Cymbopogon citratus and Camellia sinensis extracts selectively induce apoptosis in cancer cells and reduce growth of lymphoma xenografts in vivo

Cory Philion
University of Windsor

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Cymbopogon citratus and Camellia sinensis extracts selectively induce apoptosis in cancer cells and reduce growth of lymphoma xenografts in vivo

By

Cory Philion

A Thesis
Submitted to the Faculty of Graduate Studies
Through the Department of Chemistry and Biochemistry
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Cymbopogon citratus and Camellia sinensis extracts selectively induce apoptosis in cancer cells and reduce growth of lymphoma xenografts in vivo

by

Cory Philion

APPROVED BY:

___________________________________________
A. Swan
Department of Biological Sciences

___________________________________________
P. Vacratsis
Department of Chemistry & Biochemistry

___________________________________________
S. Pandey, Advisor
Department of Chemistry & Biochemistry

April 25, 2018
Declaration of Co-Authorship/Previous Publication

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. John Arnason under the supervision of Dr. Siyaram Pandey. The collaboration with Dr. Arnason is covered in chapters 2 and 3.

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Abstract

Cancer is a progressive disease characterized by a biological disharmony in which damaged or mutated cells are able to survive and proliferate by escaping cell death. Moreover, these cells are reported to have elevated levels of reactive oxygen species (ROS) and are highly dependent on cellular defense mechanisms against oxidative stress. A specific cancer of the lymphatic system, lymphoma, affects our body’s infection-fighting cells while posing a great risk to all age groups. Numerous nutraceuticals and natural polyphenolic compounds have a wide range of abilities to alter cellular redox states with potential implications in various diseases. Furthermore, therapeutic options for cancers are mostly nonselective treatments including genotoxic or tubulin-targeting compounds. Some of the natural extracts, containing multiple bioactive compounds, could target multiple pathways in cancer cells to selectively induce cell death. *Cymbopogon citratus* (lemongrass) and *Camellia sinensis* (white tea) extracts have been shown to have medicinal properties, however, their activity against lymphoma, as well as mechanistic details, have not been fully characterized. Herein, we report potent anti-cancer properties in dose and time-dependent manners of ethanolic lemongrass and hot water white tea extracts in lymphoma and leukemia models. Both extracts were able to effectively induce apoptosis selectively in these human cancer cell types. Interestingly, ethanolic lemongrass extract induces apoptosis primarily by the extrinsic pathway and was found to be dependent on the generation of ROS. Conversely, apoptotic induction by hot water white tea extract was independent of ROS. Furthermore, both of these extracts caused mitochondrial depolarization and decreased rates of oxygen consumption in lymphoma and leukemia cells, leading to cell death. Most importantly, both these extracts were effective in reducing tumor growth in human lymphoma xenograft models when administered orally. Thus, these natural extracts could have potential for being nontoxic alternatives for the treatment of cancer.
This work is dedicated to my mother and grandmother for their loving support in any and all aspects of my life. In addition, I hope for this work to reach and provide some enlightenment to all families who have been affected by the debilitating nature of cancer. There is a cure.
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List of Abbreviations

ROS: Reactive Oxygen Species
ETC: Electron transport chain
CW: Cold water
HW: Hot water
ETH-F: Ethanolic-filtered
ETH-UF: Ethanolic-unfiltered
WST-1: water-soluble tetrazolium-1
AMA: Antimycin A
NAC: N-acetyl cysteine
PCD: Programmed Cell Death
FADD: Fas-associated death domain
dnFADD: double-negative Fas-associated death domain
PCNA: Proliferating cell nuclear antigen
NHP: Natural health product
LG: Lemongrass
WT: White tea
DISC: death-inducing
DMSO: Dimethyl sulfoxide
Chapter 1: Introduction

1.1 Cancer

The term cancer encompasses a broad context used to characterize a spectrum of related diseases that fundamentally share abnormalities surrounding unregulated cell division. In result of this biological disharmony, damaged cells that would typically perish under normal conditions are alternatively able to survive and proliferate. Consequently, these erroneous cells ultimately favour aberrant growth of tissues or neoplasia otherwise known as tumors (National Cancer Institute, 2015). Due to the systemic nature of this disease, cancer creates an imbalance of the body’s homeostasis facilitated by evading regulatory mechanisms as the cancerous cells continue to replicate and acquire more mutations allowing them to proliferate while often bypassing growth suppression (Weinberg, 1996). A particular set of oncogenes are frequently compromised leading to a loss-of-function thus encouraging active growth causing the cancer to thrive. In addition to the evasion of growth suppressors and sustained proliferation, cancer cells can be distinguished from non-cancerous cells by cell death resistance, induced angiogenesis, replicative immortality and achieving metastasis (Figure 1). Appropriately, this group of unique characteristics has been deemed the six hallmarks of cancer (Hanahan & Weinburg, 2000). Expanding from the list of six, additional characteristics have since emerged expanding into the realms of immunity avoidance and deregulation of cellular energetics. The homeostasis of the host is in time breached further leading to inflammation, malnutrition, immunosuppression and eventually death as the cancer metastasizes throughout the body (Hanahan & Weinberg, 2011).
In 2016 an estimated 2 in 5 Canadians developed some form of cancer, while one year later the likelihood increased to 1 in 2 Canadians (Canadian Cancer Society, 2017). The prevalence of this disease is by no means contained to one region and has emerged to be a growing problem worldwide. More concerning is the number of new cancer cases which is expected to climb by roughly 70% over the following 2 decades as the population ages. Many contributing factors exist in support of the increasing number of cancer cases and include behavioural, environmental and dietary practices ranging from but not limited to smoking/alcohol, low vegetable intake, industry pollutants and increased exposure to direct sunlight (Danaei, 2005). Factors such as these positively correlate with the acquisition of mutations present in the onset and progression of most cancers.
1.2 Existing Treatment

Physical removal of cancerous tumors by means of surgery served as an initial means of treatment and remains viable today. Following surgery, radiation treatment became increasingly prevalent and served as a primary method in the induction of cell death amongst some cancer types either independently or in combination with the physical removal of a localized tumor (DeviTa Jr. & Rosenberg, 2012). Improved medical technology and diagnosis techniques have largely contributed to improving patient survival. However, many cancers continue to present challenges (Hanahan & Weinburg, 2000). Recently, research efforts have lead to the development of immunotherapy and antibody treatments capable of treating more challenging cancer types alongside anti-VEGF/angiogenesis agents that limit blood supply to tumors (Roukos, 2009). Today’s conventional chemotherapeutics typically lead to serious side effects due to the nonselective nature of these treatments (systemic), targeting features common to both healthy and cancerous cells. An ideal treatment would possess the capacity to specifically target cancerous tissue by means of unique properties of their respective cellular pathways (Kamb, 2007). For example, Tamoxifen is a well-known chemotherapeutic that acts as a selective estrogen receptor antagonist allowing the induction of cytostatic and pro-apoptotic mechanisms in estrogen receptor-positive breast cancers. Patients undergoing chemotherapeutic regimes experience difficulties coping with the toxicity of their treatments, and cancers may develop resistance to treatment hence limiting therapeutic potential as seen with the use of Tamoxifen (Wood, 2003). The oxidative and mitochondrial vulnerabilities unique to cancer cells, such as their increased dependence on aerobic glycolysis to produce ATP rather than oxidative phosphorylation (Warburg effect), can be exploited (Vander Heiden, 2009). This process is thought to be initiated in part by mutations in mitochondrial DNA that shift oxidative phosphorylation while increasing electron leakage and facilitate the generation of reactive oxygen species (ROS) (Chen, 2007). New approaches with selective therapies that circumvent the side effects of conventional
treatments can be developed, targeting the aforementioned vulnerabilities. Therefore, the difficulty in treating cancer is becoming less challenging, however identifying improvements continue to be a direct focus of the medical community.

1.3 Programmed Cell Death (PCD)

Under typical conditions, the development and maintenance of living organisms depend on a highly regulated physiological process known as programmed cell death. Multicellular organisms are able to maintain homeostasis by riding itself of individual cells that may be damaged or present a hindrance to the overall integrity of the cell population (Elmore, 2007). Programmed cell death (PCD) exists in primarily two distinct categories. Apoptosis (PCD Type 1) is the most common form of programmed cell death whereas PCD Type II, otherwise known as Autophagy (Elmore, 2007), achieves a similar result. An energy-independent cell death mechanism known as Necrosis results in response to external stimulus or injury in which cells advocate an inflammatory response confined to a localized area (Majno & Joris, 1995). Importantly in the case of cancer’s development and progression, cell death processes are manipulated as to avoid cell casualties and encourage proliferation while fostering resistance against anti-cancer drugs (Hanahan & Weinberg, 2011). Coincidently cancers possess a tremendous capacity to either ignore or bypass cell suicide (or apoptosis) inducing signals thus allowing mutated tumorigenic cells to flourish. Therefore, when developing anti-cancer drugs, the primary focus is to exploit characteristics unique to cancer and establish selectivity in hopes of preventing apoptosis activation among normal cell populations. An attractive target often being a focus in cancer therapy is the mitochondria. Conveniently, tumorigenic cells possess not only fewer, but also morphologically different mitochondria which function to generate ATP and fuel a wide array of biochemical processes including cell death pathways (Hail, 2005). Interestingly, many proteins associated with the prevention of inducing apoptosis have been proven to be upregulated in some
cancers (Adams, 1998). An anti-apoptotic subclass of the Bcl-2 family which are typically localized to the outer membrane of mitochondria are found in higher concentrations in malignant environments (Adams, 1998). These differences in mitochondria are significant as it can help create a selective foundation when planning treatments which can impede cancer’s strong capacity of escaping cell death.

1.4 Apoptosis

Unique cellular morphological features can characterize cells that have undergone apoptosis. Such features include, nuclear and cytoplasmic condensation alongside the formation of membrane bound apoptotic bodies (Kerr, 1972). Moreover, phosphatidylserine (PS), which is typically located on the cytoplasmic side of the cell membrane, undergoes externalization, which in turn acts as a signal for the apoptotic cell to enhance elimination by phagocytes (Bratton, 1997). This act of signaling can occur without an inflammatory response and spilling of cellular components into the cell’s cytosol as seen with necrosis (Proskuryakov, 2003). Apoptosis is a process that has previously been noted to occur in three general stages: an initiation phase involving a plethora of potential apoptosis inducing agents, secondly a decision phase in which the cell makes a “choice” to die or not die, and lastly a degradation phase generally involving the activation of caspase and nuclease proteins (Costantini, 2000) which culminates in cell death by activating the apoptotic signaling cascade (Sinha Das, 2013). Caspases belong to a family of cysteine-dependent aspartic proteases that exist inactively as preforms and activated via cleavage at aspartate residues. Once active, caspases are able to find numerous substrates within the cell that once undergo cleavage propel forward apoptotic processes (Fulda, 2006). Other potential mechanisms aside from caspase activation can initiate similar apoptotic events. In the case that apoptosis undergoes improper regulation, a lack of control can lead to array of disease including neurodegenerative diseases such as Alzheimer’s (Elmore, 2007). Oppositely, cells
that fail to receive the apoptotic signal evade cell death and can become tumorigenic as in the case of cancer mentioned prior.

There are two major pathways of apoptosis, termed the intrinsic and extrinsic pathways (Figure 2). A type of intracellular stress usually activates the intrinsic pathway. This causes the mitochondrial membrane pores to open, inducing mitochondrial membrane potential dissipation and the release of apoptogenic factors like endonuclease G, Smac/DIABLO, AIF and Omi/HtrA2, and cytochrome c (Earnshaw, 1999). Cytochrome c release leads to the activation of caspase-9, which in turn activates caspase-3 and yields the degradation of internal cellular structures and apoptosis execution (Figure 2). Observably with the intrinsic pathway of apoptosis, the mitochondrial release of cytochrome c is instigated by pro-apoptotic members of the Bcl-2 family such as Bax, Bak Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk (Ghobrial, 2005). These pro-apoptotic members are involved in regulating mitochondrial membrane potential (MMP). The release of cytochrome c from the mitochondria ultimately induces the recruitment of caspase -3 to the apoptosome, through the formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex (Fulda, 2006). Knowledge of these modes of cell death compel further research in the investigation of potential chemotherapeutics that may act as agents that could target these pathways in the cancer cell. Research in the area of natural health products (NHPs), which is the primary focus of this thesis and explained in more detail throughout the paper.

By contrast, the extrinsic pathway is initiated through the interaction of a death receptor within the tumor necrosis factor (TNF) receptor superfamily which is composed of CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors, which, upon binding, causes receptor trimerization and the subsequent recruitment of the adaptor protein with death domains (e.g. Fas-associated death domain, FADD) and the TNF related associated death domain TRADD, as well as the recruitment of the death domain pro-caspase-8 which together form the death
inducing signaling complex (DISC) (Fulda, 2006). The result of this is the cleavage and activation of caspase-8, which affects the cleavage of other downstream effector caspases which affects the cleavage of other downstream effector caspases such as caspase-3. Caspase-3 initiates further cleavage of substrates that further the progression of apoptosis (Fulda, 2006).

**Figure 2:** The Intrinsic and Extrinsic Pathways of Apoptosis

*(Nimmanapalli & Bhalla, 2003)*
1.5 Reactive Oxygen Species in Cancer

Cancerous cells may use a differential pathway of metabolism; indeed, it has been reported that they generate higher amounts of reactive oxygen species (ROS). As a result of increased levels of ROS, various pro-survival mechanisms are elevated including proliferation and angiogenesis (Storz, 2005). Furthermore, they have upregulated anti-oxidative defense mechanisms, and an increase in the production of ROS can trigger apoptosis (Pignanelli, 2017). Interest in exploiting ROS to target cancer cells selectively/effectively has risen primarily since cancer cells already demonstrate higher levels of oxidative stress. Cancerous mitochondria have been shown to be selectively susceptible to certain compounds, further indicating that there are vulnerabilities unique to cancer cells (Ma, 2017). There are several processes that result in the formation of reactive oxygen species (ROS), as they are natural products of many cellular metabolic reactions. One such reaction is through oxidative phosphorylation during normal cell respiration. During normal cell respiration, where electrons are transferred down the electron transport chain (ETC), an improper reduction reaction of oxygen can occur such that a superoxide radical is formed instead of water. Usually this rare occurrence is corrected by cellular scavenger systems, however, when cell scavenger systems are overwhelmed, ROS levels become increasingly high. This imbalance of ROS and radical scavenging mechanisms is referred to as oxidative stress and can lead to severe damage and, in extreme cases, apoptosis (Schumacker, 2006). Often times, cytotoxic agents like chemotherapeutics, will either activate cell surface death receptors or will directly affect the mitochondria by disturbing its outer membrane integrity, causing an increase in ROS production. This oxidative stress in the mitochondria can cause cytochrome C to lose affinity for the intermembrane space and be released into the cytoplasm through the mitochondrial permeability transition pore (MPTP) (Sinha, 2013). It is speculated that reactive oxygen species (ROS) play a role in the formation of the MPTP as ROS may cause the activation of BIM, which then induces the activation of the pro-apoptotic protein Bax. Subsequently, Bax interacts with the...
mitochondria, causing the permeabilization of the outer mitochondrial matrix (Bustamante, 2005). In several cases, the combination of agents that increase ROS and those that suppress antioxidant defenses has been shown to be an effective treatment of different blood cancers with limited effects on normal lymphocytes (Bahlis, 2002; Kang, 2008; Zhou, 2003). This is of particular interest considering the scope of this thesis deals specifically with cancers of the lymphatic system.

1.6 Lymphoma

Lymphoma is cancer that originates in the lymphatic system and affects infection-fighting cells otherwise known as lymphocytes. The lymphatic system expands throughout the body consisting of glands (lymph nodes) and several organs such as the thymus (Lymphoma Association, 2017). Due to its expansiveness, cancerous lymphocytes can originate almost anywhere and travel to many lengths of the body while collectively hindering healthy tissues and most importantly the immune system. Overall, there are more than 80 unique subtypes of mature lymphoid malignancy while lymphoma can further be divided into two separate categories, Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL) (Ansell, 2015). The more prevalent of the two cancer types is NHL representing 8300 of 9290 total cases of lymphoma in Canada last year (CCS, 2017) Lymphomas, both Hodgkin’s and non-Hodgkin, are most commonly diagnosed in older adolescents and young adults, aged 15 – 29 with 9290 new cases in Canada during 2017 (CCS, 2017). Even more alarming are the percentages surrounding lymphomas in children aged 0-14 which accounted for 11% of new cancer cases following only leukemia and cancers of the central nervous system (CCS, 2017).

Non-Hodgkin lymphoma (NHL), are characterized by the distinct lack of Reed-Sternberg cells and are grouped based on which type of lymphocyte they originated from (Shankland, 2012). Most often, NHL starts from B-lymphocytes and to a lesser extent T-lymphocytes or natural killer cells. NHL will typically develop during B-lymphocyte maturation and activation, occurring in the bone
marrow and germinal center of the lymph nodes respectively. Both of these events involve DNA translocations and other modifications (Shankland, 2012) creating opportunity for DNA damage and mutations which favours cancer formation. NHL can also be caused by viral infections. Some viruses such as the Epstein-Barr virus are able to directly transform lymphocytes and lead to NHL’s like Burkitt’s lymphoma (Engels, 2007). HIV infections have also been shown to lead to NHL. Immunodeficiency caused by HIV creates the conditions for unregulated proliferation of B-lymphocytes, which increases the likelihood of cancer formation. In general, some infections increase the risk of NHL development by inducing chronic immune stimulation. Long-term Hepatitis C infections can cause persistent activation of lymphocytes, leading to the development of NHL (Engels, 2007).

Hodgkin lymphoma (HL) is a B-cell malignant neoplasm that represents 11% of all lymphomas and, in 2015, accounted for 9050 of new diagnosed cases of lymphoma (Siegel, 2015). As mentioned above, the critical difference between HL and NHL is that NHL is characterized by the distinct lack of Reed-Sternberg cells while HL contain these Red-Sternberg cells (Shankland, 2012). To establish a definitive diagnosis of Hodgkin lymphoma, Reed-Sternberg cells (large malignant multinucleated cells) within the biopsy specimen must be identified. Reed-Sternberg cells are commonly seen within a rich cellular environment composed of reactive lymphocytes, eosinophils, and histiocytes (Stein, 2001). Currently, the exact cause for development of HL has not been identified, but many factors have been suggested in scientific literature. Most notable criteria attributing to an increased risk for an individual to develop HL includes viral infection, familial factors, and immunosuppression (Ansell, 2015). The Epstein-Barr virus has been implicated to transform lymphocytes leading to HL development outside of the risk it presents to the causation of NHL. Immunocompromised individuals have been shown to have a higher risk for HL, additionally having a poorer prognosis due to HIV allowing for uncontrolled proliferation of lymphocytes, potentially leading to the development of cancer (Andrieu, 1993).
Although therapeutic advances have come a long way, there is a reoccurring need in understanding lymphoma pathogenesis and expediting new treatment that don’t pose significant risk especially to the younger populations who suffer from this disease.
1.7 Natural Health Products and Active Compounds

There is evidence presented by ancient human lineages that suggest the use of natural plant-based products in the treatment of disease including cancer (Ji, Li, & Zhang, 2009). Though deemed as “scientific quackery” by some medical communities, Natural Health Products (NHPs) that have rich histories in traditional medicine (such as Ayurveda and Chinese medicine) provide a foundation of knowledge and reason for continued research and scientific validation. Two NHPs of interest that were explored further in the context of this thesis have shown particular medical value in research labs across the world and will be introduced in the following paragraphs. The complexity of phytochemical constituents found in the architecture of plants is often hypothesized to play a supporting role in ameliorating some disease conditions and/or symptoms. It must also be noted that these concentrations of bioactive compounds within many types of NHPs may be uniquely equipped to combat cancer and its multifaceted deregulation of numerous genes, which therefore may require a multi-targeted approach to treatment (Prasad, 2011). Phytochemical constituents elicit various physiological and/or pathological effects that could provide advantages in such a complex state of disease. Lastly, another advantage of NHPs from a clinical standpoint is the low risk associated with human use and consumption over many years (Ji, 2009). For these reasons, NHPs continue to be recognized today with respect to significant efficacy and the ongoing search for novel safe chemotherapeutics with selective anti-cancer activity. Aside from the use of plant as their own entities, specific bioactive compounds found within plant material have contributed to the development or derivation of many available pharmaceutical agents including chemotherapy in the treatment of cancer (Foster, 2005) but exist in a wide range from analgesia all the way to medicines surrounding sedation. One of the major drawbacks of natural extract treatments is poor stability and bioavailability. The development of pharmaceutical agents from plant origin proves to be a beneficial way of bridging the gap between traditional and modern medicine. Two NHPs of
particular interest in which are also supported by both traditional and more recent targets of study are listed below.

1.7.1 Lemongrass

*Cymbopogon citratus*, more commonly known as lemongrass (LG), is an economically important perennial plant of the *Poaceae* family found in tropical environments or sub-tropical regions. The water (or aqueous) extract of lemongrass has been documented in traditional medicine showing efficacy in combatting fever, inflammation, digestive disorders and cancer (Thangam, 2014). Additionally, the aqueous extract of lemongrass has been found to be a potent free-radical scavenger (Halabi & Sheikh, 2014). Essential oil from lemongrass has various applications involving aromatherapy and includes uses in insecticides and pharmaceuticals. Published studies in the scientific literature including several review articles present a thorough breakdown of the phytochemistry belonging to stems, leaves, and even roots of the *C. Citratus* plant. Many of the biological effects ascribed to lemongrass have been attributed to primary constituents such as citral, 3,7-dimethyl-2,6-octadienal, displaying anti-proliferative and anti-parasitic activity (Christopher, 2014). Most recently, citral containing lemongrass essential oils was shown to suppress the proliferation/survival of small-cell lung cancer cells alone or in combination with chemotherapeutic agents (Takayuki, 2018). Interestingly, the results of our study evaluating anti-cancer activity of three compounds identified at higher concentrations in lemongrass (elemicin, lonicerin, and methylisoeugenol) showed very poor efficacy in reducing viability in lymphoma cells. Reasons supporting why this hypothesis may have failed are addressed in the discussion alongside possible solutions.
1.7.2 White Tea

White tea (WT) is derived from the immature leaves of the *Camellia sinensis* belonging to the tea plant family *Theaceae*. Also found in this family are variations of green, black, and oolong tea - all of which display a wide array of health benefits based on different features in composition (Sereshti, 2013). Furthermore, this assortment of health promoting benefits have been observed to be in result of biological activities linked to an unique blend of polyphenols, volatile oils, vitamins and numerous other compounds found in prepared leaves of *C. sinesis* (Sereshti, 2013). Also important to note are the different levels of processing or harvested leaf development and how this correlates to distinctions in bioactivity. White tea being the least processed preparation is known to contain a distinct group of polyphenols specifically categorized as epicatechins, which are thought to directly support the positive assets attributed to white tea. Four major epicatechins include epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (Ravindranath, 2006) which are thought to be bioactive catechins able to interact with and quench ROS *in vitro*. As ROS have been linked to several progressive disease states, it is thought that the epicatechins in white tea can be used as a possible treatment. Currently, the anti-cancer and free radical scavenging properties of these compounds are being evaluated and addressed to an extent in body of this study.

1.8 Objectives

Anecdotal evidence alongside existing scientifically validated studies, have shown that both lemongrass and white tea possess anti-cancer properties while exhibiting low or no toxicity at all. This thesis questions whether these same extracts possess significant anti-cancer properties against lymphoma cells lines primarily of the Non-Hodgkin’s lymphoma subtype. In addition, we set out to expand on further validating the mechanisms of action in which these extracts may or may not exploit cancer cells in hopes of hindering their ability to escape cell death and thus proliferate. We conducted
studies of both extracts as individual therapies alongside the exploration of possible synergistic activities in combination with each other. Our hypothesis is that the natural health products, LG & WT, contain various bioactive compounds in which can effectively target and exploit lymphoma cell vulnerabilities therefore acting as a selective alternative to current chemotherapies while exhibiting no toxicity. In support of this hypothesis, there are three main objectives associated with this work:

1. Assessing the cytotoxicity of LG and WT extracts (both aqueous and ethanolic) on lymphoma cell lines ex vivo as individual therapies and in combination.

2. Investigating the mechanism of action and selectivity of LG & WT in lymphoma cell lines in contrast to normal healthy cells ex vivo.

3. Evaluating the efficacy and toxicity of these extracts in a representative animal model (Lymphoma Xenograft Model in Immunocompromised Mice)
2.0 Chapter 2: Materials & Methods

2.1 Lemongrass and White Tea Extraction

The water and 100% ethanolic extracts of each plant were made from the stems of lemongrass and white tea leaves. The two plant products were purchased in an already pre-ground form from Premier Herbal (Toronto, Ontario, Canada) which is a wholesale supplier for various natural materials. The provided lot numbers for the lemongrass (grown in Guatemala) and white tea (grown in China) are 315872 and WT1406KIT, respectively.

For the water extracts, before extracting, water was boiled (100°C) and then allowed to cool (until ~60°C). Each plant was then extracted with a 1:10 ratio of plant material (in grams) to distilled water (in mL) for three hours. The plant material was removed using cheesecloth, and the filtrate was centrifuged. The filtrate was then gravity-filtered using a P8 filter followed by vacuum filtration using a 0.45 μm filter. Afterwards, the filtrate was then placed in a fridge at a temperature of -80°C overnight and freeze-dried. The extracted residue left behind was weighed and reconstituted to make 100 mg/mL stock solutions.

For the ethanolic extracts, each plant was extracted with a 1:10 ratio of plant material (in grams) to anhydrous ethanol (in mL). The solution was thoroughly mixed and allowed to extract for approximately 24 hours. Following extraction, the mixture was vacuum-filtered using a 0.2 μm filter, and the filtrate underwent rotary evaporation at a temperature of approximately 38-40°C. Now, with the ethanol evaporated, the weight of the dry resin was used to make a stock solution dissolved in anhydrous ethanol. The final concentration of the extract was 100 mg/mL.
2.2 Cell Culture

The MV-4-11 Chronic myelomonocytic leukemia cell line (ATCC, Cat. No. CRL-9591, Manassas, VA, USA) was cultured with Iscove's Modified Dulbecco's Medium (ATCC, Cat. No. 30-2005, Manassas, VA, USA) supplemented with 10% (v/v) FBS standard (Thermo Scientific, Waltham, MA, USA) and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada).

The U-937 non-Hodgkin’s histiocytic lymphoma cell line (ATCC, Cat. No. CRL-1593.2, Manassas, VA, USA) was cultured with Iscove's Modified Dulbecco's Medium (ATCC, Cat. No. 30-2005, Manassas, VA, USA) supplemented with 10% (v/v) FBS standard (Thermo Scientific, Waltham, MA, USA) and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada).

The L-540 Hodgkin lymphoma (Leibniz-Institut DSMZ, Cat. No. ACC 72, Braunschweig, Germany), was cultured with RPMI-1640 medium (Sigma-Aldrich Canada, Mississauga, ON, Canada) supplemented with 20% (v/v) fetal bovine serum (FBS) standard (Thermo Scientific, Waltham, MA, USA) and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada).

The HD-MYZ Hodgkin lymphoma (Leibniz-Institut DSMZ, Cat. No. ACC 346, Braunschweig, Germany), was cultured with RPMI-1640 medium (Sigma-Aldrich Canada, Mississauga, ON, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS) standard (Thermo Scientific, Waltham, MA, USA) and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada).
The KM-H2 Hodgkin lymphoma (Leibniz-Institut DSMZ, Cat. No. ACC 8, Braunschweig, Germany), was cultured with RPMI-1640 medium (Sigma-Aldrich Canada, Mississauga, ON, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS) standard (Thermo Scientific, Waltham, MA, USA) and
2.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

All experiments involving human subjects (healthy volunteer donating blood) were done with prior approval of Research Ethics Board of the University of Windsor (protocol # REB #04-147), with informed consent obtained from the subject. Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy volunteer in BD Vacutainer CPT Tubes with Sodium Heparin N (Becton, Dickinson and Company, Cat. No. 362753, Franklin Lakes, NJ, USA) at room temperature. Tubes were inverted 5 times and centrifuged for 30 minutes at room temperature at 1500-1800 x g. The PBMC layer under the plasma layer in each tube was collected, pooled together, resuspended in 50 mL of PBS, and centrifuged at room temperature at 300 x g for 15 minutes. The supernatant was aspirated without disturbing the pellet and PBMCs were suspended and cultured in RPMI-1640 medium (Sigma-Aldrich Canada, Mississauga, ON, Canada), supplemented with 10% (v/v) FBS standard (Thermo Scientific, Waltham, MA, USA) and 10 mg/mL gentamicin (Gibco BRL, VWR, Mississauga, ON, Canada) at 37 °C and at 5% CO₂. PBMCs from healthy volunteers 1 (PBMCs V1) were taken from a healthy 28-year-old male.

2.4 WST-1 Assay for Cell Viability

The WST-1 based colorimetric assay (Roche Applied Science, Indianapolis, IN, USA) was performed to quantify cell viability as a function of cellular metabolism. 96-well clear bottom tissue culture plates were seeded with cells. The cells were treated at the indicated concentrations and time points. The treated cells were incubated with WST-1 reagent for 4 hours at 37°C with 5% CO₂. In actively metabolizing cells, the WST-1 reagent is cleaved to formazan by cellular enzymes. The presence of formazan was quantified via absorbance readings at 450 nm on a Wallac Victor³ 1420
Multilabel Counter (PerkinElmer, Woodbridge, ON, Canada). Cellular viability through measured absorbance readings expressed as percentages of the solvent control group.

2.5 Annexin V Binding Assay and Propidium Iodide (PI) for Analysis of Cell Death

Annexin V binding assay and propidium iodide staining were performed to respectively monitor early apoptosis and cell permeabilization, a marker of necrotic or late apoptotic cell death. Cells were washed with phosphate buffer saline (PBS) and suspended in Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) with green fluorescent Annexin V AlexaFluor-488 (1:20) (Life Technologies Inc, Cat. No. A13201, Burlington, ON, Canada) and 0.01 mg/mL of red fluorescent PI (Life Technologies Inc, Cat. No. P3566, Burlington, ON, Canada) for 15 minutes at 37 °C protected from light. Percentage of early (green), late apoptotic cells (green and red), and necrotic cells (red) were quantified with a Tali Image-Based Cytometer (Life Technologies Inc., Cat. No. T10796, Burlington, ON, Canada). Cells from at least 18 random fields were analyzed using both the green (ex. 458 nm; em. 525/20 nm) and red (ex. 530 nm; em. 585 nm) channels. Fluorescent micrographs were taken at 400x magnification using LAS AF6000 software with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany). Cells monitored with microscopy were counterstained with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) with a final concentration of 10 μM during the 15-minute incubation.

2.6 Monitoring Mitochondria Membrane Potential

Tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR, Mississauga, ON, Canada) was used for detecting mitochondrial membrane potential (MMP), an indicator of healthy intact mitochondria. Cells monitored with microscopy were counterstained with Hoechst 33342 as previously described³. Images were taken with a Leica DMI6000 fluorescent microscope (Wetlar, Germany) at 400x magnification using LAS AF6000 software. JC-1 was also utilized for
quantification of mitochondria membrane potential. Following treatment, JC-1 (Thermo Scientific, Cat. No. T3168, Waltham, MA, USA) at a concentration of 2 micromolar was added and allowed to incubate for 30 minutes. Following incubation, cells were washed twice in 1xPBS then analyzed using the Tali Image-Based Cytometer (Life Technologies Inc., Cat. No. T10796, Burlington, ON, Canada). Cells from at least 18 random fields were analyzed using the red (ex. 530 nm; em. 585 nm) channel.

2.7 Oxygen Consumption Quantitation

Mitochondrial function was evaluated with the MitoXpress® Xtra - Oxygen Consumption Assay [HS Method] (Luxcel Biosciences Ltd., Cat. No. MX-200, Cork, Ireland). 1 000 000 cells/well were seeded in a 96-well black clear bottom tissue culture plate and incubated for an hour at 37°C and 5% CO₂. On a heat pack, 10 μL of MitoXpress® reagent was added to each well excluding the blanks, cells were treated, the plate was shaken with a plate shaker, and 2 drops of pre-warmed high sensitivity mineral oil was added to each well to seal off the air supply. Bottom read fluorescence measurements were taken at Ex. 380 nm and Em. 650, every 2 minutes for 2 hours at 37 °C using a SpectraMax Gemini XS multi-well plate reader (Molecular Devices, Sunnyvale, CA, USA). Increases in fluorescence are indicative of oxygen consumption. Oxygen consumption rates were determined by calculating the slope of the linear regions of the oxygen consumption curves using GraphPad Prism 6 software.

2.8 Quantitation of Reactive Oxygen Species (ROS)

Whole cell ROS generation was monitored with the small molecule 2’, 7’-dicholorofluorescin diacetate (H₂DCFDA). H₂DCFDA enters the cell and is deacetylated by esterases and oxidized by ROS to the highly fluorescent 2’, 7’-dicholoroflorescein (DCF) (excitation 495 nm; emission 529 nm).
Cells were pretreated with 20 μM H$_2$DCFDA (Sigma-Aldrich Canada, Cat. No. D6883, Mississauga, ON, Canada) for 30 minutes at 37°C protected from light at 5% CO$_2$. Cells were treated for the indicated durations, centrifuged at 600 x g for 5 minutes and suspended in PBS. Percentage of DCF positive cells was quantified using the Tali Image-Based Cytometer (Life Technologies Inc., Cat. No. T10796, Burlington, ON, Canada) using 12 random fields per group with the green channel (excitation 458 nm; emission 525/20 nm).

2.9 Xenograft Model (In Vivo) and Extract Administration

All experiments involving animals (mice) and animal protocols were approved by the University of Windsor Animal Care Committee (AUPP # 14–15) in accordance with the Canadian Animal Care Committee. Immunocompromised CD-1 nu/nu male mice (Charles River Laboratories, Cat. No. 086, Sherbrooke, QC, Canada) were housed in laboratory conditions of a 12-hour light/dark cycle. Mice were injected using 23-gauge needles with 1 mL syringes with equal amounts of cell solution (containing 2x10$^6$ U-937 cells) and Corning Matrigel Basement Membrane Matrix (VWR International, Cat. No. 47743-715, Mississauga, ON, Canada) to a final volume of 200 microliters. Once tumors were established, the animals were randomized into three groups of 4 mice each including a control, treatment with LG, and treatment with WT. The control group was housed with a bottle of regular drinking water while the treatment groups were given water which contained the respective treatments. The water was changed regularly twice per week and the amount drank was measured. Each mice in the treatment groups was calculated to have consumed, on average, 80mg/kg/day of the respective treatment. The volumes of the tumours were measured according to their length, width, and height twice per week and calculated using the ellipsoid formula $\pi/6 \times$ length $\times$ width $\times$ height. Changes in body mass were measured with a scale to assess for potential weight loss and determine whether treatments were well tolerated.
2.10 Cell Lysis and Western Blot

Preparation of cell lysates and Western blot analysis were performed as previously described. In brief, protein samples were run using SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for one hour with either 5% skim milk or bovine serum albumin (BSA) at room temperature. Following blocking, the membranes were incubated with one of the following primary antibodies overnight at 4 °C: anti-caspase-8 antibody (1:1000) raised in mouse (Cell Signalling, Cat. No. 9746 S, Danvers, MA, USA), anti-caspase-9 antibody (1:1000) raised in rabbit (Cell Signalling, Cat. No. 9502, Danvers, MA, USA), anti-caspase-3 antibody (1:2000) (Novus Biologicals, Cat. No. NB100-56709V2, Littleton, CO, USA), anti-β-actin antibody (1:1000) (Santa Cruz Biotechnology, Inc., Cat. No. sc-81178, Paso Robles, CA, USA), anti-p-Histone H2A.X (Ser 139) (γ-H2AX) antibody (Santa Cruz Biotechnology, Inc., Cat. No. sc-101696, Paso Robles, CA, USA), and anti-vinculin antibody (1:2000) raised in rabbit (Cell Signalling, Cat. No. 13901, Danvers, MA, USA).

2.11 Cyrosectioning and Immunohistochemistry

Following the in vivo study, U937 tumors were harvested and placed a 10% formaldehyde solution. Three days prior to sectioning, they were transferred to 30% sucrose (w/v). Tumors were then sectioned at 20µm and subjected to immunohistochemistry using either gamma H2AX (p Ser139) antibody (1:500) raised in rabbit (Novus Biologicals, Cat. No. NB100-384, Littleton, CO, USA), active/cleaved Caspase 8 antibody (1:500) raised in rabbit (Novus Biologicals, Cat. No. NB100-56116, Littleton, CO, USA), or anti-PCNA [pc10] antibody (1:400) raised in mouse (Abcam, Cat. No. ab29, Cambridge, MA, USA). Prior to overnight incubation with these primary antibodies at 4°C, the sections were incubated in 0.33% H₂O₂ for 2 minutes in order to block endogenous peroxidases, DAKO universal blocking solution (purchased from Diagnostics Canada Inc., Mississauga) for 30
minutes, and in normal goat serum (for primary antibodies raised in rabbit) or normal horse serum (for primary antibodies raised in mouse) for 30 minutes at room temperature (prepared as per instructions on anti-rabbit Vecstatin ABC Kit [Cat. No. PK-6101] or anti-mouse Vecstatin ABC Kit [Cat. No. PK-6102], Vector Laboratories) in order to block the binding of non-specific goat or horse IgG. The sections were washed in Tris buffered saline (TBS) for 5 minutes in between each blocking step to remove any excess blocking reagents. Following the overnight incubation, the sections were washed in TBS twice and were incubated in biotinylated anti-rabbit IgG (Vector Laboratories, anti-rabbit Vecstatin ABC Kit [Cat. No. PK-6101]) or biotinylated anti-mouse IgG (Vector Laboratories, anti-rabbit Vecstatin ABC Kit [Cat. No. PK-6102]) for 75 minutes at room temperature. The sections were then washed twice with TBS for 5 minutes and were incubated in avidin biotin complex (ABC reagent) for 45 minutes at room temperature. Following two additional TBS washes, the sections were subjected to the peroxidase substrate 3, 3′ diaminobenzidine (DAB) prepared as per the instructions in the kit (Vector Laboratories, DAB Peroxidase (HRP) Substrate Kit [Cat. No. SK-4100]). Sections were then counterstained with Hematoxylin Solution, Gill No. 1 (Sigma-Aldrich Canada, Cat. No. GHS116, Mississauga, ON, Canada) and washed twice in tap water for 5 minutes. The sections were then washed in Scott’s Bluing solution 5 minutes, followed by a 5-minute wash in TBS. The sections were then dehydrated in anhydrous ethanol and xylene and were cover-slipped using Permount for visualization under a microscope.
2.12 Statistics

Statistics from this study were performed with GraphPad Prism 6 statistical software. A p-value below 0.05 was considered significant. For the experiments with single variable measurements, which include quantification of MMP, and whole cell ROS, a One-Way ANOVA (nonparametric) was conducted and each sample's mean was compared to the mean of the negative control (DMSO vehicle) unless otherwise specified. For experiments that contained multi-variables (e.g. multiple group comparisons), such as the quantification of live and dead cells, Two-Way ANOVA (nonparametric) was used and each sample's mean was compared to the mean of the negative control (DMSO vehicle) unless otherwise specified.

2.13 Phytochemical Analysis

The lemongrass extract LG072416 (10 mg) was dissolved in 1 mL dimethyl sulfoxide (DMSO), and diluted with 50% methanol+50% water+0.1% formic acid to yield a final concentration of 10 \( \mu \text{mL} \). The diluted extract was sonicated for 5 min and filtered through 0.2\( \mu \) syringe filter.

2.14 Analytical Method

Analysis was performed with an ultraperformance Liquid chromatograph connected with quadrupole time of flight mass spectrometer UPLC-QTOF (Waters Acquity Xevo G2 QTOF, Waters Corp. Separation was achieved with a reverse phase column: Guard Filter, PN 289002378 + Acquity BEH C18 1.7um 2.1 x 50mm. The mobile phase solvents were Fisher Optima LC-MS grade (Fisher Scientific, Ottawa ON), flow rate: 0.8 mL/min. The column temperature was 50 °C, and autosampler: 4 °C. Sample injection (PLUNO was 5uL, with a post injection needle wash: 200 uL (50%
acetonitrile+50% water) + weak wash 600 uL (10% acetonitrile+90% water). The mobile phase: A1: water+0.1% formic acid, B1: Acetonitrile+0.1% formic acid.

The QTOF was used with the following conditions: sourcee 400 °C, cone gas (N2) flow 50 L/h, desolvation gas (N2) 1200 L/h, Cone voltage 35V, Scan time 0.08 sec. Calibration, 100-1500 Da. MassLynx software, MSe ESI+ mode. Enkephalin m/z556.2615 was used as a reference (ESI pos), source temperature 120 °C. Scans were 100-1500 Da, F1: CE, 6V, F2: CER 20-50V (both negative and positive ionization modes). A 28 min step gradient from A1:B1 = 99%:1% to 0%:100% was used. Compounds were identified definitively based on in house spectral library or tentatively based on monoisotopic mass observed in electrospray ionization on a QTOF within mass accuracy of 5 PPM.
3.0 Chapter 3: Results

3.1 Lemongrass and White Tea Extracts Reduce Viability of Lymphoma Cells

Cold water (CW), hot water (HW), ethanolic-filtered (ETH-F), and ethanolic-unfiltered (ETH-UF) extracts of lemongrass and white tea were prepared as described in the materials and methods. To evaluate the cytotoxicity of lemongrass and white tea extracts on various Hodgkin and non-Hodgkin’s lymphoma cell lines, the water-soluble tetrazolium-1 (WST-1) assay was utilized. Both lemongrass and white tea extracts reduced the viability of all the tested lymphoma cell lines in a dose-dependent manner (Fig. 1). In particular, the ETH-UF lemongrass and HW white tea extracts were the most effective. EC50 values for the different extracts and cell lines are available in Table 1. Interestingly, the non-Hodgkin’s lymphoma cell line, U-937, was sensitive to ETH-UF lemongrass extract at concentrations below 0.05 mg/mL whereas the Hodgkin’s lymphoma cell lines, KMH2, HDMYZ, and L540, were sensitive to ETH-UF lemongrass extract at concentrations below 0.1 mg/mL. Both the non-Hodgkin’s and Hodgkin’s lymphoma cell lines displayed similar sensitivity to HW and CW white tea extracts. Thus, ETH-UF lemongrass extract and HW white tea extract were the main focus for further white tea extract were the main focus for further evaluation and characterization for this study.
Table 1: EC$_{50}$ values as calculated by WST-1 cell proliferation assay

| Cell Line | Lemongrass | | | | | | | | White Tea | | | | | |
|-----------|------------|---------|--------|--------|--------|---------|--------|--------|---------|--------|--------|--------|
|           | CW         | HW      | EtOH UF | EtOH F | CW     | HW      | EtOH UF | EtOH F |
| U-937     | 0.445231   | 0.390165| 0.026015| 0.188068| 0.092383| 0.101777| 0.30335 | 0.638006|
|           | ±          | ±       | ±       | ±      | ±      | ±       | ±      | ±      |
| KMH2      | 0.143711   | 0.211937| 0.063408| 0.298615| 0.056853| 0.066717| 0.151296| 0.280231|
|           | ±          | ±       | ±       | ±      | ±      | ±       | ±      | ±      |
| L540      | 0.212369   | 0.176398| 0.072506| 0.31694 | 0.054756| 0.059655| 0.213992| 0.397054|
|           | ±          | ±       | ±       | ±      | ±      | ±       | ±      | ±      |
| HDMYZ     | 0.628508   | 0.500629| 0.071518| 0.383979| 0.133725| 0.197496| 0.236157| 0.695556|
|           | ±          | ±       | ±       | ±      | ±      | ±       | ±      | ±      |

Four different lymphoma cell lines were treated for 48 hours with different extracts at several doses as described in the materials and methods. Cell viability was measured using the WST-1 assay as described in the legend of Figure 1 and EC$_{50}$ values were calculated using GraphPrism6 and Microsoft Excel.
Figure 3: Lemongrass and white tea extracts display broad efficacy in reducing cell viability in 4 lymphoma cell lines. Cells were treated with various doses of lemongrass and white tea extracts prepared with cold water, hot water, unfiltered ethanol, and filtered ethanol for 48 hours. Following treatment, WST-1 cell proliferation assay was used and the absorbance at 450 nm was measured. Y-axis is the percent mean ± SD from three independent experiments to the control and the x-axis is the concentrations used in milligram/ milliliter. Results graphed using the log(inhibitor) vs. response - Variable slope (four parameters) curve using GraphPrism6
3.2 Induction of Apoptosis in Hodgkin and Non-Hodgkin’s Lymphoma Cells

To assess the ability of lemongrass and white tea extracts to induce apoptosis in lymphoma cells, cells were stained with Annexin V and propidium iodide, general markers of apoptosis, and subjected to image-based cytometry and microscopy following treatment with the extracts. Following treatment for 48 hours, both lemongrass and white tea extracts were effective in inducing apoptosis in lymphoma cells (Fig. 4a). Notably, lemongrass extract was able to cause significant induction of apoptosis at a level (0.05 mg/mL) comparable to the standard chemotherapeutic VP16 in non-Hodgkin’s U-937 and Hodgkin’s KMH2 lymphoma cell lines. Fluorescent microscopy following treatment with lemongrass and white tea extracts and VP16 for 24 hours also revealed these cell death markers, along with apoptotic morphology in U-937 lymphoma cells, including cell shrinkage, membrane blebbing, and nuclear condensation (Fig. 4b). Most importantly, there was minimal to no observable apoptotic induction with normal human fibroblast cells at doses of lemongrass and white tea extracts that were highly cytotoxic to lymphoma cells after 48 hours compared to the positive control staurosporin (STS) (Fig. 5a). Furthermore, in order to have a similar counterpart to lymphoma cells, we used peripheral nucleated blood cells (PNBCs) obtained from healthy individuals as a noncancerous control. These results further indicated that lemongrass extract showed minimal apoptotic effect on these cells, however white tea extract did induce apoptosis at higher doses (Fig. 5b). Therefore, lemongrass showed excellent selectivity to cancer cells whereas white tea extract was toxic to PNBCs at higher doses.
Figure 4: Lemongrass and white tea extracts induce apoptosis in several lymphoma cell lines; following treatment with specified doses, cells were stained for annexin V and PI. (A) Lymphoma cell lines tested at 48 hours. Results were obtained using image-based cytometry with the Y-axis representative of percent of cells positive for Annexin V (green), PI (red), Annexin V and PI (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. (B) U-937 micrographs at 24 hours. Top: Bright field and fluorescent merged images at 400x magnification. Bottom: Fluorescent images stained with Annexin V (green), PI (red), and Hoechst (blue) at 400× magnification. Scale bar is 50 microns. Images are representative of three independent experiments. Values are expressed as a mean ± SD from three independent experiments. Statistical calculations were performed using Two-Way ANOVA multiple comparison. *p < 0.05 vs. Control, **p < 0.01 vs. Control, ****p < 0.0001 vs. Control.
**Figure 5:** Lemongrass and White Tea Extracts Do Not Induce Apoptosis in Non-Cancerous Cells – (A) Normal human skin fibroblasts and (B) peripheral blood nuclear cells (from healthy individuals) were tested at 48 hours. Following treatment with specified doses, cells were stained for Annexin V and PI. Results were obtained using image-based cytometry with the Y-axis representative of percent of cells positive for Annexin V (green), PI (red), Annexin V and PI (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. Statistical calculations were performed using Two-Way ANOVA multiple comparison. ****p < 0.0001 vs.Control.
3.3 Mitochondrial Depolarization and Reduced Oxygen Consumption

Mitochondria play a key role in apoptosis, which can be triggered by mitochondrial dysfunction. This can lead to the permeabilization of the mitochondrial membrane, the release of apoptogenic factors, and the induction of apoptosis\(^4\). To monitor mitochondrial stability and depolarization, the fluorescent JC-1 assay was used. At time points as early as six and 12 hours, lemongrass and white extracts were able to decrease the percentage of cells positive for the JC-1 dye, and increasingly drastic reductions were observed at the 24 and 48 hour time-point (Fig. 6a). This result indicates the collapse of mitochondrial potential in cells treated with lemongrass and white tea extracts.

To further investigate mitochondrial function directly, following treatment with lemongrass and white tea extracts, oxygen consumption was evaluated as described in materials and methods. Lemongrass and white tea extracts significantly decreased the rate of oxygen consumption in non-Hodgkin’s U-937 lymphoma cells (Fig. 6b). Antimycin A (AMA) inhibits complex III of the ETC, and so it was used as a positive control in evaluating the disruption of oxygen consumption. This finding indicates that lemongrass and white tea extracts effectively decrease oxygen consumption and, therefore, mitochondrial function.
Figure 6: Lemongrass and white tea extracts cause mitochondrial depolarization and decreased rates of oxygen consumption in lymphoma cells. (A) Lymphoma cells were plated and allowed to incubate overnight. Following overnight incubation, cells were treated for 6, 12, 24, and 48 hours. To monitor mitochondria potential cells were incubated with JC-1 for 30 minutes before analysis. Results were obtained using image-based cytometry with the Y-axis representative of percent of cells positive for JC-1 expressed as a mean ± SD from three independent experiments. (B) The MitoXpress® Xtra - Oxygen Consumption Assay was used to monitor oxygen consumption via fluorescence generation as an indicator of mitochondrial function. U-937 lymphoma cells were treated with white tea extract (WT), lemongrass extract (LG), and antimycin A (AMA), and the fluorescent MitoXpress® reagent was added monitored at Ex. 380 nm and Em. 650, every 2 minutes for 2 hours at 37°C. Oxygen consumption rates were calculated by measuring the slopes of the linear regions of the oxygen consumption curves. Values are expressed as mean ± SD from at least 3 independent experiments. *p < 0.05 vs. Control, **p < 0.01 vs. Control, ***p < 0.001 vs. Control, ****p < 0.0001 vs. Cont
3.4 Oxidative Stress Dependence of Lemongrass Extract to Induce Apoptosis

Further investigating the mechanism of induction of apoptosis by these extracts, we observed that lemongrass extract induced production of ROS in several blood cancer cell lines, such as non-Hodgkin’s lymphoma U-937 cells and E6-1 Jurkat cells, as indicated by an increase in percent of cells positive for DCF, similar to the positive control piperlongumine (PL) (Fig. 7a). In contrast, white tea extract showed minimal to no increase of ROS production in these cell lines (Fig. 7a). We wanted to investigate if this increase in oxidative stress is essential in the downstream effects of lemongrass extract for the induction of apoptosis. When pre-treated with the potent antioxidant N-acetyl cysteine (NAC), there was a near complete inhibition of markers for apoptosis and mitochondrial destabilization across numerous cancerous cell lines following treatment with lemongrass extract, similar to the positive control paraquat (PQ). The markers for apoptosis were however unaffected following treatment with white tea extract (Fig. 7b). Further experimentation with lemongrass extract investigated the role of oxidative stress in mitochondrial destabilization after treatment of three blood cancer cell lines (MV-4-11, E6-1, U-937) with lemongrass extracts and NAC, as indicated by a decrease in percent cells positive for JC-1 relative to the control (Fig. 7c). When pre-treated with NAC, leukemia cells showed stabilized mitochondrial membrane potential in a qualitative microscopy study as indicated by the presence of red JC-1 dye around the blue Hoechst-stained DNA (Fig. 7d). Furthermore, these results are complemented by the reduction in cleavage of caspase-8 and -9 by NAC co-treatment (Fig. 7e). These results indicate that lemongrass and white extracts induce cell death by different mechanistic pathways; lemongrass extract appears to be dependent on oxidative stress for the induction of apoptosis whereas white tea extract is independent of oxidative stress.
Figure 7: Lemongrass extract is dependent on the production of oxidative stress to induce apoptosis. (A) MV-4-11, E6-1, and U-937 cells were treated with piperlongumine (PL), LG, or WT with or without the antioxidant NAC for 48 hours. Following treatment, cells were stained for Annexin V and PI. Results were obtained using image-based cytometry with the Y-axis representative of percent of cells positive for Annexin V (green), PI (red), Annexin V and PI (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. (B) U-937 and E6-1 cells were treated with H2DCFDA following treatments with paraquat (PQ), LG, or WT with or without the antioxidant NAC for 3 hours. Results were obtained using the image-based cytometry with the Y-axis representative of percent of cells positive for DCF. Values are expressed as a mean ± SD from three independent experiments. (C) MV-4-11, E6-1, and U-937 cells were plated and allowed to incubate overnight. Following overnight incubation, cells were treated for 48 hours with or without NAC. To monitor mitochondria potential cells were incubated with JC-1 for 30 minutes before analysis. Results were obtained using image-based cytometry with the Y-axis representative of percent of cells positive for JC-1 expressed as a mean ± SD from three independent experiments. (D) U-937 micrographs at 48 hours. Top: Fluorescent images of cells without NAC stained with JC-1 (red) and Hoechst (blue) at 400x magnification. Bottom: Fluorescent images of cells with NAC stained with JC-1 (red) and Hoechst (blue) at 400x magnification. Scale bar is 25 microns. Images are representative of three independent experiments. (E) E6-1 cells were treated for 3 hours with LG with or without NAC, lysed, and subjected to SDS-PAGE. Cells were then transferred to a PVDF membrane and probed for the specific proteins. Bands were visualized with a chemiluminescence reagent. Statistical calculations were performed using Two-Way ANOVA multiple comparison for (A) and One-Way ANOVA multiple comparison for (B–C). **p < 0.01 vs. Control, ****p < 0.0001 vs. Control.
3.5 FADD Protein Involvement in Cancer Cells Treated with Lemongrass Extract

In order to investigate the role of specific death pathways, we probed for caspase activation in non-Hodgkin’s U-937 lymphoma cells and E6-1 Jurkat cells lacking a functional Fas-associated protein with Death Domain (dnFADD Jurkat). Western blot analysis indicated that treatment with lemongrass extract activated caspase-8 in both cell lines, but caspase-3 was only activated in U-937 cells (Fig. 8a, 7b). As a result, cell death by lemongrass extract was inhibited in the dnFADD cells (Fig. 8c). Contrastingly, white tea extract maintained its ability to induce apoptosis in the dnFADD cell line and without caspase-3 activation (Fig. 8b, 6c). Interestingly, lemongrass extract maintained its ability to produce ROS and white tea extract continued to show no increase in ROS production in the dnFADD cells (Fig. 8d). These results indicate that lemongrass extract requires a functioning FADD protein for its apoptotic activity whereas white tea extract exhibits apoptotic activity independent of the FADD protein, and further corroborate the hypothesis that lemongrass and white tea extract induce cell death by different mechanistic pathways.
Figure 8: Functioning FADD protein is required to induce apoptosis in cancer cells treated with lemongrass extract. (A) U-937 and (B) DN FADD Jurkat cells were treated for 12 hours and 48 hours, respectively, with the specified treatments, lysed, and subjected to SDS-PAGE. Cells were then transferred to a PVDF membrane and probed for the specific proteins. Bands were visualized with a chemiluminescence reagent. (C) DN FADD Jurkat cells were treated for 48 hours with the specified doses and stained with Annexin V and PI. Results were obtained using image-based cytometry with the Y-axis representative of percent of cells positive for Annexin V (green), PI (red), Annexin V and PI (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. (D) DN FADD Jurkat cells were treated with H2DCFDA following treatments with paraquat (PQ), LG, or WT for 3 hours. Results were obtained using the image-based cytometry with the Y-axis representative of percent of cells positive for DCF. Values are expressed as a mean ± SD from three independent experiments. Statistical calculations were performed using Two-Way ANOVA multiple comparison for (C) and One-Way ANOVA multiple comparison for (D). ***p < 0.001 vs. Control, ****p < 0.0001 vs. Control.
3.6 Lemongrass and White Tea Extracts Reduce Tumor Size in Lymphoma Xenograft Model in Immunocompromised Mice

Having seen effective induction of cell death by lemongrass and white tea extracts in several cell lines, we wanted to evaluate if this compound has the ability to inhibit growth of histiocytic lymphoma xenografts in mice. U-937 cells were transplanted subcutaneously in immunocompromised mice as described in the materials and methods. After palpable tumors were established, each treatment group was orally administered with either lemongrass or white tea extracts via supplemented drinking water over the course of three weeks. Both extracts were able to decrease the growth of the xenograft as determined by tumor volume relative to the vehicle control (Fig. 9a, 9b). Over the three-week study, there was no apparent change in body weight of mice in each group compared to the control, indicating that the animals were able to tolerate the administered treatments (Fig. 9b). Furthermore, immunohistochemical staining was performed for tumor tissues treated by both lemongrass and white tea extracts. There was an increase in γ-H2AX phosphorylation, an indicator of DNA breaks, as well as a reduction in positive staining for PCNA, a marker for cellular proliferation, in both the lemongrass and the white tea extract-treated groups compared to the control group in the tumor sections (Fig. 9c). Thus, these findings illustrate that lemongrass and white tea extracts are effective in reducing tumor growth in vivo when administered orally.
Figure 9: Orally administered lemongrass and white tea extract reduce tumor size in lymphoma xenograft model in immunocompromised mice. Immunocompromised mice were subcutaneously injected with cancerous cells and tumors were allowed to establish. Treatments occurred every other day and the studied compound or the equivalent vehicle control administered orally for three weeks. (A, B) The tumors were photographed before and after extraction from the animals. (C) Tumor volume and mass were measured two times per week. (D) Immunohistochemistry analysis of sectioned tumor tissues from the lymphoma study. Each section was subjected to the specified antibody followed by a biotinylated secondary antibody. Detection was done using a DAB Peroxidase HRP Substrate Kit (brown) followed by Hematoxylin counterstaining (purple). Images were obtained using inverted bright field microscopy. Sectioning results are representative of three individual tumors. Scale bar is 50 microns. Statistical analysis using One-Way Anova. *p<0.05 vs tumour volume of the control.
3.7 Phytochemical Analysis of Ethanolic Lemongrass Extract

As shown in the results above, since the unfiltered, ethanolic lemongrass extract has the most effective and selective anti-cancer activity, we performed a phytochemical analysis of this particular extract. Commonly occurring compounds (34 in all) were selected for detection based on published literature searched with the key word Cymbopogon. Elimicin and lonicerin were identified based on a spectral library of over 900 phytochemicals, which we have recorded in UNIFI software (Table 2). Methyl isoeugenol was putatively identified based on monoisotopic mass observed in electrospray ionization on a Q-TOF within mass accuracy of 5 PPM and compared to published literature. Spectra of an additional 283 phenolic compounds in our in-house library of standards were matched with the spectra obtained in positive and negative modes. Commonly known phenolics were not present in the extract.

Three compounds in the extract (elemicin, lonicerin, and methylisoeugenol) were identified in addition to the existing phytochemicals in the spectral library. These compounds were evaluated for their anti-cancer activity, both alone and in combination, as these were hypothesized to potentially be the active compounds contributing to the anti-cancer activity of lemongrass extract. However, our results show very poor anti-cancer activity, if at all, at very high doses (Fig. 10). Further work with other phytochemical classes will be required with purification of each compound and activity analysis.
Figure 10: Cytotoxic activity of three compounds identified in lemongrass extract individually and in combination on a lymphoma cell line.
Table 2: Three unique compounds identified in lemongrass extract

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4.0 Chapter 4: Discussion

4.1 Evaluation of Natural Health Product (NHP) Extracts

The primary aim of this project began with the evaluation of various NHPs that have established a level of medical promise in existing scientific literature or accompanied by strong anecdotal evidence. More specifically for the purpose of this study, NHP extracts (aqueous and ethanolic) were screened as potential non-toxic alternatives for lymphoma treatment either as individual treatments or in combination. Important to note is the concept of using only water and ethanolic extraction protocols for the purpose of this study. In communities of natural health and NHP development, both aqueous and ethanolic extracts are recognized to be two most accepted biocompatible solvents and were the only extraction protocols used for this study. Exploring different extraction methods such as Supercritical Fluid Extraction (SFE) may be an area of future interest amongst other research groups. In total, we tested 28 extracts from different plant materials (used traditionally in various cultures) and found that two extracts were among the few that showed the most significant anti-cancer activity against human lymphoma cells at reasonably low concentrations. Although lemongrass and white tea were chosen for the purpose of this thesis, other lab members further studied some honourable mentions including Hibiscus, Siberian Ginseng and Sage extracts. Due to the complex composition of NHPs, a lot of work was done in depicting accurate conclusions surrounding efficacy, mechanisms, and selectivity in order to measure and properly validate each respective extract as safe alternatives to other current lymphoma treatments.
4.2 Anti-Cancer Efficacy and Selectivity of Lemongrass and White Tea Extracts

4.2.1 Effect on Viability in Lymphoma Cells

To assess the efficacy of LG and WT we began with a cell viability assay, WST-1, by following a standard protocol in measuring the respective ability of these two extracts in impeding cell metabolism of 4 lymphoma cell lines. Although the results aren’t shown in this paper, a trypan blue assay was carried out in support of the cell viability assay. Ethanolic, unfiltered (ETH-UF) lemongrass (LG) extract in particular had the greatest cytotoxic properties on these cancers compared to the other extraction methods (cold water, hot water and filtered, ethanolic) with EC$_{50}$ values well below 0.08mg/mL for all four commercially available lymphoma cell lines while the cold and hot water extractions (CW and HW) for white tea (WT) appeared to be slightly more effective compared to their ethanolic counterparts with EC$_{50}$ values below 0.2mg/mL. These results illustrate how the extraction procedure is an important factor to consider when examining the use of plant extracts for the purpose of developing disease therapies. Furthermore, this would also imply that the yield of different bioactive molecules are extraction-method dependent and ultimately affect the resultant potency of each extract, a phenomenon observed previously in literature (Gil-Chávez, 2013) Such molecules could be specific primary (e.g. amino acids, fatty acids, sugars) or secondary (e.g. carotenoids, terpenoids, alkaloids) metabolites, which may be acquired or lost through the type of solvent used for extraction (water or ethanol) and if the extract is filtered or not. Only ETH-UF-LG and HW-WT were studied further as they were found to be the most efficacious of the four different extracts.

4.2.2 Destabilization of Mitochondrial Membrane Potential and Induction of PCD in Lymphoma Cells

Moving forward with the characterization of two plant extracts, ethanolic lemongrass and aqueous (hot water) white tea, we chose to further test activity against lymphoma cell lines using an
Annexin-V binding assay. As previous results demonstrated with WST-1, both ETH-UF-LG and HW-WT were effective at inducing cell death by apoptosis in a dose-dependent manner in 3 lymphoma cell lines as indicated by the presence of Annexin V and propidium iodide staining. Moreover, the lowest effective doses of LG (0.05 & 0.1 mg/mL) and WT (0.1 & 0.25mg/mL) were unable to induce cell death appreciably in a normal human fibroblast cell line which is a vital quality in developing selective chemotherapies. Surprisingly LG extract was also well tolerated in peripheral nucleated blood cells (PNBCs) whereas white tea induced apoptosis (of PNBCs) at higher doses. Despite this potential obstacle in the case of white tea, both extracts were well tolerated by mice when administered orally over a period of three weeks.

The induction of apoptosis has been shown to be achieved by extrinsic and intrinsic mechanisms, both which converge towards the destabilization of the mitochondria. Indeed, we have demonstrated that both ETH-UF-LG and HW-WT cause mitochondrial dysfunction in a dose and time-dependent manner as seen by monitoring the mitochondrial membrane potential dissipation as shown in Figure 4A and a decrease in mitochondrial oxygen consumption relative to the control caused by these natural extracts (Figure 5B). The reduced oxygen consumption reflects dysfunctional complexes of electron transport chain. Interestingly, despite both natural extracts converging towards mitochondrial collapse and the induction of apoptosis, the initial requirement of the generation of ROS for cytotoxicity is extract-specific. HW-WT does not appreciably induce ROS in lymphoma and leukemia cells in contrast to ETF-UF-LG. ETH-UF-LG causes ROS generation and requires the generation of oxidative stress to yield mitochondrial membrane potential collapse and the cleavage of intrinsically and extrinsically related capases-8 and -9 leading to the subsequent execution of apoptosis. The initiation of both the intrinsic and extrinsic pathways have been associated with ROS production (Fulda, 2006) and these results offer further confirmation. Thus, it is plausible to propose
that several bioactive molecules found within ETH-UF-LG increase the production of ROS and/or decrease the antioxidant capacity of the cancer cells leading to the activation of apoptosis.

4.2.3 Ramification of ROS Induced Apoptosis and Caspase Involvement

ROS levels in cancer cells have been reported to be markedly higher in comparison to their normal counterparts. Through the stimulus of growth-competence genes and metabolic alterations, ROS can promote cell growth and proliferation, and may be a key factor in the abnormal rates of proliferation observed in cancer cells (Earnshaw, 1999). The exact mechanism of how this occurs is not clear, but it is hypothesized that these high levels of ROS are able to activate a variety of pathways related to a cellular stress mechanism. Consequently, this may lead to the ability of cancer cells to propagate more freely within their environment (Trottier, 2010). It is important to note that these high levels of ROS may in fact render cancer cells more sensitive to additional ROS production and can lead to apoptotic events (Manian, 2008). Indeed, our results have indicated that lemongrass extract treatment leads to the increased generation of ROS followed by cell death. Furthermore, the increase in the production of ROS is a critical step for the induction of apoptosis by lemongrass extract as pre-treatment with antioxidant N-acetylcysteine (NAC) blocked the induction of apoptosis. However, HW-WT was still able to cause cleavage of caspase-8, and double-stranded DNA breaks as indicated by positive staining for λ-H2AX independent of ROS generation, thus, illustrating how different natural extracts can yield apoptotic induction by different means (ROS vs. non-ROS mediated).

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work has demonstrated multiple roles that FADD protein may have in inflammatory or pro-death pathways via cell surface receptor activation (DeBerardinis, 2008). Therefore, ROS may be able to induce the activation of protein(s) involved in the recruitment of FADD to oligomerize, forming active DISCs.

4.3 Lymphoma Xenograft Model in Immunocompromised Mice

Due to the activity of both ETH-UF-LG and HW-WT with their ability to induce cell death in various blood cancer cell lines, we wanted to explore the ability of these natural extracts to reduce xenograft tumors in nude mice. A major drawback of natural extract treatments is their bioavailability and stability of the bioactive compounds (Fang, 2009). Furthermore, these treatments cannot be administered intravenously due to the potential for immune reactions from these complex mixtures of primary and secondary metabolites. Therefore, it is important to evaluate these natural extracts for their efficacy in reducing tumor growth when administered orally. Indeed, our results demonstrated that orally administered and supplemented drinking water with ETH-UF-LG and HW-WT remarkably reduced tumor growth, decreased PCNA expression, and caused double-stranded DNA breaks in the tumors cells relative to the control. Interestingly, lemongrass extract has been shown to have inhibitory effect on carcinogen-induced colon cancer (Trachootham, 2009). Furthermore, there was no observable decrease in the activity of the mice as well as no effect on their weight profile during the course of treatment, indicating that these natural extracts are well tolerated. This illustrates the ability of these natural extracts to be effective not only in vitro, but in vivo as well. These results also indicate that the bioactive components of these extracts are absorbed and distributed to the tumor sites to affect tumor growth.
4.4 Conclusions and Future Work

The purpose of this thesis sought to investigate various natural health products as potential alternatives to current lymphoma therapies, providing both safe and efficacious properties. Of the 28 original extracts, two standouts emerged - lemongrass and white tea – in which over the course of the study were further explored at the level of mechanistic action, selectivity, bioavailability and even tolerability in animal models. The results presented in this study are consistent with and help expand, to an extent, the scientific validation in existing literature. Moreover, it provides exciting support of the anecdotal evidence and reasoning behind many of the traditional uses including a safe pant based approach to treating disease. This could in turn provide lymphoma cancer patients with an additional option when considering treatment that is both viable as a standalone, in combination with other NHPs, or even as an adjunct to chemotherapy – under an oncologist/physician’s surveillance. Families of younger patients, such as children, may in fact be more keen on an alternative which is less taxing on a developing body.

Future studies should aim to depict an even clearer understanding of cell death mechanisms in the context of these extracts as it relates to reactive oxygen species. Seemingly, there are some varying results with respect to how these multifaceted NHPs activate or inactivate specific pathways that ultimately however lead to the same end result- effective reduction of lymphoma cell viability and proliferation. More experiments looking at the activation of the various caspases could be carried out by means of more western blotting and would have been done here had time not been of the essence. Personally, I think it would be interesting to see the activity of these NHPs using different extractions techniques that although deviate from the scope of “natural health” could prove to be quite useful in enhancing bioactivity while continuing to display low toxicity. With respect to lemongrass in specific, more standalone studies should be explored to gauge whether individual compounds are responsible for the promising results shown in this study or if all of the many different properties of a plant material
requires to be present in the same matrix. In our study, individual bioactive compounds did not appear to be affective in the treatment of lymphoma. Lastly, it would be essential to replicate the animal study as not only was it cut short due to ethical reasons, a follow-up is crucial for scientifically validating the xenograft model. Several suggestions have been identified that would strengthen the conclusions of the in vivo work of this study. This would include evaluating both extracts for their efficacy as preventative therapy before introducing the animals to cancerous colonies. Moreover, extending the length and concentration of the extracts to access tolerability and whether toxicological issues arise if a max dose is surpassed.
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Vita Auctoris

NAME: Cory Philion

PLACE OF BIRTH: Windsor, ON

YEAR OF BIRTH: 1988

EDUCATION: Kingsville District High School, Kingsville, ON, 2007

  University of Guelph, B.Sc. Honours
  [Nutritional and Nutraceutical Sciences (NANS) - Guelph, ON, 2012]

  University of Windsor, M.Sc. Honours
  [Chemistry & Biochemistry – Windsor, ON, 2018]