells without the activation of caspases 8 and 9 seen when PAC and FLAV fractions are used alone. The possible induction of caspase-independent cell death upon treatment with higher concentrations of cranberry is discussed in Chapter 2.

In summation, the work presented in this thesis has provided the proof of concept that *Vaccinium macrocarpon* extracts contain phytochemical that may be of potential value in the development of novel chemopreventative strategies, particularly with regards to the induction of apoptosis and the potential inhibition of invasion and metastasis via MMP inhibition. Further work needs to be conducted to evaluate the hypotheses generated from this data, and future *in vivo* studies are

recommended to determine the efficacy of cranberry consumption on the development and progression of cancer.

LITERATURE CITED

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Xiaodong, W., Akey, C.W. 2002. Three dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell.* 9(2) 423-432.

Adams, J.M., Cory, S. 2001. Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem Sci.* 26: 61-66.

Agarwal, C., Sharma, Y., Zhao, J., Agarwal, R. 2000. A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB-468 cells by inhibiting mitogen-activated protein kinases and inducing G1 arrest and differentiation. *Clin Cancer Res.* 6: 2921-2930.

Agarwal, C., Sharma, Y., Agarwal, R. 2000. Anticarcinogenic Effect of a Polyphenolic Fraction Isolated From Grape Seeds in Human Prostate Carcinoma DU145 Cells: Modulation of Mitogenic Signaling and Cell-Cycle Regulators and Induction of G1 Arrest and Apoptosis. *Mol Carcinog.* 28: 1-10.

Ahn, Y.J., Kwon, J.H., Chae, S.H., Park, J.H., Yoo, J.Y. 1994. Growth-inhibitory responses of human intestinal bacteria to extracts of oriental medicinal plants. *Microbial Ecol Health Dis.* 7: 257-261.

American Cancer Society. *Cancer Facts & Figures 2008.* Atlanta: American Cancer Society; 2008.

Andersson, D., Liu, J.J., Nilsson, A., Duan, R.D. 2003. Ursolic acid inhibits proliferation and stimulates apoptosis in HT29 cells following activation of alkaline sphingomyelinase. *Anticancer Res.* 23: 3317-3322.

Arak, E.K., Raal, A.E., Peknk, T.I., Myaeorg, U.Y. 1988. Components of *Matricaria discoidea* – geranyl isovalerate, trans-β-farnesene, and heriarin. *Khimiya Prirodynkh Soedinenii.* 6: 804-806.

Arteaga, C.L. 1992. Interference of the IGF system system as a strategy to inhibit breast cancer cell growth. *Breast Cancer Res Treat.* 22(1): 101-106.

Baek, J.H., Lue, Y.S., Kang, C.M., Kim, J.A., Kwon, K.S., Son, H.C., Kim, K.W. 1997. Intracellular Ca^{2+} release mediates ursolic acid-induced apoptosis in human leukemic HL-60 cells. *Int J Cancer.* 37: 725-728.

Bakhtiarova, A., Taslimi, P., Elliman, S.J., Kosinski, P.A., Hubbard, B., Kavana, M., Kemp, D.M. 2006. Resveratrol inhibits firefly luciferase. *Biochem Biophys Res Commun.* 351(2): 481-484.

Barnes, S. 2001. Role of phytochemicals in prevention and treatment of prostate cancer. *Epidemiol Rev.* 23: 102-105.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M.A., Bernardi, P. 2005. Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem.* 13: 18558-18561.

Bemis, D.L., Capodice, J.L., Costello, J.E., Vorys, G.C., Katz, A.E., Buttyan, R. 2006. The use of herbal and over-the-counter dietary supplements for the prevention of prostate cancer. *Curr Urol Rep.* 7: 166-74.

Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., Hanahan, D. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol.* 2(10): 737-744.

Bergmann, M., Zukoc-Zivojnov, N., Chow, K.U., Trepohl, B., Hoelzer, D., Weidmann, E., Mitrou, P.S., Boehrer, S. 2004. Prostate apoptosis response gene-4 sensitizes neoplastic lymphocytes to CD95-induced apoptosis. *Ann Hematol.* 83(10): 646-653.

Berridge, M.V., Tan, A.S. 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys.* 303(2): 474-482.

Black, W.C., Welch, H.G. 1993. Advances in diagnostic imaging and overestimations of disease prevalence and the benefits of therapy. *N Engl J Med.* 328(17): 1237-1243.

Blavier, L., Henriet, P., Imren, S., Declerck, Y.A. 1999. Tissue inhibitors of matrix metalloproteinases in cancer. *Ann N Y Acad Sci.* 878: 108-119.

Boatright, K.M., Renatus, M., Scott, F.L., Sperandio, S., Shin, H., Pedersen, I.M., Ricci, J.E., Edris, W.A., Sutherlin, D.P., Green, D.R., Salvesen, G.S. 2003. A unified model for apical caspase activation. *Mol Cell.* 11(2): 529-41.

Boehrer, S., Chow, K.U., Beske, F., Kukoc-Zivojnov, N., Puccetti, E., Ruthardt, M., Baum, C., Rangnekar, V.M., Hoeizer, D., Mitrous, P.S., Weidmann, E. 2002. In lymphatic cells par-4 sensitizes to apoptosis by down-regulating bcl-2 and promoting disruption of mitochondrial membrane potential and caspase activation. *Cancer Res.* 62(6): 1768-1775.

Boivin, D., Blanchette, M., Barrette, S., Moghrabi, A., Beliveau, R. 2007. Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NF-κB by edible berry juice. *Anticancer Res.* 27(2): 937-948.

Bomser, J., Madhavi, D.L., Singletary, K., Smith, M.A. 1996. *In vitro* anticancer activity of fruit extracts from *Vaccinium* species. *Planta Med.* 62: 212-216.

Bossy-Wetzel, E., Newmeyer, D.D., Green, D.R. 1998. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J.* 17(1): 37-49.

Boujrad, H., Gubkina, O., Robert, N., Krantic, S., Susin, S.A. 2007. AIF-mediated programmed necrosis: a highly regulated way to die. *Cell Cycle.* 6(21): 2612-2619.

Bratton, S.B., Walker, G., Srinivasula, S.M., Sun, X.M., Butterworth, M., Alnemri, E.S., Cohen, G.M. 2001. Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *EMBO J.* 20: 998-1009.

Brown, T.R., Lee, C. 2001. Conference summary on prostate growth and aging, 13-15 September 2000. *Prostate.* 48(1): 54-65.

Burger, O., Ofek, I., Tabak, M., Weiss, E.I., Sharon, N., Neeman, I. 2000. A high molecular mass constituent of cranberry juice inhibits *Heliobacter phylori* adhesion to human gastric mucus. *FEMS Immunol Med Microbiol.* 29: 295-301.

Burgoyne, L.A., Hewish, D.R., Mobbs, J. 1974. Mammalian chromatin substructure studies with the calcium-magnesium endonuclease and two-dimensional polyacrylamide-gel electrophoresis. *Biochem J.* 143(1): 67-72.

Bustin, S.A., Benes, V., Nolan, T., Pfaffl, M.W. 2005. Quantitative real-time RT-PCR – a perspective. *J Mol Endocrin.* 34: 597-601.

Cailleau, R., Olive, M., Cruciger, Q.V.J. 1978. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In vitro.* 14(11): 911-915.

Calabrese, E.J., Baldwin, L.A. 2003. Chemotherapeutics and hormesis. *Crit Rev Toxicol.* 33(3-4): 305-353.

Canadian Cancer Society/National Cancer Institute of Canada. 2008. Canadian Cancer Statistics. 2008. Toronto, Canada.

Cao, J., Rehmentulla, A., Bahou, W., Zucker, S. 1996. Membrane type matrix metalloproteinase 1 activates pro-gelatinase A without furin cleavage of the N-terminal domain. *J Biol Chem.* 1996; 271: 30174-30180.

Caudroy, S., Polette, M., Nawrocki-Raby, B., Cao, J., Toole, B.P., Zucker, S., Birembaut, P. 2002. EMMPRIN-mediated MMP regulation in tumor and endothelial cells. *Clin Exp Metastasis.* 19: 697-702.

Cauwe, B., Van den Steen, P.E., Opdenakker, G. 2007. The biochemical, biological and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit Rev Biochem Mol Bio.* 42: 113-185.

CCCGA (Cape Cod Cranberry Growers' Association). History of Cranberries. www.cranberries.org/cranberries/history.html. Copyright 2002-2008. Website accessed. 12.05.08

Cha, H.J., Bae, S.K., Lee, H.Y., Lee, O.H., Sato, H., Seiki, M., Park, B.C., Kim, K.W. 1996. Anti-invasive activity of ursolic acid correlates with the reduced expression of matrix metalloproteinase-9 (MMP-9) in HT1080 human fibrosarcoma cells. *Cancer Res.* 56: 2281-2284.

Chaiswing, L., Zhong, W., Cullen, J.J., Oberley, L.W., Oberley, T.D. 2008. Extracellular redox state regulates features associated with prostate cancer cell invasion. *Cancer Res.* 68(14): 5820-5826.

Chakraborty, M., Qiu, S.G., Vasudevan, K.M., Rangnekar, V.M. 2001. Par-4 drives trafficking and activation of Fas and Fasl to induce prostate cancer cell apoptosis and tumor regression. *Cancer Res* 61: 7255-7263.

Chang, C., Werb, Z. 2001. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol.* 11(11): S37-43.

Chao, Y., Shiozaki, E.N., Srinivascula, S.M., Rigotti, D.J., Fairman, R., Shi, Y. 2005. Engineering a dimeric caspase-9: a re-evaluation of the induced proximity model for caspase activation. *PLoS Biol.* 3(6): 1079-1087.

Chatelain, K., Phippen, S., McCAbe, J., Teeters, C.A., O'Malley, S., Kingsley, K. 2008. Cranberry and grape seed extracts inhibit the proliferative phenotype of oral squamous cell carcinomas. *Evid Based Complement Alternat Med.* (ePub) 1-12.

Chen, X., Yu, Haining, Shen, S., Yin, J. 2007. Role of Zn^{2+} in epigallocatechin gallate affecting the growth of PC-3 cells. *J Trace Elem Med Biol.* 21(2): 125-131.

Chipuk, J.E., Bouchier-Hayes, L., Green, D.R. 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. *Cell Death Differ.* 13: 1396-1402.

Choi, Y.H., Baek, J.H., Yoo, M.A., Chung, H.Y., Kim, N.D., Kim, K.W. 2000. Induction of apoptosis by ursolic acid through activation of caspases and down-regulation of c-IAPs in human prostate epithelial cells. *Int J Oncol.* 17: 565-571.

Choi, J., Kim, J., Lee, J., Kang, C., Kwon, H., Yoo, Y., Kim, T., Lee, Y., Lee, S. 2001. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *Int J Oncol.* 19: 837-844.

Chung, H., Seo, S., Moon, M., Park, S. 2007. IGF-1 inhibition of apoptosis is associated with decreased expression of prostate apoptosis response-4. *J Endocrin* 194: 77-85.

Cook, L.S., Goldoft, M., Schwartz, S.M., Weiss, N.S. 1999. Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants. *J Urol.* 161: 152-155.

Coussens, L.M., Fingleton, B., Matrisian, L.M. 2002. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. 295: 2387-2392.

Decker, T., Lohmann-Matthes, M.L. 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods.* 115(1): 61-69.

de Jong, D., Prins, F.A., Mason, D.Y., Reed, J.C., van Ommen, G.B., Kluin, P.M. 1994. Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. *Cancer Res.* 54(1): 256-260.

Dhanalakshmi, S., Agarwal, R., Agarwal, C. 2003. Inhibition of NF-κB pathway in grape seed extract induced apoptotic death of human prostate carcinoma DU145 cells. *Int J Oncol.* 23(3): 721-727.

Donaldson, M.S. 2004. Nutrition and cancer: A review of the evidence for an anti-cancer diet. *Nutrition J.* 3(19): 1-21.

Du, C., Fang, M., Li, Y., Li, L., Wang, X. 2000. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell.* 102: 33-42.

Egeblad, M., Werb, Z. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer.* 2: 161-174.

Eguchi, Y., Shimizu, S., Tsujimoto, Y. 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res.* 57(10): 1835-1840.

Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer.* 2001; 1: 34-45.

Ferguson, P.J., Kurowska, E., Freeman, D.J., Chambers, A.F., Koropatnick, D.J. 2004. Flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. *J Nutr.* 134(6): 1529-1535.

Fernandez-Resa, P., Mira, E., Quesada, A.R. 1995. Enhanced detection of casein zymography of matrix metalloproteinases. *Anal Biochem.* 224: 434-435.

Folkman, J. 1972. Anti-angiogenesis: new concept for therapy of solid tumors. *Ann Surg.* 175(3): 409-416.

Foo, L.Y., Lu, Y., Howell, A.B., Vorsa, N. 2000. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli. J Nat Prod.* 63: 1225-1228.

Fraenkel, G.S. 1959. The raison d'être of secondary plant substances. *Science.* 129(3361): 1466-1470.

Gabison, E.E., Hoang-Xuan, T., Mauviel, A., Menashi, S. 2005. EMMPRIN/CD147, an MMP modulator in cancer, development and tissue repair. *Biochimie.* 87: 361-368.

Garbisa, S., Sartor, L., Biggin, S., Salvato, B., Benelli, R., Albini, A. 2001. Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. *Cancer.* 91(4): 822-832.

Genini, D., Budihardjo, I., Plunkett, W. Wang, X., Carrera, C.J., Cottam, H.B. Carson, D.A., Leoni, L.M. 2000. Nucleotide requirements for the *in vitro* activation of the apoptosis protein-activating factor-1 –mediated caspase pathway. *J Biol Chem.* 275(1): 29-34.

Gohji, K., Fujimoto, N., Hara, I., Fujii, A., Gotoh, A., Okada, H., Arakawa, S., Kitazawa, S., Miyake, H., Kamidono, S., Nakajima, M. 1998. Serum matrix metalloproteinase-2 and its density in men with prostate cancer as a new predictor of disease extension. *Int J Cancer.* 79(1): 96-101.

Goldstein, J.C., Waterhouse, N.J., Juin, P., Evan, G.I., Green, D.R. 2000. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nature Cell Biol.* 2 156-162.

Gomez, D.E., Alonso, D.F., Yoshiji, H., Thorgeirsson, U.P. 1997. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol.* 74: 111-122.

Gonzalez, R.J., Tarloff, J.B. 2001. Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. *Toxicol In Vitro.* 15(3): 257-259.

Goswami, A., Burikhanov, R., de Thonel, A., Fujita, N., Goswami, M., Zhao, Y., Eriksson, J.E., Tsuruo, T., Rangnekar, V.M. 2005. Binding and phosphorylation of par-4 by akt is essential for cancer cell survival. *Mol Cell.* 20(1): 33-44.

Gray, C.W., Ward, R.V., Karran, E., Turconi, S., Rowles, A., Viglienghi, D., Southan, C., Barton, A., Fantom, K.G., West, A., Savopoulos, J., Hassan, N.J., Clinkenbeard, H., Hanning, C., Amegadzie, B., Davis, J.B., Dingwall, C., Livi, G.P., Creasy, C.L. 2000. Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. *Eur J Biochem.* 267: 5699-5710.

Griffin, L., Rego, S., Correiro, E., Neto, C., Hart, P. 2005. *Induction of apoptosis in tumor cell lines by polyphenolic compounds isolated from Vaccinium macrocarpon.* American Society for Cell Biology, San Diego, CA.

Gringberg, M., Sarig, R., Zaltsman, Y., Frumkin, D., Grammatikakis, N., Reuveny, E., Gross, A. 2002. tBID homooligomerizes in the mitochondrial membrane to induce apoptosis. *J Bio Chem.* 277(14): 12237-12245.

Gross, A., McDonnell, J.M., Korsmeyer, S.J. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* 13: 1899-1911.

Guo, M., Perez, C., Wei, C., Rapoza, E., Gregory, S., Bou-Abdallh, F., Chasteen, N.D. 2007. Iron-binding properties of plant phenolics and cranberry's bio-effects. *Dalton.* 4951-4961.

Gurumurthy, S., Rangnekar, V.M. 2004. Par-4 inducible apoptosis in prostate cancer cells. *J Cell Biochem* 91: 504-512.

Gurumurthy, S., Goswami, A., Vasudevan, K.M., Rangnekar, V.M. 2005. Phosphorylation of Par-4 by protein kinase A is critical fro apoptosis. *Mol Cell Biol.* 25(3): 1146-1161

Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R., Bullock, P. 2004. Comparision of alamar blue and MTT assays for high through-put screening. *Toxicol In Vitro.* 18(5): 703-710.

Hanahan, D., Weinberg, R.A. 2000. The hallmarks of cancer. *Cell.* 100(1): 57-70.

Harris, C.C. 1996. p53 tumour suppressor gene: from the basic research laboratory to the clinic-an abridged historical perspective. *Carcinogenesis.* 17. 1187-1198.

Hockenbery, D.M., Zutter, M., Hickey, W., Nahm, M., Korsmeyer, S.J. 1991. BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA.* 88: 6961.

Howell, A., Vorsa, N., Der Marderosian, A., Foo, L. 1998. Inhibition of adherence of P-fimbricated *Escherischia coli* to uroepithelial-cell surfaces by proanthocyanidin extracts from cranberries. *N Engl J Med.* 339: 1085-1086.

Hui, L., Zheng, Y., Yan, Y., Bargonetti, J., Foster, D.A. 2006. Mutant p53 in MDA-MB-231 breast cancer cells is stabilized by elevated phospholipase D activity and contributes to survival signals generated by phospholipase D. *Oncogene.* 25: 7305-7310.

Huser, J., Rechenmacher, C.E., Blatter, L.A. 1998. Imaging the permeability pore transition in single mitochondria. *Biophys J.* 74: 2129-2137.

Hyun-Jeong, R., Parks, W.C. 2007. Control of matrix metalloproteinase catalytic activity. *Matrix Biol.* 26(8): 587-596.

IARC (International Agency for Research on Cancer). 2008. World Cancer Report.

Isaacs, W.B., Carter, B.S., Ewing, C.M. 1991. Wild-type p53 suppresses growth of human prostate cancer cells containing mutant p53 allelles. *Cancer Res.* 4716-4720.

Jeffery, E.H., Keck, A-S. 2008. Translating knowledge generated by epidemiological and *in vitro* studies into dietary cancer prevention. *Mol Nutr Food Res.* 52: S7-S17.

Jiang, X., Wang, X. 2000. Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem.* 275(40): 31199-31203.

Joe, A.K., Liu, H., Suzui, M., Vural, M.E. Xiao, D., Weinstein, I.B. 2002. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res.* 8(3): 893-903.

Jurgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., Reed, J.C. 1998. Bax directly induces release of cytochrome c from isolated mitochondria. *Proc Natl Acad Sci USA.* 95(9): 4997-5002.

Kahkonen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J., Pihlaja, K., Kujala, T.S., Heinonen, M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem.* 47: 3954-3962.

Kandil, F.E., Smith, M.A., Rogers, R.B., Pepin, M.F., Song, L.L., Pezzuto, J.M., Seigler, D.S. 2002. Composition of a chemopreventive proanthocyanidin-rich fraction from cranberry fruits responsible for the inhibition of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity. *J Agric Food Chem.* 36: 1023-1031.

Kao, G.D., Devine, P. 2000. Use of complementary health practices by prostate carcinoma patients undergoing radiation therapy. *Cancer.* 88: 615-9.

Kass, G.E., Eriksson, J.E., Weis, M., Orrenius, S., Chow, S.C. 1996. Chromatin condensation during apoptosis requires ATP. *Biochem J.* 318: 749-752.

Kaur, M., Agarwal, R., Agarwal, C. 2006. Grape seed extract induces anoikis and caspase-mediated apoptosis in human prostate carcinoma LNCaP cells: possible role of ataxia telangiectasia mutated – p53 activation. *Mol Cancer Ther.* 5(5): 1265-1274.

Kerr, J.F., Wyllie, A.H., Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* 26: 239-257

Kim, D.K., Baek, J.H., Kang, C.M., Yoo, M.A., Sung, J.W., Kim, D.K., Chung, H.Y., Kim, N.D., Choi, Y.H., Lee, S.H., Kim, K.W. 2000. Apoptotic activity of ursolic acid may correlate with the inhibition of initiation of DNA replication. *Int J Cancer.* 87(5): 629-636.

Kim, H., Hall, P., Smith, M., Kirk, M., Prasain, J.K., Barnes, S., Grubbs, C. 2004. Chemoprevention by grape seed extract and genistein in carcinogen-induced mammary cancer in rats is diet dependent. *J Nutr.* 134: 3445S-3452S.

Kim, H., Kong, H., Choi, B., Yang, Y., Kim, Y., Lim, M.J., Neckers, L., Yung, Y. 2005. Metabolic and pharmacological properties of rutin, a dietary quercetin glycoside, for treatment of inflammatory bowel disease. *Pharm Res.* 22: 1499-1509.

Kim, T-H., Zhao, Y., Ding, W-X., Shin, J.N., He, X., Seo, Y-W., Chen, J., Rabinowich, H., Amoscato, A.A., Yin, X-M. 2004. Bid-cardiolipin interaction at mitochondrial contact site contributes to mitochondrial cristae reorganization and cytochrome c release. *Mol Biol Cell.* 15: 3061-3072.

Kim, Y.A., Rhee, S.H., Park, K.Y., Choi, Y.H. 2003. Antiproliferative effect of resveratrol in human prostate carcinoma cells. *J Med Food.* 6(4): 273-280.

Kim, Y.L., Im, Y.J., Ha, N.C., Im, D.S. 2007. Albumin inhibits cytotoxic activity of lysophosphatidylcholine by direct binding. *Prostaglandins Other Lipid Mediat.* 83(1-2): 130-138.

APPENDICES

Appendix A

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

Early Black cranberry fruit was sampled in November of 2006 and stored at -20° C. In a blender, 1.060 kg of the Early Black cranberries were sequentially extracted with 200-300 mL portions of 40/40/19/1 methanol/acetone/water/ formic acid until the pulp was yellow. Berries were then sequentially extracted with 200-300 mL portions ethyl acetate until the filtrate was colorless. All filtrates were combined, rotovapped, and lyophilized. The crude extract was desugared from the elution of a 4.5 x 31cm diaion HP-20 column with distilled water. The extract was eluted with methanol and acetone, then rotovapped and lyophilized to dryness. The crude extract weighed about 10.5 g.

The different classes of flavonoids predominant in cranberries, including, but not limited to, phenolic acids anthocyanins, flavonols, and proanthocyanidins, were purified with a 3 x 22cm Sephadex LH-20 column. The sample (about half a gram at a time) was dissolved in a minimum amount of distilled water and was loaded on the column. The column was eluted with 70/30 methanol/water, from which the first fraction collected was the polar phenolics (yellow/orange), the second fraction was the anthocyanins (red), and the third fraction was the flavonols (yellow). When the

eluate was colorless, the column was washed with 70/30 acetone/water, from which the proanthocyanidins were collected (tan). Finally, the column was eluted with 100% acetone to collect any remaining nonpolar compounds from the column. Multiple fractions were obtained from the sephadex separation of about half gram batches and the fractions that were similar were combined. From about 500 mg of crude extract, about 30 mg of anthocyanins were obtained, as well as about 35 mg of flavonols, and 90-100 mg of proanthocyanidins.

All cranberry samples were analyzed with a Waters HPLC chromatograph equipped with an ultraviolet photodiode array detector and Millenium® software. The crude extracts and anthocyanin fractions were analyzed with a C18 (reversed phase) 4.6 x 250 mm Waters column. The program was a gradient elution. Solvent A was 4% aqueous acetic acid and solvent B was 4% acetic acid in methanol. The sample of crude extract for HPLC analysis was dissolved in 100% solvent A and run at 20mg/mL, and the sample of the anthocyanin fractions for HPLC analysis was also dissolved in 100% solvent A and run at 5mg/mL concentration. The linear gradient elution program was as follows, starting at 0 minutes with 99% solvent A (1% solvent B), gradient to 80% solvent A (20% solvent B) at 30 minutes, to 70% solvent A at 55 minutes, to 60% solvent A at 70 minutes, and finally to 100% solvent B from 80-90 minutes. The flow rate was 0.80 mL/min. The HPLC chromatogram for the crude extract is shown in Figure A.1 and that for the anthocyanin fraction is shown in Figure A.2. The four anthocyanin glycosides were quantified by as cyanidin-3-galactoside by standard curve method using the above program, measuring peak absorbance at 520 nm.

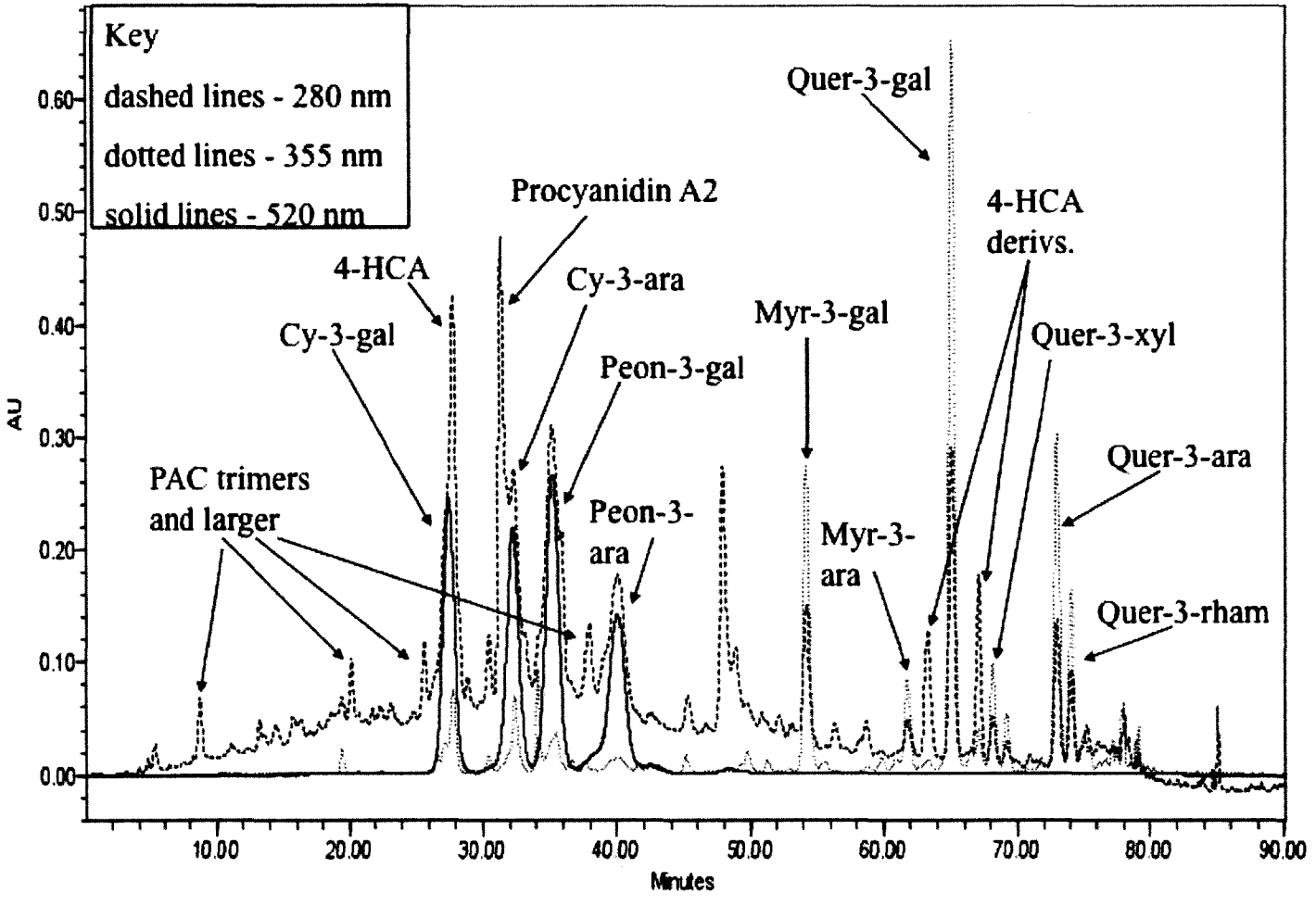

Figure A.1: HPLC analysis of Early Black cranberry (20 μg/ml)

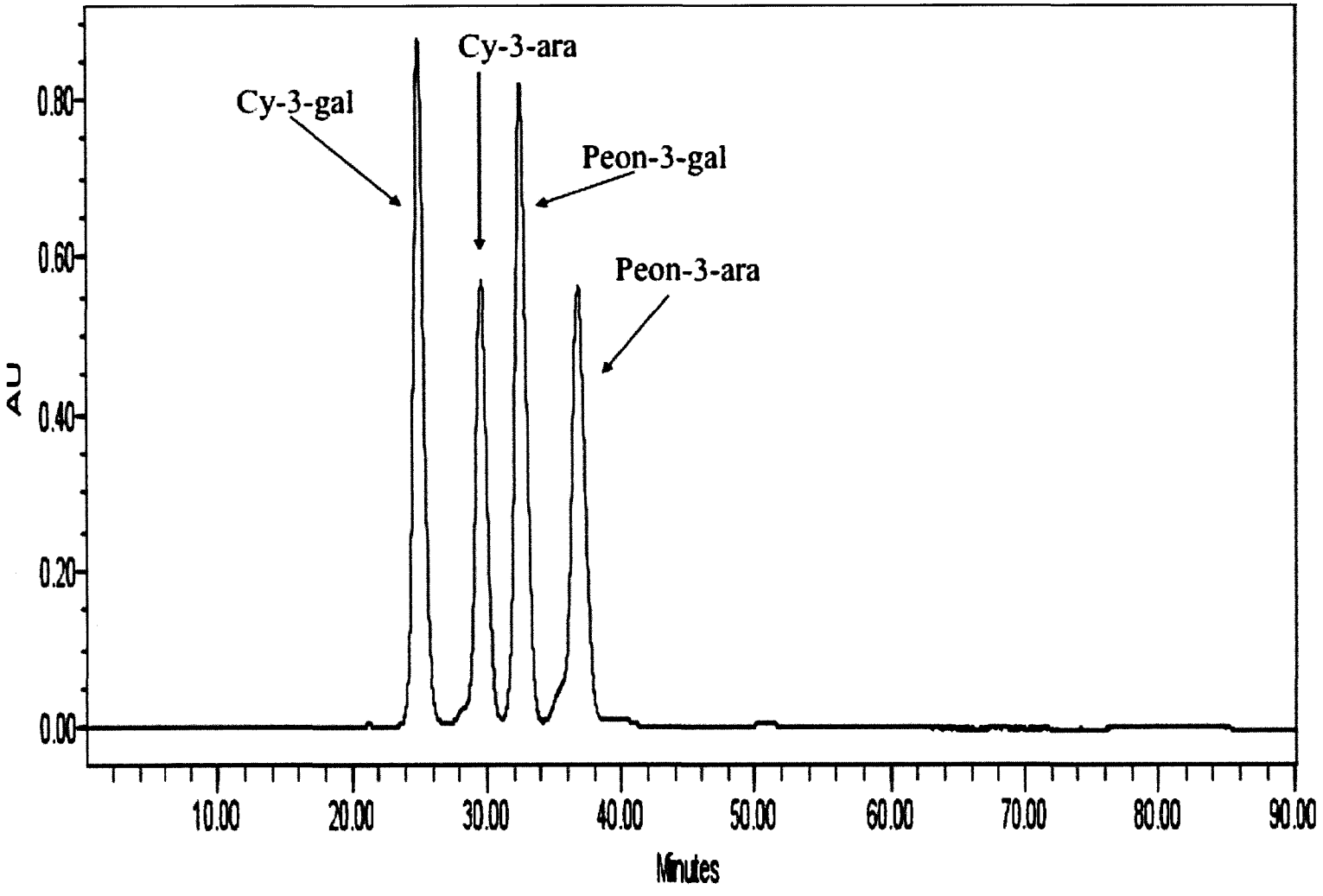

Figure A.2: HPLC analysis of Early Black anthocyanin fraction (520 nm).

The flavonol fractions were analyzed with a Waters C18 3.0 x 150 mm column. The HPLC program used a gradient elution. Solvent A was 2% aqueous acetic acid and solvent B was 2% acetic acid in methanol. The sample of the flavonol fractions for HPLC analysis was dissolved in 75/25 solvent A/solvent B and was run at 3 mg/mL concentration. The linear gradient was as follows, starting at 0 minutes with 95% solvent A (5% solvent B), gradient to 75% solvent A at 5 minutes, to 65% solvent A at 25 minutes, to 60% solvent A at 35 minutes, to 5% solvent A at 45 minutes. The HPLC chromatogram is shown in Figure A.3. Flavonol glycosides were quantified as quercetin-3-galactoside by the standard curve method using the above HPLC program, measuring peak absorbance at 355 nm.

The proanthocyanidin fractions were analyzed with an XTerra C8 4.6 x 250 mm column. The program used gradient elution. Solvent A was 2% aqueous acetic acid and solvent B was methanol. The sample of the proanthocyanidin fractions for HPLC analysis was dissolved in 50/50 solvent A/solvent B and was run at 10 mg/mL concentration. The gradient was as follows, at a flow rate of 0.8 mL/min, starting at 0 minutes with 100% solvent A, to 5 minutes with 100% A, 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 (absorbance maximum for epicatechin-based proanthocyanidins). The composition of the proanthocyanidin fraction was verified by MALDI-TOF MS analysis by Christan Krueger at the University of Wisconsin. The PAC fraction contains PACs ranging in size from 2-6 epicatechin units with at least one A-type linkage.

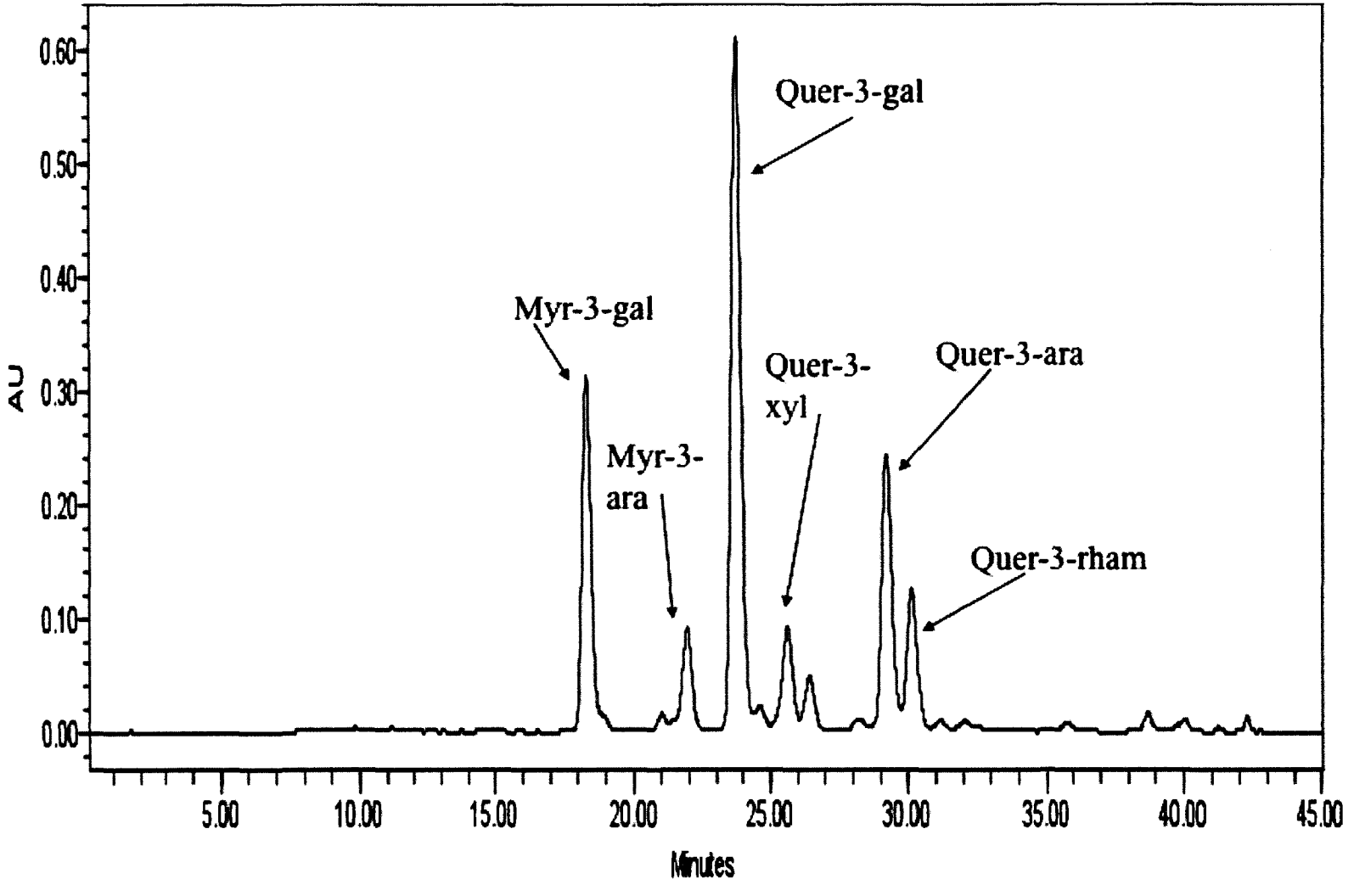

Figure A.3: HPLC analysis of Early Black flavonol fraction (355nm)

Appendix B

Aqueous extracts of pineapple weed (*Matricaria matricarioides*) increases the secretion of MMP-9 from H-*ras* transformed mouse fibroblasts.

B.1 Introduction

Matricaria discoidea DC (*M. matricarioides, M. suaveolens, Chamomilla suavelens*) is a ubiquitous urban weed that thrives in the worst environments, such as at the edges of sidewalks or other heavily traveled areas with poor quality, highly compacted soil. Growing 7.5 – 30 cm tall, the plant is topped with cone-shaped flower heads composed of densely packed yellow-green corollas. When crushed, the flowers have a rich pineapple/chamomile aroma. The flowers are edible, and can be used in salads and in the preparation of herbal tea. Pineapple weed has a number of traditional medicinal uses. When applied externally, the plant can soothe itching and wounds. When consumed as a tea, it acts to expel gas from the digestive tract and calm the nerves and stomach (Loomis *et al.*, 2004). Pineapple weed and wild chamomile are two of the most frequently used medicinal herbs in the Baltic countries and are traditionally used in the treatment of a variety of infectious inflammatory processes (Turi *et al.*, 1999).

There is little scientific literature available to describe the medicinal properties of pineapple weed. Several studies have been conducted, however, that

investigated the antimicrobial effects of aqueous extracts of pineapple weed flowers on *Escherichia coli.* Studies have shown that the antimicrobial properties of pineapple weed is not based on the ability of the medicinal to actually kill microbes or inhibit their growth (Ahn *et al.*, 1994), but are instead based on the ability of the plant to modify the organism's surface structures. Short exposures of *E. coli* strains to pineapple weed decrease the ability of the microbial cells to stick to each other and form conglomerates (aggregation). The aggregation ability of *E. coli* is directly correlated with its ability to bind to the receptors of host microorganisms (adhesion). This, in turn, correlates with the virulence of the *E. coli* strain (Turi *et al.*, 1999), indicating that pineapple weed attenuates the virulence of *E. coli.*

Despite the scant amount of research on the properties of pineapple weed, there has been a surprising amount of work done to quantify the chemical constituents of this plant. An Estonian study investigated the phenolic content of pineapple weed and reported that the quantity of total phenolics present in pineapple weed is approximately $1/5^{th}$ that of cranberry (*Vaccinium oxycoccus*). This study also indicated that the antioxidant activity of pineapple weed is likewise $1/5^{th}$ that of cranberry (Kohkonen *et al.*, 1999). This information must be taken with a grain of salt however; as a review of the literature indicates that the chemical composition of pineapple weed depends greatly on the site where it is grown (Arak *et al.*, 1988). Fortunately, the most recent work conducted into the chemical nature of *M. matricarioides* was conducted at Memorial University under the direction of Dr. Daneshtalab. Column chromatography of a methanol extract of pineapple weed yielded nine different compounds, which were subsequently analyzed by 2D-

Nuclear Magnetic Resonance spectroscopy. Amongst this group of compounds, two were identified as spiroethers, three as coumarins, two as cinnamic acid derivatives, one as a flavonoid, and the last was a diterpene (Loomis *et al.*, 2004).

This investigation focused on the potential of an aqueous extract of *M. matricariodies* to affect the secretion of matrix metalloproteinases (MMPs) from H-*ras* transformed mouse fibroblast cells (NR3s), and the activity of those secreted MMPs. The relevance of MMPs to processes such as invasion, metastasis and cancer progression is extensively reviewed in chapter 3, as are the mechanisms responsible for the regulation of MMP secretion and activity. It was hypothesized that *M. matricariodies* would prove able to inhibit the secretion of MMPs 2 and 9 from NR3 cells. It was furthermore hypothesized that decreased expression of EMMPRIN and increased TIMP-1/2 expression would account for the decreased gelatinolytic activity of MMPs 2 and 9 (as measured by gelatin zymography).

B.2 Materials and Methods

B.2.1 Materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Oakville, ON).

B.2.2 Preparation of the *Matricaria matricarioides* extract

An aqueous extract of pineapple weed (hereby referred to as pineapple weed tea [PAWT]) was prepared by boiling dried pineapple weed flowers in ultrapure water. Briefly, 12.0g of dried pineapple weed flowers were loaded into a tealeaf infuser. The infuser was placed into 150mL Millipore ultrapure water, which was brought to a rolling boil for 10 minutes. The tea was then allowed to cool to room temperature over 45 minutes, then filter sterilized though a Nalgene vacuum filter with a 0.2 μm pore size (VWR Canlab, Mississauga, ON).

B.2.3 Cell culture

H-*ras* transformed murine fibrosarcoma cells (NR3 cells) were used in this study. This cell line was produced at the Manitoba Institute of Cell Biology by stable overexpression of the H-*ras* gene in 10T1/2 cells (ATCC; Manassas, VA). These NR3 cells express low levels of H-*ras* and are capable of forming benign tumors in syngeneic mice (Egan *et al.*, 1988). NR3 cells were cultured on 20-mm Falcon plastic tissue-culture dishes (Falcon, Mississauga, ON) in alpha minimal essential medium (αMEM, Gibco, Burlington, ON) supplemented with 1% (vol/vol) antibiotic-antimycotic (Gibco) and 10 % (vol/vol) Fetal Clone III fetal bovine serum replacement (Hyclone/VWR Canlab, Mississauga, ON) at 37°C and 5% CO_2. Once cells reached a confluency of 70%, the previously described medium was removed and cells were grown in 5 mL of serum-free defined medium (DM). DM is composed of αMEM supplemented with 2.0μg/mL insulin and 4.0μg/mL transferrin. After 24 hours incubation, the DM was supplemented with PAWT. The PAWT dose is

described as the percent (vol/vol) of the supplemented DM that is composed of PAWT, with doses ranging from 0% (control) to 25%. Cells were exposed to pineapple weed-supplemented DM for 24 hours.

B.2.4 Alamar Blue assay

Please refer to Chapter 2 (section 2.2.4) for an overview of the Alamar blue assay protocol.

B.2.5 Zymography (gelatin-gel electrophoresis)

Please refer to Chapter 3 (section 3.2.4) for an overview of gelatin-gel electrophoresis.

B.2.6 Immuoblot analysis

Please refer to Chapter 2, section 2.2.8 (Immunoblot analysis) for a detailed overview of the Western immunoblot analysis protocol.

B.2.7 Image capture and gel quantification

Please refer to Chapter 2, section 2.2.9 (Image capture and gel quantification) for a detailed overview of these methods.

B.3 Results

B.3.1 Aqueous extracts of pineapple weed do not exert notable cytotoxic effects against NR3 cells.

The potential effects of pineapple weed on the viability of NR3 cells were evaluated by the Alamar Blue assay. Such an evaluation was required to ensure that cytotoxicity wasn't a complicating factor in any observed effects of PAWT treatment on the secretion of MMPs from NR3 cells. The Alamar Blue assay revealed that treating NR3 cells with pineapple weed for 24 hours had a negligible effect on cell viability (Figure B.1). Cytotoxicity was only observed upon treatment with 25% (vol/vol in cell culture medium) PAWT in serum-free conditions. The data presented in Figure B.1 was obtained from three independent experiments, with four assay replicates in each experiment.

B.3.2 Aqueous extracts of pineapple weed regulate MMP secretion from NR3 cells and the activity of secreted MMPs.

Two different experiments were conducted in order to evaluate the effects of PAWT on the secretion of MMPs from NR3 cells and the activities of those secreted MMPs. In the first experiment, NR3 cells were treated for 24 hours with increasing amounts of PAWT. Aliquots of serum-free defined cell culture medium was supplemented either with aqueous extract of *M. matricariodies*, distilled water, or a combination of PAWT and water such that the final volume of PAWT in each aliquot

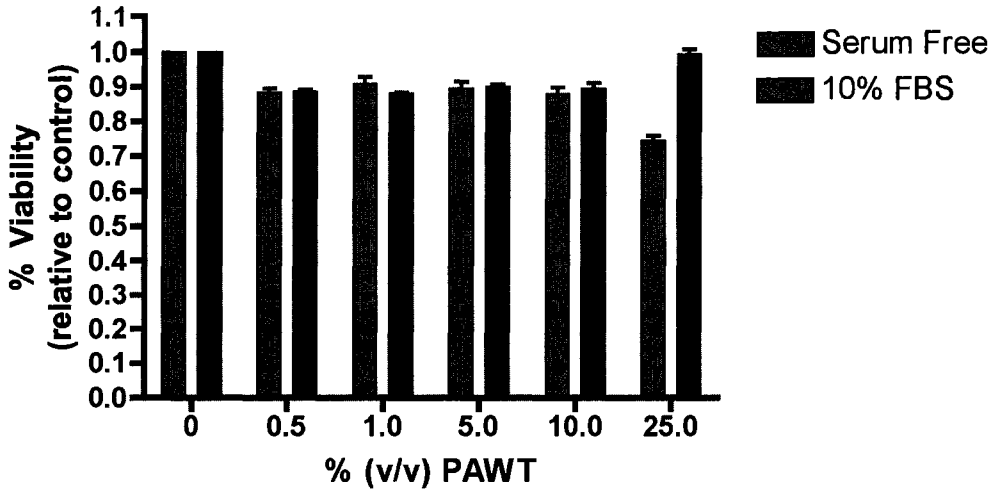

Figure B.1: Treating NR3 cells with increasing doses of an aqueous extract of *M. matricariodies* has little adverse effect on NR3 cell viability (as measured by the Alamar Blue assay). Some cytotoxicity is seen at the highest tested dose (25% [vol/vol]), though only in serum-free medium. Results from three independent experiments, with four assay replicates for each biological replicate.

ranged from 0% to 25% (vol/vol; 0%, 1%, 5%, 10%, 25%). Medium was supplemented with PAWT and water to ensure that the cell culture medium in each treatment was equally "diluted" with water, such that the observed effects could be attributed solely to *M. matricariodies* phytochemicals, rather than water. Following a 24-hour treatment with PAWT-conditioned cell culture medium, the medium was collected and analyzed by gelatin zymography. It was revealed that PAWT treatment caused a notable dose-dependent increase in the secretion of MMP-9, and an increase in the secretion of pro-MMP-2 and MMP-2 at lower concentrations (1%-10%), followed by a decrease to basal levels at the highest concentration (25%) (Figure B.2). This data was attained from three independent experiments, with four assay replicates for each biological replicate.

A second experiment was conducted to determine whether aqueous extracts of *M. matricariodies* were capable of affecting the activity of MMPs that had already been secreted by NR3 cells. NR3 cells were seeded at $5x10^5$ cells/dish and following a 24-hour incubation, cell culture medium was replaced with serum-free defined medium. NR3 cells were allowed to produce MMPs for 24 hours, at which point cell culture medium was removed. This medium was then aliquoted into vials, and supplemented with mixtures of PAWT and distilled water such that the final volume of each NR3-conditioned medium aliquot was equal and the "doses" of PAWT ranged from 0-25% (vol/vol; 0%, 1%, 5%, 10%, 25%). Cell culture medium was incubated at 37 °C for 3 hours, and was then subjected to gelatin zymography, which revealed a dramatic dose-dependent decrease in the activity of secreted MMP-9 at 5% and higher doses (Figure B.3). This trend is the opposite of that seen when NR3 cells are

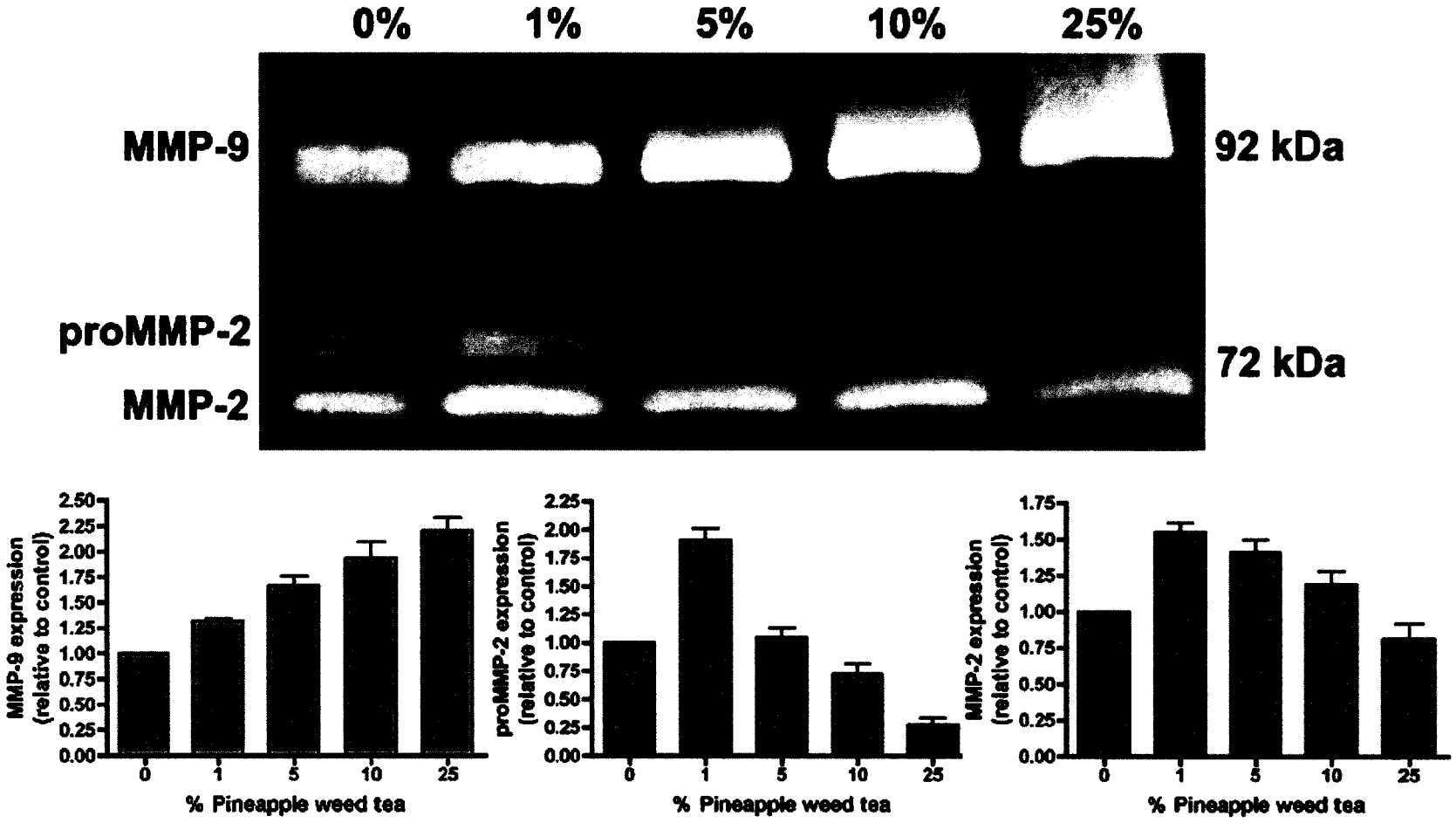

Figure B.2: Gelatin-gel electrophoresis (zymography) revealed that treating NR3 cells with increasing doses of pineapple weed caused a dose-dependent increase in the secretion of MMP-9. An increase in proMMP-2 and MMP-2 secretion is also seen at the lowest tested doses, decreasing to basal levels at the higher doses. Results from three independent experiments, with three assay replicates for each experiment.

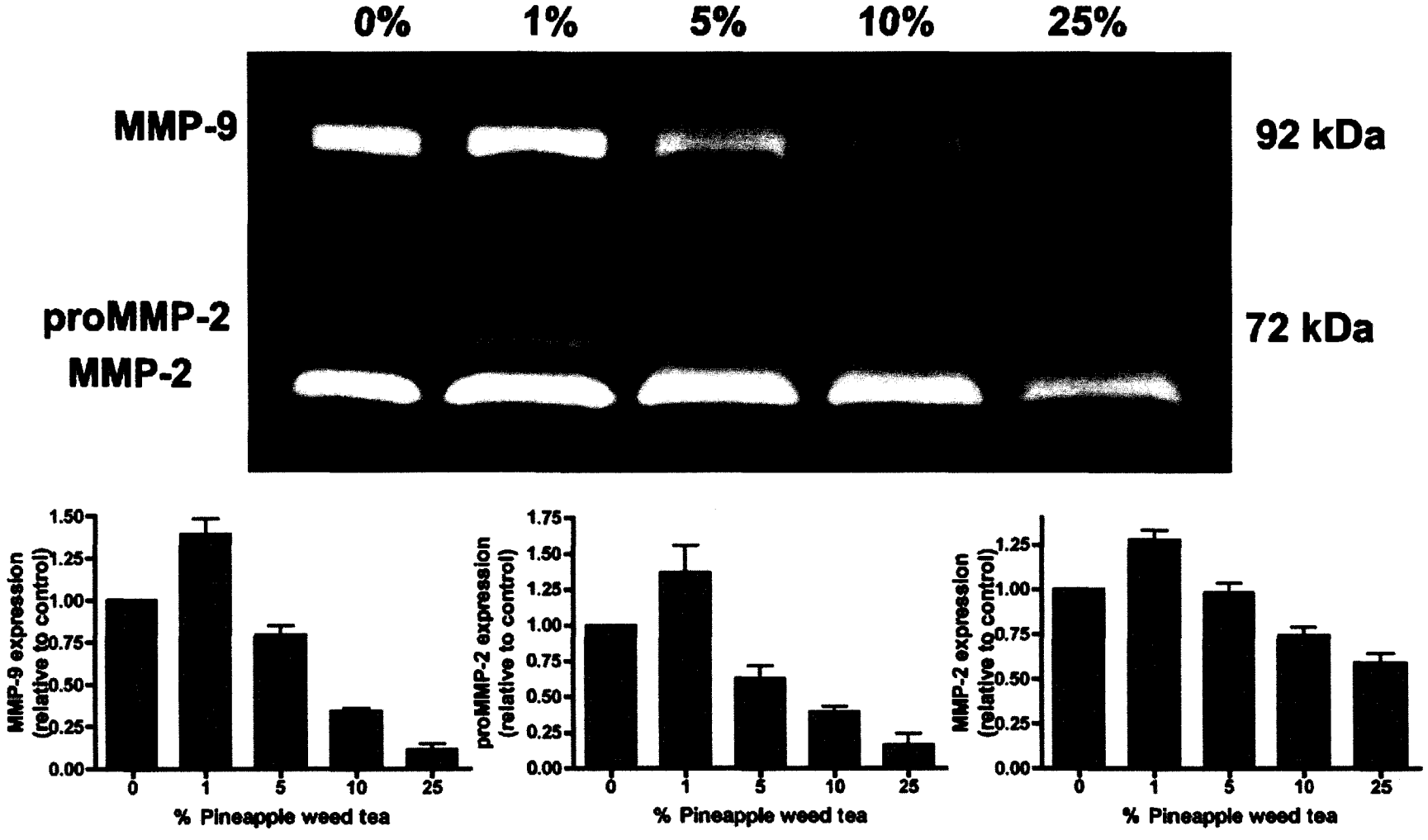

Figure B.3: Increasing doses of pineapple weed decreases the gelatinolytic activity of MMP-9 secreted from NR3 cells (in a cell-free system). The overall trend in proMMP-2 and MMP-2 activity is very similar to that seen when NR3 cells are treated with pineapple weed tea. Results are from three independent experiments (three assay replicates for each experiment).

treated with PAWT for 24 hours (where an increase in secretion is observed; Figure B.2). It thus appears that the observed increase in MMP-9 secretion from PAWT-treated NR3 cells is more dramatic than initially thought, given the observation that PAWT also inhibits the gelatinolytic activity of MMP-9. Treating NR3-conditioned medium with PAWT causes a slight increase in the gelatinolytic activities of proMMP-2 and MMP-2 at lower concentrations (1%, 5%), and a slight decrease in gelatinolytic activities at 10% and 25% (vol/vol). This trend is similar to that seen when NR3 cells are treated with PAWT. It would thus appear that PAWT treatment exerts little to no effect on the secretion of MMP-2, as the observed effects can be attributed to alterations to the gelatinolytic activity of MMP-2.

B.3.3 Treating NR3 cells with aqueous extracts of *M. matricariodies* alters the expression of MMP-regulating proteins EMMPRIN, TIMP-1, TIMP-2.

The potential of *M. matricariodies* to regulate the expression of key MMP-regulating enzymes was evaluated in NR3 cells to attempt to elucidate the mechanisms whereby treatment increases the secretion of MMP-9 from NR3 cells. The first target of this investigation was EMMPRIN (extracellular matrix metalloproteinase inducer), an enzyme found on the cell surface of tumour cells known to stimulate the production and secretion of MMP-9 from nearby tumour and stromal cells. Given the observed increase in MMP-9 secretion, it was hypothesized that treating NR3 cells with PAWT would cause a dose- and time-dependent increase in the protein expression of EMMPRIN, a hypothesis that was confirmed by western

blot analysis. The protein expression of EMMPRIN increases at the lowest tested dose of PAWT (0.5% vol/vol, 24 hours) and increases at all higher tested concentrations (up to 25% vol/vol, 24 hours) (Figure B.4).

As discussed in Chapter 3, there are many mechanisms whereby the activity of MMPs can be regulated. One such mechanism is the reversible inhibition of MMP activities through direct binding to the tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 preferably binds and inhibits MMP-9, while TIMP-2 has a stronger affinity for MMP-2. TIMP-2 also works in concert with MT1-MMP to catalyze the proteolytic cleavage of proMMP-2 to fully active MMP-2 (Cao *et al.*, 1996; Hyun-Jeong and Parks, 2007). Treating NR3 cells with increasing doses of PAWT for 24 hours revealed no clear trends in TIMP-1 expression, excepting a possible slight decrease in expression (Figure B.4B). TIMP-2 expression increases in response to PAWT treatment, increasing to a ~2.5 fold induction over control levels at 1% PAWT and gradually decreasing to baseline levels upon treatment with higher concentrations of PAWT (Figure B.4C).

B.4 Discussion

The data presented in this appendix clearly demonstrates that treating NR3 cells with an aqueous extract of *M. matricarioides* causes a dose-dependent increase in MMP-9 secretion (as measured by gelatin zymography [Figure B.2]). The observation that, in a cell-free system, pineapple weed is able to inhibit the gelatinolytic activity of MMP-9 (Figure B.3) suggests that the observed increase in

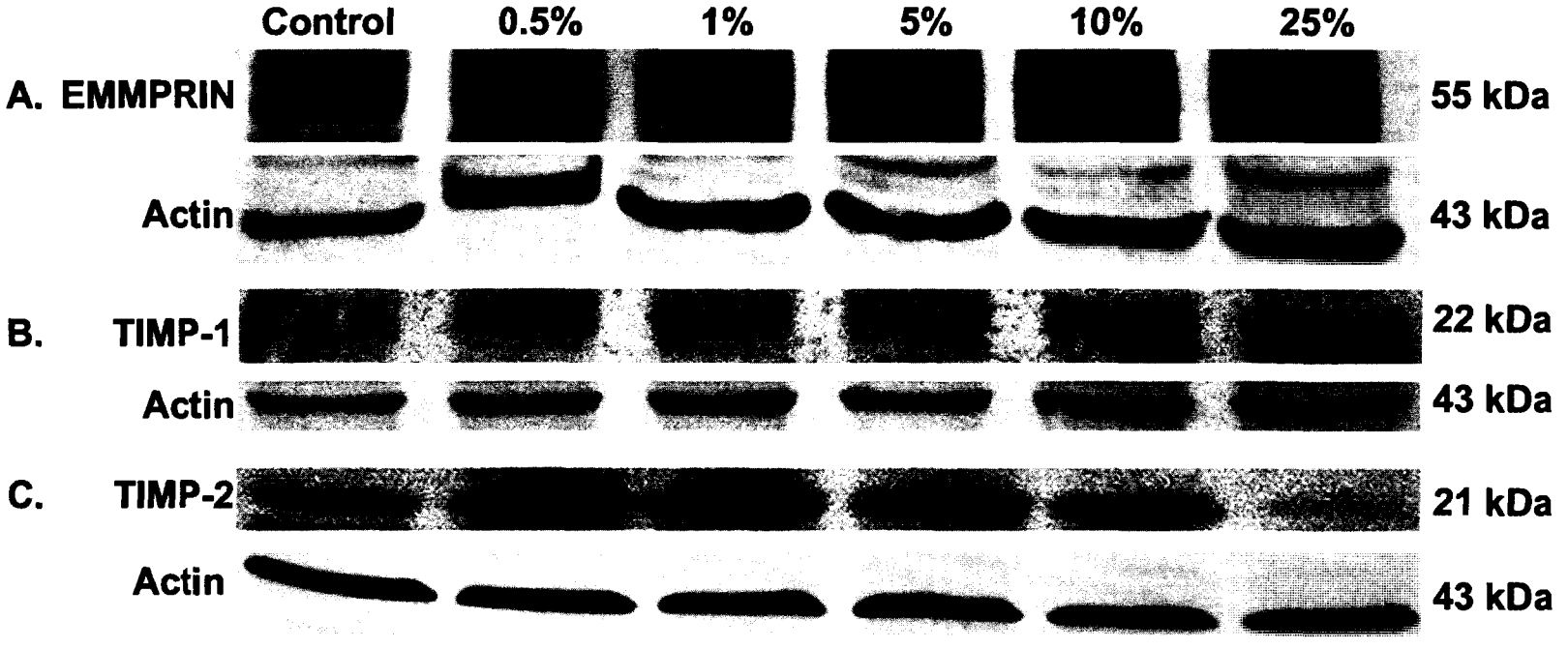

Figure B.4: Western blot analysis of pineapple weed-treated NR3 cells (24 hours) for EMMPRIN (A), TIMP-1 (B), and TIMP-2 (C). Treating NR3 cells with aqueous extracts of *M. matricariodies* causes a dose-dependent increase in the protein expression of EMMPRIN (A), though has no effect on the protein expression of TIMP-1 (B). The increase in TIMP-2 expression (C) reaches a maximum at 0.5% and 1% (vol/vol) PAWT, though remains higher than basal levels up to the 10% (vol/vol) dose. Western blots are representative of those seen in two independent experiments (three-four assay replicates for each experiment).

MMP-9 secretion is even more dramatic than that suggested by Figure B.2. The concurrent increase in secretion and inhibition of activity is quite interesting, though remains unexplained. As shown in Figure B.2 and B.3, it appears that the changes in proMMP-2 and MMP-2 secretion (Figure B.2) can be explained solely by the direct effect of pineapple weed on the gelatinolytic activity of already secreted proMMP-2 and MMP-2 (Figure B.3) (as the trends are almost identical). It thus appears that pineapple weed doesn't alter the secretion of either MMP-2 or its zymogen, but is able to increase the gelatinolytic activity of these proteases at low concentrations, with activity decreasing to basal levels as pineapple weed doses increase.

While no attempt was made to explore the direct regulation of MMP activity (cell free system), the protein expression of three key MMP-regulating proteins were evaluated to attempt to explain the changes in MMP secretion in response to pineapple weed treatment. The dramatic increase in MMP-9 secretion appears to be related to the increase in EMMPRIN protein expression, as EMMPRIN induces MMP-9 secretion from nearby tumour cells.

In comparing the two zymography-based experiments, it was determined that there was little effect of pineapple weed treatment on the secretion of proMMP-2 and MMP-2 from NR3 cells. That stated, the effect of pineapple weed treatment on TIMP-2 suggests a potential mechanism whereby pineapple weed *may* regulate the secretion of MMP-2. In addition to inhibiting active MMP-2 through reversible binding, TIMP-2 can interact with MT1-MMP to catalyze the activation of MMP-2

from its zymogen from. That the change in TIMP-2 expression in response to pineapple weed tea is quite similar to that of MMP-2 suggests that pineapple weed *may* be regulating the activation of MMP-2 via TIMP-2 upregulation.

What components of *Matricaria matricarioides* are responsible for the regulation of MMP activity? This question is beyond the scope of this project, but a review of the literature provides some compelling insights. The chemical constituents of pineapple weed have been elucidated through extensive chemical analysis (Loomis *et al.*, 2004; Ma *et al.*, 2007). Umbelliferone, one of the phytochemicals found in *M. maricariodies* has been previously shown to inhibit the activity of MMP-2 (Ngameni *et al.*, 2006). Luteolin-7-glucoside, a flavonoid isolated from *M. matricarioides*, was recently reported to have inhibitory effects on both MMPs 2 and 9 (Ende and Gebhardt, 2004). Both umbelliferone and luteolin-7-glucoside are potential phytochemicals responsible for pineapple weed-mediated regulation of MMP activity.

A few specific changes to the experimental design of this project may help provide more compelling results in future work. First, higher quality extracts of pineapple weed should be prepared for treatments. Without any background in natural products chemistry, this researcher was unable to prepare anything more elaborate than a simple tea. Supercritical fluid extraction (SFE) of dried pineapple weed flowers would yield a more concentrated and potent pineapple weed treatment. A more regimented extraction protocol would also improve the degree of reproducibility between trials.

This study clearly illustrated that the effects of pineapple weed on MMP activity are quite varied. The observation that PAWT simultaneously increases MMP-9 secretion and inhibits MMP-9 activity makes a strong case for fractionation, as it is likely that different chemical constituents are responsible for this phenomenon, as well as the disparate regulation of MMP 2 and 9. Fractionation of PAWT will certainly clarify which chemical components are responsible for which observed bioactivities.