

2014

ANTICANCER ACTIVITY OF NATURAL
HEALTH PRODUCTS (DANDELION ROOT
& LONG PEPPER EXTRACTS); EXTENSIVE
STUDY OF EFFICACY AND MECHANISM
OF ACTION

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**ANTICANCER ACTIVITY OF NATURAL HEALTH PRODUCTS
(DANDELION ROOT & LONG PEPPER EXTRACTS);
EXTENSIVE STUDY OF EFFICACY AND MECHANISM OF
ACTION**

By

PAMELA OVADJE

A Dissertation
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor
Windsor, Ontario, Canada
2014

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Anticancer Activity of Natural Health Products (Dandelion Root & Long Pepper Extracts);
Extensive Study of the Efficacy and Mechanism of Action

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DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATION

I. DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates materials that are a result of joint research. This thesis includes work carried out in collaboration with Dr. Caroline Hamm, Dr. Jose-Antonio Guerrero and Dr. John Thor Arnason, under the supervision of Dr. Siyaram Pandey.

The collaboration with Dr. Hamm is covered in chapters 2 – 6

The collaboration with Dr. Guerrero and Dr. Arnason are covered in chapters 5 – 6.

In all cases, the experimental design, execution, data analysis, interpretation and manuscript preparation were performed by the author and all listed authors read and approved the final manuscript, prior to submission.

Contributions from no-authors came in form of proofreading manuscripts, and this was covered in the acknowledgements sections of the published work, where appropriate.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis.

I certify that, with the above qualification, this thesis and the research to which it signifies, is the product of my own work.

II. DECLARATION OF PREVIOUS PUBLICATIONS

This thesis incorporates five original papers that have been published or submitted for publication in peer-reviewed journals, listed below;

Thesis Chapter	Publication Title/Full Citation	Publication Status
Chapter 2	Selective Induction of Apoptosis Through Activation of Caspase-8 In Human Leukemia Cells (Jurkat) By Dandelion Root Extract. <i>Journal of Ethnopharmacology</i> , 133 (1): 86-91	<i>Published</i>
Chapter 3	Selective Induction Of Apoptosis And Autophagy Through Treatment With Dandelion Root Extract In Human Pancreatic Cancer Cells. <i>Pancreas</i> , 41 (7): 1039-1046	<i>Published</i>
Chapter 4	Efficient Induction Of Extrinsic Cell Death By Dandelion Root Extract In Human Chronic Myelomonocytic Leukemia (CMML) Cells, <i>PloS one</i> , 7 (2): e30604	<i>Published</i>
Chapter 5	Fractionation, Phytochemical & Gene Expression Analysis of the Selective Anticancer Activity of Dandelion Root Extract in Human Cancer Cells	<i>In Progress</i>
Chapter 6	Evaluation Of The Efficacy & Biochemical Mechanism Of Cell Death Induction By Long Pepper Extract Selectively In <i>In-Vitro</i> And <i>In-Vivo</i> Models Of Human Cancer Cells. <i>PloS one</i> , MS#: PONE-D-14-20706	<i>Under Revision</i>

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ABSTRACT

The incidence of cancer worldwide is continuously on the rise, and the death toll associated with cancer, is constantly increasing. Available cancer therapy is unable to combat this ever-changing disease, and the severe side effects associated with various forms of therapy, indicate a serious need for the development of more effective and safer alternatives to currently available treatment.

The use of natural health products in disease treatment has contributed to the development of over 75% of available chemotherapy. In this thesis, we study the potential anti-cancer effect of two natural health products, dandelion root and long pepper extracts.

The major objectives of this work were to;

- Evaluate the efficacy and mechanism of these extracts in various cancer models,
- Assess any potential safety and toxicity issues associated with the use of these extracts and,
- De-convolute and identify the pharmacologically active components that contribute to the anti-cancer activity of these extract

Using standard biochemical and morphological assays, the induction of various programmed cell death processes was assessed, following treatment in cancer and non-cancer cell models, as well as in animal models.

The results obtained indicate that dandelion root and long pepper extracts were efficacious in selectively inducing apoptosis and pro-death autophagy in various

cancer cell models. Dandelion root extract rapidly activated the extrinsic pathway of apoptosis, a situation that was crucial to apoptosis induction in leukemia cells, but not required for colorectal cancer cells. Gene expression analysis showed that dandelion root extract efficiently targets multiple pathways to promote its anti-cancer activity.

The generation of reactive oxygen species by long pepper extract treatment, appeared to be partially responsible for the induction of apoptosis in cancer cells. Furthermore, the lack of toxicity observed in animal models, on oral administration regimens of dandelion root and long pepper extracts, further confirm the safety of these extracts. Interestingly, the same regimen of oral administration of these extracts was successful in halting the growth of colon tumors in xenograft models.

These findings provide scientific validation concerning the safe and effective use of natural health products as non-toxic and potentially more efficacious forms of therapy.

DEDICATION

This work is dedicated to the memory of Mr. Kevin Couvillon, whose fight with Leukemia ended in 2010. You are forever in our hearts and continually inspire us to work harder in the fight against cancer.



ACKNOWLEDGEMENTS

With much gratitude, I would like to acknowledge the efforts of my supervisor, Dr. Siyaram Pandey, for his continued guidance, support and mentorship for the duration of my studies. His enthusiasm and willingness to share his extensive knowledge is always inspiring. His love for mangoes (and other fruits) is a testament to his love for keeping the Pandey Lab healthy.

I would also like to acknowledge my committee members, Dr. Michael Boffa, Dr. Otis Vacratsis and Dr. Andrew Swan for all the help and input over the years. Furthermore, I would like to extend my gratitude to all the members of the Chemistry & Biochemistry department, who have made the last few years memorable.

To all the members of the Pandey lab, both past and present, I would like to thank you all for all the support, advices, laughs and frustrating times. And to my collaborators, thank you for all the advice and support. All of this support was essential to the successful completion of this work and I am forever grateful.

The support from the community, local and national, has been tremendous and has increased motivation for the continuous progression of this work; the Knights of Columbus, Chapter 9671, led the charter and was instrumental in the funding the commencing experiments for this project and their continued support over the years has been extremely motivating. I would especially like to acknowledge the contributions from the Couvillon family, in memory of Mr. Kevin Couvillon, who lost his battle with leukemia in 2010. The contributions and continuous support from this family has encouraged further community awareness for this project.

The contributions of P. Drew and her family members, as well as those of other community members are awe-inspiring and have continuously provided further motivation for this work.

Further funding for this project was provided in part by The Pajama Angels, The Knights of Columbus, Chapter 9671, Seeds4Hope Foundation, Lotte & John Hecht Memorial Foundation and the Jesse & Julie Rasch Foundation.

Most importantly, I would like to thank my family and friends for their continuous support and encouragement, without which, I would not have achieved any of these accomplishments.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
3-PG	3-Phosphoglycerate
ADEs	Adverse Drug Events
ADEPT	Antibody-Directed Enzyme-Prodrug Therapy
AIF	Apoptosis Inducing Factor
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
APAF1	Apoptotic Protease Activating Factor 1
ATG	AuTophagy related proteins
BAX	BCL-2 Associated X-Protein
BAK	BCL-2 Antagonist/Killer
Bcl-2	B-cell Lymphoma
BECN1	Beclin-1
BID	BH3 Interacting domain Death Agonist
BSA	Bovine Serum Albumin
CAD	Caspase Activating DNase
CAM	Complementary & Alternative Molecules
CAMs	Cell-Adhesion Molecules
CARD	Caspase Recruitment Domain
CCNSC	Cancer Chemotherapy National Service Center
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myelogenous Leukemia
CMMML	Chronic Myelomonocytic Leukemia
CON-A	Concanavalin-A
COX-2	Cyclooxygenase-2
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DNA	Deoxyribonucleic Acid
DFE	Dandelion Flower Extract
DRE	Dandelion Root Extract
EDTA	Ethylenediaminetetraacetic acid
ENDO-G	Endonuclease G
EGFR	Epidermal Growth Factor Receptor
FADD	Fas-Associated Death Domain
FAS	Fatty Acid Synthetase
FBS	Fetal Bovine Serum
FDA	Food & Drug Administration
FLICE	FADD-like interleukin-1 β -converting enzyme

FLIP	FLICE Inhibitory Protein
GIT	Gastrointestinal Tract
HIF	Hypoxia Inducible Factor
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
IAP	Inhibitors of Apoptosis Protein
LAMP1	Lysosomal-associated membrane protein 1
LC3	Microtubule-associated protein 1 light chain 3
LDHA	Lactate Dehydrogenase A
MDC	Monodansylcadaverine
MDR	Multidrug Resistance
MDS	Myelodysplastic Syndrome
MMP	Matrix Metalloproteinases
MPD	Myeloproliferative Diseases
mTOR	Mammalian Target of Rapamycin
NAC	N-acetylcysteine
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
NF-KB	Nuclear Factor kappa B
NFF	Normal Fetal Fibroblasts
NHF	Normal Human Fibroblasts
NHP	Natural Health Product
NHPD	Natural Health Product Directorate
NO	Nitrogen Oxidase
NPs	Natural Products
NSCLC	Non-Small Cell Lung Carcinoma
OMI/HtrA2	HtrA2 Serine Peptidase 2
OXPHOS	Oxidative Phosphorylation
PARP	Poly (ADP-ribose) Polymerase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3-kinase
PIP3	phosphorylated phosphatidylinositol
PL	Piperlongumine
PLX	Long Pepper Extract

PMSF	Phenylmethanesulfonyl fluoride
PQ	Paraquat
PS	Phosphatidylserine
PTEN	Phosphate Tensin homolog 10
REB	Research Ethics Board
RFU	Relative Fluorescence Units
Rb	Retinoblastoma
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SAIs	Single-Active Ingredients
SDHA	Succinate Dehydrogenase A
SLs	Sesquiterpene Lactones
SMAC/DIABLO	
SQSTM-1	Sequestosome 1
TAM	Tamoxifen
TBST	Tris Buffered Saline with Tween
TCA	Tricarboxylic Acid cycle
TCM	Traditional Chinese Medicine
TMRM	Tetramethylrhodamine Methyl Ester
TMS	Tetramethylsilane
TNF	Tumor Necrosis Factor
TPD	Therapeutic Product Directorate
TRADD	TNF Receptor type1 Associated Death Domain
TRAIL	TNF-related Apoptosis Inducing Ligand
TrkB	Tropomyosin related kinase B
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
QoL	Quality of Life
UPLC	Ultra performance liquid chromatography
WHO	World Health Organization
WRCC	Windsor Regional Cancer Centre
WST-1	Water Soluble Tetrazolium salt
VDAC	Voltage Dependent Anion Channel
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

GENERAL INTRODUCTION

CANCER

Cancer is mainly characterized a disease characterized by uncontrolled cell growth and proliferation, however, it is a term that describes over a hundred forms the disease, as almost every body tissue is capable of developing tumors (Weinberg, 1996). Cancer arises from one cell, usually initiated by a program of inappropriate cell division, with cells that have accumulated multiple mutations, specifically in classes of genes (proto-oncogenes and tumor suppressors), usually meant to control the cell growth and division processes; for instance mutations in proto-oncogenes encourage their conversion to overly active oncogenes, which drive excessive growth and multiplication, while mutations in tumor suppressors are inactivating mutations, preventing their ability to halt excessive growth and multiplication of cells with mutations. These mutations result in uncontrolled cell proliferation, the very definition of cancer, and as these cells continually divide, they accumulate more mutations that confer further proliferative advantages, as well as safeguards against growth suppression (Weinberg, 1996; Hanahan & Weinberg, 2000). This thesis will focus on targeting some of the major “hallmarks” of cancer cells that enhance their proliferative signaling, especially in leukemia, pancreatic cancers and colorectal cancers.

According to the World Health Organization, in 2012, cancer led the charge as the second leading cause of death worldwide, with 8.2 million cancer-related deaths in that year alone, a rise from 2008, which saw 7.6 million deaths (Jemal et al., 2011; Siegel et al., 2014). Canada alone saw a 3700 increase in the incidence of cancer from 2013 to 2014; in 2013, there were 187,600 new cases versus 191,300 in 2014. Along with this increase in the incidence of cancer, a

corresponding increase in cancer related deaths were observed, with 76,600 deaths estimated in 2014, compared to 75,500 deaths in 2013 (Statistics Canada, 2013; Statistics Canada, 2014).

Not only is cancer a personal burden (on patients and loved ones), it also has major economic consequences. The costs associated with cancer care, including healthcare costs (physicians and hospital expenses) as well as costs associated with lost productivity and premature death, have steadily increased over the years and due to the rising increase in cancer incidence and related death, these costs are predicted to continue to rise (Statistics Canada, 2014). This begs the dire need to develop non-toxic, preventative and therapeutic regimens for cancer.

HALLMARKS OF CANCER

The hurdles associated with finding an effective mode of chemotherapy are generally caused by the acquired capabilities that define a cancer cell. These hallmarks are essential to cancer cell growth and survival and enable cancer cells evade routine safeguard mechanisms and therapeutic agents. The multistep theory governing the process of tumorigenesis reflects the genetic alterations that promote progressive transformation of normal human cells into their malignant counterparts and the hallmarks of cancer can be associated with each step (Hanahan & Weinberg, 2000). In 2000, Hanahan and Weinberg outlined the basic hallmarks that are associated with cancer cell survival and this list was modified in later years (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). The hallmarks are outlined below and in figure 1. They include;

- i. The ability to sustain growth signals by generating many oncogenes, which act as growth signals and remove dependence on normal cell signaling, as observed in the up-regulation of growth factors and growth factor receptors (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).
- ii. The ability to evade growth suppressive signals, for instance, in the disruption of the retinoblastoma (Rb) signaling pathway, which allows the liberation of E2F to promote cell proliferation and render cancer cells insensitive to anti-growth stimuli (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).
- iii. The ability to metastasize and invade surrounding tissues, allowing tumor cells escape primary site to a secondary site, where they can take up residence. This is usually due to altered protein functions of proteins like the cell - cell adhesion molecules (CAMs), which are involved in securing cells to their surroundings (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). Processes like anoikis, a form of cell death caused by loss of contact with the extracellular matrix and/or neighboring cells, are usually down-regulated in cancer cells, due to up-regulation of proteins, such as TrkB (Liotta & Kohn, 2004). Such alterations provide cancer cells with the ability to metastasize from their primary location to a distant site, without the accompanying cell death.
- iv. The ability of cancer cells to maintain replicative signaling, usually as a result of up-regulating the expression of the telomerase enzyme, so as to inhibit telomere shortening and maintain telomere length (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

- v. The ability to develop new blood vessels (angiogenesis), which allows for the continuous supply of oxygen and nutrients, required for the survival of tumor cells. Angiogenesis signals include the up-regulation of proteins like the vascular endothelial growth factor (VEGF) (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).
- vi. The ability to bypass cell death signal, such as apoptosis, anoikis and autophagic cell death. This is usually observed in collaboration with the overexpression of pro-survival proteins, such as the pro-survival members of the Bcl-2 family (Frankin & McCubrey, 2000; Ghobrial et al., 2005).

Further research into the hallmarks of cancer cells that provide growth advantage led to the introduction of new enabling characteristics and additional hallmarks (Figure 1). These emerging hallmarks have been shown to be involved in the development and progression of some, perhaps all cancers. The first newly emerging hallmark is one that involves the ability of cancer cells to reprogram their cellular metabolism, by deregulating cellular energetics, in order to continuously promote cell proliferation and the other allows cancer cells evade immune destruction, where the T and B lymphocytes, as well as macrophages and natural killer cells would normally identify cancer cells and target them for destruction. These hallmarks are still under investigation but however, are supported by the emerging characteristics of genomic instability (which allows multiple genetic alterations drive tumor progression) and inflammatory response by innate immune cells, normally designed to fight infections and heal wounds. These emerging characteristics and hallmarks therefore supports the previously

described hallmarks of cancer cells and have proven to promote tumor progression (Hanahan and Weinberg, 2011).

CANCER METABOLISM

Originally thought to be the root cause of carcinogenesis, the “Warburg effect” has gained a lot of notoriety, as a key metabolic hallmark of cancer cells (Warburg, 1956; Hsu & Sabatini, 2008). The Warburg effect focuses on the ability of cancer cells to bypass regular mitochondrial oxidative phosphorylation (OXPHOS) for less favorable energy production by aerobic glycolysis (Warburg, 1956). Several studies have further explored the various aspects of cancer cell metabolism and how it affects cancer cell proliferation and survival. Generally, proliferating cancer cells have two main metabolic challenges to overcome; the “challenge of meeting the bioenergetics and biosynthetic demands of increased cell proliferation” and the “challenge of surviving environmental fluctuations in nutrients and oxygen availability” when tumor growth exceeds the delivery capacities of existing structures. Evidence has shown that many mutated genes, through oncogenic activation, loss of tumor suppression function, as well as altered cellular metabolism, lead to the induction of the Warburg effect and all of these mutations combined, are intricately involved in carcinogenesis and metabolic regulation (Jones & Thompson, 2009; Chandel, 2014).

In agreement with the Warburg hypothesis, one of the primary metabolic changes associated with rapidly proliferating cancer cells is the induction of aerobic glycolysis, instead of OXPHOS for the generation of ATP (Figure 2), while providing essential carbon sources for the biosynthesis of other essential macromolecules. Over 90% of the pyruvate produced from aerobic glycolysis in cancer cells is converted to lactate by lactate dehydrogenase (LDHA), to recover NAD⁺ required for continuous glycolytic process, a step that has been shown to

be necessary for the continuous proliferation of tumor cells (Cairns et al., 2011). Although the glycolytic process is less favorable for the generation of ATP molecules, it does have the ability to produce ATP at a faster rate, while generating metabolic intermediates that promote cell growth and proliferation, such as 3-phosphoglycerate (3-PG), which serves as a carbon source for amino acid and lipid synthesis. The remaining 10% of produced pyruvate (which does not get converted to lactate) can be transported into the mitochondria but forced out of the tricarboxylic acid cycle (TCA), which leads to a partial loss of mitochondrial integrity, making the mitochondria a viable target for mitochondria targeting drugs, MITOCANS (Kroemer, 2006; D'Souza et al., 2011).

It has been established that this deregulated cellular metabolism is associated with drug resistance to some of the available cancer therapies (Zhao et al., 2013). A common regulator of cell metabolism, both transformed and non-transformed cells, is the lipid kinase, phosphatidylinositol-3-kinase (PI3K), which regulates levels of phosphorylated phosphatidylinositol (PIP3) at the plasma membrane and downstream signaling cascade involved in metabolic processes that promote cell proliferation. Non-transformed cells have a tight control on this signaling pathway by dephosphorylation and thereby, inactivation of PIP3, by the tumor suppressor, phosphate tensin homolog 10, PTEN. In transformed cancer cells, however, mutations in PTEN support over-activation of the PI3K pathway and the promotion of cell proliferation (Jones & Thompson, 2009). These findings suggest that the mitochondria and the differential metabolic activity of cancer cells, present potential viable and selective targets in cancer therapy (Zhao et al., 2013).

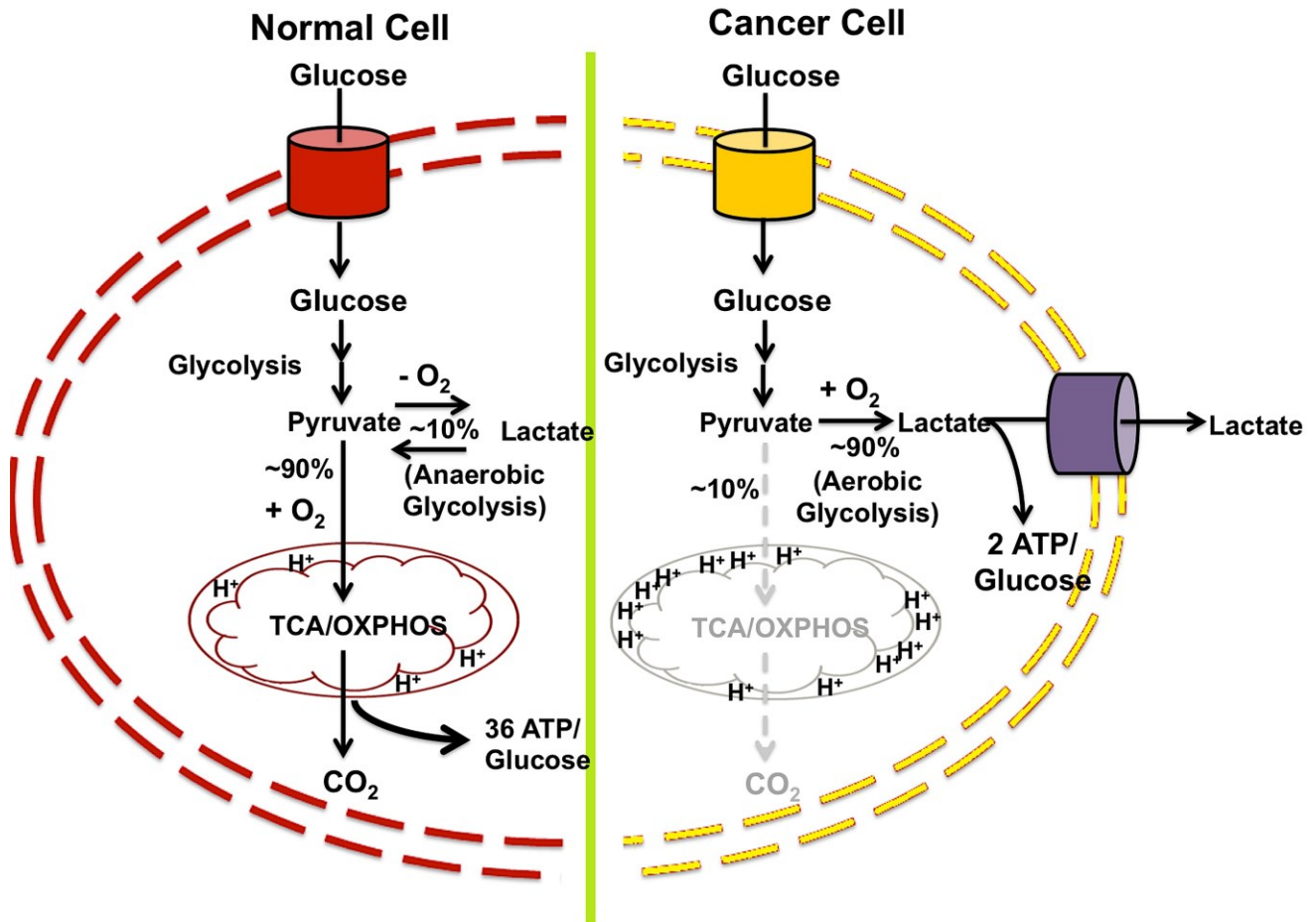


Figure 2: The metabolism of a Cancer vs. Non-Cancer cell show the differences between cancer cell and a normal cells that could contribute to the strengths and vulnerabilities of cancer cells, compared to that of their normal cell counterparts. Cancer cells up-regulate the expression of hexokinase II to promote glycolysis and even under normal aerobic conditions, cancer cells convert most of the product formed from glycolysis, pyruvate, into the lactate. Lactate promotes cancer environment acidification, evasion of growth suppressors, invasion and metastasis and the evasion of cell death programs. Furthermore, the dependence of cancer cells on glycolysis leads to a reduction in the rate of mitochondrial ATP production through the electron transport chain (OXPHOS), leading to the hyperpolarization of the mitochondria in cancer cells, a vulnerability that could be targeted for cancer therapy. (Adapted from Kroemer, 2006; D'Souza et al., 2011).

PROGRAMMED CELL DEATH (PCD)

Cell death programs occur naturally in multicellular organisms as a way to maintain tissue homeostasis (Ziegler, 2004). Therefore, it can be inferred that a disruption in cell death processes can play a role in the development of various diseases. One of the main hallmarks of cancer cells is their ability to evade programmed cell death programs, even in the face of severe cellular damage (Hanahan & Weinberg, 2011). These damaged cells are still able to replicate and proliferate, leading to an increase in the amount of damaged cells. As these cells continuously replicate, they accumulate more mutations allowing survival of cancer cells, their translocation to a different location in the body (metastasis), and the development of other characteristics found in cancer cells (Weinberg, 1996; Hanahan & Weinberg, 2011). These findings indicate that PCD play a significant role in preventing carcinogenesis, and that defects in such processes contribute significantly to the development of cancers, as well as other diseases.

Initially, necrosis and apoptosis were the major forms of cell death studied, with apoptosis being distinguished from necrosis, as apoptosis was considered physiological, programmed and inconspicuous, while necrosis was considered pathological, and a response to traumatic situations and external injury (Ziegler, 2004; Ghobrial et al., 2005). More recently, other forms of PCD have been discovered, including autophagy, necroptosis and oncosis, to name a few (Fadeel & Orrenius, 2005; Coates et al., 2010). The introduction of new forms of PCD have enabled further understanding of the role of cell death in the development of diseases, especially cancer. This thesis focuses on the two main forms of PCD, apoptosis and autophagy, and their roles in carcinogenesis.

APOPTOSIS (PROGRAMMED CELL DEATH TYPE I) - αποπτωση

The significance of PCD to cancer initiation and progression has been a major hot button in cancer research. Kerr and colleagues studied a distinct mode of cell death, separate from necrosis, and have shown that this process plays a significant role in cancer. As with the hallmarks of cancers, they observed a distinct lack of apoptosis, also known as “cellular suicide” or PCD in cancer cells, which was reversed when these cells were exposed to cytotoxic agents and/or irradiation (Kerr et al., 1994). The morphological characteristics of this mode of cell death were characterized and by the end of the 20th century, much was known about the process of apoptosis (Ziegler, 2004).

Apoptosis, a term derived from the Greek word that describes the “falling off of petals from a flower or leaves from a tree”, was coined in order to describe a physiological, energy-dependent and highly regulated process of controlled cellular self-destruction. This is distinct from necrosis, which is a passive, degradative and energy-independent mode of cell death (Gewis, 2003; Fadeel & Orrenius, 2005; Elmore, 2007). It is a process that occurs normally during development and aging, and as a defense mechanism against immune reactions and damage to the cell, induced by noxious agents, such as ionizing radiation and some of the available forms of chemotherapy (Elmore, 2007).

Although both apoptosis and necrosis can occur independently, sequentially or simultaneously, the identification of the morphological characteristics of both forms of cell death can be used to distinguish apoptosis from necrosis and other forms of PCD (Elmore, 2007). Apoptosis occurs in a

highly established sequence of events, often seen with the condensation of the nuclear and cytoplasmic material, the activation of cysteine-dependent aspartic proteases (caspases) and the reorganization and blebbing of the cell membrane to externalize phosphatidylserine. This externalized phosphatidylserine (PS) is essential in the apoptotic process, as it aids in the recognition of compact membrane-enclosed structures, called apoptotic bodies, by phagocytes or neighboring cells (Fadeel & Orrenius, 2005; Coates et al., 2010). The taking up of the apoptotic bodies prevents the triggering of an inflammatory response, which is also a distinguishing characteristic from necrosis. During necrosis, the swelling of the plasma membrane and its eventual disruption, allows the release of the cytoplasmic content into the surrounding tissue, eventually leading to the recruitment of pro-inflammatory signals and inflammatory cells (Gewis, 2003; Elmore, 2007).

There have been conflicting reports on the reversibility of apoptosis, following apoptotic stimuli receipt and induction. Studies have shown that, although thought to be a reversible process, blocking the engulfment of apoptotic bodies by phagocytic cells in *C. elegans*, can enhance cell survival (Elmore, 2007). This is in direct contrast to other findings that show that once apoptotic stimuli are received, the downstream cascade of events inevitably leads to the permeabilization of the mitochondrial membrane, which leads to the release of pro-apoptotic factors and the activation of caspases. The activated caspases cleave downstream molecules, including the caspase activating DNase (CAD), which cleaves DNA into fragments, and poly (ADP-ribose) polymerase (PARP), which is involved in DNA repair, caused by cleavage. The cleavage of PARP

prevents its repair mechanism and the process of apoptosis remains ongoing (Wyllie, 2010). Once these events occur, the cell is usually committed to apoptosis, hence no reversal in the process of apoptosis is possible (Coates et al., 2010). The focus of cancer therapy is therefore aimed at providing enough of an apoptotic stimulus that would cause the permeabilization of the mitochondrial membrane, the release of apoptogenic factors and the downstream caspase cascade in cancer cells.

For any of the aforementioned morphological characteristics to be observed, the downstream signaling cascade of apoptosis has to commence, following the receipt of apoptotic stimulus. There are two major pathways of apoptosis; the first being the extrinsic (cytoplasmic) pathway, which is triggered through receptors belonging to the Tumor Necrosis Factor (TNF) superfamily of receptors and the second is the intrinsic (mitochondrial) pathways, which is triggered by internal stress, such as irradiation or noxious agents (Figure 3). Both pathways are linked by several factors and, as such, inhibitors of apoptosis tend to inhibit both pathways (Ghobrial et al., 2005). These pathways involved in apoptosis are discussed below;

THE EXTRINSIC PATHWAY OF APOPTOSIS

The extrinsic pathway, also known as the death receptor mediated pathway of apoptosis, involves proteins in the TNF receptor superfamily (Figure 3). These proteins share a cysteine-rich extracellular domain, as well as a cytoplasmic domain of about 80 amino acids, called the death domain (Elmore, 2007). These death domains play a significant role in transmitting the death signal from the cell surface to intracellular signaling pathways (Ghobrial et al.,

2005). This process commences with the binding of a specific death ligand to its corresponding death receptors (e.g. Fatty acid synthetase [FasL/FasR]; Tumor Necrosis Factor [TNF- α /TNFR1] and TNF-related Apoptosis Inducing Ligand [TRAIL/TRAIL-R1, R2]) (Fulda & Debatin, 2006). Once the ligand is bound to its receptor, cytoplasmic adapter domains with corresponding death domains that bind the receptors, such as the Fas-associated death domain (FADD) and the TNF receptor type-1 associated death domain (TRADD), are recruited to the cytoplasmic side. This interaction recruits pro-caspase-8, an initiator caspase, by its death effector domain (DED) to the death inducing signaling complex (DISC), which is a complex of the membrane receptor, death domains and pro-caspase-8. Once bound, several pro-caspase-8 molecules are in close proximity to one another, resulting in the auto-catalytic cleavage and activation of caspase-8. The activated caspase-8 then triggers the execution phase of apoptosis, by the cleavage of downstream effector caspases, such as caspase-3 (Ghobrial et al., 2005; Fulda & Debatin, 2006; Coates et al., 2010). If the activating signal in extrinsic apoptosis is strong enough, the activated caspase-8 will can directly activate the effector caspase-3, however if this signal is not strong enough for the execution of apoptosis, the signal is usually amplified by linking the extrinsic pathway to the intrinsic pathway. This occurs when the activated caspase-8 cleaves a member of the Bcl-2 family of proteins, Bid (BH3 interacting domain death agonist), to its truncated form, tBID. tBID translocates to the mitochondria and activates intrinsic apoptosis (Gewis, 2003).

THE INTRINSIC PATHWAY OF APOPTOSIS

In apoptosis regulation, the mitochondria are not only effective in the induction of apoptosis following Bid cleavage, but also plays an integral role in the transmission of positive or negative death signals, following apoptotic stimuli. The positive signals for mitochondrial apoptosis include radiation, toxins, some chemotherapeutic agents, hypoxia, hyperthermia, viral infections, and free radicals, while the negative signals include loss of growth factors and starvation (Gewis, 2003; Elmore, 2007). These non-receptor mediated stimuli produce intracellular signals that act on the Bcl-2 family of proteins, which then directly on the mitochondria, to disrupt the mitochondrial inner transmembrane potential ($\Delta\psi_m$) and the mitochondrial permeability transition pore (Fadeel & Orrenius, 2005). The Bcl-2 family of proteins includes pro-survival members, such as Bcl-XL, Bcl-w, A1, and Mcl-1, which all possess the domains BH1, BH2, BH3, and BH4, and pro-apoptotic members, including Bax, Bak, and Bok, all with the BH1, BH2 and BH3 domains, whereas Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk only possess the BH3 domains, which is sufficient for its apoptotic inducing activity (Gewis, 2003; Fulda & Debatin, 2006; Plötz et al., 2013).

Upon activation of the pro-apoptotic Bcl-2 members and the disruption of the mitochondrial membrane potential, a series of biochemical events occur, which include the halting of ATP synthesis, the oxidation of NADH, NADPH and glutathione, the generation of reactive oxygen species (ROS) and the release of pro-apoptotic factors from the inner mitochondrial membrane into the cytosol (Ghobrial et al., 2005). These factors include cytochrome c, Smac/Diablo, Omi/HtrA2, AIF and Endonuclease G and once released into the cytosol, can

trigger caspase-mediated or caspase-independent apoptosis. Cytochrome c release from the mitochondria has been extensively studied. Once this protein is released, it binds to an adaptor molecule, Apoptotic Protease Activating Factor 1 (APAF1), which then oligomerizes and recruits pro-caspase-9, in the presence of ATP, through the caspase recruitment domain (CARD); this allows auto-activation of caspase-9 and this complex is known as the apoptosome, which is essential for the activation of downstream effector caspases, like caspase-3 (Fulda & Debatin, 2005; Elmore, 2007).

Intrinsic apoptosis is able to follow a caspase-independent pathway, following the release of the apoptosis-inducing factor (AIF) and the endonuclease G (EndoG) from the inner mitochondrial space. AIF translocates to the nucleus, where it participates in DNA degradation, while EndoG triggers caspase-independent DNA fragmentation in cells following apoptotic stimuli (Ghobrial et al., 2005; Fulda & Debatin, 2006). Both pathways have various inhibitors that prevent the downstream signaling involved in apoptosis, including inhibitors of apoptosis proteins (IAPs) and Flice inhibitory proteins (FLIPs) (Fadeel & Orrenius, 2005).

Evasion of apoptosis is considered a major hallmark of cancer cells and therefore the understanding of this process has enabled the development of several forms of therapy. However, as cancer cells are notorious for developing resistance mechanisms (Rebucci & Michiels, 2013), it is also essential to further assess other forms of PCD, in the hopes of better developing better forms of cancer therapeutics.

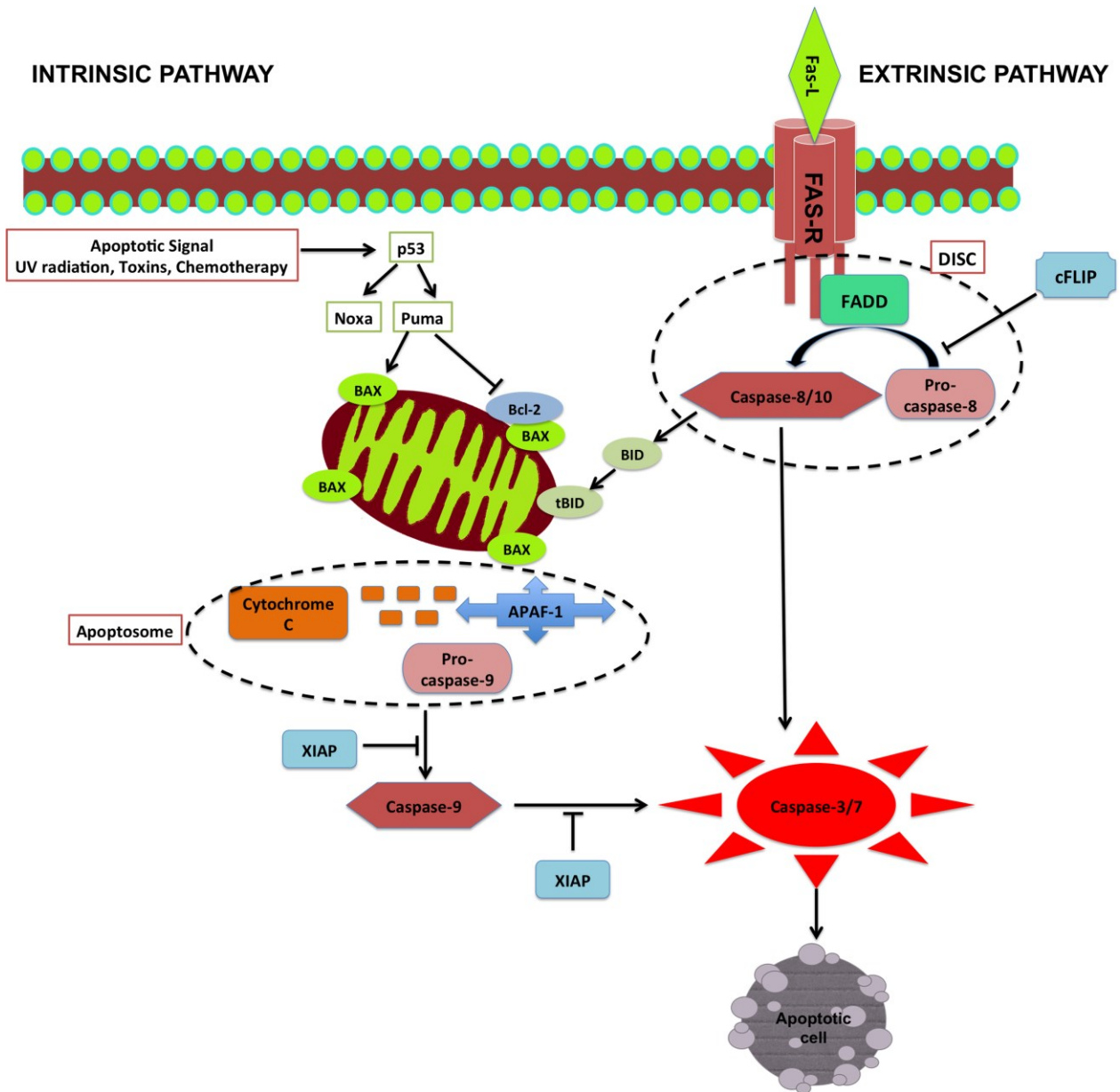


Figure 3: The Pathways of Apoptosis that can be induced, following differential apoptotic stimuli. The two main pathways of apoptosis include the intrinsic pathway that requires an internal stimulus, such as toxins and DNA damaging agents. The stimulus promotes the expression of proteins like p53, which lead to downstream signaling that permeabilizes the mitochondrial membrane and allows the release of pro-apoptotic factors, such as cytochrome c, the activation of caspase-9 and the subsequent activation of caspase-3. In the extrinsic pathway, a death ligand binds to its specific death receptor, recruits an adaptor domain and leads to the activation of caspase-8. Active caspase-8 could directly activate caspase-3 or could link to the intrinsic pathway through the cleavage of pro-apoptotic Bid, which ultimately leads to the permeabilization of the mitochondrial membrane. Cancer cells overexpress inhibitors of both apoptotic pathways, including IAPs (XIAP) and FLIPs (cFLIP). (*Adapted from Ghobrial et al., 2005; Fulda & Debatin, 2006; Coates et al., 2010*)

AUTOPHAGY (PROGRAMMED CELL DEATH TYPE II)

Autophagy, the process of “self-eating,” is a lysosomal degradative process, known to play a significant role as a tumor suppressive mechanism in the development and progression of cancer, as defective autophagy has been shown to be one of the numerous causes of tumorigenesis (Hippert et al., 2006). Along with apoptosis, autophagy is involved in maintaining cellular homeostasis, as it functions in nutrient recycling, energy generation, and the clearance of damaged proteins and organelles (He et al., 2013).

The ability of cancer cells to evade apoptosis is one of the true hallmarks of cancer and this property is vital to chemotherapeutic and radiotherapeutic resistance as characterized by the most aggressive forms of human cancers (McKenzie & Kyriakou, 2006). The introduction of autophagy in research has introduced an alternative way to induce cell death in cancers that are resistant to apoptosis and therefore chemotherapy. Autophagy is induced by a variety of stimuli, including stress, starvation and the inhibition of the mTOR pathway and is responsible for non-selective degradation of long-lived proteins, large aggregates and defective organelles (Kim et al., 2008). This process involves the formation of a double membrane vesicle around the protein/organelle to be degraded. This double membrane vesicle, known as the autophagosome, then fuses with the lysosome and the contents of the autophagosome are degraded (Figure 4) (Hippert et al., 2006). This formation of the autophagosome and the subsequent degradation of proteins and organelles are dependent on the selective combination of several Atg (AuTophagy) proteins. Some of these proteins play similar roles to the enzymes in the ubiquitination cascade (Lee et al., 2012),

suggesting that the autophagic process and the ubiquitination process are not mutually exclusive.

The emergence of the process of autophagy is controversial. Depending on the cell type and conditions, autophagy is believed to have dual roles (Hippert et al., 2006; Dalby et al., 2010). Autophagy is a catabolic process that serves as both a quality control process and a mechanism to replenish intracellular nutrients and provide materials needed for protein synthesis, under conditions of poor nutrient availability (Dalby et al., 2010; Yecies & Manning, 2011). These suggest a pro-survival role in normal homeostasis and that autophagy could also play a similar role in cancer, protecting tumor cells under stressful conditions (like hypoxia and acidity, through overproduction of lactate), by providing the necessary materials needed for required protein synthesis. For instance, there are known anticancer agents, like tamoxifen and etoposide, which induce pro-survival autophagy (Dalby et al., 2010). This pro-survival form of autophagy is usually accompanied by an inhibition of other forms of cell death, including apoptosis (Hippert et al., 2006; Thorburn, 2008). On the other hand, many studies have focused on the pro-death role of autophagy, especially in cancer cells, as it has been observed that defects in autophagic processes promote carcinogenesis (Yecies & Manning, 2011).

Furthermore, important connections between apoptosis and autophagy have been identified, indicating an important link between the two main forms of PCD (Figure 5) (Rosenfeldt & Ryan, 2011). For instance, p62 (also known as sequestosome-1, SQSTM-1) has been found to interact with death receptors, DR4 and DR5, for the activation of TRAIL induced extrinsic apoptosis. The fact

that it also interacts with the autophagy regulator, Atg8/LC3, through its LC3-interacting region, also indicates its importance in autophagy. The involvement of p62 in the process of autophagy has been well established, and although autophagic degradation is considered non-selective, the ability of p62 to specifically recognize polyubiquitinated, misfolded and aggregated proteins and organelles for selective autophagic degradation points to the importance of this protein in autophagy. These findings provide substantial evidence of a molecular link between autophagy and apoptosis, through the modulation of p62 (Moscat & Diaz-Meco, 2009).

The Bcl-2 family of proteins not only have important roles in the progression of apoptosis, but they also play a role in autophagic initiation and progression (Thorburn, 2008). Pro-survival Bcl-2 interacts with pro-apoptotic Bax to inhibit the induction of apoptosis and it has also been found to interact with Beclin 1, to inhibit its role in the formation of the autophagosome (Figure 5). Furthermore, the autophagic protein, Atg5, an E3 ubiquitin ligase-like protein, involved in the elongation of the autophagosome, has also been found to interact with FADD, resulting in apoptotic cell death through extrinsic apoptosis. Cleavage of Atg5 by calpain leads to the translocation of the truncated Atg5 to the mitochondria, where it promotes the release of cytochrome c (Thorburn, 2008; Zhivotovsky & Orrenius, 2010; Kuang, Qi, & Ronai, 2013).

Overall, there is overwhelming evidence that autophagy functions as PCD type II in some cancer cells where apoptosis is defective and, as such, it is not difficult to infer that the induction of pro-death autophagy selectively in cancer cells could be used as an anti-cancer therapeutic strategy.

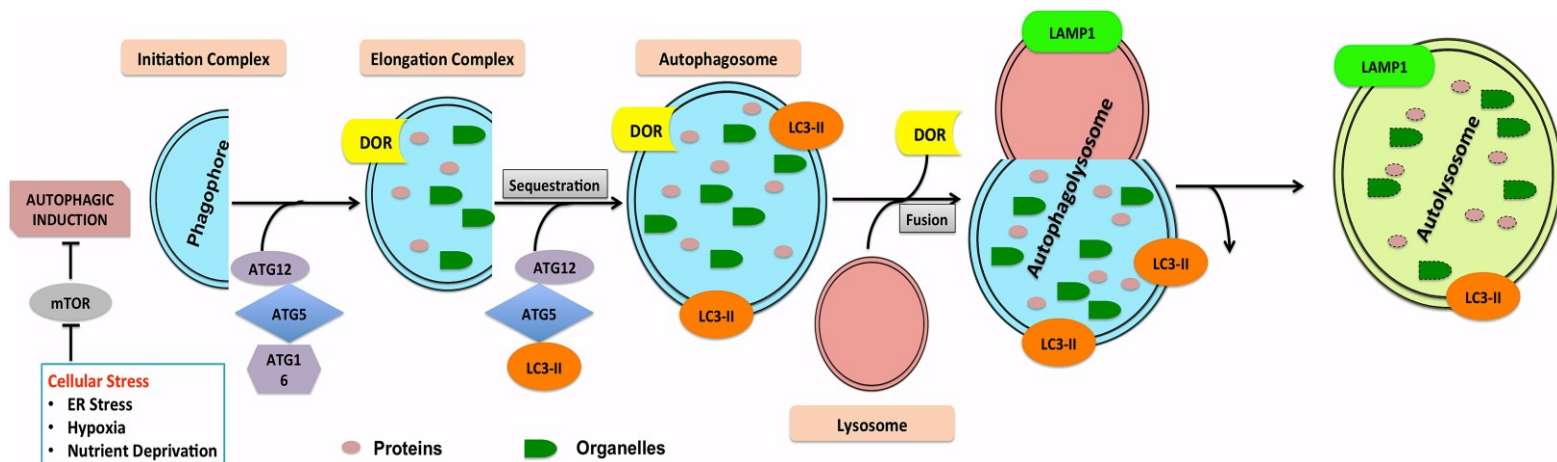


Figure 4: The Process of Autophagy that leads to autophagic degradation, through the interaction with the lysosome. Cellular stresses, such as ER stress and starvation, lead to the inhibition of the mammalian target of rapamycin (mTOR), which leads to the induction of autophagy. The AuTophagy related proteins (ATGs) and Beclin-1 and Beclin-2 trigger downstream events that leads to the complex assembly of the membrane structure of the phagophore. The formation of this complex facilitates the conjugation of LC3-I onto phosphatidylethanolamine in the lipid bilayer of the membrane, converting it to LC3-II, and allowing the phagophore elongate and engulf target proteins and organelles, via adaptor proteins like p62 and form the autophagosome. The autophagosome fuses with the lysosome and acidic compartment that promotes the fusion and degradation of the contents, through the aid of lysosomal enzymes, such as cathepsins. (Adapted from Hippert et al., 2006).

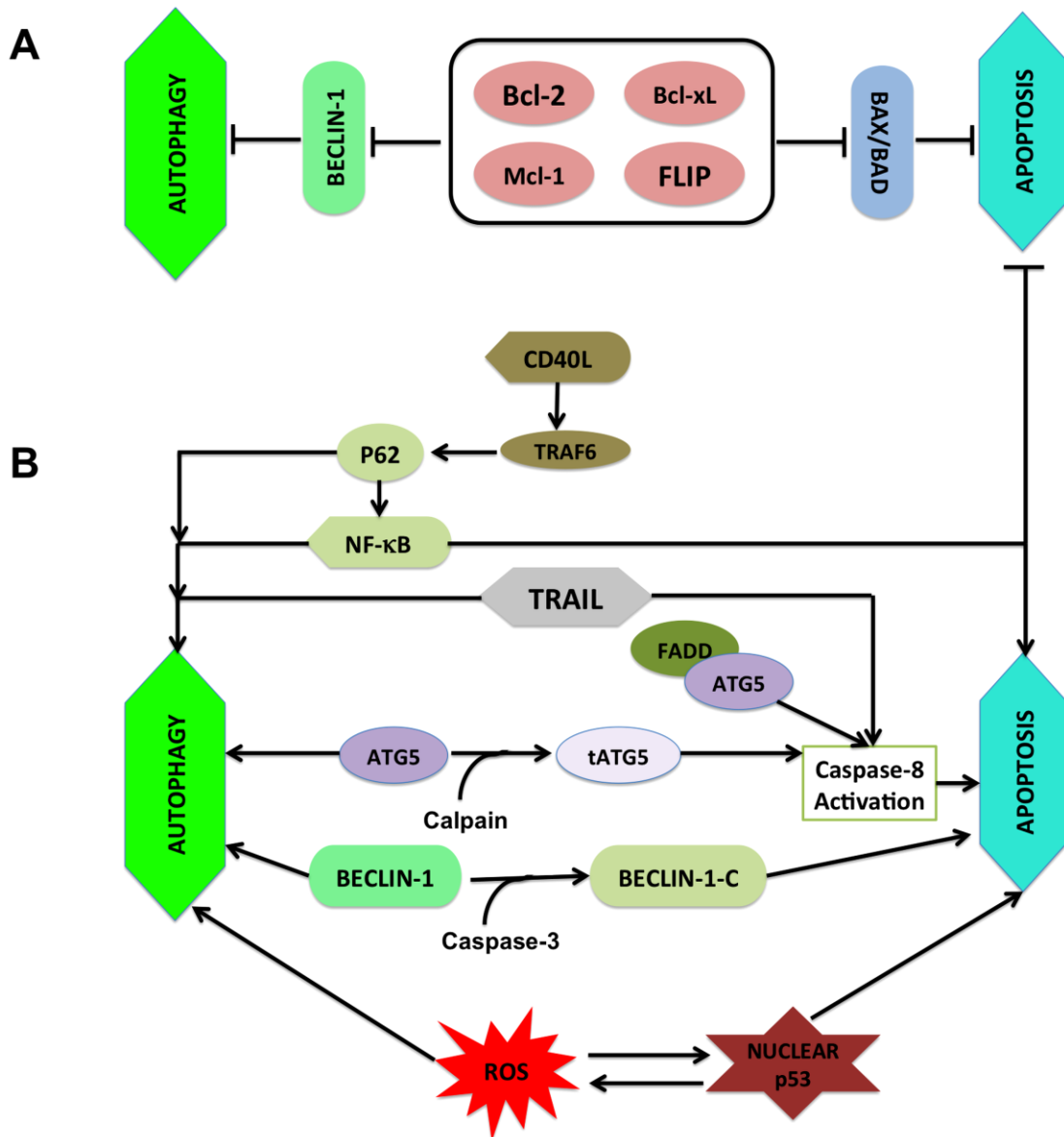


Figure 5: The Connections Between Apoptosis & Autophagy: Regulation of apoptosis and autophagy is required to prevent deregulation of these processes and their roles in disease initiation and progression. A) The Bcl-2 family of proteins regulate both autophagy and apoptosis, where Bcl-2, Mcl-1 and Bcl-xL inhibit apoptosis by blocking Bax activation to prevent mitochondrial permeabilization and the release of pro-apoptotic factors. Alternatively, these proteins interact with Beclin-1's BH3 domain to inhibit mTOR and activate autophagy. B) Several key players in autophagy (ATG5, Beclin-1 and p62) also play a significant role in apoptotic induction, through the activation of caspases and the permeabilization of the mitochondrial membrane. Some death ligands, like TRAIL have also shown significant effect in targeting the autophagic pathways for the induction of autophagic degradation. (Adapted from Thorburn, 2008; Moscat & Diaz-Meco, 2009; Rosenfeldt & Ryan, 2011).

THE ROLE OF PROGRAMMED CELL DEATH IN CANCER

Both apoptosis and autophagy have been implicated in the initiation and progression of carcinogenesis. Cancer cells are notorious for up-regulating pro-survival signaling pathways that inhibit the induction of apoptosis, while mutations in tumor suppressors, e.g. p53, and pro-apoptotic proteins are rampant (Zhivotsovsky & Orrenius, 2010). Although it has been recognized that autophagy could play dual roles, as a pro-survival or pro-death mechanism, it has been well established that pro-survival autophagy plays an integral role in carcinogenesis, providing cancer cells with the nutrients and energy they require for survival. Furthermore, this form of autophagy has been correlated with a decrease in the levels of other forms of PCD, especially apoptosis (Hippert et al., 2006).

The importance of both forms of PCD, especially in carcinogenesis is well established. Various anti-cancer therapies have been shown to, not only induce apoptosis in cancer cells, but to be efficacious in the induction of autophagy; for instance, tamoxifen induces pro-survival autophagy in breast cancer cells in *in-vitro* models and ionization radiation has been found to induce pro-death autophagy in various cancer cell type, to name a few (Coates et al., 2010). This suggests that further understanding of PCD processes (type I and II) could be beneficial anti-cancer strategies.

PCD, especially apoptosis, is designed as a safeguard against carcinogenesis in normal cells. Most chemotherapeutic treatments attempt to reignite apoptosis in cancer cells. Unfortunately, available therapies also target non-cancerous cells (as described below) and as such, there is a need to develop more selective and thus safer forms of therapy.

CURRENT CANCER THERAPY

The incidence of cancer is constantly increasing as the aging population increases (Jemal et al., 2011). Substantial research has gone into developing effective forms of treatments to halt the progression of cancer, usually by stimulating the induction of various forms of programmed cell death (PCD) in cancer cells by targeting mitotic machinery (Chabner & Roberts, 2005, Zhou & Giannakakou, 2005); in more recent years, the focus has shifted to targeting cancer cell metabolism (Zhao et al., 2013). Advances in research have successfully introduced various forms of therapy into cancer care and their effectiveness and drawbacks will be discussed further below;

SURGERY

Currently, cancer therapy designs are usually based on the organ in which the cancer originates and the localization of that organ or cancer in the body (Cortés et al., 2013). In the case of solid tumors, early detection is essential for successful treatment, as it enables surgical removal, as primary treatment, of the tumor or the organ in which it is located (if possible). Over the past decade, substantial advances have been made to improve the early detection of various cancers, thereby improving the outcome of surgical resection of tumors, as has been observed with improved colorectal cancer screenings (Desantis et al., 2014). Studies show that survival rates for non-small cell lung cancer (NSCLC) greatly decrease as the cancer stages increase, going from 80% at stage I to less than a 20% 2-year survival rate at stage III (Hussain & Nguyen, 2014).

Although surgery is associated with an increase in the survival rate of some cancer patients, there are still cases where surgery, as a mode of therapy,

is still not effective. Surgical resection is limited to patients in the early detection category, usually before the tumor has invaded and metastasized into other tissue locations. Furthermore, surgery is not without adverse effects. Long-term side effects include tingling and tightness at the site of surgery along with later development of chronic pain; although in some cases, this pain is not considered severe. In prostate cancer patients, surgery is associated with urinary incontinence, erectile dysfunction and bowel complications, while in thyroid tissues, surgery can damage the nerves in the larynx. Furthermore, it has been established that cancer recurrence is common in patients that underwent surgery, with almost half the patients having a recurrence within 3 years of surgery; hence many cancer patients usually undergo some other form of therapy, usually chemotherapy and radiation therapy, alongside surgery (Desantis et al., 2014).

RADIATION THERAPY

Due to the aggressiveness and poor prognosis associated with many cancers, the addition of other therapeutic forms, before or after surgical resection, has been implemented in several cancer types. Radiation therapy has often been employed to improve response and survival rates in cancer patients (Shaw et al., 2002; Desantis et al., 2014). This form of therapy, along with surgery, improves survival of cancer patients by potentially inhibiting further implantation of metastasized tumors, allowing for better focused treatment to the localized site of the primary tumor (Jagsi, 2014).

Normally, double stranded DNA breaks (for instance, as caused by radiation, hypoxia and the presence of free radical scavengers), induces cell cycle arrest and an enzymatic repair program for the repair of DNA damage.

Inability to repair this damage leads to one of two pathways, the induction of senescence and the induction of apoptosis or necrosis, through the up-regulation of the tumor suppressor, p53 (Ross, 1999). Rapidly dividing cancer cells are targeted by increasing doses of ionizing radiation, triggering apoptotic induction. Due to the common target that is DNA and other mitotic machinery, however, ionizing radiation also causes DNA damage in normal, non-cancerous cells, leading to severe side effects observed in cancer patients in this treatment regimen. As radiation therapy does not trigger cell death programs immediately following treatment, it has been shown to induce cell death programs days or even weeks following treatment. Slow growing tissues can remain unaffected for weeks or months following treatment and, as such, the side effects in these tissues are only observed months after the treatment regimen has been concluded (Pawlik & Keyomarsi, 2004).

Genomic instability, as a result of DNA damage, is usually a contributing factor to carcinogenesis. Coupled with the fact that cancer cells are notorious for having mutations in the tumor suppressor p53, it can be concluded that DNA damage and the inability to repair the damage and evade PCD processes contributes to the process of carcinogenesis (Gorgoulis et al., 2005). As ionization radiation induces DNA damage in order to trigger these cell death processes, it can therefore be inferred that a resulting effect of ionizing radiation is the accumulation of new tumors in a patient already undergoing treatment. This suggests that a more effective mode of therapy is required, one that does not come with the chances of inducing further carcinogenesis.

CHEMOTHERAPY

Prior to the 1940's, surgery and radiation therapies dominated the world of cancer therapy. Since the beginning of the 20th century, various forms of chemotherapy have also been in play (Chabner & Roberts, 2005; DeVita & Chu, 2008). Chemotherapy began with the use of nitrogen mustards and anti-folates, the latter of which was used to inhibit the effect of folic acid on the proliferation of leukemia cells; and over the years, has evolved to include several classes of drugs (Chabner & Robert, 2005).

Cancer chemotherapy was developed to circumvent some of the side effects observed in patients undergoing radiation therapy, particularly the DNA damaging aspect that leads to PCD or development of secondary malignancies. Newer chemotherapy introduced agents that target microtubules, which are a major component of all eukaryotic cells and are vital for the maintenance of cell shape, polarity, cell division and intracellular transport. Vinca alkaloids (e.g. vincristine) and taxanes (e.g. paclitaxel) are the major groups of microtubule targeting drugs, obtained from natural products, that have shown success as microtubule destabilizing agents (Vinca alkaloids) and microtubule stabilizing agents (taxanes). Both classes of microtubule targeting agents inhibit the progression of cells through the cell cycle, inhibit mitosis and lead to the induction of PCD. Besides their abilities to affect microtubule formation, these agents have also been found to inhibit the formation of new blood vessels, a process known as angiogenesis (Zhou & Giannakakou, 2005).

Although there have been major advances in the development of these chemotherapies, the principles and limitations that have governed the discovery

of new chemotherapeutic agents still apply today (Chabner & Robert, 2005). For instance, neurological and hematological side effects are usually observed in a dose-dependent effect in patients undergoing treatment with microtubule targeting agents. Immunosuppression, the development of secondary malignancies, and even death resulting from other side effects is usually associated with majority of the currently available chemotherapies (Zhou & Giannakakou, 2005; Desantis et al., 2014). Altogether, this information indicates that a better target for cancer therapy is needed, in order to bypass some of the severe side effects that are observed in cancer patients undergoing treatment.

TARGETED THERAPY

The limitations associated with the previously discussed forms of cancer therapy, along with the many molecular defects in cancer cells, led to the introduction of targeted therapies. These therapies were introduced in a bid to target the multiple hallmarks of cancer (Hanahan & Weinberg, 2011), as well as the metabolic changes within cancer cells (Zhao et al., 2013), in order to develop more effective treatment regimens that do not come with such severe side effects. These targeted therapies include targets such as growth factors, signaling molecules and pathways, cell-cycle proteins, modulators of apoptosis and proteins involved in angiogenesis (Chabner & Robert, 2005).

One of the most famous examples of targeted therapy was the development of the Bcr-Abl tyrosine kinase inhibitor, imatinib (Gleevec), for the treatment of chronic myeloid leukemia, with the characteristic Philadelphia chromosome (Chabner & Robert, 2005; DeVita & Chu, 2008). This innovation was a stepping-stone for the introduction of monoclonal antibodies against

receptors and growth factors in cancer therapy, such as those against the epidermal growth factor receptor (EGFR), like cetuximab (Martinelli et al., 2009). Unfortunately, targeted therapies are not without their own obstacles. Cancer cell resistance to chemotherapy is still a major limitation to cancer therapy development; intrinsic and acquired resistance is usually observed following numerous genetic and epigenetic changes in cancer cells (Rebucci & Michiels, 2013). Common resistance mechanisms involve drug efflux, using the multidrug resistance (MDR) transporter, changes in DNA damage responses and mutations in several pathways, allowing cancer cells develop the previously discussed hallmarks of cancer (Chabner & Robert, 2005; Rebucci & Michiels, 2013). Cancer cell resistance to targeted therapies have led to the introduction of combination therapies, in a bid to overcome the resistance associated with targeted therapies, while still attempting to reduce therapy-associated toxicity (Li et al., 2014).

The huge toxicity associated with cancer cell therapy is due to non-selective targets and the “by-stander” effects. Emerging evidence indicates a role of natural health products in the possible development of non-toxic alternatives for cancer therapy. This begs further research and scientific validation of such products for cancer treatment.

NATURAL HEALTH PRODUCTS (NHPs)

Natural health products (NHPs) and natural products (NPs) play a leading role in the discovery and the development of drugs for the treatment of human diseases. Traditional medicines in the Native American, Chinese, and Indian cultures have utilized numerous natural products, including dozens of spices and plant extracts. NHPs are becoming increasingly popular and are used widely for their promising therapeutic effects and fewer side effects. Scientific research into the validity of these traditional products has shown that many do indeed have potent anticancer effects (Zhi et al., 2007; Ganesan, 2008; Newman & Cragg, 2012). For instance, an extract from the Mayapple, *Podophyllum peltatum*, was traditionally used by Native Americans to combat skin cancers and other malignant neoplasms, as well as a host of other ailments. The major component of this extract was podophyllotoxin, which was the first in a series of effective anticancer agents called podophyllins (Mann, 2002). Likewise, numerous natural products used by traditional Indian Ayurvedic medicine have been shown to have strong anti-inflammatory and anticancer properties.

Even with all the incoming evidence, herbal drugs and other NHPs and NPs are usually shunned during systemic therapy because of herb–drug interaction and exaggeration of therapy-related toxicity. Current research is focused on the development of new and more effective therapeutic agents that have little to no associated toxicity to the patient. Lately, this focus has been centered on NHPs and herbal formulations, mainly in the form of plants and other biological sources around the world. NHPs have been used for centuries by a variety of cultural backgrounds for various illnesses, some of which continually

provide new medicinal applications and intriguing anecdotal evidence, which merit further investigation. Today, there are numerous natural products that fall under the umbrella of alternative medicine (Aggarwal et al., 2007; Ganesan, 2008; Kumar et al., 2011).

NHPs, from various traditional medicines, have been used for centuries for the treatment of various diseases and anecdotal evidence suggests that there is some benefit to the claims that have been reported. Furthermore, the use of these NHPs and NPs have not been associated with many side effects and toxicities (Aggarwal et al., 2006; Efferth et al., 2007). NHPs are complex mixtures, containing multiple components that could potentially have bioactivity in the face of different diseases. The complexity of NHPs makes them a viable option for the treatment of diseases, especially chronic diseases like cancer, as they might be able to target multiple pathways in a disease, while acting together to potentially reduce any toxicities associated with the use of the NHP or NP (Mishra et al., 2000; Tabas & Glass, 2013).

Studies on various natural products have shown some efficacy and lack of toxicities associated with the use of these herbal formulations. However, as with many other forms of therapies, combinations of NHPs have also been studied, focusing on the combination of NHPs and NPs at lower doses, to further reduce chances of toxicities and improve efficacy (Huang et al., 2003; Mueller et al., 2004). Such studies provide sufficient evidence for the introduction of NHPs to the public, in order to provide safer and cheaper complementary treatments for diseases, especially in chronic diseases like cancer.

NATURAL HEALTH PRODUCTS IN CANCER

The toll of cancer on the population is continuously on the rise and the efficacy and safety of available anti-cancer therapies has not yet caught up. The role of NHPs in cancer treatment has been evaluated in several scientific studies, leading to the development of a total of 27 anti-cancer drugs from natural sources between 1940 and 2010 (Figure 6) (Newman & Cragg, 2012). A common example is Camptothecin, a quinolone alkaloid, has been studied and used for its anti-tumour activity in targeting and inhibiting DNA topoisomerase I. This compound was isolated from extracts of the *Camptotheca acuminata*, which has been used for years in traditional medicine. In the last decade, this NP has been found to possess anti-cancer activity, and its derivatives, topotecan and irinotecan, are routinely used in the treatment of ovarian and colon cancers (Efferth et al., 2007).

The discovery of the anticancer activities of so many traditional medicines and natural products has been supported by scientific evidence and validation. This was in part successful due to the initiation of the Cancer Chemotherapy National Service Center (CCNSC) in 1955, by the National Cancer Institute (NCI). The mandate of this program was to “screen for anti-tumor agents on a larger scale by establishing a strict standardized protocol” for testing potential anticancer compounds (DeVita et al., 2008).

Since the 1980s, research into the anti-cancer effects of natural products has yielded many promising results. For example, resveratrol, a polyphenol present in grapes, has been under investigation for years and these studies show the potential of resveratrol as both a preventative and an anti-tumor agent (Lu et

al., 1999; Kallantari & Das, 2010). Similarly, piperlongumine, extracted from *Piper longum* and identified in the 1960s around the same time as Taxol, was found to selectively induce generation of reactive oxygen species in cancerous cells by targeting the oxidative stress response and leading to apoptotic cell death (Raj et al., 2011). In the 1980s, Kenneth Bagshawe and Peter Senter developed a novel use for natural products: the antibody-directed enzyme-prodrug therapy (ADEPT). This technique used tumor-specific antibodies bound to an enzyme that would convert non-cytotoxic prodrugs into their cytotoxic forms once in contact with the tumor. Many natural products were successfully used as prodrugs, including doxorubicin and Taxol (Mann, 2002).

The different hallmarks of cancer and tumor cells, which include evading growth suppression signals, evading programmed cell death processes, inducing angiogenesis and sustaining proliferative signaling, to name a few (Hanahan & Weinberg, 2001; Hanahan & Weinberg, 2011), provide multiple means to target cancer cells selectively. The study of NHPs against cancer cells and xenograft models has therefore focused on identifying NHPs that can target pathways that convey survival protection to cancer cells, so as to selectively and effectively eradicate cancer cells.

These earlier studies have paved the way for the introduction of more NHPs from traditional medicine to the forefront of modern medicine and anecdotal evidence indicate that these NHPs merit further investigation and scientific validation. The scientific validation of these NHPs in terms of their efficacy, safety and mechanism of actions will seal their position in modern medicine, especially in the field of cancer research and therapy.

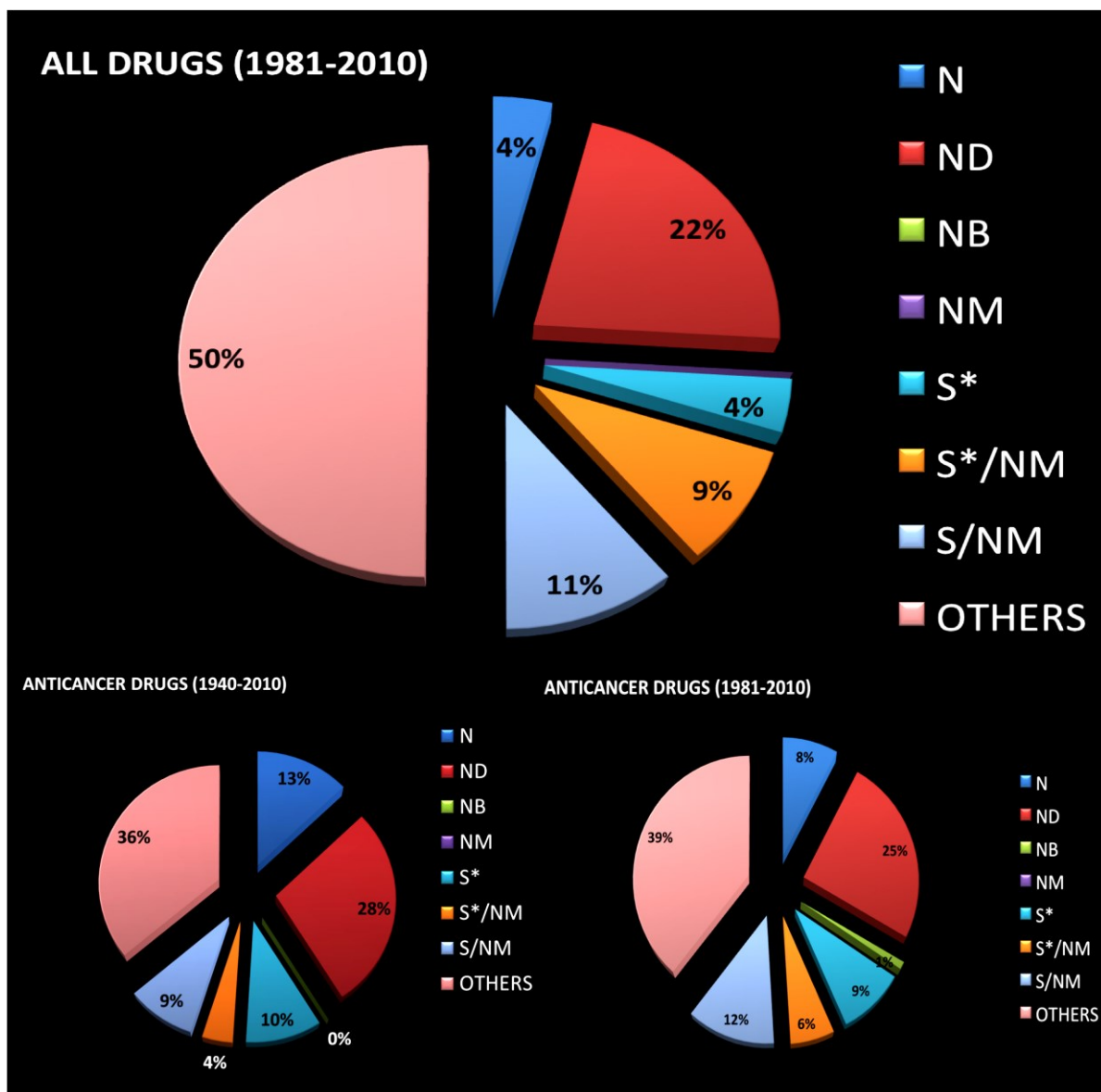


Figure 6: Sources of Anticancer Drugs from the 1940s to 2010.

* Legend: Natural product (N); Derived from a natural product-usually a synthetic derivative (ND); Natural product "Botanical (NB); Natural product mimic (NM); Totally Synthetic Drug (S); Made by total synthesis, but the pharmacophore is/was from a natural product (S*). (Adapted from Ganesan, 2008; Newman & Cragg, 2012).

DANDELIONS (*TARAXACUM spp.*)

Dandelions are perennial “weeds” that belong to the Asteraceae family of plants and are widespread throughout warmer temperate zones. This plant has been used for centuries as a form of remedy for various diseases, including treating abscesses, reducing inflammation and promoting diuresis in traditional Chinese medicine (TCM). These health benefits are not only limited to Chinese medicine; folk use and traditional medicinal uses for dandelions extend across Europe and Africa (Sigstedt et al., 2008; Yarnell & Abascal, 2009). The various health benefits attributed to the use of dandelions has been credited to specific *Taraxacum* species, including *T. japonicum*, *T. monogolicum*, *T. coreanum* and *T. officinale* (Sigstedt et al., 2008). Various parts of this plant have been used in the treatment of different ailments, with the root having been used in gastrointestinal diseases and the leaves, as a diuretic and digestive stimulant. The whole plant has been taken as a cure for hepatitis and anorexia as well, although some of the claims associated with this weed have gone unsubstantiated (Schütz et al., 2006; Yarnell & Abascal, 2009).

Some preclinical research on dandelion has introduced this plant with numerous properties to the scientific community. Research has shown the anti-inflammatory, prebiotic, anti-angiogenic and anti-neoplastic properties of dandelion root (Zhi et al., 2007; Yarnell & Abascal, 2009). Some of these studies contradict each other, leading to the publication of conflicting reports on the efficacy of dandelions, however, the one thing all these studies agree on is that dandelions can induce cytotoxicity in several types of cell types, due to its effect on oxidative stress, inflammatory response, the secretion and expression of TNF-

α and IL- α , the down-regulation of nitric oxide (NO) and cyclooxygenase-2 (COX-2) (Hu & Kitts, 2003; Koo et al., 2004; Hu & Kitts, 2005; Schütz et al., 2006; Jeon et al., 2008). The flower extract of *T. officinale* was found to be both pro-oxidant and anti-oxidant, as at low doses, anti-oxidant behaviour was observed, and attributed to the presence of luteolin and luteolin-7-glucoside, while at higher doses, this extract possess pro-oxidant activities in colon cancer cells (Hu & Kitts, 2003). It is essential to note that the production of ROS can have both pro-apoptotic and anti-apoptotic effects, depending on the conditions of the cells, as well as the cell types (Simon et al., 2000). The investigation of the mechanism of action of dandelion root extract in cancer cells is under study, with focus on the identification of the possible apoptotic pathway in which this extract is selective to cancer cells. The mechanistic efficacy of DRE will be discussed in later chapters of this thesis.

The selective anticancer efficacy of dandelion root extract has been attributed to its ability to induce death-receptor mediated extrinsic apoptosis in cancer cells selectively, and this activity can be attributed to the presence of sesquiterpene lactones (Zhang et al., 2005; Ghantous et al., 2010), and the suppression of cellular FLICE- like inhibitory protein (cFLIP), which is highly expressed in several cancer cell types, including pancreatic cancer cells, by the triterpene, lupeol (Murtaza et al., 2009). This compound is one of the bioactive components of dandelion extracts (Hata et al., 2000; Chatterjee et al., 2011). This inhibition of cFLIP has been shown to render TRAIL-resistant cancer cells sensitive to TRAIL therapy (Murtaza et al., 2009).

These are important findings that show the versatility of natural health products, especially dandelion root extract, in targeting several cellular pathways and under different situations and conditions; they also provide significant evidence for the efficacy use of NHPs, especially dandelions, in the fight against cancer.

LONG PEPPER (*PIPER Spp.*)

Long pepper, from the Piperaceae family, have been used for centuries, for the treatment of various diseases. The identification of several bioactive species of long pepper have been identified and they include *P. longum*, *P. betle* and *P. retrofactum*. A long list of benefits have been attributed to the extracts of the different *Piper spp*, with reports indicating their efficacy in different ailments (Kumar et al., 2011). Historically, long pepper has been used as a topical treatment for muscle inflammation but has shown efficacy in a number of diseases and conditions including diabetes, cancer and obesity, as well as its use as an analgesic, and digestive stimulant, without having any toxic effects (Kumar et al., 2011; Bao et al., 2013). More recently, the plant has been studied as an anti-inflammatory for Carrageenan-induced paw edema in rats, where researchers found significant decrease in paw inflammation of rats treated with long pepper indicating its efficacy in acute and sub-acute inflammation (Kumari et al., 2012).

In addition to this study, other work has been done on Piperlongumine (PL), an important component of the long pepper fruit, as a therapy against atherosclerosis. This study found that the anti-inflammatory and anti-platelet aggregation properties of PL prevented artherosclerotic plaque formation in mice,

demonstrating the efficacy of PL as a possible therapy for this inflammatory disease (Iwashita et al., 2007; Tabas & Glass, 2013). Furthermore, studies in various cancer cell lines showed the anticancer effect of PL in these cells, where PL targeted the oxidative stress response of these cells, increased the levels of reactive oxygen species (ROS) and activated the expression of several key pro-apoptotic proteins; PL's effect on the oxidative stress response in cancer cells led to its ability to induce apoptosis in these cancer cells, in a dose and time dependent manner. This anticancer effect was confirmed in *in vivo* models of breast adenocarcinoma (Raj et al., 2011). More importantly, this effect on ROS generation and oxidative stress pathway targeting was not observed in the non-cancerous cells, suggesting a dependence on the oxidative stress response pathway in cancer cells. These results confirm what has been previously known: that targeting the mitochondria could provide a better selective target in cancer cells (D'Souza et al., 2011) for more efficacious treatment.

Further studies have also shown the ability of PL to target receptors, including the platelet derived growth factor (PDGF) receptors, for the inhibition of angiogenesis, and treatment with this compound led to the depletion of androgen receptor in prostate cancer cells, through a proteasome-mediated ROS dependent pathway (Bezerra et al., 2012; Golovine et al., 2013). The results from these studies not only suggests a role of PL as a receptor antagonist, but also provides a link between oxidative stress and receptor targeting, for an alternative path to cancer cell specific targeting, proving this usefulness of NHPs and NPs in the fight against cancer.

The anti-inflammatory response associated with the use of long pepper

extract is attributed to the presence of PL; however, the effect of this compound alone is significantly decreased, when compared to the efficacy of the whole plant extract in reducing inflammatory response. Some reports indicate that the use of the whole extract of long pepper show better efficacy, when used at lower concentrations, compared to individual components of the extract, like PL, which had an effective concentration $\geq 10 \mu\text{M}$ (Vedhanayaki et al., 2003; Iwashita et al., 2007; Raj et al., 2011).

There are, however, very few scientific validation studies to prove the benefits associated with the use of long pepper extracts; most of these studies have been carried out on the components present within the extract, including piperines. Piperines have been shown to inhibit many enzymatic drug biotransforming reactions and plays a significant and specific role in the metabolic activation of carcinogens, as well as in metabolic and mitochondrial energy production (Golovine et al., 2013; Jarvius et al., 2013; Meghwal & Goswami, 2013). The presence of piperidine alkaloids show potent fungicidal activity (Lee et al., 2001; Bao et al., 2013). Some of these compounds, including PL, have shown potent anti-cancer activity (Bezerra et al., 2007), suggesting that long pepper extract contains multiple bioactive components that could be beneficial in the development of more efficacious therapies for the treatment of various diseases, including cancer.

HYPOTHESIS

There is sufficient evidence indicating the importance of NHPs in the development of drugs. Anecdotal evidence, bring to the forefront, a need for scientific validation of more NHPs. This research focused on scientifically validating the mechanistic efficacy of two NHPs, Dandelion Root Extract (DRE) and Long Pepper extract (PLX) in cancer cells.

Our hypothesis is that natural health products (NHPs), such as DRE and PLX, contains multiple bioactive components that can efficiently target multiple vulnerable aspects of cancer cells to act as a selective treatment against cancer and possibly improve the quality of life (QoL) of diagnosed patients.

OBJECTIVES

To study this hypothesis, three major objectives were put forward;

- Evaluation of the selective efficacy of Dandelion Root Extract (DRE) and Long Pepper extract (PLX) in several *in-vitro*, *in-vivo* and *ex-vivo* models
- Investigation into the mechanism of action and selectivity of DRE and PLX in cancer cell models
- Fractionation & phytochemical analysis of DRE and PLX to identify the bioactive compound(s) involved in providing the selectivity and efficacy.

OVERALL SIGNIFICANCE

The toll of cancer on the human body and the society as a whole, indicates a serious need for a better selective, more effective and relatively cheaper mode of treatment. Unfortunately, at this time, most of the available forms of therapy induce apoptosis or other forms of programmed cell death in both cancer and non-cancer cell types. The purpose of this study is to identify NHPs with better

selective efficacy against cancer cells, while attempting to provide a stepping stone for further validation studies of NHPs. The scientific studies carried out with dandelion root and long pepper extracts, as natural health products, will provide evidence for introduction of these NHPs to the public, in order to provide alternative, safer and cheaper complementary treatments for cancer therapy and improve QoL in cancer patients.

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CHAPTER 2

SELECTIVE INDUCTION OF APOPTOSIS THROUGH ACTIVATION OF CASPASE-8 IN HUMAN LEUKEMIA CELLS (JURKAT) BY DANDELION ROOT EXTRACT

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Journal of ethnopharmacology (2011), 133(1): 86–91

ABSTRACT

Dandelion extracts have been used in traditional Native American Medicine and Traditional Chinese Medicine (TCM) for treatment of leukemia and breast cancer; however, the mechanism of action remains unknown. Today, DRE is mainly marketed for management of gastrointestinal and liver disorders. The current study aims to determine the anti-cancer activity of dandelion root extract (DRE) against human leukemia, and to evaluate the specificity and mechanism of DRE-induced apoptosis.

The effect of DRE on cell viability was evaluated using the colorimetric-based WST-1 assay. Apoptotic cell death was monitored by nuclear condensation and confirmed by exposure of phosphatidylserine to outer leaflet of plasma membrane. Activation of caspases was detected using a fluorogenic substrate specific to either caspase-8 or -3. Loss of mitochondrial membrane potential was observed by microscopy using JC-1 dye. The apoptotic effect of DRE was also evaluated on a dominant negative FADD (Fas-associated death domain) cell line and non-cancerous peripheral blood mononuclear cells (PBMCs).

Aqueous DRE effectively induces apoptosis in human leukemia cell lines in a dose and time dependent manner. Very early activation of caspase-8 and the subsequent activation of caspase-3 indicate that DRE may be inducing extrinsic or receptor-mediated apoptosis. Caspase inhibition rendered this extract ineffective, thus DRE-induced apoptosis is caspase-dependent. Moreover, the dominant-negative FADD cells that are unable to form a complete DISC (death-inducing signaling complex) were resistant to DRE treatment, which further

confirms our hypothesis that DRE induces receptor-mediated apoptosis. Interestingly, non-cancerous peripheral blood mononuclear cells (PBMCs) exposed to aqueous DRE under the same treatment conditions as leukemia cells were not significantly affected.

Our results suggest that aqueous DRE contains components that act to induce apoptosis selectively in cultured leukemia cells, emphasizing the importance of this traditional medicine and thus presents a potential novel non-toxic alternative to conventional leukemia therapy.

INTRODUCTION

Dandelions are one of the most common and recognizable weeds and are found in almost every part of the world (Koo et al., 2004; Yarnell and Abascal, 2009). The *Taraxacum spp.* includes the species *Taraxacum japonicum*, *Taraxacum mongolicum* and *Taraxacum officinale*, although the differences in composition of each of these species remain vague (Jeon et al., 2008). This plant has been used in Traditional Chinese Medicine (TCM) and traditional Native American Medicine for its medicinal activity as treatment of diseases ranging from diarrhea and digestive diseases to hepatitis and cancer (Jeon et al., 2008; Yarnell and Abascal, 2009). More specifically, they have traditionally been used for treatment of breast cancer and leukemia (Sweeney et al., 2005).

Dandelions are perennial weeds composed of a variety of chemical compounds that are thought to act alone or in combination to increase the activity of the plant extracts (Schutz et al., 2006). The chemical composition of dandelion extracts has been observed and some of the important components of the extract include sesquiterpene lactones and phenylpropanoids, which are believed to have anti-inflammatory, anti-oxidative and anti-cancer properties leading to the diverse observed effects of dandelion extracts (Yarnell and Abascal, 2009). Other components have not been fully characterized and therefore their activities remain unknown (Schutz et al., 2006).

There are limited scientific studies investigating the anti-cancer activity of dandelion extracts and very little is known about the mechanism of action; reports on the effect of dandelion extracts and the production of cytokines have remained ambiguous to date (Kim et al., 2000; Koo et al., 2004).

Recently research has been focused on developing anti-cancer drugs that interfere with metabolic pathways of cancer cells to induce apoptosis, a highly regulated physiological process of cell death that has a wide range of implications in disease (Kekre et al., 2005; Ghobrial et al., 2005). It is an important mechanism by which cells undergo cell death to control cell proliferation (Kerr et al., 1972; Gewies, 2003; Fadeel and Orneius, 2005). Apoptosis is characterized by DNA fragmentation, cell shrinkage and nuclear condensation, and phosphatidylserine flipping from the inner to the outer leaflet of the plasma membrane (Kekre et al., 2005).

Development of an effective chemotherapy that selectively induces apoptosis in cancer cells is of great importance. One major pitfall of current chemotherapeutics is that the majority cause severe side effects in non-cancerous cells. In the current study, the activity of dandelion root extract (DRE) against a human acute T-cell leukemia cell line (Jurkat) was evaluated in parallel to its effect on non-cancerous peripheral blood mononuclear cells (PBMCs).

Our novel findings indicate that DRE is capable of inducing apoptosis at low concentrations specifically in cancer cells with no toxicity to PBMCs. DRE treatment caused very early activation of caspase-8 and subsequent activation of caspase-3. Moreover, it was observed that Jurkat cells expressing a dominant-negative FADD (Fas-associated death domain) protein were insensitive to apoptosis induced by DRE, indicating involvement of the extrinsic pathway of cell death (Hueber et al., 2000).

MATERIALS & METHODS

Standardized dandelion root extraction

Freshly obtained dandelion roots (from open grassy areas) were thoroughly and repeatedly washed with water; final wash with distilled water. Dandelion roots (100 g) were homogenized in 200 ml of distilled water at 25 °C using a domestic blender. Total homogenate was filtered through a NITEX nylon mesh filter (LAB PAK; Sefar BDH Inc., Chicoutimi, QC, CA) and the filtrate was centrifuged at 8000 x g for 5min at 25°C. The supernatant was filtered using 0.45 µm filters, followed by filtration through 0.22 µm filters. The final filtrate was used directly or lyophilized and reconstituted as needed into stock solutions of 100 mg/ml in water.

Cell culture

The Human acute T-cell leukemia (Jurkat clone E6-1) cell line and dominant-negative FADD Jurkat cells (clone I 2.1) were purchased from ATCC (Manassas, VA, USA). These cells were cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS) and 40 mg/ml gentamicin (Life Technologies, Mississauga, ON, CA). These cells were maintained at 37°C and 5% CO₂. Nucleated blood cells were isolated from peripheral blood obtained from healthy volunteers (University of Windsor REB# 04-060). Whole blood (12 ml) was collected in a BD Vacutainer CPT Tube (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 2900 x g for 30 minutes at 25°C. Mononuclear cells, platelets and plasma was collected and cultured in a 1:1 dilution of RPMI media, supplemented and maintained in similar conditions as the Jurkat cell lines.

Cell treatment

Cells were grown to 70% confluence and then treated with freshly prepared water extract or lyophilized extract at concentrations ranging from 0.2 mg/ml, the lowest dose with minimal activity, to 1mg/ml, the most effective dose.

Cell staining and viability assay

Viability of Jurkat cells after DRE treatment was examined by Trypan Blue Exclusion assay. Trypan Blue stain (Life Technologies, Carlsbad, CA, USA) was added to cell Suspension and live cells (Trypan negative) were counted using a Fisher Hemacytometer. Percent viability was calculated as the number of live cells per ml. Cell proliferation was determined in Jurkat cells following exposure to DRE using the colorimetric WST-1 assay (Roche, Laval, QC, Canada) according to previously published protocols (Maekawa et al., 2003; Ngamwongsatit et al., 2008). Absorbance readings were obtained using a Perkin Elmer Victor Spectrophotometry machine; a decrease in absorbance indicates reduced proliferation. To examine apoptotic morphology, cells were treated with DRE and stained with cell-permeable Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA), at a final concentration of 10 μ M and incubated for 10 minutes at 37°C. Nuclei were examined using a fluorescent microscope (Leica DM IRB, Germany) and corresponding phase-contrast and fluorescence images were taken.

Annexin-V binding assay

To observe phosphatidylserine exposure, an early marker of apoptosis, Jurkat cells were treated and collected after indicated periods then washed 2X

with PBS and resuspended in Annexin-V binding buffer (10mM HEPES/NaOH pH 7.5, 140mM NaCl, 2.5mM CaCl₂), containing 1:50 Annexin-V Alexa Fluor 488 conjugate (Molecular Probes) for 15 minutes at 25°C. Cells were then examined under a fluorescent microscope; cells that bind Annexin-V in a calcium-dependent manner are considered apoptotic.

Apopercentage apoptosis assay

Apoptosis was also detected using the apopercentage Apoptosis Kit (Biocolor Life Science Assays, County Antrim, United Kingdom). This is an additional assay based on the surface exposure of phosphatidylserine, which permits the uptake of the apopercentage dye into the apoptotic cell. Cells were grown in a gelatin layer and then treated as described above. After treatment of Jurkat cells, cells were collected, washed in PBS and incubated with the apopercentage dye for 30 minutes and multiple representative pictures were taken for each well using a light microscope.

Measurement of mitochondrial membrane potential

To monitor mitochondrial membrane depolarization in cells following DRE treatment, JC-1 dye (5,5'-6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolylcarbocyanine iodide from Invitrogen, Burlington, ON, CA) was used. JC-1 forms aggregates in healthy mitochondria, and fluoresces red. In apoptotic and necrotic cells, JC-1 does not aggregate and the monomeric form (green fluorescence) indicates loss of mitochondrial membrane potential. Jurkat cells were incubated with 2 µL/ml JC-1 dye for 15 minutes at 37°C and examined with a fluorescent microscope.

Caspase activity

Caspase-8 and -3 activity assays were performed using a previously published method (Naderi et al., 2003). Briefly, total protein from Jurkat cell lysates was incubated with fluorogenic substrates corresponding to the substrate cleavage site specific for each caspase (DEVD-AFC for caspase-3 and IETD-AFC for caspase-8). Fluorescence was measured at Ex. 400nm and Em. 505nm with a Spectra Max Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA, USA). Caspase activity was calculated per μg protein and protein concentration was determined with BioRad protein reagent (BioRad, Mississauga, ON, CA) using bovine serum albumin as a standard.

Statistical analysis

All experiments were performed in triplicates and the results expressed as mean \pm S.D. Statistical analysis was performed with GraphPad Prism 5.0 288 software.

RESULTS

Induction of Apoptosis in Human Leukemia Cells by Aqueous Dandelion Root Extract in A Dose and Time Dependent Manner

In order to determine the effect of DRE on cancer cells, human leukemia (Jurkat) cells were exposed to increasing concentrations of dandelion extract (either as freshly prepared extract or lyophilized powder dissolved in water), for 48 h. Hoechst dye was used to detect apoptotic nuclei, which are condensed and brightly stained. Increasing percentage of apoptosis corresponded with increasing concentrations of DRE (Figure 1A and B). The fresh water extract showed better efficiency at inducing apoptosis in Jurkat cells than the lyophilized extract, although the lyophilized extract had a significant effect on these cells even at 0.2 mg/ml (Figure 1B). For further confirmation of the ability of DRE to induce apoptosis in Jurkat cells, WST-1 cell proliferation assay was carried out at 0.4 mg/ml and 0.6 mg/ml DRE after 48 h (Figure 1D). DRE was effective in reducing cellular viability by approximately 60% of the control untreated cells at 0.6 mg/ml. Figure 2A indicates that at 96 h treatment, ~70% of Jurkat cells had undergone apoptosis. In order to study the time course of the DRE effect, Jurkat cells were exposed to 0.6 mg/ml DRE. As shown in Figure 2A and B, there was increased apoptosis with ~80% of cells were apoptotic after 96 h with DRE.

To address whether non-apoptotic cells remaining after 48 h DRE treatment are able to resume cell division after removal of DRE, Jurkat cells were exposed to 0.6 mg/ml DRE over 48 h then washed and incubated with fresh media (without DRE) for another 48 h. Figure 3 shows that cells initially exposed to DRE were unable to grow after media refreshment.

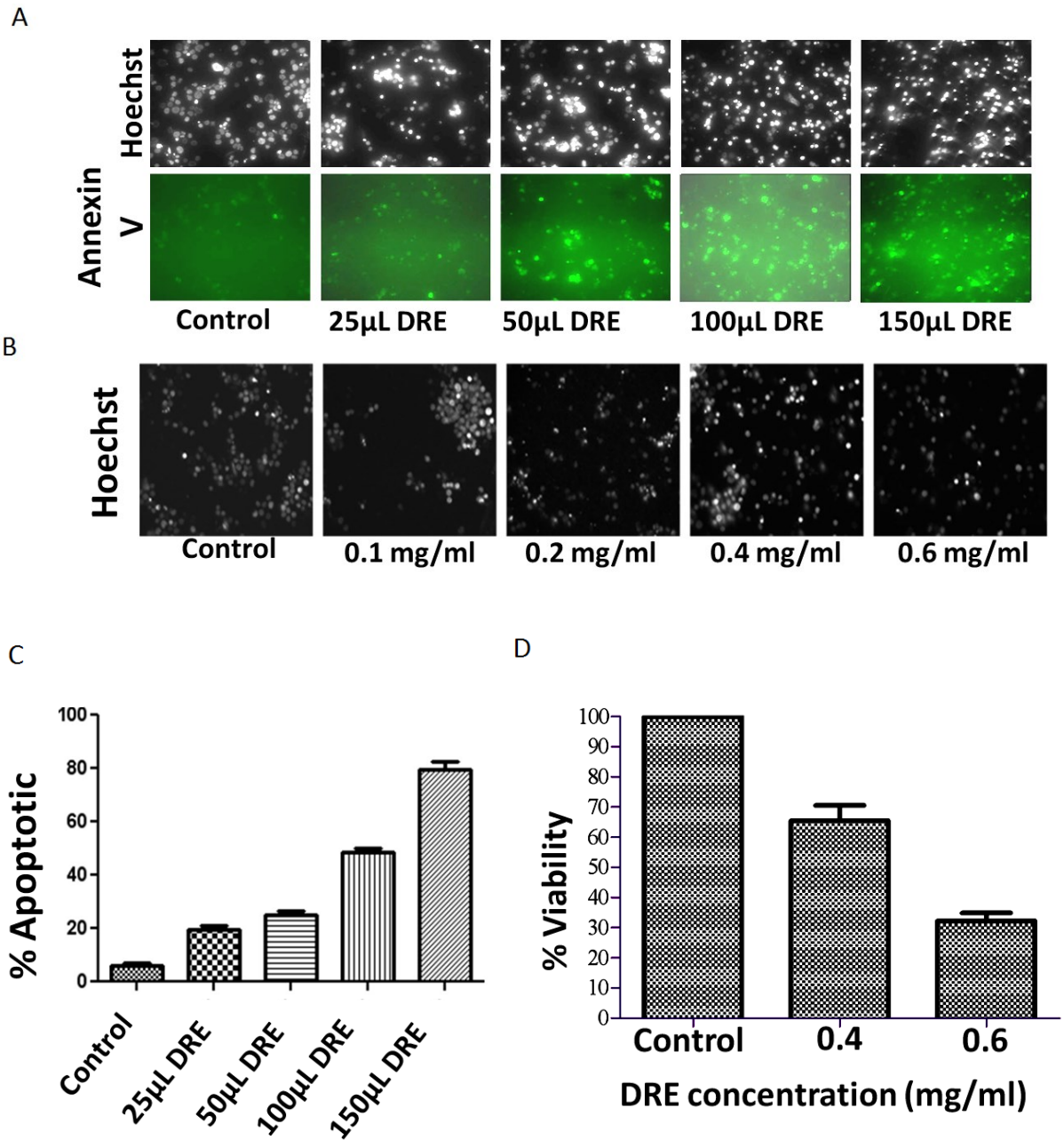
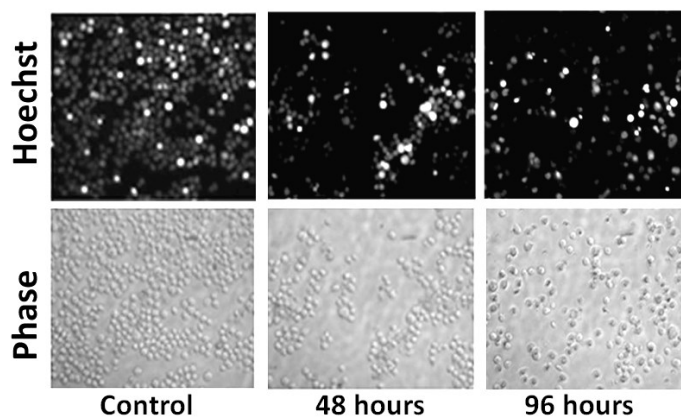


Figure 1: Induction of apoptosis by DRE in Jurkat cells. (A) Jurkat cells stained with Hoechst and Annexin V after 48 h treatment with increasing concentrations of dandelion root extract. (B) Increasing concentrations of lyophilized dandelion extract induced more marked apoptosis in Jurkat cells after 48 h treatment. (C) Crude dandelion extract 100 μL induced apoptosis in approximately 50% of the cells as determined by manual counting of Hoechst images. (D) The effect of DRE on the viability of Jurkat cells at 0.4 and 0.6 mg/ml as determined by WST-1 cell proliferation assay, as a measure of absorbance at 450 nm. Decreased cell metabolic viability (as a % of control) is observed with increasing concentrations of DRE.

A



B

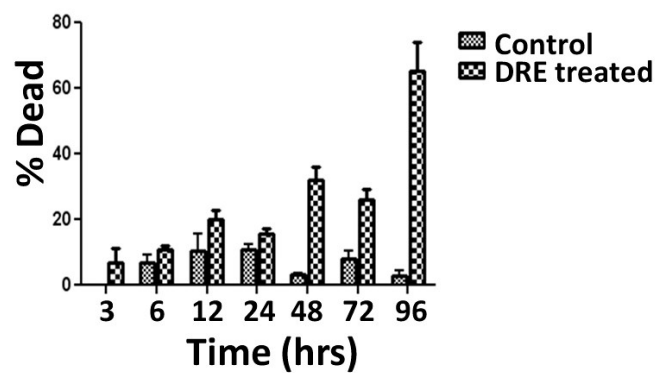


Figure 2: Effect of dandelion extract on Jurkat cells over time. (A) Effect of dandelion extract on morphology of Jurkat cells exposed to DRE as can be seen by fluorescence and phase contrast pictures after incubation with 0.6 mg/ml DRE for extended periods. (B) Apoptosis increased with time from 3 to 96 h with ~40% of cells being apoptotic after 48 h and almost 80% after 96 h. Cell death was quantified using the Trypan Blue exclusion assay.

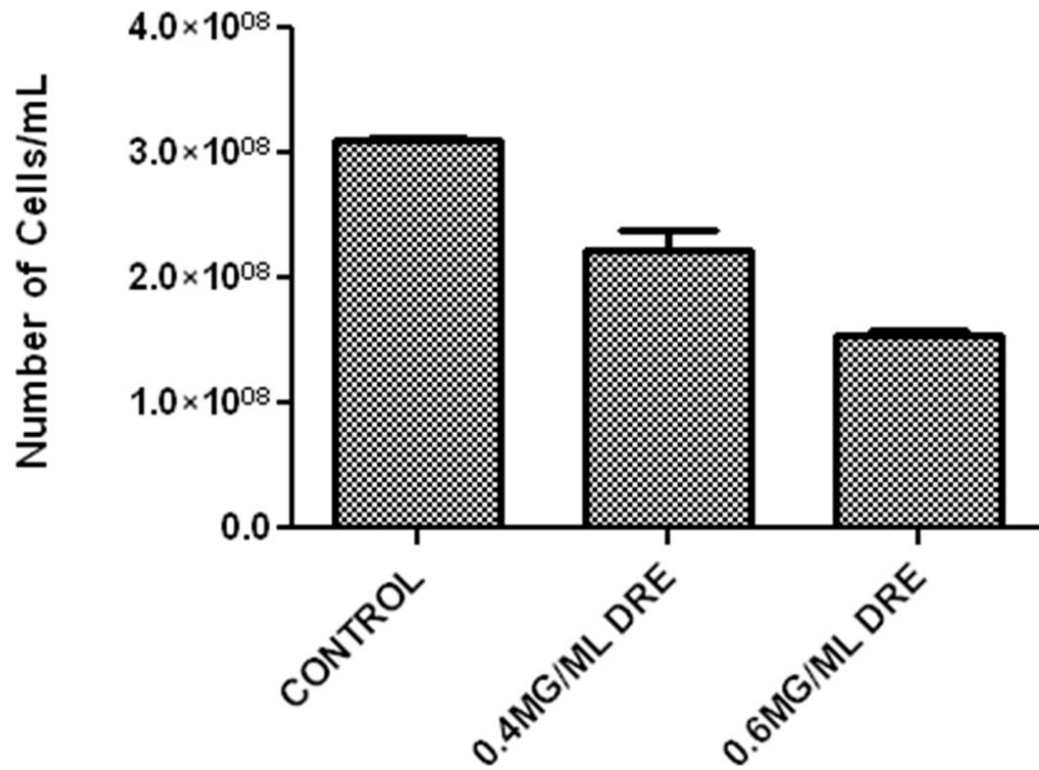


Figure 3: Exposure of Jurkat cells to DRE for 48 h inhibits growth even after removal of DRE. Jurkat cells were exposed to 0.4 mg/ml and 0.6 mg/ml DRE for 48 h. The cells were then incubated in DRE free media and were allowed to grow for 48 h. Trypan Blue exclusion assay was used to quantify the growth of Jurkat cells after fresh media replacement for 48 h.

Confirmation of Apoptosis by Annexin-V and Apopercantage Assays

In order to further confirm the apoptotic mode of cell death, additional biochemical characteristics were monitored. During apoptosis, there is reorganization of the cell membrane, which leads to the flipping of phosphatidylserine from the inner to the outer leaflet of the cell membrane (Martin et al., 1995). This feature of apoptotic cells enabled us detect and confirm the induction of apoptosis after exposure to DRE. Jurkat cells were exposed to 0.6 mg/ml DRE for 48 h and were either assayed using Annexin-V binding or apopercantage dye, both of which take advantage of membrane reorganization. Annexin-V binds with high affinity to exposed phosphatidylserine on apoptotic cells (Martin et al., 1995). Apopercantage dye is selectively imported into the cells undergoing apoptosis. Results shown in Figure 4 indicates that indeed, there is an increased number of Annexin-V and apopercantage positive cells after DRE exposure compared to control, thus further confirming that DRE induced apoptosis in Jurkat cells.

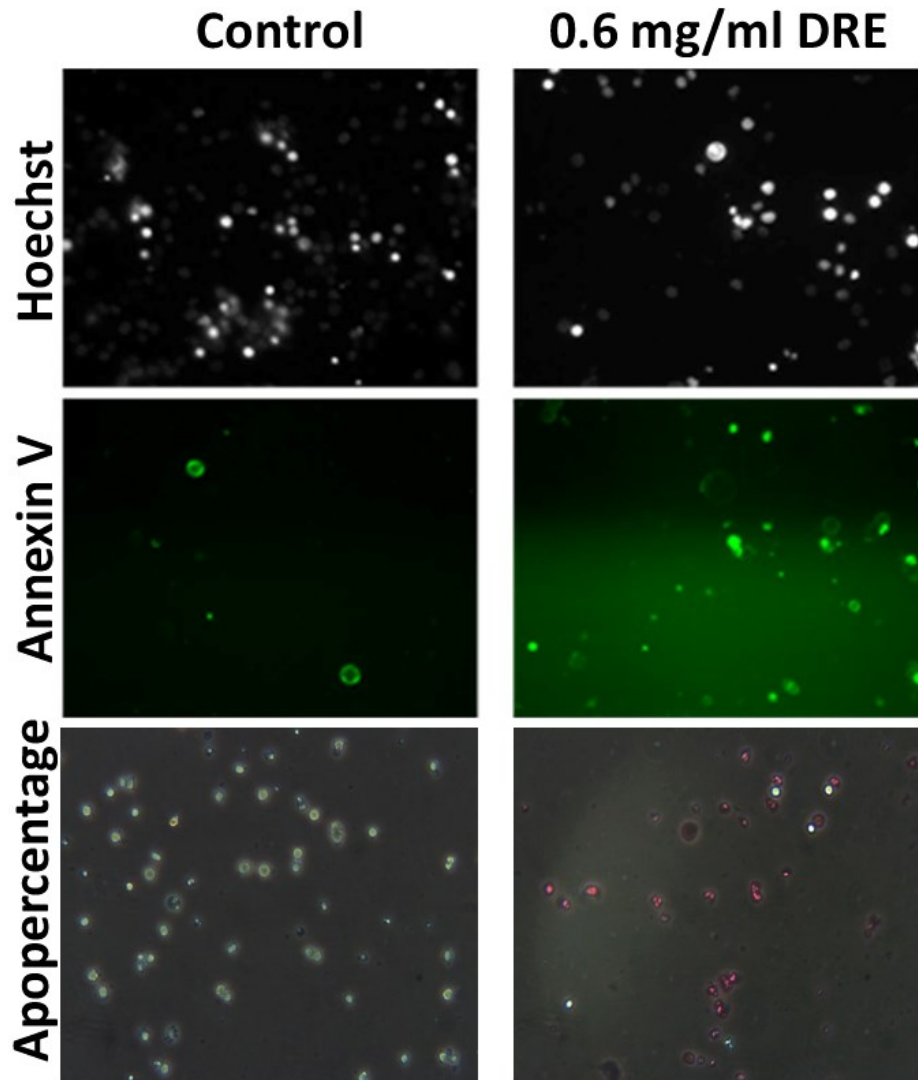


Figure 4: Confirmation of apoptosis by Annexin-V and apopercentage. Annexin-V and apopercentage assays were carried out to confirm apoptosis by the flipping of the phosphatidylserine to the outer leaflet of the cell membrane during apoptosis as described in the materials and methods. Both assays indicate that DRE treatment caused externalization of phosphatidylserine as observed by Annexin V (green fluorescence) and apopercentage (pink cells).

Dandelion Root Extract Induces Activation of Caspase-8 Followed by Caspase-3 in Jurkat Cells

Activation of aspartate-specific cysteine proteases also known as caspases, is an important biochemical event of apoptosis (Fadeel and Orrneius, 2005; Kekre et al., 2005). Different caspases are activated at the initiation and execution phases of apoptosis. To determine the effect of DRE on the activation of caspases, the activities of caspase-8 and caspase-3 were assayed in Jurkat cellular extracts at different time periods following DRE treatment. Results shown in Figure 5A indicates that DRE treatment caused very early activation of caspase-8 at 6 minutes following DRE exposure followed by subsequent activation of caspase-3 at 6h (Figure 5B).

Generally caspase-8 is activated following assembly of death inducing signaling complex (DISC) in receptor-mediated apoptosis and thus our result indicated that receptor-mediated apoptotic pathway might be involved in DRE-induced apoptosis. The Fas associated death domain (FADD) is a cytosolic adaptor protein that binds to Fas-receptor following its engagement with Fas ligand, and recruits pro-caspase-8 leading to activation of caspase-8. Dominant-negative FADD Jurkat cells are unable to assemble functional DISC, and thus are resistant to receptor-mediated apoptosis. To determine if DRE requires the FADD receptor for activation of caspase-8, we tested dominant-negative FADD Jurkat cells under the same conditions as wild-type Jurkat cells. Results indicated that there was no response to increasing concentrations of DRE at 48 h compared to the control untreated cells (see figure 5, chapter 4). This suggested that indeed, DRE-induced apoptosis is receptor mediated.

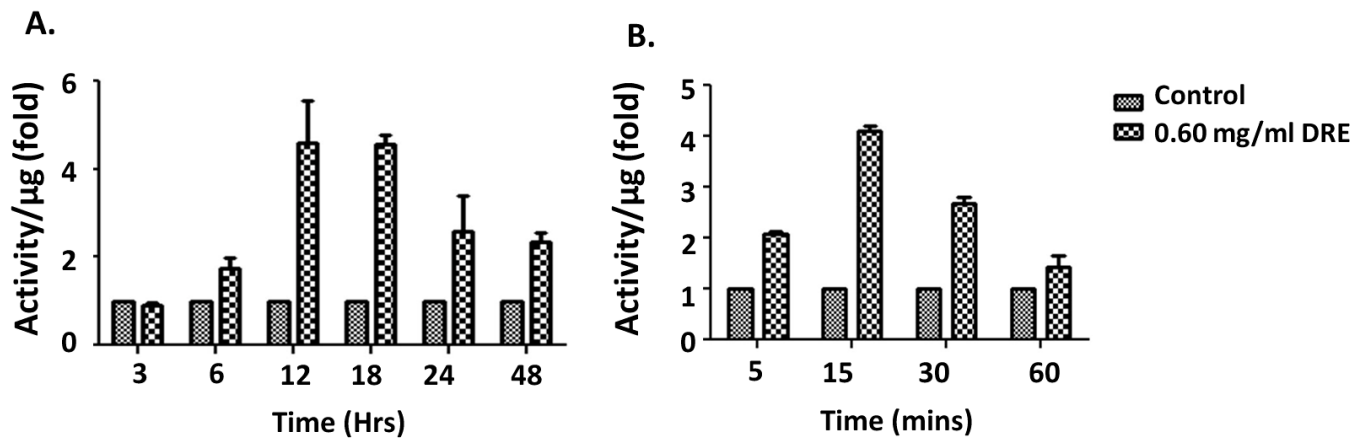


Figure 5: Dandelion root extract induces early activation of caspases in Jurkat cells. Fluorometric caspase activity assay using caspase-3 (A) and caspase-8 (B) specific peptide was performed as described in the methods section. The results here are reported as activity per μg of protein (in fold) and the average of three experiments are shown. Early activation of caspase-8 and ensuing activation of caspase-3 in Jurkat cells upon exposure to DRE at various time intervals.

Destabilization of Mitochondrial Membrane Potential by Dandelion Root Extract

The mitochondria are known as the powerhouse of the cell and play an essential role in apoptosis (Armstrong, 2006). Recent research has been focused on mitochondria as a target for chemotherapy because destabilization of mitochondria membrane potential leads to the release of apoptotic factors thereby allowing apoptosis to occur (Bell et al., 2008; Ralph and Neuzil, 2009). To determine the effect of DRE on Jurkat mitochondria, we exposed these cells to 0.4 mg/ml DRE for 48 h. Mitochondrial destabilization was observed by JC-1 staining with increasing concentrations of DRE (Figure 6).

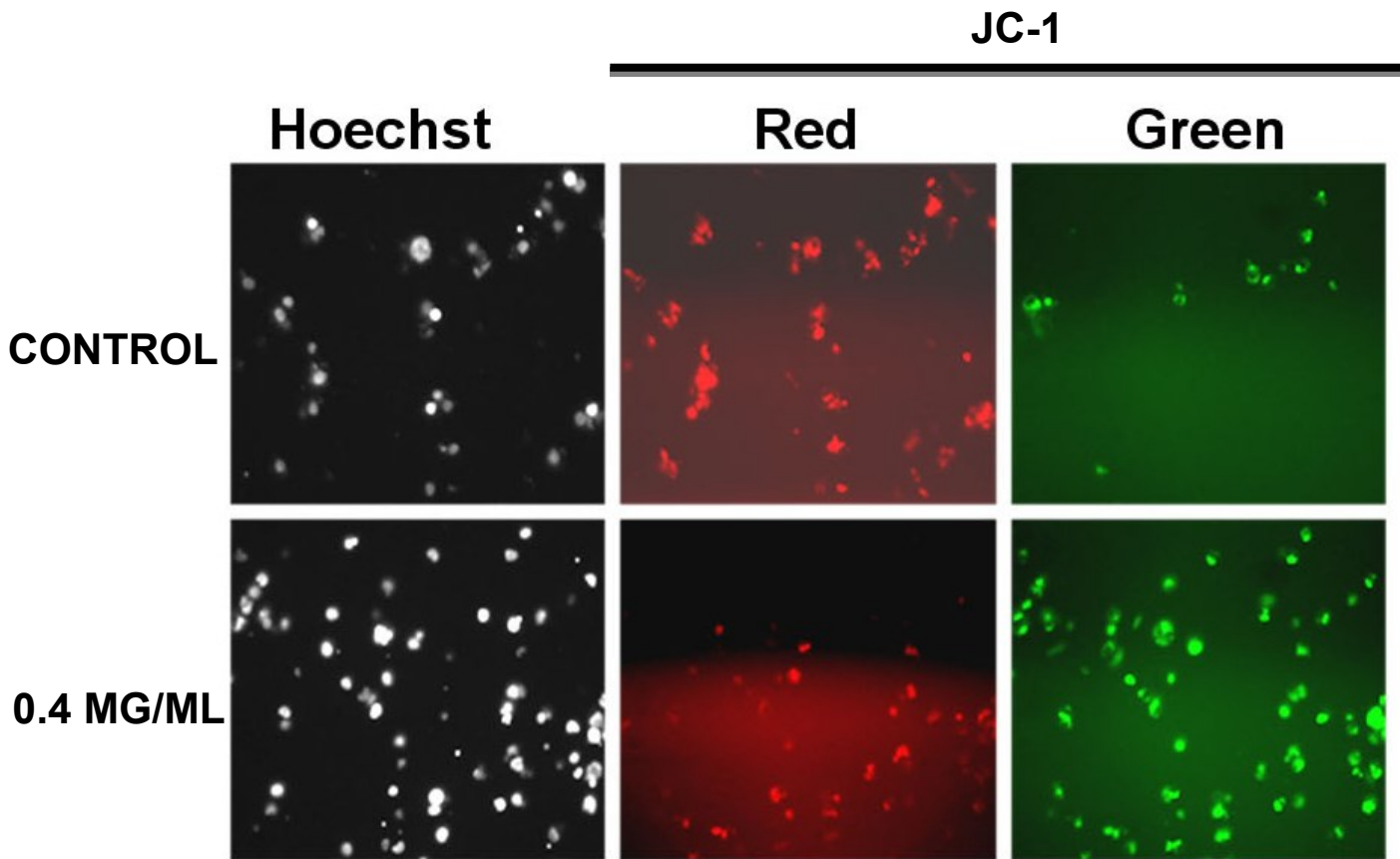


Figure 6: Destabilization of mitochondrial membrane potential by dandelion extract. Jurkat cells were treated with DRE at 0.4 mg/ml and incubated with JC-1 dye. Red fluorescence indicates cells with intact mitochondrial potential. Exposure to 0.4 mg/ml DRE led to the loss of mitochondrial membrane potential.

Dandelion Root Extract is Non-Toxic to Non-Cancerous Peripheral Blood Mononuclear Cells (PBMCs)

Dandelion extracts have been traditionally used for various ailments and no toxic effects have been reported; we wanted to investigate its effect on non-cancerous peripheral blood mononuclear cells (PBMCs). PBMCs were incubated with various concentrations of DRE for 48 h, under the same conditions as Jurkat cells and apoptotic nuclei were monitored by Hoechst staining. Results indicated that there were no differences between control untreated cells and DRE treated cells (Figure 7).

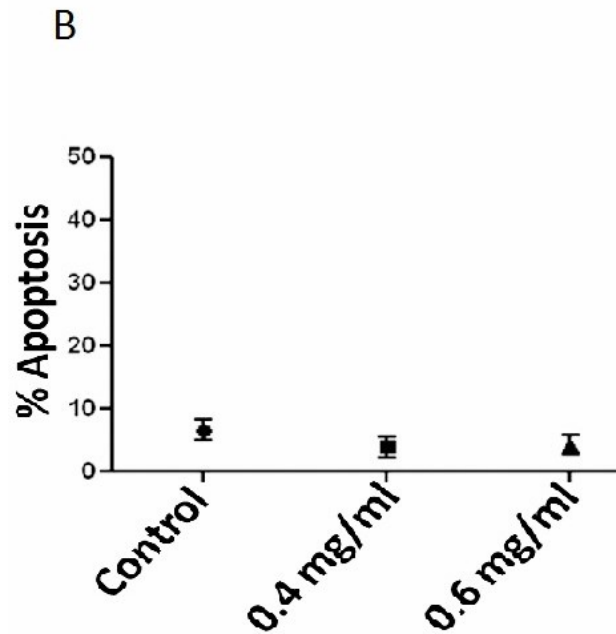
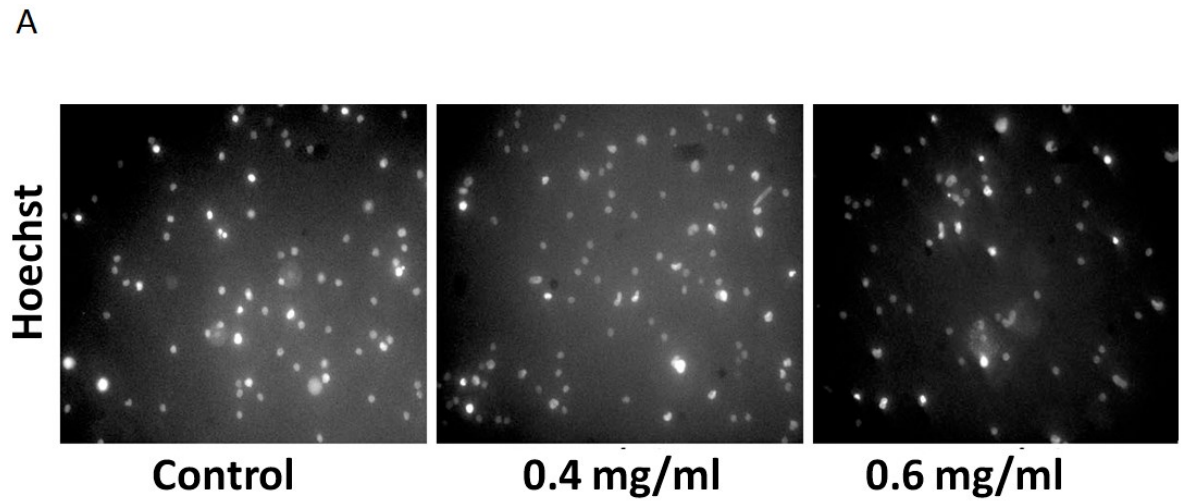


Figure 7: Effect of DRE on non-cancerous peripheral mononuclear blood cells (ncPBMCs). ncPBMCs, from healthy volunteers, were treated at the indicated concentrations for 48 hours before being stained with Hoechst 33342 dye and imaged on a fluorescence microscope (A). (B) Nontoxic effect of DRE on PBMCs. PBMCs treated in (A) were quantified by manual counting of brightly stained apoptotic nuclei.

DISCUSSION

The major setbacks of most available chemotherapies are that they cause severe side effects due to toxicity of non-cancerous tissue, and often become ineffectual due to chemo-resistance (Kekre et al., 2005; Ma et al., 2009). The holy grail of cancer therapy is to develop a novel alternative that has high specificity to induce apoptosis in cancer cells. In the last decade there has been a surge of anti-cancer drugs introduced, with about half of them derived from natural sources (Koo et al., 2004; Lee, 1999).

Although aqueous dandelion extracts have been used in traditional medicine throughout Asia, Europe, and North America (Yarnell and Abascal, 2009) for treatment of many diseases including leukemia and breast cancer, the mechanism by which the root extract acts is unknown. Most of the components of dandelion extract have been isolated and identified but their functions remain uncharacterized (Schutz et al., 2006). Reports on the scientific use of this extract in the treatment of cancer have been unclear and/or conflicting (Sweeney et al., 2005). In a study conducted by Koo et al. (2004), dandelion extract was shown to induce cytotoxicity in the HepG2 human hepatocellular carcinoma cell line by inducing production of cytokines such as TNF- α and IL-1 β . Conversely, Kim et al. (2000) reported that dandelion extract has anti-inflammatory activity in astrocytes through inhibition of IL-1 β and TNF- α production. In a recent study by Sigstedt et al. (2008) both dandelion leaf and root extracts were shown to inhibit proliferation and invasiveness of cancer cell lines by decreasing MMP-2 and MMP-9 expression, indicating a possible mechanism by which dandelions act to inhibit the progression of cancer.

Here we report the cancer-specific apoptosis activity of dandelion root extract (DRE) and the possible mechanism by which this extract works. A previous study has shown some effect on the viability of HepG2 cells, with minimum effect using 0.2 mg/ml (Koo et al., 2004). We used a range of concentrations between 0.2 mg/ml and 1.0 mg/ml for the induction of apoptosis in Jurkat cells and the EC₅₀ was observed at 0.6 mg/ml DRE. Apoptosis was evident within 48 h at 0.6 mg/ml of DRE by chromatin condensation and exposure of phosphatidylserine on the outer leaflet of the cell membrane (Figures 1 and 4). Increase in apoptosis, confirmed by a WST cell viability assay, was observed in a dose and time-dependent manner (Figures 2 and 3).

Interestingly, we observed very rapid activation of initiator caspase-8 (within minutes) in Jurkat cells treated with DRE (Figure 5A), which was followed by activation of executioner caspase-3 within 12 h of treatment (Figure 5B). This indicated that DRE induces the death receptor mediated extrinsic pathway of apoptosis (Figure 5A). To confirm this mechanistic hypothesis dominant-negative FADD Jurkat cells, which have a truncated non-functional FADD protein, were exposed to DRE. These cells were found to be resistant to DRE-induced apoptosis; DnFADD cells are unable to assemble the death-inducing signaling complex responsible for activation of caspase-8 and thus unable to undergo extrinsic apoptosis (see figure 5, chapter 4; Marini et al., 2003).

Furthermore, we demonstrated that DRE-induced apoptosis is caspase dependent as pre-treatment with the pan-caspase inhibitor ZVAD-fmk inhibited cell death in Jurkat cells (Malyshev et al., 2004). Exposure to DRE (0.4 and 0.6 mg/ml) for 48 h led to the destabilization of mitochondrial membrane potential, as

observed by a decrease in JC-1 dye accumulation (Figure 6). Decreased mitochondrial membrane potential is thought to result from truncation and activation of the pro-apoptotic protein Bid caused by activated caspase-8 (Belka et al., 2000). Truncated BID protein binds to pro-apoptotic protein Bax, which inserts into the mitochondrial outer membrane causing collapse of the mitochondrial membrane potential (Li et al., 1998).

Over the years there has been no report of toxicity in people taking dandelion extracts. To test the selectivity of our aqueous dandelion root extract, non-cancerous peripheral blood mononuclear cells were treated with DRE under the same conditions as Jurkat cells and examined for apoptotic morphology. Interestingly, these normal non-cancerous cells were unaffected by DRE treatment (Figure 7). This outcome may be due to the fact that the expression of Fas receptors is far greater in human leukemia cells than in normal cells (Kekre et al., 2005). This increased expression in leukemia cells may be the sensitizing factor for apoptosis induction in Jurkat cells by DRE, which is consistent with our results that DRE induces the death-receptor mediated pathway of apoptosis.

Our results clearly indicate that components of dandelion root extract act either alone or in combination with each other to induce formation of the death inducing signaling complex and subsequent extrinsic apoptosis selectively in human leukemia cells (Figure 8). The mechanism by which this extract induces apoptosis is being studied further to determine possible genomic interactions and to delineate the active component of DRE. These *in-vitro* results are a stepping-stone for the development of a more efficient mode of therapy and present a novel non-toxic alternative to conventional leukemia therapy.

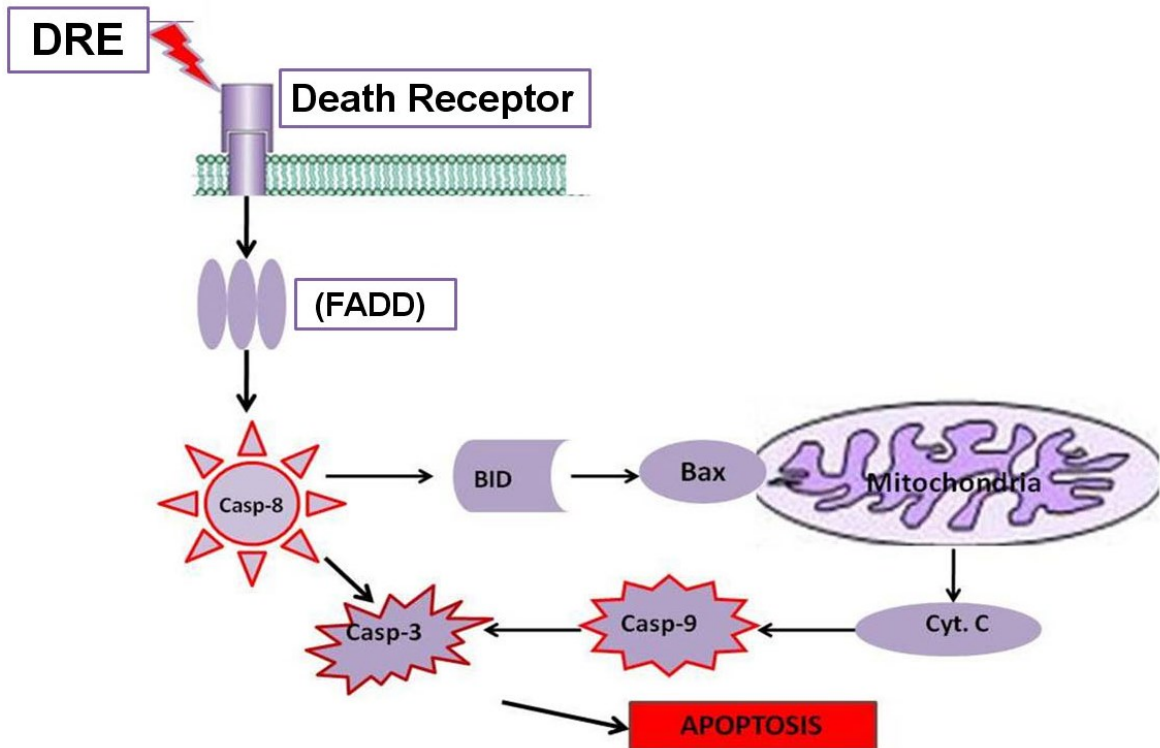


Figure 8: Graphical Representation of the Mechanism of Action of DRE in Human T Cell Leukemia (JURKAT). Following DRE treatment, there was rapid activation of caspase-8 in Jurkat cells, indicating the targeting of the death ligand/receptor dynamic. This presence of the Fas-Associated Death Domain (FADD) was required for the activation of caspase-8, which led to the activation of caspase-3 (directly or indirectly, through the truncation of Bid and the destabilization of the mitochondrial membrane). The subsequent activation of caspase-3 preceded the induction of apoptosis, characterized by known morphological and biochemical features of apoptosis.

Acknowledgements

We gratefully acknowledge Knights of Columbus Council 9671 for funding of this research project.

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CHAPTER 3

SELECTIVE INDUCTION OF APOPTOSIS AND AUTOPHAGY THROUGH TREATMENT WITH DANDELION ROOT EXTRACT IN HUMAN PANCREATIC CANCER CELLS

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Pancreas (2012), 41(7), 1039 – 1046

ABSTRACT

Pancreatic cancer has a 100% mortality rate; the aim of this study is to evaluate the efficacy of Dandelion Root Extract (DRE) in inducing apoptosis and autophagy in aggressive and resistant pancreatic cancer cells. The effect of DRE was evaluated using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) assay. Apoptotic cell death was confirmed by nuclear condensation by Hoechst staining, and externalization of phosphatidylserine to the outer leaflet of the plasma membrane by Annexin-V binding assay. Loss of mitochondrial membrane potential was observed using the JC-1 (5, 5', 6, 6'-tetrachloro-1,1', 3,3' tetraethylbenzimidazolylcarbocyanine iodide) dye. The induction of autophagy was detected using an MDC assay and this was confirmed by immunofluorescence for LC3-II.

BXPC3 and PANC-1 pancreatic cells were sensitive to aqueous DRE. This extract induces selective apoptosis in a dose and time-dependent manner. DRE caused the collapse of the mitochondrial membrane potential, leading to pro-death autophagy. Normal Human and Fetal Fibroblasts were resistant at similar doses.

In this study, we demonstrate that DRE has the potential to induce apoptosis and autophagy in human pancreatic cancer cells with no significant effect on non-cancerous cells. This will provide a basis upon which further research in the field of cancer treatment through DRE can be executed.

INTRODUCTION

In Canada, it is estimated that approximately 173 800 new cases of cancer will be diagnosed and 76 200 Canadians will die of cancer in 2010 (Canadian Cancer Society, 2010). The risk of developing this disease increases with age; nonetheless it can affect all ages. Pancreatic cancer, a very aggressive and highly resistant form of cancer, is a cancer that originates in the pancreas (Canadian Cancer Society, 2010). It is the fourth leading cause of cancer-related deaths in the world today (Castellanos et al., 2011). In 2010, it was estimated that 4000 new cases would be diagnosed and 3900 deaths due to pancreatic cancer in Canada.

Treatment for pancreatic cancers include the already known forms of chemotherapeutics such as 5-fluorouracil and gemcitabin (Skinner, 2010), surgery and radiation therapy. Although they show initial efficacy, these treatments do not remain effective for extended periods of time and are usually accompanied by severe side effects (Poplin et al., 2009; Kindler et al., 2010). Furthermore, in the early stages of pancreatic cancer there are no clear signs or symptoms (Tuvesan & Hanahan, 2011). Depending on the location of the tumour in the pancreas, signs and symptoms begin to appear once surrounding tissues become affected and surgical resection of the tumour is only successful if the disease is diagnosed in its early stages when the cancer has not metastasized (Castellanos et al., 2011). Early prognosis is therefore one of the leading areas of research in this field due to its difficult identification (Canadian Cancer Society, 2010). Most commonly, pancreatic cancer is diagnosed in very late stages and survival is dismal at best. With one of the highest mortality to incidence rate ratios

(Tempero et al., 2011), there is an important need to introduce a safe and effective mode of targeting both early and late stage pancreatic cancers.

One of the most common hallmarks of cancer involves the ability to evade the physiological process of programmed cell death, also known as apoptosis (Johnstone et al., 2002; Hanahan & Weinberg, 2011). Cancer cells have also been found to utilize autophagy, a catabolic process used to degrade cytoplasmic materials, as a pro-survival technique to overcome stressors such as starvation and chemotherapy (Rosenfeldt & Ryan, 2011). There has been a lot of controversy surrounding the field of autophagy, where it has been shown to play dual roles, as both a pro-survival and pro-death phenomenon (Rosenfeldt & Ryan, 2011). Recent studies have shown that pro-death autophagy and apoptosis have interconnected pathways and can be regulated by the same proteins, including the pro-apoptotic Bcl-2 family of proteins (Thorburn, 2008). It is therefore necessary to investigate these pro-death pathways so as to harness their properties for targeting various types of cancers.

In current cancer therapies, natural compounds such as Taxol (Paclitaxel) and Navelbine, have been widely utilized. However, many of these compounds are genotoxic or non-selective and therefore cause damage to normal cells as well (Mukherjee et al., 2001). Dandelions are very common weeds found in almost every part of the world (Koo et al., 2004), the leaves and root of which, have been studied for their effects on digestion and gastrointestinal diseases (Yarnell and Abascal, 2009). Recent investigations have shown that dandelion root extract (DRE) has the ability to selectively induce apoptosis in human melanoma and leukemia cells, with no toxicity to non-cancerous peripheral blood

mononuclear cells (Chatterjee et al., 2011; Ovadje et al., 2011). Whether DRE can induce programmed cell death in highly aggressive and resistant human pancreatic carcinoma cell lines remains unknown.

In this study, we show the efficacy of Dandelion Root Extract in inducing apoptosis in a dose and time dependent manner in aggressive human pancreatic cell lines (BxPC-3 and PANC-1). In parallel, similar experiments in non-cancerous Normal Human Fetal Fibroblasts indicate that DRE selectively targets human pancreatic cancer cells, confirming results from previous studies (Chatterjee et al., 2011; Ovadje et al., 2011). Early activation of caspase-8 and subsequent activation of caspase-3 indicate that apoptosis induction by DRE is due to activation of the extrinsic pathway of apoptosis. Interestingly, we observed that DRE induced a pro-death form of autophagy in human pancreatic cancer cells. This induction of autophagy corresponds with the destabilization of the mitochondrial membrane potential, which was observed after treatment with DRE. Through revival experiments, it was shown that the signal to commit suicide was retained once the cells had been exposed to DRE. Although we are unsure of the active ingredients in DRE responsible for the induction of cell death, our work provides novel evidence of the selective anti-cancer effects of DRE in human pancreatic cancer cells.

MATERIALS & METHODS

Dandelion Root Extraction and Cell Treatment

Dandelion root extract (DRE) was prepared from the roots of collected dandelion weeds found in localized open grasslands. Following repeated washing with water, the roots were air dried and ground to a fine powder. The powder was then homogenized in 200 mL of distilled water and filtered at room temperature using NITEX nylon mesh filters (LAB PAK; Sefar BDH Inc., Chicoutimi, Quebec, Canada). The filtrate was then spun down at 8000 x *g* at 25°C for 5 minutes and the supernatant was filtered by 0.45 µm and 0.22 µm filters. The final filtrate was lyophilized and 100mg/ml stock aqueous extract was prepared.

Cell Lines and Cell Culture Maintenance

The cancer cell line used in this study was human pancreatic cancer BxPC3 and PANC-1 which were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. The normal cell line used were normal human fibroblasts (NHF) cells, which were obtained from Coriell Institute for Medical Research, USA. BxPC3 cells were grown in RPMI-1640 media supplemented with 15% fetal bovine serum (FBS) and 10 mg/mL Gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). The normal human fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) obtained from Sigma Chemical Company, Mississauga, Ontario, Canada, supplemented with 10% FBS, 2mM L-Glutamine, and 10 mg/mL Gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). PANC-1 cells were grown in DMEM media supplemented with 15% fetal bovine serum (FBS) and 10 mg/mL Gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada). All cell lines were grown and maintained at 37°C,

95% humidity, and 5% CO₂ in a Forma Scientific CO₂ incubator equipped with a HEPA filter (Forma Scientific Inc., Marietta, Ohio, USA). All cell lines were grown to approximately 50% confluence and were treated with either Fresh water Dandelion Root Extract or lyophilized extract (0.5 mg/mL to 7.5 mg/mL). Treated cells were allowed to grow over time up to 96 hours after treatment and was examined using several staining assays as described below.

CELL STAINING

Hoechst Staining

To visualize apoptosis in the cells, a photosensitive DNA-binding stain called Hoechst 33342 (Sigma Chemical Company, Mississauga, Ontario, Canada) was incubated with a final concentration of 10 µM with cells at room temperature for approximately 10 minutes. Following incubation, the cells were viewed with a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany) and Northern Eclipse Version 8.0 Imaging software at 100X and 400X objective. Apoptotic cells are characterized by brightly stained, condensed nuclei as compared to larger, round and less-brightly stained non-apoptotic cells. The average percent apoptosis was calculated by counting and averaging the number of brightly condensed nuclei over the total number of cells observed over 5 fields at 400X objective.

Annexin-V Binding Assay

An early marker of apoptosis is the flipping of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane, which can be visualized through the use of an Annexin-V binding assay. Cells treated with various doses of DRE were scraped using a rubber policeman to removed adherent cells from

the plate bottom and washed twice in phosphate buffered saline (PBS). Following washing with PBS, the cellular pellet was resuspended in Annexin-V binding buffer (10mM HEPES, 10mM NaOH pH 7.5, 140mM NaCl, 2.5mM CaCl₂, 50 nM Sucrose), along with the Annexin-V Alexa Fluor® 488 conjugate (Sigma Chemical Company, Mississauga, Ontario, Canada), which binds to phosphatidylserine, at 1:50 with respect to the buffer. This was allowed to incubate in low light conditions at room temperature for approximately 15 minutes. Hoechst 33342 dye was also incubated with the cells for the final 10 minutes in order to visualize nuclear morphology of positively stained Annexin-V cells. The cells were then visualized and images were taken using a fluorescence microscope (Leica DM IRB) at 400X objective.

Trypan Blue Exclusion assay

In order to quantify the number of viable Pancreatic Cancer cells, Trypan blue staining was used to visualize the cells. Trypan blue is a dye, which stains only dead cells whose plasma membranes are permeable to the dye. A 1:1 mixture of cell suspension and 0.4% Trypan blue dye (Sigma Chemical Company, Mississauga, Ontario, Canada) was loaded onto a haemocytometer (Hausser Scientific, USA) where non-viable blue-stained cells and viable unstained cells were counted. The average number of viable cells was expressed as number of cells/mL.

Cell Viability Assay

In order to determine cell viability, a colorimetric dye called WST-1 ([2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, Roche diagnostics, Mannheim, Germany) was used. In metabolically active, viable cells,

WST-1 produces formazan, which can be measured for absorbance. First, a Trypan blue count was done in order to seed equal number of cells into 96 well plates. Approximately 5000 cells were seeded with a total volume of 200 μ L in each well and treated following attachment of the cells overnight. Following treatment, WST-1 dye was added to each well (20 μ L per 200 μ L) and incubated at 37°C for 4 hours. Absorbance was read at 590nm with a Victor™ Plate Reader (Wallac, Turku, Finland). Absorbance values of the treated cells were calculated as a percentage of absorbance values of control.

Mitochondrial Membrane Potential

In order to visualize mitochondrial membrane permeability in BxPC-3 cells, JC-1 dye (5, 5, 6, 6-tetrachloro-1, 1, tetraethylbenzimidazolocarbo-cyanine iodide, Sigma Chemical Company, Mississauga, Ontario, Canada) was incubated with treated the cells. JC-1 forms aggregates in mitochondria with intact membrane potential. The dye was diluted 2:50 in PBS to a concentration of 0.5 μ M and was incubated with treated cells for 45 minutes at 37°C. During the final 10 minutes of incubation, Hoechst dye was added to the cells. Following incubation, the cells were then visualized and images were taken using a fluorescence microscope at 400x objective. Tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR, Mississauga, ON, Canada) was used for PANC-1 cells to measure Mitochondrial Membrane Potential. PANC-1 cells were grown on coverslips. After treatment period, the cells were incubated for 45 minutes with 200 nM TMRM at 37°C. The coverslips were then placed on microscope slide and pictures were taken at 40x objective using inverted fluorescence microscope (Leica DM IRB).

MDC Assay

To detect autophagy, an MDC (monodansylcadaverine, Sigma Chemical Company, Mississauga, Ontario, Canada) assay was performed. MDC is a fluorescent compound that is incorporated into autophagic vacuoles and produces a bright punctate stain. Propidium iodide (PI) is used as a co-stain with MDC to visualize dead cells. First, 100 μ M MDC is diluted 1:25 in PBS and added to the cells along with PI, then incubated at 37°C for 15 minutes. Following incubation, the cells were visualized and images were taken using a fluorescence microscope at 400X objective.

Immunocytochemical Analysis

BxPC3 cells were plated on poly-L-lysine coated coverslips and treated with DRE for 48 hours to stimulate the induction of autophagy. Treated cells were fixed in cold methanol, then cold acetone for 5 seconds and allowed to air dry. Following fixation, the cells on the coverslips were incubated with PBS containing 0.05% Tween 20 and 5% Normal Goat Serum for 10 minutes. Cells were then incubated with anti-LC3 antibody (1:500 dilution) (Novus Biologicals, Cat. No. NB100-2220, Littleton, CO, USA), overnight at 4°C. The following day the cells were subjected to two five-minute washes with PBS containing 0.05% Tween20 and then incubated with anti-rabbit antibody, Alexa Fluor[®] 488 conjugate (1:1000) (Cell signaling Technologies, Cat. No. 4412, Pickering, ON. CA), for an hour at room temperature. Following two washes with PBS-Tween20 for 5 minutes, cells were counterstained with Hoechst for 10 minutes and visualized and images were taken using a fluorescence microscope at 400x objective.

Caspase Activity

The Caspase assays were performed using a previously published method (Naderi et al., 2003). To determine caspase activity, the total protein from BXP-3 and PANC-1 cell lysates were incubated with the fluorogenic substrates corresponding to the substrate cleavage site, specific for each caspase, DEVD-AFC for Caspase-3 and IETD-AFC for Caspase-8 and 9. The fluorescence was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm using a Spectra Max Gemini XS (Molecular Devices, Sunnyvale, California). Caspase activity was calculated as activity per μg protein and protein concentration was determined with BioRad protein assay reagent (BioRad, Mississauga, Ontario) using bovine serum albumin (BSA) as a standard. Readings were analyzed using GraphPad Prism 5.0 288 software.

Statistical analysis:

All experiments were performed in triplicates and the results expressed as mean \pm S.D. Statistical analysis was performed with GraphPad Prism 5.0 288 software.

RESULTS

Effect of Dandelion Root Extract on Highly Aggressive Pancreatic Cancer Cells.

In order to determine the effect of DRE on pancreatic cancer cells, two different pancreatic cancer cell lines (BxPC-3 and PANC-1) were treated with DRE at doses of 0.5, 1, 2.5, 5, and 7.5 mg/mL and examined at different time points. To visualize morphological features of apoptosis induction in BxPC3 and PANC-1 following DRE treatment, Hoechst, a dye that binds to the minor groove of DNA, was used to observe nuclear condensation. An increase in nuclear condensation was observed with an increase in concentration, as well as an increase in the time of exposure to DRE (Figure 1A). Apoptotic cells and non-apoptotic cells were manually counted and quantified as average percentage apoptosis in both cell lines. The average percent apoptosis was found to increase with increasing concentration of DRE over time (Figure 1B).

An early marker of apoptosis is the reorganization of the cell's membrane, which leads to externalization of the phosphatidylserine from the inner leaflet to the outer leaflet of the cell membrane. This can be observed with Annexin V binding assay. Following 72-hour treatment with DRE, there is an increase in bright green fluorescence indicative of apoptosis in a dose and time dependent manner in DRE treated BxPC-3 cells. Annexin-V positive staining was observed as early as 24 hours in DRE treated PANC-1 cells (Figure 1C).

Quantitative cell viability as a function of cell metabolism was assessed through WST-1 assay. DRE led to a 60% decrease in cell viability 48 hours following treatment with only 20% of BxPC-3 cells remaining viable after 96 hours

of exposure to DRE. Similar results were observed in PANC-1 cells; with a gradual decrease in cell viability with an increase in dose of DRE compared to untreated control cells (Figure 1D).

According to results of both qualitative and quantitative analyses, the EC_{50} for both pancreatic cancer cell lines is around 5.0 mg/mL.

In order to investigate the fate of cells exposed to DRE but show no apoptotic morphology following treatment for 48 hours (40%), BxPC-3 cells were plated and treated for 48 hours at 5.0mg/ml and 7.5mg/ml DRE. After 48 hours, equal numbers of BxPC-3 cells were seeded and allowed to grow in drug-free media, with growth observed every 24 hours by trypan blue exclusion assay. The untreated control cells were able to maintain constant growth over time. However, the DRE treated cells were unable to revive growth in fresh media as time progressed. Analysis reveals a significant decrease in the number of cells per mL at 96 hours post-treatment from 700 000 to 10 000 cells between the untreated control cells and the cells treated with DRE, respectively (Figure 1E). Similar results were observed for the PANC-1 cell line following revival after treatment with DRE for 48 hours.

These results indicate that DRE led to the loss of cell viability and effectively induced apoptosis in human pancreatic cancer cells in a dose and time dependent manner. More importantly, cells that had been exposed to DRE retained the signal to commit suicide even after the removal of DRE.

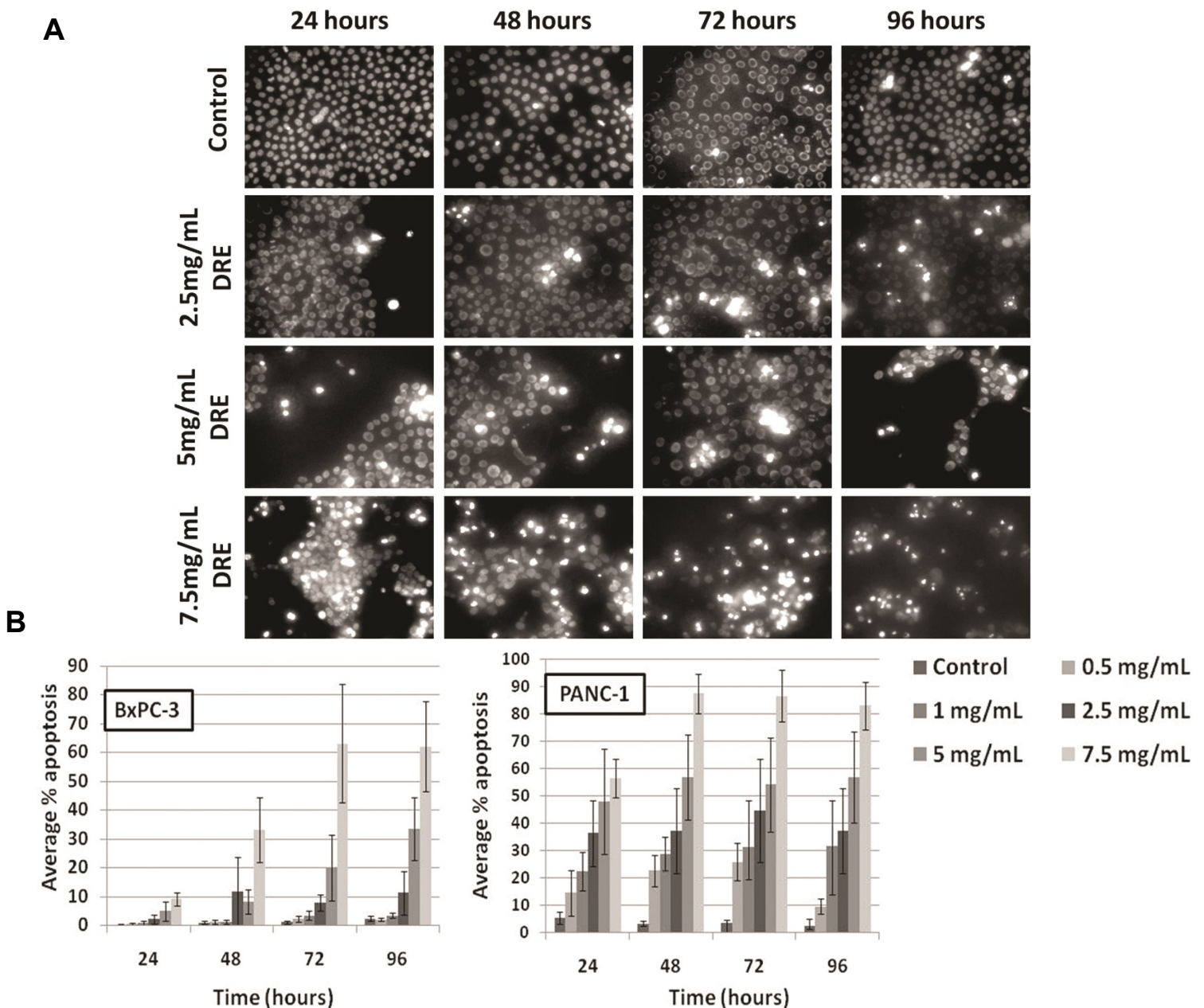


Figure 1: Efficacy of Dandelion Root Extract in inducing apoptosis in human Pancreatic cancer cells. (A). An increase in brightly stained, condensed nuclei indicative of apoptosis, is seen with increasing doses and time periods following treatment with DRE. (B). Manual quantification of Hoechst pictures. In comparison to the results seen in A, there is an increase in the average % apoptosis in a dose and time dependent manner.

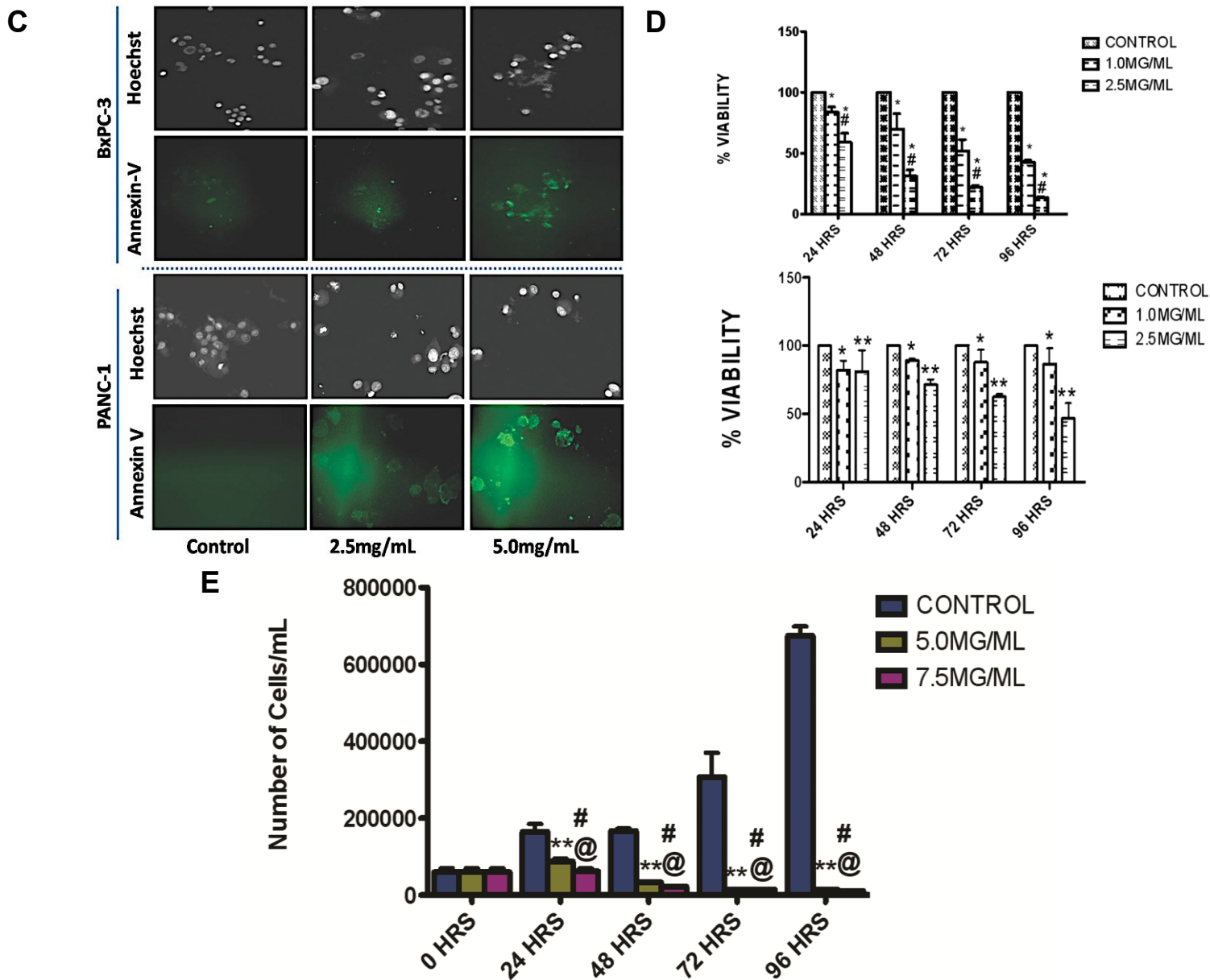


Figure 1: Efficacy of Dandelion Root Extract in inducing apoptosis in human Pancreatic cancer cells. (C). Brightly stained apoptotic cells seen in Hoechst images are visualized by Annexin-V binding to exposed phosphatidylserine in the lower panel of images in both BxPC-3 and PANC-1 cells. (D). Treatment with DRE led to a drastic decrease in the percent of metabolically viable cells (as a % of control) after treatment at the indicated time points; (i) BxPC-3 (* $p < 0.05$; *# $p < 0.0001$) and (ii) PANC-1 (* $p < 0.05$; ** $p < 0.0001$) hours with DRE. (E). Exposure to of BxPC-3 cells to DRE for 48 hours halted the cell growth following removal of treatment. Cells were unable to revive growth after exposure to DRE and retained the signal to commit suicide after removal of DRE (**, #@ $p < 0.0001$).

Repeated Doses of DRE at Lower Concentrations is More Effective than a Single Low/High Dose in Inducing Apoptosis in Human Pancreatic Cancer Cells

Any treatment at high doses can be toxic and as can be observed from our previous results in figure 1, higher doses of DRE were required to induce apoptosis in both pancreatic cancer cell lines. Treatment of these cells with repeated doses of low concentrations of DRE showed a higher efficacy than one single high dose. Exposure of BxPC3 (Figure 2A) and PANC-1 (Figure 2B) to low concentration (0.5, 1.0, and 2.5mg/mL) of DRE for 48 hours and then a second treatment for another 48 hours reveals that two treatments are more effective than treating once with a high dose. Comparing the control and 2.5mg/mL treated phase images shows a drastic difference in the number of dead cells following the second 48 hour treatment (Figure 2). These results indicate multiple doses of low concentrations of DRE are more effective in inducing apoptosis than one single high dose in human pancreatic cancer cells.

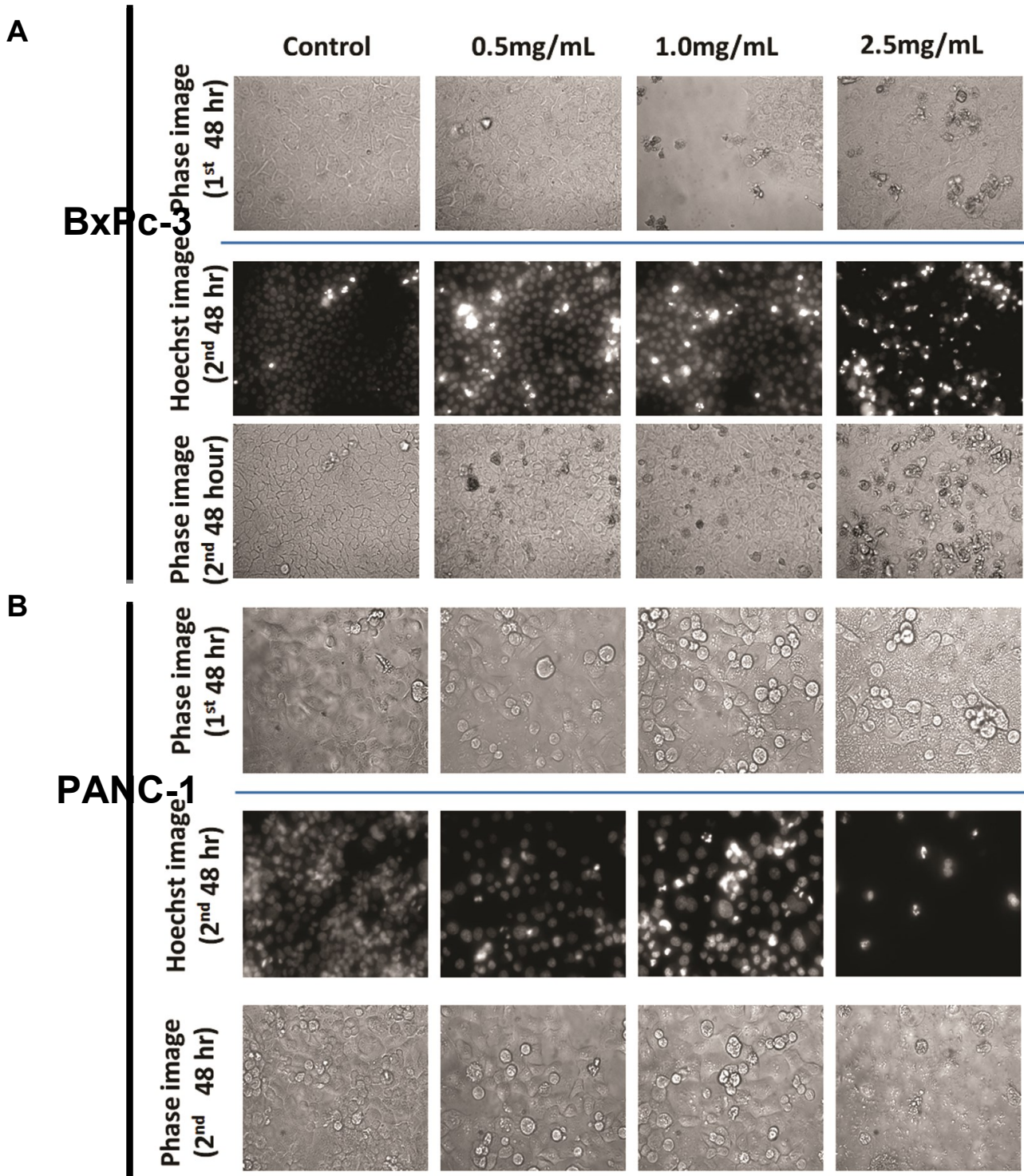


Figure 2: Repeated doses of DRE at lower concentrations in human pancreatic cancer cells: Treating BxPC-3 (A) and PANC-1 (B) twice for 48 hours at doses of 0.5, 1, and 2.5mg/mL has a significantly higher effect than a single treatment for 48 hours. Following the second 48 hour treatment, cells were stained with Hoechst 33342 and fluorescence images were obtained at 400X magnification.

DRE Destabilizes the Mitochondrial Membrane Potential in Human Pancreatic Cancer Cell Lines

In order to determine the mechanism of action of DRE, we wanted to observe the effect of DRE on the mitochondria of human pancreatic cancer cells. Both the extrinsic and intrinsic pathways of apoptosis converge on and permeabilize the mitochondria leading to the release of pro-apoptotic factors (Fadeel & Orrenius, 2005). Evaluation of mitochondrial membrane potential is done through visualization of red fluorescence of JC-1 or TMRM mitochondria permeable dyes in healthy, intact mitochondria. DRE led to the destabilization of the mitochondrial membrane potential in BxPC-3 cells as observed by the loss of red aggregates of JC-1 dye in DRE treated cells after 48 hours of treatment (Figure 3A). Similar results were observed in DRE treated PANC-1 cells, stained with TMRM (Figure 3B). These results indicate that the mitochondria of highly aggressive pancreatic cells are vulnerable to DRE, leading to permeabilization of the mitochondrial membrane potential, characteristic of apoptosis.

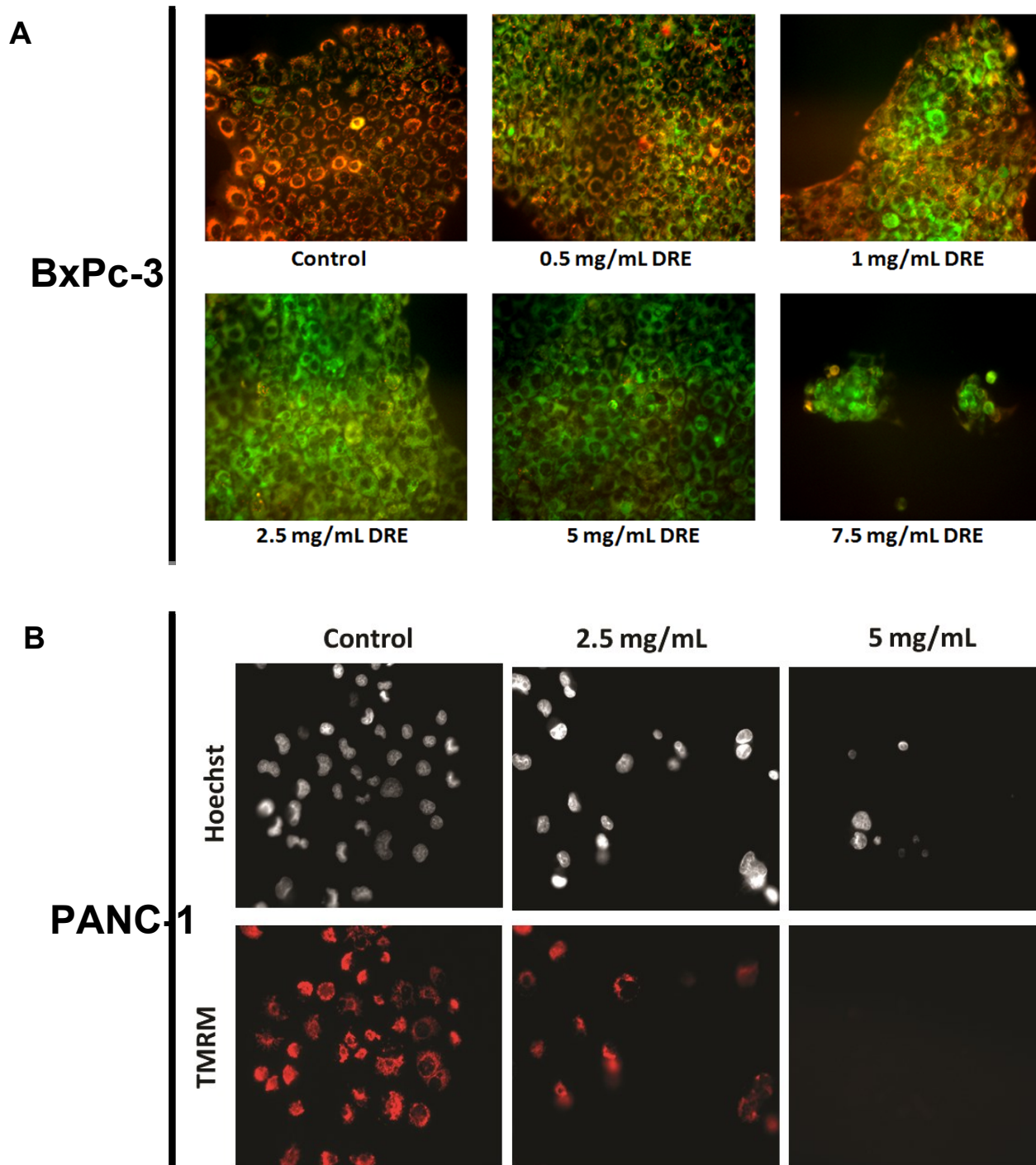


Figure 3: DRE destabilized the mitochondrial membrane potential of human pancreatic cancer cells. Pancreatic cancer cells were grown on coverslips and treated with DRE for 48hours and stained with either JC-1 (A) or TMRM (B), which are mitochondrial permeable dyes that aggregate in healthy mitochondria and fluoresce red or remain in the cytosol in their monomeric form (Green). Fluorescence microscopy was used to assess membrane potential. The loss of dye aggregation is observed in DRE-treated BxPC-3 (A) and PANC-1 (B) cells.

DRE activates the death-receptor mediated extrinsic pathway of apoptosis

The loss of mitochondrial membrane potential in itself does not provide us with information regarding the pathway of apoptosis induction. In order to get more information on the pathway of apoptosis induction, which would shed more light on the mechanism of induction of apoptosis by DRE, we analyzed the chronological activation of caspases. BxPC-3 cells were treated with 2.5 mg/ml DRE, at the indicated time points, and subsequently analyzed for the activation of caspases, using substrates specific to each type of caspase. Our results show that there is a very rapid activation of caspase 8 in BxPC-3 cells (Figure 4), suggesting an extrinsic mode of cell death. Similar results were observed in PANC-1 cells.

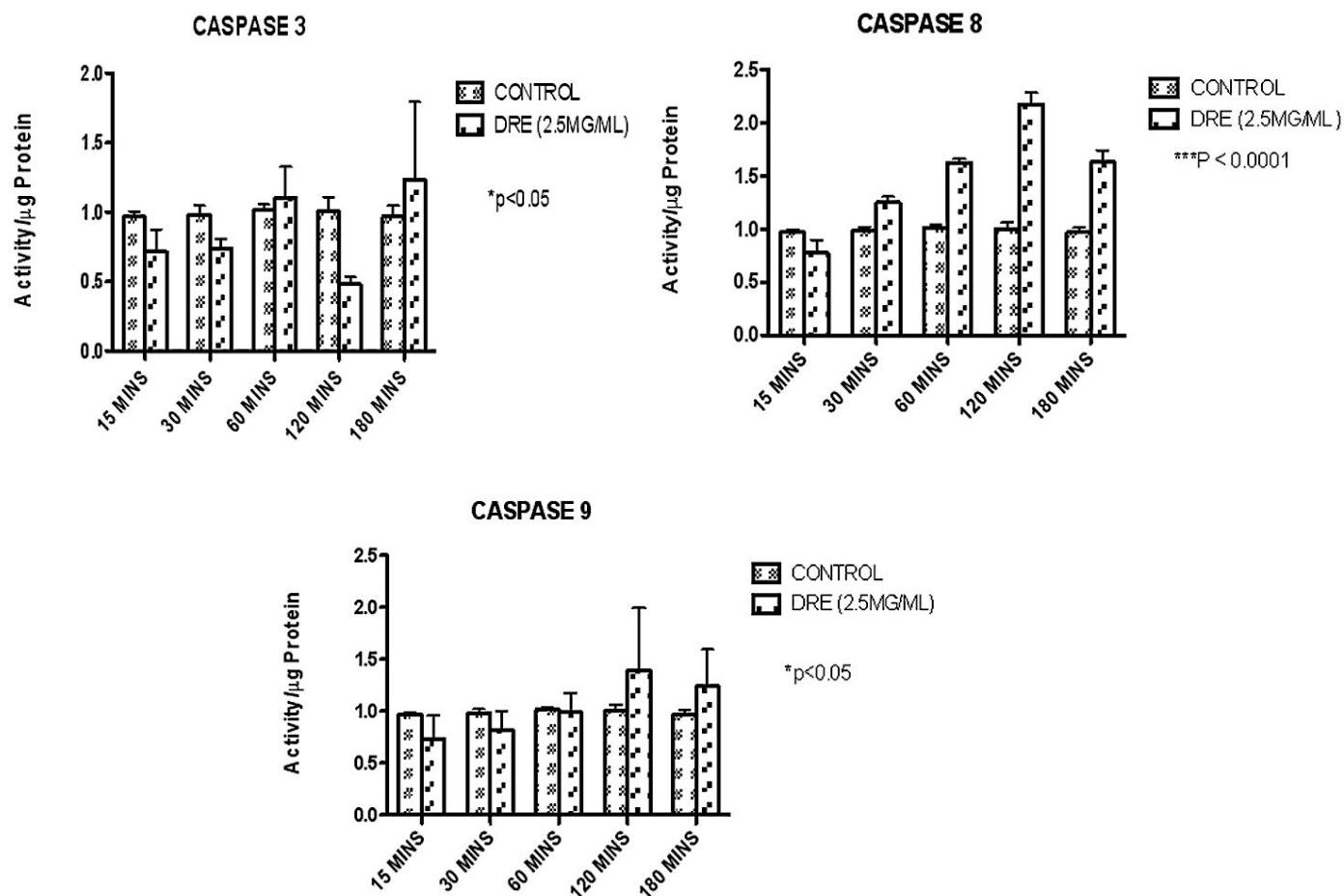


Figure 4: DRE induces extrinsic apoptosis in human pancreatic cancer cells. BxPC-3 cells were treated with DRE at the indicated time points. Subsequently, cells were collected, washed and incubated with lysis buffer to obtain cell lysate. The cell lysate was incubated with caspase substrates, specific to each caspase (3, 8 and 9) and incubated for an hour. Fluorescence readings were obtained using a spectrofluorometer. An average of 6 readings per well and a minimum of three wells were run per experiment. The results here are reported as activity per μg of protein (in fold) and the average of three experiments are shown

DRE Induces Pro-Death Autophagy in Human Pancreatic Cells

Along with apoptosis, autophagy is a physiological process of cell death involved in the maintenance of cellular homeostasis (Gottlieb et al., 2009). Cells undergoing autophagy form autophagic vacuoles, which can be visualized through the use of MonoDansylCadaverine stain (Niemann et al., 2000). The corresponding PI stains reveals which of the autophagic cells are dead, suggesting a pro-death capacity of DRE in pancreatic cancer cells.

Tamoxifen is known to induce pro-survival autophagy (Qadir et al., 2008), and therefore is used as a positive control. We observed a clear induction of autophagy in the treated cells at increasing doses of DRE over time as compared to the positive tamoxifen control in BxPC-3 cells. (Figure 5A). When autophagy is induced, microtubule-associated protein 1 light chain 3 (LC3-I), usually localized in the cytosol, is conjugated to phosphatidylethanolamine. This conjugation results in the lipidated protein LC3-II that is recruited to autophagosomal membranes and can be used as another marker for autophagy detection (Ma et al., 2011).

To confirm the results of autophagy seen by MDC staining, immunocytochemical analysis was used to detect the conversion of LC3-I to LC3-II. BxPC-3 treated with DRE at 2.5mg/mL had a similar effect on the conversion of LC3-I to LC3-II, compared to the tamoxifen treated cells. Both the tamoxifen and DRE treated cells incubated with antibody against LC3-II gave positive results, which confirmed the autophagy observed in 5a (Figure 5B). Treated PANC-1 cells with DRE doses of 2.5 mg/mL showed similar results.

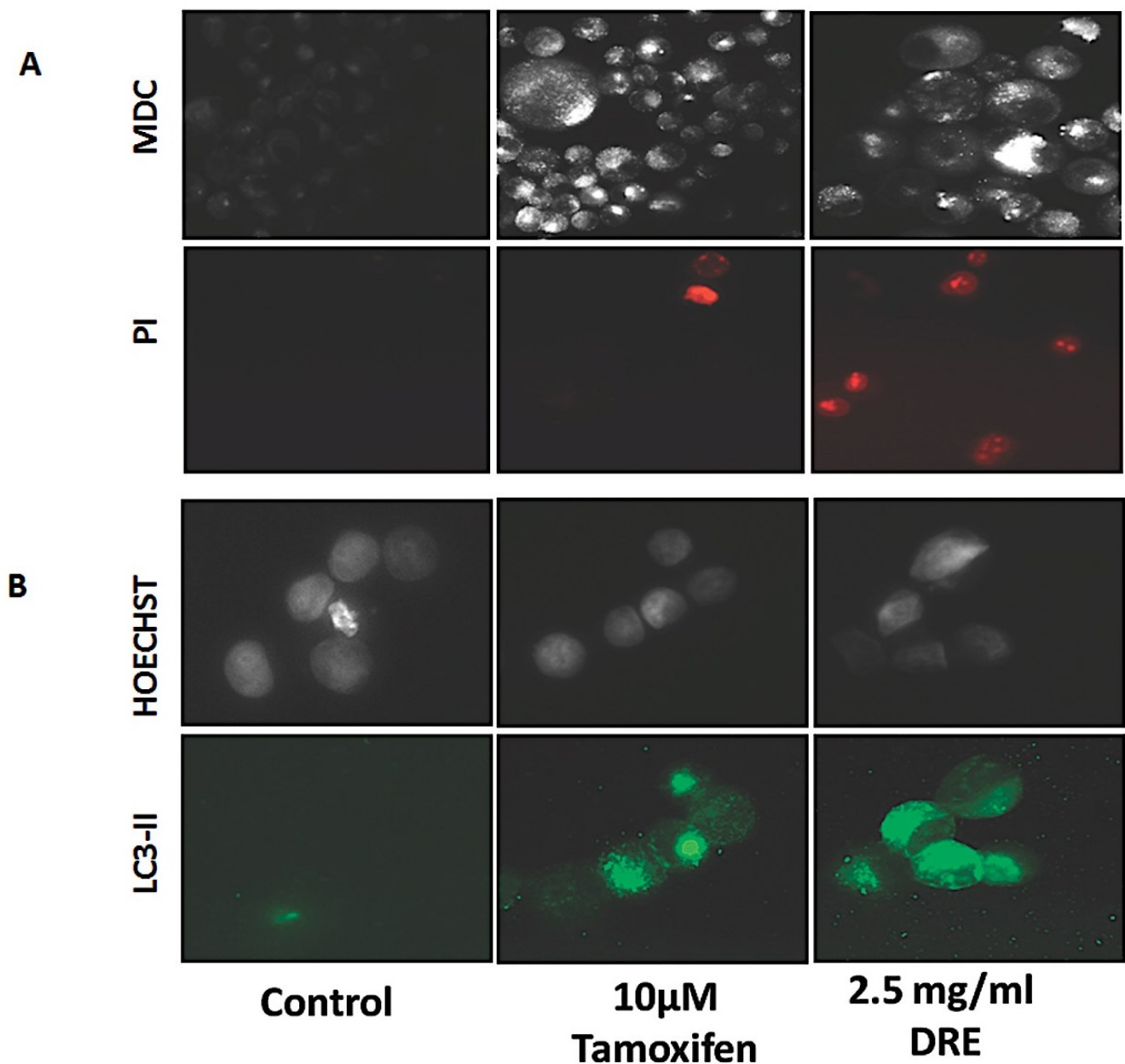


Figure 5. DRE induces pro-death autophagy in human pancreatic cancer cells. (A). BxPC-3 cells were seeded onto coverslips in six-well plates and treated with DRE at 2.5mg/mL for 48 hours. Tamoxifen is a known inducer of pro-survival autophagy and was used as a positive control for autophagic induction in pancreatic cancer cells. Following treatment, cells were stained with monodansylcadaverine and counterstained with Propidium Iodide to indicate the dead cells. (B). BxPC-3 cells were plated on coverslips and treated with DRE or Tamoxifen for 48 hours. Following treatment, coverslips were incubated with primary antibody specific to LC3-II overnight at 4°C, then incubated with appropriate secondary antibody for an hour at room temperature. The cells were counterstained with Hoechst and imaged.

DRE is not Toxic to Non-Cancerous Normal Human Fibroblasts

Dandelions have been used as an herbal medicine for centuries and there has been no reported evidence of toxicity (Kemper et al., 1999). In order to further investigate whether DRE selectively targets cancerous cells, Normal Human Fibroblasts (NHF) were treated with DRE under the same conditions as the pancreatic cancer cells. Treatment of non-cancerous NHFs with DRE at doses of 1.0, 2.5 and 5 mg/mL for 96 hours reveals no morphological signs or characteristic of apoptosis as seen by Hoechst staining (Figure 6; top panel). In comparison to pancreatic cancer cells treated with DRE, NHFs retain their viability in the presence of DRE as seen through WST-1 cell viability assay (Figure 6; bottom panel). However, the treated cells are not as viable as the untreated control cells and plateaus at approximately 70% cell viability. The non-cancerous NHF cells do not undergo apoptosis in the presence of DRE. These results reveal that DRE is non-toxic to non-cancerous cells and selectively targets cancerous cells.

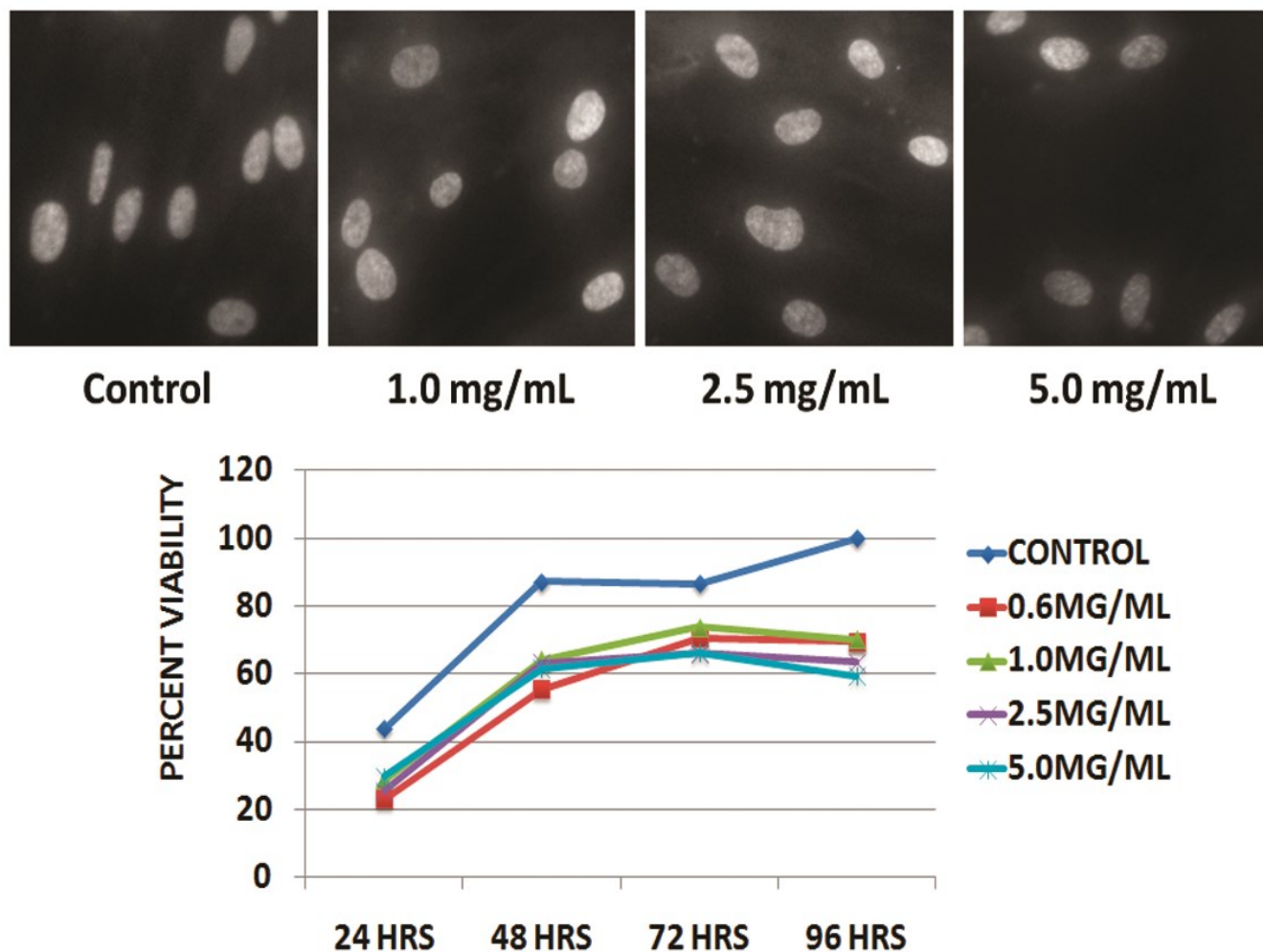


Figure 6: DRE has selective toxicity to human pancreatic cancer cells: Normal Human Fibroblasts were treated with DRE at increasing concentrations (1.0 to 5.0 mg/ml) for 96 hours. The cells were then stained with Hoechst dye for nuclear morphology and fluorescent images were obtained. No nuclear condensation, characteristic of apoptosis, is observed in NHF treated cells (Top panel). NHFs were seeded in a 96-well plate at 4000 cells/well and treated with increasing concentrations of DRE, before incubating with the WST-1 viability dye. WST-1 cell viability assay was then used to monitor the viability of NHFs after treatment with DRE every 24 hours, to a maximum of 96 hours subsequent to treatment (Bottom panel). Unlike in pancreatic cancer cells, increasing doses of DRE did not drastically affect the viability of normal human fibroblasts.

DISCUSSION

Natural compounds have shown significant anti-cancer activity, with reports showing their potential to inhibit the progression of several cancers by interfering with several key mechanisms employed by cancer cells (Boik, 2000). In this study, we have shown that the root extract of the very common weed, dandelions, have the ability to selectively induce apoptosis and autophagy in very aggressive human pancreatic cancer cells.

The high mortality rate of pancreatic cancer patients is most likely due to the fact that the disease is usually asymptomatic until it metastasizes and becomes invasive, at which point it is incurable (Koostra et al., 2008; Maitra & Hruban, 2008). Therefore, a majority of pancreatic cancer patients presented in this state of advanced and/or metastatic condition are inoperable (Maitra & Hruban, 2008). A lot of chemotherapeutic agents have been tested for their efficacy in treating this disease but only a few of them have shown moderate activity and produce very little survival benefit and severe side effects (Longo et al., 2008).

We observed that the exposure of pancreatic cancer cells to Dandelion Root Extract (DRE) led to the loss of cell viability followed by the induction of apoptosis in a dose and time dependent manner. More importantly, we looked at the effect of initial exposure to DRE on the ability of these cells to revive growth. After treatment for 48 hours, cells were removed from the treated media and replated in fresh, drug-free media and allowed to grow. We observed that this initial exposure to DRE halted the ability of the cells to revive growth, indicating that these aggressive pancreatic cells retained the signal to commit suicide, induced

by DRE (Figure 1).

Over the ages, people have relied on dandelions for therapeutic benefits in the treatment of various diseases ranging from diarrhea and other gastrointestinal diseases to more serious diseases, like hepatitis. To date, there has been no report of toxicity linked to usage of dandelion extracts (Sigstedt et al., 2008; Yarnell & Abascal, 2009). Previous studies in our lab have shown that DRE is non-toxic to non-cancerous peripheral blood mononuclear cells obtained from healthy volunteers (Chatterjee et al., 2011). To confirm these results, as well as corroborate what has been seen for centuries, we tested DRE on non-cancerous Normal Fetal Fibroblasts (NFF) in parallel experiments. As expected, there was no significant effect on the viability of NFFs. These cells did not show apoptotic morphology, characterized by nuclear condensation and fragmentation (Figure 6). These results indicate that DRE is specific in its targeting of human cancer cells, without affecting the normal cells tested.

It is very well known that a lot of chemotherapies have very severe side effects, mostly due to the fact that these drugs are not specific to cancer cells and are sometimes only effective at very high doses. Also, cancer cells, especially pancreatic cancer cells, develop resistance to conventional chemotherapies (Maitra & Hruban, 2008). As mentioned earlier, the EC_{50} for DRE in highly aggressive pancreatic cancer cells is between 1.0 (BxPC-3) and 2.5 mg/mL (PANC-1) between 24 and 48 hours. Interestingly, we found that repeated treatment of pancreatic cancer cells with lower doses of DRE led to a greater induction of apoptosis than the single high dose (Figure 2A and B). This might indicate that the active ingredient in the extract are not stable or effective for long

periods in the cells and a second dose may be required to effectively induce apoptosis in the cells that remain after the first treatment. In this way, repeated treatment with low dose may overcome the aggressiveness and resistance of these cells to DRE. Although DRE has shown no significant toxicity to non-cancerous cells, these results indicate that giving a repeated dose of DRE at lower concentrations is more effective as well as reducing the chance of side effects (if any) seen in high dose chemotherapies.

Not many studies have been done to elucidate the mechanism of action of natural extracts, specifically DRE, for their efficacy for disease treatment. We have previously shown that DRE targets the death receptor mediated pathway of apoptosis in human leukemia (Ovadge et al., 2011) and aggressive human melanoma cells (Chatterjee et al., 2011). Previously, pancreatic cancer cells have been shown to express Fas and TRAIL receptors (Hasel et al., 2001; Siegmund et al., 2007). We hypothesize that some components of DRE may imitate death ligands and might interact with the death receptors, activating the extrinsic pathway of apoptosis. In this study, we also confirmed this by observing the activation of caspases after DRE treatment (Figure 4). These results corroborate the previous results obtained. Both the extrinsic and intrinsic pathway of apoptosis converges on the mitochondria (Fulda & Debatin, 2006).

The activation of both pathways leads to the permeabilization of the mitochondrial membrane for the release of pro-apoptotic factors such as apoptosis inducing factor (AIF), cytochrome C and endonuclease G, which are involved in the execution of apoptosis (Fulda & Debatin, 2006). In this study, we observe the loss of mitochondrial membrane potential after caspase-8 activation,

following treatment with DRE (Figure 3A and B). This loss was seen as early as 48 hours, before we see the bulk of apoptotic cells, indicating that DRE may indirectly involve mitochondria destabilization in pancreatic cancer cells for the execution of apoptosis. Mitochondrial destabilization could lead to increased Reactive Oxygen Species (ROS) (Fulda & Debatin, 2006).

Autophagy could be induced by the presence of such dysfunctional mitochondria, a process of “self-eating” activated under conditions of starvation and stress. Under these conditions, the cells attempt to deal with stress (such as damaged organelles and proteins) by engulfing and degrading these organelles and proteins and recycling the materials obtained from the degradation. Prolonged exposure to stressors lead to prolonged degradation of organelles and proteins eventually leading to cell death caused by autophagy (Rosenfeldt & Ryan, 2011). There are also studies that have shown that molecular pathways involved in programmed cell death type I (apoptosis) and type II (autophagy) are interconnected and proteins involved in one pathway, could be involved in the other (Thorburn, 2008). Indeed, we observed the induction of autophagy by DRE in both pancreatic cancer cells, concurrent with mitochondrial membrane destabilization (Figure 5).

We therefore hypothesize that components found in DRE activate the death receptor mediated extrinsic pathway of apoptosis, which involves the early activation of caspase-8, followed by activation of the executioner caspase-3 as well as cleavage of BID and mitochondrial destabilization. Damaged mitochondria and the generation of ROS could therefore lead to the induction of pro-death autophagy, coupled with apoptosis for the cell death we observe 96 hours after

exposure of highly aggressive and resistant human pancreatic cancer cells to DRE.

At this point of the study, we are aware of some limitations to this work. The active ingredient responsible for the anticancer activity in DRE has not yet been identified; although natural products are usually considered to be complex botanicals and activity is not due to a single active ingredient (Foster et al., 2005). It is possible that the crude aqueous dandelion root extract contains multiple compounds working together in unison to promote its selective anticancer activity. We have standardized a protocol for extraction and after every extraction process, we assay for activity of the extract. The different batches of root extracted have shown similar activity throughout. More importantly, preliminary HPLC analysis of our extract has shown the presence of compounds (triterpenes and sesquiterpenes) previously reported to be present in dandelions (data not shown). Since natural aqueous DRE has been used as a traditional medicine for other ailments, and is not associated with any toxicity, it represents a safer potential form of therapy for pancreatic cancer treatment. In an attempt to ensure that our experiments are well controlled, we have standardized an extraction procedure so as to ensure we have approximately equal amounts of extracts in each treatment. Enrichment of the apoptosis inducing fraction of DRE, characterization of compounds in this fraction and evaluating its efficacy in an *in-vivo* model of pancreatic cancer, are the obvious next steps currently in progress in our laboratory.

Acknowledgement:

We would like to dedicate this work to the memory of Kevin Couvillon who lost his fight to cancer in 2010. This work is supported by the Knights of Columbus Chapter 9671 (Windsor, Ontario), Seeds 4 Hope grant from Windsor and Essex County Cancer Centre Foundation for their financial support to this project. We would like to acknowledge the hard work of Ms. Sabrina Ma for editing and proof-reading this manuscript

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CHAPTER 4

EFFICIENT INDUCTION OF EXTRINSIC CELL DEATH BY DANDELION ROOT EXTRACT IN HUMAN CHRONIC MYELOMONOCYTIC LEUKEMIA (CMML) CELLS

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PloS One (2012), 7(2), e30604. doi:10.1371/journal.pone.0030604

ABSTRACT

Chronic Myelomonocytic Leukemia (CMML) is a heterogeneous disease that is not only hard to diagnose and classify, but is also highly resistant to treatment. Available forms of therapy for this disease have not shown significant effects and patients rapidly develop resistance early on in therapy. These factors lead to the very poor prognosis observed with CMML patients, with median survival duration between 12 and 24 months after diagnosis. This study is therefore centered around evaluating the selective efficacy of a natural extract from dandelion roots, in inducing programmed cell death in aggressive and resistant CMML cell lines.

To confirm the induction of programmed cell death in three human CMML cell lines, nuclear condensation and externalization of the phosphatidylserine, two main characteristics of apoptosis, were detected using Hoechst staining and annexin-V binding assay. The induction of another mode of cell death, autophagy, was determined using a monodansylcadaverine (MDC) stain, to detect the formation of autophagy vacuoles.

The results from this study indicate that Dandelion Root Extract (DRE) is able to efficiently and selectively induce apoptosis and autophagy in these cell lines in a dose and time dependent manner, with no significant toxicity on non-cancerous peripheral blood mononuclear cells. More importantly, we observed early activation of initiator caspase-8, which led to mitochondrial destabilization and the induction of autophagy, suggesting that DRE acts through the extrinsic pathway of apoptosis. The inability of DRE to induce apoptosis in dominant-negative FADD cells, confirms the mechanism of action of DRE in in vitro models

of CMML.

The results from this study indicate that natural products, in particular Dandelion Root Extract, have great potential, as non-toxic and effective alternatives to conventional modes of chemotherapy available today.

INTRODUCTION

The risk of developing cancer increases with age; nonetheless it can affect all ages (Attia et al., 2008). Chronic Myelomonocytic Leukemia (CMML) is a highly aggressive and resistant form of leukemia with a highly variable natural course of progression. The difficulty in diagnosing this disease contributes to its aggressive and resistant nature. In recent years, the World Health Organization (WHO) has classified this disease as both a myeloproliferative disease (MPD) and a myelodysplastic syndrome (MDS) (Ramshaw et al., 2002; Foucar, 2009). This classification system has improved diagnosis of CMML, although it can still prove difficult.

Even with advances in chemotherapy research, the prognosis for CMML still remains very poor with median survival of 12 to 24 months (Foucar, 2009). The available forms of treatment come with very severe side effects, with patients developing resistance early on during treatment. The only effective form of treatment that has shown significant success in CMML patients is Hematopoietic Stem Cell Transplantation, although very few studies have been done with CMML MDS/MPD (Steensma & Bennett, 2006). It is therefore of utmost importance to develop more modes of treatment that are not only safe, but also effective in targeting CMML cells specifically.

Natural products have been used for centuries as a source of nutrition, nourishment and for various forms of therapy for a wide range of diseases. Recent reports indicate that over the past sixty years, more than 60% of the approved anti-cancer drugs have been either natural or derived from natural products (Gordaliza, 2008).

Taraxacum officinale, commonly known as Dandelions, are perennial weeds of the Asteraceae family, thought to have originated in central Asia, but now known to grow almost anywhere in the world today. Traditional medicine, especially Traditional Chinese Medicine, has encouraged the use of dandelion extracts for the treatment of various diseases, ranging from diarrhoea and digestive diseases to more serious ailments like hepatitis and anorexia (Schütz et al., 2006; Yarnell & Abascal, 2009). There have been very few scientific studies to ascertain many claims of the use of dandelion extracts in the treatment of diseases, but recently, studies indicate this plant has anti-inflammatory, anti-oxidant and anti-carcinogenic activities (Schütz et al., 2006).

Our group is studying the anticancer effects of dandelion root extract (DRE), by evaluating its ability to induce physiological programs of cell death in aggressive, resistant CMML cells. Previous work done by our group showed that DRE effectively induced apoptosis in human T cell leukemia (Jurkat) and melanoma cells, by rapidly activating the death-receptor mediated extrinsic pathway of apoptosis (Chatterjee et al., 2011; Ovadje et al., 2011).

In this study, the efficacy of DRE in more aggressive leukemia cell lines was assessed to determine its selectivity and efficacy in inducing apoptosis/autophagy in CMML cells. Results indicate that DRE effectively induces apoptosis and autophagy in a dose and time dependent manner. The rapid activation of caspase-8, through the activation of the extrinsic pathway of apoptosis, was observed in these cells, comparable to levels found in Jurkat cells (Ovadje et al., 2011). Non-cancerous peripheral blood mononuclear cells (ncPBMCs), treated with dandelion root extract in parallel, were not susceptible to

apoptosis, demonstrating the selectivity of dandelion root extract in cell culture. Results from this study indicate that dandelion root extract could potentially represent a novel non-toxic alternative to conventional cancer therapy available today.

MATERIALS & METHODS

Standardized Dandelion Root Extraction

Freshly obtained Dandelion roots (from open grassy areas) were processed according to a previously published protocol (Ovadge et al., 2011). The roots were thoroughly washed with distilled water several times. One hundred grams of dandelion roots were homogenized in 200 ml of distilled water at room temperature using a domestic blender. Total homogenate was filtered through a NITEX nylon mesh filter (LAB PAK; Sefar BDH Inc. Chicoutimi, Quebec CA) and the filtrate was spun down, 8000 x g for 5 mins at 25°C. The supernatant was filtered using 0.45 µm filters, followed by lyophilisation. The dry powder (% yield: 7.34%) was reconstituted in water to give a stock solution of 100 mg/ml DRE. This was then filtered through 0.22 µm filters and used in the treatment of Chronic Myelomonocytic leukemia cells.

Cell Culture and Treatment

Human CMML cell lines (MV-4-11, HL-60 and U-937 cells), as well as a dominant negative FADD Jurkat cell line was purchased from ATCC (Manassas, VA.). MV-4-11 and HL-60 cells were cultured in Iscove's Modified Dulbecco's Medium, supplemented with 15% fetal bovine serum (FBS) and 40 mg/ml gentamicin (Life Technologies, Mississauga, Ontario). The DnFADD cells were cultured in RPMI-1640 medium supplemented with 15% FBS and 40 mg/ml gentamicin. These cells were grown and maintained in an incubator set at 37°C with an atmosphere containing 5% CO₂ and 95% humidity.

Human nucleated blood cells were purified from whole blood obtained from healthy volunteers, modified from a previously published protocol (Griffin et

al., 2010), and as approved by the University of Windsor ethical committee, REB# 04-060. Whole blood (12 ml) was collected into a BD Vacutainer CPT Tube (Cell Preparation Tube) obtained from Becton Dickinson (Franklin Lakes, N.J.). The whole blood was spun down in a tabletop lowspeed centrifuge at 2900 x g for 30 mins at 25°C. The red blood cells went through the polyester gel and the top layer containing mononuclear cells, platelets and plasma was collected. These cells were cultured under the same conditions as the CMML cells (37°C, 5% CO₂ and 95% humidity) and cultured in AIM-V medium from Invitrogen (Burlington, ON). For the induction of apoptosis and autophagy by treatment with Dandelion Root Extract (DRE), cells were grown to 50–70% confluence and then treated either with different concentrations of lyophilized extract (0.6 mg/ml to 5.0 mg/ml). At different times after treatment, cells were analyzed for apoptotic markers as described below.

Trypan Blue Exclusion Assay

To examine the ability of MV-4-11 cells to revive growth after previous exposure to DRE, these cells were treated with DRE for 48 hours, after which the cells were removed from media containing extract and equal number of cells were re-plated and examined every day. After treatment, a cell suspension was added (1:1) to Trypan Blue stain (Life Technologies, Mississauga, ON). Using a hemacytometer (Fisher Scientific, Horsham, PA.), live cells (trypan blue negative) were counted three times, calculated and tabulated as number of cells per ml using GraphPad Prism 5.0 288 software.

Hoechst Staining

Cells were grown and treated, and then stained with cell-permeable

Hoechst 33342 (Molecular Probes, Eugene, Ore.), at a final concentration of 10 mM and incubated for 10 mins at 37°C. The cells were then examined under a fluorescent microscope (Leica DM IRB, Germany) and fluorescent pictures were taken. Five fields of fluorescent pictures were used to count apoptotic versus live cells (where brightly stained cells with condensed nuclei were considered apoptotic). These results were then calculated and tabulated as percentage of apoptotic cells using GraphPad Prism 5.0 288 software.

Annexin-V Binding Assay

To confirm the induction of apoptosis by staining for phosphatidylserine exposed on the outer leaflet of the plasma membrane (a characteristic feature of apoptosis), CMMML cells were grown and treated with different concentrations of DRE. The cells were collected after given periods as indicated, washed in phosphate-buffered saline (PBS) and resuspended in annexin-V binding buffer (10 mM HEPES/NaOH pH 7.5, 140 mM NaCl, 2.5 mM CaCl₂), containing 1:50 annexin-V Alexa Fluor 488 conjugate (catalog no. A13201, Molecular Probes) for 15 mins at room temperature. Cells were then examined under a fluorescent microscope (Leica DM IRB), and pictures were taken. All pictures were processed using Adobe Photoshop 7.0 software.

Assessing Mitochondrial Membrane potential

JC-1 Mitochondrial Membrane Potential Detection Kit was applied to monitor mitochondrial membrane destabilization in cells undergoing apoptosis. In non-apoptotic cells with healthy mitochondria, JC-1 (5, 5' 6, 6'-tetrachloro-1, 1', 3, 3' tetraethylbenzimidazolylcarbocyanine iodide) (Catalog. No. M34152, Burlington, ON) exists as a monomer in the cytosol and also accumulates as

aggregates in the mitochondria which fluoresce red. In apoptotic and necrotic cells, JC-1 exists in its monomeric form, diffused in the cytosol where it fluoresces green. MV-4-11 and DnFADD cells were collected after treatment and incubated with 100 μ L per ml JC-1 dye for 15 min at 37°C. The cells were examined under fluorescent microscope (Leica DM IRB, Germany) and fluorescent pictures (six fields per sample) were taken. All pictures were processed using Adobe Photoshop 7.0 software.

Monodansylcadaverine (MDC) Staining

Monodansylcadaverine (MDC) (Sigma-Aldrich Canada, Mississauga, ON, Canada) was used to visualize autophagic vacuoles. CMML cells were plated in six-well plates and treated with different concentrations of DRE for 48 hours. After treatment, cells were incubated with 0.1 mM MDC for 30 minutes. Fluorescent pictures were acquired at 400X magnification on a Leica DM IRB inverted fluorescence microscope.

Propidium Iodide Staining

Cell death was detected by cell lysis observed by staining with propidium iodide (PI) dye (Sigma Aldrich, Canada, Mississauga, ON. Canada). After incubating with MDC dye for 30 minutes, cells also co-stained with PI at a concentration of 1.0 mg/mL for the last 15 minutes of incubation with MDC. Following incubation, the cells were visualized and images were taken using a fluorescence microscope at 400X objective.

TUNEL staining

After treating MV-4-11 cells with DRE for the indicated time points, the

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's protocol (Molecular Probes, Eugene, OR) and a previously published protocol (Negoescu et al., 1996), in order to detect DNA damage. Cells were treated with DRE or VP-16 (as a positive control) at indicated concentrations and time points and analyzed for the fragmentation of DNA. Following treatment, cells were fixed by suspending them in 70% (v/v) ethanol and stored at -20°C overnight. The sample was then incubated with a DNA labeling solution (10 µL reaction buffer, 0.75 µL TdT enzyme, 8 µL BrdUTP, 31.25 µL of dH₂O) for 1 hour at 25°C. Each sample was exposed to an antibody solution (5 µL Alexa Fluor 488 labeled anti-BrdU antibody and 95 µL rinse solution). The cells were incubated with the antibody solution for 20 minutes and pictures were taken using a fluorescent microscope (Leica DM IRB, Germany).

Cell viability Assay

To examine the viability of CMML cells after treatment, cells were incubated with cell proliferation reagent WST-1 (Catalog No. 05 015 944 001, Roche Diagnostics) for 4 h at 37°C, following treatment with DRE at indicated doses and time points, using manufacturers protocol. WST-1 works by reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye. The WST-1 reagent produces a water-soluble formazan product (Ngamwongsatit et al., 2008). Fluorescence readings were obtained at 450 nm using a Perkin Elmer Victor Fluorescence instrument. Viability readings were analyzed using GraphPad Prism 5.0 288 software and expressed as percentages of the control untreated groups.

Assessment of Caspase Activity

The Caspase assays were performed using a previously published method (Naderi et al., 2003). To determine caspase activity, the total protein from MV-4-11, HL-60 and DnFADD cell lysates were incubated with the fluorogenic substrates corresponding to the substrate cleavage site, specific for each caspase, DEVD-AFC for Caspase-3 and IETD-AFC for Caspase-8. The fluorescence was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm using a Spectra Max Gemini XS (Molecular Devices, Sunnyvale, California).

Caspase activity was calculated as activity per μg protein and protein concentration was determined with BioRad protein assay reagent (BioRad, Mississauga, Ontario) using bovine serum albumin (BSA) as a standard. Readings were analyzed using GraphPad Prism 5.0 288 software.

To further confirm the role of caspases in apoptosis induction by DRE, the broad caspase inhibitor, ZVAD-fmk (Catalog No. 219007, EMD4Biosciences, San Diego, California), was incubated with MV-4-11 cells an hour before treatment, as described above. Some cells were collected, washed in PBS and stained with Hoechst 33342 and annexin V. Others were analyzed for the activation of caspases, using fluorogenic substrates described above, after DRE treatment.

Mitochondrial Isolation and Measurement of ROS Production

Mitochondria were isolated from untreated MV-4-11 and HL-60 cells for analysis of ROS production. Cells were washed twice in cold PBS, resuspended in hypotonic buffer (1 mM EDTA, 5 mM Tris-HCl, 210 mM mannitol, 70 mM sucrose, 10 mM Leu-pep, 10 mM Pep-A, and 100 μM PMSF) and immediately

homogenized, and centrifuged at 600 x *g* for 5 minutes at 4°C. The supernatant was centrifuged at 15,000 x *g* for 15 minutes at 4°C. The resulting cytosolic supernatant was discarded and the mitochondrial pellet was resuspended in cold hypotonic buffer.

To measure the production of ROS, Amplex Red (Molecular Probes, Eugene, OR) was used. The isolated mitochondria were resuspended in cold hypotonic buffer and ≥ 20 μg of protein was added to wells of a 96-well opaque plate. The isolated mitochondria was treated with 1.0 mg/ml DRE and as a positive control, 250 μM paraquat (PQ) (Sigma Aldrich, Mississauga, ON, Canada) was used. Amplex Red reagent was added to each well for a final concentration of 50 μM ; horseradish peroxidase (HRP) was added in the ratio of 6units per 200 μL . Fluorescence readings were taken every 5 minutes for a total time of 4 hours at Ex. 560 nm and Em. 590 nm.

RESULTS

DRE effectively induces apoptosis in Chronic Myelomonocytic Leukemia cells:

To assess the effect of Dandelion Root Extract in aggressive and resistant human chronic myelomonocytic leukemia cells, MV-4-11, HL-60 and U-937 cells were treated with increasing concentrations of DRE for increasing time points. These cells were analyzed for the induction of apoptosis, using Hoechst and Annexin-V binding assay. Apoptosis was characterized by condensation of the nuclear chromatin and the externalization of the phosphatidylserine to the outer leaflet of the cell's membrane (Ziegler, 2004). Results indicate that DRE efficiently induced apoptosis in these highly aggressive and resistant leukemia cells in a dose- and time-dependent manner (Figure 1), as an increase in the percentage of apoptotic cells corresponded to an increase in the dose and time of exposure.

Another characteristic feature of apoptosis is nuclear DNA fragmentation that can be observed after cleavage of caspase-3 (Fadeel & Orrenius, 2005). To further confirm the induction of apoptosis, TUNEL assay was used to quantify DNA fragmentation in these cells after exposure to DRE. Etoposide, a known chemotherapy that targets topoisomerase II to induce double stranded DNA breaks (Ziegler, 2004), was used as a positive control for DNA fragmentation. Results indicate that DRE activated the apoptotic pathways leading to late stage DNA fragmentation, compared to etoposide, which caused DNA fragmentation that led to the induction of apoptosis (Figure 2).

For a more quantitative assessment of the effect of DRE on CMML cells,

these cells were treated with DRE and analyzed for the effect of this extract on the viability of these cells in a time-dependent manner. Results observed indicate that treatment with DRE led to a decrease in the viability of MV-4-11 cells as a function of metabolic activity, using a WST-1 cell proliferation assay (Figure 3A). This extract led to a 60% decrease in the viability of treated cells compared to the control, untreated cells, indicating that DRE could potentially affect the metabolic activity of CMML cells, ultimately leading to cell death observed in figure 1A. Since we observed an approximate decrease in metabolic activity of 60% of MV-4-11 cells 96 hours after treatment with DRE, the next step was to determine if DRE has any effect on the other 40% of cells that did not show any apoptotic morphology 96 hours after treatment. MV-4-11 cells were treated with DRE and after 48 hours the cells were removed, from media containing DRE, washed and equal numbers of cells (200,000 cells/sample) were re-plated (for control and treated cells) in fresh media without the extract. These cells were then analyzed for their ability to revive growth after previous exposure to DRE.

Although MV-4-11 cells continued to grow in the absence of DRE, it was observed that cells previously treated with DRE had reduced ability to revive growth and therefore grew at a slower pace than the cells not treated with DRE for 48 hours. More importantly, it was observed that treatment with a second dose of DRE, after 48 hours, could induce apoptosis successfully in all the cells that do not show apoptotic morphology after the first treatment (Figure 3B).

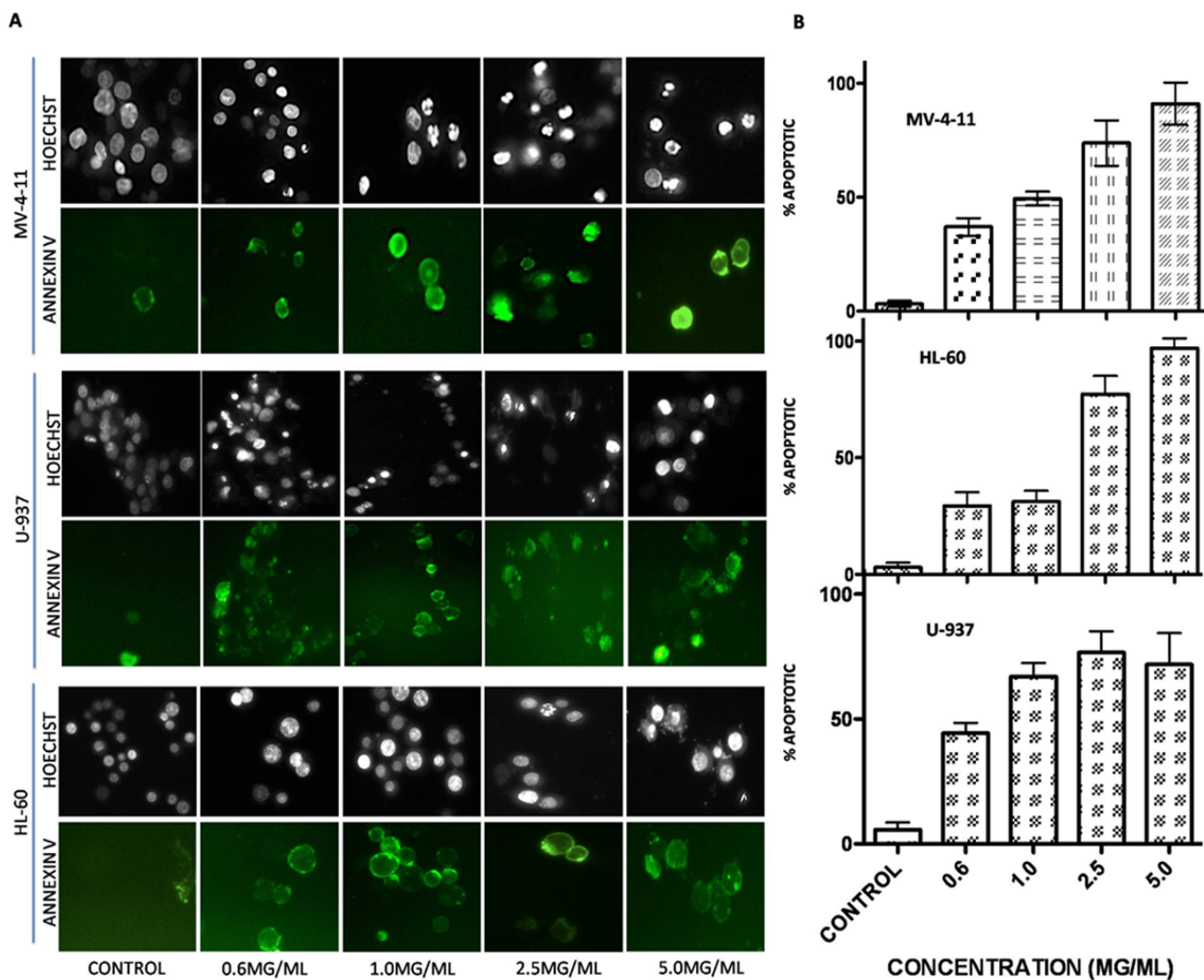


Figure 1. DRE induces apoptosis in CMML cells in a dose dependent manner: MV-4-11, HL-60 and U-937 cells were treated with increasing doses of DRE and analyzed for the induction of apoptosis. (A) Hoechst and Annexin-V staining of DRE-treated CMML cells 48 hours after treatment. Magnification 400X. (B) Manual quantification of three individual experiments of DRE-treated cells, 48 hours after. 6 pictures were obtained for every sample and live versus dead cells were counted to ascertain the percent apoptotic cell count for every sample. Experiment was performed three times and the mean and standard deviation of the three experiments were obtained

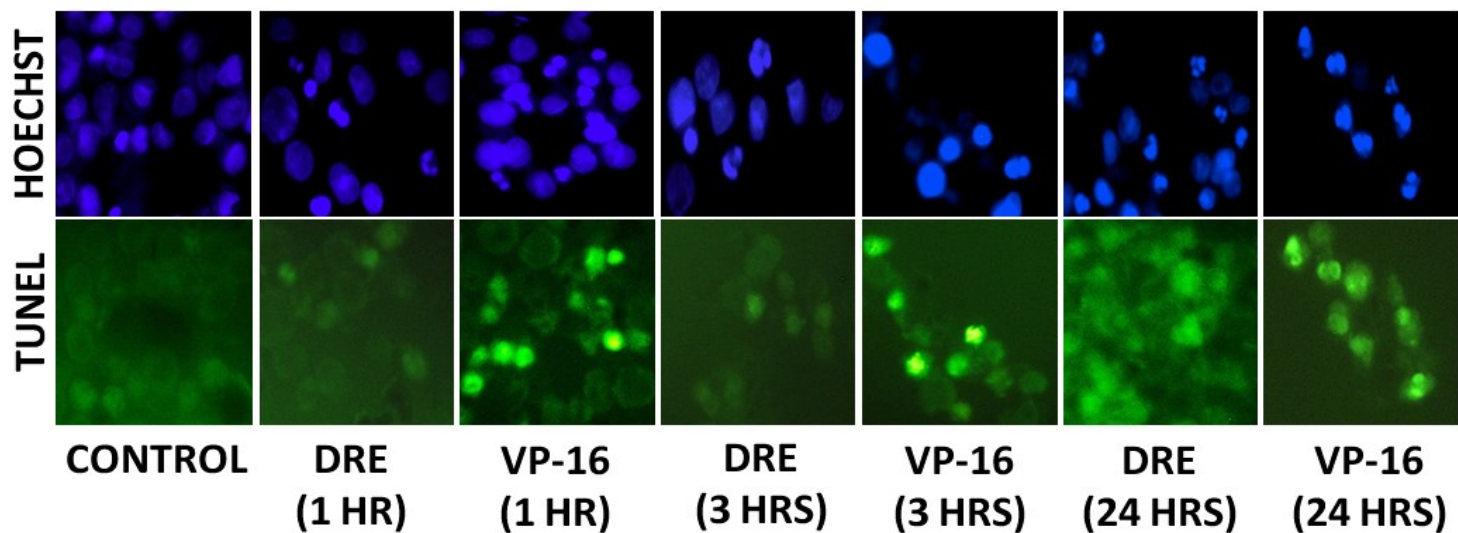


Figure 2. DRE induces late stage DNA damage in MV-4-11 cells: MV-4-11 cells were treated with 1.0 mg/ml DRE and 10 μ M VP-16 at the indicated time points to determine if DNA damage was the cause or effect of apoptosis observed in CMML cells. Following treatment, cells were fixed and immunostained with anti-BrdU antibody to observe DNA damage. Increase in TUNEL positive staining corresponds with an increase in levels of DNA damage following treatment. More positively stained cells are observed in the final stages of apoptosis caused by DRE, compared to the DNA targeting drug, VP-16. Magnification: 200X

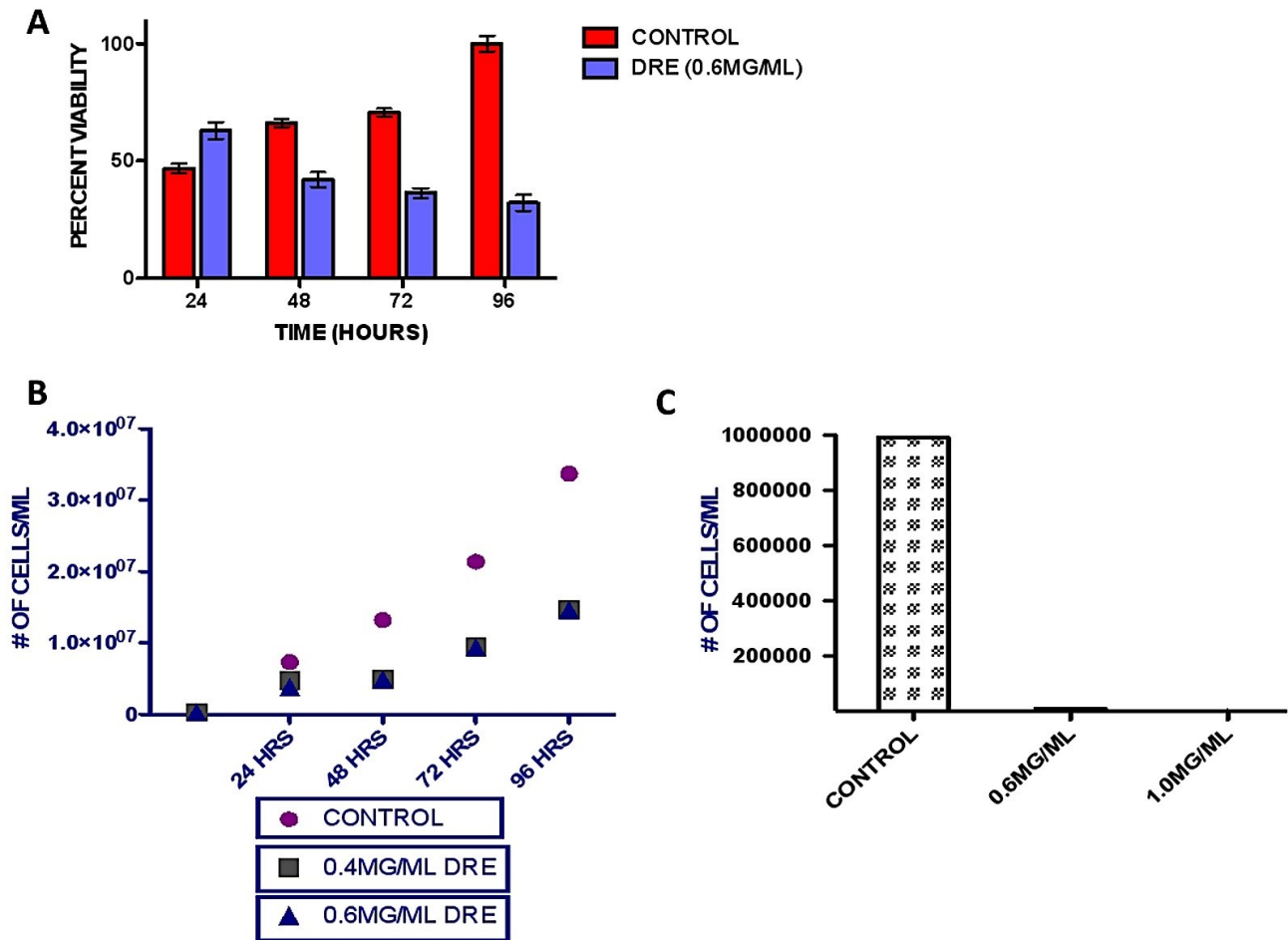


Figure 3. DRE reduces the viability of MV-4-11 cells in a time dependent manner: (A). MV-4-11 cells were plated in 96-well clear bottom plates and treated with DRE at indicated time points and analyzed for the ability of DRE to reduce the metabolic viability of these cells, measured by a decrease in metabolic activity. Following treatment, the WST-1 reagent was added to each well, the absorbance readings were taken at 450 nm, and expressed as a percentage of the control. The absorbance readings were analyzed using GraphPad Prism version 5.0 and values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. (b). Following treatment with DRE for 48 hours, MV-4-11 cells were removed from media containing DRE and equal number of cells were replated in fresh media, with no extract and allowed to grow for 96 hours, observing growth every 24 hours by trypan blue exclusion assay (left). As a single dose of DRE slowed down growth of MV-4-11 cells but did not completely halt this growth, MV-4-11 cells were treated with DRE for 48 hours, then treated with a second dose of DRE after the first 48 hours. Subsequent to the second treatment, cells were observed for growth ability (right), using trypan blue exclusion assay.

DRE activates the extrinsic pathway of apoptosis in CMML cells

The first step in determining the mechanism of DRE-induced apoptosis in human CMML was to assess the chronological activation of caspases in DRE-treated cells. This should give some insight to the pathway by which apoptosis is induced in these cells. In order to observe the activation of caspases, MV-4-11 cells were treated with DRE for various time points, following which, caspase activity was assayed, using substrates specific to caspases-3, -8 and -9. Results indicate that DRE rapidly activates caspase-8, followed by subsequent activation of caspase-3 at later time points (Figure 4A and B) similar results were observed in HL-60 cells.

In order to determine if caspase activation is required for the induction of DRE-induced cell death in CMML cells, a pan-caspase inhibitor, ZVAD-fmk, was used to pre-treat the cells for an hour and then the cells were treated with DRE for different time points and assayed for caspase-8 activation. Figure 4C confirms the inhibition of caspase-8 activation by ZVAD-fmk and this inhibition correlates with the inability of DRE to induce apoptosis in MV-4-11 cells following pre-treatment with the pan-caspase inhibitor (Figure 4D). These results indicate that caspases are required for the apoptosis-inducing effects of DRE, more importantly, caspase-8 and -3.

From these results, we hypothesize that DRE activates the extrinsic pathway of apoptosis and that the activation of caspases (caspase-8 and subsequent activation of caspase-3) are required for this process. To confirm this effect on the activation of the extrinsic pathway of apoptosis, we wanted to

observe the effect of DRE on cells with a dominant-negative mutation in the Fas-Associated Death Domain (FADD) protein, an essential component of the death inducing signaling complex (DISC) (Fulda & Debatin, 2006). Dominant negative FADD (DnFADD) cells were treated with DRE for 96 hours at increasing concentrations and analyzed for the induction of apoptosis by nuclear condensation and change in morphology. Results show that DRE did not induce apoptosis in cells with a mutant FADD domain, nor did it lead to a change in the morphology of these cells (Figure 5A). A trypan blue exclusion assay was used to evaluate the effect of DRE on the number of cells after exposure to this extract, compared to cells not treated with DRE. In figure 5B, we observe no significant difference between the control untreated cells and the DRE-treated cells. Furthermore, activation of caspase-8 and 3 were not observed in DRE treated DnFADD cells (Figure 5D). These results indicate that DRE requires the Fas-Associated Death Domain for its activation of the extrinsic pathway of apoptosis.

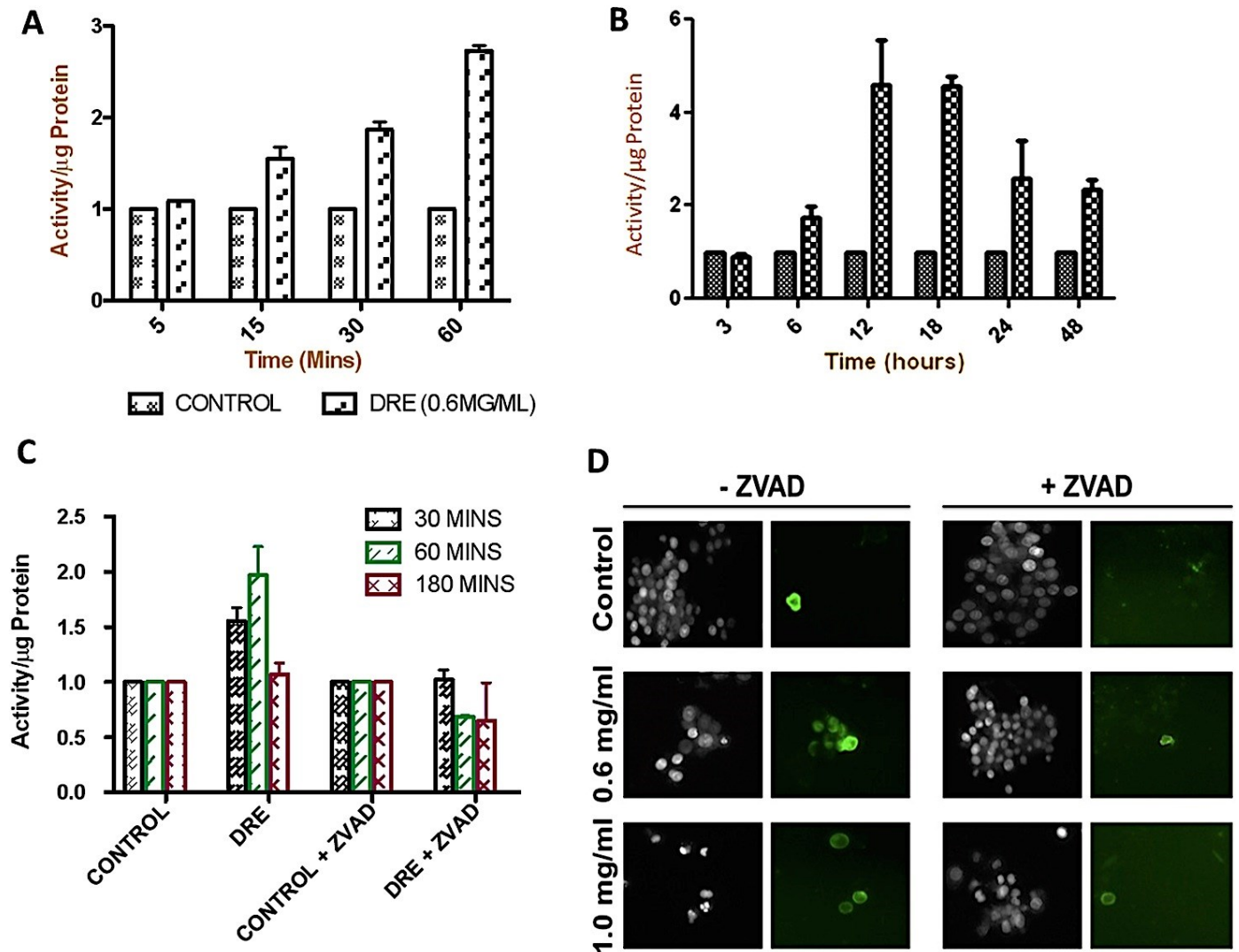


Figure 4. DRE activates the death-receptor-mediated extrinsic pathway of apoptosis: Following treatment with DRE, at indicated time points and concentrations, MV-4-11 cells were collected, washed and incubated with lysis buffer to obtain cell lysate. The cell lysate was incubated with caspase substrates, specific to each caspase (3,8 and 9) and incubated for an hour. Fluorescence readings were obtained using a spectrofluorometer. An average of 6 readings per well and a minimum of three wells were run per experiment. The results here are reported as activity per μg of protein (in fold) and the average of three experiments are shown (a,b). (c). Prior to DRE treatment, MV-4-11 cells were pre-treated with a pan-caspase inhibitor, Z-VAD-fmk for an hour and then treated with DRE at the indicated concentration for indicated time points. These cells were then incubated with caspase-8 substrate and fluorescence readings were obtained. These cells were also analyzed for the induction of apoptosis by Hoechst and annexin-V staining (d). Magnification: 400X.

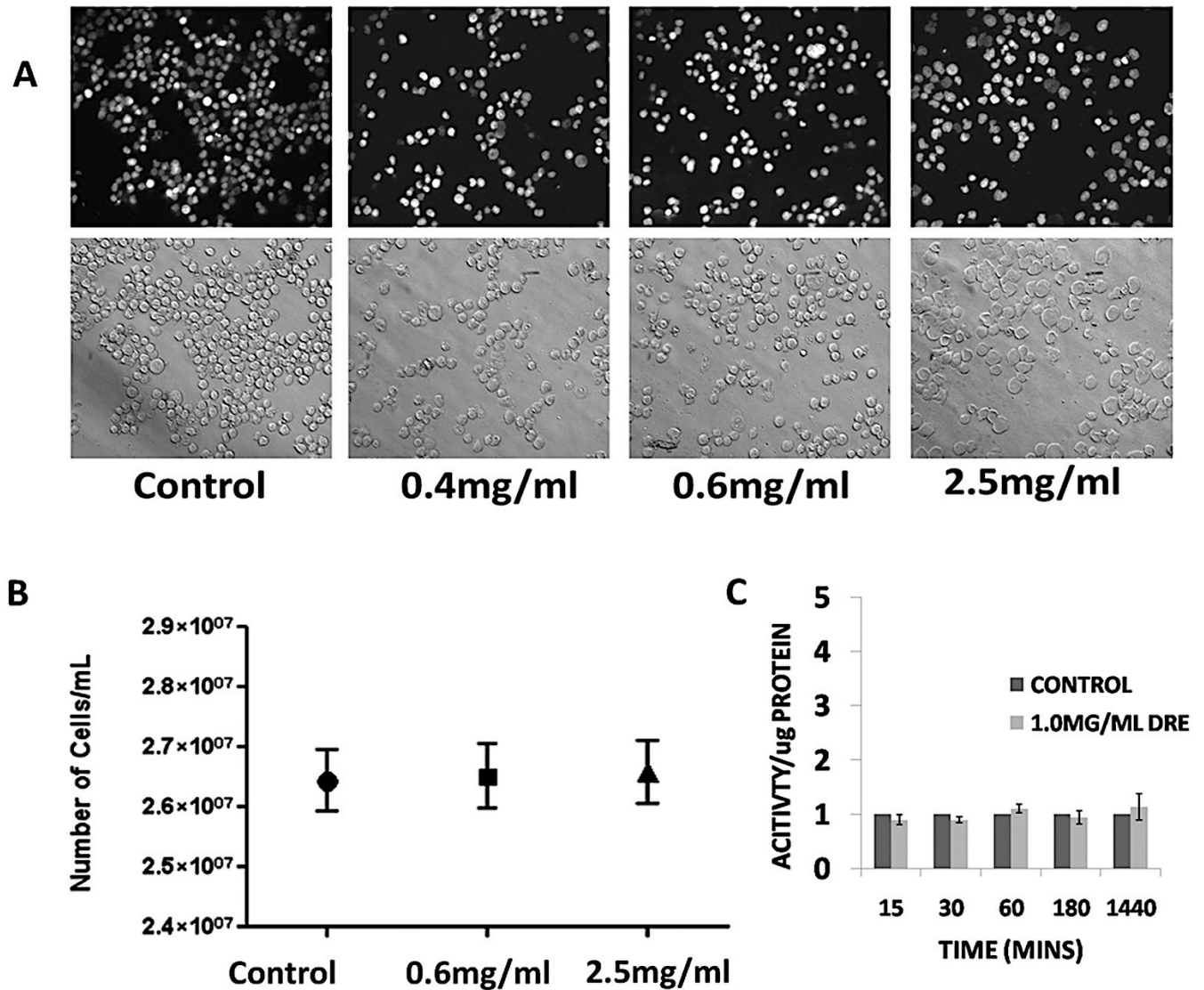


Figure 5. DRE required the Fas-Associated Death Domain (FADD) for its activity: (A). Dominant-negative FADD (DnFADD) cells were treated with DRE at indicated concentrations, for 96 hours and analyzed for the induction of apoptosis by nuclear condensation (Hoechst) and change in morphology (Phase contrast). Magnification: 400X. (B). Following treatment with DRE, DnFADD cells were collected and cell number was obtained using the trypan blue exclusion assay. Live cells were impermeable to the trypan blue dye. (C). DnFADD cells were treated with DRE for the indicated time points and analyzed for the activation of caspase-8, using caspase-8 specific substrate and fluorescence readings were obtained. An average of 6 readings per well and a minimum of three wells were run per experiment. The results here are reported as activity per μg of protein (in fold) and the average of three experiments are shown.

DRE destabilizes the mitochondrial membrane of CMML cells

Due to the fact that the two different pathways of apoptosis can target the mitochondria, the next step was to observe the effect of DRE on the mitochondria of CMML cells affected by DRE. MV-4-11 cells were treated with DRE for 24 hours and analyzed for the destabilization of the mitochondrial membrane potential, using a cationic lipophilic dye, JC-1, which aggregates in healthy mitochondria with intact potential, to give off red fluorescence. A loss or decrease in red fluorescence is therefore indicative of a loss of mitochondrial membrane potential (MMP). Figure 6A shows the effect of DRE on the mitochondrial membrane of MV-4-11 cells.

Here, we observed a loss of red fluorescence, indicative of a loss of intact mitochondrial membrane potential, indicating that DRE has an indirect effect on the mitochondria of CMML cells, by causing the dissipation of MMP. To further implicate the extrinsic pathway of apoptosis in DRE-induced apoptosis, we wanted to observe the effect on the mitochondria of DnFADD cells treated with DRE. Figure 6B shows the effect of DRE on the mitochondria of DnFADD cells. Here, we observed a consistency in the red fluorescence, indicating that the mitochondria of DnFADD cells remained intact and were unaffected by treatment with DRE. These results confirm that DRE does require FADD for its induction of apoptosis.

Mitochondria are considered the major players in the production of reactive oxygen species (ROS) under physiological conditions. The levels of the ROS increase drastically under pathological conditions, thereby leading to the induction of cell death (Adam-Visi & Chinopoulos, 2006). Furthermore, the

production of ROS is known to have dual roles; it could be the cause or effect of the induction of apoptosis. ROS has been shown to induce the activation of certain death receptors, such as Fas and TNF (Simon et al., 2000). Therefore, these points of evidence implicate the mitochondria in the initiation and/or execution of apoptosis.

To further assess the effect of DRE on the mitochondria of CMML cells, the production of ROS was studied. To do this, the mitochondria were isolated from MV-4-11 and HL-60 cells and were treated directly with DRE. ROS production was assessed by incubation with Amplex Red dye over 4 hours, with readings taken at 5-minute intervals. Paraquat (PQ), is a known inducer of ROS production in the mitochondria [14] and this was used as a positive control of ROS production in both MV-4-11 and HL-60 cells. In both cell lines, it can be observed that there is an increase in the production of ROS in DRE-treated cells compared to the control untreated cells; in the MV-4-11 cells, this increase surpasses that of the PQ treated cells (Figure 7A and B). Therefore, these results are indicative of the mitochondria as an indirect target of DRE.

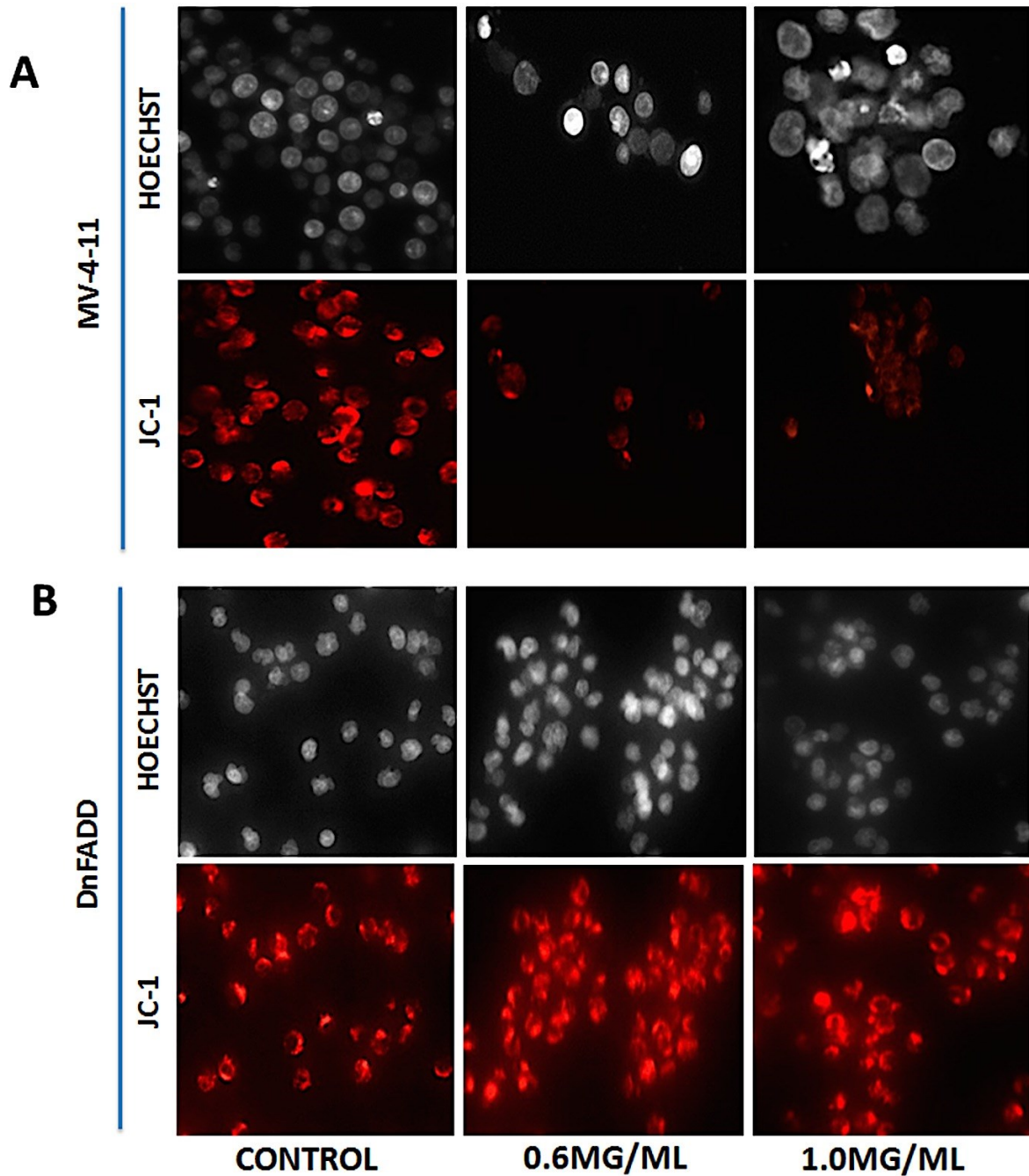


Figure 6. DRE destabilizes the mitochondria membrane potential of MV-4-11 cells: (A). Following treatment with DRE, MV-4-11 cells were incubated with JC-1 dye to detect the loss of mitochondrial potential (ref to Materials and Methods). Red fluorescence indicates only cells that have healthy mitochondria. The mitochondria of MV-4-11 cells are completely destabilized by DRE treatment. On the other hand, the mitochondria of DnFADD cells remained unaffected by DRE treatment (B). Magnification: 400X

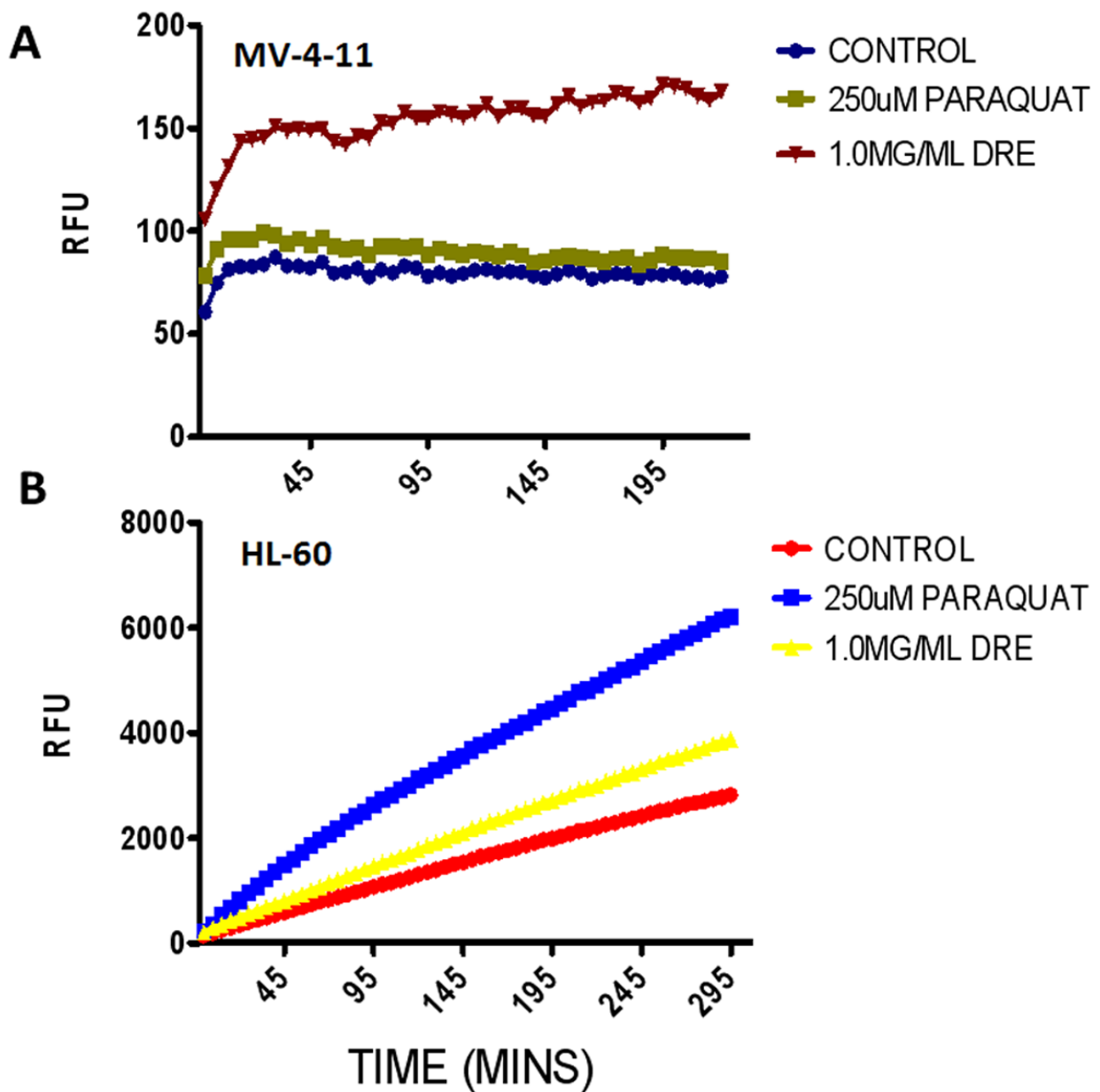


Figure 7. DRE increases ROS production in isolated mitochondria from CMML cells: Isolated mitochondria from (A) MV-4-11 and (B) HL-60 cells were treated directly with 1.0 mg/ml DRE and ROS production was measured using Amplex Red substrate in the presence of horseradish peroxidase (HRP). Results were compared to control untreated mitochondria and positive control, paraquat (PQ). Fluorescence readings were taken in 5 min intervals for 4 h at Ex. 560 nm and Em.590 nm and expressed as relative fluorescence units (RFU). Analyses of results were performed using GraphPad Prism version 5.0 and results shown are representative of 3 independent experiments demonstrating similar trends.

DRE induces pro-death autophagy in MV-4-11 cells

Autophagy, also known as “self-eating”, is an evolutionary conserved process that is known to play a significant role in the maintenance of cellular homeostasis. It is the primary degradation process by which cells can get rid of long-lived or defective proteins, as well as, defective organelles (Thorburn, 2008). It is reported to play a dual role, as it supports cell survival and cell death (in extreme cases of “self-eating”) (Rosenfeldt & Ryan, 2011). Various cellular stressors, such as hypoxia, starvation, protein aggregation and ROS production, usually induce this process. The presence of these stressors leads to the formation of the autophagosome, a double membrane vesicle, around the protein or organelle to be degraded. The autophagosome will fuse to the lysosome and its contents can be degraded (Liu & Lenardo, 2007; Dalby et al., 2010).

In order to assess the induction of autophagy in CMML cells, MV-4-11 cells were treated with DRE for 48 hours, then stained with monodansylcadaverine (MDC) dye and counterstained with propidium iodide to observe cell death. Tamoxifen (TAM), a known inducer of pro-survival autophagy (Qadir et al., 2008), was used as a positive control. Results indicate that, unlike TAM, DRE does induce a pro-death form of autophagy, as characterized by the bright punctate MDC staining, as well as the propidium iodide positive staining, in DRE-treated cells, comparable to the TAM-treated cells. These cells were more brightly stained than the control untreated cells (Figure 8).

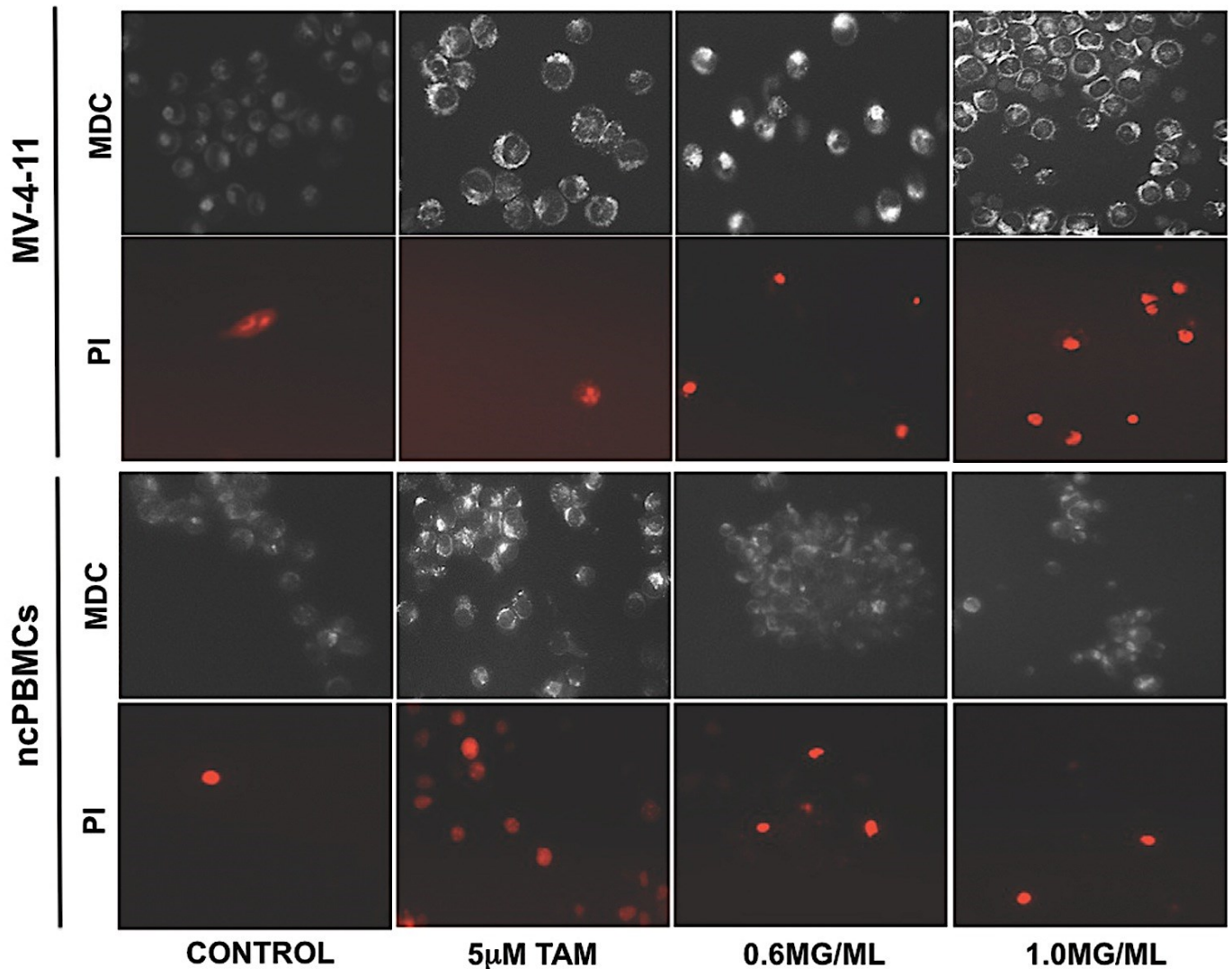


Figure 8. DRE triggers pro-death autophagy in MV-4-11 cells but not in non-cancerous Peripheral blood mononuclear cells (ncPBMCs): MV-4-11 and ncPBMCs cells were treated with DRE for 48 hours and analyzed for the induction of autophagy; Tamoxifen (TAM) was used as a positive control for the induction of pro-survival autophagy. Following treatment, the cells were stained with MDC to detect autophagic vacuoles and counterstained with PI to detect cell death. Fluorescence images were captured. Magnification: 400X.

DRE is selective to CMML cells, with little toxicity on normal, non-cancerous cells

Dandelions have been used for centuries for nourishment and therapy, with few reports of little to no toxicity, due to the lack of toxins and alkaloids present in this plant. High doses have been reported to cause allergic contact dermatitis, although this was shown in studies using dandelion extract as a topical treatment [6]. In order to assess toxicity of DRE in the lab, non-cancerous peripheral blood mononuclear cells (ncPBMCs) were isolated from the blood of apparently healthy volunteers, according to a previously published protocol (Fuss et al., 2009).

In assessing for the induction of autophagy in CMML cells, we observed a distinct lack of autophagic vacuole in ncPBMCs, as well as a corresponding lack of propidium iodide positive cells in these treated samples, compared to MV-4-11 cells, where we observed the induction of pro-death autophagy (Figure 7). Following this comparison, we wanted to confirm this selectivity, by assessing for the induction of apoptosis. Following isolation of PBMCs from healthy volunteers, cells were plated into six-well culture plates and treated with DRE at increasing concentrations for 96 hours, then analyzed for the induction of apoptosis. Results show that DRE does not induce apoptosis in ncPBMCs, even at high doses (Figure 9A and B). As ncPBMCs are not actively proliferating in culture, some isolated PBMCs were pre-incubated with concanavalin A (conA), a plant mitogen known to stimulate T cell proliferation *in-vitro* (Dwyer & Johnson, 1981). These cells were then exposed to DRE treatment and analyzed for the induction of apoptosis. As with the ncPBMCs not pre-treated with conA, those pre-incubated

with conA and subsequently treated with DRE remained unsusceptible to DRE-induced apoptosis (data not shown). These results have also been shown in other non-cancerous cell lines, for example, normal human fibroblasts (NHFs) (Chatterjee et al., 2011).

To further evaluate the selective induction of cell death in ncPBMCs, these cells were treated with DRE for 48 hours and stained with MDC and PI to assess the induction of pro-death autophagy, using tamoxifen as a positive inducer of autophagy. Figure 8 (bottom panel) indicates that tamoxifen effectively induced autophagy in ncPBMCs, ultimately leading to the cell death, observed with propidium iodide staining. Importantly, there was no induction of autophagy in these cells, as compared to the control, untreated cells. These DRE treated cells were also impermeable to propidium iodide, confirming the lack of cell death in ncPBMCs treated with DRE. These results suggest that DRE is non-toxic to non-cancerous blood cells, making it selective to CMML cells.

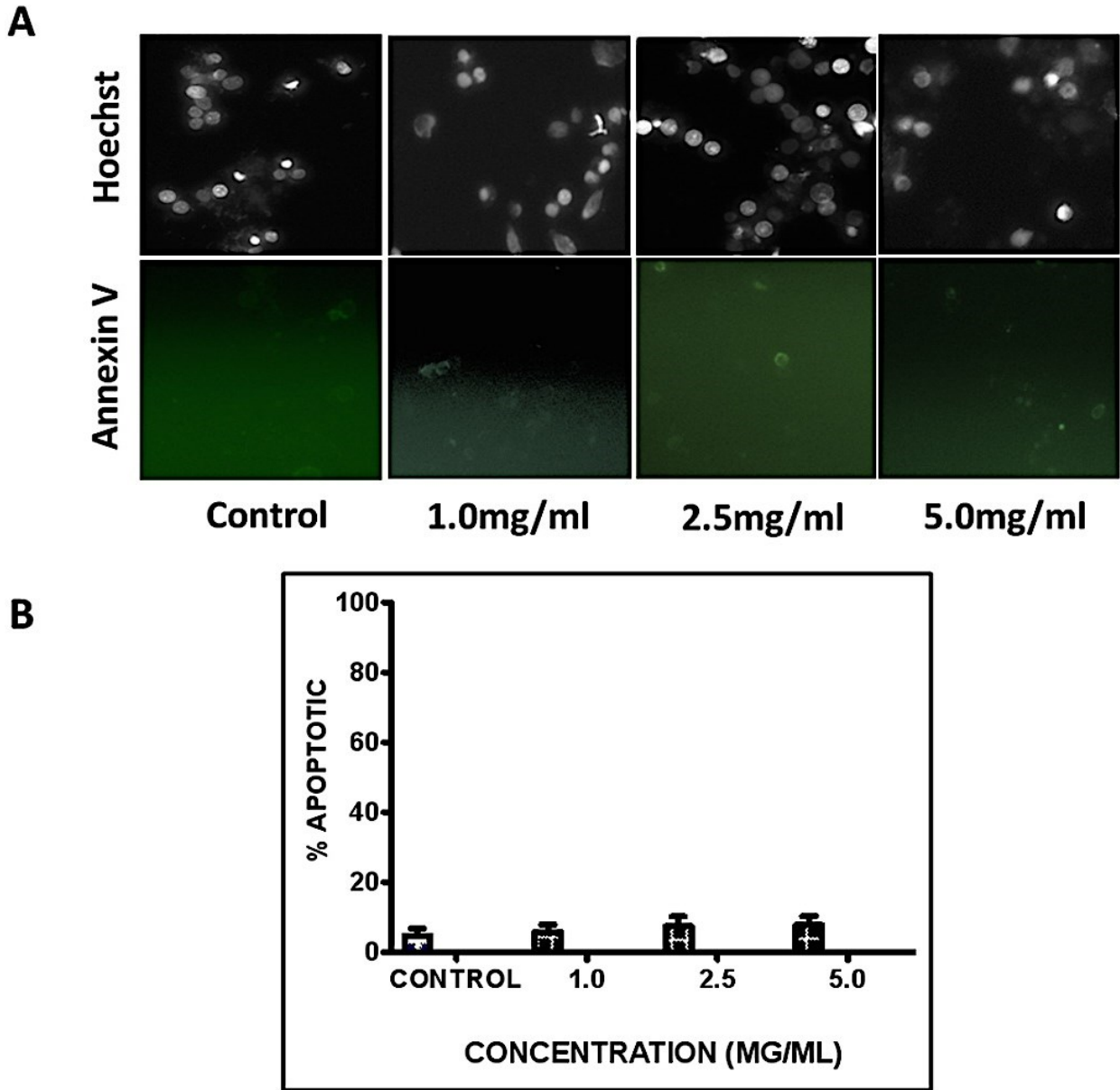


Figure 9. DRE does not target normal non-cancerous peripheral blood mononuclear cells (ncPBMCs): (A). ncPBMCs were isolated from healthy volunteers and plated in six-well plates. These cells were treated with DRE at increasing concentrations for 96 hours and analyzed for the induction of apoptosis by nuclear condensation (Hoechst) and externalization of the phosphatidylserine (Annexin-V binding). Magnification: 400X. (B). Results from three different experiments were quantified to determine the percentage of apoptosis occurring in ncPBMCs treated with DRE.

DISCUSSION

The poor prognosis for Chronic Myelomonocytic Leukemia (CMML) patients is a major indication that there is a serious need for a more effective and non-toxic alternative to the conventionally available forms of chemotherapy and surgical procedures (Steensma & Bennett, 2006). With the introduction of natural products, not only as sources of nourishment, but also for their therapeutic benefits, it is therefore necessary to study the vast array of natural products as non-toxic and less expensive alternatives for the treatment of CMML. Apoptosis and autophagy, two necessary modes of programmed cell death, are important mechanisms, which cells utilize for the maintenance of cellular homeostasis (Zhivotovsky & Orrenius, 2010). Cancer cells, however, have developed mechanisms to evade these programs, so as to enable enhanced proliferation, aggressiveness and resistance (Hanahan & Weinberg, 2000; Ghobrial et al., 2005). In this study, we demonstrate the selective efficacy of dandelion root extract in inducing apoptosis and autophagy in highly aggressive and resistant CMML cell lines. We observed the induction of apoptosis in three of the CMML cell lines used for this study (Fig. 1). More importantly, this effect was selective, as non-cancerous PBMCs and NHFs remained unsusceptible to DRE-induced apoptosis (Figure 9A and B).

Although various extracts of dandelions have been used for centuries for the treatment of various diseases, there have been very few scientific studies done to ascertain the mechanism by which these extracts act and although some of the compounds present in dandelion extracts have been isolated and identified, they still are not fully characterized (Schu"tz et al., 2006; Yarnell &

Abascal, 2009). The major aim of the study was therefore to determine the mechanism of DRE-induced cell death in CMML cells.

In this study, we report a rapid activation of caspase-8 (within minutes) and subsequent activation of caspase-3 in human CMML cells, indicative of a rapid activation of the death receptor mediated extrinsic pathway of apoptosis (Figure 4A and B). This activation has been shown to be necessary for the induction of apoptosis observed after DRE treatment, as the inhibition of caspase activity, using a pan-caspase inhibitor, ZVAD-fmk, prevented the induced cell death observed (Figure 4C and D). These results suggest that caspases, more importantly, caspase-8 and 3, are required for the induction of apoptosis by DRE. The Fas-Associated Death Domain (FADD), as well as other death domains, are highly important for activation of caspase-8 through extrinsic apoptosis (Elmore, 2007). The binding of the death ligand to its corresponding death receptor leads to the recruitment of specific receptor death domains for the formation of the death inducing signaling complex (DISC) (Fulda & Debatin, 2006). The absence of FADD should therefore prevent the activation of the extrinsic pathway of apoptosis through inhibition of the activation of caspase-8 (Shakibaei et al., 2010).

To further confirm the effect of DRE on the extrinsic pathway of apoptosis, DnFADD cells were treated with DRE and analyzed for apoptosis induction. According to our results, no nuclear condensation and morphological changes were observed with the treatment of DRE. There was no change in the number of cells treated with DRE, compared to the control untreated cells, suggesting that DRE did not have a significant effect on the cells with a truncated FADD protein

(Figure 5A and B). Furthermore, these cells were not susceptible to DRE-induced rapid activation of caspases, specifically caspase-8 (Figure 5C). As we observed late activation of caspase-3, we wanted to observe the connection between the intrinsic and the extrinsic pathway of apoptosis.

It is known that the activation of caspase-8 could yield two main results. First, the activated caspase-8 could lead directly to the activation of the effector caspase, caspase-3 or it could lead to mitochondrial changes, through the cleavage of pro-apoptotic protein, Bid. The truncation of Bid causes mitochondrial membrane destabilization, which leads to the release of pro-apoptotic proteins for the activation of caspase-3 [26]. In this study, we observed the destabilization of the mitochondrial membrane potential 24 hours after treatment with DRE. This destabilization of mitochondrial membrane potential was only observed in MV-4-11 cells, not in DnFADD cells (Figure 6A and B). These results suggest that DRE indirectly targets the mitochondria after the activation of the extrinsic pathway of apoptosis. It confirms the requirement of the adapter domain for DRE-induced apoptosis, as we do not observe a similar loss of mitochondrial membrane potential in DnFADD cells treated with DRE. As the mitochondria are major players in the production of ROS (Chen et al., 2003), the next step was to observe the effect of DRE on the production of ROS. Results confirm the production of ROS from isolated mitochondria (Figure 7A and B), further confirming the effect of DRE on the mitochondria of CMML cells.

The integrity of the mitochondria is essential for the maintenance of cellular homeostasis and defects in the mitochondria caused by treatment with DRE could provide sufficient signals for the induction of programmed cell death

type II, also known as Autophagy, a process induced by the presence of defective organelles, proteins and starvation (Gottlieb & Carreira, 2010).

Autophagy has been shown to play a significant role in cancer survival and its role in programmed cell death has been controversial. In response to stressors, cells undergo “self-digestion” as a means of temporary survival, where macromolecules are digested in order to provide an alternate energy source. However, excessive exposure to stressors could lead to excessive autophagy, ultimately resulting in cell death (Yu et al., 2006; Dalby et al., 2010). The loss of mitochondrial membrane potential, observed after treatment with DRE, therefore led us to determine if the stress induced by our extract, leading to mitochondrial membrane destabilization was a sufficient signal for the induction of autophagy.

Our results indicate that treatment with DRE triggered the induction of a pro-death form of autophagy in MV-4-11 cells (Figure 8; top panel). Comparing this to treatment with tamoxifen, which is known to induce pro-survival autophagy in cancer cells, treatment with DRE not only triggered autophagy, comparable to TAM, the cells were also propidium iodide positive, indicative of cell death. Moreover, ncPBMCs stained with MDC and PI showed no induction of autophagy in these cells after DRE treatment (Figure 8; bottom panel). These results suggest that DRE is effective in selectively inducing both forms of programmed cell death in human CMML cells.

In conclusion, Dandelion Root Extract has shown selective efficacy in inducing two forms of programmed cell death in highly aggressive and resistant CMML cell lines. The rapid activation of caspase-8 not only activated the extrinsic pathway of apoptosis, but also triggered pro-death autophagy selectively in these

cells, suggesting that this extract has components that enhance its selective efficacy in targeting CMML cells. These results indicate that within the vast array of available natural products and compounds, there are non-toxic alternatives to conventional chemotherapy that are safe and effective.

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CHAPTER 5

PRECLINICAL EVALUATION OF THE SELECTIVE ANTICANCER ACTIVITY OF DANDELION ROOT EXTRACT; FRACTIONATION, PHYTOCHEMICAL & GENE EXPRESSION ANALYSIS IN HUMAN CANCER CELLS

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(2014) In Preparation

ABSTRACT

Natural health products have formed the basis for the introduction of many identified drugs in the treatment of various diseases, especially as anti-cancer agents. Dandelion extracts have been studied extensively in recent years for its anti-depressant and anti-inflammatory activity. Recent work in our lab, with *in-vitro* systems, have shown the anti-cancer potential of an aqueous extract of dandelion root in several cancer cell models, with no toxicity to non-cancer cells. The detailed analyses of the efficacy and toxicity of this extract in *in-vivo* and *ex-vivo* models, as well as, the mechanism(s) of action remains unexplored. Furthermore, the pharmacologically active components within this extract, involved in the anti-cancer activity are unknown.

In this study, we examine the role of an aqueous extract of dandelion root in highly aggressive colon cancer cell lines, as well as its anti-cancer efficacy in patient-derived *ex-vivo* samples of leukemia. Aqueous DRE induced significant and selective programmed cell death (apoptosis) in colon cancer cells, irrespective of their p53 status (HT-29 – p53 mutant and HCT116 – p53 wild type). The anti-cancer efficacy of this extract was confirmed in *in-vivo* studies, where the oral administration of DRE-supplemented drinking water halted the continued growth of human colon tumors in xenograft models. More importantly, we observed this cell death inducing ability of DRE in peripheral blood mononuclear cells (PBMCs), isolated from the blood of newly diagnosed leukemia patients in the Windsor-Essex area.

In this study, we also hypothesized that the complexity of this natural extract is able to target more than one signaling pathway, and hence, more than

one cancer cell vulnerability, in order to have a potent anti-cancer effect. We determined the presence of bioactive ingredients, including α -amyirin, β -amyirin and lupeol in bioactive fractions of aqueous DRE. This anti-cancer effect of DRE hinged on the activation of multiple pathways, including the rapid activation of extrinsic apoptosis, the destabilization of the mitochondrial membrane potential and the induction of pro-death autophagy, following an increase in the expression of genes involved in programmed cell death type I and II (apoptosis and autophagy, respectively).

Our results show that aqueous DRE can potentially represent a non-toxic, effective alternative, which could possibly reduce the occurrence of cancer cells resistance, as it contains multiple bioactive components that can target multiple signaling pathways, specifically in cancer cells. Furthermore, these findings have led to the approval of dandelion root extract, as the first NHP in Canada, for Phase I clinical trials, for hematological cancers in Canada.

INTRODUCTION

The increase in the world's aging population, as well as the adoption of cancer-causing behaviours, are the major contributors to an escalation in the incidence of different forms of cancers globally. There are over 12 million new cancer cases arising annually and over 7 million cancer-related deaths worldwide, and even with the introduction of many chemotherapy and chemopreventative approaches, cancer is still one of the leading causes of deaths in the world today, with a statistic of one in four deaths being attributed to cancer alone (Jemal et al., 2011; Siegel et al., 2013).

Despite the progress made in the development and introduction of many chemotherapy agents, the accompanying toxicities and side effects (Jemal et al., 2011), indicate that further research is required to reduce the incidence of cancer rates, and the amount of cancer-related deaths, as well as improve the quality of life of patients already diagnosed with the disease.

Natural health products (NHPs) and natural products (NPs) have been essential in the development of many drugs, with over 75% of the currently available chemotherapies having been derive from natural sources (plants, microbes and marine sources), with the common example being paclitaxel (Mann, 2002). These NHPs have been used in various traditional medicines and recent scientific studies in the use of these NHPs for specific diseases have yielded some scientific validation to the use of these products (Ganesan, 2008; Newmann & Cragg, 2012). Even with all the incoming evidence, herbal drugs and other NHPs and NPs are usually shunned during systemic chemotherapy

because of herb-drug interaction and the exaggeration of chemotherapy-related toxicity (Foster et al., 2005; Nobili et al., 2009).

Dandelions (*Taraxacum spp*) have been used for centuries for the treatment of various ailments; surprisingly enough, it has received little research attention. Some scientific studies have shown the efficacy of various parts of this plant as anti-inflammatory, anti-oxidative and as a diuretic agent (Yarnell & Abascal, 2009).

Recent studies in our lab have shown the anti-cancer activity of an aqueous extract of dandelion root, selectively in cancer cells. In these studies, we show that dandelion root extract (DRE) is able to selectively induce the rapid activation of the death-receptor mediated extrinsic pathway of apoptosis in a dose and time dependent manner, in several cancer cell types (Chatterjee et al., 2011; Ovadje et al., 2011; Ovadje et al., 2012a; Ovadje et al., 2012b). Furthermore, we observed a caspase-8 dependence, following DRE treatment. This result showed that, following the inhibition of caspase activation, using a pan-caspase inhibitor, zVAD-fmk, in chronic myelomonocytic leukemia cells, there was a corresponding inhibition of the induction of apoptosis (Ovadje et al., 2012b). These findings have led us to further investigate the mechanism of DRE in cancer cells that enhance its selectivity, as evidenced by the lack of toxicity in non-cancer cells.

In this study, we hypothesize that there are bioactive component(s) within aqueous DRE that are able to target multiple vulnerabilities within a cancer cell, in order to induce selective programmed cell death processes. In this study, we delve further into the mechanism of action of DRE in highly aggressive, drug

resistance colon cancer cells, where we observe the activation and localization of active caspase-8 to the mitochondria and the peri-nuclear space. However, this caspase-8 activation was not essential to the mechanistic efficacy of DRE in colon cancer cells, as inhibition of caspase-8 activation did not have an effect on the apoptosis inducing activity of DRE in these cell types, demonstrating the versatility of this extract in *in-vitro* models. Further assessment of DRE in *in-vivo* and *ex-vivo* models (with patient-derived samples of leukemia) suggests that this extract could potentially represent a novel non-toxic alternative to conventional cancer therapy available today, as it was well-tolerated in mice models but was efficient in inducing apoptosis in *ex-vivo* samples. More importantly, we have been able to identify three bioactive components, α -amyrin, β -amyrin and lupeol, within this extract that could contribute to its overall bioactivity, demonstrating the benefits of utilizing the whole complex extract of dandelion root.

These results further scientifically validate the use of NHPs, especially the dandelion root species, as a potential anti-cancer agent, worthy of further investigation.

MATERIALS & METHODS

Dandelion Root Extraction & Preparation

The dandelion roots used for this study were obtained from Premier Herbal Inc. (Lot No. 318121). The root extract was prepared, modified from a previously published protocol (Ovadje et al., 2011). Dried dandelion root was immersed in liquid nitrogen for about 5 to 10 minutes, until thoroughly frozen. The frozen pieces were ground up in an impingement grinder to an average particle size of $\leq 45 \mu\text{m}$. Following grinding, dandelion root powder was extracted in boiling water on low heat for 3 hours. The total extracted material was filtered through a NITEX nylon mesh filter (LAB PAK; Sefar BDH Inc. Chicoutini, Quebec CA) and the filtrate was spun down at $800 \times g$ for 5 minutes at room temperature. The supernatant was filtered through a $0.45 \mu\text{m}$ filter, followed by lyophilization. The dried extracted material was reconstituted in water to give a final stock solution of 100 mg/ml and then passed through a $0.22 \mu\text{m}$ filter, in a biological safety cabinet and stored at 4°C . this material was used for the experiments described in this study.

Cell Culture and Treatment

Human colon cancer cell lines (HT-29, p53 mutant and HCT116, p53 WT (Muller et al., 2013) were purchased from ATCC (Manassas, VA). These cells were cultured in McCoy's 5a medium (ATCC, Catalog no. 30-2007), supplemented with 10% fetal bovine serum (FBS) and $40 \mu\text{g/ml}$ gentamicin (Life Technologies, Mississauga, ON). Normal human colon mucosal epithelial cell line (NCM460, Incell Corporation, LLC, San Antonio TX) were subcultured in RPMI 1640 medium (Sigma Aldrich, Mississauga, ON), supplemented with 10% FBS

and 40 µg/ml gentamicin. These cells were grown and maintained in an incubator, set at 37°C, with an atmosphere containing 5% CO₂ and 95% humidity.

Peripheral blood mononuclear cells (PBMCs) were purified from whole blood samples, obtained from either healthy volunteers (non-cancerous PBMCs – ncPBMCs) or from newly diagnosed leukemia patients (prior to the administration of chemotherapy). These cells were prepared according to a previously published protocol (Griffin et al., 2010; Ovadje et al., 2012b) and as approved by the University of Windsor's research ethical committee, REB# 04-060. Whole blood (~12ml) was collected into a BD Vacutainer CP Tube (Cell Preparation Tube) obtained from Becton Dickinson (Franklin Lakes, NJ). The whole blood was spun down by density gradient centrifugation in a tabletop lowspeed centrifuge at 2900 x g for 30 minutes at 25°C. The red blood cells went through the polyester gel and the top layer containing mononuclear cells, platelets and plasma was collected. These cells were washed twice in AIM-V medium (Invitrogen, Burlington, ON), following which, cells were maintained in an incubator set at 37°C with an atmosphere containing 5% CO₂ and 95% humidity. The cells were cultured in AIM-V medium.

To assess the efficacy of DRE in our cell culture models, cells were plated and grown to 50 – 70% confluence prior to treatment with dandelion root extract (DRE), at increasing concentration (0.5 mg/ml – 6.0 mg/ml). Subsequent to treatment, cells were analyzed for efficacy of DRE, as described below. All cells were cultured for ≤ 4 months, before being discarded and fresh frozen cells were used to continue studies, lasting longer than the 4-month period.

Assessment of Cellular Metabolic Activity & Viability

To examine the viability of colon cancer and normal colon mucosal epithelial cells after treatment, cells were incubated with cell proliferation reagent WST-1 (Catalog No. 05 015 944 001, Roche Diagnostics) for 4 hours at 37°C, following treatment with DRE at indicated doses and time points, using the manufacturers protocol. WST-1 works by reacting with the mitochondrial succinate-tetrazolium reductase, forming the water-soluble formazan product (Ngamwongsatit et al., 2008). Fluorescence readings were obtained 450 nm using a Perkin Elmer Victor Fluorescence instrument and viability readings were analyzed using GraphPad Prism 6.0 288 software and expressed as a percentage of the control untreated groups.

Assessment of Programmed Cell Death Induction

An early marker of apoptosis is the reorganization of the cell membrane to expose phosphatidylserine from the inner leaflet of the cell membrane to the outer leaflet. This allows apoptotic cells to be taken up by phagocytic cells (Fadeel & Orrenius, 2005). This characteristic can also be exploited to assess the induction of apoptosis, according to a previously published protocol (Rieger et al., 2011). Following treatment with DRE, cells were trypsinized (0.15% trypsin) to lift adherent cells from the plates. The cells were washed twice in phosphate buffered saline (PBS). After washes with PBS, the pellet was resuspended in Annexin-V binding buffer (10 mM HEPES, 10 mM NaOH, PH 7.5, 140 mM NaCl, 2.5 mM CaCl₂ and 50nM sucrose) and Annexin-V Alexa Fluor 488 conjugate (Catalog No. A13201, Life Technologies, Burlington, ON), which binds to the exposed phosphatidylserine, at a 1:50 ratio, with respect to the binding buffer.

This reaction was incubated at room temperature for 15 minutes. Hoechst 33342, a photosensitive DNA binding dye (Molecular Probes, Eugene, OR), was used as a counterstain, at a final concentration of 10 μ M, in the last 10 minutes of the incubation at room temperature. Apoptotic cells will be characterized by brightly stained, condensed nuclei, as compared to the larger, rounder and less brightly stained non-apoptotic cells. At the time of Hoechst staining, cells were counterstained with propidium iodide, a cell impermeable photosensitive DNA binding dye (Sigma Aldrich, Mississauga, ON), at a final concentration of 1 μ g/ml (added at the same time as Hoechst 33342). Following the incubation, cells were visualized and images were obtained using a fluorescence microscope (Leica DMI 6000 fluorescence microscope, with a Leica DFC 360FX camera and Leica STP6000 control board).

The images were obtained at 400X magnification and fluorescence quantification was also carried out using a TALI image-based cytometer (Catalog No. T10796, Life Technologies, Burlington ON), using a previously published protocol (Remple & Stone, 2011; Chan et al., 2012).

Evaluation of Mitochondrial Membrane Potential

JC-1 Mitochondrial Membrane Potential Detection Kit was used to monitor membrane destabilization in cells undergoing apoptosis. In non-apoptotic cells, with healthy mitochondria, JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzamidazolylcarbocyanine iodide) (Catalog No. M34152, Life Technologies, Burlington ON) exists as a monomer, which fluoresces green, in the cytosol and also accumulates as aggregates in the mitochondria, which fluoresces red. In apoptotic and necrotic cells, with defective mitochondria, JC-1

only exists in its monomeric form, diffused in the cytosol, with no accumulation in the mitochondria, leading to a distinct lack of red fluorescence. Treated cells were incubated with JC-1 dye, at a final concentration of 200 nM, for 45 minutes at 37°C. the cells were counterstained with Hoechst staining and were examined by fluorescent microscopy and fluorescent images were taken at 400X magnification. Samples were also observed using image-based cytometry.

Mitochondrial Isolation and Measurement of ROS Production

Mitochondria were isolated from untreated HT-29 and NCM460 cells for analysis of reactive oxygen species (ROS) production. Cells were washed twice in cold PBS, resuspended in hypotonic buffer (1 mM EDTA, 5 mM Tris-HCl, 210 mM mannitol, 70 mM sucrose, 10 μ M Leu-pep, 10 μ M Pep-A and 100 μ M PMSF) and immediately homogenized, before centrifuging at 600 x g for 5 minutes, at 4°C to get rid of nuclear pellet and unbroken cell debris. The resulting cytosolic supernatant was discarded and the mitochondrial pellet was resuspended in cold hypotonic buffer.

To measure the production of ROS, Amplex Red (Molecular Probes, Eugene, OR) was used. Following resuspension in cold hypotonic buffer, \geq 20 μ g of protein was added to wells of a 96-well opaque plate. Treatment was done with 2.5 mg/ml DRE, a positive control, 250 μ M paraquat (PQ) (Sigma Aldrich, Mississauga ON) and a negative control, 3 mM N-Acetylcysteine (N-AC) (Sigma Aldrich, Mississauga ON). Amplex Red reagent was added to each well from a final concentration of 50 μ M; horseradish peroxidase (HRP) was added in the ratio of 6units per 200 μ L. Fluorescence readings were taken every 5 minutes for a total time of 5 hours at Ex. 560 and Em. 590 nm on a spectrofluorometer (SpectraMax

Gemini XS, Molecular Devices, Sunnyvale, CA). The readings were analyzed on GraphPad Prism 6.0 288 software and expressed as relative fluorescence units (RFU) per μg protein.

Evaluation of Caspase Activation

Cysteine-Aspartic Proteases (Caspases) are a major player in the initiation and execution of apoptosis (Fadeel & Orrenius, 2005). The caspase assays were performed and modified from a previously published method (Naderi et al., 2003). Caspase activation was confirmed using an Image-iT Live caspase detection kit (Catalog No. I35105, Life Technologies, Mississauga ON). Following treatment for an hour with DRE, cells were stained with a cell permeable FLICA dye for the detection of active caspases, at 150X dilution and incubated for 45 minutes at 37°C. Following incubation with the dye, cells were washed in 1X wash buffer and counterstained with propidium iodide. The stained cells were visualized by TALI image-based cytometry. To further confirm the role of caspases in apoptosis induction by DRE, a caspase-8 specific inhibitor, IETD-fmk (Catalog No. 218759, EMD4Biosciences, San Diego, CA) or a pan-caspase inhibitor, ZVAD-fmk (Catalog No. 219007, EMD4Biosciences) were incubated with HT-29 cells, an hour before DRE treatment, as described above. Cells were analyzed for metabolic viability by the WST-1 viability assay.

Immunocytochemical Analysis of Caspase Activation

To confirm the activation of caspases and their localization upon activation, HT-29 cells were plated onto poly-L-lysine (Sigma Aldrich, Canada) coated coverslips in a 6-well plate and allowed to attach to the coverslips for 24 hours. These cells were then treated with DRE for different time points, ranging

from 30 minutes to 24 hours, following which the cells were incubated with a mitochondrial specific dye, MitoTracker (Invitrogen, Canada) for 45 minutes, before the cells were obtained for immunocytochemical analysis. Cells were fixed in 3.7% paraformaldehyde (PFA) solution for 5 minutes at room temperature, followed by a 1.4% formaldehyde/0.1% NP-40 solution for 1.5 minutes at room temperature. Subsequent to fixation, cells were incubated with a blocking solution of 5% goat serum in PBS, followed by incubation overnight at 4°C with anti-mouse primary antibody, specific to active caspase-8 (Santa Cruz Biotechnology, CA). The following day, coverslips were incubated with a goat anti-mouse secondary antibody, conjugated to AlexaFluor 488 (CellSignalling Technology, MA) for an hour at room temperature. The coverslips were washed in PBS and incubated with Hoechst 33342 for 10 minutes, at a final concentration of 10 µM and mounted on slides, using 80% glycerol. The slides were stored at 4°C until ready for visualization by fluorescent microscopy.

Gene Expression Profiling of DRE Treated Cells:

RNA extraction and cDNA synthesis

Following treatment of chronic myelomonocytic leukemia (CMML) cells, HT-29 and NCM460 cells, with DRE, total cellular RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Inc.), according to the manufacturer's protocol. The RNA quality was examined using gel electrophoresis and measuring the A280/A260 ratio (NanoDrop 2000). The cDNA was synthesized from 500 ng of total RNA by using the RE3 Reverse Transcriptase Mix first-strand synthesis system. Following a denaturation step of 5 minutes at 42°C, RNA was reverse transcribed to a single stranded cDNA using oligo(dT) primers (Qiagen, Inc.). The

reverse transcription reaction was performed in a total volume of 20 μ L at 42°C for 15 minutes, immediately followed by 95°C for 180 minutes.

PCR Array (Cell Death Signaling Pathway).

The polymerase chain reaction (PCR) for the cell death signaling pathway was performed, following the reverse transcription of isolated RNA, using the RT²Proiler PCR array system from Qiagen, Inc. The PCR array was performed to combine the quantitative performance of the SYBR Green based system, with multiple profiling abilities of the pathway-focused gene expression (Ornatowska et al., 2007). 384-well (4 x 96-well) plates containing gene-specific primer sets for 84 relevant genes in the major programmed cell death pathways (Apoptosis, Autophagy and Necrosis), 5 housekeeping genes, a positive control and a negative control gene set was used (Catalog No. PAHS-212Z, Qiagen, Inc. Toronto, ON).

Amplification of specific gene products was detected using the SYBR Green PCR mastermix and the real time amplification data was gathered using the ABI 7900HT software. The samples were amplified for 40 cycles for 15 s at 95°C and 60 c at 60°C. each curve was completed with a melting curve analysis, to confirm the specificity of amplification. Gene expression was normalized to internal controls (housekeeping genes) to establish fold change in gene expression between the controls and treated samples by C_T method (Qiagen RT² Profiler PCR Array Analysis program).

Fractionation & Phytochemical Analysis of Dandelion Root Extract

Analyses were carried out in collaboration with Dr. JT. Arnason and his group, at the University of Ottawa, Ottawa ON. The protocol was carried out following a previously published protocol (Guerrero et al., 2010). Ground dandelion root (1.5 kg) was extracted twice in ethanol (95% in H₂O) with a 24-hour incubation for both extractions, at room temperature. The first extraction was made using 10 L of solvent and the second extraction, using 8.0 L. the combined extracts were evaporated in a vacuo and lyophilized to yield 93.3 g of a brown residue (crude extract; 6.2% yield). Dried extract was chromatographed on a glass column packed with silica gel (1.0 kg) eluting with 5% increments of polarity gradients, starting from 100% hexane, to 100% ethylacetate, to 100% methanol (hexanes-EtOAc (1:0 f 0:1) and EtOAc-MeOH (1:0 f 9:1). This yielded 210 fraction, which were then pooled to yield 25 primary fraction, by thin-layer chromatography (TLC) analyses performed on a silica gel 60 F254 plate (Merck), an visualization of plates was carried out using a ceric sulfite (10%) solution in H₂S. Bioactivity analyses led to the identification of 5 bioactive fractions. Secondary fractionation yielded 24 secondary subfractions. Pre-fractionation of secondary fractions with significant bioactivity was carried out and separated on a preparative scale 1200 series Agilent preparative scale HPLC using a reversed-phase Gemini Axia 250 x 21.2 mm column, particle size 10 µm (Phenomenex Inc., Torrance CA), using an isocratic mobile phase composition of 45% THF in 55% water at 37.5mL.min to afford uvaol (12.0 mg, 0.0008%), botulin, (100 mg, 0.007%), α-amyrin (6.5 mg, 0.0004%), and betulinic acid (5.0 mg, 0.0003%) at the monitoring wavelength of 210 nm, bandwidth 4, reference off.

The final preparative scale isolation of the phytochemicals was undertaken using a reversed-phase Gemini Axia 250 x 21.2 mm column, particle size 10 μm (Phenomenex Inc., Torrance CA), on an Agilent 1200 Series preparative HPLC system comprising a binary pump, an autosampler with a 2 mL loop, a diode array detector, with a flow cell (Path length 3 mm and maximum pressure limit, 120 bar), and a fraction collector (40 μL collection tubes). IR spectra were recorded on a Shimadzu 8400-S FT/IR spectrometer. Optical rotations were registered on a Perkin Elmer 241 digital polarimeter. NMR spectra were recorded on a Bruker Avance 400 spectrometer in $\text{C}_5\text{D}_5\text{N}$, at either 400 MHz (^1H) or 100 (^{13}C) MHz, using tetramethylsilane (TMS) as an internal standard. EIMS and HREIMS were obtained on a Kratos Concept IIH mass spectrometer.

Western Blotting Analysis

SDS-PAGE was performed on the protein samples. Treated cells were lysed in lysis buffer (0.1% NP40, 20 mM Tris-HCl, 100 mM NaCl and 5 mM EDTA), following which, the total protein was measured by Bradford assay. Proteins were separated on a 10% gel and then transferred to a nitrocellulose membrane. Following transfer, the membranes were blocked in a milk solution (5% w/v milk in Tris-Buffered Saline with Tween-20 (TBST)) for 90 minutes. Subsequent to the blocking step, membranes were probed with primary antibodies, overnight at 4°C ; anti-cFLIP, raised in rabbit (1:1000), (Catalog No. GTX28421, GeneTex Inc. Irvin, CA) and anti-actin, raised in mouse (1:1000), (Catalog No. sc-81178, Santa Cruz Biotechnology, CA). Following incubation with the primary antibodies, membranes were washed in TBST (1X – 15 minute wash and 2X – 5 minute wash) and following the washes, membranes were incubated

with anti-mouse or anti-rabbit horseradish peroxidase conjugated secondary antibody (1:1000) – (Catalog No. ab6728 and ab6802, respectively, Abcam, Cambridge MA) for an hour at room temperature. The membranes were then washed for 3 X 5 minutes in TBST, followed by band visualization with a Visiglo Select HRP chemiluminescent substrate kit (Catalog No. CA11027-138, VWR International, Mississauga ON). Densitometry analyses were performed using Image J software.

In-vivo Assessment of Dandelion Root Extract:

Toxicity Assessment

Six week old Balb/C mice were obtained from Charles River Laboratories and housed in constant laboratory conditions of 12-hour light/dark cycle, in accordance with the animal protocols outlined in the University of Windsor research ethics board (AUPP #10-17). Following acclimatization, mice were divided into two groups (4 animals/control (untreated) and 4 animals/treatment group). The control untreated group was given plain filtered water, while the second group was given 5 mg/ml dandelion root in drinking water, resulting in approximately 40 mg/kg/day of DRE in their drinking water, respectively for 75 days. During the period of the study, toxicity was measure by weighing mice twice a week and urine was collected for protein urinalysis by urine dipstick and Bradford assays. Following the duration of the study, mice were sacrificed and their organs (Livers, kidneys and hearts) were obtained for immunohistochemical and toxicological analysis by Dr. Brooke at the University of Guelph.

Efficacy of DRE in Tumor Xenograft Models of Immunocompromised Mice

Six week old male CD-1 nu/nu mice were obtained from Charles River Laboratories and housed in constant laboratory conditions of 12-hour light/dark cycle, in accordance with the animal protocols outlined in the University of Windsor research ethics board (AUPP #10-17). Following acclimatization, mice were subcutaneously injected in the right and left hind flanks with a colon cancer cell suspension (in PBS) at a concentration of 2×10^6 cells/ mouse (HT-29, p53^{-/-} in the left flank and HCT116, p53^{+/+} in the right flank). Tumors were allowed to develop (approximately a week), following which, the animals were randomized into treatment groups of 4 mice per group, as outlined in the toxicity studies above. The mice were allowed to drink *ad libitum*, and treatment was replaced everyday. All mice were assessed for toxicity, as well as efficacy of oral administration on the growth of tumors. The tumors were assessed every other day by measuring the length, width and height, using a standard caliper and the tumor volume was calculated according to the formula $\pi/6 \times \text{length} \times \text{width}$. The mice were also assessed for any weight loss for the duration of the study, which also lasted 75 day. Following the study, mice were sacrificed and their organs and tissues (livers, kidneys, hearts and tumors) were obtained and stored in formaldehyde for immunohistochemical and toxicological analysis.

Hematoxylin & Eosin (H & E) Staining

Mice organs were fixed in 10% formaldehyde, following which the organs were cryosectioned into 10-micron sections and placed on a superfrost/Plus microscope slides (Fisherbrand, Fisher Scientific). Sections of organs were stained according to a standardized H & E protocol (Fischer et al., 2008).

Statistical Analyses

All experiments were repeated at least three independent times. Statistical analysis was performed using GraphPad Prism 6.0 288 software. Statistical tests included the Students T-test, one- and two-way Anova.

RESULTS

Efficacy of Dandelion Root Extract in Various Cancer Cell Models:

Dandelion Root Extract Induces Apoptosis in Aggressive Colorectal Cancer Cells

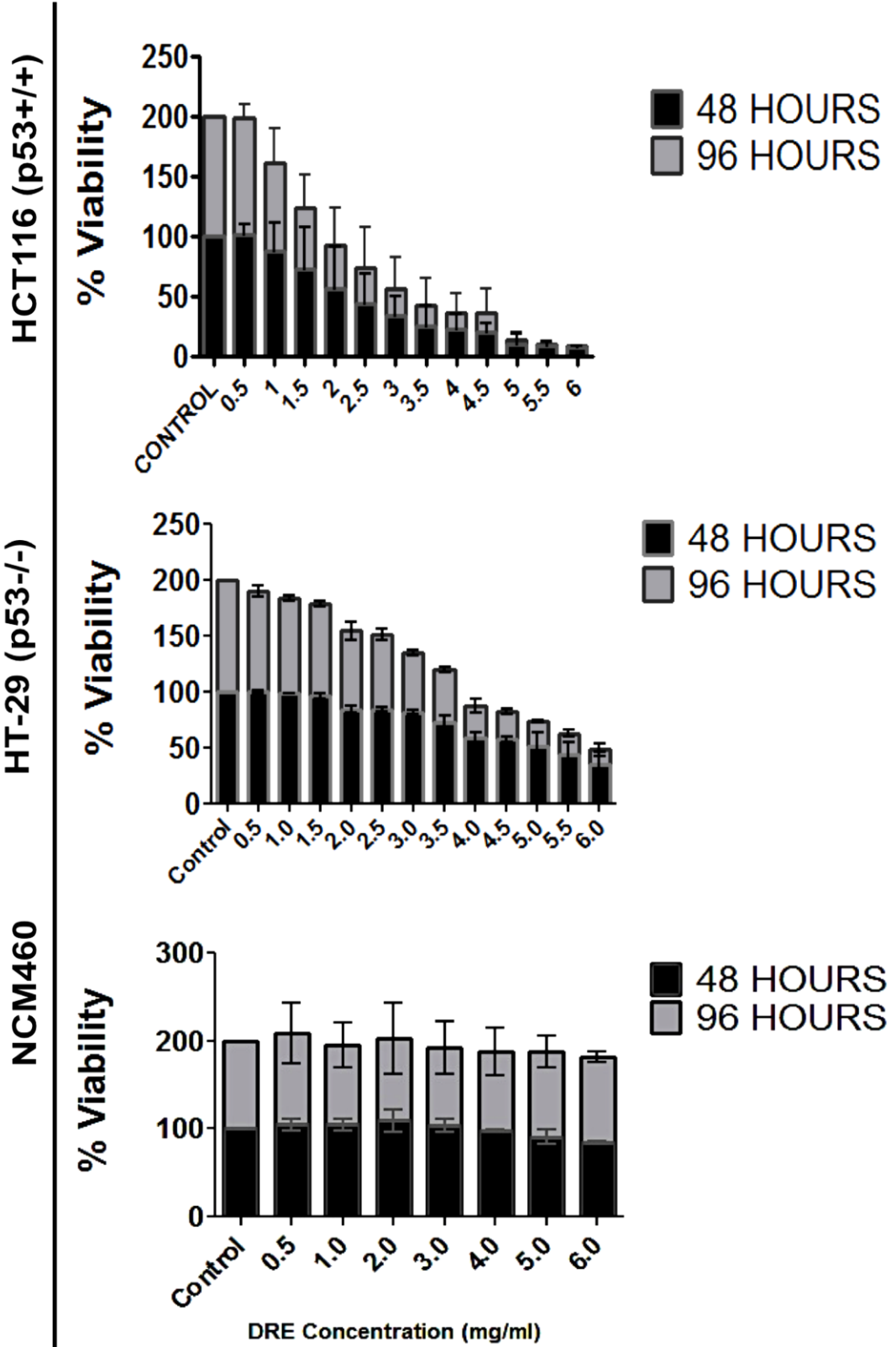
Previous results with dandelion root extract (DRE) have implicated this extract as a possible anti-cancer agent in various leukemia, melanoma and pancreatic cancer cell models (Chatterjee et al., 2011; Ovadje et al. 2011; Ovadje et al., 2012a; Ovadje et al., 2012b). The capacity of DRE's apoptosis-inducing activity prompted further studies into the efficacy of this aqueous extract in highly aggressive colorectal cancer cells.

To determine if this water extract of dandelion root shows potency against colon cancer cells, HT-29 and HCT116, were used in this study. Along with these cell types, normal mucosal colon epithelial cells (NCM460) were used to assess any levels of toxicity associated with DRE treatment. Following treatment with DRE, at increasing concentrations and time points, we observed a resultant decrease in the viability of both HT-29 and HCT116 colorectal cancer cells, irrespective of p53 status (Figure 1A). Employing the WST-1 cell viability assay, we determined the EC₅₀ of DRE in both colon cancer cell lines; 2.0 mg/ml in HCT116 cells and 3.5 mg/ml in HT-29 cells. The selectivity of DRE to cancer cells was once again confirmed, as we did not observe a similar trend in the response of NCM460 cells, treated in parallel with the colon cancer cells. (Figure 1A).

Further investigation into the anti-cancer efficacy of DRE in colon cancer cells indicates that DRE efficiently induces apoptosis in colon cancer cells selectively. Following treatment, cells were stained with Hoechst 33342,

propidium iodide and Annexin V dye to observe the nuclear morphology, cell membrane integrity and externalization of phosphatidylserine respectively. Following DRE treatment, we observed a corresponding increase in propidium iodide and Annexin V positive staining, by fluorescence microscopy, indicative of apoptosis in colon cancer cells, while NCM460 cells again remained unaffected (Figure 1B). Image- based cytometry was used to quantify the amount of apoptosis induced by DRE. Treatment with DRE led to an approximately 40% increase in Annexin V positive cells and a corresponding 97% increase in propidium iodide cells (Figure 1C). These results confirm the anti-cancer potential of dandelion root extract and demonstrates its efficacy in aggressive colorectal cancer cells.

A



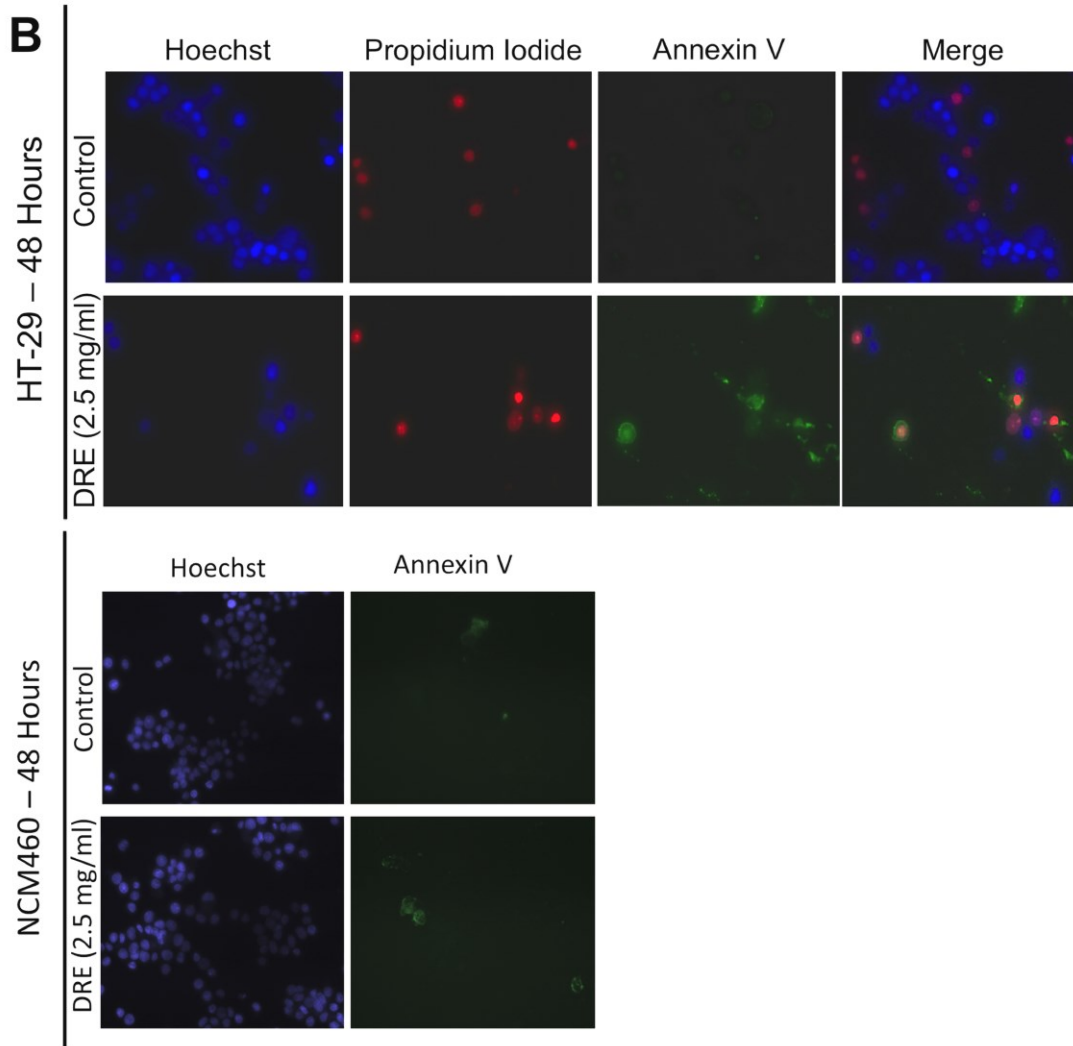


Figure 1: Dandelion Root Extract Induces Apoptosis in Aggressive Colorectal Cancer Cells: Colon Cancer cells (HT-29 [p53^{-/-}] and HCT116 [p53^{+/+}]) and normal colon mucosal epithelial cells (NCM460) were treated with increasing doses of DRE and analyzed for anticancer effects. A) Cells were plated in 96-well clear bottom plates and treated with DRE at indicated time points and analyzed for the ability of DRE to reduce the viability of these cells, measured by a decrease in metabolic activity. Following treatment, the WST-1 reagent was added to each well, the absorbance readings were taken at 450 nm, and expressed as a percentage of the control. The absorbance readings were analyzed using GraphPad Prism version 5.0 and values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. B) Hoechst (Blue), Annexin V (Green) and Propidium Iodide (PI) (Red) staining of DRE-treated cells, 48 hours after treatment. Fluorescence images were obtained at 400X magnification.

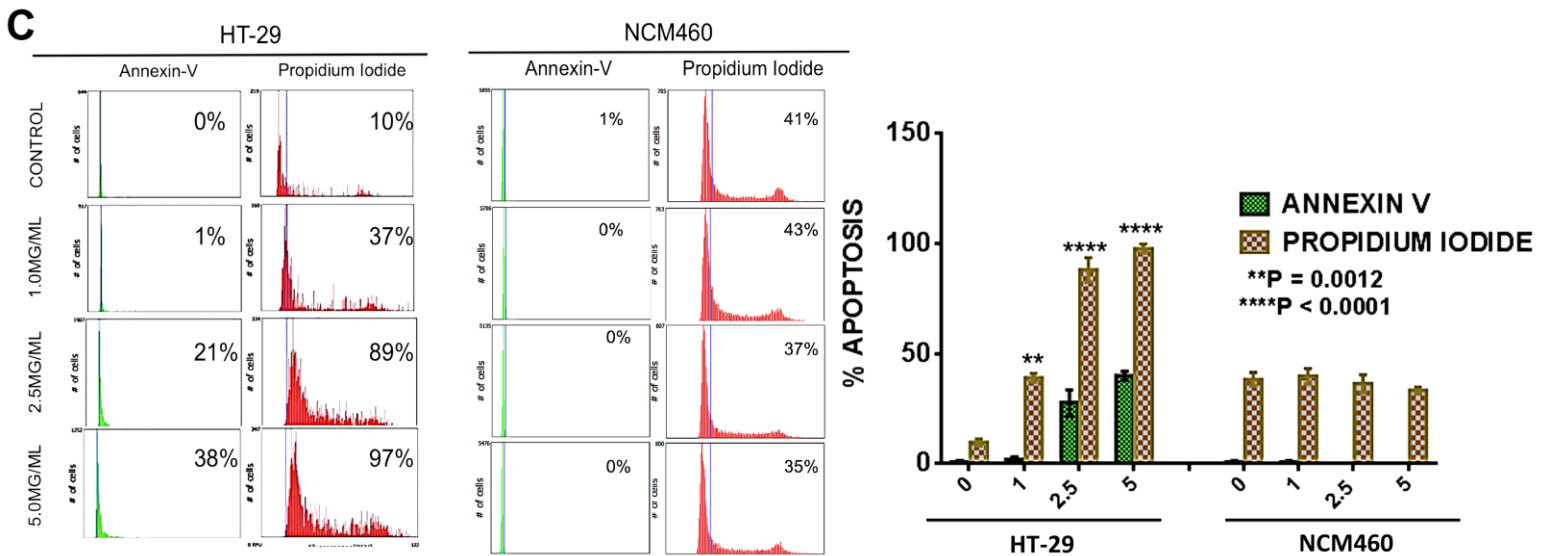


Figure 1: Dandelion Root Extract Induces Apoptosis in Aggressive Colorectal Cancer Cells: Colon Cancer cells (HT-29 [p53^{-/-}] and HCT116 [p53^{+/+}]) and normal colon mucosal epithelial cells (NCM460) were treated with increasing doses of DRE and analyzed for anticancer effects. C) Following treatment, cells were stained with Annexin V and PI and image-based cytometry was used to assess the induction of apoptosis. Experiments were repeated three independent times and the data was quantified to determine the percentage of apoptotic and necrotic cells, as measured by annexin V and PI.

Dandelion Root Extract Can Halt the Growth of Colon Tumors in Xenograft Models

As alluded to previously, the anticancer efficacy of DRE has been observed in various cancer cell types, including the reported colorectal cancer cell types mentioned above. The *in-vitro* results indicated a need for further studies into the anticancer efficacy of DRE. Dandelions have been used for centuries for the treatment of various diseases; and the different parts of the plant have been used to treat ailments, including dyspepsia, spleen and liver complaints, hepatitis and anorexia (Schütz et al., 2006; Yarnell & Abascal, 2009). These reports also outline a lot of the anecdotal evidence surrounding the use of various parts of the plant. However, there is a distinct lack of reports indicating toxicities associated with its use. Our *in-vitro* studies further prove that DRE is able to distinguish between cancer and non-cancerous cells to cause a decrease in the viability of cancer cells and induce apoptosis, with no toxicity to non-cancerous cells.

To further scientifically evaluate and validate the safety of DRE, the first step was to assess the toxicity to regular mice in a toxicological study. Balb/c mice were orally administered DRE, at a dose of 40 mg/kg/day, in their drinking water and allowed to drink *ad libidum*, for a period of 75 days; these mice were observed for signs of toxicity for the duration of the study. To measure toxicity, mice were weighed twice a week and urine was collected, for protein urinalysis studies, to determine any damage to the kidneys of the animals on DRE regimen. Results showed no significant difference between the control, untreated group and the DRE supplemented water group in terms of weight of the mice in each

group. No significant difference was observed between the control and DRE-fed groups, suggesting a lack of toxicity (Figure 2A). These results were further confirmed by a distinct lack of difference in protein levels in the urine of mice collected from each group in the last four weeks of the study. The protein urinalysis was performed using a urine dipstick kit, as well as a Bradford protein concentration assay. The results indicate that there were trace amounts of protein in the urine of mice from both groups (Figure 2B).

Following the duration of the study, mice were sacrificed and their organs were obtained for histopathological analysis, by a certified pathologist at the University of Guelph. Hematoxylin and eosin staining of the hearts, livers and kidneys showed no gross morphological differences in tissue slices between the control and the DRE treated group (Figure 2C). These results indicate a lack of toxicity associated with DRE, through oral administration in our animal studies and results from the pathologist further confirmed our results. The results from the pathologist were accompanied by a statement, indicated that the presence of any lesions in the tissues are minimal or mild and interpreted as either background or incidental lesions and the lack of lesion type and frequency was sufficient to conclude no toxicological effect of DRE to the balb/c mice (Table 1).

Table 1: Summary of Histological Lesions in Balb/C Mice on DRE regimen

	No Treatment (Plain-filtered water)					Treatment group (DRE-Supplemented water)			
	M1	M2	M3	M4	M5	M1	M2	M3	M4
<u>Liver:</u>									
-Infiltration, leukocyte, predominantly mononuclear, minimal		X	X		X				
-Focal mineralization, minimal						X			
-Hepatocyte necrosis, minimal									
-Focus of cellular alteration, eosinophilic, minimal			X	X		X			
-Hepatocyte vacuolation, lipid type, minimal			X	X				X	
- Hepatocyte vacuolation, lipid type, mild	X			X		X			X
Fibrin thrombus			X						
<u>Heart:</u>									
-Infiltration, leukocyte, predominantly mononuclear, minimal		X				X			
Myofiber separation and vacuolation, minimal (suspect artifact)		X	X					X	
<u>Kidney:</u>									
- Infiltration, leukocyte, predominantly mononuclear, minimal	X	X		X				X	X
Tubule vacuolation, minimal					X		X		X
Fibrin or other extracellular matrix, glomerulus									

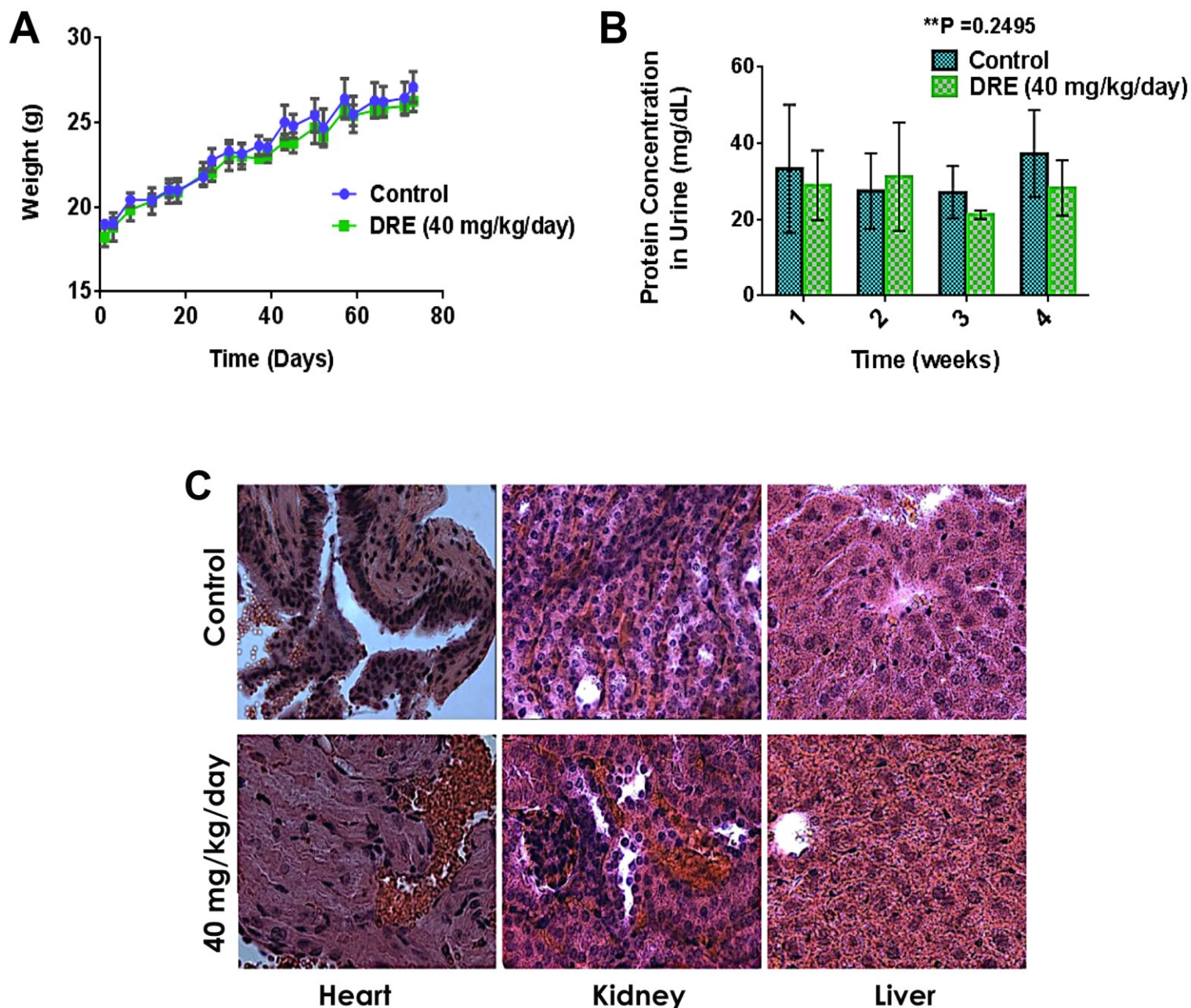
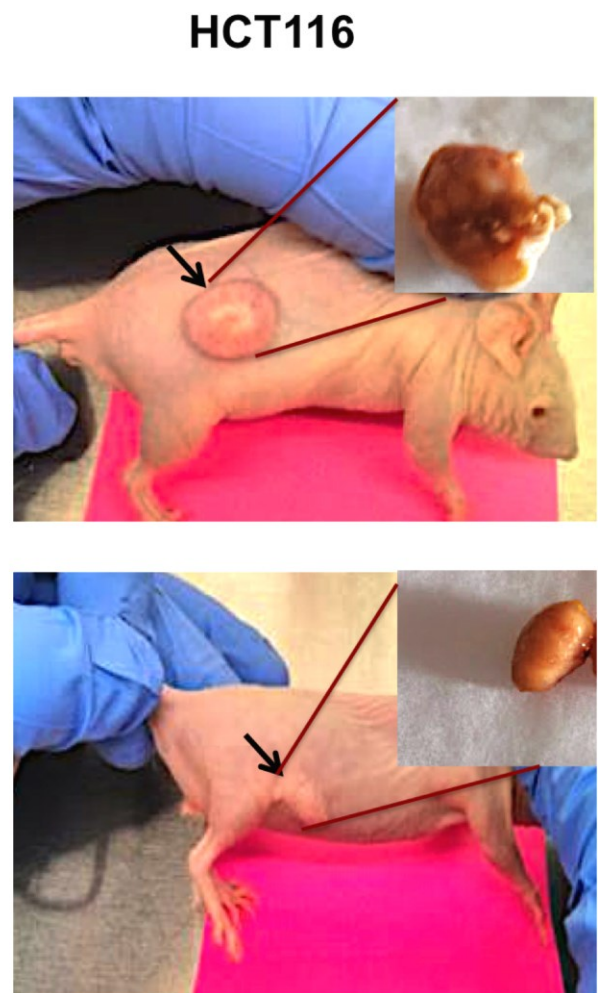
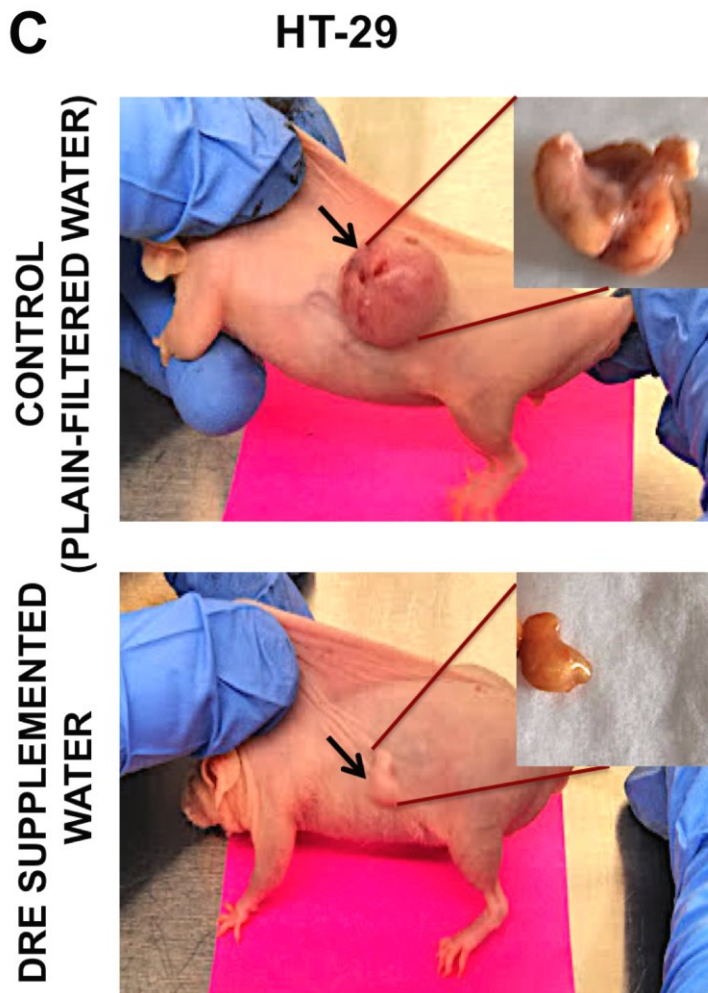
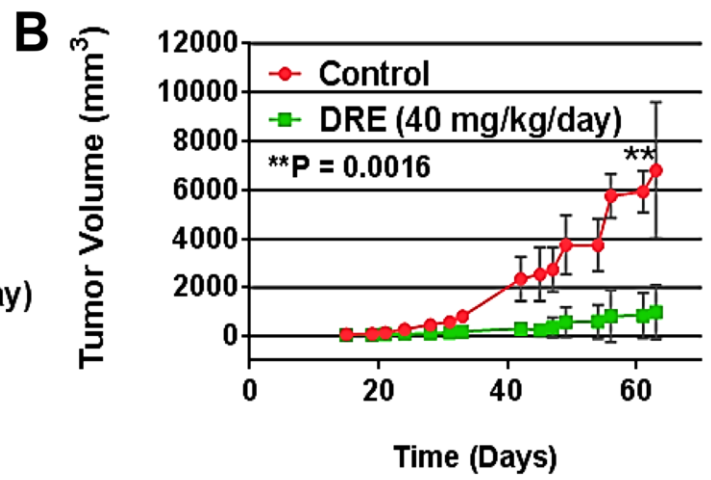
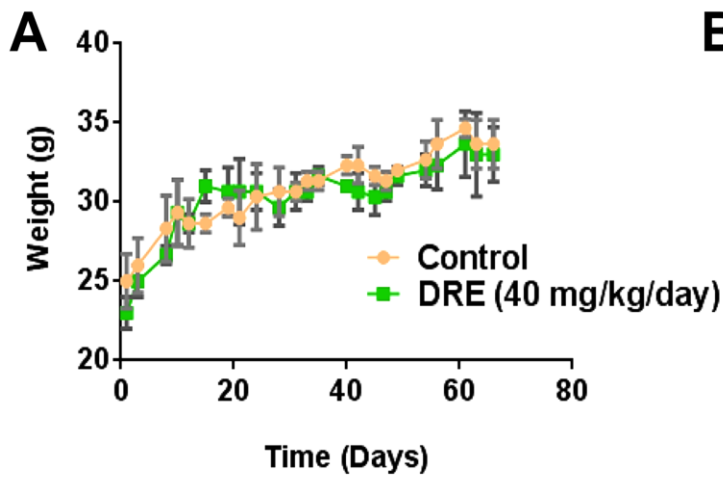


Figure 2: Dandelion Root Extract is Well Tolerated in Animal Models: Balb/C mice were separated into two groups, one group on plain filtered water and the other, on DRE-supplemented water (40 mg/kg/day) for a period of 75 days. Mice were assessed for toxicity of DRE. A) Weight of mice in each group for the duration of the study. B) Urine was obtained from mice every week for the last 4 weeks of the study. Protein urinalysis was carried out using urine dipstick and the Bradford protein assay. C) Following the period of the study, mice were sacrificed and their tissues (hearts, kidneys and livers) obtained for histopathological studies; Hematoxylin and Eosin staining of tissues. Images were obtained on a brightfield microscope at 63X Objective.

Following toxicity studies, we wanted to further evaluate the efficacy of DRE in animal tumor models. For this part of the study, CD-1 nu/nu immunocompromised mice were subcutaneously injected with HT-29 cells (left flank) and HCT116 cells (right flank). Following the establishment of colon tumors, mice were separated into two groups (same as the toxicity study). Mice were observed for the duration of 75 days, while the weights and tumor volumes were measured twice a week. The results obtained demonstrate that oral administration of 40 mg/kg/day of DRE in the drinking water did not have an effect on the weights of the mice (as observed in the toxicity studies) and was efficient in suppressing the growth of both p53 WT (HCT116) and p53 mutant (HT-29) tumors in-vivo (Figure 3A – C). Additionally, H & E staining revealed less nuclei in the DRE treated group, compared to the control group, however, as observed in the toxicity studies, there were no gross morphological differences in the livers, kidneys and hearts of the control and DRE treated groups (Figure 3D). These results not only indicate that oral administration of DRE is not toxic to animal models, but is efficacious in halting the growth of colon tumors in xenograft models.



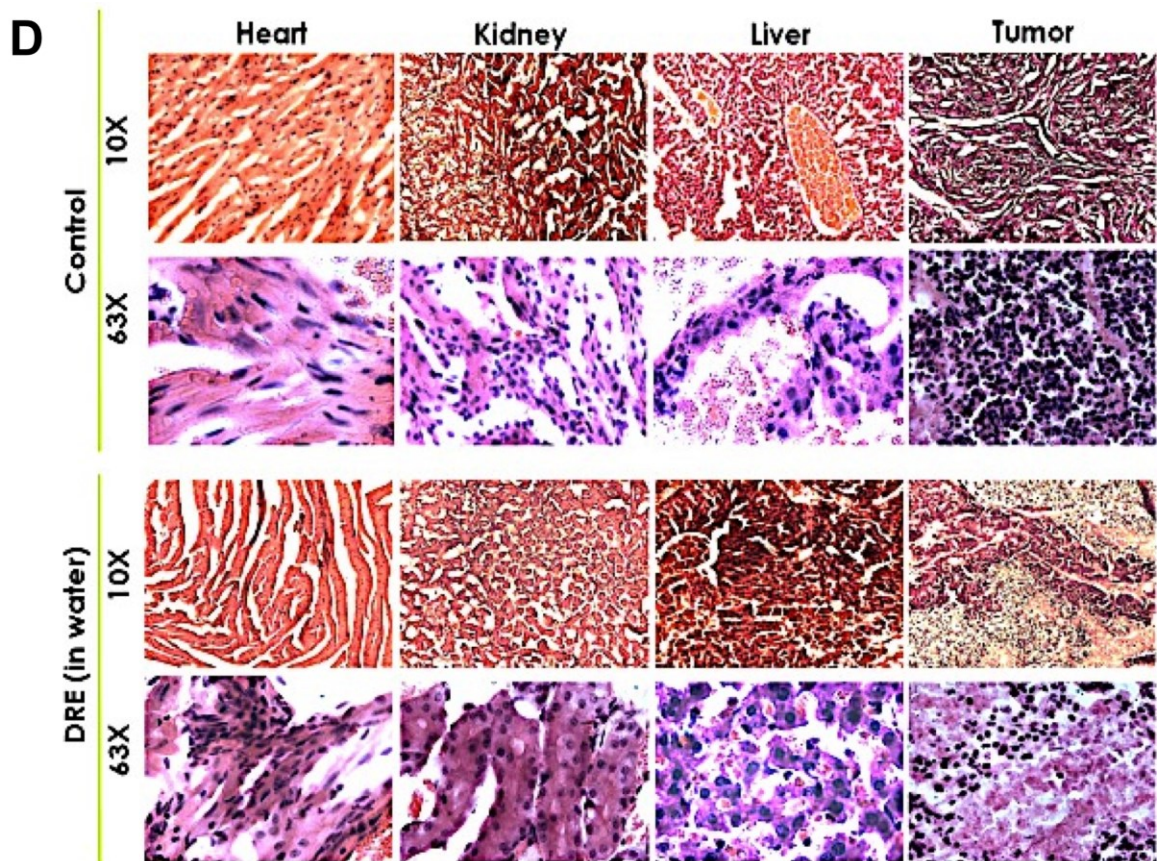


Figure 3: Dandelion Root Extract Halts the Growth of Colon Tumor Xenografts: Following the toxicity studies, immunocompromised CD-1 nu/nu mice were subcutaneously injected with 2×10^6 colon cancer cells (HT-29 on the left flank and HCT116 on the right flank). Following the establishment of tumors, mice were separated into the same groups, as described in the toxicity studies. A) Mice were weighed every other day for the duration of the study. B) The tumor volumes were measured using a standard caliper and the tumor volumes were calculated according to the formula $\pi/6 \times \text{length} \times \text{width}$. C) Images of mice tumors week 9 of the study, showing differences in the tumor sizes between the control, untreated group and the DRE group. D) Following the period of the study, mice were sacrificed and their tissues (hearts, kidneys and livers), as well as tumors, were excised for histopathological studies; Hematoxylin and Eosin staining of tissues. Images were obtained on a brightfield microscope at 63X Objective.

Dandelion Root Extract Induces Apoptosis in Clinical Samples of Patient-Derived Peripheral Blood Mononuclear Cells

The next phase of this study was to determine if DRE could be taken further, as a potential anticancer agent in a clinical setting. Blood samples were obtained from newly diagnosed leukemia patients. Table 2 provides details about patient age, leukemia type at time of diagnosis, along with their response to DRE treatment in ex-vivo conditions. All of the diagnoses are first occurrence, unless otherwise stated. Patients were diagnosed in the clinic with: Acute Myeloid Leukemia (AML), subtyped as M0 - M7 or MDS (myelo- dysplastic syndrome); Acute Lymphoblastic Leukemia (ALL), sub- typed as Burkitt's (L3); Chronic Myelogenous Leukemia (CML); or Chronic Myelomonocytic Leukemia (CMML) and Chronic Lymphocytic Leukemia (CLL). All samples (n = 11) showed an increase in apoptosis (by Hoechst, Annexin-V and propidium iodide staining in fluorescence microscopy and image-based cytometry) in a dose and time-dependent manner, with > 80% of apoptotic cells at the highest dose of 5.0 mg/ml DRE at 48 hours (Figure 4A-C). These results were distinctly dissimilar to results obtained with ncPBMCs, which showed no influence on apoptotic induction by DRE treatment (Ovadge et al., 2012b). These results demonstrate that DRE is selectively toxic to cancer cells in *in-vitro*, *in-vivo* and *ex-vivo* models, irrespective of cancer type, with little to no associated toxicity to non-cancerous cell models.

Table 2: Patient-Derived Samples of Leukemia, with Response to DRE Treatment

Patient #	Age	Clinical Diagnosis	Time of Exposure to DRE	% Apoptosis
1	42	CLL	48 H	44 ± 10.2
2	72	AML	48 H	56 ± 7.8
3	78		48 H	97.9 ± 1.1
4	58	ALL	48 H	70 ± 3.9
5	56	AML – M4	48 H	37.7 ± 5.3
6	69		48 H	39.03 ± 5.4
7	52	ALL	48 H	76 ± 10.4
8		AML	48 H	57.9 ± 3.6
9			48 H	87.2 ± 13.7
10			48 H	66.4 ± 3.2
11			48 H	89.3 ± 4.5

****Spaces; Information was withheld.**

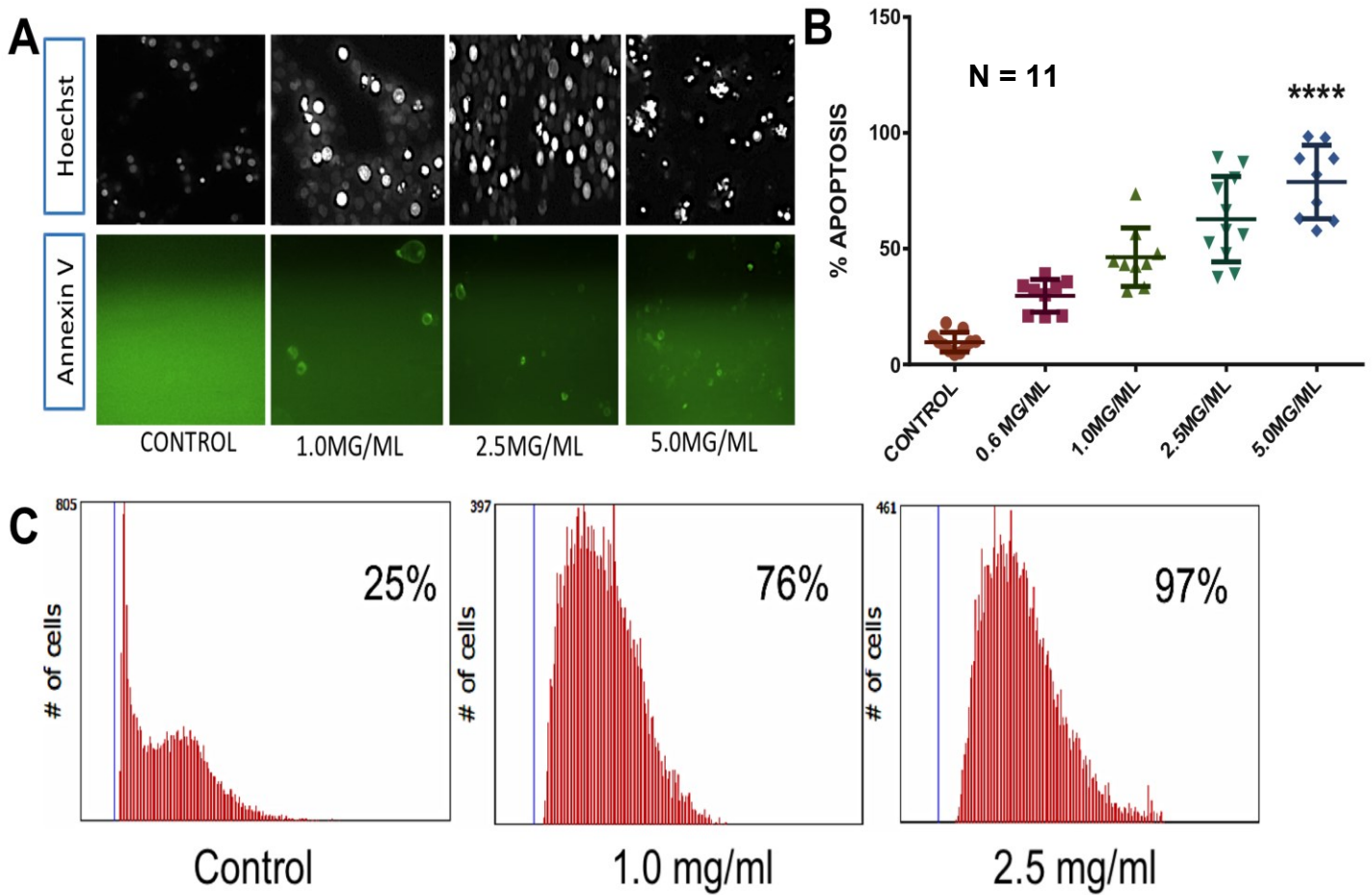


Figure 4: Dandelion Root Extract Induces Apoptosis in Clinical Samples of Patient-Derived Peripheral Blood Mononuclear Cells (PBMCs): PBMCs were isolated from the blood of newly diagnosed patients. Cells were treated with increasing concentrations of DRE and analyzed for the induction of apoptosis. A) Representative Hoechst (white), Annexin V (Green) staining of DRE-treated cells, 48 hours after treatment. Fluorescence images were obtained at 400X magnification. B) Quantification of apoptotic cell death in PBMCs treated DRE (n = 11). C) Following treatment of PBMCs, cells were stained with PI and analyzed by image-based cytometry. Representative results are shown here.

Mechanism of Dandelion Root Extract in Selectively Targeting Cancer Cell:

Dandelion Root Extract Targets the Mitochondria of Colon Cancer Cells

Few scientific studies have attempted to delineate the mechanism of dandelion extracts, especially in cancer cells. Some studies have suggest that dandelion flower extracts (DFE) have antioxidant, pro-oxidant and cytotoxic activity in colon adenocarcinoma cells, Caco-2, while being able to scavenge reactive oxygen species in mouse macrophage cells, RAW264.7 (Hu & Kitts, 2003; Hu & Kitts, 2005). These effects have been attributed to the presence of flavonoids within the water and ethylacetate extracts of the flower. However, there is still a lot to be done regarding the presence of other compounds within these extracts and how they influence the activities. Previous studies in our lab have shown that aqueous DRE does not induce its cytotoxicity by inducing double stranded DNA breaks, by TUNEL labeling, in chronic myelomonocytic leukemia (CMML) cells, in order to induce apoptosis in these cell types (Ovadge et al., 2012b). These results have a confirmation in already published work, by other groups, indicating that DFE was efficient at reducing DNA damage, by decreasing the breakage of supercoiled DNA strands, even in the presence of oxidative stress (Hu & Kitts, 2003). These results therefore suggest a lack of genotoxicity associated with DRE use. However, as with previous studies, we observed a disruption of the mitochondrial membrane potential following DRE treatment, by JC-1 staining, in HT-29 cells. On the other hand, the mitochondria of NCM460 cells remained unaffected (Figure 5A). These results were quantified and confirmed by image-based cytometry, where we observed a decrease in the intensity of red fluorescence, indicative of a loss of mitochondrial membrane

potential in HT-29 cells, with no difference between the control and DRE treated samples in NCM460 (Figure 5B). These data suggest that DRE is able to target multiple significant vulnerabilities in cancer cells selectively and that such targets might include the mitochondria. To further investigate the role of the mitochondria in DRE induced PCD, mitochondria was isolated from HT-29 and NCM460 cells and the isolated mitochondria was directly treated with 2.5 mg/ml DRE. In the presence of Amplex Red and HRP, fluorescence readings were obtained every 5 minutes. Results obtained showed that DRE treatment led to a significant increase in the levels of reactive oxygen species (ROS) produced in HT-29 cells, compared to the control, untreated mitochondria. In parallel, it can be observed that treatment of isolated mitochondria from NCM460 cells, with DRE, did not lead to the same increase in the levels of ROS (Figure 5C), further confirming that DRE has multiple targets in cancer cells, leading to the selective targeting to self-destructive processes. These results further suggest that, like DFE, DRE might also contain both antioxidant and pro-oxidant properties (Hu & Kitts, 2003).

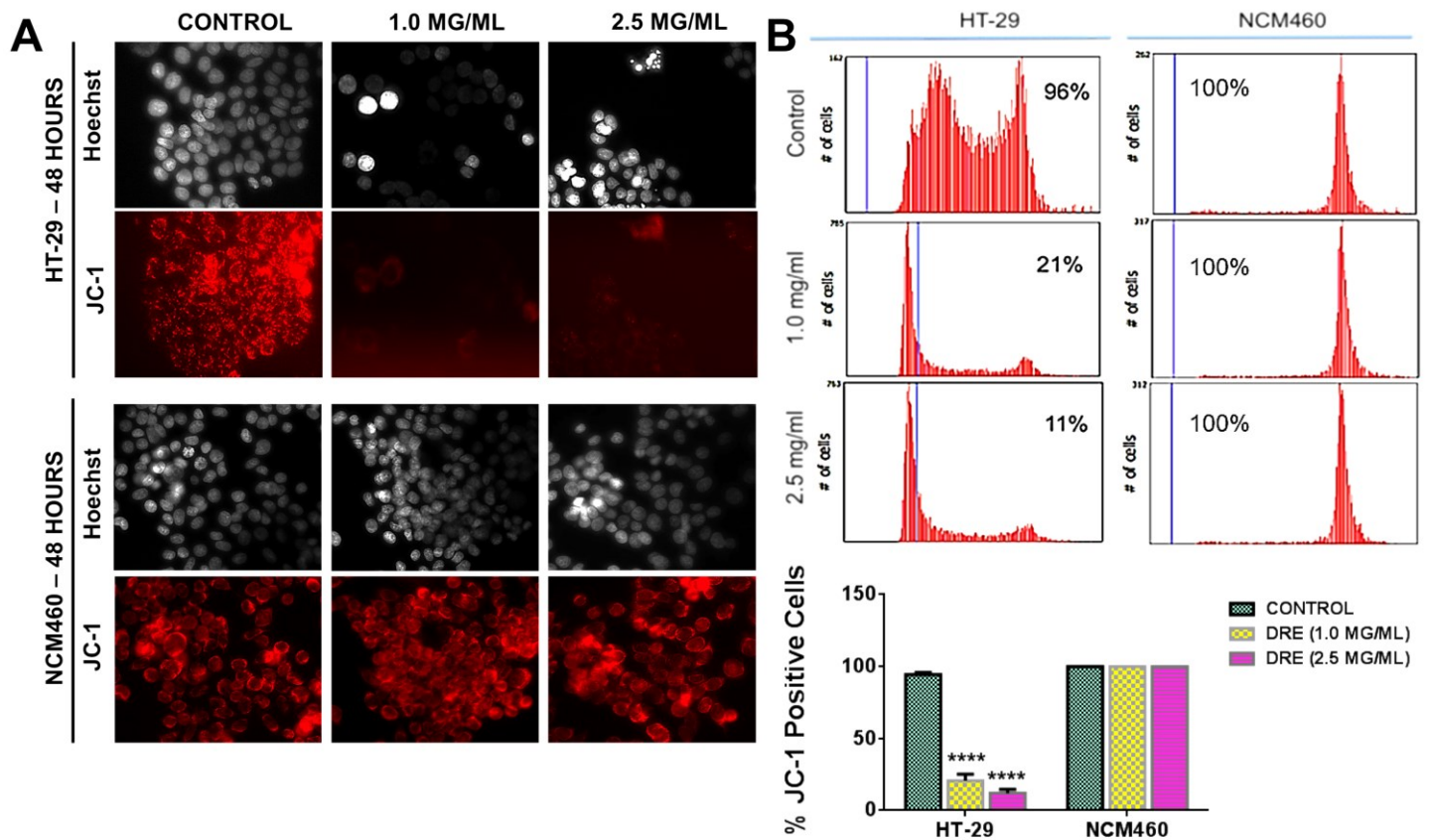


Figure 5: Dandelion Root Extract Targets the Mitochondria of Colon Cancer Cells: Following treatment with DRE, HT-29 and NCM460 cells were incubated with JC-1 dye. Red fluorescence intensity was measured by fluorescence microscopy (Magnification: 400X) (A) and image based cytometry (B) to detect the loss of the mitochondrial membrane potential. The mitochondria of HT-29 cells were completely destabilized, while those of the NCM460 cells remained intact.

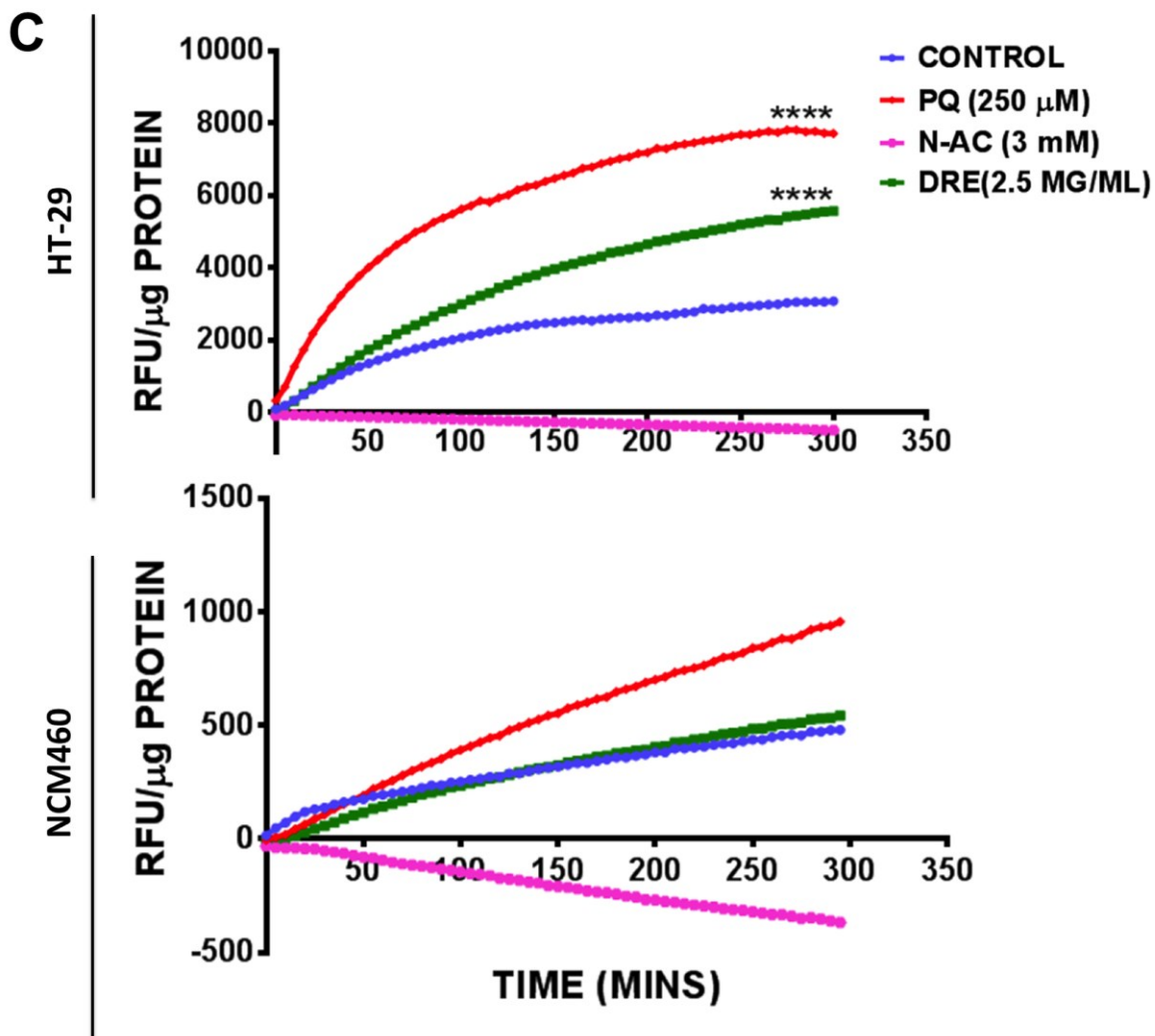


Figure 5: Dandelion Root Extract Targets the Mitochondria of Colon Cancer Cells: C) Isolated mitochondria from HT-29 and NCM460 were treated directly with DRE; ROS production was measured using Amplex Red substrate in the presence of horseradish peroxidase (HRP). Results were compared to the control, untreated mitochondria, the positive control, Paraquat (PQ) and the negative control, N-Acetylcysteine (N-Ac). Fluorescence readings were taken in 5-minute intervals for 5 hours at Ex. 560 nm and Em. 590 nm and expressed as relative fluorescence units (RFU) per μ g protein. Analyses of results were performed using Graphpad Prism and results shown are a representative of 3 independent experiments.

Dandelion Root Extract Activates Caspase-8 in Human Colorectal Cancer Cells

Previous studies in our lab have shown that DRE efficiently and rapidly activates the extrinsic pathway of apoptosis in leukemia and pancreatic cancer cells, with a dependence on the activation of caspase-8 for the subsequent induction of apoptosis, as cells with a dominant-negative Fas-Associated Death Domain (Dn-FADD) were unresponsive to DRE treatment (Ovadje et al., 2012b). This part of this study was aimed at further investigating the role of the extrinsic pathway in DRE-induced apoptosis. Using substrates specific fluorescent labeled inhibitors of caspases (FLICA) for each caspase (DEVD – Caspase-3; IETD – Caspase-8), the chronological activation of caspases could be monitored in HT-29 and NCM460 cells that had been treated with DRE for increasing time points, ranging from 15 minutes to 48 hours. Our results by image-based cytometry show that DRE induced a 68% increase in caspase-8 activation in HT-29 cells treated with 2.5 mg/ml DRE for an hour, with a corresponding 30% increase in propidium iodide staining. Furthermore, these results were specific to HT-29 cancer cells, as NCM460 normal cells did not respond to DRE treatment in the same manner (Figure 6A). Our results show that NCM460 cells had no increase in caspase-8 activation or propidium iodide staining, confirming the selectivity of DRE to cancer cells. These results also further confirm that DRE acts through the extrinsic pathway of apoptosis in cancer cells.

To further investigate the role of extrinsic apoptosis in DRE's mechanism of action, the localization of activated caspases, following DRE treatment, was studied. Caspases are crucial for the initiation, propagation and execution of

apoptosis (Loo et al., 2002), and therefore their activation and localization has been a focus of much research, with many conflicting reports. Some reports suggest that before activation, pro-caspases are located in the cytosol, and following apoptotic stimuli and activation, these caspases remain in the cytosol (Loo et al., 2002). Some other reports on the other hand have determined that following activation, caspases localize to the mitochondria, where they interact with other pro-apoptotic proteins for the progression of apoptosis (Chandra et al., 2004). A third option also indicates that inactive caspases are kept in the mitochondria, and following apoptotic stimuli and activation, these caspases are released out of the mitochondria into the cytoplasm and surround the peri-nuclear space (Qin et al., 2001). The aim of this part of the study was to confirm the activation of caspase-8, following DRE treatment and to determine the localization of caspase-8, before and after apoptotic stimuli and activation.

In order to do this cells that were plated onto coverslips were treated with DRE for 30, 60, 180 and 360 minutes and assessed for the activation and localization of caspase-8. These cells were incubated with MitoTracker dye for 45 minutes, prior to immunocytochemical analysis, described in the materials and methods. The results show that there was a progressive destabilization of the mitochondrial membrane following treatment of DRE, as early as 30 minutes, but more importantly, it was observed that pro-caspase-8 (green) localized with the mitochondrial dye (red) in the control untreated samples and following activation after DRE treatment, the now active caspase-8 was released out of the mitochondria as indicated by the dispersed green dye associated with caspase-8 (Figure 6B). These results indicate that, not only is DRE able to efficiently

activate the extrinsic pathway of apoptosis, by the rapid activation of caspase-8, it is also leads to the destabilization of the mitochondrial membrane in treated samples.

In our previous studies, we show that the activation of caspases was essential to the induction of apoptosis by DRE. To confirm this dependence of caspase activation, cells were pre-incubated with either a pan-caspase inhibitor, Z-VAD-fmk or a caspase-8 specific inhibitor, IETD-fmk for an hour, before DRE treatment. Following treatment, cells were analyzed by WST-1 viability assay. Results show that in the presence of caspase inhibitors, Z-VAD-fmk (results not shown) and IETD-fmk, there was still a corresponding decrease in the viability of HT-29 cells, in response to an increase in dose and time of exposure to DRE treatment. These results were comparable to the same studies carried out in the absence of caspase inhibitors (Figure 6C), suggesting that in HT-29 colorectal cancer cells, DRE's mechanism of action is caspase independent.

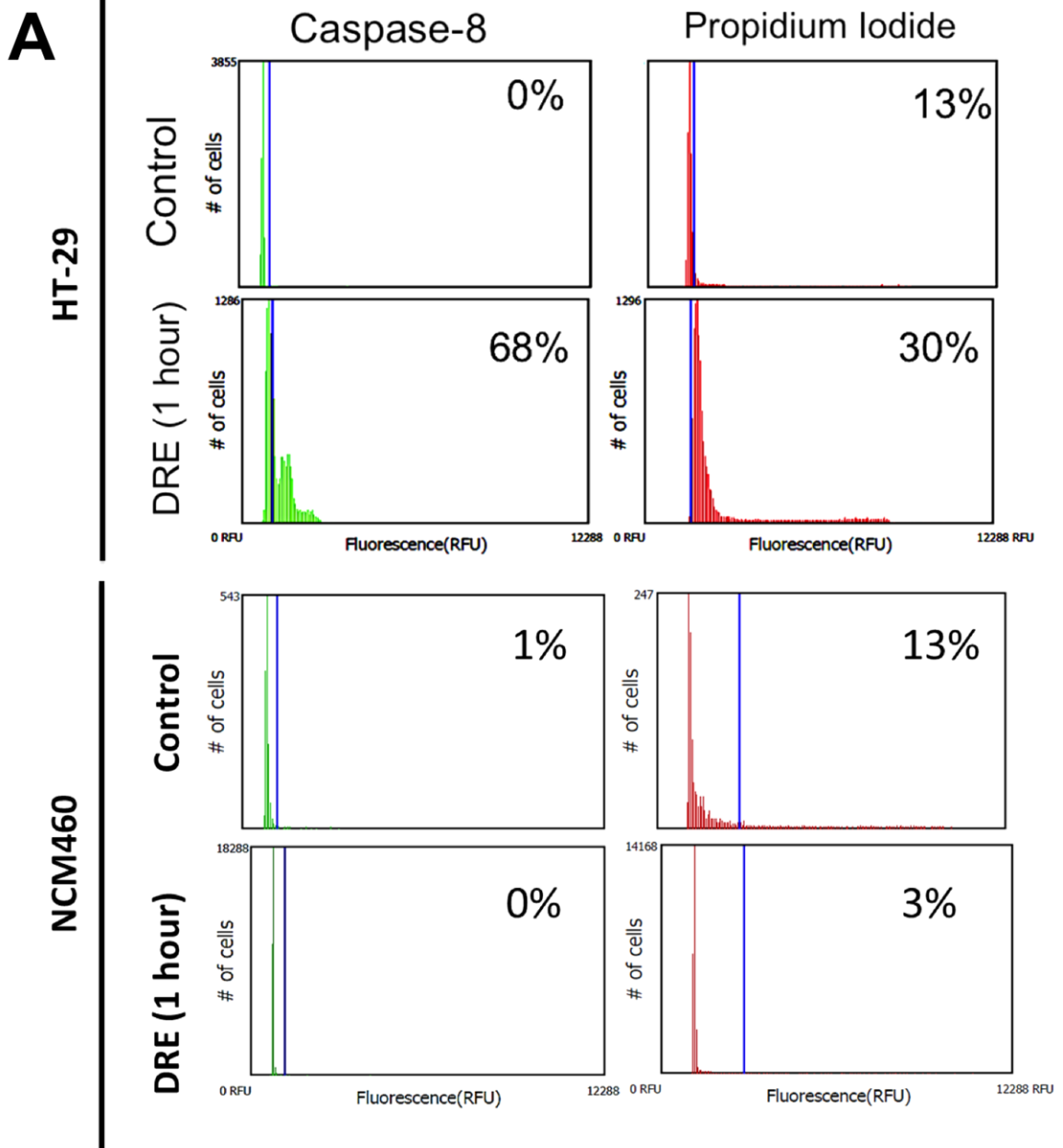
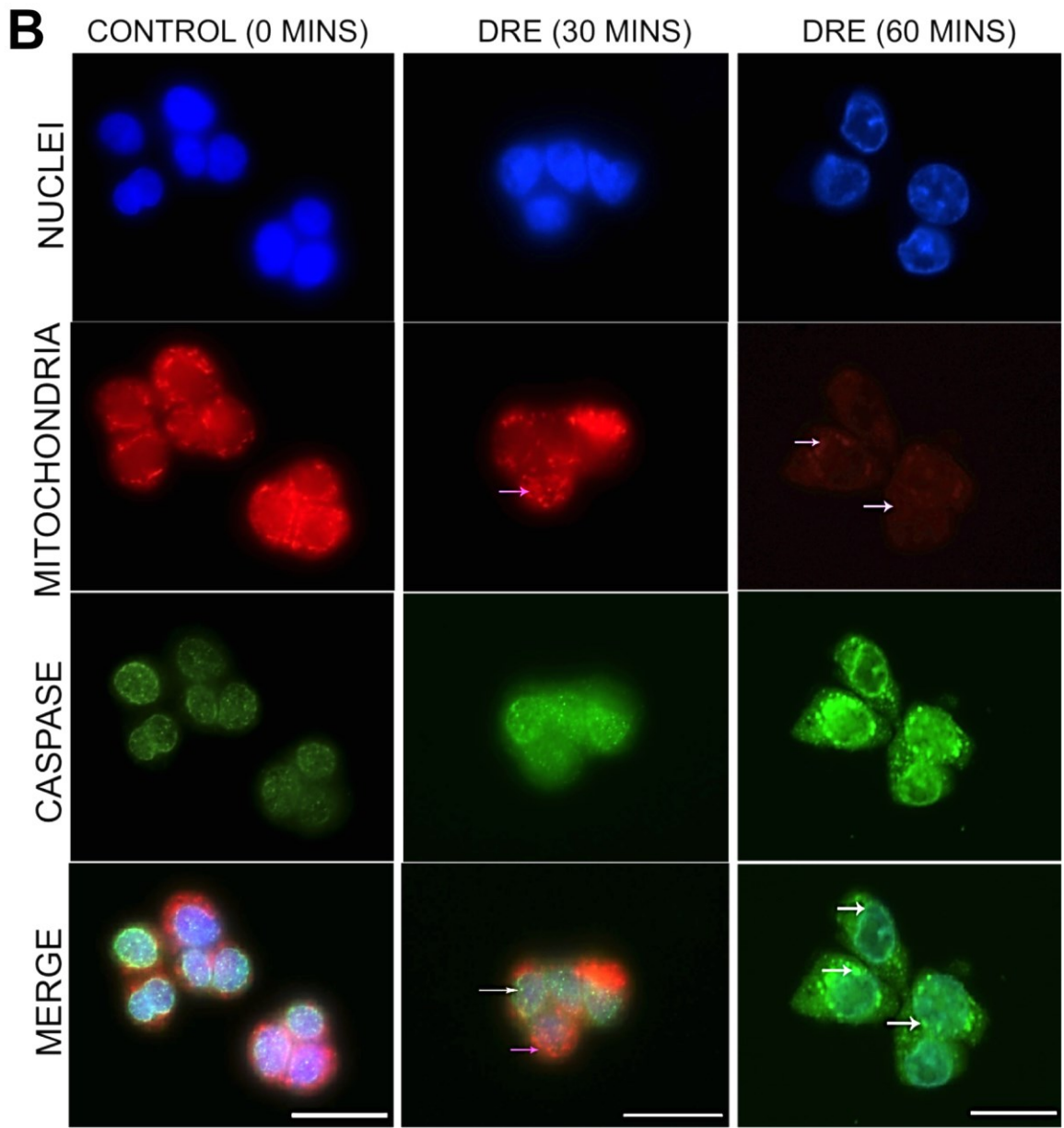


Figure 6: Dandelion Root Extract Triggers the Activation of Caspase-8: A) Following DRE treatment, HT-29 and NCM460 cells were incubate with FLICA substrates specific to caspase-8 and incubated for an hour and counterstained with propidium iodide. Fluorescence intensity was measured with image-based cytometry.



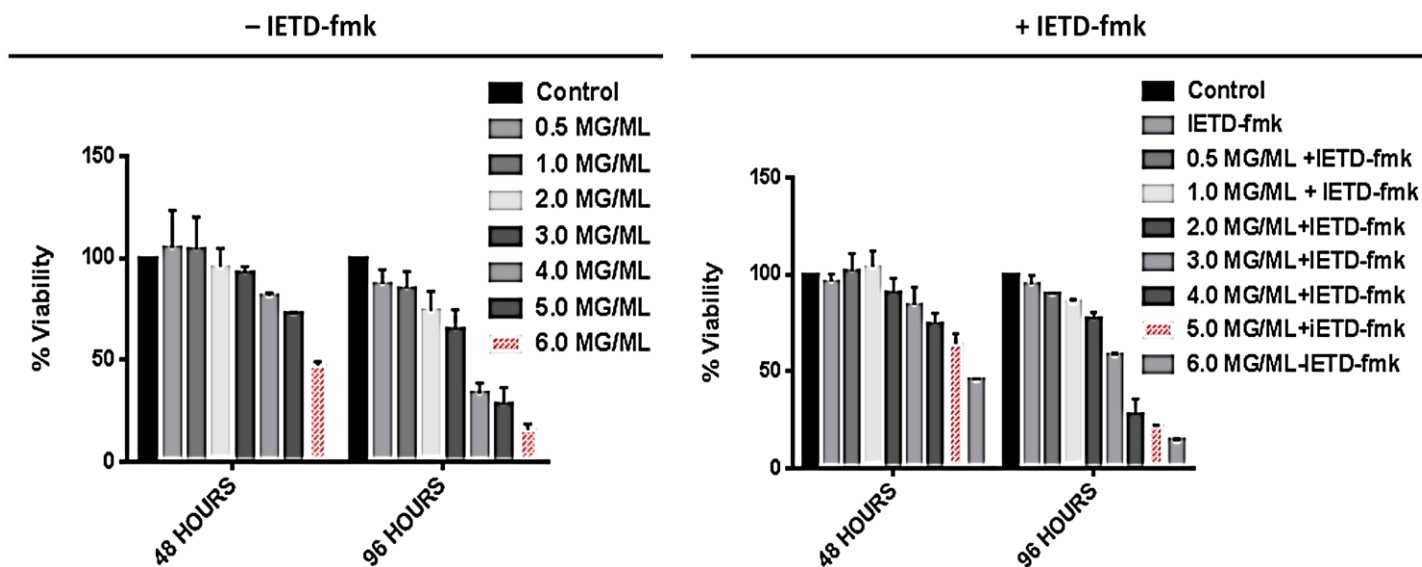


Figure 6: Dandelion Root Extract Triggers the Activation of Caspase-8: B)

To determine the localization of caspase-8 following activation, treated cells were incubated with MitoTracker (Red), fixed and then incubated with the anti-caspase-8 antibody, followed by a secondary antibody conjugated to AlexaFluor 488 (Green). Cells were also counterstained with Hoechst 33342 (blue) to detect the nuclear morphology. Images were obtained at 63X objective. White arrows indicate the dispersion of MitoTracker Red, indicative of a loss of mitochondrial membrane potential and the magenta arrows show the presence of active caspase-8 in the nucleus following DRE treatment. Scale bars = 20 μ m. C). Prior to DRE treatment, HT-29 cells were pre-treated with a caspase-8 specific inhibitor, IETD-fmk, for an hour at indicated concentrations and analyzed for cell viability, using the WST-1 assay as a measure of absorbance at 450 nm. Decreased cell metabolic viability (as a % of control) is observed with increasing concentrations of DRE.

Dandelion Root Extract Promotes the Expression of Cell Death Genes in Cancer Cells

Due to the inability of caspase-8 specific inhibitors to prevent the anticancer activity of DRE in HT-29 colon cancer cells, which was contrary to the results observed in our previous studies with leukemia and pancreatic cancer cells, the next step was to further investigate the different aspects of cell death processes that could be induced/activated, following DRE treatment. Using the RT2 Profiler Cell Death Pathway Finder PCR system, we were able to profile the expression of 84 key genes in the central mechanisms for apoptotic, autophagic and necrotic cell death. In CMML cells, we observed that DRE treatment led to high expression of several cell death genes, with $\geq 70\%$ of genes being expressed before cycle 30 in the treatment group, compared to $\geq 90\%$ of genes over cycle 30 (Figure 7A). Further analysis of the data showed 22 genes with over a 10-fold increase in expression, following DRE treatment (Table 3; Figure 7B). Table 3 further lists the gene functions of these highly expressed genes. It is important to note that treatment of CMML cells with DRE led to increased expression of $\geq 70\%$ of genes (≥ 59 out of 84 genes) in this cell death pathway finder. The results reported in this study are for the genes that had over a 5-fold increase in gene expression, as compared to the control untreated sample. This increase in gene expression was not confined to one major pathway, however, we observed increases in expressions of genes involved in apoptosis and autophagy, indicating that DRE has multiple targets in cancer cells, especially leukemia cells. This further confirms the connections between the programmed cell death processes of apoptosis and autophagy.

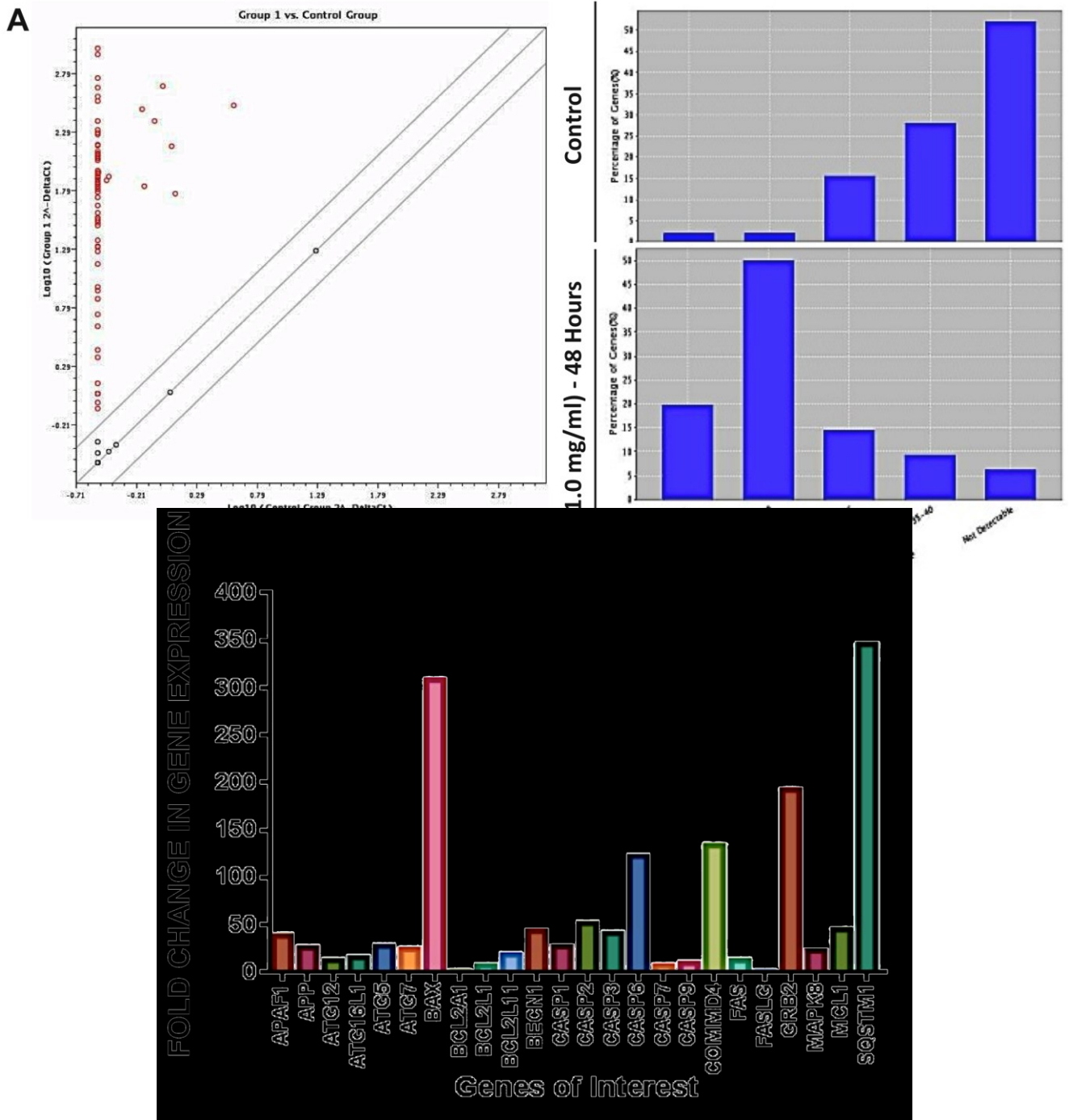


Figure 7: Fold Change in Expression of Cell Death Genes in Chronic Myelomonocytic Leukemia (CMML) Cells – (MV-4-11): The effect of DRE on cell death gene expression in CMML cells were evaluated using the RT2 profiler PCR assay. Following treatment of CMML cells with DRE, RNA was isolated, converted to cDNA and incubated with oligodT primers. PCR microarray analysis was carried out on an ABI 7900HT. A) Percentage of genes highly expressed or not detected in the control and DRE treated samples of CMML cells. B) Fold change in gene expression of cell death genes in CMML cells treated with DRE, compare to the control untreated cells (Fold expressions in control are set at 1 and treated samples are expressed as fold increases from the control).

Table 3: Fold Change in Expression of Cell Death Genes in Chronic Myelomonocytic Leukemia (CMML) Cells – (MV-4-11)

<u>Gene</u>	<u>Fold Change</u>	<u>Gene Function</u>
APAF 1 (Apoptotic Protease Activating Factor 1)	38.54	A central component of the intrinsic pathway of apoptosis. Forms the apoptosome with cytochrome c and pro-caspase-9 (Yoshida et al., 1998; Gu et al., 2014)
APP (Amyloid beta (A4) Precursor Protein)	26.02	Increased expression of APP is involved in caspase-3 dependent and independent apoptosis (Nishimura et al., 2002)
ATG12 (Autophagy related 12)	12.41	Plays a distinct role in the early steps of autophagosome formation during autophagic degradation, especially mitophagy (Radoshevich et al., 2010)
ATG16L1 (Autophagy related 16-like 1)	15.72	Essential for the elongation of isolation membranes during autophagy; interacts with ATG12-ATG5 to determine the site of LC3 lipidation (Fujita et al., 2008; Ishibashi et al., 2011)
ATG5 (Autophagy related 5)	27.70	Interacts with ATG12 and ATG16L1 for the formation of the autophagosome in autophagic degradation (Fujita et al., 2008; Radoshevich et al., 2010; Ishibashi et al., 2011)
ATG7 (Autophagy related 7)	24.09	An E1-like enzyme; Activates ATG12 and ATG8; Essential for the ATG conjugation system and the formation of the autophagosome (Xue et al., 2010)
BAX (BCL-2 Associated X-Protein)	308.06	Pro-apoptotic member of the Bcl-2 family; interacts with the mitochondrial voltage-dependent anion channel (VDAC), leading to the loss of membrane potential. (Pawlowski et al., 2000)
BCL2L1 (Bcl-2 like 1)	7.00	Also known as Bcl-xL; Considered an anti-apoptotic member of Bcl-2 family. Its phosphorylation (as a result of increase in ROS) is associated with a decrease in its anti-apoptotic and anti-autophagic activity (Song et al., 2012; Kim et al., 2014)
BCL2L11 (Bcl-2 like 1)	18.57	Apoptosis facilitator, also known as Bim; Activates Bax and Bad (other pro-apoptotic proteins); Can override apoptotic resistance caused by overexpression of anti-apoptotic Bcl-2 members (Plötz et al., 2013)
BECN1 (Beclin 1)	43.44	Caspase-mediated cleavage of BECN1 is

		involved in the cross-talk between apoptosis and autophagy; dysfunction is BECN1 is implicated in many disorders, including cancer (Kang et al., 2011).
CASP1 (Caspase-1)	27.30	Cleaves a variety of substrates (121 substrates), including pro-inflammatory cytokine pro-interleukin (IL)-1 β to induce apoptosis (pyroptosis) (Denes et al., 2012)
CASP2 (Caspase-2)	51.48	Can be activated in the absence of extrinsic and intrinsic apoptotic stimuli, although it is also a direct downstream target of caspase-8, even though it is an initiator caspase (Olsson et al., 2009; Soo-Hyun & Crispin, 2012)
CASP3 (Caspase-3)	41.10	Effector caspase; involved in the cleavage of Poly-(ADP-ribose) Polymerase (PARP) (Olsson et al., 2009)
CASP6 (Caspase-6)	122.45	Considered an effector caspase of the extrinsic pathway, but could also be an activator of caspase-8 for a positive feedback loop (Cowling & Downward, 2002; Olsson et al., 2009)
CASP9 (Caspase-9)	9.45	Initiator caspase in the intrinsic pathway of apoptosis; forms the apoptosome with APAF1 and cytochrome c; activates effector caspases, such as caspase-3 (Yoshida et al., 1998; Olsson et al., 2009)
COMMD4 (COMM domain containing 4)	133.81	Inhibits NF- κ B activation (Jin et al., 2012)
FAS (Fatty acid synthetase receptor)	12.34	The extrinsic pathway of apoptosis requires the binding of death ligands, e.g. Fas ligand, to its specific death receptor, triggering downstream effects, which include the activation of initiator caspase (e.g. caspase-8) and the subsequent activation of effector caspases (Elmore, 2007)
FASLG (Fatty acid synthetase ligand)	1	
GRB2 (Growth factor receptor-bound protein 2)	192.01	Has a dual role in receptor kinase signaling. Can actively inhibit the phosphorylation of receptor tyrosine kinases involved in cell proliferation (Belov & Mohammadi, 2013)
MAPK8 (Mitogen Activated Protein Kinase 8)	22.60	Also known as BCL2-associated agonist of cell death; involved in a variety of cell processes, including cell proliferation and apoptosis, especially in intrinsic apoptosis (Show et al., 2008; Bjørkøy et al., 2009)
MCL1 (Myeloid Cell Leukemia 1)	44.70	Alternative splicing results in multiple variants; a longer variant which is anti-apoptotic and a shorter variant, which promotes apoptosis

		RefSeq, Oct 2010
SQSTM1 (p62) (Sequestosome 1)	345.39	Autophagy receptor; shown to activate caspase-8 mediated apoptosis; interacts with ubiquitinated proteins/organelles and facilitates that translocation to the lysosome for autophagic degradation (Zhang et al., 2013)

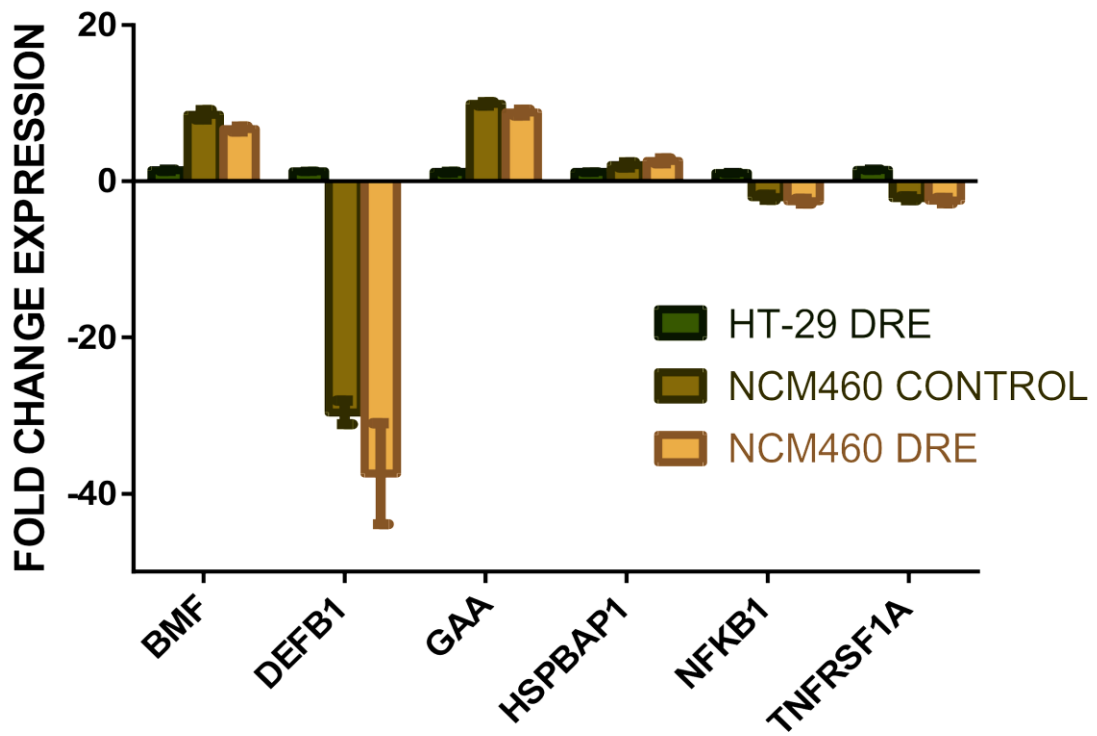
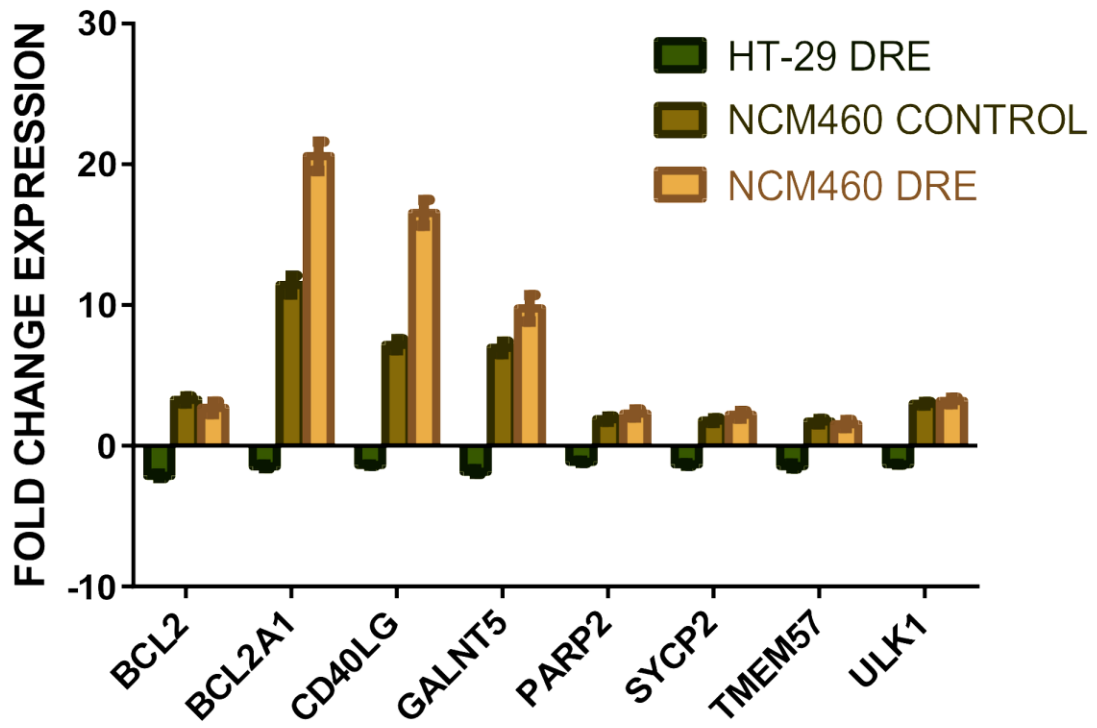
To further determine the differences in how colorectal cancer cells respond to DRE, and to further investigate the selectivity of DRE to cancer cells, the gene expression study was carried out in HT-29 and NCM460 cells. Table 4 outlines the results obtained from this part of the study, emphasizing the differential expression of genes between HT-29 colon cancer cells and NCM460 normal colon epithelial cells. It was observed that treatment with DRE had opposite effects in cancer and non-cancerous cells, when it came to how they express cell death genes, as cell death genes overexpressed in HT-29 cells, following treatment, with down-regulated in NCM460 cells, under the same conditions and vice versa (Figure 8). These results further show that DRE is able to distinguish between cancer and non-cancer cells (colon) in order to induce selective cytotoxicity to cancer cells, without associated toxicity to the non-cancer cells.

Table 4: Differential Fold Change in Expression of Cell Death Genes in Human Colorectal Cancer (HT-29) Versus Normal Colon Mucosal Epithelia Cells (NCM460)

<u>Gene</u>	<u>Fold Change (HT-29)</u>	<u>Fold Change (NCM460)</u>	<u>Gene Function</u>
Bcl-2 (B-Cell Lymphoma 2)	-2.2 ± 0.23	+3.2 ± 0.44	An integral outer mitochondrial membrane protein that blocks the apoptotic death by interacting and inhibiting pro-apoptotic proteins, e.g. BAX, APAF1 (Tsujimoto & Croce, 1986)
Bcl-2A1 (Bcl-2 related protein A1)	-1.5 ± 0.21	+15.5 ± 1.1	BCL2A1 is a target of NF-κB activation, in response to inflammatory signals. This gene exerts pro-survival functions and is generally overexpressed in several cancers (Vogler, 2012)
CD40LG	-1.4 ± 0.11	+11.2 ± 0.7	An integral membrane protein and a member of the TNF superfamily, overexpressed in several carcinomas. Enhances cytokine production and promotion of cell proliferation (Banchereau et al., 1994)
GALNT5 (polypeptide N-acetylgalactosaminyltransferase 5)	-1.8 ± 0.23	+8.5 ± 0.75	A member of the TNF superfamily. They are responsible for the altered O-linked glycosylation occurring during the development of various cancers and their progression via altering O-glycan biosynthesis (He et al., 2014)
PARP2 (poly (ADP-ribose) polymerase 2)	-1.2 ± 0.15	+2.2 ± 0.25	Induced by double stranded DNA breaks, as a cellular response to DNA damage. It is involved in DNA repair and transcriptional regulation. PARP inhibitors are an emerging field in cancer therapy (Liang et al., 2013; Yelamos et al., 2014)
SYCP2 (Synaptonemal Complex 2)	-1.3 ± 0.16	+2.0 ± 0.22	A major component of synaptic complexes during meiosis (prophase). May be involved in the organization of chromatin by temporarily binding to DNA scaffold attachment regions (Schalk et al.,

			1999)
TMEM57 (Transmembrane Protein 57)	-1.4 ± 0.25	+1.6 ± 0.25	A target of Jun Kinase signaling. Has been shown to interact with several proteins, including the transcription regulators HTT and SMAD9). Its function is still being studied
ULK1 (Unc-51 Like Autophagy Activating Kinase 1)/ ATG1	-1.3 ± 0.16	+3.1 ± 0.2	A serine/threonine protein kinase involved in autophagy in response to starvation by phosphorylating Beclin-1. Transcriptional activation of ULK1 is involved in cancer cell survival (Pike et al., 2013; Russell et al., 2013)
BMF (Bcl-2 modifying factor)	+1.4 ± 0.23	+7.2 ± 0.55	A Bcl-2 family membrane that might activate apoptosis and anoikis. Interacts with other members of the Bcl-2 family for apoptosis induction (Grespi et al., 2010; Hausmann et al., 2011)
DEFB1 (Defensin, Beta 1)	+1.3 ± 0.09	-40 ± 7.54	Found to be down-regulated in several cancer types and plays a significant role in innate and adaptive immune response to promote cytotoxicity (Donald et al., 2003)
GAA (Acid alpha glucosidase)	+1.2 ± 0.22	+9.9 ± 0.34	Active in the lysosomes for the breakdown of glycogen into glucose. Plays a role in autophagic induction by encouraging the degradation of p62 conjugated cargo (Nascimbeni et al., 2012)
HSPBAP1 (Heat shock protein 27kDa associated protein 1)	+1.1 ± 0.11	+2.5 ± 0.3	May play a role in cellular stress response. Knockdown of this gene is associated with an increase in caspase-3/7 activation (Smirnov et al., 2012)
IFNG (Interferon Gamma)	+3.3 ± 0.25	+1.5 ± 0.5	Coordinate various cell response programs, including macrophage activation, inhibition of cell proliferation and activation of apoptosis (Schroder et al., 2004)
NFKB1 (Nuclear	+1.1 ± 0.11	-2.3 ± 0.3	Has both pro- and anti-apoptotic activities; a major transcription

factor kappa B)			factor in various cell signaling pathways (Kaltschmidt et al., 2000; Stark et al., 2007)
TNFRSF1A (Tumor Necrosis Factor Receptor Superfamily, Member 1A)	+1.5 ± 0.15	-2.2 ± 0.3	Member of the TNF superfamily. Recruits adapter domains such as FADD and TRADD for activation of extrinsic apoptosis (Schall et al., 2014)
CASP1 (Caspase-1)	+12.3 ± 2.1	-2.5 ± 0.25	Cleaves a variety of substrates (121 substrates), including pro-inflammatory cytokine pro-interleukin (IL)-1 β to induce apoptosis (pyroptosis) (Denes et al., 2014)
SNCA (Alpha synuclein)	+3.0 ± 0.14	-4.8 ± 0.45	A biomarker of colorectal cancer, and overexpressed in various other cancers; might be involved in cell death activation in certain cell types (Devine et al., 2011; Bethge et al., 2014)
TNF	+8.3 ± 0.32	-7.5 ± 0.55	A cytokine that binds to its receptors (TNFR1, TNFRSF1A) and promotes extrinsic apoptosis under ideal conditions (Hassan et al., 2014)



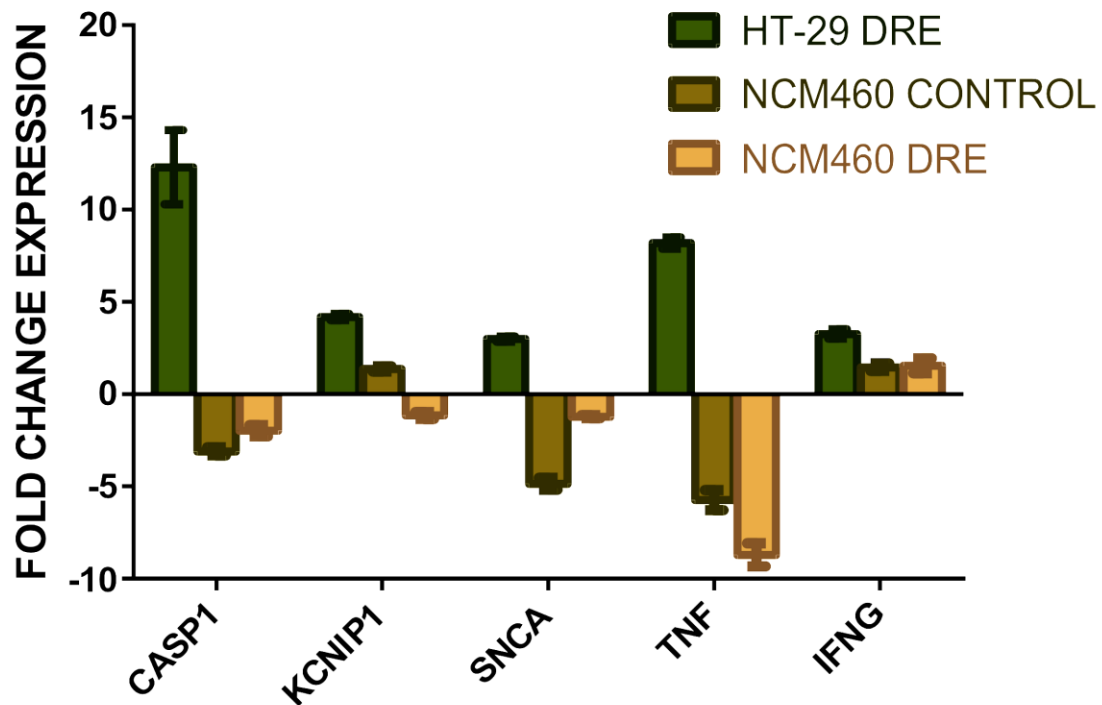


Figure 8: Differential Fold Change in Expression of Cell Death Genes in Human Colorectal Cancer (HT-29) Versus Normal Colon Mucosal Epithelial Cells (NCM460): The differential effect on gene expression in colon cancer and normal colon epithelial cells was evaluated using the RT² profiler array. Following 48-hour treatment with DRE, RNA was isolated from HT-29 and NCM46 cells, converted into cDNA and incubated with oligodT primers. Following PCR analysis, data was analyzed, using Qiagen PCR data analysis software. A) Cell death genes down-regulated in HT-29 cells and up-regulated in NCM460 cells, following treatment. B) Cell death genes, with no significant change in fold expression in HT-29 cells, with a corresponding increase or decrease in fold expression in NCM460 cells. C) Cell death genes overexpressed in HT-29 cells, but down regulated or unchanged in fold expression in NCM460 cells. **** P<0.0001

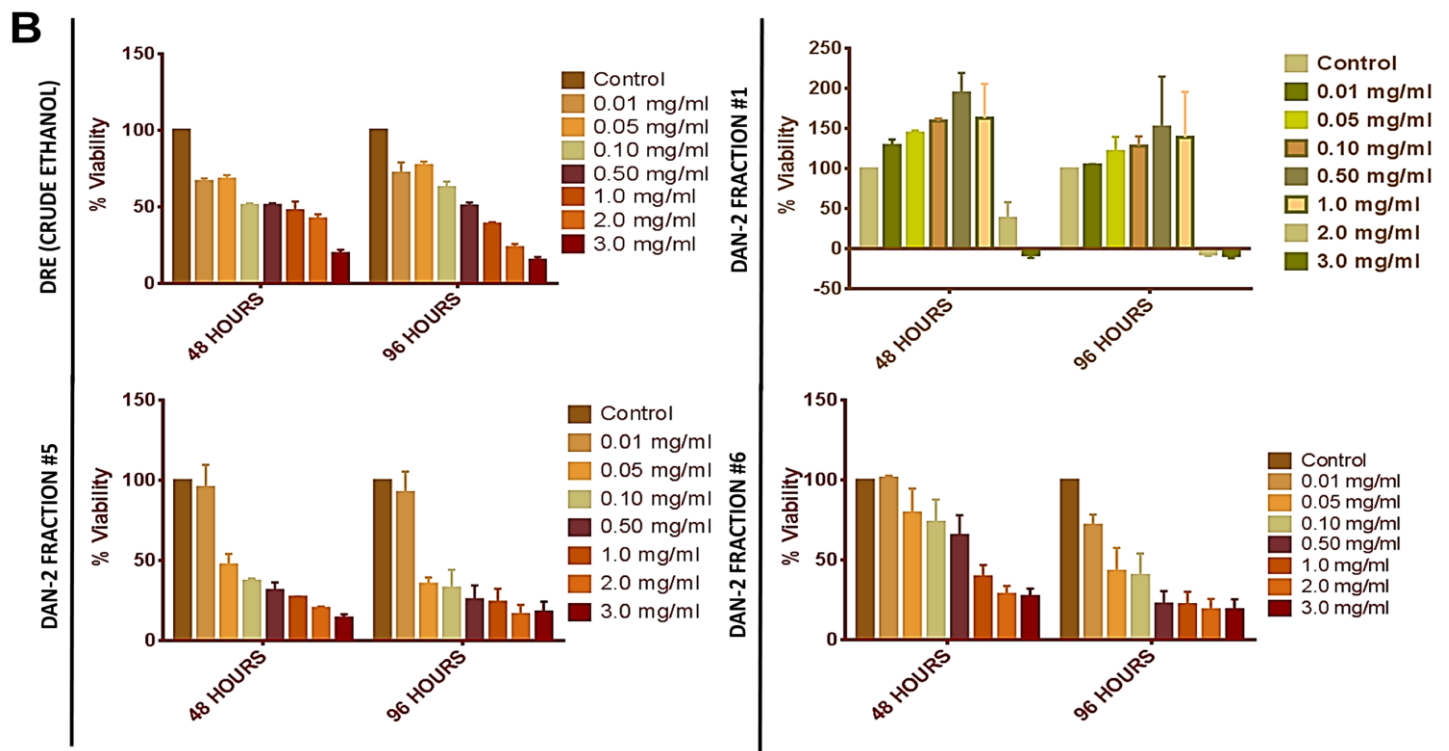
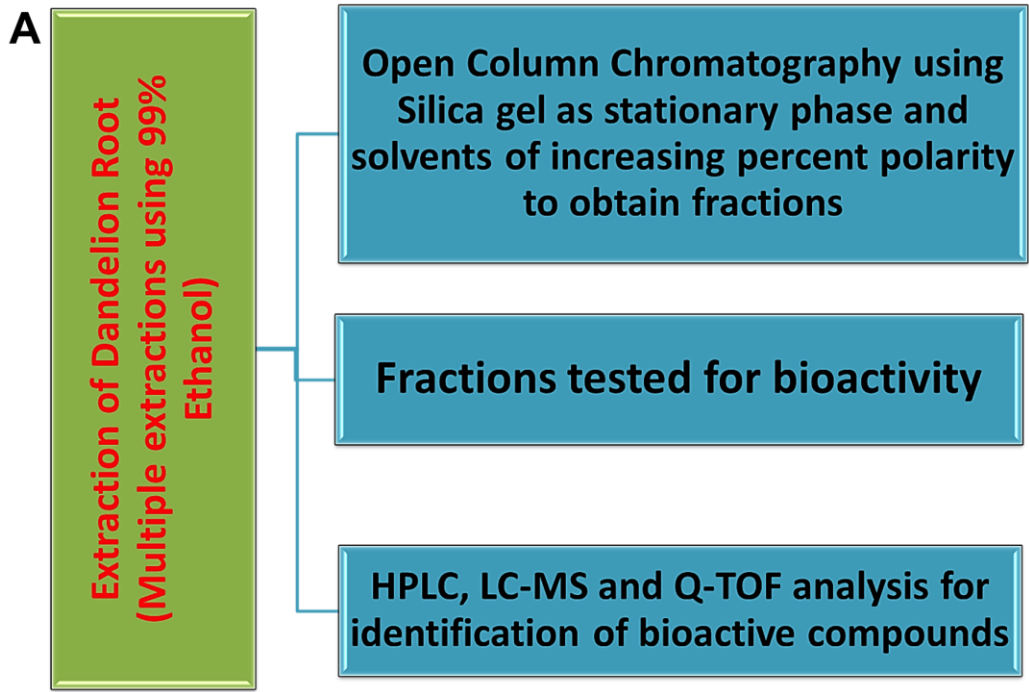
Fractionation and Phytochemical Analysis of Dandelion Root Extract:

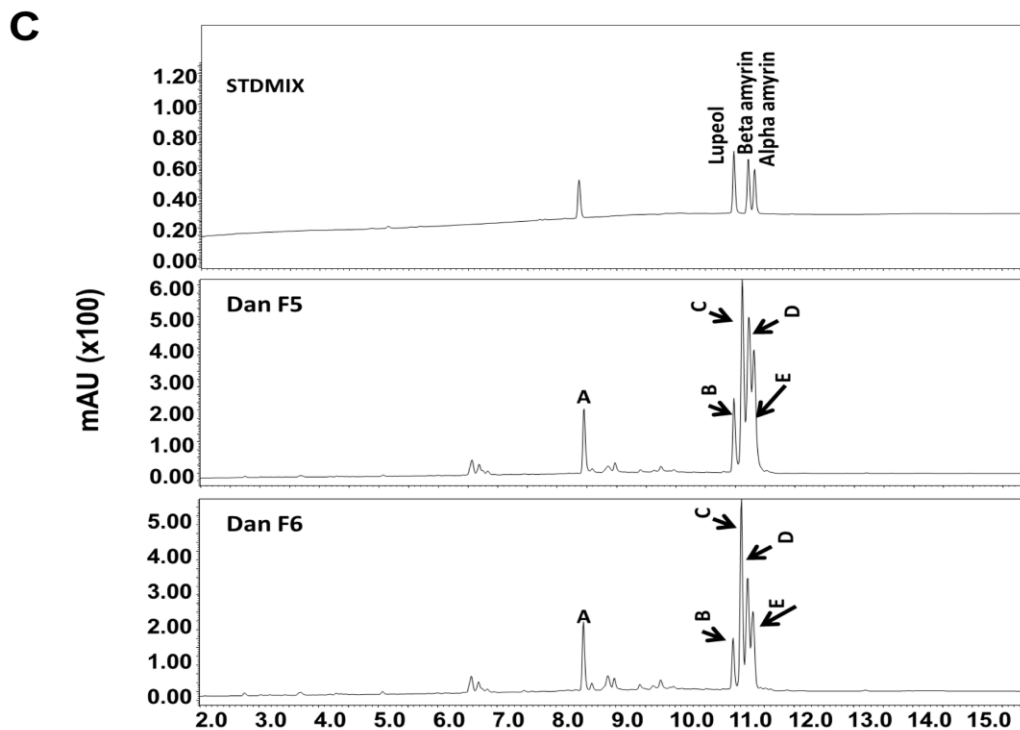
Identification of Bioactive Fractions & Components within Dandelion Root Extract

To commence the phytochemical analysis, dandelion root was extracted twice in anhydrous ethanol and rotor evaporated to obtain the extracted material and analysis was carried out according to the flowchart (Figure 9A). Some of this material was kept aside for bioactivity testing, while the rest of the extracted material was fractionated in a glass column packed with silica gel. The primary fractions (210) obtained were pooled together by thin-layer chromatography, based on similarities in their profiles to obtain 26 fractions. These fractions were then tested for bioactivity, by WST-1 cell viability assay. The results from the fraction studies were compared to those obtained from the bioactivity testing of the ethanolic extract of dandelion root. Bioactivity testing identified 6 out of 26 fractions with significant activity, comparable to the whole ethanolic extract (Figure 9B – shows results from two of the bioactive fractions and results from a fraction with barely adequate bioactivity).

Following the identification of bioactive fractions, these active fractions were further fractionated to obtain secondary fractions (25) by a preparative HPLC column. Further bioactivity testing narrowed down the number of bioactive fractions. The identified bioactive fractions were analyzed by HPLC-MS, using a standard mix, to identify the different compounds within the fractions (Figure 9C-D). This led to the identification of three major components within two of the bioactive fractions (Fraction #5 and #6); α -amyrin, β -amyrin and lupeol (Figure 10A). These data confirms the presence of multiple pharmacologically active

components within DRE. Assessment with WST-1 indicate that further analysis is essential to determine if any of the identified components could potentiate the anti-cancer activity of DRE observed with the whole complex mixture in cancer cells.



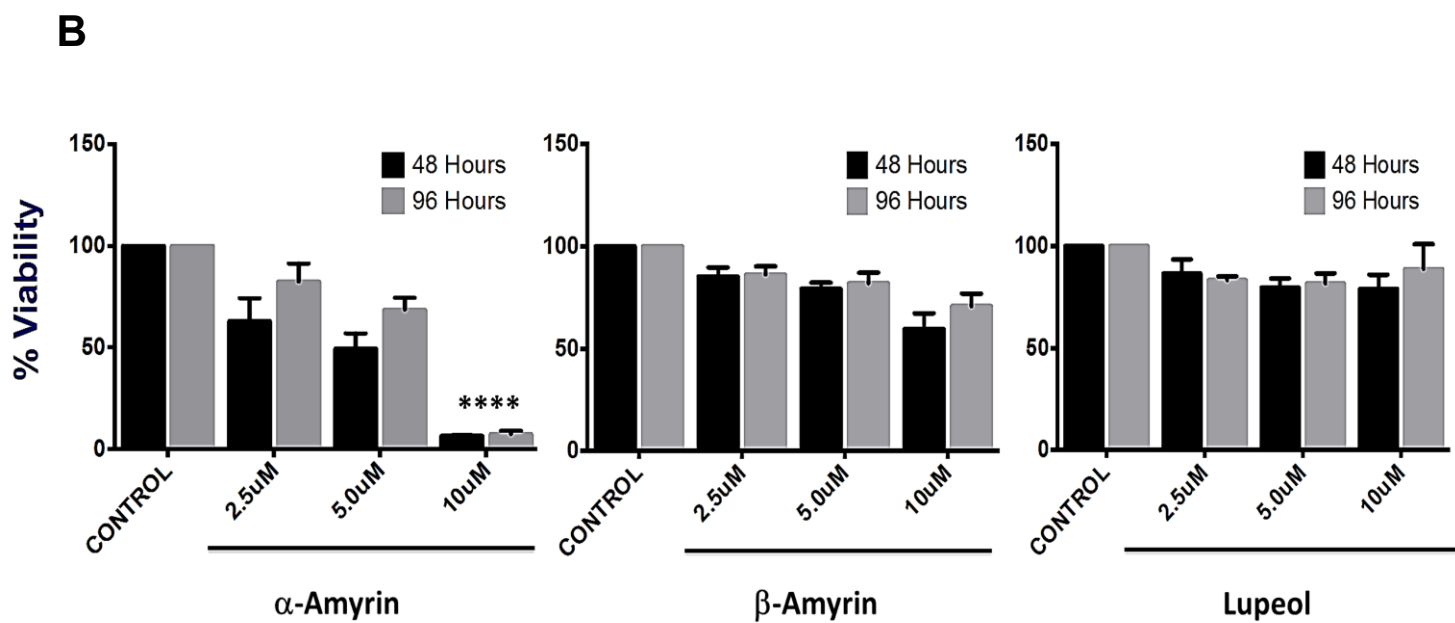
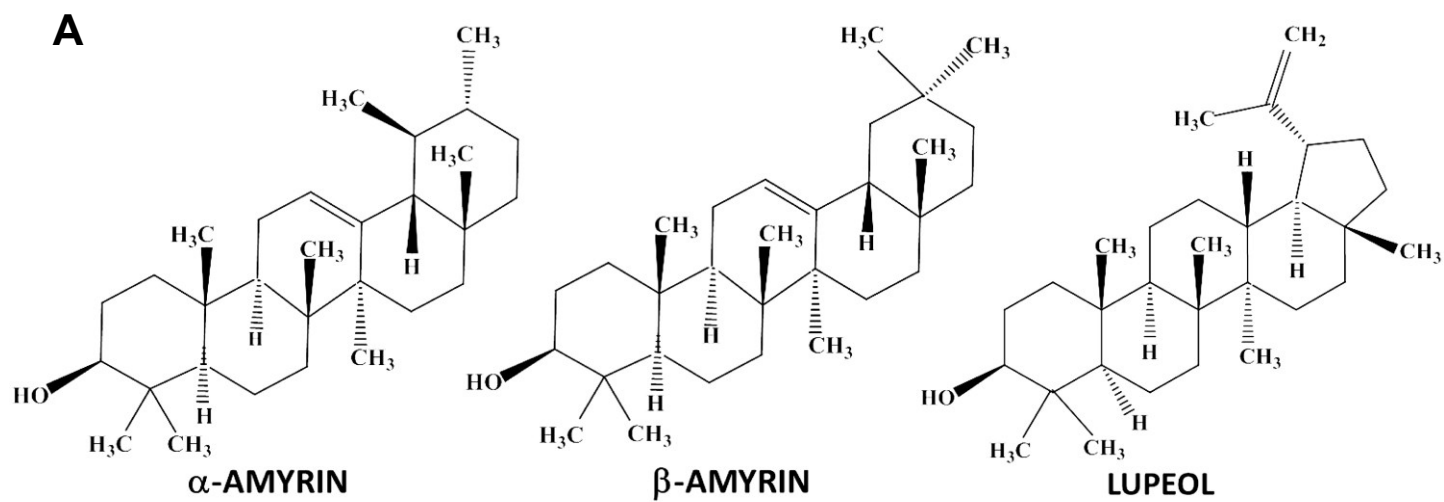


Sample	Major Peak	RT (min)	M/Z	Identification
Standard compound mix (STDMIX)	Lupeol	10.98	409 (M-OH)-	NA
	β -Amyrin	11.22	409 (M-OH)-	NA
	α -Amyrin	11.32	409 (M-OH)-	NA
Fractions 5 and 6	A	8.45	279 (negative mode)	In progress
	B	10.98	409 (M-OH)-	Lupeol
	C	11.11	409 (M-OH)-	In progress
	D	11.22	409 (M-OH)-	β -Amyrin
	E	11.31	409 (M-OH)-	α -Amyrin

Figure 9: Identification of Bioactive Fractions & Components within Dandelion Root Extract: A) Flowchart of the steps outlining the fractionation and phytochemical analysis of DRE. Following ethanolic extraction of DRE, fractionation, followed by chromatographic analyses, were carried out to identify bioactive compounds within DRE. B) Effect of crude ethanolic extracts and secondary fractions on the metabolic viability of HT-29 colon cancer cells. 5 active fractions were further analyzed to identify the components within. C) UPLC-DAD-MS chromatogram of bioactive fraction #5 and #6, along with a standard mix of known compounds.

Anticancer Activity of Identified Bioactive Compounds within Dandelion Root Extract

To test the hypothesis that DRE contains multiple bioactive compounds that could potentiate its cytotoxicity, and following the identification of 3 compounds, within 2 of the bioactive fractions, the next phase of this study was to determine if any of these compounds had significant cytotoxicity to cancer cells, comparable to DRE as a whole. Each compound was tested alone in HT-29 cells, at increasing concentrations and for increasing time points, and the viability of these cells, as a function of their metabolism was measured, using the WST-1 viability assay. We observed a decrease in the viability of cells treated with α -amyirin, with 10 μ M as the most effective concentration. At the concentrations used ($\leq 10 \mu$ M), β -amyirin and lupeol did not show significant viability reducing properties (Figure 10B). To further test our hypothesis that the components within DRE are able to act in synergy to provide better efficacy at lower doses, we tested these components, in combination studies (α -amyirin + β -amyirin; α -amyirin + lupeol; β -amyirin + lupeol). Results show that α -amyirin and β -amyirin, when used in combination, showed a combined additive effect on the ability to affect the metabolic activity of HT-29 cells, than when used individually (Figure 10C). These results strongly suggest that the multiple bioactive components within DRE could act in synergy to show better anticancer efficacy, especially in highly aggressive cancer cell types



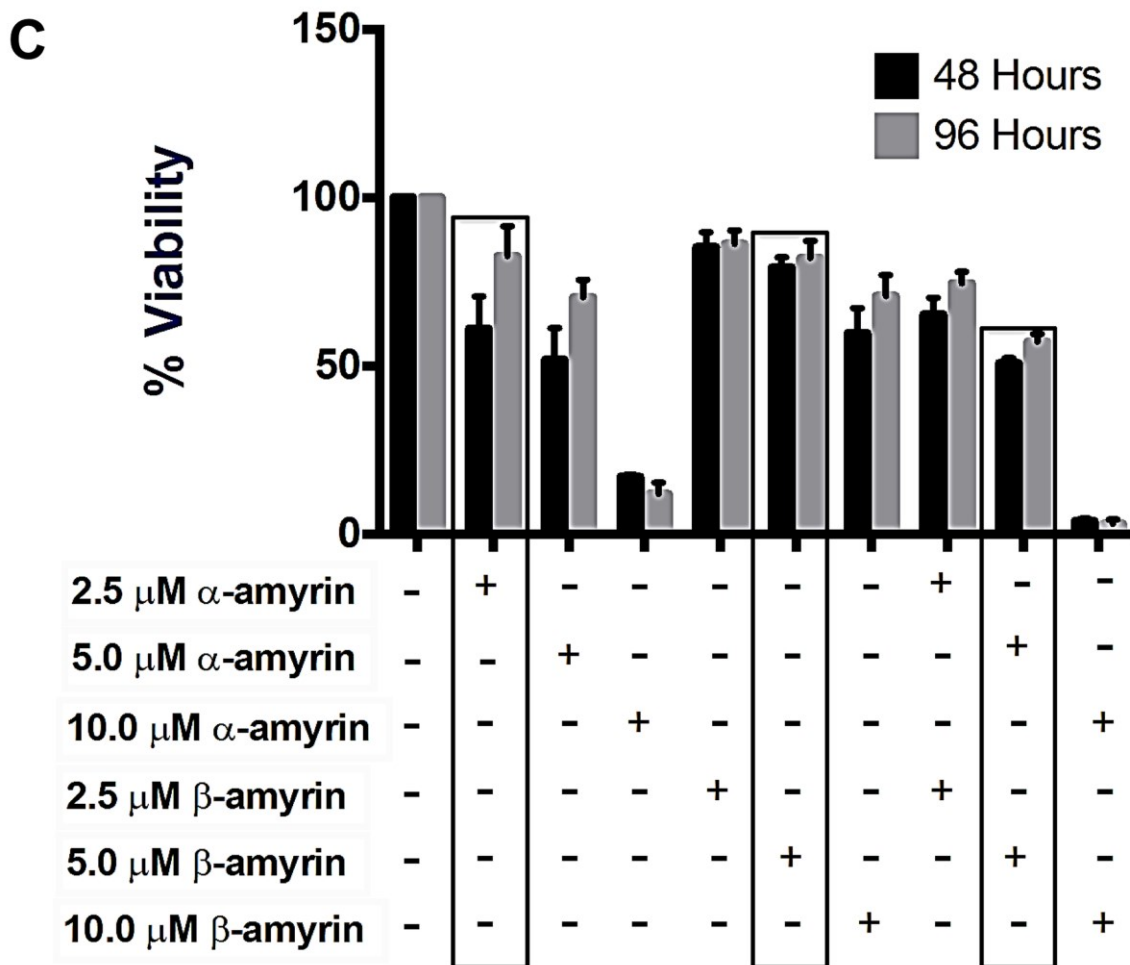


Figure 10: Anticancer Activity of Identified Bioactive Compounds within Dandelion Root Extract: A) Identified compounds (by ChemDraw) in bioactive fractions #5 and #6. These triterpenes are isomers (MW = 427 g/mol). B) Following treatment, HT-29 cells were analyzed for the ability of α -amyrin, β -amyrin and lupeol to reduce the viability of these cells, measured by a decrease in metabolic activity. Following treatment, the WST-1 reagent was added to each well, the absorbance readings were taken at 450 nm, and expressed at a percentage of the control. C) Compounds were analyzed alone and in combination with each other to assess combined effect in cancer cells. α -amyrin and β -amyrin showed an additive effect at slightly lower doses than the effective concentrations.

Effect of Dandelion Root Extract on the Expression of cFLIP

Although lupeol did not show any significant effect on the viability of HT-29 cells (and BxPC-3 cells [data not shown]), there have been studies that indicate that lupeol plays a role in TRAIL-mediated apoptosis (Murtaza et al., 2009). This study showed that lupeol, at 40 μ M, suppressed the expression of cellular FLICE-like inhibitory protein (cFLIP), an inhibitor of the extrinsic pathway of apoptosis, which prevents the formation of the death inducing signaling complex (DISC) and the subsequent activation of caspase-8 (Safa, 2012). To further confirm the role of extrinsic pathway in DRE mediated apoptosis, we compared the effect of DRE to that of lupeol, in terms of their abilities to suppress the expression of cFLIP. Western blotting analysis showed that DRE, at 2.5 mg/ml, was sufficient to inhibit the expression of cFLIP in cancer cells. There was a slight additive effect when DRE was combined with the lower dose of lupeol, at 10 μ M, again confirming the beneficial effect of multiple components within DRE, compared to the use of individual components. These results further confirm the activity of DRE through the extrinsic pathway of apoptosis.

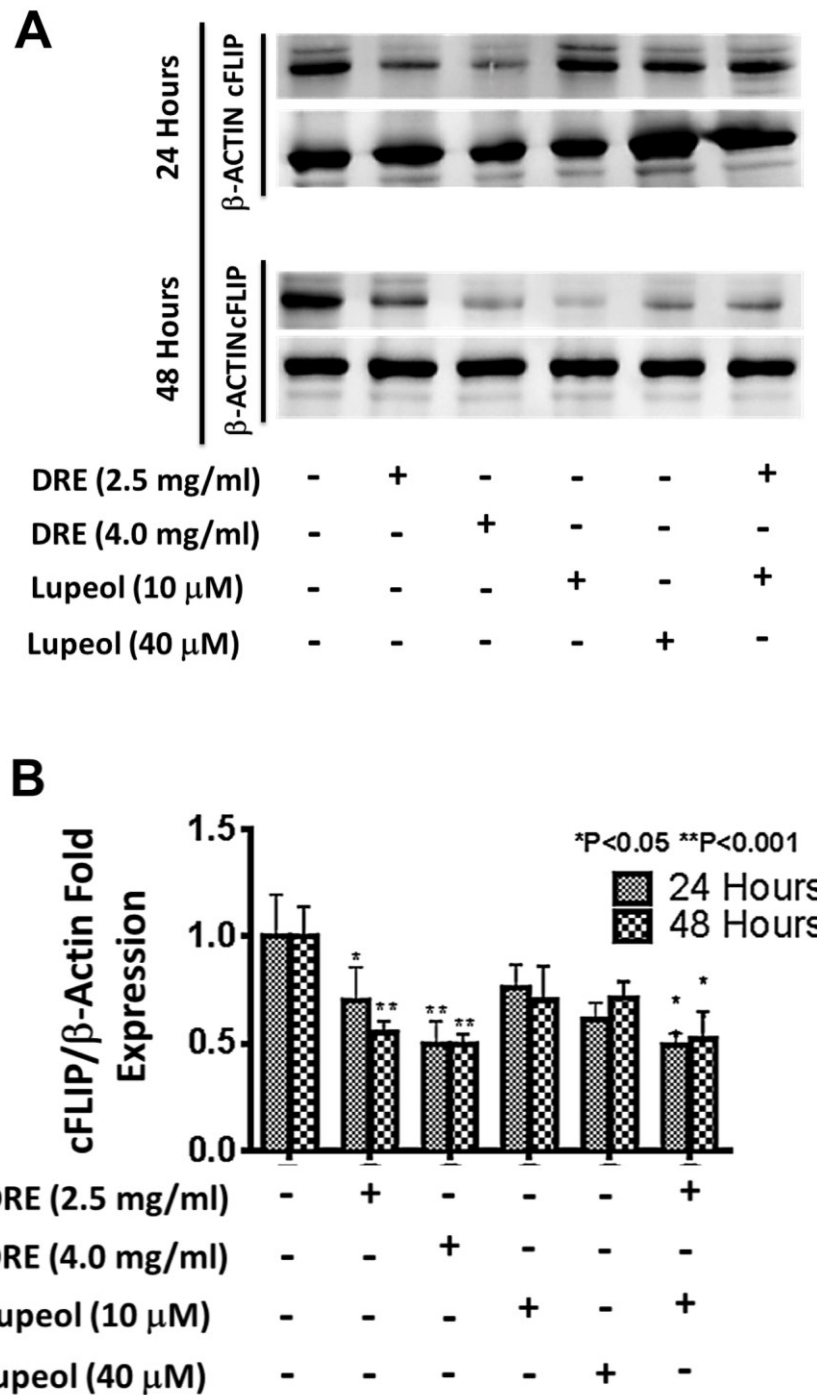


Figure 11: Effect of Dandelion Root Extract on the Expression of cFLIP: Comparing the effect of DRE and lupeol on the expression of cFLIP. Following treatment, cell lysates were obtained and proteins were separated on a gel. The proteins were transferred onto a PVDF membrane and probed for cFLIP levels, using β -actin as a loading control. A) Imaged blot of cFLIP expression levels. B) Densitometry analysis of expression levels, compared to β -actin loading control.

DISCUSSION

Over 38% of Canadians diagnosed with colorectal cancer will succumb to death from it. This is the third leading cause of cancer related deaths, only surpassed by lung and prostate cancers (Sigstedt et al., 2008). The lack of safer, non-toxic modes of therapy reveals a need for better alternatives. The use of dandelion extracts has been documented, as anecdotal evidence, for centuries. It has been used in traditional medicine in various cases of diseases, especially as a detoxifying agent in digestive disorders (Sigstedt et al., 2008), implying that it does not have significant toxic effects associated with its use. The results presented in this study, along with our previous published work, back the conclusion that an aqueous extract of dandelion root, still under the umbrella of a natural health product (NHP), can provide a safer, more efficacious mode of treatment, to current chemotherapy. In this study, we show that DRE can selectively and effectively reduce the viability of aggressive colon cancer cells, irrespective of p53 status. Furthermore, treatment of these cells with DRE triggered the process of apoptosis in colon cancer cells, without having a similar effect on the viability and survival of non-cancerous colon mucosal epithelial cells (Figure 1), confirming the lack of reports of toxicity. Further confirmation of the lack of toxicity associated with DRE use was done in animal models of toxicity and efficacy.

In this study, we show that there is no toxicity associated with the use of DRE, as animals on oral administration of DRE for over a 3-month period, showed no decrease/significant change in their body weights, no kidney damage, as measured by protein urinalysis and no gross morphological changes in tissue

appearance (Figure 2). This lack of toxicity permitted further investigation into the anticancer potential of DRE in animal xenograft models of colon tumors. Following the establishment of tumors, mice were provided with DRE in their drinking water, at a final concentration of approximately 40 mg/kg/day, and allowed to drink *ad libidum*, for the duration of the study. We found that mice in the treatment group had much slower tumor growth than those in the control, untreated group (Figure 3). More importantly, we show the efficacy of DRE in *ex-vivo* samples of leukemia, from newly diagnosed patients.

We report novel findings that DRE selectively triggers apoptosis in clinical samples in a dose and time dependent manner, based on nuclear morphology and reorganization of the cell membrane to expose phosphatidylserine (Figure 4), with no effect on non-cancerous peripheral blood mononuclear cells, from healthy volunteers (Ovadjie et al., 2012b). Interestingly, the differences in the clinical diagnosis of each leukemia type did not have a deterrent effect on the activity of DRE. Further investigations into the different sub-populations of the different leukemia blood samples is needed. These results therefore provide sufficient evidence for the anticancer potential of DRE and therefore a need for further studies in the understanding of this extract, and its benefits as a potential anticancer agent.

Our previous work aimed at understanding the mechanism of action of DRE in cancer cells, to better understand how this extract is able to distinguish between a cancer cell and a non-cancer cell, in order to selectively trigger programmed cell death programs. This study was designed to delve further into the mechanism of action of DRE, as well as identify the components responsible

for its anti-cancer activity. As with our previous works, we observed that DRE is able to target the vulnerable mitochondria in cancer cells, which may be due to reduced oxidative phosphorylation and reduced flux through the electron transport chain (Ralph & Neuzil, 2009). Treatment with DRE led to a decrease in the mitochondrial membrane potential (Figure 5A) and an increase in ROS levels, from isolated mitochondria (Figure 5B). These results further confirm our hypothesis that DRE is able to target the vulnerable aspects of cancer cells, like the mitochondria, in order to induce the selective cytotoxicity we have observed.

In this study, we further established the rapid activation of the extrinsic pathway of apoptosis selectively in colon cancer cells, following DRE treatment (Figure 6A). To further understand the mechanism of extrinsic apoptosis, following the activation of caspase-8 and look at the effect of DRE on the mitochondria, HT-29 cells were incubated with MitoTracker dye, as well as stained with an anti-caspase-8 antibody for immunocytochemical analysis. The results confirm the activation of caspase-8 by fluorescence microscopy, while showing the destabilization of the mitochondrial membrane, indicated by the dispersion of the red cationic dye that will normally aggregate around the mitochondrial membrane in healthy mitochondria. More importantly, due to the controversy surrounding the localization of caspases, both the inactive zymogens and the active forms, this staining was used to determine the localization of caspase-8 before apoptotic stimuli and following activation. In the control untreated cells, it was observed that pro-caspase-8 localized in the membrane of the mitochondria, where the green fluorescence co-localized with the MitoTracker red in the healthy mitochondria. Following treatment and activation of caspase-8,

it was observed that the now active caspase-8 was released from mitochondrial membrane space into the rest of the cells, as was observed with the co-localization of the nuclear stain with the caspase-8 staining under fluorescence. This result suggests that pro-caspase-8, and possibly other caspases, reside in the mitochondria and upon activation, translocate to other locations in the cells, where they lead to the progression of apoptosis. These results correspond to the results published by Qin and colleagues, where they showed the localization of caspases to the mitochondria before activation and their release from the mitochondria, following activation (Qin et al., 2001).

However, unlike our previous study, where we show that the activation of caspases was essential to DRE's mechanism in leukemia cells (Ovadge et al., 2012b), our colon data suggests otherwise. In this study, we find that the inhibition of caspase activation through a broad caspase inhibitor, Z-VAD-fmk (data not shown) and a caspase-8 specific inhibitor, IETD-fmk did not discourage the viability reducing ability of DRE in these cells (Figure 6C). This differential requirement of caspase-8 activation in the mechanism of DRE's activity follows in line with our hypothesis that DRE can target multiple pathways in a cancer cell.

To further assess the effect of DRE in cancer cells, we studied the gene profiles, using a pathway finder RT² Profiler assay, it was observed that DRE treatment in both CMML and HT-29 cells, induced the expression of several cell death genes, involved in both the apoptotic and autophagic cell death pathways (Figure 7 and 8). More importantly, there was clear differential gene expression observed in normal NCM460 cells, as genes that were up-regulated in cancer cells, were down-regulated or not expressed in normal cells and vice versa. It is

also observed that the gene expression in the leukemia and the colon cancer cell, following DRE treatment, overlapped slightly. However, these results reveal that DRE is able to target multiple cell death inducing pathways in several cancer cells, while having the opposite effects in non-cancer cell models. There was no major difference between the control, untreated gene expression and the DRE treated gene expression in NCM460 cells, further confirming that DRE is able to selectively target cancer cells, activate multiple pathways and induce cytotoxicity in cancer cells alone.

To further study our hypothesis of the complexity of DRE and the multiple components that could target the multiple vulnerable aspects of cancer cells, we carried out fractionation and phytochemical analysis of an ethanolic extract of dandelion root, in order to identify the bioactive components within DRE. Following primary and secondary fractionation, we identified three of the bioactive compounds, found in two out of the six fractions, with significant bioactivity, comparable to that of the ethanolic DRE (Figure 9B). These compounds include α -amyrin, β -amyrin and lupeol (Figure 9C, 10A). At high doses, $\geq 10 \mu\text{M}$, α -amyrin efficiently reduced the viability of colon cancer cells; however, β -amyrin and lupeol did not show similar bioactivity at these doses (Figure 10B). Continued studies using the identified compounds, in combination with each other, showed an additive effect of α - and β -amyrin, when used in combination, than individually (Figure 10C). Such an effect was not observed in the combinations with lupeol, therefore indicating that the multiple components, within this complex extract of dandelion root, can act together, in synergy or in addition, to trigger the cytotoxicity that we observe in cancer cells, following treatment.

A previous study with lupeol shows that this compound, albeit at high doses of 40 μ M, was able to sensitize pancreatic cancer cells to TRAIL induced apoptosis, by inhibiting the expression of cFLIP (Murtaza et al., 2009), which is an inhibitor of the extrinsic pathway of apoptosis. Studying DRE further, we found that DRE, as a whole complex mixture, has a better effect on reducing the expression of cFLIP in both HT-29 (Figure 11), and BxPc-3 pancreatic cancer cells (data not shown). This was a dose and time dependent response, as by 48 hours following treatment, there was a significant reduction in the expression of cFLIP, in the treated cells, compared to the control, untreated cells. These results further confirm that DRE does activate the extrinsic pathway of apoptosis and that, even though its mechanism of action is cell type specific, the complex mixture of this extract efficiently and selectively imparts its cytotoxicity to various cancer cell types, in a dose and time dependent response.

CONCLUSIONS

Our results show that a complex mixture of aqueous dandelion root extract (DRE) is capable of efficiently and selectively triggering multiple programmed cell death pathways in a variety of cancer cells. These results further confirm our hypothesis that DRE contains multiple bioactive components, not limited to α -amyryn, β -amyryn and lupeol that could target the multiple vulnerable aspects of cancer cells, such as the vulnerability of cancer cell mitochondria. Overall, these findings indicate that a complex mixture of an NHP, such as dandelion root extract, could provide an alternative to currently available chemotherapy, to improve the quality

of life of cancer patients and possibly provide a more efficacious mode of treatment while doing so.

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CHAPTER 6

EVALUATION OF THE EFFICACY & BIOCHEMICAL MECHANISM OF CELL DEATH INDUCTION BY LONG PEPPER EXTRACT SELECTIVELY IN *IN-VITRO* AND *IN-VIVO* MODELS OF HUMAN CANCER CELLS

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PloS One, 2014 (Under Revision)

ABSTRACT

Currently chemotherapy is limited mostly to genotoxic drugs that are associated with severe side effects due to non-selective targeting of normal tissue. Natural products play a significant role in the development of most chemotherapeutic agents, with 74.8% of all available chemotherapy being derived from natural products. To scientifically assess and validate the anticancer potential of an ethanolic extract of Long pepper (PLX), a plant of the *piperaceae* family that has been used in traditional medicine, especially Ayurveda and investigate the anticancer mechanism of action of PLX against cancer cells.

Following treatment with ethanolic long pepper extract, cell viability was assessed using a water-soluble tetrazolium salt; apoptosis induction was observed following nuclear staining by Hoechst, binding of annexin V to the externalized phosphatidyl serine and phase contrast microscopy. Image-based cytometry was used to detect the effect of long pepper extract on the production of reactive oxygen species and the dissipation of the mitochondrial membrane potential following Tetramethylrhodamine or 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride staining (JC-1). Assessment of PLX *in-vivo* was carried out using Balb/C mice (toxicity) and CD-1 nu/nu immunocompromised mice (efficacy). HPLC analysis enabled detection of some primary compounds present within our long pepper extract.

Our results indicated that an ethanolic long pepper extract selectively induces caspase-independent apoptosis in cancer cells, without affecting non-cancerous cells, by targeting the mitochondria, leading to dissipation of the mitochondrial membrane potential and increase in ROS production. Release of

the AIF and endonuclease G from isolated mitochondria confirms the mitochondria as a potential target of long pepper. The efficacy of PLX in *in-vivo* studies indicates that oral administration is able to halt the growth of colon cancer tumors in immunocompromised mice, with no associated toxicity. These results demonstrate the potentially safe and non-toxic alternative that is long pepper extract for cancer therapy.

INTRODUCTION

The continuing increase in the incidence of cancer signifies a need for further research into more effective and less toxic alternatives to current treatments. In Canada alone, it was estimated that 267,700 new cases of cancer will arise, with 76,020 deaths occurring in 2012 alone. The global statistics are even more dire, with 12.7 million cancer cases and 7.6 million cancer deaths arising in 2008 (Jemal et al., 2011; Canadian Cancer Statistics, 2012). The hallmarks of cancer cells uncover the difficulty in targeting cancer cells selectively. Cancer cells are notorious for sustaining proliferative signaling, evading growth suppression, activating invasion and metastasis and resisting cell death among other characteristics (Hanahan & Weinberg, 2011). These characteristics pose various challenges in the development of successful anticancer therapies. The ability of cancer cells to evade cell death events has been the center of attention of much research, with focus centered on targeting the various vulnerable aspects of cancer cells to induce different forms of Programmed Cell Death (PCD) in cancer cells, with no associated toxicities to non-cancerous cells.

Apoptosis (PCD type I) has been studied for decades, the understanding of which will enhance the possible development of more effective cancer therapies. This is a form of cell death that is required for regular cell development and homeostasis, as well as a defense mechanism to get rid of damaged cells; cells undergoing apoptosis invest energy in their own demise so as not to become a nuisance (Canadian Cancer Statistics, 2012). Cancer cells evade apoptosis in order to confer added growth advantage and sustenance, therefore

current anticancer therapies endeavour to exploit the various vulnerabilities of cancer cells in order to trigger the activation of apoptosis through either the extrinsic or intrinsic pathways (Fadeel & Orrenius, 2005; Elmore, 2007). The challenges facing some of the available cancer therapies are their abilities to induce apoptosis in cancer cells by inducing genomic DNA damage. Although this is initially effective, as they target rapidly dividing cells (Fulda & Debatin, 2006), they are usually accompanied by severe side effects caused by the non-selective targeting of normal non-cancerous cells, suggesting a need for other non-common targets for apoptosis induction without the associated toxicities.

Natural health products (NHPs) have shown great promise in the field of cancer research. The past 70 years have introduced various natural products as the source of many drugs in cancer therapy. Approximately 75% of the approved anticancer therapies have been derived from natural products, an expected statistic considering that more than 80% of the developing world's population is dependent on the natural products for therapy (Davidson et al., 2013). Plant products especially contain many bioactive chemicals that are able to play specific roles in the treatment of various diseases. Considering the complex mixtures and pharmacological properties of many natural products, it becomes difficult to establish a specific target and mechanism of action of many NHPs. With NHPs gaining momentum, especially in the field of cancer research, there are numerous new studies on the mechanistic efficacy and safety of NHPs as potential anticancer agents (Newman & Cragg, 2012).

Long pepper, from the Piperaceae family, has been used for centuries for the treatment of various diseases. Several species of long pepper have been

identified, including *Piper longum*, *Piper betle*, *Piper retrofactum*, extracts of which have been used for years in the treatment of various diseases. A long list of uses and benefits are associated with extracts of different *Piper spp*, with reports indicating their effectiveness as good digestive agents and pain and inflammatory suppressants (Bao et al., 2013). However, there is little to no scientific validation, only anecdotal evidence, for the benefits associated with the use of long pepper extracts. There are scientific studies have been carried out on several compounds present in extracts of long pepper, including piperines, which has been shown to inhibit many enzymatic drug bio-transforming reactions and play specific roles in metabolic activation of carcinogens and mitochondrial energy production (Raj et al., 2012; Golovine et al., 2013; Jarvius et al., 2013; Megwal & Goswami, 2013) and various piperidine alkaloids, with fungicidal activity (Lee et al., 2001; Bao et al., 2013). Some of these compounds have shown potent anticancer activity (Bezerra et al., 2007), suggesting that Long pepper extracts could represent a new NHP, with better selective efficacy against cancer cells.

In this study, we examine the efficacy of an ethanolic extract of Long Pepper (PLX) against various cancer cells, as well as attempt to elucidate the mechanism of action, following treatment. Results from this study demonstrate that PLX reduced the viability of various cancer cell types in a dose and time dependent manner, where apoptosis induction was observed following mitochondrial targeting. Due to the low doses of PLX required to induce apoptosis in cancer cell, it was easy to find the therapeutic window of this extract. The induction of apoptosis was found to be caspase-independent, although there

was activation of both the extrinsic and intrinsic pathways and the production of ROS was not essential to the mechanism of cell death induction by PLX. The ability of PLX to target multiple vulnerabilities of cancer cells and still act to induce apoptosis in the presence of different types of inhibitors suggest the potential application of PLX in safe and efficacious cancer therapy.

MATERIALS & METHODS

Cell Culture

A malignant melanoma cell line G-361, human colorectal cancer cell lines HT-29 and HCT116 (American Type Culture Collection, Manassas, VA, USA Cat. No. CRL-1687, CCL-218 & CCL-247, respectively) were cultured with McCoy's Medium 5a (Gibco BRL, VWR, Mississauga, ON, Canada) supplemented with 10% (v/v) FBS (Thermo Scientific, Waltham, MA, USA) and 40 mg/ml gentamicin (Gibco, BRL, VWR). The ovarian adenocarcinoma cell line OVCAR-3 (American Type Culture Collection, Cat. No. HTB-161) was cultured in RPMI-1640 media (Sigma-Aldrich Canada, Mississauga, ON, Canada) supplemented with 0.01 mg/mL bovine insulin, 20% (v/v) fetal bovine serum (FBS) standard (Thermo Scientific, Waltham, MA, USA) and 10 mg/mL gentamicin. The pancreatic adenocarcinoma cell line BxPC-3 (American Type Culture Collection, Cat. No. CRL-1424) was cultured in RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS) standard and 40 mg/mL gentamicin. Normal-derived colon mucosa NCM460 cell line (INCELL Corporation, LLC., San Antonio, TX, USA) was grown in INCELL's M3Base™ medium (INCELL Corporation, LLC., Cat. No. M300A500) supplemented with 10 % (v/v) FBS and 10 mg/mL gentamicin.

All cells were grown in optimal growth conditions of 37°C and 5 % CO₂. Furthermore, all cells were passaged for ≤ 6 months.

Long Pepper Extraction

Indian long pepper seeds were obtained from Quality Natural Foods limited, Toronto Ontario. The plant material was ground up and extracted in

anhydrous ethanol (100%) in a ratio of 1:10 (1g plant material to 10 ml ethanol). The extraction was carried out overnight on a shaker at room temperature. The extract was passed through a P8 coarse filter, followed by a 0.45 µm filter. The solvent was evaporated using a RotorVap at 40°C and the dried extracted material was reconstituted in dimethylsulfoxide (Me₂SO) at a stock concentration of 450 mg/ml.

Cell Treatment

Cells were plated and grown to 60-70% confluence, before being treated with Long Pepper Extracts (PLX), N-Acetyl-L-cysteine (NAC) (Sigma-Aldrich Canada, Cat. No. A7250), and broad-spectrum caspase inhibitor, Z-VAD-FMK (EMD Chemicals, Gibbstown, NJ, USA) at the indicated doses and durations. NAC was dissolved in sterile water. Z-VAD-FMK was dissolved in dimethylsulfoxide (Me₂SO). PLX was extracted as previously described, reconstituted in Me₂SO and cells were treated either crude long pepper extract, before evaporation or Me₂SO reconstituted extract and control cells were treated with corresponding concentrations of Me₂SO.

ASSESSING THE EFFICACY OF LONG PEPPER EXTRACT (PLX) IN CANCER CELLS:

WST-1 Assay for Cell Viability

To assess the effect of PLX on cancer cells, a water-soluble tetrazolium salt (WST-1) based colorimetric assay was carried out as per manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA), to quantify cell viability as a function of cellular metabolism. Equal number of cells were seeded onto 96-well clear bottom tissue culture plates then treated with the indicated treatments

at the indicated concentrations and durations. Following treatment, cells were incubated with the WST-1 reagent for 4 hours at 37° C with 5 % CO₂. The WST-1 reagent is cleaved to formazan by cellular enzymes in actively metabolizing cells. The formazan product was quantified by taking absorbance readings at 450 nm on a Wallac Victor³ TM 1420 Multilabel Counter (PerkinElmer, Woodbridge, ON, Canada). Cellular viability was expressed as percentages of the solvent control groups.

Nuclear Staining

Subsequent to treatment, the nuclei of cells were stained with 10 µM Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA) to monitor nuclear morphology for apoptosis induction at designated time points. Cells were incubated with 10 µM Hoechst dye for 10 minutes and micrographs were taken with a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany) at 400X magnification.

Annexin V Binding Assay

To confirm the induction of apoptosis, the binding of Annexin V to externalized phosphatidylserine on the outer cellular surface, was assessed. Following treatment with PLX, cells were washed twice in phosphate buffer saline (PBS). Subsequently, cells were resuspended and incubated in Annexin V binding buffer (10 mM HEPES, 10 mM NaOH, 140 mM NaCl, 1 mM CaCl₂, pH 7.6) with Annexin V AlexaFluor-488 (1:50) (Invitrogen, Canada, Cat No. A13201) for 15 minutes. Micrographs were taken at 400X magnification on a Leica DM IRB inverted microscope (Wetzlar, Germany).

Whole Cell ROS Generation

Following treatment with PLX, cells were incubated with 2',7'-Dichlorofluorescein diacetate H₂DCFDA (Catalog No. D6883, Sigma Aldrich, Mississauga ON, Canada) for 45 minutes. Cells were collected, washed twice in PBS and green fluorescence was observed using a TALI image-based cytometer (Invitrogen, Canada). NAC was used to assess the dependence of PLX on ROS generation and viability.

ASSESSMENT OF MITOCHONDRIAL FUNCTION FOLLOWING PLX TREATMENT:

Tetramethylrhodamine Methyl Ester (TMRM) Staining

To monitor mitochondrial membrane potential (MMP), tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR, Mississauga, ON, Canada) or 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride (JC-1) (Invitrogen, Canada) were used. Cells were grown on coverslips, treated with the indicated concentrations of treatments at the indicated time points, and incubated with 200 nM TMRM for 45 minutes at 37° C. Micrographs were obtained at 400X magnification on a Leica DM IRB inverted fluorescence microscope (Wetzlar, Germany). To confirm the results obtained by fluorescence microscopy, image-based cytometry was used to detect red fluorescence. Cells were seeded in 6-well plates and following treatment, cells were incubated with TMRM for 45 minutes, washed twice in PBS and placed in TALI slides. Red fluorescence was obtained using a TALI image-based cytometer (Invitrogen, Canada).

Mitochondrial Isolation to Assess Mitochondrial Targeting

Cells were collected by trypsin, washed once in cold PBS, resuspended in cold hypotonic buffer (1 mM EDTA, 5 mM Tris-HCl, 210 mM mannitol, 70 mM sucrose, 10 μ M Leu-pep and Pep-A, 100 μ M PMSF), and manually homogenized. The homogenized cell solution was centrifuged at 3000 rpm for 5 minutes at 4 °C. The supernatant was centrifuged at 12,000 rpm for 15 minutes at 4 °C and the mitochondrial pellet was resuspended in cold reaction buffer (2.5 mM malate, 10 mM succinate, 10 μ M Leu-pep and Pep-A, 100 μ M PMSF in PBS). The isolated mitochondria were treated with PLX at the indicated concentrations and incubated for 2 hours in cold reaction buffer. The control group was treated with solvent (ethanol). Following 2 hour incubation with extract, mitochondrial samples were vortexed and centrifuged at 12,000 rpm for 15 minutes at 4°C. The resulting supernatant and mitochondrial pellets (resuspended in cold reaction buffer) were subjected to Western Blot analysis to assess for the mitochondrial release/retention of pro-apoptotic factors.

Western Blot Analyses

Protein samples were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked with 5% w/v milk TBST (Tris-Buffered Saline Tween-20) solution for 1 hour. Membranes were incubated overnight at 4°C with an anti-endonuclease G (EndoG) antibody (1:1000) raised in rabbits (Abcam, Cat. No. ab9647, Cambridge, MA, USA), an anti-succinate dehydrogenase subunit A (SDHA) antibody (1:1000) raised in mice (Santa Cruz Biotechnology, Inc., sc-59687, Paso Robles, CA, USA), or an anti-apoptosis inducing factor (AIF) antibody raised in rabbits (1:1000) (Abcam, Cat. No.

ab1998, Cambridge, MA, USA). After primary antibody incubation, the membrane was washed once for 15 minutes and twice for 5 minutes in TBST. Membranes were incubated for 1 hour at room temperature with an anti-mouse or an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000) (Abcam, ab6728, ab6802, Cambridge, MA, USA) followed by three 5-minute washes in TBST. Chemiluminescence reagent (Sigma-Aldrich, CPS160, Mississauga, ON, Canada) was used to visualize protein bands and densitometry analysis was performed using ImageJ software.

IN-VIVO ASSESSMENT OF LONG PEPPER EXTRACT:

Toxicity Assessment

Six week old Balb/C mice were obtained from Charles River Laboratories and housed in constant laboratory conditions of a 12-hour light/dark cycle, in accordance with the animal protocols outlined in the University of Windsor Research Ethics Board- AUPP 10-17). Following acclimatization, mice were divided into three groups (3 animals/control (untreated), 3 animals/gavage control (vehicle treatment) and 4 animals/treatment group). The control untreated group was given plain filtered water, while the second and third group was given 50 mg/kg/day vehicle (DMSO) or PLX, by gavage, respectively for 75 days. During the period of study, toxicity was measured by weighing mice twice a week and urine was collected for protein urinalysis by urine dipstick and Bradford assays. Following the duration of study, mice were sacrificed and their organs (livers, kidneys and hearts) were obtained for immunohistochemical and toxicological analysis by Dr. Brooke at the University of Guelph.

Efficacy of PLX in Tumor Xenograft Models of Immunocompromised Mice

Six week old male CD-1 nu/nu mice were obtained from Charles River Laboratories and housed in constant laboratory conditions of a 12-hour light/dark cycle, in accordance with the animal protocols outlined in the University of Windsor Research Ethics Board- AUPP 10-17). Following acclimatization, the mice were injected subcutaneously in the right and left hind flanks with a colon cancer cell suspension (in Phosphate buffered saline) at a concentration of 2×10^6 cells/mouse (HT-29, p53^{-/-}, in the left flank and HCT116, p53^{+/+}, in the right flank). Tumors were allowed to develop (approximately a week), following which the animals were randomized into treatment groups of 4 mice per group, a control group, a gavage control group given plain filtered sterile water, as well as gavage regimen of the vehicle (5 μ L Me₂SO in PBS) twice a week. The final group was given filtered water supplemented with long pepper extract at a concentration of 100 μ g/ mL, as well as gavage regimen of long pepper extract (5 μ L extract in PBS), twice a week, corresponding to 50 mg/kg/day. The tumors were assessed every other day by measuring the length, width and height, using a standard caliper and the tumor volume was calculated according to the formula $\pi/6 \times \text{length} \times \text{width}$. The mice were also assessed for any weight loss every other day for the duration of the study, which lasted 75 days, following which the animals were sacrificed and their organs and tissues (liver, kidneys, heart and tumors) were obtained and stored in 10% formaldehyde for immunohistochemical and toxicological analysis.

Hematoxylin & Eosin (H & E) Staining

Mice organs were fixed in 10% formaldehyde, following which they were

cryosectioned into 10 μ m sections and placed on a superfrost/Plus microscope slides (Fisherbrand, Fisher Scientific). Sections of organs were stained according to a standardized H & E protocol²⁵.

Analysis of Long Pepper Extract by HPLC

HPLC analysis of the long pepper crude extract was carried out at University of Ottawa in the Arnason lab. A total of five well-known piperamides were analyzed and compared to the crude long pepper extract. The extracts and piperamide standards were analyzed on a Luna C18-5u-250 x 4.6 mm column at 45°C at a flow rate of 1.0 mL/min with a mobile phase constituted of H₂O and methanol as outlined below;

Time (mins)	H ₂ O (%)	MeOH (%)
0.0	37.5	62.5
15.0	35.0	65.0
35.0	0.0	100.0
45.0	0.0	100.0
46.0	37.5	62.5

Chromatogram profiles were used to detect the any differences between a sample standard of known piperamides in the crude long pepper extracts.

RESULTS

Ethanollic Extract of Long Pepper (PLX) Effectively and Selectively Reduces the Viability of & Induces Apoptosis in Cancer cells in a Dose & Time Dependent Manner

The first step in understanding the effect of long pepper extract in this study was to assess the effect of PLX on the viability of cancer cells. Following treatment with increasing concentration of PLX at increasing time points, cells were incubated with a water soluble tetrazolium salt, which gets metabolized to a red formazan product by viable cells with active metabolism. This product can then be quantified by absorbance spectrometry. We observed the efficacy of crude PLX in reducing the viability of cancer cells, including colon (HCT116), pancreatic (BxPC-3), ovarian cancer (OVCAR-3) and melanoma cells. This effect was dose and time dependent (Figure 1).

To further evaluate the anticancer activity of PLX, we wanted to assess its role in cell death and its selectivity to cancer cells. Our results demonstrate that PLX is able to selectively induce cell death in cancer cells (colon, pancreatic and leukemia) in a dose and time dependent manner, as characterized by the increase in propidium iodide positive cells in cancer cells treated with PLX (Figure 2). Furthermore, this effect was selective, as normal colon epithelial cells remained unaffected by this treatment, at the same concentrations and time-points (Figure 2B). Additionally, apoptosis induction in various cancer cells, melanoma (G-361), ovarian and colon cancer (HT-29) cells, was confirmed by Annexin-V binding assay. This induction of apoptosis was confirmed to be selective to cancer cells, as normal colon cells (NCM460) remained unaffected by

PLX treatment. This was indicated by nuclear condensation, cell morphology and externalization of phosphatidyl serine to the outer leaflet of the cell membrane, as indicated by Hoechst staining, phase contrast images and binding of annexin V dye respectively (Figure 3A and B).

The selectivity of PLX to cancer cells was further confirmed by the WST-1 cell viability assay that showed that PLX was highly effective at low doses, a therapeutic window was easily observed (Figure 3C). Treatment of HT-29 with 0.20 mg/ml effectively reduced the viability by approximately 90%, while NCM460 cells remained at 100% viability at the same dose. This indicates that PLX can be more effective at low doses, further reducing the chances of toxicity associated with treatment.

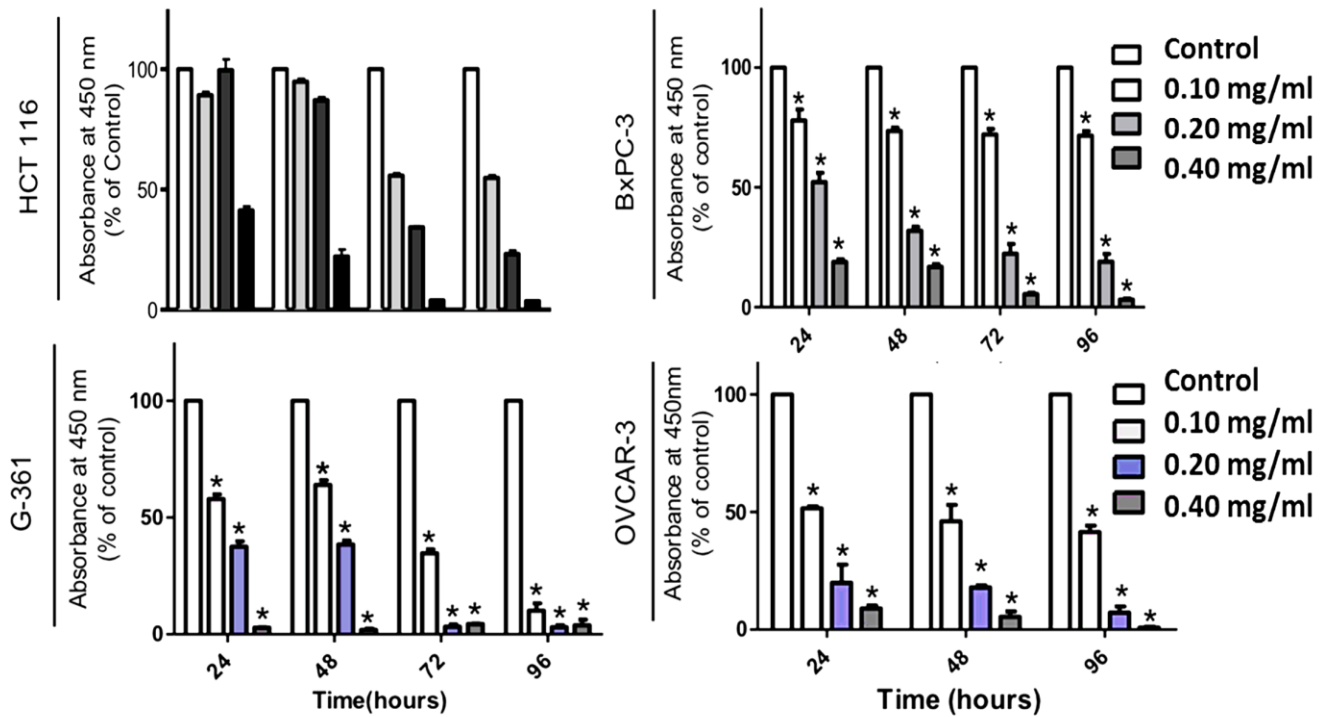
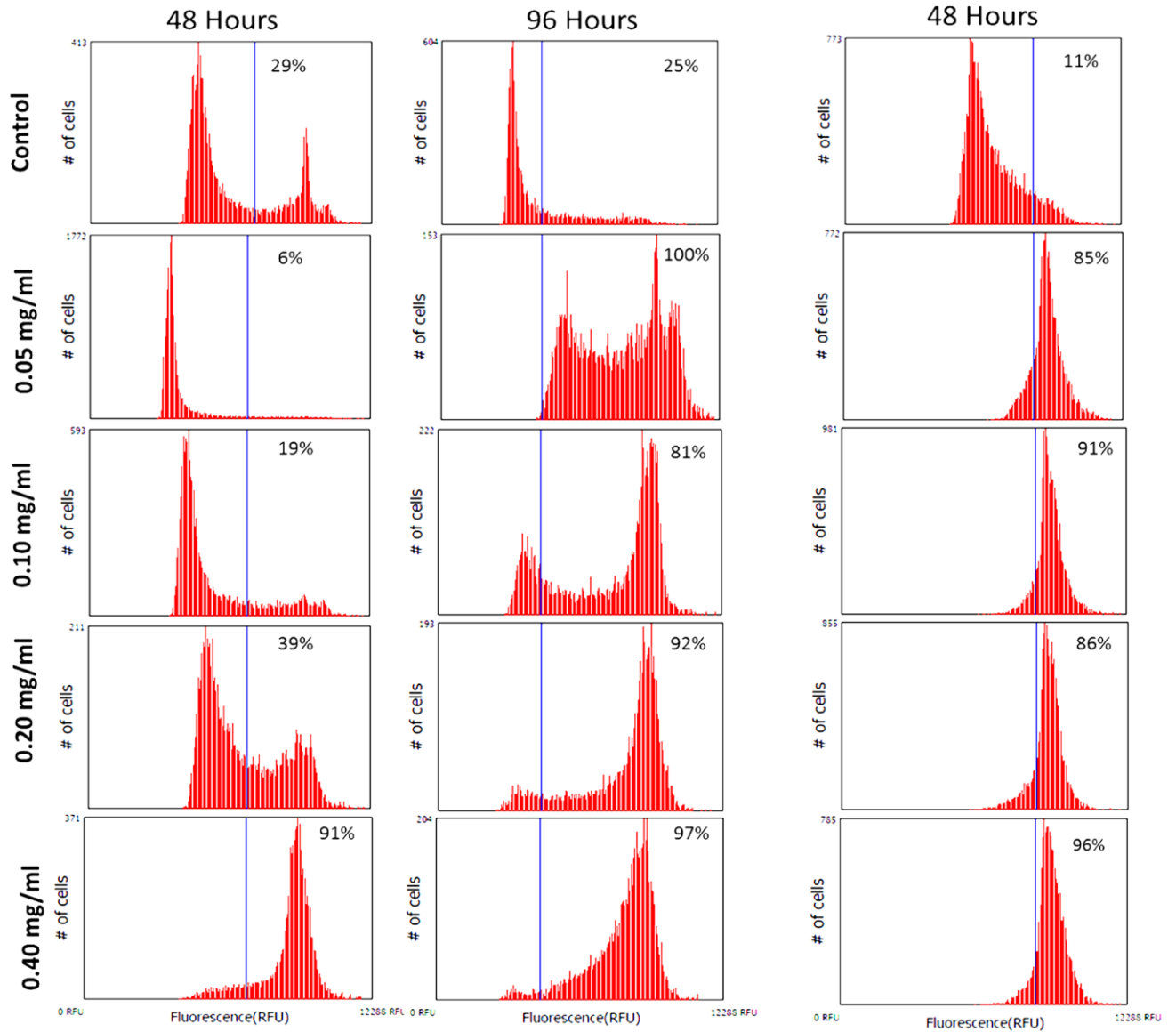


Figure 1: Crude Ethanolic Extract of Long Pepper (PLX) Effectively Reduces the Percentage of Viable Cancer cells in a Dose & Time Dependent Manner: Colon (HCT116), Ovarian (OVCAR-3), Pancreatic (BxPC-3) cancer and Melanoma (G-361) cells were treated with a crude ethanolic extract of long pepper (PLX), following which they were incubated with WST-1 cell viability dye for 4 hours. Absorbance was read at 450nm and expressed as a percent of the control. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. **P<0.0001

A

BxPc-3

E6-1



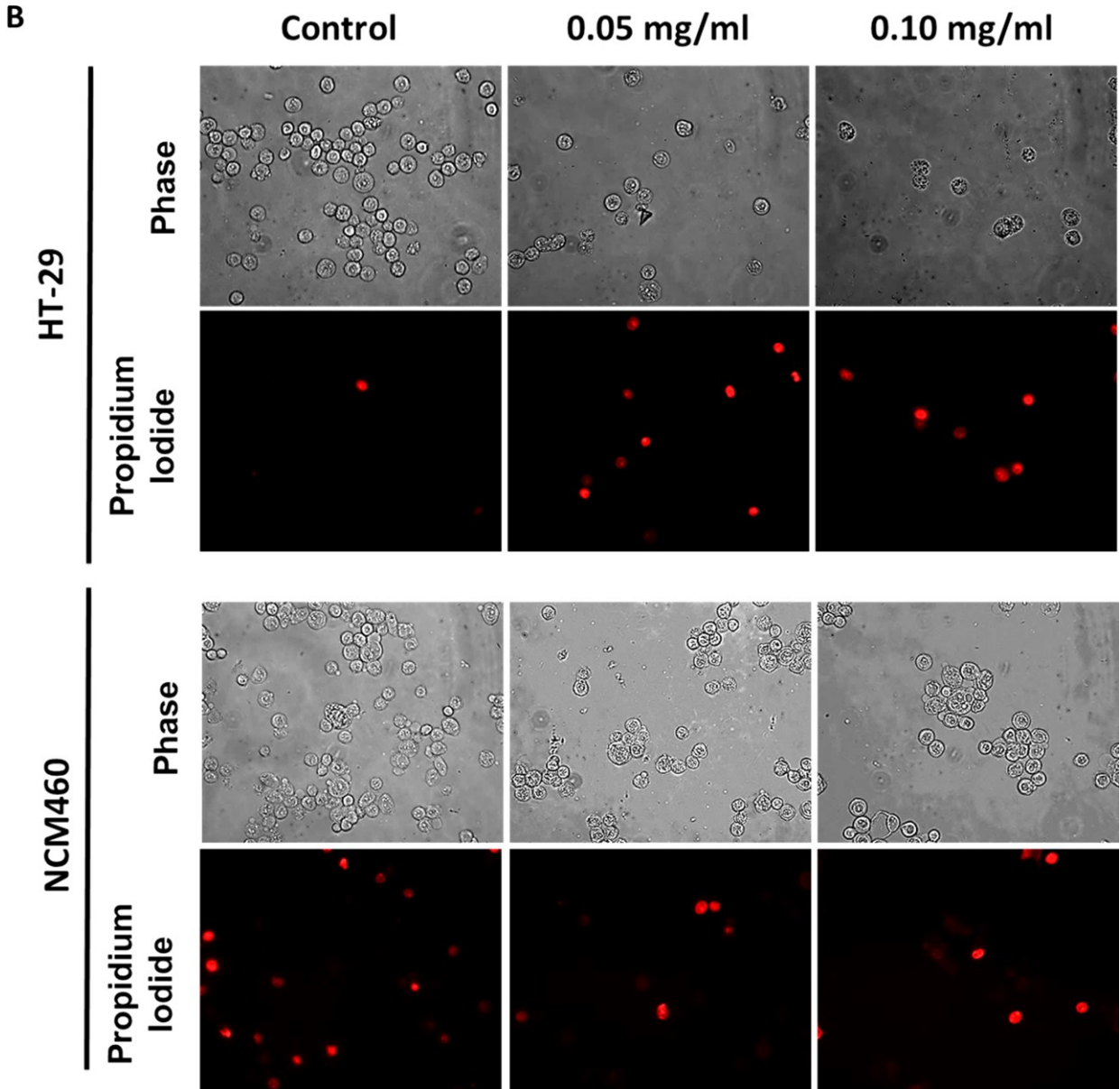


Figure 2: PLX Selectively Induces Cell Death in Human Cancer Cells in a Dose & Time Dependent Manner: (A) Following treatment of Human pancreatic (BxPc-3) cancer and T cell leukemia cells with PLX, at indicated time points, cells were incubated with propidium iodide and assessed for the induction of cell death by image-based cytometry. (B) Similar experiments were carried out in human colon cancer cells (HT-29) and normal colon epithelial cells (NCM460). Fluorescence microscopy was used to assess the induction of cell death as characterized by presence of propidium iodide positive cells. Images were taken at 400x magnification on a fluorescent microscope. Scale bar = 15 μ m

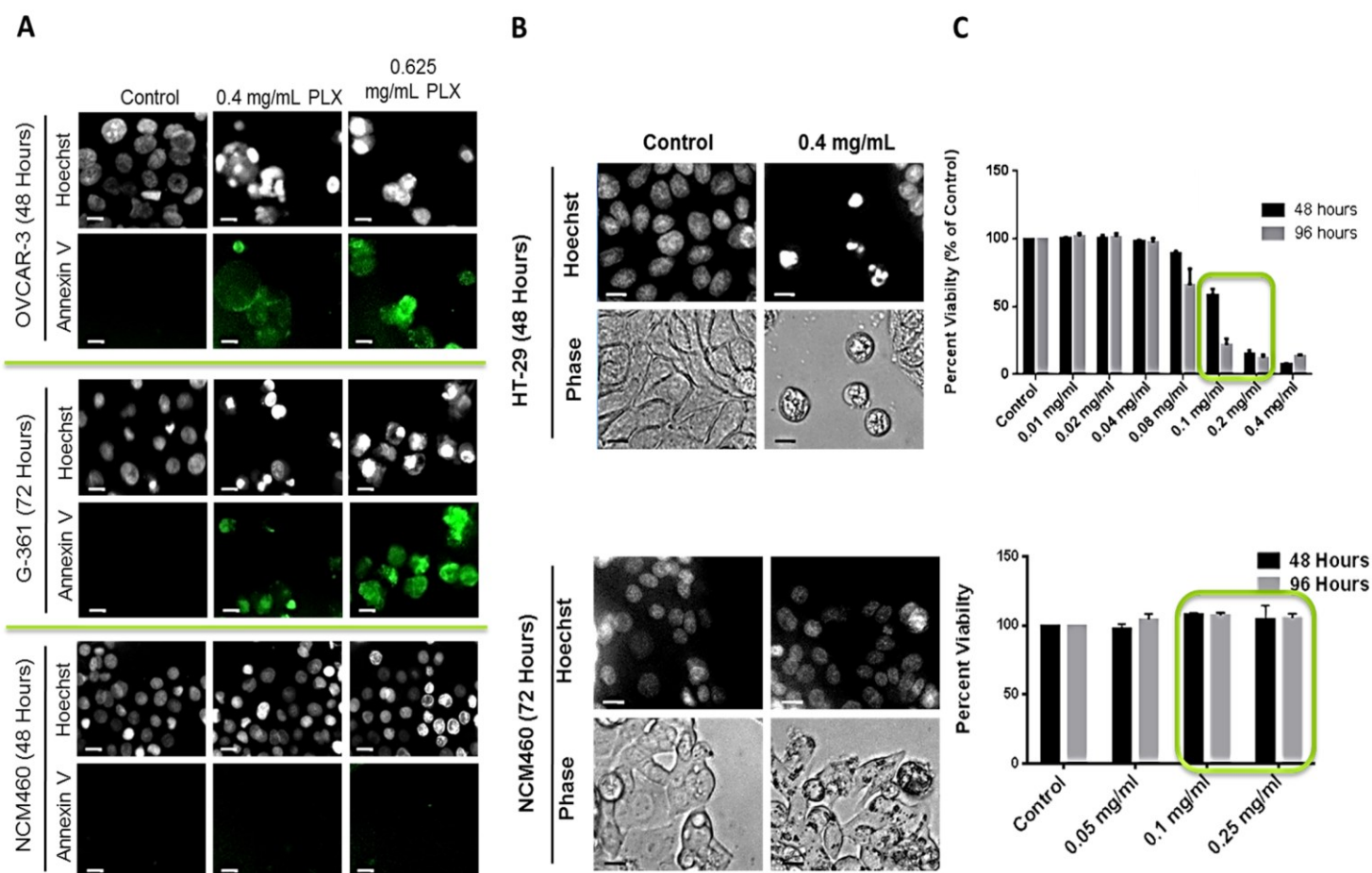


Figure 3: PLX Selectively Targets Cancer Cells for Apoptosis Induction: Subsequent to treatment with PLX, cells (Ovarian; OVCAR-3, Melanoma; G-361 and Normal Colon Epithelia cells (NCM460) were stained with Hoechst to characterize nuclear morphology and Annexin-V to detect apoptotic cells (A) and cellular morphology by phase contrast microscopy (B); Images were taken at 400x magnification on a fluorescent microscope. Scale bar = 15 μ m. (C) Following PLX treatment, HT-29 colorectal cancer cells and non-cancerous NCM460 cells were incubated with WST-1 cell viability dye for 4 hours and absorbance was read at 450nm and expressed as a percent of the control. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. **P<0.0001.

PLX Induces Caspase-Independent Apoptosis in Human Cancer Cells

Caspases are cysteine aspartic proteases that play a predominant role as death proteases (Earnshaw et al., 1999). Their roles in various cell death processes remains controversial, as their activation or inhibition could be essential to the progression of inhibition of cell death pathways (Thorburn, 2008; Zhivotovsky & Orrenius, 2010).

To assess the role of caspases in our study, BxPc-3 cells treated with PLX at 0.10 mg/ml at indicated time points. Following treatment, cells were collected, washed and incubated with lysis buffer to obtain cell lysate. The cell lysate was incubated with caspase substrates, specific to each caspase (3, 8 and 9) and incubated for an hour. Fluorescence readings were obtained using a spectrofluorometer. Our results indicate that PLX is able to activate both pathways (extrinsic and intrinsic apoptosis) in a time dependent manner. This was observed as rapid activation of caspases-3, 8 and 9 were observed as early as an hour, following treatment (Figure 4A).

To determine the importance of these activated caspases to the apoptosis-inducing effect of PLX, colon (HCT116) and pancreatic (BxPc-3) cancer cells were pre-treated with a pan-caspase inhibitor, Z-VAD-fmk (20 μ M), for an hour before treatment with PLX. Following treatments, the WST-1 cell viability assay was used to assess for viability and efficacy of PLX. Our results indicate that the inhibition of caspases could not prevent the reduction of viability (Figure 4B), signifying that the effect of PLX in cancer cells is caspase independent.

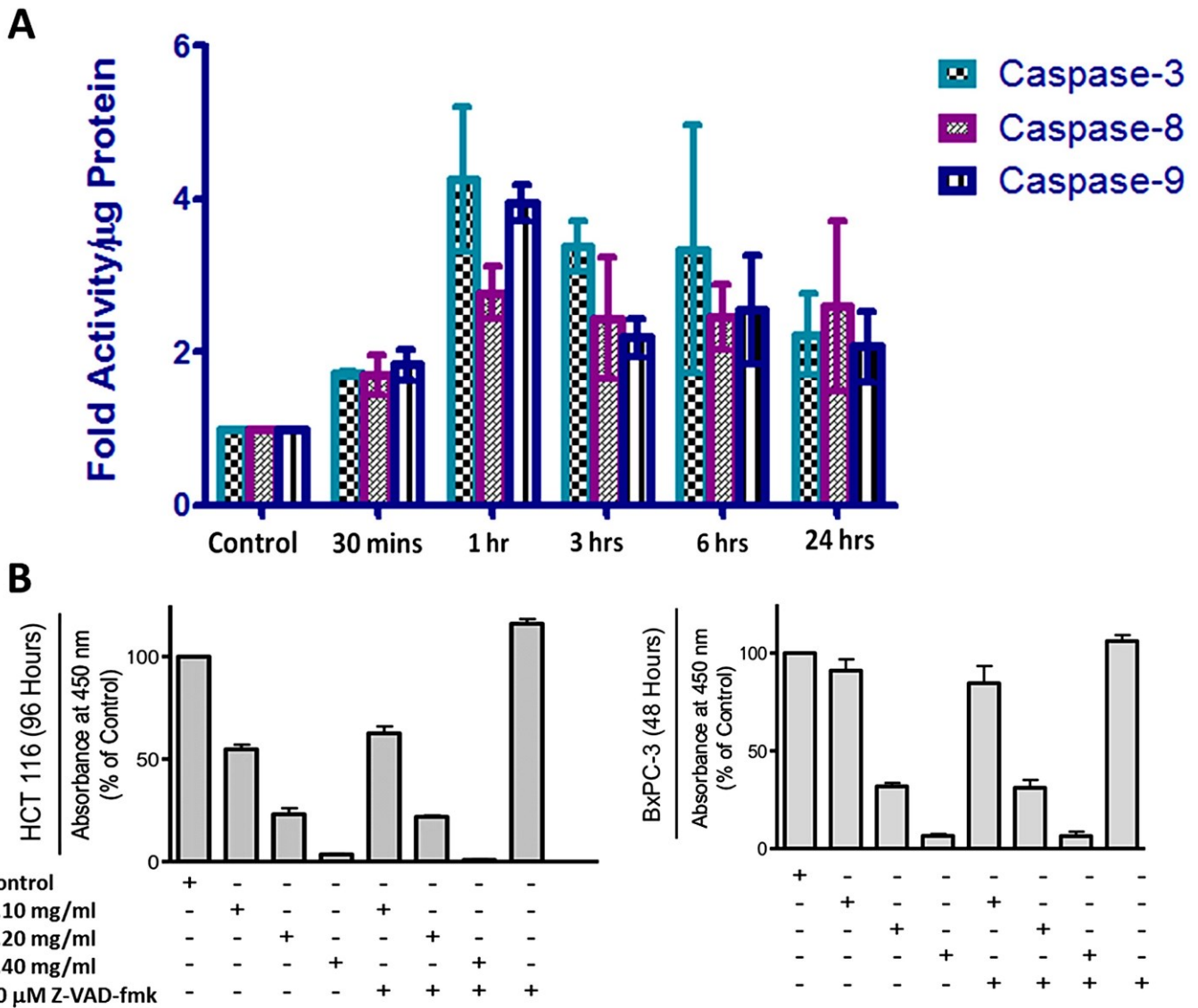


Figure 4: Long Pepper Extract (PLX) Activates the Extrinsic & Intrinsic Pathways of Apoptosis: Following treatment with 0.10 mg/ml PLX, at indicated time points, BxPc-3 cells were collected, washed and incubated with lysis buffer to obtain cell lysate. The cell lysate was incubated with caspase substrates, specific to each caspase (3, 8 and 9) and incubated for an hour. Fluorescence readings were obtained using a spectrofluorometer. An average of 6 readings per well and a minimum of three wells were run per experiment. The results here are reported as activity per μg of protein (in fold) and the average of three independent experiments is shown. **(B)** The reduction in viability was caspase independent, as a pan-caspase inhibitor, Z-VAD-fmk could not prevent the loss of viability induced by PLX treatment in colon and pancreatic cancer cells. Absorbance was read at 450 nm and expressed as a percent of the control. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. ****P<0.0001**

Long Pepper Extract Induces Oxidative Stress and Targets the Mitochondria of Cancer Cells

Generation of oxidative stress has been well established as a major player in the induction of several cell death processes, especially apoptosis (Simon et al., 2000; Madesh & Hajnóczy, 2001). The next part of our study focused on the role of oxidative stress in PLX induced apoptosis. Following treatment with PLX for 48 hours, cells were incubated with 2',7'-Dichlorofluorescein diacetate H₂DCFDA for 45 minutes. The resulting green fluorescence histograms were obtained using a TALI image-based cytometer. From the results, it was observed that PLX induced extensive generation of whole cell reactive oxygen species (ROS) in HT-29 colon cancer cells, while acting to suppress any ROS present in the non-cancerous cell lines, NCM460 and normal human fibroblasts (NHF) (Figure 6A & B). This confirms our results of selectivity and indicates that PLX might act as a pro-oxidant in cancer cells in order to induce apoptosis.

To determine if this oxidative stress was essential to PLX activity, HCT116 colon cancer cells were pre-treated with N-acetyl-L-cysteine (NAC), a well-established anti-oxidant, used extensively in vitro studies (Dekhuijzen, 2004; Dodd et al., 2008), before treatment with PLX. Subsequent to PLX treatment, cells were analyzed for effect of PLX on viability, using the WST-1 viability assay. The results suggest that although PLX acts to induce oxidative stress to cause apoptosis, this oxidative stress is not essential to its activity. Both the cells treated with PLX alone and NAC followed by PLX showed a reduction in their viability (Figure 6C).

The mitochondria have also been shown to play a major role in the

progression and execution of apoptosis. The permeabilization of the mitochondrial membrane usually leads to the release of pro-apoptotic factors, including cytochrome c, apoptosis inducing factor (AIF) and endonuclease G (EndoG) (Earnshaw, 1999; Elmore, 2007). These factors cause a caspase-independent pathway for apoptosis to pass through and could bypass the antioxidant effects of NAC observed in figure 6C.

To assess the efficacy of PLX on the mitochondria of cancer cells, OVCAR-3, HT-29 and NCM460 cells were stained with TMRM, a cationic dye that accumulates in healthy mitochondria. Mitochondrial membrane potential (MMP) dissipation was only observed in OVCAR-3 and HT-29 cells as seen with the dissipation of red TMRM fluorescence, by fluorescence microscopy and image-based cytometry (Figure 5A, B & C). Following mitochondrial membrane collapse, we wanted to determine if there was release of some pro-apoptotic factors. Western blot analysis was used to monitor for the release of AIF and EndoG from isolated OVCAR-3 mitochondria. Results demonstrate that PLX directly caused the release of both AIF and EndoG from the mitochondria of OVCAR-3 cells (Fig 5D). These results provide an insight to the mechanism of PLX action, where the mitochondria appears to be a direct target of PLX for the reduction of viability and the induction of apoptosis

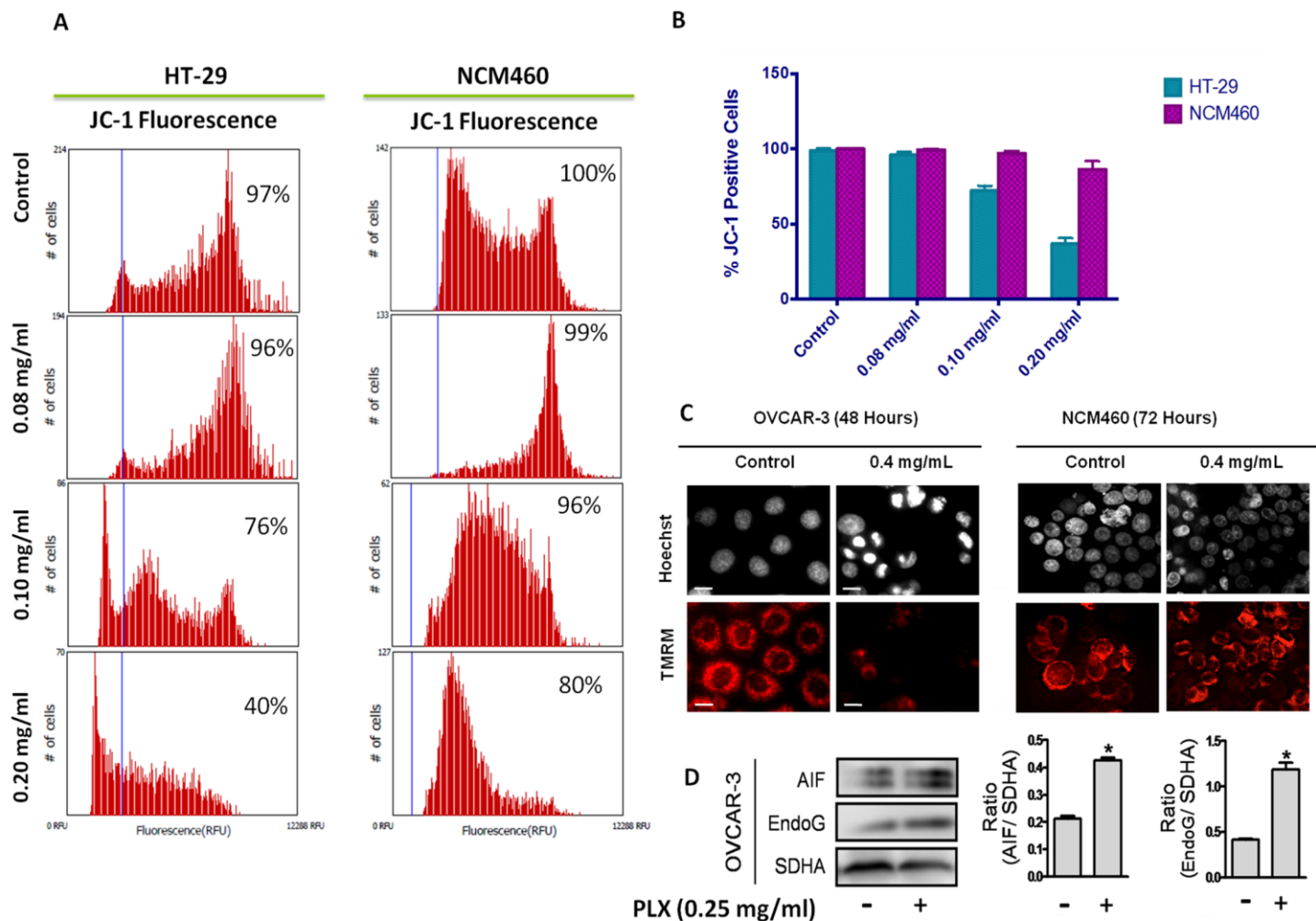


Figure 5: PLX Destabilizes the Mitochondrial Membrane of Cancer Cells: Colon cancer (HT-29), Ovarian cancer (OVCAR-3) and Normal Colon Epithelial (NCM460) cells were treated for 48 hours with PLX, following which, they were incubated with JC-1 (A) or TMRM (C) cationic mitochondrial membrane permeable dyes. Fluorescence readings were obtained using image based cytometry (A) and fluorescence microscopy; corresponding Hoechst dye images are also shown (C). Images were taken at 400x magnification on a fluorescent microscope. Scale bar = 15 μ m. (D) Isolated mitochondria of OVCAR-3 cells were treated directly with PLX or solvent control (ethanol) for 2 hours. Following treatment, samples were centrifuged, to obtain mitochondrial supernatants, which were examined for the release of pro-apoptotic factors, AIF and EndoG via western blot analyses, and mitochondrial pellets which were probed for SDHA to serve as loading controls. Image is representative of 3 independent experiments demonstrating similar trends. Values are expressed as mean \pm SD of quadruplicates of 1 independent experiment; * p <0.01 versus solvent control (ethanol).

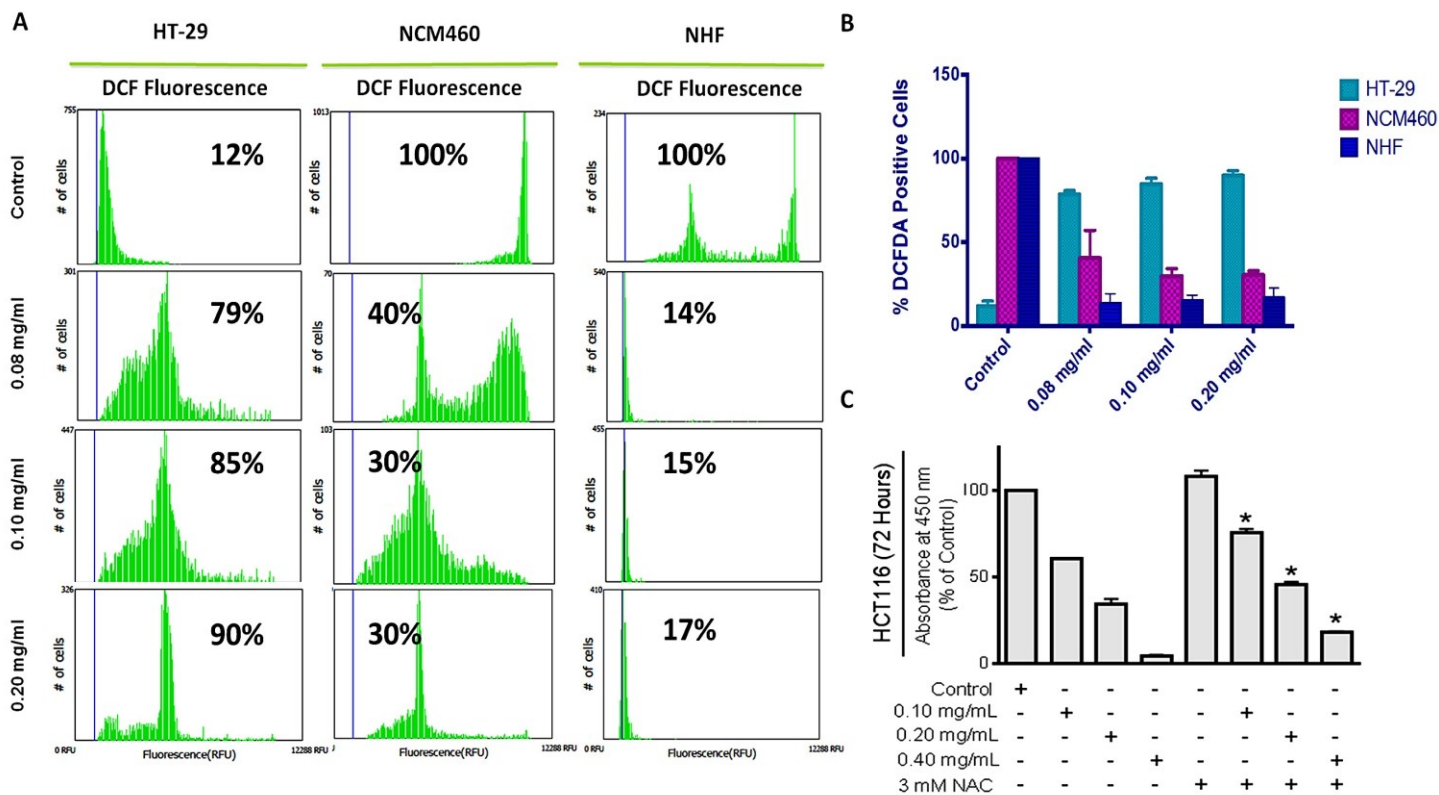


Figure 6: PLX Causes but is Not Dependent on the Production of Reactive Oxygen Species (ROS): (A) Colon cancer (HT-29), Normal Colon Epithelial (NCM460) and Normal Human Fibroblast (NHF) cells were treated with PLX for 48 hours, following which, they were incubated with H₂DCFDA and fluorescence results were obtained using an image based cytometer. Results were quantified using Graphpad prism 6.0 (B). (C) HCT116 colon cancer cells were treated with 3 mM N-acetylcysteine for an hour prior to PLX treatment. Cells were then treated PLX at indicated concentrations for 72 hours, following which the WST-1 assay was performed. Absorbance readings were taken at 450 nm and expressed as a percent of the control. Values are expressed as mean \pm SD from quadruplicates of 3 independent experiments. * $p < 0.05$

Long Pepper Extract is Well-Tolerated in Animal Models

Long pepper extracts (mainly water extracts) have been used for centuries and have been associated with various benefits (Bao et al., 2013). With all these anecdotal reports of benefits, there have been no reports of toxicities associated with its use. To further scientifically evaluate and validate the safety of PLX, balb/c mice were orally gavaged with 50 mg/kg/day vehicle (DMSO) or PLX for 75 days and the mice were observed for signs of toxicity. To assess for toxicity, mice were weighed twice a week, urine was collected for protein urinalysis studies and following period of treatment, mice were sacrificed and their organs were obtained for pathological analysis by a certified pathologist at the University of Guelph (Dr. Brooke). Results from this part of the study demonstrate that there was no weight loss overall in mice that were given PLX supplemented water (Figure 7B).

To further assess toxicity, urine was collected from mice once a week and protein urinalysis was performed using a urine dipstick and a Bradford protein concentration assay. Protein urinalysis results indicate that there were trace amounts of protein in the urine of mice both from the control and the PLX group, with trace readings corresponding to protein concentrations between 5 and 20 mg/dL. Bradford assays confirm the results obtained by dipstick urinalysis (Figure 7A). There was no major difference between the control group and PLX group, confirming the lack of toxicity associated with oral administration of PLX in drinking water.

Furthermore, the hearts, livers and kidneys were obtained following the toxicity study, sliced and stained with hematoxylin and eosin. Results show no

gross morphologic difference between the control and the treatment group, confirming the lack of toxicity associated with PLX treatment. Results from the pathologist indicate that the presence of any lesions in the tissues are minimal or mild and interpreted as either background or incidental lesions and the lack of lesion type and frequency was enough to conclude no toxicological effect of PLX to the balb/c mice (Table 1).

Table 1: Summary of Histological Lesions in Balb/C Mice on PLX regimen

	No Treatment		Vehicle (Gavage Control)			Long Pepper Extract (Treatment group)			
	M1	M2	M1	M2	M3	M1	M2	M3	M4
<u>Liver:</u>									
-Infiltration, leukocyte, predominantly mononuclear, minimal		X	X		X	X			X
-Focal mineralization, minimal									
-Hepatocyte necrosis, minimal									X
-Focus of cellular alteration, eosinophilic, minimal			X	X				X	
-Hepatocyte vacuolation, lipid type, minimal			X	X			X		
- Hepatocyte vacuolation, lipid type, mild	X			X				X	X
Fibrin thrombus			X						
<u>Heart:</u>									
-Infiltration, leukocyte, predominantly mononuclear, minimal		X				X			X
Myofiber separation and vacuolation, minimal (suspect artifact)		X	X						X
<u>Kidney:</u>									
- Infiltration, leukocyte, predominantly mononuclear, minimal	X	X		X		X		X	
Tubule vacuolation, minimal					X				X
Fibrin or other extracellular matrix, glomerulus								X	

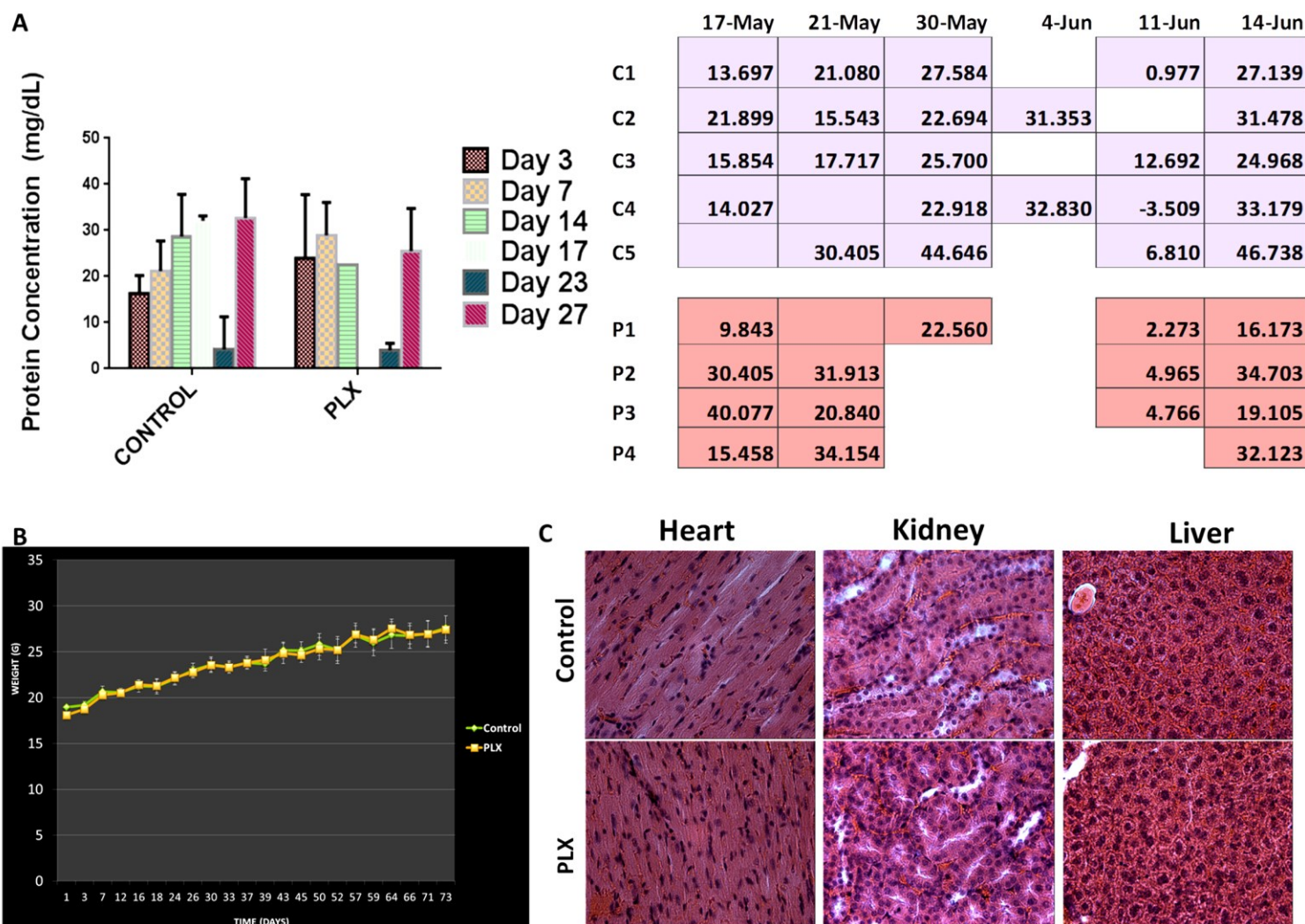
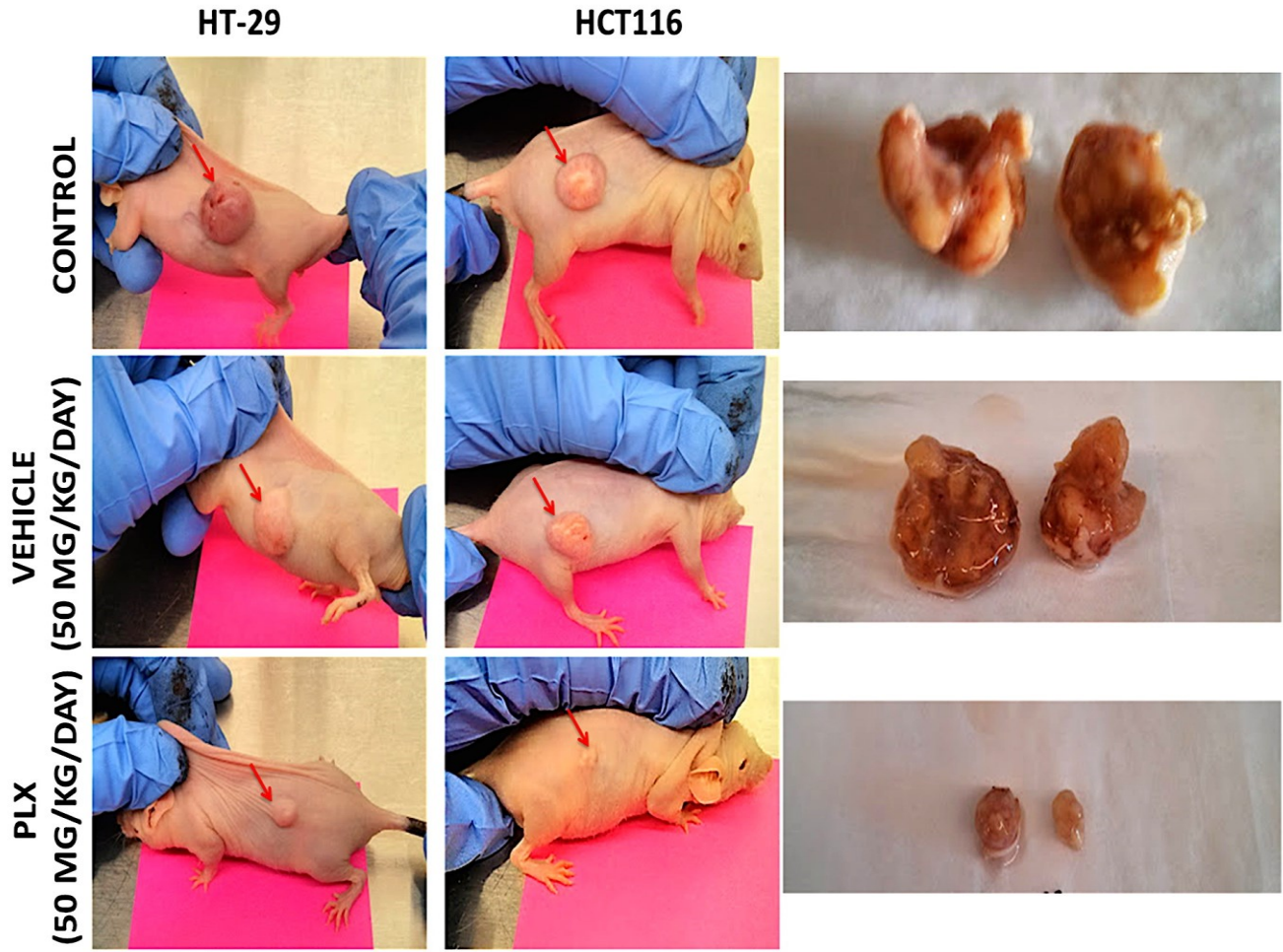


Figure 7: PLX is Well-Tolerated in Mice Models: Balb/C mice were divided into three groups (3 animals/control (untreated), 3 animals/gavage control (vehicle treatment) and 4 animals/treatment group). The control untreated group was given plain filtered water, while the second and third group was given 50 mg/kg/day vehicle (DMSO) or PLX, respectively. Mice were assessed for toxicity with protein urinalysis by Bradford Assay and dipstick analysis (A) and weight changes (B). (C) Hematoxylin and Eosin stained tissue sections of the liver, heart and kidney of control versus PLX treated group. Images were obtained on a bright field microscope at 63X objective.

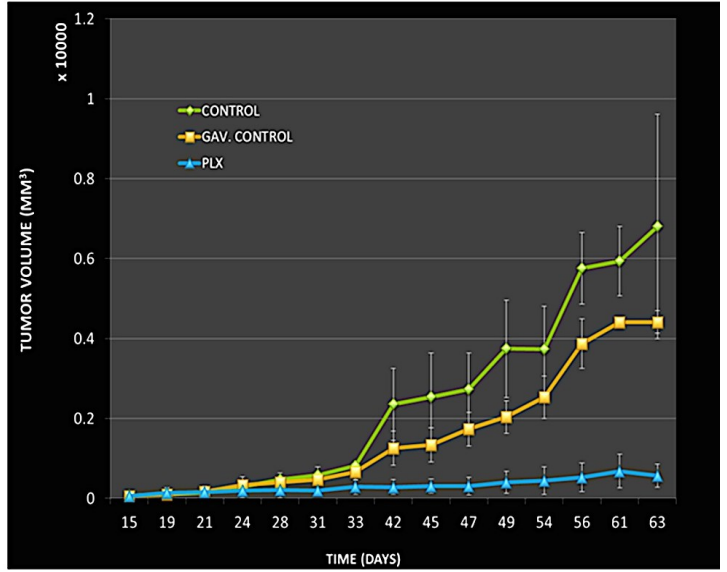
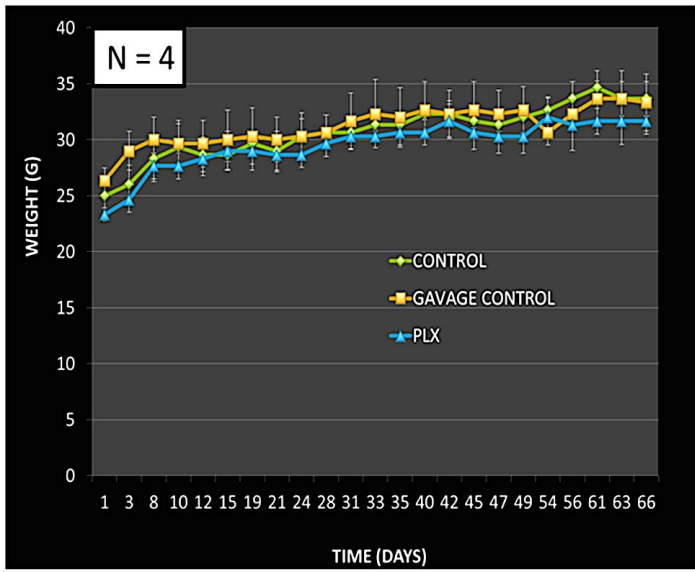
Oral Administration of Long Pepper Extract Halts the Growth of Human Colon Cancer Xenografts in Immunocompromised Mice

Following efficacy studies, we wanted to further study the efficacy of PLX. For this study, CD-1nu/nu immunocompromised mice were subcutaneously injected with HT-29 cells (left) and HCT116 cells (right). Following the establishment of tumors, mice were separated into three groups, a control group, a vehicle (Me₂SO) group and a PLX treated group. Mice were observed for 75 days, with weights and tumor volumes measured twice a week. Results demonstrate that oral administration of PLX could suppress the growth of both p53 WT (HCT116) and p53 mutant (HT-29) tumors *in-vivo*. There were no signs of toxicity, as indicated by increasing weights during the study (Figure 8A & B). Furthermore, H & E staining revealed less nuclei in the PLX treated group, compared to the control group, however, as observed in the toxicity studies, there were no gross morphological differences in the livers, kidneys and hearts of the control and PLX groups (Figure 8C).

A



B



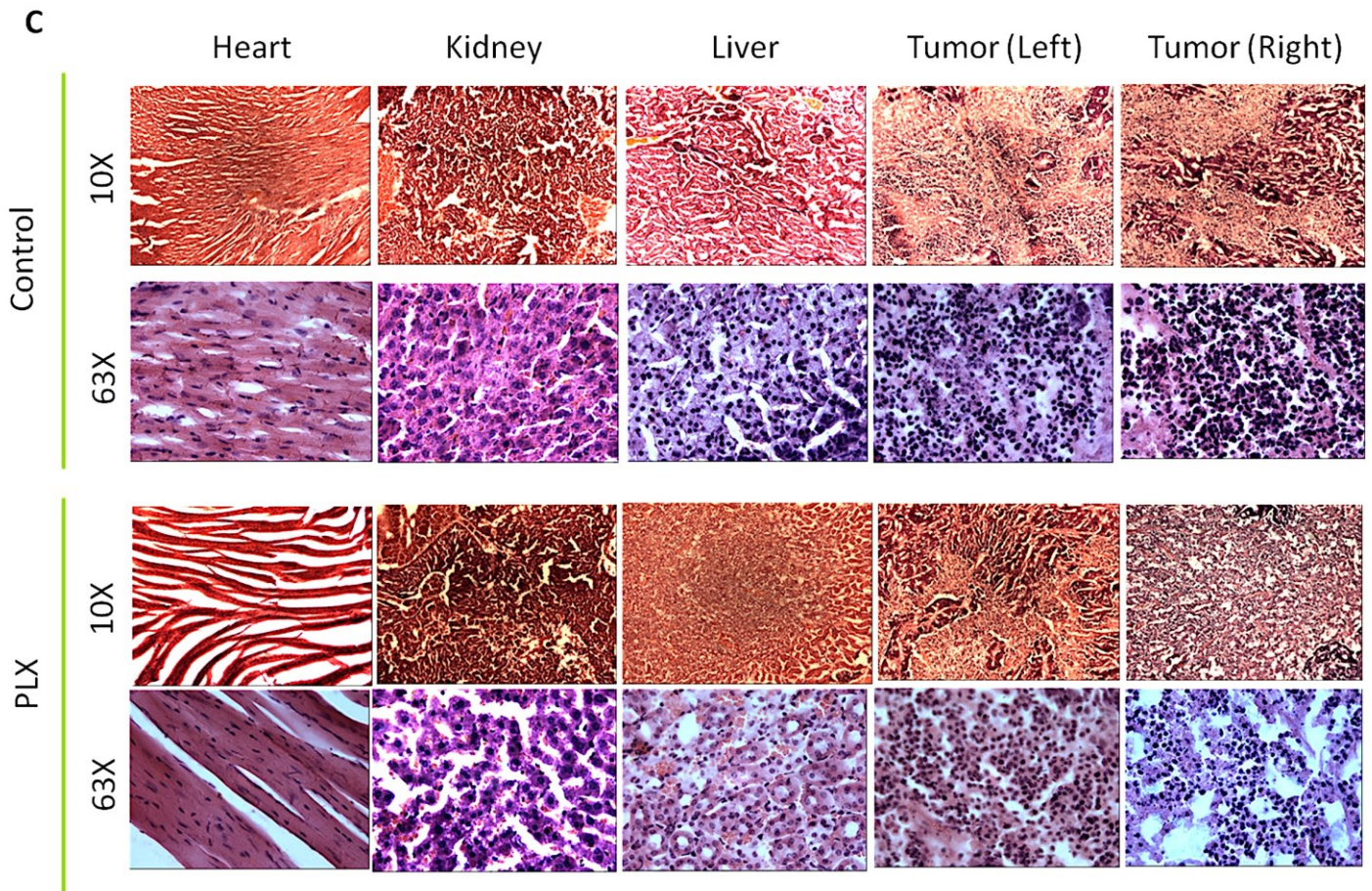


Figure 8: PLX Halts Growth of Colon Tumors in Xenograft Models: CD-1 nu/nu mice were subcutaneously injected with colon cancer cells; HT-29 (p53^{-/-}) on the left flank and HCT116 (p53^{+/+}) on the right flank. (A) Representative tumor size control mice and 50 mg/kg/day vehicle or PLX treated mice, respectively. PLX halted the growth of both HT-29 and HCT116 tumors *in-vivo*. (B) Average body weights of control and PLX treated mice. The body weights did not vary significantly during the study. Tumor volumes were measured and tumor curve shows the efficacy of 50 mg/kg/day oral administration of PLX. (C) Histopathological analysis of tissue samples obtained from control and PLX-treated animals. Hematoxylin and Eosin stained tissue sections of the livers, hearts, kidneys and tumors. Images were obtained on a bright field microscope at 10X and 63X objective.

Analysis of Long Pepper Extract

The availability of several species of long pepper and the host of compounds present within them make it essential to characterize the long pepper extract that has shown potent anticancer activity, both in *in-vitro* and *in-vivo* studies. HPLC profile studies were carried out on the crude ethanolic extracts, compared with a piperamide standard mix. The chromatogram profile show that our PLX extract contained several classes of compounds known to be present in piper species, including piperines and dihydropiperlongumine, with a peak corresponding to the presence of piperlongumine(Figure 9A & B). We hypothesize that the presence of this compound, along with the other compounds observed in this profile, could lend to the anti-cancer activity of PLX that we observe in the various cancer cell models. Further studies and characterization would provide more information about the roles of these compounds in cancer cell death induction.

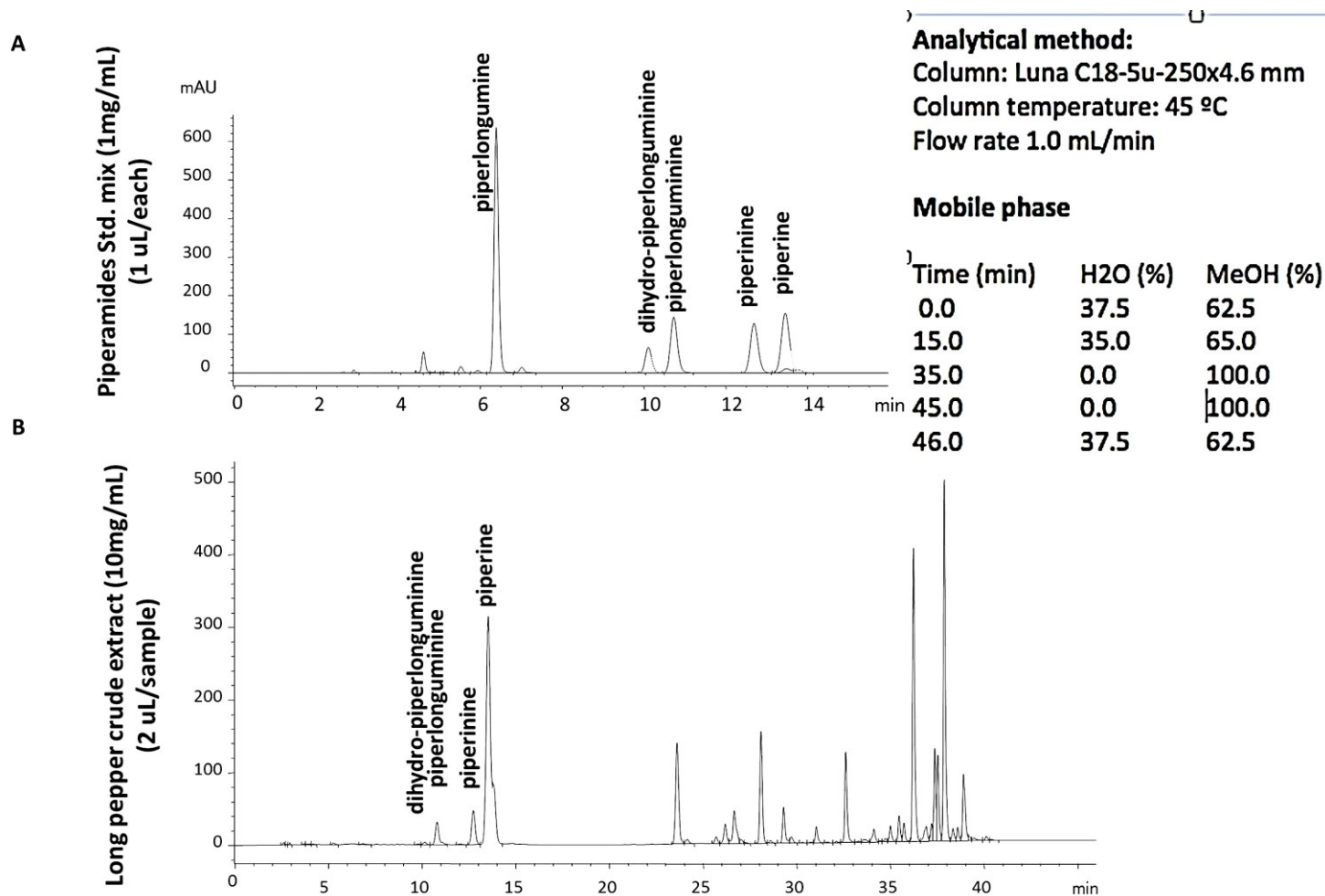


Figure 9: HPLC Analysis of PLX: Chromatograms of Long pepper crude extract (PLX) used for this study (10mg/mL at 2 μ L/Sample) (B) compared to Piperamides Standard mix (1mg/mL at 1 μ L/standard) (A).

DISCUSSION

In this report we demonstrate for the first time, the selective anti-cancer potential of an ethanolic extract of long pepper (PLX) in several cancer cell lines. PLX effectively reduced the viability of cancer cells and induced apoptosis in a dose- and time-dependent manner, at low doses, allowing for a greater therapeutic window in *in-vitro* studies (Figure 1 – 3). This apoptosis inducing effect was found to be independent of caspases, cysteine aspartic proteases that play a role in the progression and execution of apoptosis (Figure 4B). These results suggest that PLX is not toxic to non-cancerous cells at such low doses, as was observed in the cancer cells. Selectivity and lack of toxicity was confirmed with *in-vivo* toxicological studies. Damage to the kidneys is a common occurrence during various types to toxic therapies. This damage to the kidney results in large amounts of protein (>3.5 g/day) leaking into the urine (Bleske et al., 2013; Fang et al., 2013), and this can be measured by various assays. Lack of toxicity was confirmed by the lack of increased protein concentration in the urine samples collected from both the control group and PLX treated group, by two different assays. The urine dipstick method indicated that all urine samples from the control and PLX groups had trace amounts of protein, corresponding to concentrations between 5 mg/dL and 20 mg/dL, well within the acceptable concentration range. Bradford protein assay showed a concentration of approximately 30 mg/dL most days urine was collected (Figure 7A). This is still within the acceptable range of protein concentration in urine. These results confirm anecdotal studies that suggest no associated toxicity or side effects observed with take long pepper extracts. The efficacy of PLX in *in-vivo* models

also showed that not only was PLX well-tolerated, it was also effective at halting the growth of human tumor xenografts of colon cancer in nude mice (Figure 7A and B).

The next step in understanding the effect of PLX on cell death induction in cancer cells was to identify the mechanism of apoptosis induction observed following PLX treatment.

The role of oxidative stress in cell death processes has been well characterized. It is well established the reactive oxygen species (ROS) could be the cause or effect of apoptosis induction in cells (Simon et al., 2000). Some studies have suggested cancer cells to be more dependent on cellular response mechanisms against oxidative stress and have exploited this feature to selectively target cancer cells (Raj et al., 2012). The role of ROS generation in PLX-induced apoptosis was assessed following treatment. In this study, we found that PLX induced whole cell ROS production in a dose dependent manner, as indicated by the increase in green fluorescence of H₂DCFDA dye, cleaved by intracellular esterases and oxidized by ROS present (Figure 6A & B). However, we observed that ROS generation was not completely essential to PLX activity, as the presence of N-acetylcysteine could not entirely hamper the ability of PLX to reduce the viability of colon cancer cells (Figure 6C).

The caspase-independence observed in figure 4B, suggest that PLX is acting through pro-apoptotic factors other than caspases. The mitochondria play a major role in the progression and execution of apoptosis. The permeabilization of the mitochondrial membrane usually leads to the release of pro-apoptotic factors, including cytochrome c, apoptosis inducing factor (AIF) and

endonuclease G (EndoG) (Earnshaw, 1999; Elmore, 2007). AIF and EndoG execute apoptosis in a caspase-independent possibly leading to the caspase- and partial ROS-independence observed. We show here that PLX caused MMP dissipation in cancer cells, while non-cancerous NCM460 cell mitochondria remained intact following treatment (Figure 5A – C). The dissipation of the mitochondrial membrane led to the release of AIF and EndoG (Figure 5D), allowing for the progression and execution of apoptosis in the absence of caspases and oxidative stress, providing insight to the mechanism of PLX action in cancer cells. Cancer cells differ from non-cancerous cells in variety of ways, which could enhance the selectivity of PLX to cancer cells. The Warburg effect is characterized by the high dependence of cancer cells on glycolysis and low dependence on mitochondria for energy production in cancer cells, therefore creating a more vulnerable target in cancer cell mitochondria (Warburg, 1956). Moreover, various anti-apoptotic proteins associated to the mitochondria have been reported to be highly expressed in cancer cells. Such proteins could serve as targets for selective cancer (Mathupala, 1997; Casellas et al., 2002; Green & Kroemer, 2004).

Unlike isolated natural compounds, there are usually more benefits to using a whole plant extract than the isolated compound. Multiple components within extracts have many different intracellular targets, which may act in a synergistic way to enhance specific activities (including anticancer activities), while inhibiting any toxic effects of one compound alone. Additionally, the presence of multiple components may possibly decrease the chances of developing chemoresistance (Foster et al., 2005). Moreover, natural extracts can

be administered orally to patients, as a safe mode of administration. Some known compounds of the long pepper plants have been isolated and studied for their various activities (Lee et al., 2001; Raj et al., 2012; Bao et al., 2013; Golovine et al., 2013; Jarvius et al., 2013; Megwal & Goswami, 2013).

In this study, we show some signature compounds that are present in our PLX extract, including piperlongumine, dihydropiperlongumine and piperine. Notably, piperlongumine, a compound from the *Piper longum* plant, has previously been shown to have selective anticancer activity (Raj et al., 2011). The small peak of piperlongumine observed in the HPLC chromatogram in Figure 9, as piperlongumine may be due to the reduction of piperlongumine to the larger dihydropiperlongumine peak that we observe. In a previous study that showed the efficacy of piperlongumine, high concentrations of 10 μ M was required for significant cell death induction in cancer cells (Raj et al., 2012). In this study, a very low amount of the complex mixture of the ethanolic extract of long pepper (that contains many bioactive compounds) was sufficient in inducing apoptosis in cancer cells selectively. This indicates that the individual bioactive compounds (present in nanomolar concentrations within the extract) could act synergistically to induce apoptosis in cancer cells at very low concentrations. These findings highlights that the *Piper spp.* contain novel compounds with potent anticancer activity, in addition to piperlongumine.

In conclusion, our results demonstrate that long pepper extract (PLX), with a long historical use in traditional medicine, is selective in inducing cell death in cancer cells by targeting non-genomic targets. It is well tolerated in mice models

and effective in reducing the growth of human tumor xenotransplants in animal models, when delivered orally. This could open a window of opportunity to develop a novel, safer cancer treatment, using complex natural health products from the Long Pepper.

CHAPTER 6 REFERENCES

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CHAPTER 7

GENERAL DISCUSSION

The distinct lack of readily available, efficacious and non-toxic cancer therapies prompts further investigation and research into other available forms of treatment, in a bid to promote better quality of life for cancer patients. Natural health products (NHPs) and natural products (NPs) have been a major focus of many forms of therapies for centuries, in various traditional medicines and are often regarded as low risk due to their long history of human use. Anecdotal and literature reports are inconclusive and sometimes contradictory, therefore, these reports are not very useful for clinicians and patients (Foster et al., 2005; Jain, 2013). Although NHPs and herbal products are not considered toxic, because of their long use, there are some reports of adverse drug events (ADEs), as these are not single-active ingredients (SAIs), but contain multiple pharmacologically active components that could affect multiple signaling pathways within the system.

The bioactive components of complex natural products may be associated with low inherent risks of usage or a higher risk, when there is drug interaction with pharmacologically active compounds or enzymes that could affect drug disposition (Foster et al., 2005). Thus, there is a lack of rigorous scientific investigation into the use of many NHPs, and as such, diligent and rigorous assessment of potentially efficacious NHPs, NPs and herbal products are required. This is in a bid to ensure the safe and effective use of these products by potential users. Over 75% of the anticancer drugs approved since the 1940's have been obtained from natural products or are synthetic derivatives of natural products (both plants and marine/biological sources) (Mann, 2002; Newman & Cragg, 2012). This signifies that within the vast array of health and herbal

products, there are pharmacologically active components and whole complex mixtures that could show better efficacy, while lacking toxicity or reducing toxicities associated with other treatment options.

The major accomplishment of my work was the identification of natural health products (NHPs) with significant anti-cancer activity, the determination of the mode of action, contributing to their anti-cancer activities and the assessment of the safety of these NHPs in preclinical settings, which led to the clinical development of one NHP. My work focuses on two NHPs, the water extract of dandelion root (DRE) and the ethanolic extract of long pepper (PLX), and the stepwise characterization of their anti-cancer activities and potential toxicities.

Dandelions have been used for centuries, with the first reported evidence of the therapeutic use of dandelions mentioned by Arabian physicians during the 10th and 11th centuries in the treatment of liver and spleen diseases (Schütz et al., 2006). Anecdotal evidence shows the efficacy of various parts of this plant for the treatment of various diseases. For instance, the roots are used as a gastrointestinal remedy to support digestion and liver function, while the leaves are used as a diuretic and a bitter digestive stimulant. The roots have been investigated for demulcent and immune-modulating effects, while the leaves have been investigated for its role in inflammation (Yarnell & Abascal, 2009). There are various classes of pharmacologically active compounds within dandelions that have been predicted to lend to its therapeutic activity (Table 1), but the most important classes are triterpenoids and sesquiterpene lactones (SLs) (Takasaki et al., 1999; Schütz et al., 2006; Yarnell & Abascal). Biological studies on 11 triterpenoids found in various *Taraxacum* species and other plant sources show

that this class of compounds have significant anti-carcinogenic activity, with taraxasterol having the best anticancer activity (Takasaki et al., 1999). Studies on SLs have shown that this class of compounds have both anti-inflammatory and anti-cancer effects. This class of compounds was originally identified in an initial screen by the National Cancer Institute (NCI), the same screening that led to the identification of Taxol, and SLs have proven themselves worthy of continuous investigations, up to the levels of clinical trials, as anti-inflammatory and anti-cancer agents (Zhang et al., 2005; Ghantous et al., 2010). Owing to these characteristics, plant extracts, such as dandelions, with high amounts of SLs are becoming more important and are being given considerable interest, especially in cancer and inflammatory diseases. They have been shown to selectively target cancer stem cells and some have proceeded successfully to Phase I and II clinical trials (Jeon et al., 2008; Ghantous et al., 2010). All of these findings, along with the fact that dandelion extracts are complex mixtures, containing both triterpenoids and SLs, along with other classes of compounds, suggest the importance of this NHP in the fight against cancer.

The use of long pepper extract is not as well documented as that of dandelions. Of more interest are some of the components of long pepper, for instance, piperlongumine (PL), and its role in oxidative stress response, especially in cancer cells (Raj et al., 2011). PL was also found to induce cell cycle arrest, induce apoptosis by down-regulating the expression of pro-survival proteins and up-regulating the expression of pro-apoptotic proteins, as well as inducing DNA damage in cancer cells (Bezerra et al., 2012). There are other pharmacologically active compounds, within long pepper extract that have been

studied for their various activities. Piperidine alkaloids have shown fungicidal activities, while piperines affect enzymatic drug bio-transforming reactions and plays specific roles in metabolic activation of carcinogens and mitochondrial energy production (Jarvius et al., 2013; Megwal & Goswami, 2013). These studies provide sufficient substantiation that the whole complex mixture of long pepper extract, as well as dandelion root extract, could provide safer, non-toxic alternative to conventional modes of treatment, as a potential anti-cancer agent.

In light of this information, my findings of the complex mixture of DRE and PLX, with very clear anti-cancer activities, are novel and extremely important. During the period of this work, we found that aqueous dandelion root extract selectively induced apoptosis (PCD type I) and autophagy (PCD type II) in a variety of cancer cell models (Table 2). These results were significant, as we observed the versatility of a simple water extract of dandelion root in different cancer cell types, each with different and multiple mutations that promote their ability to proliferate in cell culture. Using various biochemical and morphological markers, we confirmed the induction of different modes of cell death in cancer cells. The induction of apoptosis was observed, following the rapid activation the death receptor mediated extrinsic pathway and the destabilization of the mitochondrial membrane potential, right before the formation of apoptotic bodies and the externalization of phosphatidylserine, indicative of apoptosis. The induction of pro-death autophagy in some of these cell types confirms the connection between autophagy and apoptosis (Chapter 1, Figure 5), as well as indicating the versatility of DRE in targeting multiple pathways in a cancer cells selectively.

Compound class	Examples	Comments
Sesquiterpene Lactones	Taraxacosides, taraxinic acid, ainsliosides	Generally believed to have anti-inflammatory and anti-cancer activity. Has antioxidant effects
Triterpenes	Taraxasterol, alpha- and beta- amyrin	Also believed to have anti-inflammatory and anti-tumor activity
Phytosterols	B-sitosterol	Thought to be useful in cancer prevention
Phenylpropanoids	Monocaffeoyltartaric acid, caffeic acid	Has inflammation modulatory effects

Table 1: Classification of Pharmacologically Active Components in DRE

Cancer cells	Non-cancerous cells
Human T cell leukemia (Jurkat) (<i>Ovadge et al., 2011</i>)	Normal human fibroblasts
Chronic myelomonocytic leukemia (<i>Ovadge et al., 2012a</i>)	Peripheral blood mononuclear cells (from apparently healthy volunteers)
Pancreatic cancer (<i>Ovadge et al., 2012b</i>)	Human Umbilical Vein Endothelial Cells
Colon cancer	Normal colon epithelial cells
Melanoma (<i>Chatterjee et al., 2011</i>)	Normal colon fibroblasts
Patient-derived leukemia samples	
Breast cancer (SUM149 & MCF-7)	

Table 2: List of Cancer and Non-cancer Cell Lines Analyzed for the Anti-cancer Efficacy of DRE

Following further studies, we observed that DRE efficiently targets multiple pathways in cancer cells, and is able to distinguish between cancer and non-cancerous cells in this ability. We observed this differential targeting in colorectal cancer cells, compared to non-cancerous colon mucosal epithelial cells, by gene expression analysis. These analyses, combined with fractionation and phytochemical analysis, suggest the ability of DRE to target multiple cell death pathways, in a dose and time dependent manner, due to the presence of multiple components within the extract (discussed in Chapter 5). The identification of three triterpenoids in DRE, α -amyrin, β -amyrin and lupeol, aid in further understanding of the mechanism of DRE in cancer cells.

Previous studies have attributed the anticancer activity to some of these triterpenoids, although the main triterpenoid with the anti-cancer effect, taraxasterol (Takasaki et al., 1999), has not yet been identified in our extract of dandelion root. On their own, it was observed that α -amyrin, β -amyrin and lupeol had a minimal cytotoxic effect on colon cancer cell proliferation, at the doses used. However, a study by Murtaza and colleagues provided evidence that lupeol, at 40 μ M, induced apoptosis in pancreatic cancer cells, by inhibiting the expression of cFLIP, thereby activating the extrinsic pathway of apoptosis. This was a confirmation of another study by Hata and colleagues, showing the efficacy of lupeol as an anti-cancer agent by activating extrinsic apoptosis (Hata et al., 2000; Murtaza et al., 2009). Furthermore, sesquiterpene lactones have also been implicated in the activation of the extrinsic pathway of apoptosis (Zhang et al., 2005; Ghantous et al., 2010). These reports supports our recent findings that

dandelion root extract, even at low doses, efficiently and rapidly activates caspase-8, which is essential for the induction of apoptosis in leukemia cells (Ovadje et al., 2012a), while in colon cancer cells, this activation was not required for apoptosis induction, as inhibition of caspase-8 activation did not deter the induction of PCD by DRE treatment (Chapter 5, Figure 6C). This activation of extrinsic pathway of apoptosis also corresponded to the inhibition of cFLIP following DRE treatment in a dose and time dependent manner. The drawback of the anti-cancer effect of the purified compounds is the requirement of significantly high concentrations, which may not be achievable physiologically and could be toxic at such levels. In contrast, DRE as a complex mixture of many of these compounds, and some yet unidentified components, where the individual concentrations of these compounds will be very low (potentially sub-micromolar concentrations), show very high efficacy. This indicates that, in DRE, various components might act in synergy, to promote the targeting of multiple pathways of cell death.

All of these findings and previously published data confirm the role of such complex NHPs in combating a complex disease like cancer, with multiple vulnerabilities that can be exploited for the development of a more efficacious form of therapy.

To go one step further to understanding the efficacy, *in-vivo* studies were carried out. Although dandelion extracts have been used for centuries, with no reports of adverse effects, proper scientific validation of the lack of toxicity was required. In this work, we show, through several biochemical and pathological markers, that DRE, when administered orally over a long period, was not toxic to

mice. More importantly, efficacy studies in xenograft models of colon cancer in immunocompromised mice showed that oral administration of DRE halted the growth of colon tumors and improved survival of the mice on this regimen. These results showed that the complex mixture of DRE are absorbed through the gastrointestinal tract (GIT) and are able to get to the tumor site and halt further growth. These findings not only provide further evidence of the efficacy of DRE as an anti-cancer agent, but also proving that this NHP is a safer alternative to current chemotherapy standards.

Evaluation of the anti-cancer efficacy of therapeutics on cancer tissues obtained from cancer patients, in *ex-vivo* model, takes pre-clinical studies one step close to human testing in clinical trials and could be an indicator of patient response during clinical studies. Interestingly, we observed the induction of apoptosis and pro-death autophagy by DRE in several patient derived samples of leukemia, following the caspase-8 activation. Moreover cancer-selective response was further confirmed, as PBMCs from healthy volunteers were not susceptible to the induction of programmed cell death. These are important findings as they confirm the efficacy of DRE in *ex-vivo* models, bringing us one step close to using this complex mixture as a therapeutic anti-cancer agent in actual cancer patients, especially those with haematological cancers. These findings, along with the previous efficacy, safety and mechanistic studies presented in our application to Health Canada, convinced the review panel and dandelion root extract was approved for Phase I clinical trials in hematological cancers.

The thorough scientific validation of the anti-cancer efficacy of dandelion root extract in various cancer cell models, provide the necessary background evidence and guide to further validate and assess other NHPs with potential efficacies.

The efficacy of piperlongumine as a possible anti-cancer agent, even at high doses ($\geq 5 \mu\text{M}$), along with the efficacy studies on other components of long pepper, suggest that this NHP could possess better effectiveness, and less associated toxicity. Part of this work was dedicated to laying the groundwork for studying the anti-cancer efficacy of long pepper extract. In this work, we show that an ethanolic extract of long pepper extract led to a caspase-independent mode of apoptosis in several cancer cells. This induction of apoptosis was associated with the permeabilization of the mitochondrial membrane and the generation of reactive oxygen species (ROS). The mechanism of action of this extract was found to be partially dependent on the oxidative stress, as pre-treatment with N-acetylcysteine led to a partial rescue of the cells that were later treated with long pepper extract. These findings are in agreement with previous reports with piperlongumine, where it was observed that this compound targeted the oxidative stress response mechanisms, led to the production of ROS and induced apoptosis selectively in cancer cells (Raj et al., 2011).

Furthermore, the use of this complex mixture of PLX was required at low doses, compared to the high doses of a single component, providing a significant therapeutic window for its use. Its efficacy in cell culture and the lack of toxicity in animal models, further provides the necessary evidence for its safety and efficacy, as anti-cancer agent. These results are essential for further clinical

development of this NHP. Furthermore, HPLC analysis of the ethanolic extract of long pepper showed a small peak corresponding to the presence of piperlongumine, along with peaks representing other components within long pepper extract. The miniscule amount of piperlongumine in long pepper extract suggests that this compound may not be the most essential component that lends to the anti-cancer activity of this extract.

These findings further provides sufficient evidence that backs up the use of complex mixtures, with multiple pharmacologically active components, that work in synergy to promote efficacy and possibly reduce any toxicity associated with any one component. They further provide the essential scientific validation behind the use of common natural health products to deliver more efficacious modes of treatment, that are safer and mostly inexpensive, compared to currently available forms of treatment.

FUTURE DIRECTIONS & CONCLUSIONS

The results presented in this thesis afford some scientific evidence for the use of NHPs in disease treatment, especially in developing better cancer therapies. However, substantial work is still needed to move these NHPs from where they are right now to where they could be beneficial to patients.

Future work on this project should involve further determination and characterization of the pharmacologically active components within dandelion root and long pepper extracts. 6 of 28 fractions obtained from the secondary phytochemical fractionation of dandelions were found to have significant bioactivity, comparable to whole complex DRE and further fractionation of two of those fractions (with similar profiles) led to the identification of three out of five compounds. Considering these compounds, on their own, were required at high doses and only additive effects were observed when α -amyirin and β -amyirin were used in combination, it is of utmost importance that the remaining active components with DRE be identified and their anti-cancer activity be evaluated, as single agents and in combination. The identification all the bioactive components within DRE will be beneficial, as it can ensure that better extraction processes can be employed to extract yield higher amounts of the pharmacologically active components. HPLC analysis of long pepper, using a standard mix of known piperines and piperine alkaloids, led to the identification some of the common components of this extract. Further phytochemical and fractionation analysis of long pepper will be needed to get all the components.

Determining the mechanism of action(s) of complex NHPs in cancer cells, and the ability of these NHPs in particular to identify specific vulnerabilities in cancer cells, has proven challenging. Due to the presence of multiple pharmacologically active components that could individually target multiple pathways, it becomes a puzzle to identify a single mechanism of action of such a complex mixture. However, as with the expression analysis of genes involved cell death programs, we identified several key genes that were influenced by DRE treatment. These findings have helped narrow down the mechanistic efficacy of this extract. Further investigation into the roles of these genes in promoting the hallmarks of cancer cells, or in the initiation and progression of PCD is required. Understanding the roles of these genes in relation to cancer cell growth and death will be required to better comprehend the differences between cancer cells and non-cancer cells, in an attempt to better develop more efficacious cancer therapies.

During the Phase I clinical trials for the use of dandelion root extract in haematological cancers, blood samples will be collected from patients in the trial, and assessed for the induction of apoptosis and autophagy. Caspase-8 activation will also be used as a marker for apoptosis induction. While histopathological analysis will be carried out by the clinic on patient samples, gene expression profiling should also be carried out on each sample obtained, before and after treatment commences. This will enable identification of patients that will benefit from using DRE, as a treatment option.

HEALTH AGENCIES & REGULATORY BODIES INVOLVED WITH NHPs

The whole purpose for the scientific validation of NHPs against diseases, especially cancer, is to provide awareness for these NHPs and NPs that have been used for centuries in various traditional medicines. Published scientific studies carried out provide of the necessary evidence regarding the efficacy of these NHPs, their indications and contra-indications, as well as information on their safe and effective use. In Canada, Health Canada is the governing agency for the introduction of drugs and NHPs to the public, with a division completely dedicated to NHPs, the Natural Health Product Directorate (NHPD). This division was generated to assist and ensure that Canadians have access to NHPs that are “safe, effective and of high quality, while respecting freedom of choice and philosophical and cultural diversity”.

Regulations for NHPs came into effect in 2004 and take into account the unique nature and characteristics of NHPs. At the end of 2012, the NHPD published information that outline how NHPs are assessed, with a focus on health claims, the use of risk information and the use of NHPs in combination; these include the “Pathway for Licensing NHPs Making Modern Health Claims”, Pathway for Licensing NHPs making Traditional Health Claims” and “Quality of Natural Health Products Guide”, which summaries the requirement for standardization of high quality NHPs. Even after clinical trials and progression to the market, Health Canada continues to collect information on, track and analyze adverse reaction reports for NHPs through the Canada Vigilance Program and other regulatory agencies, like the World Health Organization (WHO), thus allowing constant monitoring of NHPs to ensure continuous safety and efficacy

associated with their use. More information on Health Canada's requirements for NHPs can be found at their website:

<http://www.hc-sc.gc.ca/dhp-mps/prodnatur/index-eng.php>

Dandelion root extract, as a therapeutic anti-cancer agent, is leading the movement of natural health products in Canada and is one of the first NHP approved as a therapeutic complex mixture treatment for Phase I clinical trials in cancer therapy. In fact, the NHPD is now becoming the Therapeutic Health Directorate (TPD) in Health Canada, tasked with the duty of assessing the scientific validation of health claims of NHPs with potential efficacy in the treatment of diseases, especially cancer, that are still lacking effective forms of treatment.

In the United States, the Food and Drug Administration (FDA) is the agency in charge of the regulating the production and provision of NHPs to the public. NHPs are referred to as complementary and alternative medicine (CAM), which are divided into 5 main domains –

- a) Whole medical systems; Ayurveda, homeopathic medicine and traditional Chinese medicine (TCM). This is the most common domain of NHPs/CAMs
- b) Mind-Body Medicine; meditation, prayer and creative therapies
- c) Biologically based practices with herbs, foods, vitamins and dietary supplements
- d) Manipulative and Body-based practices; chiropractic and osteopathic manipulation and massage
- e) Energy medicine; including therapeutic touch.

These domains undergo the same levels of rigorous review, as described in the Health Canada review aspect above. More information of application to the FDA and their requirements can be found at their website: <http://www.fda.gov/regulatoryinformation/guidances/ucm144657.htm>

These regulatory agencies ensure that health claims made by traditional medicine have scientific validations for anecdotal evidence presented for centuries. They ensure proper standardizations involved in the production and usage of NHPs/NPs/CAMs to maximize the benefits of these products and medicines.

CONCLUSIONS

The work presented in this thesis has significantly contributed to the scientific validation of natural health products and their roles in disease prevention and treatment. It has laid the groundwork for the validation of other NHPs that could potentially be used in the development of other cancer treatments, and even other diseases, with no treatment options available. This work also hinges on the platform that the complex mixtures of dandelion root and long pepper extracts is much more beneficial than a single identified component, allowing for the multiple events that are observed in *in-vitro* studies. Collectively, this work emphasizes the importance of natural health products, especially dandelion root and long pepper extracts in the development of safer, more efficacious anti-cancer agents, as preferable alternatives to the currently available forms of therapy.

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APPENDIX A

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I. JOURNAL OF ETHNOPHARMACOLOGY

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APPENDIX B

CHEMICAL REAGENTS	COMPANY NAME	CATALOG NO.
2',7'-dichlorodihydrofluorescein diacetate (H ₂ DCFDA)	Sigma Aldrich	D6883
5,5',6,6'-tetrachloro-1,1',3,3' – tetraethylbenzimidazolyl carbocyanine iodide (JC-1)	Invitrogen	T3168
Amplex Red	Molecular Probes	90101
Annexin-V AlexaFluor 488	Invitrogen	A13201
Anti-β-actin (Ms mAb)	GeneTex	GTX628802
Anti-cFLIP (Ms mAb)	GeneTex	GTX28421
Anti-LC3 (Rb pAb)	Novus Biologicals	NB100-2220
Anti-caspase-8 (Ms mAb)	Santa Cruz Biotechnology	sc-70501
Anti-EndoG (Rb mAb)	Abcam	ab9647
Anti-SDHA (Ms mAb)	Santa Cruz Biotechnology	sc-59687
Anti-AIF (Rb pAb)	Abcam	ab1998
BioRad Protein reagent	BioRad	500-0006
Bovine Serum Albumin	Sigma Aldrich	A7906
Dandelion Roots	Premier Herbal Inc.	Lot#: 318121
DEVD-AFC	Calbiochem	218829
Eosin	Sigma Aldrich	E4382
Etoposide	Sigma Aldrich	E1383
First Strand cDNA synthesis kit	Qiagen, Inc	330401
Gentamicin	Life Technologies	157-0-072
Hematoxylin	Sigma Aldrich	HHS16
Hoechst 33342	Molecular Probes	H1399
Horseradish Peroxidase	Sigma Aldrich	P6782
IETD-AFC	Calbiochem	368059
IETD-fmk	calbiochem	218773

Image-iT Live Caspase-8 kit	Invitrogen	I35105
Leupeptin	Sigma Aldrich	L2023
Long pepper Seeds	Quality Natural Foods Ltd	
MitoTracker	Invitrogen	M7513
Monodansylcadaverine	Sigma Aldrich	30432
N-acetylcysteine (NAC)	Sigma Aldrich	A9165
Normal Goat Serum	Sigma Aldrich	G9023
PARAQUAT	Sigma Aldrich	856177
Pepstatin A	Sigma Aldrich	77170
Phenylmethanesulfonyl Fluoride	Sigma Aldrich	P7626
Poly-L-lysine	Sigma Aldrich	P4707
Propidium Iodide	Sigma Aldrich	P4170
Qiagen RT ² Profiler Pathway kit	Qiagen, Inc	PAHS-212Z
RNeasy Kit	Qiagen, Inc	74106
SYBR Green MasterMix	Qiagen, Inc	330502
Tamoxifen (TAM)	Sigma Aldrich	T9262
Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) Assay Kit	Invitrogen	A23210
TMRM	Life Technologies	T-688
Trypan Blue	Sigma Aldrich	T8154
WST-1	Roche Diagnostics	11644807001
ZVAD-fmk	EMD4Biosciences	219007

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