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Anticancer Activity of Natural Health Products (Dandelion Root, Lemongrass, and Hibiscus Extracts); A Study of Efficacy, Interaction, and Mechanism of Action

By

CHRISTOPHER NGUYEN

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2019
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Declaration of Co-Authorship/Previous Publication

I. Declaration of Co-Authorship

I hereby declare that this thesis incorporates materials that is the result of contributions of various researchers. While this thesis was done independently of collaboration with other research labs under the supervision of Dr. Siyaram Pandey, other student researchers were involved in this project. In all cases, the experimental design, execution, analysis, interpretation, and manuscript preparation were conducted by authors listed and statements of author contribution can be located in Appendix B. All listed authors have read and approved the final manuscript prior to submission and these statements can be found in Appendix C. Contributions from those who proofread manuscripts were covered in the acknowledgement sections of published work where appropriate and were not listed as authors.

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This thesis incorporates three original research papers and one review paper that have been either submitted for publication in peer-reviewed journals or are in preparation. They are listed below with the appropriate thesis chapter associated with the publication:

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<td>Nguyen C, Pandey S. Exploiting mitochondrial vulnerabilities to trigger apoptosis selectively in cancer cells. <em>Oxid Med Cell Longev.</em> Submitted 2019 11 Apr. MS#: 7193936</td>
<td>Submitted</td>
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<td>Chapter 4</td>
<td>Lemongrass (<em>Cymbopogon citratus</em>) effectively induces apoptosis in colon cancer cells and interacts with FOLFOX <em>in vitro</em> and <em>in vivo</em></td>
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Abstract

Cancer continues to be the leading cause of death in Canada. Many conventional chemotherapies have indicated side effects due to a lack of treatment specificity and are thus not suitable for long-term usage. Natural health products (NHPs) are well-tolerated and safe for consumption, and some have pharmaceutical uses particularly for their anti-cancer effects.

We have previously investigated the anti-cancer efficacy of dandelion (*Taraxacum officinale*) root, lemongrass (*Cymbopogon citratus*), and hibiscus (*Hibiscus syriacus*) extracts. However, their efficacy on prostate, colorectal, and breast cancer as well as their interactions with standard chemotherapeutics have not been studied to determine if they will be suitable for adjuvant therapies. If successful, these extracts could potentially be used in conjunction with chemotherapeutics to minimize the risk of drug-related toxicity and enhance the efficacy of the treatment. This work aimed to evaluate the efficacy and mechanism of apoptotic induction in various cancer cells, assess the drug-drug interactions of NHPs and chemotherapeutics, and investigate the effects of these NHPs on tumour xenografted mice models.

Using standard biochemical and morphological assays, we have demonstrated that dandelion root extract (DRE), lemongrass extract (LGE), and hibiscus extract (HE) exhibit selective anti-cancer activity. These extracts were also able to enhance the anticancer efficacy of common chemotherapeutics and protect normal healthy cells from toxicity. In mice xenografted with human cancer cells, DRE and LGE were able to reduce tumour burden and LGE was able to enhance FOLFOX activity. Thus, the implementation of these well-tolerated extracts in adjuvant therapies could be a selective and efficacious approach to cancer treatment. These findings provide scientific validation to support the safe and effective use of NHPs as well-tolerated and effective forms of cancer treatment.
This work is dedicated to the memory of my grandfather, Domenic Vu, who lost his battle to cancer in 2011, and my grandmother, Hoa Vu, who passed away in 2017.

This work is also dedicated to the memory of Mr. Kevin Couvillon, whose fight with leukemia ended in 2010. Thank you for inspiring us and motivating us in the fight against cancer.
Acknowledgements

I would like to thank Dr. Pandey for giving me the opportunity to be able to work in your lab for the past five years. Your enthusiasm and excitement for the work that every student in your lab does is contagious and inspires me to work as hard as possible to share the same enthusiasm for research. You have been a great supervisor and professor as well as a great mentor.

I would also like to thank Dr. Karpowicz and Dr. Marquardt for being a member of my thesis committee. Your time, effort, and mentorship has been invaluable.

Thank you to Marlene Bezaire, Cathy Wilson, and Jayne Pierce. You guys are one of the hardest working administration teams I have ever had the pleasure of working with. Your enthusiasm for helping out all students is contagious for the entire department.

I would like to thank the members of my cancer group for always working hard and motivating me to match your enthusiasm and drive for research. Thank you to Ali Mehaidli, Kiruthika Baskaran, Ivan Ruvinov, Ola Zaitoon, Ben Scaria, Krishan Parashar, Sahibjot Grewal, Alaina Pupulin, Johan Pushani, Rahul Jaychandiran, Jana Khanafer, Kyle Wong, Chris Raad, Siddh Sood, Lauren Goddard, and Manaav Mehta specifically for being great team members. I would also like to thank Caleb Vegh for his amazing guidance and friendship through the last few years, as well as his amazing team members including Darcy Wear, Lauren Culmone, Iva Okaj, Simon Pupulin, and many others for their friendship and help.

Thank you to Dr. Dennis Ma, Chris Pignanelli, Cory Philion, Dr. Pamela Ovadje, Mike Stanesic, and Krithika Muthukumaran for being mentors as well as great role models in the lab through my undergraduate experience with the Pandey Research Lab.
Thank you to all past and present Pandey Lab members for making the lab a welcoming environment and a very special place for all members. Every member has helped to make Pandey lab a very unique place.

I would like to extend a large debt of gratitude for all parties involved in helping to fund the research that has led to this thesis. The Couvillon family has provided an immense amount of support and interest in our research, and it is with great pride that our research project has been named after their son, Kevin. I would also like to thank the Prostate Cancer Fight Foundation: Ride for Dad program, Knights of Columbus, Windsor Mold Group, 100 Who Care Windsor, Jesse & Julie Rasch Foundation, Lotte & John Hecht Memorial Foundation, the Seeds4Hope Foundation, the Palmer family, and the Rodrigues family for their contributions to the project.

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<tr>
<td>5-FU</td>
<td>5-fluouracil</td>
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<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
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<tr>
<td>Apaf-1</td>
<td>Apoptosis protease activating factor 1</td>
</tr>
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<td>AV</td>
<td>Annexin V</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecules</td>
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<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>ddH$_2$O</td>
<td>Double distilled water</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DRE</td>
<td>Dandelion root extract</td>
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<tr>
<td>EndoG</td>
<td>Endonuclease G</td>
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<tr>
<td>ER+</td>
<td>Estrogen-receptor positive</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>H$_2$DCFDA</td>
<td>2′, 7′-dichlorofluorescin diacetate</td>
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<tr>
<td>HE</td>
<td>Hibiscus extract</td>
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<tr>
<td>HKII</td>
<td>Hexokinase II</td>
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<tr>
<td>LGE</td>
<td>Lemongrass extract</td>
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<tr>
<td>LV</td>
<td>Folinic acid</td>
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<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
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<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<td>NAC</td>
<td>N-acetylcycteine</td>
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<tr>
<td>NHP</td>
<td>Natural health product</td>
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<tr>
<td>OxPhos</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PCA</td>
<td>Protocatechuic acid</td>
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<td>PCD</td>
<td>Programmed cell death</td>
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<td>PI</td>
<td>Propidium iodide</td>
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<td>PST</td>
<td>Pancrastatin</td>
</tr>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>TRADD</td>
<td>TNF receptor type-1 associated death domain</td>
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<tr>
<td>VDAC1</td>
<td>Voltage-dependent anion channel 1</td>
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Chapter 1

General Introduction

Containing work from:

Exploiting mitochondrial vulnerabilities to trigger apoptosis selectively in cancer cells

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¹Department of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario N9B 3P4, Canada

1.1 Cancer

Despite many advances in cancer treatment approaches, cancer continues to remain the leading cause of death in both Canada and the United States [1,2]. In Canada, of 206,200 projected cancer incidences, 1 in 4 diagnosed patients were expected to succumb to the disease in 2018 [1]. In the United States, 1,762,450 new cancer cases are projected to be diagnosed, with about 1 in 3 diagnosed patients expected to succumb to the disease in 2019 [2].

Cancer is a disease characterized by uncontrolled cell growth and proliferation resulting from evasion of regulatory mechanisms in cells [3]. Cancer is a multi-step process involving many genetic changes due to mutation of the genome. Genetic changes to 350 of the 22,000 protein coding genes have been determined to be involved in and contribute to cancer development [4]. These genes can be categorized into two categories, tumour suppressor genes and oncogenes. Tumour-suppressor genes encode proteins that are able to inhibit cell proliferation, and thus their knockdown via mutation may lead to development of cancer [5]. For example, p53 encodes a protein able to transmit a variety of stress-inducing signals to induce different anti-proliferative cellular responses leading to cell death. Inactivation of p53 in cancer is a characteristic checkpoint in cancer, allowing for uncontrolled cancer cell proliferation [6]. Oncogenes encode proteins that are able to transform cells supporting the progression of cancer development. For example, the Ras oncogene is responsible for causing a metabolic swap from oxidative phosphorylation to glycolysis to enable reprogramming of cancer cell metabolism [7].

Hanahan and Weinberg proposed hallmarks of cancerous cells that allow for these cells to be distinguished from non-cancerous cells (Figure 1) [3]. In later years, this list was modified to include additional characteristics (Figure 2) [8]. These hallmarks are essential to allow for cancer cell growth and survival, allowing them to evade safeguarding mechanisms from the body. This
theory identifies the genomic changes and defining characteristics that promote progressive transformation of normal healthy cells into malignant cancer cells. These hallmarks include:

I. Sustaining proliferative signaling through the generation of oncogenes which are able to eliminate normal cell signaling dependence through their growth signals capabilities.

II. Evading growth suppressors that negatively regulate cell proliferation. This is through the suppression or disruption of tumour suppressor genes capable of regulating proliferation.

III. Bypassing cell death signaling to resist cell death through the overexpression of pro-survival proteins such as the Bcl-2 family of proteins.

IV. Enabling replicative immortality to maintain replicative signaling usually by up-regulating the expression of telomerase enzyme to inhibit telomere shortening.

V. Inducing the development of new blood vessels (angiogenesis) to allow for a continuous supply of oxygen and nutrients through the up-regulation of proteins such as vascular endothelial growth factor.

VI. Activating metastasis and invading surround tissue, allowing tumour cells to migrate to a secondary site to establish. This can be done due to alteration of certain protein functions such as cell adhesion molecules (CAMs) and avoidance of cell-death mechanisms when metastasizing.

Hanahan and Weinberg have proposed two emerging hallmarks that have been shown to be involved in the development and progression of some, perhaps all, cancers:

VII. Reprogramming of energy metabolism in order to fuel cell growth and division. This allows for the increased energetic needs of cancer cells to be met through an increased dependence on aerobic glycolysis.
VIII. Evading immune destruction from lymphocytes, macrophages, and natural killer cells that would normally identify cellular dysfunction and react to kill cancer cells. This may also be done by avoidance of detection by immune system.

Figure 1: The hallmarks of cancer. Six different properties of cancer cells have been attributed to the progression of cancer in humans. Targeting of these hallmarks may lead to the development of improved cancer therapeutics (Hanahan & Weinberg, 2011).

Figure 2: Emerging hallmarks of cancer. Further research has suggested that two additional hallmarks of cancer are now involved in pathogenesis of potentially all cancers (Hanahan and Weinberg, 2011).
As normal cells progressively move towards a neoplastic state of abnormal cell growth, they will develop these hallmark capabilities based on the abilities of these traits to allow them to ultimately become malignant [8]. As cancerous cells progressively express these hallmarks, they will form tumours, become malignant, undergo metastasis to spread, and eventually lead to mortality due to cancer [9]. However, these hallmarks additionally provide researchers with interesting targets when developing treatments that are able to exploit cancer cell dependencies and induce cell death [3].
1.2  Cancer Metabolism: Mitochondria and Cancer

Cancer cells, through the accumulation of many genetic mutations, are notorious for their enhanced ability to proliferate in suboptimal host conditions. Cancer cells are able to manipulate metabolic and immunogenic pathways to adapt and operate under difficult conditions [10]. Indeed, metabolic reprogramming in cancer cells has been identified as a hallmark of cancer and a potential vulnerability that can be targeted to fight this disease [8].

In order to proliferate in hostile conditions, cancer cells undergo metabolic reprogramming to meet their energy needs. In the 1920s, Otto Warburg observed the production of excessive lactate in cancer cells in the presence of oxygen due to ‘oxidative glycolysis’, with the phenomenon eventually termed the ‘Warburg effect’ [11,12]. However, this has led to the wrong belief that there is mitochondrial damage and inefficiency in cancer cells, forcing them to rely on excessive glycolysis for their energy needs [13]. Contrary to this, a large breadth of work has shown that mitochondria respiration remains undamaged in many cancers, and many cancer cells that exhibit the Warburg effect retain mitochondrial respiration [14]. Interestingly, in ovarian cancer, the microenvironment of cancer cells directly impacts their metabolic reprogramming [15]. Peripheral cells of the tumour spheroid exposed to normal oxygen levels relied heavily on aerobic glycolysis and were proliferative cells. In contrast, internal cells were poorly vascularized, leading to cell quiescence and heavy dependence on mitochondrial oxidative phosphorylation (OxPhos) for the majority of their ATP production [16,17]. This study highlights that the fact that, while standard chemotherapy was effective at killing proliferative cells, the quiescent cell population is resistant and causes tumour regeneration. In order to properly combat cancer, a multi-faceted approach including mitochondria vulnerabilities should be considered to ensure complete tumour elimination. In fact, recent observations have indicated that mitochondria support and play a
critical role in tumourigenesis through their metabolic reprogramming, oxidative signaling, reactive oxygen species generation, and production of oncometabolites [18-21].

Cancer cells have higher metabolic needs and antioxidant defenses compared to healthy cells. Cancer cells rely heavily on aerobic glycolysis to meet their energy needs, and as a result upregulate glucose transporters to meet these demands [22,23]. Furthermore, due to aerobic glycolysis and production of large amounts of lactate and pyruvate leading to increased acidity in the cytoplasm in cancer cells, the mitochondria are hyperpolarized compared to those of normal cells [23]. This can also be due to increased intracellular Ca\(^{2+}\) levels and upregulation of anti-apoptotic Bcl2 protein [24,25]. The hyperpolarization of the mitochondria has also been attributed to increased apoptosis evasion as well [25,26].

While the mitochondria are the organelle responsible for ATP generation, they contain a number of pro-apoptotic factors such as cytochrome c, endonuclease G, and apoptosis inducing factor (AIF) which can induce cell suicide program if they are released outside. This occurs due to the depolarization of the mitochondrial membrane potential leading to membrane permeabilization. Thus, mitochondria in the cell are also a death machinery that can be activated by its leakage. Release of cytochrome c in the cytosol leads to its association with APAF and Caspase-9 eventually thus forming the apoptosome which activates Caspase-3 eventually executing apoptotic cell death [27]. In contrast, AIF-initiated apoptosis is caspase-independent, through chromatin condensation and DNA fragmentation [28]. The presence of such pro-apoptotic proteins in the mitochondria highlights the organelle as an interesting target for cancer therapy research.

Mitochondria play a central role in the apoptosis induction process. It would be appropriate to say that each mitochondrion acts as a self-destructing button for the cell. In non-cancerous cells,
the mitochondria are assembled properly, and are generally difficult to permeabilize. However, in cancer cells, mitochondria could be vulnerable to certain agents that can permeabilize the membrane [29]. Our research group has hypothesized that, due to rapid proliferation, cancer cells face a limited resource of lipids, and the assembly of the mitochondrial membrane may be compromised compared to normal healthy cells. While healthy cells may be resilient to mitochondrial targeting agents, cancer cells may be especially susceptible to them due to hyperpolarization of the membrane as well as exposure of certain proteins to these agents resulting from poor assembly. These overall differences between cancerous and non-cancerous mitochondria can be targeted to allow for selective apoptosis induction in cancer cells.
1.3 Targeting Mitochondrial Vulnerabilities

Cancer cells may rely on OxPhos heavily in addition to aerobic glycolysis for their excessive energy needs [30,31]. Furthermore, the mitochondrial membranes are hyperpolarized and poorly assembled [32], targeting the mitochondrial vulnerabilities of cancer cells could be an interesting approach to selectively kill cancer cells. Weinberg and Chandel postulate that, while cancer cells may simply turn to aerobic glycolysis, there are other reasons supporting mitochondrial targeting [16]; The first being that poorly perfused tumours may have limited access to aerobic conditions and glucose, thereby focusing on mitochondrial ATP generation, posing an excellent target for such drugs [33,34]. Due to their limited access to glucose, cancer cells will turn to OxPhos because of the increased yield of ATP per glucose molecule. The second being that some cancer cells indeed show a heavy dependence on OxPhos for their ATP needs [34,35]. Finally, drugs targeting and inhibiting mitochondrial ATP production may synergize positively with those targeting aerobic glycolysis. In doing so, these drugs may potentially sensitize cancer cells to aerobic glycolysis-targeting drugs and enhance their action. Further, when the mitochondria are targeted, leakage of pro-apoptotic factors from mitochondria will result into activation of apoptotic regardless.

Some vulnerabilities that may be targeted include the induction of oxidative stress on cancerous mitochondria, targeting modified mitochondrial metabolism, and to sensitize or inhibit chemoresistant cancer cells.

Induction of oxidative stress as a target for cancer treatment

Reactive oxygen species (ROS) are radicals containing a single unpaired electron in their outermost electron shell [36]. At lower levels, ROS can be advantageous in promoting proliferation and signaling [37,38]. However, at higher levels, ROS can induce oxidative stress leading to cell
death [37]. Cancer cells are believed to require a high concentration of ROS to supplement their increased proliferation rates, and it has been hypothesized that cancer cells can take advantage of the resulting augmented DNA-damage to promote further mutation and tumourigenesis [38,39]. The induction of oxidative stress via chemotherapies or radiation therapy on cancer cells may result in DNA damage-induced cell death [40-43].

The production of ROS in the mitochondria is an inevitability due to its generation as a by-product in OxPhos, and it plays a role in alteration of mitochondrial dynamics [44]. The production of ROS in mitochondria is efficiently quenched in normal cells using antioxidative defense system include superoxide dismutase and glutathione peroxidase [45,46]. If not quenched or eliminated, the excess oxidative stress can cause further dysfunction of mitochondrial proteins leading to increased production of ROS, creating a vicious cycle of mitochondrial damage and oxidative stress, leading to the collapse of the mitochondrial membrane potential (MMP), permeabilization, and the induction of apoptosis [44].

It would be worthwhile combining mitochondrial targeting agents with oxidative stress inducers to target cancer cells.

**Mitochondrial reprogramming as a target for cancer treatment**

Cancer cells rely heavily on aerobic glycolysis for the bulk of their energy needs and turn to oxidative phosphorylation only in situations such as nutrient deficiencies. Upregulation of aerobic glycolysis is a result of increased expression of oncogenes (such as MYC and KRAS) and deregulation of the P13K signaling pathway [47-49]. Due to excessive glycolysis and lactic acid production, mitochondria are hyperpolarized [23]. By exploiting these changes compared to normal mitochondrial characteristics, selective and effective treatments can be developed.
Recent work into targeting of mitochondrial reprogramming vulnerabilities has focused on many different approaches including inhibition of upregulated metabolic proteins found only in cancer cells [50], targeting mitochondrial oxidative phosphorylation or respiration [51,52] or through the induction of antibiotics-induced mitochondrial dysfunction [53].

**Sensitization and reversal of chemoresistance by targeting the mitochondria**

Cancer cell mitochondria contain higher amounts of anti-apoptotic Bcl2 family of proteins to evade apoptosis and thus are resistant to anti-cancer drugs. The depletion of mitochondrial DNA (mtDNA) has been reported in numerous cancers *in vivo* and has been implicated in increasing the expression of anti-apoptotic genes such as Bcl2, activating pro-survival enzymes ultimately leading to resistance to chemotherapy-mediated apoptosis [54-58]. Due to mitochondrial reprogramming to rely on aerobic glycolysis, cancer cells are able to further resist apoptosis through upregulation of regulatory enzymes such as mitochondrial hexokinase 2 [59]. Hexokinase 2 has been shown to increase lactate production, cell proliferation, resistance to drugs, and invasion [60]. Lactate production allows for cancer cells to maintain a slightly acidic micro-environment and enhance their survival through pathways such as using lactate as an antioxidant [61-63]. Targeting these mitochondrial proteins could remove this resistance, and cancer cells may be susceptible to cell death [54].
1.4 Apoptosis

The programmed cell death (PCD) process of apoptosis is able to impede and stop cancer development and progression and triggered by physiological stressors or extracellular signals [8]. One hallmark of cancer is the ability of cancer cells to avoid these PCD processes through various mechanisms allowing them to continue proliferation under the same physiological stressors or signals that would normally induce cell death in healthy cells [8].

Apoptosis is the complex and physiological process of cell death that is used to remove cells with damaged DNA or unwanted cells. Thus, apoptosis is a safeguard against cancerous development of a cell containing damaged DNA. Apoptosis can be triggered by DNA damage, oxidative stress, growth factor deprivation, and mitochondrial depolarization [64]. The knowledge of biochemical mechanisms of apoptosis have led to developments of therapies targeting cancer cells to induce cell death. [64]. The exploitation of cellular vulnerabilities in cancerous cells, including oxidative stress and mitochondrial membrane destabilization, should be used to develop novel therapeutic agents that could trigger apoptosis and eradicate the disease [65,66]. Since the discovery of apoptosis, researchers have aimed to exploit cancer cell vulnerabilities to induce apoptosis selectively in cancer cells. ranging from identification of tumour-suppressor genes, discovery of novel compounds, and development of technologies such as nanoparticles to selectively identify targets which will be efficacious and selective for cancer [67-69]. Unfortunately, cancer cells develop resistance to many chemotherapies by resisting apoptosis and/or exporting the drugs outside the cell. Cancer cells achieve this by disrupting pro-apoptotic proteins, reducing the function of caspases, and impairing death receptor signalling [64].
There are two pathways by which apoptosis can initiate and progress – the intrinsic pathway and the extrinsic pathway, both triggered by different signals, but leading to apoptosis. The process of apoptosis, both intrinsically and extrinsically are shown in Fig. 3 [70].

The intrinsic pathway (Figure 3) is triggered by internal stressors, including DNA damage, oxidative stress, developmental cues, lack of growth factors, radiation, toxins, and chemotherapeutics. These may come via a negative or positive intracellular signal. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis whereas positive signals involve the introduction of a stressor such as radiation, toxins, viral infections, and free radicals [71]. The intrinsic pathway is regulated in part by the Bcl2 family of proteins, all containing a BH-3 domain. The proteins in the Bcl2 family go on to activate Bax-like proteins, which will permeabilize the outer membrane of the mitochondria [72]. This releases the pro-apoptotic factors such as cytochrome c into the cytoplasm. The released cytochrome c can then lead to the activation of the initiator caspase-9 by binding Apoptosis Protease Activating Factor 1 (Apaf-1) and pro-caspase-9 to form the apoptosome [71]. Caspase-9 will set off a caspase cascade of subsequent executioner caspase activations such as caspase-3, leading to the induction of apoptosis in the cell [72]. The appearance of phosphotidylserine on the outer leaflet of apoptotic cells then facilitates phagocytic recognition, allowing for their early uptake and disposal [73].

Intrinsic apoptosis is additionally able to follow a caspase-independent pathway. The release of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) from the inner mitochondrial membrane space results in the degradation and fragmentation of DNA in cells following apoptotic stimuli [74].
In the extrinsic pathway (Figure 3), the signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily [75]. In the FasL/FasR model of the extrinsic pathway, when the ligand binds to the Fas receptor, this leads to the recruitment of the adapter protein Fas-associated death domain (FADD), as well as the binding of TNF ligand to TNF receptor. This results in the binding of the adapter protein TNF receptor type-1 associated death domain (TRADD) with recruitment of FADD [76]. FADD will associate with procaspase-8 to form the death-inducing signaling complex (DISC). This formation of DISC will set off a caspase cascade, leading to apoptosis by the activation of initiator caspase-8 and caspase-10 through adaptor proteins, namely FADD [72].
Figure 3: The intrinsic and extrinsic pathways of apoptosis. The intrinsic pathway is found on the left, while the extrinsic pathway is found on the right. Initiation factors will lead to the recruitment of pro-apoptotic members of the Bcl2 family to the mitochondria, permeabilizing the membrane to allow for releasing of other pro-apoptotic proteins such as cytochrome C. The activation of death receptors will then lead to the activation of the extrinsic pathway. The activation of both pathways leads to a caspase cascade eventually leading to apoptosis (Zhang et al, 2016).
1.5 Current Cancer Treatments

Current approaches to cancer treatment include chemotherapy, surgery, and radiation therapy. Although effective, these treatment types may cause inadvertent side effects due to their lack of selectivity for cancer cells and toxicity to normal healthy cells [77]. Chemotherapy-based regimens seem to have reached a therapeutic plateau due to dosage limitations stemming from the risk of fatal side effects [78]. Surgical removal of the tumour has been excellent in cases of early detection, and substantial advances have been made to improve detection and diagnostics of various cancers [79]. However, the effectiveness of this method is limited to cases in which the tumour has not yet invaded or metastasized to other locations. It has also been established that cancer relapse is common in patients undergoing surgery, with nearly 50% of cases having recurrence within 3 years of surgery [80]. Radiation therapy has been implicated in genomic instability after treatment, ultimately leading to further cancer development, due to their ability to induce mutations in the genome that may lead to upregulation of oncogenes or inhibition of tumour suppressor genes [81]. Thus, these treatments designed to destroy cancer cells may end up leading to toxicity, eventual relapse, or further cancer development.

Once cancers have progressed to the metastatic stage, treatment approaches tend to focus on the usage of chemotherapies to eradicate the primary tumour while fighting any secondary metastases. Chemotherapies including DNA damaging therapeutics, e.g. cisplatin, doxorubicin, or 5-fluorouracil, and tubulin modifying agents, e.g. paclitaxel, have been developed to induce apoptosis in cancer cells. However, many issues and limitations have arisen with these drugs due to their non-selective nature and extreme toxicity to healthy tissues. Furthermore, cancer cells develop resistance to these drugs and patients may end up experiencing severe side effects without any effect on cancer [82-85]. In addition, these genotoxic treatments lead to DNA damage in
normal tissues with increasing risk of cancer development. Thus, we are risking the use of cancer-inducing compounds in our attempts to treat cancer.

**Targeted Treatments**

Due to lack of selectivity and potential toxicity in cancer treatments, it is critical to discover and development targeted therapeutics that are able to induce selective cancer cell death. Of particular interest in our research group is the targeting of cancer cell mitochondria and metabolism due to their inherent differences as described in section 1.2. To exploit these vulnerabilities in cancer cells, current advances have designed compounds capable of targeting these differences. Of particular interest to our group was the ability of mitochondria-targeting agents able to induce selective apoptosis (mitocans) to target the metabolism and oxidative stress defense mechanisms of cancer.

Cancer cells rely heavily on aerobic glycolysis and, as a result, over-express hexokinases (HK) which catalyze the first step of glucose metabolism [86]. Targeting hexokinase II (HKII) or voltage-dependent anion channel 1 (VDAC1) can uncouple their interaction with each other, potentially stunting aerobic glycolysis, leading to cancer cell death [87,88]. Current treatments have not only selectively induced cell death cancer cells but have led to metabolic stimulation of healthy cells [89-92].

An interesting approach to cancer treatment lies in engineering mitocans that are able to target electron transport chain (ETC) complex proteins [93]. Treatments designed to target cancer cell OxPhos may shut down an important machinery in cancer cell metabolism, sensitizing them to aerobic glycolysis targeting agents or potentially facilitate to the leakage of mitochondrial pro-apoptotic proteins. Our group has previously demonstrated that pancreatin (PST) analogs induced apoptosis at extremely low EC\(_{50}\) levels in a wide variety of cancers with a much higher
efficacy than standard chemotherapeutics [94]. PST analogs were additionally shown to be interacting with complex II or III in order to exert its anti-cancer effects. It may be possible that PST analogs interact or bind with complex II or III in order to exploit an unidentified mechanism of cancer cell metabolism, or potentially affect downstream pathways of these complexes.

Finally, increased oxidative stress can affect mitochondrial proteins leading to further dysfunction of mitochondria, generating more ROS thus starting a vicious cycle of oxidative stress and mitochondrial dysfunction. Thus, targeting mitochondria or oxidative stress defense mechanisms can initiate this vicious cycle leading to cell death [95]. By generating excessive ROS, cancer cell defense mechanisms may be unable to cope with increased oxidative stress leading to apoptosis. Indeed, many recent studies have demonstrated selective apoptosis induction in cancer cells [96-99].
1.6 Natural Health Products

Natural health products (NHPs) are materials isolated from various food and plant sources that have been shown to have medicinal properties [100]. In fact, many current therapies for a wide variety of diseases originate from natural compounds. For example, the commonly used chemotherapeutic taxol was isolated from the bark extract of the Pacific yew tree, *Taxus brevifolia*, when the extract was shown to have a cytotoxic effect on cancer [101]. It is possible that well-tolerated, highly potent anticancer compounds are still left to be discovered and developed into a novel cancer therapeutics.

The traditional application of natural health products (NHPs) as medicinal products have been documented throughout history in numerous applications – likely dating back thousands of years before recorded history [102]. NHPs are compounds derived from natural sources such as plants and thus must have been some of the first sources of medicinally active materials available for traditional treatments and therapies [102]. The ancient Egyptian document *Ebers Papyrus*, dated around 1550 BC, contained 800 complex prescriptions and 700 natural agents including *Aloe vera* (aloe), *Boswellia carteri* (frankincense) and the oil of *Ricinus communis* (castor) [103]. Numerous natural products have been used by the Indian *Ayurveda*, a medical system using natural products shown to have strong anti-inflammatory and medicinal properties [104].

Continued investigation of NHPs has allowed for their identification as potential cancer therapies due to their high efficacy and ability to be selective for cancer cells. This has led to NHP and NHP derivatives to come to the forefront in research to find alternative approaches to cancer treatment [105]. The anti-cancer activity of NHPs has been shown to be a result of multiple bioactive compounds targeting various vulnerabilities of cancer cells, rather than the effects of
one bioactive compound. These whole plant materials have been widely used for the extent of recorded history, and placing a focus on whole plant extracts may allow for the development of an efficacious treatment that is well-tolerated and may be taken for a long period of time to avoid cancer relapse and improve the quality of life of diagnosed patients [105].

Recently there have been many advances in NHP efficacy on cancer. NHPs have been shown to target many cancer cell mitochondrial vulnerabilities. Recent studies have shown that NHP and NHP derivatives are able to selectively target cancer cell metabolism and induce cell death [106-110]. Our own research on natural extracts of dandelion root [111], long pepper [112], lemongrass and white tea [113] were shown to be highly effective in inducing apoptosis via the generation of excessive ROS and dissipation of the MMP.

Currently, oncologists and clinicians rightfully do not recommend specific natural products for general consumption alongside of chemotherapy treatments [114]. This is primarily due to the risk of chemotherapy inhibition by NHPs that are known as powerful antioxidants, leading to reduced efficacy in treatment [115]. Many clinicians encourage their patients to use proven chemoprevention strategies to reduce their cancer risk due to the lack of solid scientific backing the usage of NHPs in cancer treatment. Further, they encourage patients to enroll in regulated clinical trials of NHPs to help build the sample data to potentially bring NHPs to market [114]. Thus, this thesis and recent NHP research in the last decade has focused on developing and investigating NHP in a standard, reproducible, and unbiased manner to validate them as effective anticancer agents.

Three NHPs have been studied for this thesis due to our previous experimental results showing promise as anticancer agents. *Taraxacum officinale*, more commonly regarded as the dandelion plant, has been used for centuries to treat abscesses, reduce inflammation, and treat
gastrointestinal diseases [116]. *Cymbopogon citratus*, commonly known as lemongrass, has traditionally been used to treat fever, inflammation, digestive disorders and cancer [117]. *Hibiscus rosa-sinesis*, the hibiscus flower, has traditionally been used and has been shown to have high pharmacological potential to treat disorders such as hypertension and pyrexia [118].

**Dandelion Root**

Preclinical research on dandelion has shown that dandelion root shows anti-inflammatory, anti-angiogenic, and anti-neoplastic properties [119]. Further, dandelion root has been shown to induce cytotoxicity due to its ability to induce oxidative stress, inflammation, and the secretion of apoptotic factors to induce apoptosis [120-122]. Dandelion root and leaf extracts showed potent radical scavenging and reducing activity [123]. Our research group has established the efficacy of dandelion root extract (DRE) on leukemia and pancreatic cancer, but its effects on prostate cancer has not yet been elucidated. Further, the interactions of DRE with common chemotherapeutics have yet to be investigated to validate their usage as an adjuvant in cancer treatment.

Compounds extracted from dandelion root extract include caffeic and chlorogenic acids along with vitexin-2-rhamnoside (apigenin). Further, hesperidin and myricetin were uniquely detected in the crude powdered root extract while luteolin-7-O-glucoside and isovitexin were both present in the leaf extract [123]. These compounds have been shown to be strong antioxidants while having anti-inflammatory properties [124-126]. However, the specific anti-cancer effects of these compounds have yet to be tested.

**Lemongrass**

Preclinical research on lemongrass has demonstrated the antiproliferative and antioxidative abilities of lemongrass extract (LGE). LGE showed anticancer efficacy on colon, breast, and ovarian cancer at very low EC$_{50}$ concentrations ranging from 68 μg/mL to 104.6 μg/mL while
having no significant effect on normal liver cells [127]. Lemongrass essential oils were shown to suppress the proliferation and survival of lung cancer cells, additionally enhancing the anticancer effect of chemotherapeutics [128]. Further, these oils showed antioxidative and anti-inflammatory properties on prostate and glioblastoma cell lines [129].

Our research on lemongrass has substantiated LGE validity by showing their significant anticancer efficacy on lymphoma using an *in vitro* and *in vivo* model [113]. Three compounds identified at higher concentrations in lemongrass (elemicin, lonicerin, and methylisoeugenol) showed very poor efficacy in reducing viability in lymphoma cells when treated individually. We believe that this could potentially be due to the fact that multiple phytochemicals may work together in natural extracts. Thus, the usage of whole plant extracts may allow for the targeting of multiple vulnerabilities of cancer.

**Hibiscus**

Preclinical research has shown that hibiscus extract (HE) exhibits significant anticancer efficacy on prostate cancer, leukemia, gastric cancer, and human squamous cell carcinoma [130-133]. Extracts of hibiscus have shown antioxidant, cholesterol-lowering, anti-obesity, insulin resistance reduction, anti-hypertensive, and skin cancer chemopreventative properties [134]. A previous study of *Hibiscus syriacus* observed that several triterpenoids, including betulin and betulinic acid, from HE inhibited triple-negative breast cancer cell viability with limited toxicity on normal cells [135]. Aqueous HE has also been shown to inhibit melanoma growth in an *in vitro* model [136]. In addition to botulin and betulinic acid, protocatechuic acid (PCA) has been shown to demonstrate significant antiviral activity, but no work on cancer has been conducted [137]. Hibiscus has shown high promise as a therapeutic, and merits further investigation as an anti-cancer treatment option.
1.7 Hypothesis, Objectives, Significance

Hypothesis

There is significance evidence supporting the importance of NHPs in drug development as well-tolerated treatments for cancer. As well, there is a necessity for the scientific validation to promote the usage of these NHPs alongside current treatment options in adjuvant therapy to mitigate chemotherapy-related toxicity and improve patient prognosis. This research focused on analyzing the anti-cancer efficacy of three NHPs, dandelion root extract (DRE), lemongrass extract (LGE), and hibiscus extract (HE), on cancer as well as determining the drug-drug interactions of these extracts.

Our hypothesis is that DRE, LGE, and HE are able to selectively target cancer cell vulnerabilities to induce apoptosis in a selective manner, and will be able to enhance the anticancer efficacy of chemotherapeutics when used in combination in cellular and animal models of cancer.

Objectives

To study this hypothesis, three major objectives were identified:

1. Evaluation of the ability of DRE, LGE, and HE to induce selective cell death in several in vitro and in vivo cancer models.

2. Evaluation of the adjuvant ability of DRE, LGE, and HE to enhance the anticancer efficacy of common chemotherapeutics in in vitro and in vivo cancer models.

3. Investigation into the mechanisms of action of DRE, LGE, and HE in cancer cell models
Significance

The collective burden of cancer and current cancer therapeutics indicate a critical need for the development of treatments which are more effective, selective, and financially reasonable. Many conventional cancer treatments are unable to selectively target cancer cells, and unfortunately will induce apoptosis in non-cancerous healthy cells. The purpose of this study is to develop NHPs as cancer therapeutics able to selectively induce apoptosis against cancer cells. Many oncologists are rightfully hesitant to promote the usage of NHPs alongside chemotherapeutics due to a lack of reproducible scientific validation. The scientific studies carried out with DRE, LGE, and HE will provide evidence allowing for the introduction of these NHPs as an efficacious anticancer therapy as well as a suitable treatment partner for many current therapeutics. In doing so, we will be able to provide a safer and cheaper complementary treatment for cancer to improve patient prognosis.
1.8 Chapter 1 References


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Chapter 2

Dandelion root and lemongrass extracts induce apoptosis, enhance chemotherapeutic efficacy, and reduce tumour xenograft growth \textit{in vivo} in prostate cancer

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2.1 Introduction

Prostate cancer remains one of the most commonly diagnosed cancers among men worldwide, accounting for 1 in 5 new diagnoses [1]. Advances in prostate cancer detection including improvement of prostate specific antigen detection methods [2], and functional MRI techniques [3, 4] have resulted in reduced mortality rates due to early diagnosis and treatment of the disease. However, prostate cancer related fatality is associated with late diagnoses, and progression to the metastatic castration-resistant stage [5]. At this stage, common treatment approaches involve chemotherapy treatment including first-line therapies such as mitoxantrone and docetaxel [5].

Current treatments for prostate cancer have included chemotherapy, radiation therapy, hormonal therapy, and cryosurgery among others [8]. Many chemotherapeutic treatment approaches have been developed, targeting vulnerabilities in cancerous cells to induce programmed cell death, also known as apoptosis [6, 7]. Unfortunately, although these treatments have shown efficacy, many adverse side effects have been observed in patients undergoing these treatments [8]. For example, cardiac toxicity has been observed in the usage of mitoxantrone treatment [9, 10]. These side effects can be attributed to the nonspecific nature of the treatment for cancer, resulting in the targeting of healthy noncancerous cells. However, aggressive metastatic cancers pose a constant threat of relapse following remission by chemotherapy treatment. Randomized phase 3 trials have shown that androgen deprivation therapy along with docetaxel improved relapse-free survival in prostate cancer patients [11], the long-term usage of chemotherapies is highly undesirable due to the inadvertent side effects. Thus, there is a great need for the development of treatment approaches that can avoid treatment-related toxicity and able to be used on a long-term basis.
The potential of natural health products (NHPs) in cancer treatments presents an interesting option that can be well-tolerated and could be used over a long period of time. NHPs are materials that have been extracted from plant sources that have exhibited medicinal properties [12]. Interestingly, many of the current chemotherapeutic compounds have been isolated from plant-based materials, for example paclitaxel (otherwise known as taxol) from the Pacific yew tree (*Taxus brevifolia*) bark extract [13]. It could be possible that there are plant extracts that are well-tolerated, have selective toxicity for cancer cells, and target multiple vulnerabilities of cancer cells.

Our research into dandelion root, lemongrass, long pepper, and hibiscus extracts have shown that these NHPs have the potential to induce apoptosis selectively in cancer cells [14-17]. These extracts have also been used widely and traditionally as food or medicinal products [18]. Thus, these extracts are well-tolerated and have the potential for long-term consumption to help prevent cancer remission. Although we have conducted research on dandelion root extract and lemongrass extract on colon cancer, leukemia, and lymphoma with high levels of success, their specific effect on prostate cancer is yet to be investigated. Furthermore, the interaction of DRE and LGE with standard cancer treatments has not yet been studied.

Dandelion (*Taraxacum officinale*) root has traditionally been used in Chinese, Arabian and Native American traditional medicine for the purpose of treating diseases including digestive ailments to cancers [19]. Lemongrass (*Cymbopogon citratus*) has also been used traditionally and has been shown to anti-inflammatory, anti-microbial, and radical scavenging anti-oxidant properties in many diseases [20-22]. As mentioned previously, both of these extracts have been investigated in colon cancer, leukemia, and lymphoma cells, but their effect on prostate cancer remain undetermined [14, 15, 17, 23]. Our objective was to investigate dandelion root extract (DRE) and lemongrass extract (LGE) to assess their ability to induce apoptosis in prostate cancer
cells *in vitro* and *in vivo*. More importantly, we wanted to assess the interaction of DRE and LGE with current chemotherapies to assess their potential use in adjuvant therapies.

In this study, we have shown that aqueous dandelion root extract (DRE) and ethanolic lemongrass extract (LGE) are able to induce apoptosis in prostate cancer in a dose and time-dependent manner. Further, we have demonstrated that both treatments are selective for prostate cancer with no significant effect on normal healthy cells. Importantly, we have shown for the first time that DRE and LGE display positive interactions with current chemotherapeutic agents (i.e. they are able to enhance the efficacy of chemotherapies) on prostate cancer. The efficacy of DRE and LGE were further demonstrated in prostate cancer xenografts in immunocompromised mice, showing a reduction of tumour burden by oral administration of DRE and LGE. These results support the potential of DRE and LGE as viable alternatives to current treatments, as well as their validity in adjuvant therapies. This in turn may lead to the development of a well-tolerated long-term treatment option.
2.2 Materials and Methods

Dandelion root aqueous extraction and Lemongrass ethanolic extraction

Asian dandelion (*Taraxacum officinale*) root were obtained from Premier Herbal Inc. (Toronto, ON, Canada). The flowers were ground using a coffee grinder into a fine powder. The powder was extracted in boiled double distilled water (ddH$_2$O) (1 g leaf powder to 10 mL ddH$_2$O) at 60°C for 3 hours. The extract was then run through a cheese cloth and then filtered via gravity filtration with a P8 coarse filter, followed by vacuum filtration with a 0.45 µm filter (PALL Life Sciences, VWR, Mississauga ON Cat No. 28148-028). The water extract was frozen at -80°C, freeze dried using a lyophilizer and then reconstituted in ddH$_2$O in order to obtain a final stock concentration of 100 mg/mL. Prior to use, the water extract was passed through a 0.22 µm filter (Sarstedt, Montreal, QC, CA Cat No. 83.1826.001) in a biosafety cabinet.

Lemongrass (*Cymbopogon citratus*) was obtained from Premier Herbal Inc. The lemongrass was ground using a coffee grinder into a fine powder. The powder was extracted in 100% anhydrous ethanol (1 g leaf powder to 10 mL anhydrous ethanol) at room temperature overnight. The extract was filtered via gravity filtration with a P8 coarse filter, followed by vacuum filtration with a 0.45 µm filter. The extract was evaporated using a RotoVap at 40°C and reconstituted in ethanol to obtain a final stock concentration of 200 mg/ml. The ethanolic extract was then passed through an Acrodisc® 0.2µm DMSO-safe syringe filter in a biosafety cabinet.

Cell culture

The prostate cancer cell line DU-145 (ATCC® HTB-81™) was cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) supplemented with 10% (v/v) fetal bovine serum (FBS, Catalog No. 12484-020, Thermo Scientific, Waltham, MA, USA) and 0.4% (v/v) gentamicin (Catalog No. 15710-064, Gibco BRL, VWR, Mississauga, ON, CA).
The prostate cancer cell line PC-3 (ATCC® CRL-1435™) was cultured in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) (ATCC® 30-2004™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.

The normal colon mucosa cell line (ATCC® CRL-1831™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.

All cells were maintained in an incubator at 37°C with 5% CO₂ and 95% humidity. All cells were passaged for less than 6 months.

**Trypan Blue exclusion assay**

Equal amounts of cells were seeded onto a 6-well clear bottom tissue culture plate and treated with various concentrations of aqueous dandelion root extract and chemotherapeutics. 48- and 96-hours post-treatment, cells were collected and washed with phosphate buffer saline (PBS). Cells were then re-suspended in 100µL of PBS and an equal amount (100µL) of Trypan Blue solution (Catalog No. T8154, Sigma-Aldrich, Mississauga, ON, CA). The number of viable cells were counted using the Countess® II FL automated cell counter (Life Technologies, Burlington, ON, CA). Results were analyzed using the GraphPad Prism 6 software and expressed as “Number of viable cells/mL” and expressed over a growth curve of the number of viable cells over the treatment period.

**Analysis of cell death: Annexin V (AV) binding assay and propidium iodide (PI)**

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.

**Reactive oxygen species (ROS) scavenging**

To assess the dependence on apoptotic induction on oxidative stress, the ROS scavenger N-acetylcysteine (NAC) was used to rescue cells. Cells were pretreated with 10 μL NAC (Sigma-Aldrich Canada, Cat. No. A7250, Mississauga, ON, Canada) for 30 minutes at 37°C at 5% CO₂. Cells were then treated for the indicated durations, collected, washed with phosphate buffer saline (PBS), and suspended in Annexin V binding buffer with green fluorescent Annexin V AlexaFluor-488 (1:20) and 0.01 mg/mL of red fluorescent PI 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. 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.

**Caspase inhibition**

To assess the caspase dependence of apoptotic induction, the caspase inhibitor Z-VAD-FMK was used to rescue cells. Cells were pretreated with 4 μL Z-VAD-FMK (Sigma-Aldrich
Canada, Cat. No. V116, Mississauga, ON, Canada) for 30 minutes at 37°C at 5% CO₂. Cells were then treated for the indicated durations, collected, washed with phosphate buffer saline (PBS), and suspended in Annexin V binding buffer with green fluorescent Annexin V AlexaFluor-488 (1:20) and 0.01 mg/mL of red fluorescent PI 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. 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.

**In vivo assessment of the dandelion root and lemongrass extract efficacy**

Immuno-compromised CD1 nu/nu mice, aged six weeks old, were obtained from Charles River Laboratories. Mice were housed and the protocols were followed using relevant guidelines and regulations that were approved by the University of Windsor Animal Care Committee (AUPP #17-15) in accordance with the Canadian Animal Care committee in a laboratory setting with 12-hour light/dark cycles. Following an acclimatization period, mice were injected subcutaneously with prostate cancer cells (DU-145, PC-3) cell lines suspension in Matrigel® at a concentration of 1.0x10⁶ cells per mouse in the hind flanks. Upon tumour formation, mice were randomly separated into four groups (control, DRE drinking water, and LG drinking water). Control and chemotherapeutic mice were given normal water, while DRE treatment groups received 40 mg/kg/day while LGE treatment groups received 16 mg/kg/day for 8 weeks. Mice were then sacrificed using CO₂ chamber followed by cervical dislocation, and tumours were harvested. Tumour volumes (using the formula ½*(L*W²) to calculate approximate volume) and body weights of each mouse were measured every other day throughout the length of the study.

**Statistical Analysis**

All statistical analysis was done using the GraphPad 6.0 Prism software. To test for
statistical significance a two-way analysis of variance (ANOVA) was used. All trials were conducted at least three independent times.
2.3 Results

Dandelion root and lemongrass extracts induce apoptosis in a dose- and time-dependent manner in prostate cancer cells

Hot water extract of dandelion root and ethanolic extract of lemongrass were prepared as described in the materials and methods. To investigate the anti-cancer efficacy of dandelion root extract (DRE) and lemongrass extract (LGE), prostate cancer cells were stained using Trypan Blue dye to assess cell permeability via image-based cytometry following 48- and 96-hour treatments. Prostate cancer cells (DU-145, PC-3) were additionally fluorescently stained with the apoptotic markers Annexin V (AV) and propidium iodide (PI). The cells were then subjected to fluorescent image-based cytometry following 48- and 96-hour treatments.

LGE was observed to be effective at inducing cell death through the Trypan Exclusion assay while DRE showed efficacy only at 96-hour treatment (Figure 1A). These observations were corroborated by fluorescence image cytometry results indicating that LGE and DRE are effective in inducing apoptosis as early as 48-hours and 96-hours post-treatment, respectively (Figure 1B). Specifically, significant apoptosis was observed in both prostate cancer cell lines for LGE beginning at dosages of 0.05 mg/mL (0.05 mg of crude extract in 1 mL of DMSO) at both timepoints. For DRE, significant apoptosis induction was observed beginning at 4 mg/mL treatments. Dosage and time dependent apoptosis induction was further observed in both cell lines as increased treatment concentration and treatment times increased apoptotic induction.

Both prostate cancer cell lines were further treated with taxol in order to compare the induction of apoptosis between standard chemotherapeutic treatment of taxol and DRE/LGE treatment. In both cell lines, DRE and LGE treatments resulted in comparable or enhanced apoptotic induction when compared to taxol treatment (Figure 1B).
Figure 1: Dandelion root and lemongrass extracts induce apoptosis in prostate cancer cells. (A) Prostate cell line DU-145 was treated with various treatments of dandelion root extract (DRE), lemongrass extract (LGE), and chemotherapeutics taxol and mitoxantrone and assessed at 48 hours and 96 hours. Results were obtained using image-based cytometry to assess the percentage of live cells compared to a vehicle control. (B) Prostate cell lines DU-145 (top panels) and PC-3 (bottom panels) were treated with various treatments of DRE, LGE, and chemotherapeutics and assessed at 48 hours and 96 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), propidium iodide (PI, red), both (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 < 0.05 vs. Control, **p < 0.01 vs. Control, ****p < 0.0001 vs. Control.
Dandelion root extract and lemongrass extract are selective for prostate cancer cells

If DRE and LGE treatments are selective for prostate cancer, the risk of drug-related side effects could be minimized. To assess whether DRE and LGE affects normal healthy non-cancerous cells, normal colon mucosa (NCM-460) cells were treated with DRE and LGE and assessed using fluorescent image-based cytometry as described above. Compared to an untreated control, DRE and LGE treatments showed no significant increase in apoptotic induction (Figure 2). The highest dosages used in this experiment (4 mg/mL DRE, 0.05 mg/mL LGE) were able to induce significant apoptosis when used to treat prostate cancer cells, but not NCM-460 cells. Further, while DRE and LGE treatments showed no effect on normal healthy cells, taxol induced apoptosis on these cells in a similar manner as on prostate cancer cells.

To further investigate the benefit of combinatorial treatments of DRE and LGE, the effect of DRE or LGE addition to chemotherapy treatment on NCM-460 cells was investigated. If protective, DRE or LGE would be able to minimize the amount of apoptotic induction on normal healthy cells by taxol. Indeed, we have observed that the addition of DRE and LGE to standard taxol chemotherapeutic treatment reduced the induction of apoptosis when compared to taxol treatment alone (Figure 2). These results indicate that DRE and LGE are selective for prostate cancer and can potentially protect normal healthy cells from being targeted by chemotherapy.
Interactions of dandelion root and lemongrass extracts with standard chemotherapies taxol and mitoxantrone

In standard treatments today, many chemotherapies are utilized in conjunction with other drugs. To properly assess the potential of DRE and LGE to be used in adjuvant or combination therapies in a novel treatment regimen, combination treatment assays were conducted to determine the interactions between DRE or LGE with taxol and mitoxantrone. These NHPs may either enhance, inhibit, or not affect chemotherapy efficacy. DU-145 prostate cancer cells were treated with taxol or mitoxantrone in the presence or absence of 4 mg/mL DRE (Figure 3) or 0.01 mg/mL LGE (Figure 4). As described in the materials and methods, both image-based cytometry and...
fluorescence microscopy were used to analyze the ability of these treatments to induce apoptosis in prostate cancer cells.

The addition of 4 mg/mL DRE to mitoxantrone treatments was able to significantly increase the induction of apoptosis when compared to mitoxantrone treatment alone (Figure 3A). However, DRE did not seem to have any interaction with taxol and did not inhibit its action. The addition of 0.01 mg/mL LGE to both taxol and mitoxantrone treatments were able to significantly increase apoptosis induction compared to individual treatments (Figure 4A). Interestingly, the lowest combination dosage of taxol treatment (0.01 μM with 0.01 mg/mL lemongrass extract) showed comparable induction of apoptosis to the highest individual treatment dosage of taxol (0.5 μM). This indicates that LGE combination treatment was able to show a similar apoptosis induction to individual treatment with a 50-fold decrease in chemotherapeutic concentration.

Morphological assays were conducted to assess the effect of treatments on cell morphology. Fluorescent microscopy following Annexin V and propidium iodide staining after combination DRE treatments (Figure 3B) and combination LGE treatments at 48 hours (Figure 4B) confirmed apoptosis induction as described above. These apoptotic markers were observed in treated prostate cancer cells as expected, along with apoptotic morphology including cell shrinkage, membrane blebbing, and nuclear condensation.
Figure 3: Dandelion root extract enhances chemotherapeutic anti-cancer efficacy when treated in combination on prostate cancer cells. (A) DU-145 cells were treated with chemotherapeutics taxol (top panel) and mitoxantrone (bottom panel) individually and in combination with 4 mg/mL dandelion root extract (DRE) and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), propidium iodide (PI, red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. The percentage of viable cells were graphed for both individual chemotherapeutic and combination chemotherapeutic treatments (graphs on right). (B) Fluorescence microscopy images of individual and DRE combination chemotherapeutic treatments. Top panels: Brightfield 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. 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, @p<0.05 vs. Individual Chemotherapy Treatment.
Figure 4: Lemongrass extract enhances chemotherapeutic anti-cancer efficacy when treated in combination on prostate cancer cells. (A) DU-145 cells were treated with chemotherapeutics taxol (top panel) and mitoxantrone (bottom panel) individually and in combination with 0.01 mg/mL lemongrass extract (LGE) and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), propidium iodide (PI, red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. The percentage of viable cells were graphed for both individual chemotherapeutic and combination chemotherapeutic treatments (graphs on right). (B) Fluorescence microscopy images of individual and LGE combination chemotherapeutic treatments. Top panels: Brightfield and fluorescent merged images at 400x magnification. Bottom: Fluorescent images stained with Annexin V (green), PI (red), and Hoechst (blue) at 400x magnification. Scale bar is 50 microns. Images are representative of 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, @p<0.05 vs. Individual Chemotherapy Treatment.
Dandelion root extract-induced apoptosis is caspase-dependent, whereas lemongrass extract-induced apoptosis is dependent on oxidative stress

DRE and LGE are extracts composed of a wide variety of compounds that can interact and function in complex manners. In order to further understand how these complex extracts exhibit their anti-cancer effects, the mechanism of apoptotic induction should be investigated. In order to determine if apoptosis is induced through oxidative stress, N-acetylcysteine (NAC) is used. NAC is a reactive oxygen species (ROS) scavenger and thus is able to rescue cells from apoptosis if the induction is primarily due to oxidative stress. LGE treatments on prostate cancer cells along with NAC were able to significantly reduce the amount of apoptosis induction when compared to individual LGE treatment whereas DRE treatments appear unaffected (Figure 5).

Further, to determine if apoptotic induction is caspase dependent or independent, Z-VAD-FMK, a commonly used broad-range caspase inhibitor, is used. If dependent, Z-VAD-FMK would be able to rescue cells and indicate dependence as described above. Indeed, DRE treatments were rescued when treating with DRE and Z-VAD-FMK, showing significant reduction of apoptotic induction when compared to individual DRE treatment (Figure 5). LGE treatments did not respond to caspase inhibition.
Oral administration of dandelion root and lemongrass extracts reduce the tumour burden in prostate cancer xenograft models in immunocompromised mice

To confirm and further support the anti-cancer efficacy of DRE and LGE, we wanted to investigate if these extracts have the ability to inhibit the growth of prostate cancer cells xenografted in immunocompromised mice. DU-145 and PC-3 cells were transplanted subcutaneously in mice. After tumour establishment, treatment groups were orally administered either DRE or LGE supplemented drinking water for eight weeks. Indeed, DRE and LGE were able to reduce the tumour burden of the xenografted mice as determined by tumour volume and weight compared to vehicle controls (Figure 6A-B). Over the course of treatment, there was no apparent change in the mice body weight gain in each treatment group when compared to controls, indicating that these treatments were well tolerated (Figure 6C). Thus, DRE and LGE were effective and well-tolerated in reducing tumour burden and growth in vivo when administered orally.

Figure 5: Dandelion root extract apoptosis induction is caspase dependent and lemongrass extract apoptotic induction is oxidative stress dependent. DU-145 cells were pre-treated with either N-acetylcysteine (NAC, reactive oxygen species scavenger) or Z-VAD-FMK (caspase inhibitor), treated with various dosages of dandelion root extract (DRE), lemongrass extract (LGE) and DRE or LGE combination treatments with taxol, and assessed at 48- and 96-hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), propidium iodide (PI, red), both (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 < 0.05 vs. Control, **p < 0.01 vs. Control, ****p < 0.0001 vs. Control, #p<0.05 vs. Individual DRE Treatment, @p<0.05 vs. Individual LGE Treatment.

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Figure 6: Dandelion root and lemongrass extracts administered orally reduce the tumour burden on prostate cancer xenograft mice. DU-145 (A) and PC-3 (B) prostate cancer cells were xenografted onto immunocompromised mice hind flanks subcutaneously. After tumour formation, these mice were orally fed dandelion root (DRE) and lemongrass (LGE) extract for 8 weeks. After mice were sacrificed, tumours (top panels) were excised and measured for tumour volume (using the formula \( \frac{1}{2}L \times W^2 \)) to calculate approximate volume and tumour weight. (C) Mouse body mass was measured two times a week and averaged to compare between control, DRE, and LGE groups. Statistical calculations were performed using One-Way ANOVA multiple comparison. \(*p < 0.05\) vs. Control, \(**p < 0.01\) vs. Control, \(****p < 0.0001\) vs. Control. No markings indicate non-significance vs. Control.
2.4 Discussion and Conclusions

We have demonstrated in this report that dandelion root extract (DRE) and lemongrass extract (LGE) induce cell death in prostate cancer cells in a time and dosage dependent manner (Figure 1). In contrast to chemotherapeutics like taxol, apoptosis induction by these extracts is very selective for prostate cancer cells without any significant effect on normal colon mucosa (NCM-460) (Figure 2). Importantly, we have shown that adjuvant treatment with DRE and LGE in combination with taxol or mitoxantrone enhanced the apoptosis induction capacities of these drugs (Figure 3, 4). Furthermore, we have evaluated the anti-cancer efficacy of both DRE and LGE in vivo where we have shown that these extracts were able to significantly and drastically reduce the tumour burden in mice with human prostate cancer xenografts. These extracts were administered orally, were well-tolerated, and did not affect mouse appetite or weight. These results support the potential of DRE and LGE supplementation in chemotherapeutic regimens due to observed enhancement of activity.

Previous work on DRE has shown its efficacy in inducing cell death in leukemia and colon cancer cells [14, 17]. However, the effects of DRE and LGE on human prostate cancer cells remained to be studied. Indeed, both DRE and LGE induced apoptosis in the two prostate cancer cell lines in a time- and dose-dependent manner. These cells displayed characteristic features of apoptosis including Annexin V binding. These extracts have been prepared using either water or ethanol, both being biocompatible solvents thus they fall in the category of food supplements. They have been traditionally consumed as teas or medicinal foods and have shown to be well-tolerated. Although the dosages for DRE and LGE appear higher compared to pure compound treatments used in therapeutics, it is important to note that these are crude extracts of dandelion root and lemongrass. These extracts may contain many common compounds (salts and sugars) in...
addition to bioactive phytochemicals responsible for anti-cancer activity. We have previously shown through phytochemical analysis on extracts of dandelion root, lemongrass, and long pepper (Piper longum) that the active compound concentration is very low [14-16]. Further, we have shown that the active components of DRE and long pepper extract were ineffective in inducing apoptosis when used alone [16] indicating that these extracts may be effective through multiple compounds working together and targeting multiple pathways.

Generally, oncologists are cautious in advising the usage of natural health products (NHPs) alongside chemotherapies. This is primarily due to the potential of NHPs to interact negatively with chemotherapeutic treatment, reducing the efficacy of these drugs. Therefore, it is extremely important to investigate the interaction of these extracts with standard chemotherapeutics. We investigated these interactions between DRE, LGE, and common chemotherapeutics. Indeed, we have demonstrated that addition of 4 mg/mL DRE enhanced the efficacy of mitoxantrone while not significantly affecting taxol (Figure 3). Further, addition of 0.01 mg/mL LGE was able to enhance the efficacy of both taxol and mitoxantrone treatments (Figure 4). These results clearly indicate that the interaction that these NHPs have with chemotherapeutics are positive, enhancing the effect of chemotherapy. Most interestingly, addition of DRE to 1 nM mitoxantrone treatment indicated similar apoptotic induction at a concentration of 20 nM mitoxantrone alone. Similarly, addition of LGE to 0.01 μM taxol resulted in effects similar to 0.5 μM taxol. These results indicate that adjuvant therapy with DRE and LGE could potentially reduce the concentration required, thus avoiding drug-related adverse side-effects. Thus, we have shown for the first time that these NHPs can be used as adjuvants to chemotherapies and potentially enhance their effect. Natural extracts have been used in patients on chemotherapies to enhance their quality of life in some cases [24].

The mechanism of apoptosis induction by these extracts in prostate cancer is a topic of
great interest to determine the underlying cause of cell death. We have previously demonstrated that DRE and LGE were able to induce oxidative stress and decrease mitochondrial membrane potential, leading to apoptosis induction in cancer cells [14, 15]. Though the exact mechanism is not fully elucidated, it has been hypothesized that high reactive oxygen species (ROS) levels can activate cellular stress mechanisms and may sensitize cancer cells to further ROS production leading to apoptosis [25]. N-acetylcysteine (NAC), a known ROS scavenger, is able rescue cells by inhibiting apoptosis induction via oxidative stress [26]. Further, we can use the caspase inhibitor Z-VAD-FMK in order to assess the dependency of apoptosis induction on caspase action in a similar manner. This assay allows for the assessment of whether our treatments are ROS-dependent or caspase-dependent to induce apoptosis. Indeed, we have observed that LGE treatment along with NAC was able to rescue DU-145 cells (Figure 5) while LGE treatment with Z-VAD-FMK did not inhibit apoptosis, indicating that LGE treatment is ROS-dependent and caspase-independent. Interestingly, DRE treatment did not show any inhibition by NAC but was inhibited by Z-VAD-FMK, indicating that DRE treatment is caspase-dependent and ROS-independent. It is important to note that while these treatments showed some reduction, no cells were completely rescued, indicating that the mechanism of action of these extracts may be multi-faceted and should be further investigated. Further work could look into quantifying the amount of ROS being produced via 2’, 7’-dichlorofluorescin diacetate (H2DCFDA) treatment or assessing different mechanisms of action to further assess these extracts.

As indicated previously, taxol and mitoxantrone have shown inadvertent side-effects due to the non-selective targeting [9, 10, 27]. Indeed, we observed that taxol was toxic to normal colon mucosa (NCM-460) cells (Figure 2). We have shown in this study that LGE and DRE treatments were selectively toxic to prostate cancer cells without affecting normal non-cancerous cells (Figure
2). Interestingly, addition of DRE or LGE to taxol was able to significantly reduce the extent of apoptosis in normal healthy cells compared to taxol treatment alone. This protective effect indicates the potential for DRE and LGE use in long-term adjuvant therapy in order to reduce the drug-related toxicity often associated with chemotherapy.

Some crude natural extracts have been shown to have anti-cancer efficacy against prostate cancer, but these studies were limited to in vitro work [28-30]. It is very important to assess the efficacy of natural extracts against prostate cancer using in vivo mice models with human prostate cancer xenografts. We carried out in vivo studies in which the mice (with human prostate cancer xenografts) were given DRE and LGE orally in their drinking water after tumour establishment. Indeed, both DRE and LGE were able to reduce the tumour burden of prostate cancer tumours significantly (Figure 6A-B) while having minimal effect on the overall weight gain of the mice (Figure 6C) indicating general tolerance. These results indicated that the anti-cancer bioactive compounds in these extracts must have been absorbed and stable upon systematic consumption and eventually affecting the tumour growth on subcutaneous site. This is important as we must consider that treatment was administered orally through mouse drinking water as opposed to methods such as intravenous or intraperitoneal administration. We have already demonstrated that both DRE and LGE are well-tolerated in mouse models [14, 15]. Thus, due to the fact that DRE and LGE treatment were efficacious against prostate cancer and well-tolerated, these NHPs are safe for long-term consumption prolonging the remission period.

Conclusions

In this study, we have demonstrated the efficacy of DRE and LGE for inducing cell death selectively in prostate cancer cells. For the first time, we have shown positive interactions of DRE and LGE with standard chemotherapeutics like taxol and mitoxantrone as they enhance their ability
to induce apoptosis. Most importantly, addition of DRE and LGE led to reduced dosages of chemotherapies with same extent of apoptosis compared to individual treatments (at high doses). Thus, DRE and LGE can reduce the drug-related toxicity with chemotherapeutics by reducing the dosage required for treatment. Further, oral administration of DRE and LGE to mice with human prostate cancer xenografts was able to significantly reduce the tumour burden. These extracts were well-tolerated in these mice as indicated by normal weight gain and food intake. Thus, these results suggest that DRE and LGE could potentially be used alongside mitoxantrone and taxol as adjuvants to enhance the efficacy of these drugs as well as improve the quality of life due to reduced toxicity for prostate cancer patients. These findings also provide support for the further development of these NHPs as a promising anticancer option to treating prostate cancer.
2.5 Chapter 2 References


Chapter 3

Hibiscus flower extract selectively induces apoptosis in breast cancer cells and positively interacts with common chemotherapeutics

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3.1 Introduction

Breast cancer is the most prevalent cancer among women worldwide, accounting for 25% of cancer incidence and 15% of cancer deaths among females [1]. Current work has developed and enhanced prediction models, screening methods, diagnostic tools, and disease management [2-6]. However, breast cancer treatment approaches become more complicated once the disease progresses to the complex metastatic stage. Although surgery to remove tumours in breast cancer has a high probability of survival, the majority of breast cancer related deaths are not from the primary tumour itself, but a result of metastasis to organs [7].

Apoptosis is the complex and ordered physiological process of cell death. An understanding of cell death, particularly in relation to cancer, allows for an assessment of the pathogenesis and treatment of the disease [8]. The exploitation of cellular vulnerabilities in cancerous cells, including oxidative stress and mitochondrial membrane destabilization, by therapeutic agents could trigger apoptosis and potentially eradicate the disease [9,10]. Indeed, most therapeutics have been developed to induce cell death. However, many treatments are unfortunately nonspecific for cancer and can additionally target healthy non-cancerous cells eventually leading to inadvertent side effects and toxicity [11,12].

Current treatments for metastatic breast cancer include adjuvant chemotherapy using cytotoxic drugs including anthracyclines, taxane-based, and platinum-based drugs [13]. Although both taxane-based and platinum-based chemotherapeutics have shown effectiveness in treating breast cancer, both drugs have exhibited toxicity and lack of selectivity to support a long-term treatment plan [11,12]. One study evaluating over 1000 patients found that treatments of anthracycline and taxane-based adjuvant strategies led to a higher pathologic complete response and higher survivability. However, a high risk of tumour relapse is possible if the tumour is not
completely eradicated [14,15]. Thus, there exists a great need for a treatment that can avoid toxicity in treatments while also able to be used on a long-term basis.

Natural health products (NHPs) are materials isolated from various food and plant sources that have been shown to have medicinal properties [16]. The commonly used chemotherapeutic taxol was isolated from the bark extract of the Pacific yew tree, *Taxus brevifolia*, when the extract was shown to have a cytotoxic effect [17]. Although many treatments have been derived from natural sources, we have yet to exhaust nature’s vast variety of selection. It is possible that a well-tolerated, highly potent anticancer compound is still left to be discovered and developed into a novel cancer therapeutic. Indeed, many NHPs have been shown to induce apoptosis selectively in cancer cells, including our research into dandelion root, lemongrass, and long pepper extracts [18-20]. Traditionally, NHPs have been used widely as both medicinal and food products [21].

Hibiscus flower (*Hibiscus rosa-sinensis*) has traditionally been used and has been shown to have high pharmacological potential to treat disorders such as hypertension and pyrexia [22]. Further, hibiscus extract (HE) has been shown to have significant antioxidant and hypolipidemic effects [23]. Previous work on hibiscus has indicated that HE exhibits significant anticancer efficacy on prostate cancer, leukemia, gastric cancer, and human squamous cell carcinoma [24-27]. A previous study of *Hibiscus syriacus* observed that several triterpenoids from HE were able to inhibit triple-negative breast cancer cell viability with limited toxicity on normal cells [28]. This work lends support to the notion that a whole plant extract of hibiscus could contain anticancer compounds while being well-tolerated.

Triple-negative breast cancer accounts for approximately 15-20% of all breast cancers and is characterized by negative expression of estrogen and progesterone receptors as well as HER2 protein [29]. Many challenges arise in the treatment of triple-negative breast cancer due to poor
prognosis resulting from the lack of actionable targets in order to use a specific targeted therapy able to combat the disease [30,31]. As such, the discovery and development of therapies able to target triple-negative breast cancer is of great importance.

We aimed to investigate the efficacy of HE against breast cancer by assessing the toxicity of HE treatment on human triple-negative and estrogen-receptor positive (ER+) breast cancer cells. Further, we aimed to investigate its interaction with current chemotherapies to assess the potential of its use in adjuvant therapies.

In this study, we have shown that aqueous HE is able to induce apoptosis in breast cancer cell models in vitro in a dose-dependent manner. We have also shown that HE treatment shows selectivity for cancer cells, with minimal effect on normal non-cancerous cells. Most importantly, we wanted to investigate the potential of using HE as an adjuvant to current chemotherapeutic treatments. We have demonstrated HE treatments (when combined with chemotherapeutic treatments) enhanced the induction of apoptosis when compared to individual treatment alone. These results support the possibility of supplementing chemotherapeutic regimens with HE, which has shown to be well-tolerated in normal non-cancerous cells. This may lead to a better combined effect, reducing the chemotherapeutic dosages and related toxicity.
3.2 Materials and Methods

**Hibiscus leaf aqueous extraction**

Hibiscus flower (*Hibiscus rosa-sinensis*) were obtained from Premier Herbal Inc. (Toronto, ON, Canada). This aqueous extraction protocol is similar to that previously published with the following modifications [18,19]. The flowers were grinded using a coffee grinder into a fine powder. The powder was extracted in boiled double distilled water (ddH₂O) (1 g leaf powder to 10 mL ddH₂O) at 60°C for 3 hours. The extract was then run through a cheese cloth and then filtered via gravity filtration with a P8 coarse filter, followed by vacuum filtration with a 0.45 µm filter (PALL Life Sciences, VWR, Mississauga ON, CA Cat No. 28148-028). The water extract was frozen at -80°C, freeze dried using a lyophilizer and then reconstituted in ddH₂O in order to obtain a final stock concentration of 100 mg/mL. Prior to use, the water extract was passed through a 0.22 µm filter (Sarstedt, Montreal, QC, CA Cat No. 83.1826.001) in a biosafety cabinet.

**Cell Culture**

The breast cancer cell line MCF-7 (ATCC® HTB-22™) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™) supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo Scientific, Waltham, MA, USA, Cat No. 12484-020) and 0.4% (v/v) gentamicin (Gibco BRL, VWR, Mississauga, ON, CA Cat No. 15710-064).

The breast cancer cell line MDA-MB-231 (ATCC® HTB-26™) were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.
The normal human skin fibroblast cell line (NHF; Coriell Institute for Medical Research, Cat. No. AG09309, Camden, NJ, USA) were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.

All cells were maintained in an incubator at 37ºC with 5% CO₂ and 95% humidity. All cells were cultured for less than 6 months with regular passaging.

**Analysis of cell death: annexin V binding assay and propidium iodide**

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 treated with various concentrations of hibiscus flower extract similar to those published previously with aqueous extracts of dandelion root and white tea [18,19]. Cells were then treated individually or in combination with chemotherapeutics taxol, cisplatin, and tamoxifen as indicated in the results section. This protocol is similar to that previously published [18,19]. Cells were washed with phosphate-buffered 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, Burlington, ON, CA, Cat No. A13201) and 0.01 mg/mL of red fluorescent PI (Life Technologies Inc, Burlington, ON, CA, Cat No. P3566) 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, Burlington, ON, CA, Cat No. T10796). 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.

**Reactive oxygen species (ROS) quantification**

Whole cell ROS generation was monitored with the small molecule 2′, 7′-dichlorofluorescin diacetate (H$_2$DCFDA). H$_2$DCFDA enters the cell and is deacetylated by esterases and oxidized by ROS to the highly fluorescent 2′, 7′-dichlorofluorescein (DCF) (excitation 495 nm; emission 529 nm). This protocol is similar to that previously published [18,19]. 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, collected, centrifuged at 3500 × g for 5 minutes, and resuspended in PBS. Percentage of DCF positive cells was quantified using the Tali Image-Based Cytometer (Life Technologies Inc, Burlington, ON, CA, Cat No. T10796) using 13 random fields per group with the green channel (excitation 458 nm; emission 525/20 nm). Cells were monitored with microscopy and counterstained with Hoechst 33342. Images were taken with a Leica DMi6000 fluorescent microscope (Wetzlar, Germany) at 400x magnification using LAS AF6000 software.

**Mitochondrial potential monitoring**

Tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR, Mississauga, ON, CA, Cat No. 89139-392) was used for detecting mitochondrial membrane potential (MMP), an indicator of healthy intact mitochondria. This protocol is similar to that previously published [18,19]. Following incubation with TMRM, cells were collected, washed with 1x PBS, resuspended in PBS, and then analyzed using the Tali Image-Based Cytometer (Life Technologies Inc, Burlington, ON, CA, Cat No. T10796). Cells from 13 random fields were analyzed using the
red (ex. 530 nm; em. 585 nm) channel. Cells were monitored with microscopy and counterstained with Hoechst 33342. Images were taken with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany) at 400x magnification using LAS AF6000 software.

**Statistical Analysis**

All statistical analysis was done using the GraphPad 6.0 Prism software. To test for statistical significance a two-way analysis of variance (ANOVA) was used. All trials were conducted at least three independent times.
3.3 Results

Hibiscus extract induces apoptosis in a dosage dependent manner in triple-negative and estrogen-receptor positive breast cancer cells

Hot water extract of hibiscus flower was prepared as described in the material and methods. To assess the ability of HE to induce apoptosis in breast cancer, triple-negative and ER+ breast cancer cells were fluorescently stained with apoptosis markers Annexin V (AV) and propidium iodide (PI). The cells were subjected to fluorescent image-based cytometry and fluorescent microscopy following 48- and 96-hour treatments.

HE was effective in inducing apoptosis in both triple-negative MDA-MB-231 and ER+ MCF-7 breast cancer cells (Figure 1A). Specifically, significant apoptosis induction was observed in both breast cancer cell lines at a dosage of 2 mg/mL (2 mg of crude lyophilized extract in 1 mL of ddH₂O). Dosage dependent apoptosis induction was observed in both cell lines as increasing treatment concentration increased the amount of apoptosis observed.

Both MDA-MB-231 and MCF-7 cells were additionally treated with tamoxifen, taxol, and cisplatin to compare the induction of apoptosis between standard chemotherapeutic treatments and HE. In both cell lines, tamoxifen and cisplatin treatments did not significantly induce apoptosis and taxol significantly induced apoptosis only in MDA-MB-231 cells (Figure 1A). HE treatment at 4 mg/mL caused significant induction of apoptosis at a comparable or greater level to all chemotherapeutics tested.

Morphological assays were conducted to assess the effect of treatments on cell morphology. Fluorescent microscopy using AV and PI after hibiscus treatments at 48 hours confirmed apoptosis induction due to hibiscus. These apoptosis markers were observed in MDA-
MB-231 breast cancer cells as expected, along with apoptotic morphology including cell shrinkage, membrane blebbing, and nuclear condensation (Figure 1B).

Figure 1: Hibiscus extracts induce apoptosis in breast cancer. (A) Breast cancer cell lines MDA-MB-231 and MCF-7 were treated with various treatments of HE and chemotherapeutics and assessed at 48 hours and 96 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. (B) Fluorescence microscopy images of 1.0, 4.0 and 5.0 mg/mL HE treatment on MDA-MB-231 cells were taken at 48 hours. Top panels: Brightfield 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. 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.
**Interaction of hibiscus extract with conventional chemotherapies tamoxifen, taxol, and cisplatin in combination treatments**

Commonly today, many chemotherapeutics are utilized in conjunction with other drugs. In order to assess if HE can be combined with chemotherapeutics in a novel treatment regimen, combination assays were conducted to determine whether or not hibiscus enhances, inhibits, or has no effect on chemotherapeutic potency. MDA-MB-231 and MCF-7 breast cancer cells were treated with tamoxifen, taxol, and cisplatin in the presence or absence 1 mg/mL HE. As described above, both image-based cytometry and fluorescent microscopy were used to analyze apoptosis induction.

In the triple-negative breast cancer cell line, MDA-MB-231, combination treatments of chemotherapeutics taxol and cisplatin with 1 mg/mL HE were able to significantly increase the induction of apoptosis when compared to chemotherapeutic treatments alone (Figure 2A). Interestingly, the lowest combination concentration of taxol treatment (0.01 μM with 1 mg/mL HE) showed similar apoptosis induction to the highest individual treatment concentration of taxol (0.5 μM). This indicates that combination treatment with 1 mg/mL HE was able to show a similar apoptosis induction to individual treatment with a 50-fold decrease in chemotherapeutic concentration. Using fluorescent microscopy, this result was confirmed with combination treatments of taxol and cisplatin along with HE showing a higher incidence of apoptotic marker fluorescence and increased apoptotic morphology when compared to individual chemotherapeutic treatments (Figure 2B).

In ER+ breast cancer cell line, MCF-7, combination treatments of chemotherapeutics tamoxifen, taxol and cisplatin with 1 mg/mL HE did not show any significant change in apoptosis induction when compared to individual treatments (Figure 3A). Although we did not observe any
enhancement, there was no inhibition observed. This result was confirmed using fluorescent microscopy (Figure 3B). However, it is important to note that the chemotherapeutic treatment ranges used did not show any significant apoptosis induction in MCF-7. As shown in Figure 1, HE at a concentration of 2 mg/mL showed significant apoptosis induction while combination treatments with 1 mg/mL did not induce significant apoptosis.
Figure 2: Hibiscus extracts indicate synergy with chemotherapeutics when treated in combination on triple-negative breast cancer cells. (A) MDA-MB-231 cells were treated with chemotherapeutics taxol (top panel) and cisplatin (bottom panel) individually and in combination with 1 mg/mL HE and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. The percentage of viable cells were graphed for both individual chemotherapeutic and combination chemotherapeutic treatments (graphs on right). (B) Fluorescence microscopy images of individual and hibiscus combination chemotherapeutic treatments on MDA-MB-231 cells were taken at 48 hours. Top panels: Brightfield 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. 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, @p<0.05 vs. Individual Chemotherapy Treatment.
Figure 3: Hibiscus extracts do not interact with chemotherapeutics in combination treatment on estrogen-receptor positive breast cancer. (A) MCF-7 cells were treated with chemotherapeutics tamoxifen (top panel, taxol (middle panel), and cisplatin (bottom panel) individually and in combination with 1 mg/mL HE and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. The percentage of viable cells were graphed for both individual chemotherapeutic and combination chemotherapeutic treatments (graphs on right). (B) Fluorescence microscopy images of individual and hibiscus combination chemotherapeutic treatments on MDA-MB-231 cells were taken at 48 hours. Top panels: Brightfield and fluorescent merged images at 400x magnification. Bottom: Fluorescent images stained with Annexin V (green), PI (red), and Hoechst (blue) at 400x magnification. Scale bar is 50 microns. Images are representative of 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, @p<0.05 vs. Individual Chemotherapy Treatment, # = not significant.
Hibiscus extract is selective in inducing apoptosis for breast cancer cells

If selective for breast cancer, individual and combinatorial HE treatment could potentially minimize adverse side effects by not affecting healthy cells. In order to investigate the selectivity of HE for breast cancer, normal human fibroblast (NHF) cells were treated with HE treatments and assessed in a similar manner as described above. Compared to control treatments, there was no increase in apoptosis in HE up to 2 mg/mL at which we have observed significant apoptosis in cancer cells (Figure 1). There was minimal to no observable apoptosis induction when compared to the positive control taxol (at a high dosage known to be cytotoxic to normal human cells) using HE treatments that were highly efficacious when used to treat breast cancer cells (Figure 4A). These results were confirmed with fluorescent microscopy. Cells only began to show apoptotic marker fluorescence and apoptotic morphology at the highest HE concentration of 5 mg/mL (Figure 4C).

To further investigate the benefit of using a combination treatment of HE with chemotherapeutics, taxol and hibiscus combination treatments were compared to individual treatments of taxol on NHF cells. On their own, chemotherapeutics treatments showed toxicity (Figure 4B). They are non-selective compared to hibiscus. Most surprisingly, HE combination treatments did not lead to increased apoptosis induction when compared to individual treatments, but instead lowered the amount of apoptosis induction observed (Figure 4B). These results indicate that HE shows selectivity to breast cancer cells and potentially protects normal human healthy cells from being affected by chemotherapeutic treatments.
Figure 4: Hibiscus extracts are selective for cancer and reduce toxicity of chemotherapeutics. (A) NHF cells were treated with various dosages of HE and (B) hibiscus combination treatments with taxol and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (yellow), or negative for both Annexin V and PI (blue). Values are expressed as a mean ± SD from three independent experiments. The percentage of viable cells were graphed for both individual chemotherapeutic and combination chemotherapeutic treatments (graphs on right). (C) Fluorescence microscopy images of individual hibiscus treatments on NHF cells were taken at 48 hours. Top panels: Brightfield 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. 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, @p<0.05 vs. Individual Chemotherapy Treatment.
Hibiscus extract is able to induce apoptosis in breast cancer cells by increasing oxidative stress and targeting the mitochondria

HE is an extract composed of many compounds able to interact in a complex manner. Determining the method of apoptosis induction will allow for a greater understanding of how these complex extracts show the observed anticancer potency. In order to determine if HE is able to induce apoptosis in breast cancer through inducing oxidative stress, H₂DCFDA was used to monitor the generation of ROS in breast cancer cells treated with chemotherapeutics in the presence or absence of HE. Indeed, it was observed that individual HE treatment was able to induce significant ROS generation in treated cells (Figure 5A). Further, combination treatments on triple-negative MDA-MB-231 cells using chemotherapeutic and HE were able to significantly increase the generation of ROS in treated cells when compared to treatment in the absence of HE. These results were confirmed using fluorescence microscopy (Figure 5B).

Further, as HE is made up of multiple factors and components, some of these may also target the mitochondria. Tetramethylrhodamine methyl ester (TMRM) dye was used in order to visualize the mitochondria membrane potential (MMP) in treated cells. Interestingly, HE at 1 mg/mL did not show significant loss of the MMP but instead was able to amplify the loss of MMP in both triple-negative and ER+ breast cancer cells when present in chemotherapeutic treatment compared to when absent (Figure 6A). These results were confirmed using fluorescent microscopy (Figure 6B).
Figure 5: Hibiscus extract induces oxidative stress on breast cancer and enhances chemotherapeutic oxidative stress induction. (A) MDA-MB-231 (left) and MCF-7 (right) breast cancer cells were treated with chemotherapeutics taxol and cisplatin individually and in combination with 1 mg/mL HE and assessed at 3 hours post-treatment against a positive control of hydrogen peroxide ($H_2O_2$). Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with the generation of reactive oxygen species ($H_2DCFDA$, fluoresces green). Values are expressed as a mean ± SD from three independent experiments. (B) Fluorescence microscopy images of individual and hibiscus combination chemotherapeutic treatments on MDA-MB-231 and MCF-7 cells were taken at 3 hours. Left image in groupings: Fluorescent images stained with $H_2DCFDA$ (green) and Hoechst (blue) at 400× magnification. Right image in groupings: Fluorescent images stained with $H_2DCFDA$ (green) alone. Images are representative of 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, @p<0.05 vs. Individual Chemotherapy Treatment.
Figure 6: Hibiscus extract enhances chemotherapeutic ability to reduce mitochondrial membrane potential. (A) MDA-MB-231 (left) MCF-7 (right) breast cancer cells were treated with chemotherapeutics taxol and cisplatin individually and in combination with 1 mg/mL HE and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with mitochondrial membrane potential (TMRM, fluoresces red). Values are expressed as a mean ± SD from three independent experiments. (B) Fluorescence microscopy images of individual and hibiscus combination chemotherapeutic treatments on MDA-MB-231 and MCF-7 cells were taken at 48 hours. Fluorescent images stained with TMRM (red) and Hoechst (blue) at 400× magnification. Images are representative of 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, @p<0.05 vs. Individual Chemotherapy Treatment.
3.4 Discussion and Conclusions

In this study, we have shown that HE is able to induce apoptosis in both human ER+ and triple-negative breast cancer cell lines in vitro (Figure 2A). We have demonstrated that HE treatment is very selective in inducing cell death in cancer cells without any significant effect on NHF cells (Figure 4A). On the other hand, common chemotherapeutics like taxol were indiscriminate and induced apoptosis in both cancer and non-cancerous cells (Figure 4A). Most importantly, we have shown that addition of HE in combination with chemotherapeutic agents enhanced the induction of apoptosis in triple-negative breast cancer cells (Figure 2A). These results support the possibility of supplementing chemotherapeutic regimens with HE, which is well-tolerated in normal healthy cells. This may lead to a better combined effect, reducing the chemotherapeutic dosages needed in treatment and therefore reduce toxicity.

As indicated previously, breast cancer, primarily triple-negative, is highly resistant to chemotherapy treatment. We have shown that both triple-negative and ER-positive breast cancer cells are affected by HE treatment (Figure 2). HE has also been shown to induce apoptosis significantly around treatment of 2 mg/mL of crude extract in prostate cancer, with a similar dose-dependency [24].

A common hesitation of using natural health product extracts alongside chemotherapies is the possibility of negative drug interactions, leading to reduced efficacy in treatment. Our goal was to investigate whether or not combination HE treatments would inhibit, not affect, or enhance the efficacy of chemotherapeutic treatments. Indeed, we found that taxol and cisplatin treatments on triple-negative breast cancer cells were enhanced with the addition of 1 mg/mL (sublethal dosage in individual treatment) HE treatment (Figure 2) while unaffected in ER-positive breast cancer cells. These results clearly indicate that HE’s interaction with chemotherapeutic drugs is positive.
or has no interaction in breast cancer cells. If any effect was observed at all, HE treatment enhanced the efficacy of chemotherapeutic treatments. Moreover, HE combination treatments on NHF cells were able to reduce the toxicity of taxol (Figure 4A). Extent of apoptosis induced by 0.01 μM taxol in combination with HE was equivalent to that induced by 0.5 μM taxol alone (Figure 2A). This 50-fold decrease in effective chemotherapy concentration clearly indicates the possibility of reducing chemotherapeutic dosage to avoid adverse side-effects without sacrificing efficacy. As such, HE could serve a significant purpose in terms of adjuvant therapy.

The mechanism of apoptosis induction in breast cancer is a topic of great interest to determine the underlying cause of cell death. Previously, we have shown that ethanolic extracts of lemongrass and aqueous extracts of dandelion root were able to induce oxidative stress and decrease mitochondrial membrane potential, leading to apoptosis induction in cancer cells [18,19]. While the exact mechanism is not yet clear, it has been hypothesized that high ROS levels can activate cellular stress mechanisms and may sensitize cancer cells to further ROS production leading to apoptosis [9]. Indeed, our results indicate that HE treatment led to increased ROS generation in both triple-negative and ER+ breast cancer cells (Figure 5). Moreover, taxol and cisplatin treatments in combination with HE showed increased ROS generation when compared to individual treatments. This helps explain the increase in apoptosis induction of combination treatments compared to individual treatments as discussed above (Figure 3, 4). It should be noted that triple-negative breast cancer cells were more vulnerable to oxidative stress than ER-positive breast cancer cells. These are two different cells with varied susceptibilities, and the lowered ROS generation of ER-positive breast cancer treatment indicates either an alternate mechanism of apoptosis induction or a need for increased dosage. Further, we have demonstrated that HE
combination treatment is able to enhance the mitochondrial membrane potential reduction in breast cancer cells (Figure 6).

As indicated above, cisplatin and taxol have shown extremely toxic side effects due to a lack of selectivity in treatment. Studies have indicated that HE is well-tolerated in nude mice xenograft models while exhibiting an anti-metastatic and anti-tumour effect [24]. Hibiscus has been traditionally used and has shown to be well-tolerated when consumed by humans. Consumption has also been associated with many beneficial effects including supporting mitochondrial function, energy homeostasis and improvement of the cardiovascular health [32]. Indeed, we have shown that HE was selectively toxic to cancer cells wherein the lowest effective dose of HE on breast cancer (2 mg/mL) was unable affect NHF cells (Figure 4A). HE treatments in combination with chemotherapeutics were also able to reduce the toxicity in NHF cells and lower the amount of apoptosis induction when compared to chemotherapeutic treatments in the absence of HE (Figure 4B). As such, HE shows great potential as an adjuvant to these therapies and help render some selectivity in treatment for cancer. If HE treatment shows anticancer efficacy, it could be used over a long-term period of time without any side effects [33].

It is important to note that HE dosages may appear to be high compared to pure compound cancer therapeutics. However, it is important to note that this is an aqueous extract of the hibiscus flower, which contains mainly sugars, salts, and other naturally abundant compounds in flowers. Previous work on phytochemical analysis of many other extracts including long pepper (Piper longum) and dandelion root (Taraxacum officinale) have shown that the concentration of the active compound might be very low [18,20]. Further, our work on these NHPs showed that active compounds found in long pepper and dandelion root extract were ineffective in apoptosis induction when used alone [20]. This indicates the importance of multiple phytochemicals that work together
natural extracts. In this case, it represents a very interesting opportunity for further research into HE to identify and test the potency of active compounds in aqueous hibiscus flower extract.

Conclusions

The work presented in this study indicates great potential of NHPs such HE to treat breast cancer in combination with standard chemotherapies. HE has shown an ability to enhance apoptotic induction by chemotherapy treatments through an increase in ROS generation and mitochondrial membrane collapse on both triple-negative and ER-positive breast cancer cells. This result is significant due to the general difficulty in discovery of an effective treatment for resistant triple-negative breast cancer. Most importantly, addition of HE with chemotherapeutic treatment could produce desired level of apoptotic induction at very low dosages of chemotherapies compared to chemotherapies alone. Therefore, addition of HE can significantly reduce the drug-related toxicity of chemotherapeutics. Future work into assessment of HE can look into combinatorial effects on in vivo models to further investigate the potential of HE for human usage. We have shown that HE treatment has the potential to be used alongside tamoxifen, taxol, and cisplatin treatments without any inhibition of drug potency. Thus, these findings open up interesting opportunity for further development of NHPs as a promising anticancer treatment option.
3.5 Chapter 3 References


Chapter 4

Lemongrass (*Cymbopogon citratus*) effectively induces apoptosis in colon cancer cells and interacts with FOLFOX *in vitro* and *in vivo*

Manuscript currently in preparation for submission by Christopher Nguyen, Ivan Ruvinov, and Ben Scaria.
4.1 Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in Canada, comprising 13% of all cases in 2018. It is estimated that 1 in 13 men and 1 in 16 women in Canada will develop CRC during his or her lifetime, and of those, 28% and 19% respectively are expected to die [1]. Thus, it is critical that we develop more accurate diagnostic methods, better-researched preventative measures, and most importantly, safer treatment plans.

Chemotherapy cancer treatment consists of the usage of chemical agents able to halt the growth of tumors and potentially reduce their size. They can be used alone or in combination with other therapies such as surgery and radiation, however their therapeutic usage is nonselective and leads to toxicity in healthy noncancer cells. They often target characteristics of cells which are not unique to cancerous cells, thus additionally affecting healthy cells and resulting in serious side effects to patients [2]. The most common chemotherapy treatment for CRC is a combination of fluorouracil (5-FU), folinic acid (LV), and oxaliplatin, otherwise collectively known as FOLFOX. Although it is generally successful, this treatment does have toxic side effects including gastrointestinal and neuro-toxicities [3]. Due to the potential for further patient risk due to adverse side effects, it is advisable to develop selective treatments able to target cancer cells and induce cell death.

Natural health products (NHPs) have been used traditionally for their medicinal properties and wide applications. Although their usage has been recorded over thousands of years, they often lack scientific credibility and experimental validation and are thus overlooked by medical professionals when prescribing treatments. Regardless, many chemically synthetic drugs have derived from plants or are analogous to compounds found in plants [4]. Due to their relatively
inexpensive cost and abundancy, researchers are now shifting their attention to development of NHPs as well-tolerated and effective treatments for cancer.

Health care professionals are often hesitant to allow patients undergoing cancer treatments to experiment with NHPs due to the possibility that they might interfere with the anticancer effects of the chemotherapy agents. However, it has been reported that increases in oxidative stress may interfere with chemotherapeutic efficacy in inducing apoptosis in cancer cells [5]. Indeed, previous studies involving the treatment of cancers using NHPs have shown underlying mechanisms involving increases in reactive oxygen species (ROS) production [6,7]. It is therefore vital to conduct a scientifically sound evaluation of the drug-drug interactions between different treatments before medical professionals can comfortably allow NHP intake alongside chemotherapy treatments.

Herein, we report lemongrass extract’s ability to induce apoptosis in CRC in vitro in colon cancer cell lines and in vivo in colon cancer tumour xenograft mice models. LG was able to not only effectively cause cancer cell death on its own, but also enhance the anticancer effects of two standard chemotherapy drugs when administered in combination. These results also translated into an animal study wherein oral administration of lemongrass extract was able to drastically reduce tumor growth of xenografted mice alone and in combination with FOLFOX. More importantly, lemongrass extract treatments proved to be non-toxic to normal colon cells in cell culture and to the mice in the animal study.
4.2 Materials and Methods

Lemongrass extraction

Lemongrass (Cymbopogon citratus) will obtained from Premier Herbal Inc. The lemongrass was grinded using a coffee grinder into a fine powder. The powder was extracted in 100% anhydrous ethanol (1 g leaf powder to 10 mL anhydrous ethanol) at room temperature overnight. The extract was filtered via gravity filtration with a P8 coarse filter, followed by vacuum filtration with a 0.45 µm filter. The extract was evaporated using a RotoVap at 40°C and reconstituted in ethanol to obtain a final stock concentration of 200 mg/ml. The ethanolic extract was then passed through an Acrodisc® 0.2µm DMSO-safe syringe filter in a biosafety cabinet.

Cell Culture

The colon cancer cell line HT-29 (ATCC® HTB-38™) were cultured in McCoy’s 5A Medium (ATCC® 30-2007™) supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo Scientific, Waltham, MA, USA, Cat No. 12484-020) and 0.4% (v/v) gentamicin (Gibco BRL, VWR, Mississauga, ON, CA Cat No. 15710-064).

The colon cancer cell line HCT116 (ATCC® CCL-247™) were cultured in McCoy’s 5A Medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin. The normal colon mucosa cell line (ATCC® CRL-1831™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.

The normal colon mucosa cell line NCM-460 (ATCC® CRL-1831™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC® 30-2002™) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.4% (v/v) gentamicin.
All cells were maintained in an incubator at 37°C with 5% CO₂ and 95% humidity. All cells were cultured for less than 6 months with regular passaging.

**Analysis of cell death: annexin V binding assay and propidium iodide**

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 then treated individually or in combination with chemotherapeutics FOLFOX and taxol as indicated in the results section. Cells were washed with phosphate-buffered 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, Burlington, ON, CA, Cat No. A13201) and 0.01 mg/mL of red fluorescent PI (Life Technologies Inc, Burlington, ON, CA, Cat No. P3566) 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, Burlington, ON, CA, Cat No. T10796). 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.

**Reactive oxygen species (ROS) quantification**

Whole cell ROS generation was monitored with the small molecule 2’, 7’-dichlorofluorescin diacetate (H₂DCFDA). H₂DCFDA enters the cell and is deacetylated by esterases and oxidized by ROS to the highly fluorescent 2’, 7’-dichlorofluorescein (DCF) (excitation 495 nm; emission 529 nm). Cells were pretreated with 20 μM H₂DCFDA (Sigma-
Aldrich Canada, Cat. No. D6883, Mississauga, ON, Canada) for 30 minutes at 37°C protected from light at 5% CO₂. Cells were treated for the indicated durations, collected, centrifuged at 3500 × g for 5 minutes, and resuspended in PBS. Percentage of DCF positive cells was quantified using the Tali Image-Based Cytometer (Life Technologies Inc, Burlington, ON, CA, Cat No. T10796) using 13 random fields per group with the green channel (excitation 458 nm; emission 525/20 nm). Cells were monitored with microscopy and counterstained with Hoechst 33342. Images were taken with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany) at 400x magnification using LAS AF6000 software.

**Mitochondrial potential monitoring**

Tetramethylrhodamine methyl ester (TMRM) (Gibco BRL, VWR, Mississauga, ON, CA, Cat No. 89139-392) was used for detecting mitochondrial membrane potential (MMP), an indicator of healthy intact mitochondria. Following incubation with TMRM, cells were collected, washed with 1x PBS, resuspended in PBS, and then analyzed using the Tali Image-Based Cytometer (Life Technologies Inc, Burlington, ON, CA, Cat No. T10796). Cells from 13 random fields were analyzed using the red (ex. 530 nm; em. 585 nm) channel. Cells were monitored with microscopy and counterstained with Hoechst 33342. Images were taken with a Leica DMI6000 fluorescent microscope (Wetzlar, Germany) at 400x magnification using LAS AF6000 software.

**In vivo assessment of lemongrass extract efficacy and combinations with FOLFOX**

Immuno-compromised CD1 nu/nu mice, aged six weeks old, were obtained from Charles River Laboratories. Mice were housed and the protocols were followed using relevant guidelines and regulations that were approved by the University of Windsor Animal Care Committee (AUPP #17-15) in accordance with the Canadian Animal Care committee in a laboratory setting with 12-hour light/dark cycles. Following an acclimatization period, mice were injected subcutaneously
with colon cancer cells (HT-29, HCT 116) cell lines suspension in Matrigel® at a concentration of 1.0x10^6 cells per mouse in the hind flanks. Upon tumour formation, mice were randomly separated into four groups (control, LG drinking water, FOLFOX subcutaneous injection, LG drinking water and FOLFOX subcutaneous injection). Control and chemotherapeutic mice were given normal water, while LGE treatment groups received 16 mg/kg/day for 8 weeks. At 4 weeks, subcutaneous injections of FOLFOX commenced once per week for 4 weeks. Mice were then sacrificed using CO₂ chamber followed by cervical dislocation, and tumours were harvested. Tumour volumes (using the formula $\frac{1}{2}*(L*W^2)$ to calculate approximate volume) and body weights of each mouse were measured throughout the length of the study once per week.

**Statistical Analysis**

All statistical analysis was done using the GraphPad 6.0 Prism software. To test for statistical significance a two-way analysis of variance (ANOVA) was used. All trials were conducted at least three independent times.
4.3 Results

Lemongrass extract displays anti-cancer activity in colon cancer and does so in a dose-dependent manner in HCT116 cell line.

We assessed the ability of lemongrass extract (LGE) to induce apoptosis in two colon cancer cell models at 48 hours post-treatment. Cells were treated with varying doses of LGE and assessed for apoptotic activity using annexin V (AV) binding, which detects the externalization of phosphatidylserine. Propidium iodide (PI) was simultaneously used to detect necrotic cells given its ability to intercalate DNA, indicating cell permeabilization and death. Cells positive for AV only (green) were considered to be early apoptotic cells, necrotic cells were detected as positive for PI only. Those positive for both AV and PI reflected late apoptotic cells. Finally, those negative for both AV and PI were considered as viable cells (Figure 1).

LGE was able to induce apoptosis in both CRC model cell lines at a 48-hour treatment time point. It displayed dose-dependent anticancer activity in the less-aggressive HCT116 cell line but was still able to significantly reduce cell viability in p53(-/-) HT-29 cell line. Staurosporine (STS) was used as a positive control for its ability to induce apoptosis [8].
Figure 1: Lemongrass extract induces apoptosis in colorectal cancer cells. Colon cell lines HCT 116 and HT-29 was treated with various treatments of lemongrass (LG) extract and compared to a positive control of staurosporin at 48 hours. Results were obtained using image-based cytometry to assess the percentage of live cells compared to a vehicle control. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), propidium iodide (PI, red), both (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 < 0.05 vs. Control, **p < 0.01 vs. Control, ****p < 0.0001 vs. Control.
Lemongrass extract is non-toxic in a non-cancerous colorectal cell line.

We assessed the ability of LGE to selectively kill cells using a normal colon cell model. Cells were treated with LGE at varying doses and monitored for the induction of apoptosis and necrosis using image-based cytometry for AV binding and PI staining (Figure 2). 48-hours after treatment, LGE displayed no significant apoptotic activity in normal colon (NCM-460) cell line.

Figure 2: Lemongrass extract is selective for cancer. Normal colon mucosa (NCM-460) cells were treated with various dosages of lemongrass (LG) extract with staurosporin and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), propidium iodide (PI, red), both (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. ns = not significant, *p < 0.05 vs. Control, **p < 0.01 vs. Control, ****p < 0.0001 vs. Control.
Lemongrass extract induces apoptosis in a time-dependent manner and does not inhibit the anticancer activity of standard chemotherapies FOLFOX and taxol.

On its own, lemongrass extract was able to induce apoptosis in colon cancer cell lines at very low doses. We next wanted to investigate whether treatment with LGE in combination with synthetic chemotherapeutic drugs would have any effect on cell apoptosis of colon cancer cell lines. Additionally, we wanted to observe whether these results would vary if the treatments are left for a longer period of time before results are obtained.

To determine whether LGE interacts with standard chemotherapeutic drugs to either enhance or reduce their effect, cells were treated with LGE alone and in combination with FOLFOX and taxol and monitored for the induction of apoptosis using AV binding and PI staining after 48 hours with image-based cytometry (Figure 3). No significant changes were observed between the individual taxol and combinatorial treatments involving lemongrass extract and standard chemotherapy drugs in HCT 116 cell line. However, FOLFOX apoptotic induction was enhanced when used in combination with LGE compared to FOLFOX treatment alone. In the more aggressive HT-29 cell line, a slight increase in cell apoptosis occurred when the treatments were used in combination, however not large enough to conclusively deem significant.

Cells were also observed with these identical agents at a later time point to observe the time-dependent effects of the treatments. Once treated, this batch of cells was monitored for the induction of apoptosis after 96 hours with image-based cytometry (Figure 3). Results showed the same pattern as the 48-hour trial where combination treatments in the HCT 116 cell line showed no increase or decrease in cell apoptosis whereas combination treatments in the HT-29 cell line only slightly enhanced cell death relative to the individual treatments. However, a slightly greater
proportion of cells from both cell lines indicated to be undergoing apoptosis at 96 hours compared to their 48-hour counterpart treatments.

Figure 3: Lemongrass extracts do not inhibit chemotherapeutic efficacy when treated in combination on colorectal cancer cells. HCT 116 and HT-29 cells were treated with chemotherapeutics FOLFOX and taxol individually and in combination with 0.025 mg/mL lemongrass extract and assessed at 48 hours and 96 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with Annexin V (green), PI (red), both (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 < 0.05 vs. Control, **p < 0.01 vs. Control, ****p < 0.0001 vs. Control, @p<0.05 vs. Individual Chemotherapy Treatment.
Changes in morphology and characterization of apoptosis following treatment with lemongrass extract alone and in combination with chemotherapeutics.

Qualitative analysis of HCT 116 and HT-29 cells was performed to complement quantitative results of combination treatments. Cells were incubated with lemongrass extract alone and in combination with standard chemotherapy drugs FOLFOX and taxol for 48 hours then subjected to inverted fluorescent microscopy (Figure 4). Green (AV) and red (PI) staining were used as markers of apoptosis and are especially prominent in the treatment groups. Bright-field images also show cell shrinkage and membrane blebbing as indicators of cell death.

Figure 4: Lemongrass extracts induce a change in colorectal cell morphology. HCT 116 (A) and HT-29 (B) cells were treated with chemotherapeutics FOLFOX and taxol individually and in combination with 0.025 mg/mL lemongrass extract and assessed at 48 hours. Fluorescence microscopy images of individual hibiscus treatments. Top panels: Brightfield 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.
**Lemongrass extract is able to induce apoptosis in CRC cells by increasing oxidative stress.**

LGE is an extract composed of many compounds able to interact in a complex manner. Determining the method of apoptosis induction will allow for a greater understanding of how these complex extracts show the observed anticancer potency. In order to determine if LGE is able to induce apoptosis in CRC through induction of oxidative stress, H2DCFDA was used to monitor the generation of ROS in CRC cells treated with FOLFOX in the presence or absence of LGE. Indeed, it was observed that individual LGE treatment was able to induce significant ROS generation in treated cells (Figure 5A). The combination sample of FOLFOX and LGE showed an increased capability to generate ROS compared to FOLFOX alone.

![Figure 5: Lemongrass extract induces oxidative stress on colorectal cancer and enhances FOLFOX oxidative stress induction.](image)

HCT 116 and HT-29 colorectal cancer cells were treated with FOLFOX individually and in combination with 0.025 mg/mL lemongrass extract and assessed at 3 hours post-treatment against a positive control of hydrogen peroxide (H2O2). Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with the generation of reactive oxygen species (H2DCFDA, fluoresces green). 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, @p<0.05 vs. Individual Chemotherapy Treatment.
Lemongrass extract causes depolarization of the mitochondrial membranes in CRC cell lines.

Although cell death mechanisms involving ROS and caspase activation were eliminated as activators of apoptosis in colorectal cancer cells, the release of other apoptogenic factors from the mitochondria may result in the induction of apoptosis. To monitor mitochondrial stability and depolarization, the fluorescent tetramethylrhodamine methyl ester (TMRM) assay was used. TMRM molecules accumulate in mitochondria with an intact mitochondrial membrane potential (MMP). HCT 116 and HT-29 cells were treated with the respective treatment groups and incubated for 48-hours and results were obtained using image-based cytometry. Interestingly, lemongrass extract exhibited an even greater ability to disrupt MMP than the positive controls taxol and FOLFOX, relative to the DMSO control (Figure 6).

![Figure 6: Lemongrass extract enhances chemotherapeutic ability to reduce mitochondrial membrane potential. HCT 116 and HT-29 colorectal cancer cells were treated with FOLFOX individually and in combination with 0.025 mg/mL lemongrass extract and assessed at 48 hours. Results were obtained using image-based cytometry to assess the percentage of cells positive with fluorescence associated with mitochondrial membrane potential (TMRM, fluoresces red). 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, @p<0.05 vs. Individual Chemotherapy Treatment.](image-url)
Oral administration of lemongrass extracts reduces the tumour burden in colon cancer xenograft models in immunocompromised mice and enhances the efficacy of FOLFOX

To confirm LGE efficacy, we wanted to investigate if these extracts have the ability to inhibit the growth of colon cancer cells xenografted in immunocompromised mice and potentially enhance the effects of FOLFOX. HCT 116 and HT-29 cells were transplanted subcutaneously in mice. After tumour establishment, treatment groups were separated into groups and orally administered either vehicle supplemented drinking water (control group), or LGE supplemented drinking water for four weeks. FOLFOX and FOLFOX with LGE groups were administered weekly intraperitoneal injections of FOLFOX. Indeed, LGE was able to reduce the tumour burden of the xenografted mice as determined by tumour volume and weight compared to vehicle controls (Figure 6A-B). Interestingly, LGE supplemented FOLFOX groups showed the largest reduction in tumour burden compared to control (Figure 7A-B). Over the course of treatment, there was no apparent change in the mice body weight gain in each treatment group when compared to controls, indicating that these treatments were well tolerated (Figure 7C).
Figure 7: Lemongrass extract administered orally reduces the tumour burden on colorectal cancer xenografted mice and enhances FOLFOX efficacy. HCT 116 (A) and HT-29 (B) colorectal cancer cells were xenografted onto immunocompromised mice hind flanks subcutaneously. After tumour formation, these mice were orally administered lemongrass extract for 8 weeks. After 4 weeks, FOLFOX was administered subcutaneously once a week. After mice were sacrificed, tumours were excised and measured for volume (using the formula $\frac{1}{2} \times (L \times W^2)$ to calculate approximate volume). (C) Mouse body mass was measured once a week and averaged to compare between experimental groups. Statistical calculations were performed using One-Way ANOVA multiple comparison. *$p < 0.05$ vs. Control, **$p < 0.01$ vs. Control, ****$p < 0.0001$ vs. Control. No markings indicate non-significance vs. Control.
4.4 Discussion and Conclusions

In order to test the ability of LGE to induce apoptosis we used annexin V and PI as staining markers for early and late stage apoptosis, respectively. Two colorectal cancer (CRC) cell lines were treated with increasing doses of lemongrass and assessed at 48 hours (Figure 1). In the HCT 116 cell line, apoptosis and necrosis induction were observed at doses as low as 0.025 mg/mL in a dosage dependent manner. The more aggressive HT-29 cell line also responded to LGE with reduced cell viability but did not do so in a dose-dependent manner. Both cell lines showed comparable cell death percentages between the lowest dose of lemongrass extract (0.025 mg/mL) and 1 μM staurosporine, an agent known to induce apoptosis at low doses (Figure 1).

Importantly, we additionally tested LGE at concentrations toxic to CRC cells on a normal colon cell line, NCM-460. These cells are healthy and not cancerous and provide an in vitro model for testing to see whether the extract is selective for cancer and able to avoid having detrimental effects on healthy cells tissues. Interestingly, NCM-460 cells did show any significant decrease in viability even at the highest dose of 0.1 mg/mL (Figure 2) indicating that LGE has no significant effect on normal colon cells. However, it is important to note that staurosporine also did not cause induction of apoptosis which may indicate that this cell line could be more resistant to chemical agents and could explain why LGE did not cause induction of apoptosis. To mitigate this, other healthy cell line models should be used to confirm this result. However, the selectivity of LGE as proposed by these results supports previous findings wherein the same extract did not cause a decrease in cell viability of NCM-460 cells, normal human fibroblast cells, and peripheral blood mononuclear cells [6]. Therefore, these results indicate that lemongrass extract induces selective apoptosis in colorectal cancer cells with no significant effect on normal healthy cells.

It is unlikely that NHPs will eventually be prescribed and used as singular treatments in cancer therapy approaches. Current approaches to treating cancer rely on the usage of multiple
therapeutics to target multiple vulnerabilities of cancer cells [9]. As mentioned, oncologists are hesitant to promote the usage of NHPs alongside chemotherapeutics due to the risk of negative interactions. Thus, it is critical to examine whether LGE, if taken as an adjuvant to chemotherapeutic drugs will enhance or inhibit their anticancer effects.

After treating HCT 116 and HT-29 cells with FOLFOX and taxol alone and in combination with LGE, cells were stained with AV and PI dyes as indicators of apoptotic induction. In HCT 116 cells, LGE in combination with FOLFOX resulted in statistically significant enhancement of anticancer efficacy. However, combination treatment with taxol did not affect cell viability relative to the individual taxol treatments (Figure 3). In HT-29 cells, FOLFOX and taxol treatments in combination with LGE did not show a statistically significant difference in apoptotic induction when compared to the individual treatments (Figure 3). Both cell lines showed increased cell death at the 96-hour time point, indicating that lemongrass may act in a time-dependent manner (Figure 3). Morphological changes such as membrane blebbing and cell shrinkage in addition to AV and PI staining in treatment groups using fluorescent microscopy (Figure 4). These results complemented the results presented previously and showed no decrease in apoptosis-related markers in combination groups. In general, LGE did not cause an increase in efficacy at inducing apoptosis when used in combination with FOLFOX and taxol. However, it is important to note that LGE did not inhibit the ability to induct apoptosis of FOLFOX and taxol.

This result may seem counterintuitive due to a lack of any enhanced efficacy observed in LGE combination treatments. However, our research group has reported beneficial effects of using LGE in combination with chemotherapeutics including taxol (reported in Chapter 2). Our investigation of LGE indicates that the efficacy of taxol would not be inhibited in CRC. Thus, patients who may have cases of prostate cancer metastases may benefit from LGE treatment should
the colon be the secondary site. It is responsible to report that LGE does not have a significant ability to enhance FOLFOX and taxol in CRC cell lines.

The induction of apoptosis by LGE has not yet been characterized. The induction of apoptosis by LGE may be dependent on the extract’s ability to induce oxidative stress. Indeed, LGE treatment induced significant generation of ROS in both CRC cell lines and enhanced the ability of FOLFOX to induce oxidative stress (Figure 5). However, the difference in ROS generation could be primarily due to the action of LGE, indicating the need for further analysis to conclusively analyze the true cause of this enhancement. The induction of caspase-independent apoptosis is dependent on the permeabilization of the outer mitochondrial membrane. If LGE apoptotic induction is dependent on mitochondrial membrane permeabilization, this may shed light on the specific cancer cell targets and give insight into how these extracts are selective. HCT 116 and HT-29 cells were treated with LGE alone and in combination with FOLFOX and taxol to assess effect of LGE treatment on mitochondrial membrane potential. LGE treatment led to drastic decrease in the mitochondrial membrane potential and was able to enhance the ability of FOLFOX and taxol to dissipate the MMP (Figure 6). This indicates that the chemotherapeutics treatments do not inhibit LGE action on the mitochondria.

It is important to investigate the effects of LGE on a more complex in vivo mouse model to assess its anticancer efficacy and identify potential toxicities in treatment. Immunocompromised mice were xenografted with human CRC cells and orally administered LGE alone or alongside FOLFOX intraperitoneal injection. Interestingly, LGE was able to reduce the tumour burden on all mice at a comparable level to FOLFOX injections (Figure 7A-B). Further, LGE in combination with FOLFOX injections reduced the tumour burden compared to LGE and FOLFOX treatments alone. This result is interesting due to the lack of significant enhancement observed in vitro on
CRC cell models. This may indicate that, at the animal level, LGE’s effects may be complex and sensitize mice to FOLFOX treatment. In addition, LGE treatment groups showed similar weight gain profiles with control mice, indicating that that treatment was generally well-tolerated (Figure 7C). Looking at FOLFOX injected mice, mice weight gain halted once injection treatments began, indicating that mice suffered adverse effects to treatment such as loss of appetite. However, oral administration of LGE was able to mitigate this effect in FOLFOX injected mice, as shown by similar weight gain profiles of these mice. These results indicate that the active bioactive compounds in these extracts were absorbed and stable when consumed, inhibited tumour growth on the subcutaneous tumour xenograft site, and are well-tolerated for general consumption.

Conclusions

Lemongrass extract shows great potential as an anticancer agent in colorectal cancer models. When used alone or in combination with conventional chemotherapeutics, low dosages of lemongrass selectively induce apoptosis in CRC and do not inhibit cytotoxic effects of the other drugs. LGE was able to enhance the ability to generate oxidative stress and dissipate of the mitochondrial membrane potential when used in combination with FOLFOX and taxol, however these results require further testing to identify whether or not this happens in healthy cell lines. In xenografted mice models of colon cancer, LGE was able to significantly reduce the tumour burden in mice, enhance the efficacy of FOLFOX, and reduce drug-related side effects. Ultimately, LGE has shown to be a well-tolerated treatment option for CRC that does not inhibit the effect of conventional therapeutics.
4.5 Chapter 4 References


Chapter 5

General Discussion
5.1 General Discussion

Prior to the last decade, there has been a lack of rigorous scientific investigation into NHP usage and a heavy dependence on anecdotal evidence. This has resulted in many oncologists being hesitant to recommend the usage of NHPs due to a lack of valid scientific representation and the risk of negative interactions with other treatments. However, research into natural products has come to the forefront in part due to the ability of NHP phytochemicals to exhibited anti-carcinogenic activities by interfering with the initiation, development and progression of cancer [1]. Bioactive extracts of NHPs may be associated with reduced risk of adverse side effects due to their traditional and wide usage. If these NHPs are used in combination with other pharmacologically active compounds, they may reduce the drug-related toxicity and allow for an increase in treatment efficacy [2]. Interestingly, 3 in 4 of every anticancer drug approved in the last 80 years have been obtained either directly from natural products or as derived analogs of natural product compounds [3]. Further, over 130 natural product-derived treatments are current undergoing clinical trials to potentially be brought forward to market [4]. However, before labelling any NHPs as safe for consumption and effective against cancer, studies with strong scientific backing must be conducted.

In this body of work, the usage of NHPs as a therapeutic approach for cancer treatment was validated in a reproduceable and unbiased manner. We identified three interesting NHP extracts that merited further investigation of their anticancer effects, dandelion root extract (DRE), lemongrass extract (LGE), and hibiscus extract (HE). Our group’s previous research on these extracts indicated that these extracts induce selective cancer cell death in multiple models of cancer [5,6]. However, the effects of DRE and LGE on prostate cancer, HE on breast cancer, and LGE on colon cancer have yet to be investigated. Thus, a large portion of my research focussed on the
ability and mechanism of apoptotic induction in these extracts using \textit{in vitro} cancer cell models and more complex \textit{in vivo} mouse models. Further, the interactions of these extracts with common chemotherapeutics have not yet been determined. In order to allow for further progression of NHPs as cancer therapeutics, studies must show that these treatments are capable of being used as adjuvants in combination therapies. This is due to the fact that current treatment approaches use multiple chemotherapeutics to target various cancer cell vulnerabilities. This work also aimed to identify whether or not DRE, LGE, and HE are able to have any significant interaction with chemotherapeutics.

Our findings indicate that DRE, LGE, and HE have significant anticancer efficacy on various cancer types. We determined that DRE was less efficacious on prostate cancer compared to previous findings showing its efficacy on cancers such as leukemia, pancreatic cancer, and colon cancer. Though still capable of inducing apoptosis in prostate cancer, this effect was not seen in a significant manner until 96 hours post-treatment. This may be explained due to increased drug resistance observed in prostate cancer as the diseases progresses towards a metastatic state \cite{7}. Further, this could be explained due to differences in cancer cell vulnerabilities leading to variation in responses to certain treatments. The induction of apoptosis was determined to be caspase-dependent which is consistent with previous findings. Further research should investigate into whether or not DRE can target the mitochondrial membrane to permeabilize and release pro-apoptotic factors, eventually resulting in caspase-dependent apoptosis. LGE was shown to be extremely effective in a dosage-dependent manner on both prostate and colorectal cancer models. In both cases, apoptotic induction was heavily dependent on the generation of reactive oxygen species to induce oxidative stress on cancer cells. Many treatments targeting to induce oxidative stress are known to be capable of overcoming cancer cell oxidative stress defence mechanisms to
ultimately induce cell death [8]. Finally, HE induced potent apoptosis on both estrogen-receptor positive and triple-negative breast cancer cells. Triple-negative breast cancer cells are notoriously resistant to treatment and this discovery is substantial in validating the anticancer efficacy of NHPs.

Current chemotherapeutic treatments for cancer are not selective and will adversely affect normal healthy cells. This is due to generalized targeting of cancer cells, resulting in normal cells being affected by treatment as well. For example, tubulin-targeting therapeutics may target the tubulin of any cell showing not only potent anticancer efficacy due to the rapid growth and reliance of cancer cells on tubulin, but also in normal healthy cells which rely on tubulin as well [9]. Many of the drug-related toxicities and side effects stemming from chemotherapeutic treatments are unable to be avoided [10]. In all cases, DRE, LGE, and HE showed no significant toxicity on normal healthy cells at concentrations that would otherwise toxicity in cancer cells. This indicates that these treatments have the ability to be well-tolerated if taken by humans.

The development of well tolerated anticancer agents able to synergize with chemotherapies is essential to allow for a reduction in concentrations of toxic drugs used with no loss in therapeutic success. In prostate cancer, we identified that DRE is able to enhance the efficacy of mitoxantrone, and that LGE is able to enhance the efficacy of both taxol and mitoxantrone. In breast cancer, we identified that HE is able to enhance both taxol and cisplatin in resistant triple-negative cells. It is also important to note that treatments of DRE, LGE, and HE were able to reduce the cytotoxic effects of chemotherapy treatment on normal healthy non-cancerous cells compared to chemotherapy treatment alone. This indicates that these extracts may not only enhance chemotherapy effects on cancer but protect normal cells from cytotoxicity. In colon cancer, LGE was not determined to significantly enhance FOLFOX or taxol aside from at one timepoint for
FOLFOX. However, none of these extracts inhibited the effect of chemotherapies on cancer indicating that they should be safe to be used in combination. Interestingly, LGE and HE combination treatments with chemotherapies enhanced their ability to significantly increase ROS production and reduce mitochondrial membrane potential. This may explain why these extracts are capable of synergizing with other treatments. These extracts may play a role in sensitization of cancer cells through the generation of oxidative stress, allowing for a loss of defence mechanisms against drugs, leading to enhanced apoptosis.

To better understand anticancer efficacy, in vivo studies were carried out on tumour xenografted mouse models. Although these NHPs have been used for centuries with no adverse effects reported, proper scientific validation on a more complex model of cancer is required. In this work, we show that DRE and LGE were able to significantly reduce the tumour burden on prostate cancer xenografted mice. We further show that LGE is also able to significantly reduce the tumour burden on colorectal cancer xenografted mice. These results show that the complex mixtures of DRE and LGE are able to be absorbed through the gastrointestinal tract and can be brought to tumour sites to exert their effects. In all cases, DRE and LGE oral administration to mice did not significantly decrease their weight gain profiles, indicating that these extracts were well-tolerated. Further, LGE treatment in colon cancer mice was surprisingly able to enhance the efficacy of FOLFOX and reduce related-toxicity. This is conflicting with in vitro results indicating that LGE and FOLFOX did not show significant synergy at 96 hours. This could be explained due to the fact that readily available water may have led to increased LGE consumption by mice, indicating that higher dosages of LGE may synergize well with FOLFOX. Another explanation could be that LGE consumption boosts the appetite of mice, explaining the mitigation of weight
loss in mice injected with FOLFOX. Due to an increase in mouse health, an improved ability to reduce tumour burden may have been observed.

Natural health product extracts are made up of complex mixtures containing multiple pharmacologically compounds. We have shown in our previous work that the isolated bioactive compounds of DRE and LGE were ineffective in inducing apoptosis when used individually. This indicates that these extracts may rely on multiple phytochemicals interacting and working together to target multiple vulnerabilities of cancer cells. This lends support to the notion that due to their ability to target characteristics that are selective for only cancer, these extracts are well-tolerated and suitable to be brought to market for human consumption.

This work provides sufficient evidence that DRE, LGE, and HE are highly effective and selective anticancer therapeutics that are able to synergize and interact positively with many common chemotherapies. Further, these extracts are able to drastically reduce the tumour burden on xenografted mice while exerting no side effects and even protecting mice against toxicity by chemotherapies. These NHPs further provide the essential scientific validation required to support the development of safer and less expensive approaches to cancer treatment.
5.2 Future Directions

The results presented in this thesis indicate that NHPs are promising anticancer therapies. However, there is still much work needed to move these NHPs from the preclinical stage to the market to be used to benefit patients.

Future work on this project should involve the determination and characterization of pharmacologically active components in DRE, LGE, and HE. Although some work has been done to identify these components, investigations looking at which combinations of phytochemicals induce significant anticancer efficacy will allow for better understanding of the mechanisms of NHP action. By doing so, we can identify mixtures of phytochemicals that are able to be developed into novel cancer therapeutics and brought forward as well-tolerated chemotherapeutics.

Further work into determining the mechanism of actions of these complex NHPs will allow for the identification of novel cancer vulnerabilities, allowing for the development of targeted treatments able to improve the selectivity of these treatments. This could be accomplished with gene or protein expression analysis to identify which specific cancer cell processes are triggered via NHP-mediated apoptosis. In doing so, we can design treatments able to enhance this effect and isolate them to only cancer cells.

These NHPs should be further studied with other chemotherapies and cancer types to identify a full profile of what cancers and therapies are able to benefit from NHP usage. This requires more rigorous research into drug-drug interactions and in vivo mouse studies to assess the efficacy and toxicity of these treatments. As mentioned, many natural compounds have progressed to clinical trials. As we investigate NHPs at the pre-clinical and clinical stage, more scientifically sound research will result in a wider acceptance and development of NHPs as clinically valid treatment approaches.
5.3 Conclusions

Herein, we have presented work that significantly contributes to the scientific validation of NHPs and their roles in cancer treatment. This work has provided support that NHPs are capable of reducing cancer viability at cellular and animal model levels with no significant toxicity to healthy non-cancerous cells. These NHPs may also be used with chemotherapeutics to enhance their effect or reduce their toxicity to normal cells. Our work has indicated that the natural health products dandelion root, lemongrass, and hibiscus have the potential to be further investigated and brought to market for patient usage. Collectively, this study has demonstrated the importance of natural health products in the development of well-tolerated and effective anti-cancer treatments.
5.4 Chapter 5 References


Appendix A

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Appendix B

Author Contributions (by Chapter)

I. Chapter 1

CN was the major contributor in conducting literature review, selecting relevant literature, and preparing the review manuscript. SP was a major contributor in conducting literature review, and supported manuscript preparation and revision.

II. Chapter 2

CN co-conducted all experiments with the help from all other authors and was the major contributor in analyzing data, preparing figures and writing the manuscript. AM co-conducted all apoptotic induction assays along with CN, SG, AP, and KP. KB co-conducted all animal studies and mechanistic studies with CN, IR, and BS. AP assisted with apoptotic induction assays and cell culturing. IR and BS helped out with animal measurements and co-conducted mechanistic assays along with CN and KB. SG, AP, and KP assisted with cell culturing and various assays where applicable. CV assisted with fluorescent microscopy and apoptotic induction experiments. SP is the primary investigator of this experiment.

III. Chapter 3

CN co-conducted all experiments with the help from all other authors and was the major contributor in analyzing data, preparing figures and writing the manuscript. KB co-conducted all apoptotic induction assays along with CN, AP, and OZ and was a contributor in writing the manuscript. AP assisted with apoptotic induction assays and cell culturing. IR co-conducted mechanistic (reactive oxidation species generation and mitochondrial membrane potential) assays.
OZ assisted with apoptotic induction assays and fluorescent microscopy pictures. SG, BS, and AM assisted with cell culturing and various assays where applicable. CV assisted with fluorescent microscopy and apoptotic induction experiments. SP is the primary investigator of this experiment.

IV. Chapter 4

CN co-conducted all experiments with the help from all other authors and supported in analyzing data, preparing figures and writing the manuscript. IR co-conducted all experiments along with CN and helped to analyze data, prepare figures, and write the manuscript. BS co-conducted mechanistic and animal studies. LG, OZ, KB, and JK assisted with cell culturing and image-based fluorescent cytometry assays. CV assisted with fluorescent microscopy and apoptotic induction experiments. SP is the primary investigator of this experiment.
Appendix C

Co-author Final Manuscript Approvals

Request Email:

To Whom It May Concern:

Hello,

Thank you again for being a co-author on our papers entitled [relevant paper name] submitted to [relevant journal name] on [date submitted].

I am writing to request your permission to include these works in my Master’s thesis submitted to the University of Windsor entitled “Anticancer Activity of Natural Health Products (Dandelion Root, Lemongrass, and Hibiscus Extracts); A Study of Efficacy, Interaction, and Mechanism of Action.”

Please write back at your earliest convenience to confirm receipt and final decision.

Thank you,

Christopher Nguyen

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