Assessing the Anticancer Potential of Natural Health Products (Lemongrass, Hibiscus, and Eleuthero Ginseng) Against Breast Cancer Models

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Assessing the Anticancer Potential of Natural Health Products (Lemongrass, Hibiscus, and Eleuthero Ginseng) Against Breast Cancer Models

by

Michael Stanesic

A Thesis
Submitted to the Faculty of Graduate Studies
Through the Department of Chemistry and Biochemistry
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March 10, 2017
Author’s Declaration of Originality

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Abstract

Breast cancer is a growing problem worldwide. Currently used cancer therapy is effective against cancer, but also causes cell death in normal cells, thereby causing severe side effects in patients. There is a serious need for the development of more effective and safer alternatives to chemotherapy and radiation.

Natural health products have been used for centuries to treat various forms of diseases, although many of the therapeutic benefits have not been scientifically studied for their efficacy and safety. The use of natural health products has led to the development of most of the chemotherapy currently used today. In this thesis, we evaluated three natural health products, lemongrass, eleuthero ginseng, and hibiscus, for their efficacy as a treatment against breast cancer.

The main objectives of this thesis were the following:

i) Evaluate the efficacy of each extract on breast cancer cells by analyzing their effects on metabolism, cell proliferation, and cell death induction.

ii) Decipher each extract’s mechanism of action for cell death induction.

iii) Evaluate the effects of combining extracts together, and using individual extracts to sensitize breast cancer cells to hormonal therapy.

iv) Determine the effectiveness of these extracts in vivo.

Our results indicate that all three natural health product extracts possess anticancer activity against both ER-positive and triple negative breast cancer. The results presented in this thesis offer some scientific validation for the use of natural health products, such as lemongrass, eleuthero ginseng and hibiscus extracts, in the treatment of breast cancer.
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CHAPTER 1 - INTRODUCTION

Cancer is defined as uncontrolled cell proliferation, which is a result of an accumulation of multiple mutations in the DNA of genes that are involved in regulating cell growth, division and death (Loeb et al., 2003). Specifically, if these mutations cause a loss-of-function in tumor-suppressor proteins, and/or allow growth signals to remain active in an unregulated manner, these mutated cells will be able to proliferate indefinitely (Loeb et al., 2003). There are many factors involved in causing cancer, and these may include: tobacco use, poor diet, obesity, alcohol intake, infections, physical inactivity, environmental pollutants, and ultraviolet/ionizing radiation, including increased exposure to direct sunlight. Many of these factors contribute to DNA damage that may lead to the mutations that can cause uncontrolled cell growth and proliferation. As these cells continue to divide, they acquire more mutations that may confer further proliferative advantages and/or protection from growth suppression (Weinberg, 1996).

According to the Canadian Cancer Society, cancer is a disease that mostly affects Canadians aged 50 and older, but it can occur at any age. In 2015, an estimated 100,500 Canadian men have been diagnosed with and 41,000 men have died of cancer, while an estimated 96,400 Canadian women were diagnosed with and 37,000 women die of cancer. On average, 539 Canadians are diagnosed with and 214 Canadians die of cancer every single day (Canadian Cancer Society, 2015). According to the World Health Organization (WHO), the number of new cancer cases is expected to rise by about 70% over the next 2 decades, likely due to the rapidly aging population, as of 2030, 1 in 4 Canadians will be older than 65 (World Cancer Report, 2014). These statistics demonstrate just how severe the need for the development of non-toxic, preventative and
therapeutic regimens for cancer are. My thesis focuses on the dire effects of breast cancer and possible treatment options to reduce the statistics associated with this disease.

1.1 BREAST CANCER

Breast cancer is a specific type of cancer that originates within breast tissue. This type of cancer is significantly more common in women than in men, as indicated by statistics from the Canadian Cancer Society, which shows that in the year 2015, there were approximately 25,000 new Breast cancer cases in women, and 220 new cases in men. Furthermore, on average, 68 Canadian women are diagnosed and 14 of those diagnosed die from breast cancer every day (Canadian Cancer Society, 2015). The stage of a particular patient’s breast cancer is an important prognostic factor for determining how the cancer will affect a person and how it will respond to treatment. There are five stages of breast cancer, and they are ranked from lowest to highest grade as follows: 0, I, II, III, IV. Lower stages have less risk of the cancer recurrence and a more favorable prognosis, while the higher stages have a greater risk of recurrence and a less favorable prognosis (Canadian Cancer Society, 2015).

There are multiple risk factors involved in developing breast cancer, and these can be categorized by hormonal, non-hormonal, and genetic factors. The main hormonal risk factor for developing the disease is the amount of exposure to the class of steroid hormones known as estrogens, which include estradiol, estrone and estriol. The reason for this risk is because estrogens are able to activate the estrogen receptors (ER). When estrogens activate their receptors, specifically the nuclear ER, found in breast epithelial cells, the changes in gene expression ultimately induce cell growth and proliferation.
Hence, too much estrogenic signaling can be a major factor in the development of breast cancer. Women who undergo estrogen replacement therapy have an increased risk of developing the disease (Colditz et al., 1993). The fact that women have much higher endogenous levels of estrogens than men is a major contributing factor for the higher prevalence of breast cancer in women (Brinton et al., 2015). The higher levels of estrogen can be attributed to higher amounts of adipose tissue in women compared to men. Adipose tissue cells express higher levels of an enzyme called Aromatase (Bulun et al., 1994), which facilitates the conversion of androgens into estrogens, such as Testosterone$\rightarrow$Estradiol and Androstenedione$\rightarrow$estrone.

Non-hormonal risk factors for developing breast cancer include lifestyle choices, such as eating a non-balanced diet that lacks nutrition (or eating inadequate nutrition), consumption of alcohol, tobacco use, obesity, and physical inactivity, among others. Eating a balanced nutritional diet containing the proper amounts of vitamins and minerals, as well as dietary organic compounds with a wide array of physiological functions, such as antioxidant agents, which are known to help prevent diseases, including cancer (Dorgan and Schatzkin, 1991). Dietary antioxidants are able to scavenge free-radicals in the body and help prevent intracellular proteins and DNA from being oxidized and damaged (Pham-Huy et al., 2008). Since mutations are known to play a major role in the development of cancers as a result of mutated proteins being produced with altered functions, the essential role of a diet with adequate nutritional options will be beneficial for reducing the non-hormonal risk of developing breast cancer. Furthermore, there is a strong link between excessive consumption of alcohol and cancer. Multiple reasons for this include ethanol’s first-order metabolite, acetaldehyde, a known
carcinogen, due to its ability to bind and make protein and DNA adducts (Aberle, et al, 2004; Lambert, et al., 1988). Additionally, it has been proposed that ethanol plays a more direct role in the development of breast cancer by the following mechanisms: ethanol-induced upregulation of Aromatase (Purohit V., 2000) and ERα (Fan, et al, 2000), and down-regulation of BRCA1 (Fan, et al, 2000). Ethanol-induced upregulation of aromatase causes an increased conversion of androgens into estrogens. Ethanol-induced upregulation of ERα means that with a given serum level of estrogens, there will be increased estrogenic signaling, as a result of more abundant receptors available for activation. Additionally, ethanol-induced down-regulation of BRCA1, a tumor-suppressor gene due to its potency as a ligand-independent corepressor (i.e. inhibitor) of ERα, means that ERα signaling will continually be stimulated, since it can’t be turned off by BRCA1. Taken together, these three proposed mechanisms for how the body is affected by ethanol support the claim that alcohol consumption is an important risk factor in the development of breast cancer.

Aside from hormonal and non-hormonal factors, genetic factors also play a large role in the development of breast cancer. Two of the most important genes that are known to be risk factors are BRCA1 and BRCA2. The proteins translated from these genes are known as Breast Cancer 1 (or 2) or by their synonym name Breast Cancer Type 1 (or 2) Susceptibility Protein. These genes are tumor-suppressors that are normally expressed in breast epithelial cells, where they either help repair damaged DNA or destroy cells, if DNA cannot be repaired (Yoshida and Miki, 2004). BRCA1/BRCA2 are proto-oncogenes that code for proteins involved in regulating cell growth and proliferation, however, a mutation in these genes results in an oncogene. BRCA1 is a tumor suppressor
because it is a ligand-independent corepressor (ie. inhibitor) of the ER, androgen receptor (AR) and the progesterone receptor (PR) (Zheng et al., 2001). Females who carry germline mutations in *BRCA1* have a 60-80\% lifetime risk of developing breast cancer and a 20-40\% lifetime risk for developing ovarian cancer (Struwing et al., 1996). Much like *BRCA1*, females who carry germline mutations in *BRCA2* have a 60-85\% lifetime risk of developing breast cancer and a 10-20\% lifetime risk for developing ovarian cancer (Ford et al., 1998). However, unlike male carriers of *BRCA1* mutations, men with germline mutations in *BRCA2* have a lifetime breast cancer risk of approximately 6\%, which represents a 100-fold increase over the male population risk. *BRCA2* mutations also may be associated with an increase in colon, prostate, pancreatic, gallbladder, bile duct, and stomach cancers as well as malignant melanoma (The Breast Cancer Linkage Consortium, 1999).

In addition to *BRCA1* and *BRCA2*, two other genes that are known to play a genetic role in the susceptibility to breast cancer are Tumor protein 53 (*TP53*) and Phosphatase and tensin homolog (*PTEN*). *TP53* is a tumor suppressor gene because it codes for p53, a protein that plays a critical role in cell signaling by multiple mechanisms, such as activating DNA repair proteins, arresting cell division so that DNA damage can be repaired before moving forward in the cell division process, as well as initiating apoptosis if the DNA damage is beyond repair (Amaral et al., 2010). According to the Catalog of Somatic Mutations in Cancer (COSMIC) database, on average, *TP53* is mutated in 31\% of all tumors, and is mutated in 23\% of breast cancer tumors. However, *TP53* mutations occur more frequently in other types of tumors such as: ovarian (50\%), large intestine (43\%) and lung (36\%) cancers (Forbes S.A., et al., 2011). *PTEN* is a gene
that codes for Phosphatase and Tensin Homolog (PTEN), a protein phosphatase that plays a role in growth, proliferation, and survival. PTEN acts as a tumor suppressor by negatively regulating the PI3K/AKT signaling pathway by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the cell membrane. Inactivation of PTEN is associated with cancer because of the increased activity of the PI3K/AKT signaling pathway. Aside from mutations, p53 and PTEN activity can also be lost through other mechanisms such as epigenetic changes or post-translational modifications (Leslie and Foti, 2010). These changes make a difference in breast cancer development/progression since these proteins normally act to prevent cancer development by causing cell death in abnormal cells or by turning off growth receptor signaling, and therefore, inhibiting their activity by post-translational modifications or epigenetic changes in gene expression further promotes tumor progression by allowing these abnormal cells to continue proliferating even though the cells are damaged.

No two breast cancers are exactly alike, due to the different genetics a person inherits, as well as the accumulation of specific mutations over time (Powell et al., 2012). In order to effectively treat a patient with breast cancer, it is imperative to characterize the type of patient-specific cancer cells and the surrounding microenvironment, as this can identify potential targets for developing treatment options. Identifying hormonal players, e.g. ERα and/or ERβ, PR or Human Epidermal Growth Factor 2 Receptor (HER2) provides a target for treatment. Unfortunately, there are breast cancer subtypes that are not positive for any of the aforementioned receptors and this type that lacks all three receptors is known as a triple negative breast cancer (Foulkes et al., 2010).
Therefore, identifying different targets, particularly for triple negative breast cancer, is required in order to effectively treat the patient.

1.2 HORMONE RECEPTOR POSITIVE BREAST CANCER

1.2.1 Estrogen Receptor (ER):

There are two classes of estrogen receptors: nuclear estrogen receptors (ex. ER\(\alpha\) and ER\(\beta\)) and membrane estrogen receptors (ex. GPER, ER-X, \(G_q\)-mER, GPR30). When bound and stimulated by estrogen, the nuclear estrogen receptors induce long-term cellular changes by altering gene expression, and the membrane estrogen receptors are G-protein coupled receptors that are responsible for some of the rapid cellular changes induced by estrogens, including negative feedback, modulation of homeostasis, neuroprotection and regulation of synaptic plasticity, all of which play an important role in the actions of estrogen in the brain (Raz et al., 2008). When estrogens activate their receptors, specifically the nuclear estrogen receptors, found in breast epithelial cells, the changes in gene expression ultimately induce cell growth and proliferation.

ER+ breast cancers are the most common types of breast cancer seen amongst women, representing about 80% of all breast cancer cases (Lumachi et al., 2015). Of these, about 65% are also PR+.

1.2.2 Progesterone Receptor (PR):

PR+ breast cancers (or other types of cancers) overexpress receptors for the ovarian steroid hormone, progesterone. Progesterone binds to its receptor, and depending on the relative amount of coactivators and/or corepressors in the target cell, this signaling pathway can induce the expression of specific genes that are involved in cell growth and
proliferation. This signaling pathway is analogous to the ER signaling pathway. However, the PR is also able to associate and modulate the activity of ERα (Mohammed et al., 2015). Specifically, progesterone signaling through the PR has been shown to inhibit estrogen-mediated growth of ERα-positive breast cancer cells, thus showing antiproliferative activity, or at least a slowing of the signals that cause the cancerous cells to grow (Mohammed et al., 2015).

1.2.3 Human Epidermal Growth Factor 2 Receptor (HER2):

The HER2 receptor is a receptor tyrosine kinase (RTK) that is coded by the ERBB2 gene and is found on the cell membrane of normal breast epithelial cells. This receptor helps control how these normal cells grow, divide and repair (Goddard et al., 2011). However, in about 20-30% of all breast cancer cases, HER2 is overexpressed, which promotes breast cancer cell growth in an uncontrolled manner and causes these cancers to be more aggressive, faster-growing, and more likely to spread than tumors that don’t overexpress HER2 (Slamon et al., 1987). Since over-expression of HER2 is considered a prognostic marker for how a cancer will affect a patient and its response to a given therapy, HER2 expression is routinely used as an important biomarker and therapeutic target for many patients with breast cancer (Goddard et al., 2011).

When mitogens (e.g. EGF) bind to the extracellular domain of the HER2 receptor, two HER2 receptors dimerize, and the intracellular domain of the receptor activates multiple biochemical signaling pathways, such as the pathways involving: PI3K/Akt, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), Phospholipase C-gamma, and signal transducer and activator of transcription (STAT). These pathways
ultimately promote cell survival, proliferation, and the growth of new blood vessels by angiogenesis (Ménard et al., 2003). Normally when cells divide, there are checkpoints, governed by cyclin-dependent kinases (CDKs), whose function is to keep cell division under control. However, for cells that overexpress HER2, they can skip/deactivate these checkpoints, which allows for even greater increases in cell proliferation (Kute et al., 2004).

1.3 TRIPLE NEGATIVE BREAST CANCER (TNBCs)

About 10-20% of all invasive breast cancer cases are triple negative, which is a type of breast cancer characterized by the absence of estrogen and progesterone receptors, and also don’t overexpress the HER2 receptor (Foulkes et al., 2010). As a result of lacking those three main growth receptors, TNBCs are known to have a very poor prognosis, are very aggressive, and difficult to treat because common breast cancer treatment options target one or more of the hormonal receptors and are therefore ineffective for this type of breast cancer (Foulkes et al., 2010). Another key characteristic of TNBC is inflammation, which is the result of an upregulation of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE₂) enzymes (Kochel et al., 2016). This inflammation increases blood flow to the site of the tumor, which provides more nutrients to these fast dividing cells, and helps the tumor grow faster as well as being involved in metastasis (Coussens and Werb, 2002).
1.4 HALLMARKS OF CANCER

Cancer cells are mainly characterized by the ability of abnormal cells to proliferate indefinitely, as well as losing the ability to die by PCD mechanisms. However, cancer is much more complicated than that. As these abnormal cells continue to proliferate, they continually acquire more mutations that may provide further proliferative advantages and/or protection from growth suppressors (Hanahan and Weinberg, 2011). These mutations may also confer further advantages such as: the ability to hide/evade degradation from the immune system, other mutations that can help cells proliferate indefinitely such as increasing telomerase activity, tumor-promoting inflammation, and the ability to escape from the primary tumor site and metastasize to a secondary site (Hanahan and Weinberg, 2011). In addition, accumulation of mutations may promote the induction of angiogenesis to increase blood supply and nutrients for inner cells of tumors that are normally lacking blood supply, genome instability (which promotes further mutations), and shifting cellular metabolism towards anaerobic energy production, which allows the cells to grow even when they don’t receive enough blood oxygen (such as inner cells of a tumor) (Hanahan and Weinberg, 2011).

Collectively, all of these cellular changes are known as the “hallmarks of cancer”. Given the fact that cancer can progress to have all of these hallmarks, it makes sense that cancer should no longer be treated with just one or two drugs, since these cells may acquire mutations that promote resistance to a single drug therapy. Rather, cancer should be treated with multiple drugs, which may provide a better method of killing the cancer before the cells are able to acquire resistance to the multiple drugs.
1.5 TREATMENT OPTIONS FOR BREAST CANCER

No two cancers (even two breast cancers) are exactly alike. This is due to the different genetic make-up of the different patients, as well as the accumulation of specific mutations over time. A patient’s environment also plays a role in the differences observed within the different cancers (Boffetta and Nyberg, 2003) Therefore, treatment plans are often designed to meet the unique needs of each patient. The specific treatment plan for an individual with breast cancer will depend on the following factors: the stage of the breast cancer, the expression or lack of specific hormone receptors (i.e. ER, HER2, and PR), pre- or post-menopausal women, the risk of recurrence, overall health of the woman, and any kind of personal decision(s) about specific treatment options (Canadian Cancer Society, 2015).

One possible form of treatment for a patient with breast cancer is **Targeted Therapy**, which includes hormonal and biological therapies. Hormonal therapy is only offered for hormone receptor-positive (ER+, PR+ or both) breast cancers. If a patient with breast cancer is identified as expressing (or overexpressing) the ER, PR, or both receptors, the patient may be given a drug that can specifically target those receptors by binding to them and blocking them from being stimulated by their endogenous ligand (either estrogen or progesterone). This interaction blocks the growth-promoting effects of these receptors, thereby inhibiting the growth of the breast cancer cells (Lee W.L. et al, 2008). For example, Tamoxifen (trade name: Nolvadex), is a competitive inhibitor of the ER, and belongs to a class of drugs known as Selective Estrogen Receptor Modulators (SERMs). SERMs could act as an agonist in one target tissue and an antagonist in a different tissue. This discrepancy in biological activity is dependent on the relative
amounts of coactivator and/or corepressor proteins in the particular target tissue/cell (Dutertre and Smith, 2000). Aside from SERMs, another possible treatment option for a patient with ER+ breast cancer is aromatase inhibitors, such as anastrozole (Arimidex), exemestane (Aromasin), and letrozole (Femara). They are only used in women who have already gone through menopause. This class of medication inhibits aromatase, the enzyme that facilitates the conversion of androgens, such as testosterone, into estrogens, such as estradiol, thereby reducing the serum level of estrogens to inhibit growth-promoting estrogenic signaling. Onapristone is an example of a PR antagonist (or progesterone receptor modulator, PRM). Since both tamoxifen and onapristone do not directly induce cell death, but rather blocks estrogen- and progesterone-induced growth of the cancer cells, these drugs are known as cytostatic agents (Dutertre and Smith, 2000). On the other hand, the other form of targeted therapy is biological therapy, which includes the use of antibodies that target and block a specific receptor. For example, Trastuzumab (trade name: Herceptin) is a monoclonal antibody against HER2, and thereby blocks its activity. In about 20-30% of breast cancers, HER2 is overexpressed, which causes these breast cancer cells to grow and divide in an uncontrolled manner, as well as increasing the likelihood of recurrence (Slamon et al., 1987). With the administration of trastuzumab, these receptors can be blocked and thereby the growth and progression of breast cancer can be slowed or halted. Although trastuzumab alone does not cause cell death in breast cancer cells, combining trastuzumab with other chemotherapeutic drugs has been shown to increase both survival and response rate, in comparison to trastuzumab alone (Pegram et al., 2004).
Aside from targeted therapy (which is dependent on the receptor status of ER, PR and HER2), the main form of treatment for cancer (in general) is chemotherapy, radiation, and surgery. Surgery is the best option if the cancer is localized in one primary site where most, if not all of the cancer, can be physically removed. However, there may still be some cancer cells left in the area, which can allow the cancer to come back with time. In order to prevent this recurrence, the patient is treated with ionizing radiation and chemotherapy (e.g. Doxorubicin). Both of these forms of treatment are genotoxic, which means that they cause DNA damage, thereby inducing cell death (especially in rapidly dividing cells), because the cells can no longer replicate the genome. The problem with this form of treatment is that radiation and chemotherapy also harms the genome of normal, non-cancerous cells as well. This is the reason why these treatments are associated with a large amount of side effects such as: hair loss, gastrointestinal effects such as nausea and vomiting, lethargy, depression, just to mention a few. Hair loss and nausea/vomiting are very common side effects because hair follicles and intestinal stem cells are the most rapidly dividing normal cells of the human body. These side effects are usually so severe that they take a large toll on the overall health and well-being of the patient and care-givers as well. Some patients even die as a result of the chemotherapy-induced side effects, rather than from the cancer itself, suggesting a need for better treatment options.

When a patient is first diagnosed with cancer, they are given systemic treatments that may include one or more of the following treatments: cytotoxic drugs (i.e. chemotherapy), hormonal and targeted therapy. In the beginning, these treatments are effective in about 90% of primary breast cancers and half of metastases. However, after a
period of time, the cancer cells become resistant to therapy (Gonzalez-Angulo et al., 2007). This is a major problem for clinicians trying to treat patients. Therefore, the old one target, one drug approach is not effective in the long run. An effective approach to treating cancer should include many targets, as this will reduce the chance of the cancer becoming resistant to treatment.

1.6 PROGRAMMED CELL DEATH (PCD)

In order to maintain tissue homeostasis in multicellular organisms, cells that are no longer needed or cells that are damaged have the ability to sacrifice themselves for the betterment of the tissue or organism overall. They are able to do this by undergoing a form of programmed cell death (PCD), which is a cell death process mediated by an intracellular mechanism (Elmore, 2007). **Apoptosis** (PCD Type I) is the most common form of PCD, but programmed cell death can also occur by **Autophagy** (PCD Type II), **Necrosis**, or one of many other forms that have been characterized (Elmore, 2007). Necrosis is technically not a ‘programmed’ cell death mechanism, since it is the result of an external stimulus, such as infection or trauma to tissue, in which the injured/dying cells promote inflammation of other tissues in the surrounding area (Majno and Joris, 1995).

All characterized PCD pathways are carried out in a regulated process, and usually confers some advantage(s) to the overall organism (Elmore, 2007). For example, if a cell has become damaged or is past the point of cellular (e.g. DNA) repair, that cell may commit suicide, via apoptosis, in order to prevent this damaged cell from replicating and possibly becoming tumorigenic, which would be detrimental to the overall organism.
The work presented in this thesis will focus on apoptosis and autophagy, PCD type I and II, respectively.

1.6.1 Apoptosis:

By far, the most well-known and characterized type of programmed cell death that occurs in multicellular organisms is apoptosis. This biochemical process causes characteristic changes in cell morphology that ultimately results in cell death. These characteristic changes include: cell shrinkage, nuclear fragmentation, chromatin condensation, cellular fragmentation into membrane-bound apoptotic bodies that are eventually phagocytized by neighboring cells, such as macrophages or parenchymal cells (Elmore, 2007). These apoptotic bodies are closely packed with organelles, such as mitochondria and nuclear fragments, and can be identified inside these neighboring cells until the apoptotic bodies are eventually degraded when fused with a lysosome. It should be noted that cellular shrinkage, disassembly into apoptotic bodies and engulfment of individual cells characteristically occurs without associated inflammation, which would otherwise be the consequence of releasing intracellular contents into surrounding tissues. This characteristic of apoptosis is advantageous in comparison to necrosis. There are two major pathways by which apoptosis can occur, and they are known as the intrinsic and extrinsic pathways of apoptosis. Both apoptotic pathways are energy-dependent signaling cascades (Elmore, 2007). In any case, both pathways of apoptosis share some common features, including the: activation of specific cysteine aspartic-proteases (caspases) (although the stage at which caspases are activated differs between the two pathways), externalization of phosphatidylserine, generation of oxidative species, and many more (Saraste and Pulkki, 2000). Caspases are a group of ten proteins that play a critical role in
mediating apoptosis and inflammation. These caspases are initially made as inactive (zymogen) proteins, and subsequently become cleaved into their active form by protease activity. These proteins can be further classified into distinct subgroups based on the protein’s function. These subgroups include: initiator caspases (caspase-2, -8, -9, -10), effector caspases (caspase-3, -6, -7), and inflammatory caspases (caspase-1, -4, -5) (Elmore, 2007). Four other caspases have been identified, but they are not normally involved in apoptosis, aside from a few unique instances, such as septic shock, amyloid-β toxicity, or only involved in embryonic development and not in adult tissue (Elmore, 2007).

Another biochemical feature of apoptosis is the expression of cell surface markers that induce phagocytosis of the dying cell by a nearby macrophage. This is accomplished by the externalization of phosphatidylserine. Phosphatidylserine is a phospholipid that is normally only localized to the inner layer of the plasma membrane bilayer. However, when a cell is undergoing apoptosis, an early stage of this process is the externalization of phosphatidylserine (Lee S.H., et al., 2013). The externalization of this particular phospholipid is a well-recognized signal for phagocytes to come and eliminate this apoptotic cell (Elmore, 2007). Furthermore, it has recently been discovered that two other proteins can be exposed on the outside surface of the cell membrane to signal apoptotic cell clearance. These include Annexin I and calreticulin (Arur et al., 2003).

The Extrinsic Pathway of Apoptosis:

The extrinsic pathway of apoptosis, otherwise known as death receptor-mediated apoptosis, involves binding of a ligand to a receptor on the plasma membrane of a cell, or the trimerization of the death receptor at the membrane. These receptors belong to the Tumor Necrosis Factor (TNF) receptor superfamily. Members of this receptor family
share a cysteine-rich extracellular domain as well as a cytoplasmic domain comprised of approximately 80 amino acids, called a death domain (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting signals from the extracellular side to the intracellular signaling pathways involved in cell death (Park et al., 2007).

The binding of a death ligand to its corresponding death receptor results in the recruitment of adaptor proteins, such as the Fas-associated death domain (FADD), or a similar molecule called TRADD in the case of TNFR signaling. Following this interaction, dimerization of nearby DEDs recruits pro-caspase-8 to the receptor to form the death-inducing signaling complex (DISC), which is a combination of the death receptor, death effector domains (DEDs) and pro-caspase-8. Once the DISC is formed, auto-catalytic cleavage of pro-caspase-8 forms the active caspase-8, which can then carry out the execution phase of extrinsic apoptosis (Kischkel et al., 1995). This execution phase can occur by one or two methods; 1) caspase-8 causes the cleavage and activation of downstream executioner caspases such as caspase-3, or 2) active caspase-8 can cleave Bid, a Bcl-2 family protein that possesses a BH3 domain only. Following this cleavage, truncated Bid (ie. tBid) can now translocate to the mitochondria to cause oligomerization of Bax or Bak, which results in the release of cytochrome C (Cory and Adams, 2002). This mechanism of caspase-8 cleaving Bid is the main link between the extrinsic and intrinsic pathways of apoptosis. However, another mechanism that can link the two apoptosis pathways involves cleavage of caspase-6 downstream of the mitochondrial pathway may feed back to the death receptor pathway by cleaving pro-caspase-8 (Cowling and Downward, 2002).
The Intrinsic Pathway of Apoptosis:

The intrinsic pathway of apoptosis is centered around the mitochondria, since the mitochondria play such a vital as the main producer of ATP energy via oxidative phosphorylation, as well as by localizing and sequestering many apoptosis-inducing/pro-apoptotic proteins, some of which include: cytochrome C, AIF (apoptosis-inducing factor), Smac (second mitochondria-derived activator of caspase), DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low PI), Omi/HtrA2, and endonuclease G in the intermembrane space of the mitochondria (Cande et al., 2002).

Due to the important role of the mitochondrion in cell health and survival, the integrity of the mitochondria is regulated by the mitochondrial permeability transition pore (MPTP), a protein that is located on the inner membrane of the mitochondria (Bernardi and Forte, 2007). The MPTP is responsible for regulating matrix Ca$^{2+}$, mitochondrial transmembrane potential (proton electrochemical gradient) ($\Delta \Psi_m$), pH, and volume. This pore is triggered by Ca$^{2+}$ influx into the mitochondria (Lemasters et al., 2009). The MPT pore is known to briefly open or “flicker” to provide the matrix with a fast Ca$^{2+}$ release channel (Petronilli et al., 1999), and this may occur before $\Delta \Psi_m$ is lost and the mitochondrial membrane integrity is irreversibly lost (Lemasters et al., 2009). However, prolonged opening of the MPTP precipitates a bioenergetic crisis, by increasing the permeability of the mitochondrial membrane to molecules of less than 1500Da in molecular weight (Lemasters et al., 2009). This leads to the collapse of the $\Delta \Psi_m$, in turn halting ATP production and eventually depletion of ATP. Furthermore, the mitochondrial matrix begins to swell, causing the outer mitochondrial membrane to
rupture and release intermembrane proteins, including pro-apoptotic proteins (Lemasters et al., 1998).

Furthermore, a cell may commit suicide by the intrinsic pathway of apoptosis if it is stressed by an intracellular stimulus such as: heat, ionizing radiation-induced DNA damage, nutrient deprivation, viral infection, chemotherapy, radiation, or hypoxia, as these can all induce intracellular apoptotic signals from the mitochondria (Cotran et al., 1998). These non-receptor mediated stimuli produce intracellular signals cause upregulation of the Bcl-2 family of proteins, which act directly on the mitochondria to disrupt the mitochondrial membrane potential ($\Delta\Psi_m$) (Elmore, 2007). This family of proteins consists of two main groups, which are characterized as either pro-apoptotic (e.g. Bax, Bak, BAD, etc) or anti-apoptotic (e.g. Bcl-2, Bcl-xL, etc), both of which are involved in regulating the mitochondrial outer membrane permeability (MOMP). All members of the Bcl-2 family possess one or more of four possible Bcl-2 homology (BH) domains; BH1, BH2, BH3, and BH4. Depending on the precise combination of structural domains, a protein may be characterized as either pro-apoptotic or anti-apoptotic. For example, the pro-apoptotic members BIM, BID, and BAD possess only BH3 domain(s), whereas the anti-apoptotic members Bcl-2 and Bcl-xL possess all four BH domains (Taylor et al., 2008).

Alternatively, the proteins in the Bcl-2 family can be classified by their function; 1) BH3-only proteins that are able to sense and become activated (either directly or indirectly) by cellular stress (e.g. DNA damage) (Taylor et al., 2008), 2) multidomain pro-apoptotic proteins that are able to permeabilize the mitochondrial outer membrane (MOM), thereby releasing intermembrane proteins that subsequently activate caspases
(Taylor et al., 2008), and 3) anti-apoptotic proteins that are able to bind to and inhibit both types of pro-apoptotic proteins (Taylor et al., 2008). Group 2 pro-apoptotic proteins permeabilize the MOM by forming a mitochondrial apoptosis-induced channel (MAC). These proteins, which include Bax and/or Bak, once activated, translocate to the mitochondria and oligomerize to form the MAC, where it inserts into the outer membrane of the mitochondria and releases cytochrome C into the cytosol, which eventually leads to caspase activation and apoptosis (Martinez-Caballero et al., 2005). The anti-apoptotic proteins prevent the formation of the MAC by binding and inhibiting Bax and/or Bak (Martinez-Caballero et al., 2005).

In summary, following activation of pro-apoptotic members of the Bcl-2 family and/or loss of the MOMP, a series of biochemical events occurs, including: halting of ATP synthesis, oxidation of NADH, NADPH and glutathione, generation of reactive oxygen species (ROS), and the release of pro-apoptotic factors (cytochrome C, AIF, Smac, DIABLO, Omi/HtrA2, and Endonuclease G) from the inner mitochondrial membrane into the cytosol (Elmore, 2007). Once these factors are released, they can trigger caspase-dependent or caspase-independent apoptosis.

Caspase-dependent apoptosis occurs when cytosolic cytochrome C binds to the Apoptotic Protease Activating Factor 1 (APAF-1), following which APAF-1 oligomerizes, and this recruits pro-caspase-9 in the presence of ATP. This complex is known as the apoptosome, and allows for activation of caspase-9, an initiator caspase that is essential for activation of downstream executioner caspases, like caspase-3 (Chinnaiyan, 1999). On the other hand, cells may undergo caspase-independent apoptosis when pro-apoptotic proteins like apoptosis-inducing factor (AIF) and endonuclease G
(EndoG) are released from the mitochondrial intermembrane space. AIF and EndoG translocate to the nucleus and cause DNA fragmentation (Elmore, 2007).

As with the extrinsic pathway discussed above, there are inhibitors of the intrinsic pathway. The X-linked inhibitor of apoptosis proteins (XIAPs) inhibit the activation of caspase-9, as well as the downstream activation of caspase-3/7 from caspase-9. Interestingly, XIAPs can be inhibited by Smac/DIABLO and Omi/HtrA2, which are released from the mitochondria following loss of the mitochondrial membrane, thereby further promoting apoptosis (Saelens et al., 2004).

1.6.2 Autophagy (Programmed Cell Death Type II):

Autophagy is an intracellular process that plays an important role in a cell. This normal physiological process of ‘self-eating’ plays an important role in maintaining cellular homeostasis when active at basal levels in the cell, but can also be strongly induced in response to nutrient deprivation stress and energy regeneration (He et al., 2013). Homeostasis is maintained by degrading misfolded or aggregated proteins and eliminating damaged organelles (Glick et al., 2010). Hence, autophagy is generally thought of as a pro-survival mechanism, since accumulation of dysfunctional proteins or mitochondria can cause a lot of problems within a cell, usually leading to cell death. However, when unregulated, autophagy has been linked to non-apoptotic cell death, although the pro-death mechanism for this remains undefined (Denton et al., 2015). Therefore autophagy may provide an alternative way to induce cell death in cancer cells that are resistant to other forms of cell death like apoptosis. Autophagy is induced by a variety of stimuli, including ER stress, nutrient starvation, and inhibition of the mammalian/mechanistic target of rapamycin (mTOR) pathway (Glick et al., 2010). When
nutrient starvation occurs, mTOR is inhibited and autophagy plays a critical role in the adaptive response to promote survival until nutrients become available again. However, when nutrients are abundant, mTOR inhibits autophagy (Nazio et al., 2013).

The process of autophagy begins with an isolation membrane, known as a phagophore. This phagophore expands to engulf dysfunctional proteins (either misfolded or aggregated) or organelles (such as mitochondria), thereby trapping/localizing the cargo within a double membrane-bound vesicle called an autophagosome. This autophagosome then matures by fusing with a lysosome, which promotes the degradation of the contents inside the autophagosome by lysosomal acid hydrolases (i.e. proteases). Permeases and transporters present on the lysosome export amino acids and other degradation products back into the cytosol, where they can be reused to build new macromolecules (Mizushima, 2007).

Autophagy can be thought of as a ‘recycling system’ that helps balance energy sources and promotes damage control by removing dysfunctional organelles and proteins (Glick et al., 2010). Therefore, autophagy has a pro-survival role for normal cells and suggests that autophagy may play a similar role in cancer. By protecting cancerous cells under stressful conditions, such as those found in the center of a tumor, where hypoxia and acidity levels are much higher due to higher glycolytic activity and lactate production, autophagy may provide the necessary materials required to synthesize proteins and maintain energy levels, thereby promoting tumor-growth (Rosenfeldt and Ryan, 2009). Furthermore, autophagy has been proposed to be partly responsible for drug resistance, as inhibition of autophagy enhanced therapy-induced apoptosis in one study.
(Amaravadi et al., 2007). Ultimately, the role of autophagy in cancer may be cell type and/or stage-specific.

1.6.3 Necrosis:

Necrosis is a passive (energy independent) form of cell death, but is unplanned/accidental and usually results from tissue injury or an infection. Necrosis does not have the same regulatory mechanisms as apoptosis. The term necrosis is used to label the presence of dead cells or tissues after they have died, regardless of the prelethal processes that caused the death (Majno and Joris, 1995). The presence of necrosis indicates that a cell has died but not necessarily how the death occurred (Schwartz and Bennett, 1995). There are two prelethal pathways that may lead to necrosis: oncosis, and secondary necrosis. Oncosis is a pathway that leads to cell death and is accompanied by cellular swelling (very early), organelle swelling (including mitochondria and nucleus), blebbing, and cell membrane rupture and increased permeability (Majno and Joris, 1995). It is this rupturing of the cell membrane and leakage of cellular contents such as organelles and whole proteins into the surrounding area that causes widespread inflammation leading to unplanned cell death in surrounding cells. Oncosis ultimately leads to rapid depletion of cellular energy stores and the failure of membrane ion pumps (Majno and Joris, 1995).

On the other hand, secondary necrosis (otherwise known as apoptotic necrosis) is a secondary form of necrosis that follows apoptosis and budding. In the absence of phagocytosis by macrophages, apoptotic bodies may lose their integrity and proceed towards secondary necrosis (Silva, 2010). Secondary necrosis also causes swelling and
increased membrane permeability, except the membrane permeability is regulated (Silva, 2010).

1.7 NATURAL HEALTH PRODUCTS

Natural Health Products (NHPs) and Natural Products (NPs) are generally derived from naturally occurring plants and marine sources (Health Canada). These products include: herbal remedies, vitamins and minerals, homeopathic medicines, probiotics, traditional medicines, among others. Since they are in the category of food, these whole-plant extracts must be made using water or ethanol as the solvent for the extractions, since both solvents are known to be well-tolerated and safe when consumed. For many centuries, NHPs have been used as a form of medicine in Traditional Chinese Medicine (TCM), as well as Ayurvedic Medicine. During this time, the use of NHPs and NPs has not been associated with many side effects or toxicities (Efferth et al., 2007). These products have been used to treat a wide range of symptoms, from minor ailments such as gastrointestinal issues to much more severe problems such as pain or bacterial/viral infections. In addition, NHPs have been used to modulate immune function and prevent chronic diseases (Haddad et al., 2005).

NHPs are complex polychemical mixtures that may contain hundreds to thousands of different bioactive compounds, each of which may be able to target multiple pathways of a disease, which may potentially have an additive or synergistic effect and thereby improve efficacy and reduce any toxicities associated with the use of high doses of any single treatment. This complexity of NHPs makes them a viable treatment option for complex diseases such as cancer.
Although most of these NHPs have not been scientifically characterized for their composition or uses, these products have been used for many centuries, and are generally well-tolerated (Aggarwal et al., 2006). To date, there is little scientific research to validate the therapeutic uses for most NHPs. Although there is some research to support the claims of potential anticancer effects of some natural health products such as Lemongrass, Hibiscus and Eleuthero Ginseng (Halabi & Sheikh, 2014; Wong YH et al., 2014; Wong AS et al., 2014), these studies did not investigate the anticancer effects on breast cancer, nor did they characterize the mechanism for these anticancer effects.

1.7.1 Lemongrass:

Lemongrass, otherwise known as *Cymbopogon citratus*, is a plant that is native to India and Asia, where it is most commonly used for its medicinal properties as well as its uses in culinary. The most common medicinal uses for lemongrass in traditional therapy are to aid gastrointestinal symptoms such as upset stomach, diarrhea, and dyspepsia, as well as to relieve the symptoms of colds and fevers (Shah et al., 2011). Furthermore, lemongrass is also known to possess potent antifungal, antimicrobial, and antibacterial properties, and may also be used to help relieve fatigue, anxiety and depression (Shah et al., 2011). In order to treat the above symptoms, the whole leaves or powdered leaves may be brewed in hot or boiling water to make a decoction, or made into an essential oil when used in aromatherapy.
1.7.2 Eleuthero Ginseng:

Eleuthero Ginseng, otherwise known as Siberian Ginseng or *Eleutherococcus senticosus*, is technically not part of the ginseng family of plants. Plants that are considered part of the ginseng family possess ginsenosides (Lee and Kim, 2014), which are a class of natural steroid glycosides. However, eleuthero ginseng contains eleutherosides. Although the active components are different than a true ginseng species like American or Panax ginseng, eleutherosides are also able to stimulate the immune system, and are known to have adaptogenic effects, which alleviates symptoms of stress, anxiety, and depression (Deyama et al., 2001).

1.7.3 Hibiscus:

Hibiscus, otherwise known as *Hibiscus rosa-sinesis*, is a species of tropical Hibiscus, a flowering plant that is native to East Asia. There are a large number of varieties and hybrids available, with flower colours ranging from white, to yellow and orange, to pink and most commonly bright red. The flowers of Hibiscus Rosa-Sinesis have many uses including: hair care, pH indicator, cosmetic skin care, as well as many medicinal uses in Chinese herbology. These medicinal uses include treatment of ulcers, diabetes, and are also known to have antibacterial and wound-healing properties (Ruban and Gajalakshmi, 2012; Shivananda et al., 2007).
1.8 HYPOTHESIS

Based on anecdotal and some recent scientific evidence, three extracts (Lemongrass, Hibiscus and Eleuthero Ginseng) seem to have anticancer properties (Halabi & Sheikh, 2014; Wong YH et al., 2014; Wong AS et al., 2014).

Our hypothesis is that natural health products, such as extracts of Lemongrass, Hibiscus and Eleuthero Ginseng have the potential to be an alternative option to currently available chemotherapy in the treatment of breast cancer. These extracts may contain multiple bioactive compounds that can effectively target multiple pathways where breast cancer may be vulnerable, and thereby act as an efficient and selective treatment for cancer patients.

OBJECTIVES

In order to study this hypothesis, there were five main objectives to be investigated:

1) Assess the anticancer activities of Lemongrass, Hibiscus and Eleuthero Ginseng in breast cancer cells (both triple negative and ER+).
   I. On metabolic viability
   II. On proliferation rate
   III. On programmed cell death induction

2) Determine if there is a benefit to using these extracts in combination.

3) Assess the effectiveness of these NHPs in sensitizing breast cancer cells to improve the response to current treatment options, such as Tamoxifen and 4-OH-Tamoxifen.

4) Determine the mechanism of action of these extracts.

5) Effectiveness of these extracts in vivo.
1.9 OVERALL SIGNIFICANCE

Treatment of breast cancer patients with chemotherapy and radiation is efficacious, but comes with a large amount of side effects that can take a large toll on a patient’s physical and mental well-being, due to the genotoxic nature of these treatments to both cancer and normal cells. Therefore, there is a serious need for non-toxic, selective and cheaper treatment options, while still being as effective. The purpose of this thesis is to evaluate the efficacy of natural health product (NHP) extracts, specifically lemongrass, eleuthero ginseng, and hibiscus, in the treatment of breast cancer. Scientific evaluation of these NHPs will provide evidence for the efficacy in the treatment of breast cancer, in the hopes to provide an effective, and non-toxic alternative to current chemotherapy.
CHAPTER 2 – MATERIALS & METHODS

2.1 STANDARDIZED NATURAL HEALTH PRODUCT EXTRACTION

The water and 100% ethanolic extracts of each plant were made from the root of Eleuthero Ginseng, the stems of Lemongrass, and the leaves of Hibiscus flowers. The three plant products were purchased in an already pre-ground form from Premier Herbal (Toronto, Ontario, Canada).

i. Water Extracts

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

ii. Ethanolic Extracts

Each plant was then extracted with a 1:10 ratio of plant material (in grams) to anhydrous ethanol (in mL). The solution was thoroughly mixed and allowed to extract for approximately 24hrs. Following this, the mixture was then gravity filtered using a 0.2µm filter. Next, the filtrate was rotovaped at a temperature of approximately 38-40°C. After all of the ethanol had been removed, the weight of the dry resin was used to make a stock solution dissolved in anhydrous ethanol. The final stock concentration of each extract
was as follows: 200mg/mL for lemongrass, 250mg/mL for eleuthero ginseng, and 100mg/mL for hibiscus.

2.2 CELL CULTURE

Four breast cancer adherent cell lines were used in this study. The Michigan Cancer Foundation-7 (ie. MCF-7) cells, which are hormone receptor positive containing both the estrogen and progesterone receptors, but lack overexpression of the HER2 receptor. The other three cell lines used were MDA-MB-231, MDA-MB-468 and SUM149, all of which are triple negative, meaning that they lack the three main growth receptors (ER, PR, and HER2). The four cell lines were purchased from ATCC (Manassas, Virginia, USA). In the future, two other cell lines that should be tested with these NHP extracts are Human Mammary Epithelial Cells (HMEC) and MCF10A, both of which are normal breast epithelial cells. Experiments need to be conducted on these cell lines in order to determine if these extracts are selective to cancer cells or if they are toxic to both normal and cancer cells.

The MCF-7 and MDA-MB-468 cell lines were both cultured in RPMI-1640 media, whereas the SUM149 and MDA-MB-231 cell lines were cultured in DMEM F-12 Ham nutrient mixture with pyridoxine, 15mM HEPES, and bicarbonate, without L-glutamine (Sigma-Aldrich Canada Ltd, Mississauga, Ontario, Canada). Both media were supplemented with 10% fetal bovine serum (FBS) and 40mg/mL gentamicin (Life Technologies, Mississauga, Ontario, Canada). The cells were maintained at 37°C and 5% CO₂.

Since all four cell lines used were adherent, cells were subcultured (ie. split) through the process of media aspiration, washing of cells with PUCK’s solution (which
contains 0.8mM ethylenediamine tetraacetic acid (EDTA), 140mM NaCl, 5mM KCl, 5.5mM glucose, and 4mM NaHCO₃), followed by trypsinization for 10-15 mins at 37°C with subsequent aliquoting into flasks or plates once media was added to inhibit trypsin. Prior to subculturing, all solutions and media were pre-heated to 37°C using an ISOTEMP 210 water bath (Fisher Scientific, Waltham, Massachusetts, USA).

2.3 CELL TREATMENT

Cells were plated and allowed to adhere to the plate for approximately 24hrs before the cells (at ~70% confluency) were treated with a water or ethanolic extract. The stock solution of each extract was made as the following: 200mg/mL lemongrass extract dissolved in 100% EtOH, 250mg/mL eleuthero ginseng extract dissolved in 100% EtOH, and 100mg/mL hibiscus extract dissolved in ddH₂O. Before treating any cells, each stock solution was diluted in PBS to make a 10mg/mL standard, and this was used to make the final dose of each drug (in media) in order to treat the cells. The final concentrations of each extract used to treat ranged from 0.01-1.0mg/mL.

Tamoxifen, 4-OH-tamoxifen, staurosporine, and paraquat were used as positive controls for experiments investigating cell death, mitochondrial outer membrane depolarization and reactive oxygen species (ROS). Tamoxifen (Sigma-Aldrich Canada Ltd, Mississauga, Ontario, Canada; Cat. No. T5648) was made at a stock concentration of 100mM (in DMSO), and diluted to 1.0mM (in PBS). The latter stock solution was used to treat cells at a final concentration of 15µM. 4-OH-tamoxifen (Sigma-Aldrich Canada Ltd, Mississauga, Ontario, Canada; Cat. No. T176) was made at a stock concentration of 1mM (in PBS), and diluted to a final concentration of 1µM (in PBS) for cell treatment.
Before treatment of breast cancer cells with an NHP extract (either lemongrass, eleuthero ginseng, or hibiscus), the stock extract was diluted in PBS to make a 10mg/mL standard, before being further diluted in media to make the final dose used to treat the cells.

The majority of the objectives focused on treating breast cancer cells with one NHP extract. However, additional experiments were done to see if combinations of extracts could have an enhanced induction of cell death compared to single extract treatment. Furthermore, common breast cancer hormonal therapy, such as Tamoxifen and its metabolite 4-OH-Tamoxifen, were used to treat cells an hour before or after treatment with an NHP extract to see if the cells could be sensitized to the induction of cell death.

2.3.1 Cell Viability WST-1 Assay:

Cell viability after treatment with an NHP extract (lemongrass, eleuthero ginseng or hibiscus) was assessed by a water-soluble tetrazolium-1 (WST-1) colorimetric assay. This assay indirectly measures cell proliferation based on the formation of metabolic reducing agents (ie. NAD(P)H) being able to reduce (and cleave) the water-soluble tetrazolium-1 salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] to formazan, a product that strongly absorbs light at 450nm. Therefore, the amount of formazan product formed (ie. higher absorbance at 450nm) directly correlates with the number of metabolically active cells in the sample culture. For this assay, cells were plated in a 96-well plate at a cell density dependent on the growth rate of a particular cell line, so that the wells were approximately 70% confluent when treated. For example, MCF-7’s were plated at 6,000 cells/well, MDA-MB-231’s were plated at 10,000 cells/well, whereas SUM149s were plated at 8,000 cells/well. Following plating,
cells were given 24hrs to adhere to the well, at which point the wells were treated with various doses of extracts. Following treatment, one plate was given 48hrs and another plate was given 96hrs before 5µL the WST-1 dye was added to each well and allowed to incubate for 4±1hrs. After this incubation period, the absorbance of each well at 450nm was measured using the SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA, Part no. M5E) and the SoftMax Pro 5 software. The absorbance of each well after treatment with an extract was then calculated and graphed as a percentage of the control. A decrease in absorbance is indicative of reduced metabolic activity, which indirectly signifies reduced proliferation.

Additionally, a second experiment was performed in order to evaluate if the efficacy of NHP extract treatment on reducing metabolic viability can be increased by treating multiple times at lower doses compared to treating once with a higher dose. This ‘double dose’ experiment was carried out the same as the WST-1 assay described above, except an extra treatment group was treated a second time 48hrs after the initial treatment. At the 96hrs timepoint after the initial treatment, the three treatment groups were given the WST-1 dye, incubated for 4±1hrs, and then the absorbance of each well at 450nm was measured.
2.3.2 Trypan Blue Cell Exclusion Assay:

In order to quantify the cell proliferation rate of viable breast cancer cells following treatment was an NHP extract, trypan blue staining was used to identify/label viable vs. dead cells. Trypan blue is a cell-impermeable dye that stains only dead cells whose plasma membranes are compromised and therefore allows the blue dye to aggregate within the dying/dead cells. A 1:1 mixture of cells resuspended in PBS and 0.4% Trypan blue dye (Sigma Chemical Company, Mississauga, Ontario, Canada, Cat. No. T8154) was loaded onto a haemocytometer (Hausser Scientific, USA) and was placed inside an Invitrogen Countess II FL Automated Cell Counter (ThermoFisher Scientific, Canada, Cat. No. AMQAF1000). This countess machine quantified the number of viable (unstained) and non-viable (stained) cells of each sample. The number of viable cells per mL was measured at 24, 48, 72 and 96hrs post treatment with an extract, and this was used to create a cell proliferation curve. The initial number of cells plated was dependent on each cell line’s confluency and growth rates in order to prevent overpopulation at later timepoints past treatment.
2.3.3 Annexin-V Binding Assay and PI Staining:

In order to observe phosphatidylserine externalization from the inner to outer leaflet of the cell membrane, an early marker of apoptosis, breast cancer cells were treated with an NHP extract and collected at 24hrs and 48hrs post treatment. The cells were then washed twice with PBS, and then resuspended in Annexin-V binding buffer (10mM HEPES/NaOH, pH 7.5, 140mM NaCl, 2.5mM CaCl$_2$, 50mM Sucrose). Added to this was the Annexin-V Alexa Fluor 488 conjugate (Sigma-Aldrich Canada, Mississauga, Ontario, Canada) at a ratio of 2µL:25µL AV alexa fluor dye to AV binding buffer. This mixture was allowed to incubate for 15 mins at 25°C before the samples were analyzed. Binding of Annexin-V Alexa fluor to externalized phosphatidylserine gives off a green fluorescence, and the percentage and amount of cells that were GFP-positive was measured using the Invitrogen Countess II FL Automated Cell Counter, or visualized using fluorescence microscopy.

Furthermore, cell death was also detected by propidium iodide (PI) staining. This red-fluorescent dye is normally impermeable to cell membranes, but can cross and aggregate within dying cells whose cell membrane had been lysed during a later stage of cell death. In order to detect cell death, propidium iodide (Life Technologies Inc, Burlington, Ontario, Canada, Cat. No. P3566) was added at a final concentration of 0.01mg/mL and allowed to incubate for 15 mins before the samples were analyzed. The percentage and amount of cells that are RFP-positive (ie. marked with the red fluorescent dye) was also measured using the Invitrogen Countess II FL Automated Cell Counter, or visualized using fluorescence microscopy.
2.3.4 Evaluation of Mitochondrial Membrane Potential Using TMRM:

To monitor depolarization of the mitochondrial membrane potential ($\Delta \Psi_m$) following treatment of breast cancer cells with an NHP extract, TMRM (tetramethylrhodamine methyl ester) was utilized. TMRM (ThermoFisher Scientific, Canada, Cat. No. T668) is a cell-permeable, cationic, lipophilic, red fluorescent dye that is readily sequestered by active/healthy mitochondria. At 3, 6, 12 and 24hrs post treatment with an NHP extract, breast cancer cells were collected and incubated with TMRM at a concentration of 0.5µM for 45 mins at 37°C in the dark. The percentage and amount of cells that are RFP-positive (ie. marked with the red fluorescent dye) was measured using the Invitrogen Countess II FL Automated Cell Counter, or visualized using fluorescence microscopy. A decrease in red fluorescence (compared to the control) indicates a smaller number of healthy/active mitochondria.
**2.3.5 Monodansylcadaverine (MDC) Assay:**

To detect autophagy within breast cancer cells following treatment with an NHP extract, a MDC assay was performed. MDC (Sigma Chemical Company, Mississauga, Ontario, Canada) is an autofluorescent dye that specifically accumulates in autophagic vacuoles (more specifically, autophagolysosomes). Accumulation of MDC in autophagic vacuoles produces a bright punctate stain that can be visualized by fluorescence microscopy. Propidium iodide (PI) was used as a co-stain with MDC to label dead cells.

Breast cancer cells were plated in 6-well plates containing coverslips (coated with poly-L-lysine for 1 hr prior) and the cells were allowed to adhere to the coverslips for approximately 24 hrs. The cells were then treated with various NHP extract doses for 48 hrs. At the 48 hrs timepoint, 100µM MDC was diluted 1:25 in PBS and added to the treated cells along with PI, and allowed to incubate for 15 mins at 37°C. Following incubation, the cells were visualized and images were taken using a fluorescence microscope at 400x objective.

Tamoxifen (Sigma-Aldrich Canada Ltd, Mississauga, Ontario, Canada; Cat. No. T5648) is known to induce autophagy (Hwang et al., 2010), and thereby causes the characteristic appearance of ‘punctate’ staining. Therefore, Tamoxifen (at a concentration of 15µM) was used as a positive control for this experiment.
2.3.6 Probing Levels of (Whole Cell) Reactive Oxygen Species (ROS):

To determine if production of reactive oxygen species (ROS) was a cause of apoptosis, an \( \text{H}_2 \text{DCFDA} \) assay was performed. \( \text{H}_2 \text{DCFDA} \), 2',7'-dichlorodihydrofluorescein diacetate, is a non-fluorescent cell-permeable compound that is a reduced form of the fluorescent molecule fluorescein. \( \text{H}_2 \text{DCFDA} \) (Sigma-Aldrich Canada, Mississauga, ON, Canada, Cat. no. D6883) was used as an indicator for the production of ROS since cleavage of the acetate groups by intracellular esterases (which retains the molecule within the cell), followed by oxidation by ROS (specifically, hydrogen peroxide, hydroxyl radicals, and peroxynitrite) forms the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF), which has an excitation and emission wavelength of 513 and 530nm, respectively.

At 3 and 6hrs post treatment with an NHP extract, MCF-7 and MDA-MB-231 breast cancer cells were collected after being incubated with \( \text{H}_2 \text{DCFDA} \) for the past 40 mins (thereby allowing the cells to take up the cell-permeable dye), the levels of DCF (which are directly proportional to levels of ROS) was quantified by fluorescence microscopy using a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA, Part no. M5E) and the SoftMax Pro 5 software. Levels of ROS (measured in RFU) were normalized per 10,000 live cells in each treatment sample. The number of live cells per sample was measured by trypan blue cell exclusion assay using the Invitrogen Countess II FL automated cell counter.

Paraquat (PQ; Sigma-Aldrich Canada, Mississauga, ON, Canada, Cat. no. 856177) is known to inhibit complex I of the electron transport chain, and thereby causes
the production of ROS (Cochemé and Murphy, 2008). Therefore, Paraquat (at a concentration of 250µM) was used as a positive control for this experiment.

2.3.7 Gene Expression Profiling of NHP Treated Breast Cancer Cells:

i. *RNA Extraction and cDNA Synthesis*

Following treatment of MDA-MB-231 breast cancer cells with a 0.25mg/mL dose of an NHP extract (lemongrass, eleuthero ginseng, or hibiscus) for 24 or 48hrs depending on the extract, total cellular RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Inc.), according to the manufacturer’s protocol. Quality of the RNA was then examined by measuring the A$_{280}$:A$_{260}$ ratio (NanoDrop 2000). Using 500ng of the total RNA, cDNA was synthesized using the RE3 Reverse Transcriptase Mix first-strand synthesis system. Following a denaturation step of 5 mins at 42°C, RNA was reverse transcribed to a single stranded cDNA using oligo(dT) primers (Qiagen, Inc.). The reverse transcription reaction was performed in a total volume of 20 µL at 42°C for 15 mins, followed immediately by 95°C for 90 mins.

ii. *RT$^2$ Profiler PCR Array (Human Cancer PathwayFinder)*

Following the reverse transcription of isolated RNA, polymerase chain reaction (PCR) was performed on the Human Cancer PathwayFinder (Qiagen, Inc., Cat. no. 330231 PAHS-033ZA) using the RT$^2$ Profiler PCR array system from Qiagen, Inc. 384-well (4 x 96-well) plates containing gene-specific primer sets for 84 relevant genes representative of 9 different biological pathways involved in transformation and tumorigenesis. Positive and negative control gene sets were also used.

Amplification of specific gene products was detected using the SYBR Green PCR mastermix and the real time amplification data was gathered using the ABI 7900HT
software. The samples were amplified for 40 cycles for 15s at 95°C and 60s at 60°C. To confirm the specificity of amplification, each gene was completed with a melting curve analysis. Gene expression was normalized to controls (housekeeping genes) to establish a reference for gene expression fold changes between the controls and treated samples by C_T method (Qiagen RT^2 Profiler PCR Array Analysis program).

2.3.8 Effects of NHP Extracts in-vivo:

i. Efficacy of Extracts in Tumor Xenografts of Immunocompromised Mice

In accordance with the animal protocols outlined in the University of Windsor research ethics board (AUPP #10-17), six-week-old female CD-1 nu/nu immunocompromised mice were obtained from Charles River Laboratories and were housed in laboratory conditions of 12hrs light/dark cycles. Following acclimatization, all female mice were subcutaneously injected with a triple negative breast cancer cell line and matrigel (at a 1:1 ratio of cells in PBS to matrigel) on both the left and right hind flanks. The left side was injected with MDA-MB-231 (5x10^6, 3x10^6, and 1x10^6 cells for trials 1, 2, and 3, respectively), and the right flank was injected with SUM149 (1x10^6 cells for all 3 trials). Tumors were allowed to develop for approximately 7-10 days, following which mice were divided into groups of 3 or 4 mice (depending on total number of mice and number of treatment groups). The mice were supplied with regular drinking water and food throughout the duration of the studies. Mice were orally gavaged three times a week (Monday, Wednesday, Friday), and each time the weight of each mouse and tumor size was measured to evaluate the efficacy of NHP extract oral administration on the growth of the breast cancer tumors. Tumor size
(volume in \(\text{mm}^3\)) was approximated using the length (L), width (W), and height (H) measured with a standard caliper and was calculated by \(L \times W \times H\).

\[\text{ii. Toxicity Assessment}\]

Mice toxicity was assessed by measuring the weight of each mouse each time the mice were treated and had their tumor(s) measured. Furthermore, following the duration of the study, mice were sacrificed and their organs and tissues (liver, kidneys, heart and tumors) were collected and stored in formaldehyde for toxicity staining.

\[\text{2.3.9 Hematoxylin & Eosin (H&E) Staining:}\]

Following the mice study, mice were sacrificed and their organs and tissues (kidneys, liver, heart and tumors) were obtained and fixed in 10\% formaldehyde for toxicological analysis. Mice organs were cryosectioned into 10-micron sections and placed on superfrost/PLUS microscope slides (Fisherbrand, Fisher Scientific). These sections of organs were stained according to a standardized H&E protocol (Fisher et al., 2008).

\[\text{Statistical Analysis}\]

All experiments were completed in triplicate and the results are expressed as mean ± standard deviation. Statistical analysis was performed with GraphPad Prism 5.0 288 software.
CHAPTER 3 – RESULTS

3.1 – ASSESSING THE ANTICANCER ACTIVITY OF NHP EXTRACTS

3.1.1 – Metabolic Viability (WST-1 Assay):

In order to evaluate the effect of treating one ER-positive (MCF-7), and three triple negative (MDA-MB-231, MDA-MB-468, SUM149) human breast cancer cell lines with an NHP extract, metabolic viability was measured as a function of cell metabolism via a WST-1 assay. The four methods of extracting lemongrass (cold water, hot water, ethanol unfiltered, and ethanol filtered) all reduced metabolic viability in a dose-dependent manner, with the unfiltered ethanolic extract having the most potent activity (FIGURE 3.1.1.1). Similarly to lemongrass, the unfiltered ethanolic extract of eleuthero ginseng had the most potent activity for reducing metabolic viability of breast cancer cells (FIGURE 3.1.1.2). On the other hand, the hot and cold water extracts of hibiscus had the most potent activity and was roughly equivalent with one another. Therefore, the unfiltered ethanolic extracts of lemongrass and eleuthero ginseng, and the hot/cold water extracts of hibiscus were used in all subsequent experiments.

In a subsequent experiment, MCF-7 and MDA-MB-468 cells exposed to lemongrass or eleuthero ginseng extract at the start of treatment (time zero) and again at 48hrs after initial treatment had a more efficacious effect at 96hrs post initial treatment compared to treating once (FIGURE 3.1.1.4). This implies the cells metabolized the lemongrass extract relatively quickly, and therefore multiple treatments at low doses prolonged and enhanced the effect on reducing metabolic viability. However, treating breast cancer cells with hibiscus extract multiple times was less effective at the 96hrs timepoint compared to treating once. While conducting this subsequent experiment, the
doses used were increasing by small increments in order to find the IC$_{50}$, which was found to be approximately ~0.05, ~0.30, and ~0.25mg/mL for lemongrass, ginseng and hibiscus, respectively. These values are approximations as the EC$_{50}$ depends on the particular cell line being treated and the timepoint of interest.

Furthermore, some combinations of extracts reduced metabolic viability more effectively/efficiently than either extract alone. More specifically, the 0.1mg/mL dose of any two extracts combined had a better effect on MCF-7 cells than on their own (FIGURE 3.1.1.5). Whereas for the MDA-MB-231 cells, combinations of 0.25mg/mL ginseng and 0.1mg/mL of hibiscus or lemongrass was more effective than either extract dose on their own (FIGURE 3.1.1.6).

In order to evaluate the potential of sensitizing breast cancer cells to treatment using NHP extracts and hormonal therapy, MCF-7 cells were treated with tamoxifen or 4-OH-tamoxifen an hour before or after NHP treatment and metabolic viability was measured. Eleuthero ginseng enhanced the reduction of metabolic viability (FIGURE 3.1.1.7), whereas treatment of MDA-MB-231 cells with hibiscus before or after treatment with tamoxifen or 4-OH-tamoxifen was more effective at reducing metabolic viability at 48hrs than hibiscus alone (FIGURE 3.1.1.8). However, the enhanced reduction of metabolic viability for both MCF-7 and MDA-MB-231 cells was not statistically significant.
FIGURE 3.1.1.1. Lemongrass extracts effectively reduced metabolic viability in breast cancer cells. Breast cancer cells were plated overnight and treated for 48 or 96hrs. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. A, MCF-7 cells and B, MDA-MB-231 and SUM149 cells. Statistical analysis was performed using the 2-way ANOVA test. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
FIGURE 3.1.1.2. **Unfiltered ethanolic eleuthero ginseng extract effectively reduced metabolic viability in breast cancer cells.** Breast cancer cells were plated overnight and treated for 48 or 96hrs. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. A, MCF-7 cells and B, MDA-MB-231 and SUM149 cells treated with unfiltered ethanolic ginseng extract. Statistical analysis was performed using the 2-way ANOVA test. *p < 0.05 vs control, **p < 0.01 vs control, ***p < 0.001 vs control, ****p < 0.0001 vs control.
FIGURE 3.1.1.3. **Water extracts of hibiscus effectively reduced metabolic viability in breast cancer cells.** Breast cancer cells were plated overnight and treated for 48 or 96hrs. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. *A*, MCF-7 cells and *B*, MDA-MB-231 and SUM149 cells treated with cold water hibiscus extract. Statistical analysis was performed using the 2-way ANOVA test. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
FIGURE 3.1.1.4. Treating breast cancer cells with lemongrass and ginseng extracts multiple times was more effective at reducing metabolic viability than treating once, but the opposite was seen with hibiscus. MCF-7 and MDA-MB-468 breast cancer cells were plated overnight and treated for 48 or 96hrs, as well as an extra group treated a second time 48hrs after initial treatment (blue) to see how metabolism of NHP extracts affect metabolic viability. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results are shown as mean ± SD from three independent experiments. Statistical analysis was performed using the 2-way ANOVA test. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
FIGURE 3.1.1.5. **Combinations of NHP extracts enhanced the reduction of metabolic viability of MCF-7 cells compared to single NHP extract treatment.** Breast cancer cells were plated overnight and treated for 48 or 96 hrs. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450 nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. Statistical analysis was performed using the 2-way ANOVA test. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
FIGURE 3.1.1.6. Combinations of NHP extracts enhanced the reduction of metabolic viability of MDA-MB-231 cells compared to single NHP extract treatment. Breast cancer cells were plated overnight and treated for 48 or 96hrs. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. Statistical analysis was performed using the 2-way ANOVA test. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
FIGURE 3.1.1.7. Treatment of MCF-7 cells with tamoxifen or 4-OH-tamoxifen an hour after ginseng extract may enhance the reduction of metabolic viability. Breast cancer cells were plated overnight and treated for 48 or 96hrs. Cells were treated with eleuthero ginseng extract an hour before or after treatment with Tamoxifen or 4-OH-Tamoxifen to see if the cells can be sensitized. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. Statistical analysis was performed using the one-way ANOVA test. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
FIGURE 3.1.1.8. Treatment of MDA-MB-231 cells with tamoxifen an hour before or after treatment with hibiscus extract may enhance the reduction of metabolic viability. Breast cancer cells were plated overnight and treated for 48 or 96hrs. Cells were treated with a hibiscus extract an hour before or after treatment with Tamoxifen or 4-OH-Tamoxifen to see if the cells can be sensitized. Following treatment, the WST-1 assay was implemented to assess the cytotoxicity. Absorbance was measured at 450nm and each value was represented as a percent of control. Results shown are the mean ± SD from three independent experiments. Statistical analysis was performed using the one-way ANOVA test. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
3.1.2 – Cell Proliferation Rate (Trypan Blue Cell Exclusion Assay):

In order to assess if each NHP extract has the ability to decrease cell proliferation to compliment previously shown metabolic viability results, the effect of lemongrass, eleuthero ginseng and hibiscus on cell proliferation rates of human breast cancer cells was evaluated using a Trypan Blue cell exclusion assay. All lemongrass doses (except 0.01mg/mL) had a similar efficacy for reducing/halting proliferation rates as the positive controls 15µM Tamoxifen (for MCF-7) and 1µM Staurosporine (for MDA-MB-231, and MDA-MB-468) (FIGURE 3.1.2.1).

As for eleuthero ginseng, the two lower doses (0.1 and 0.25mg/mL) were not as effective as the positive controls for reducing/halting proliferation rates for all three cell lines. Only the highest dose ginseng (0.5mg/mL) had a similar efficacy as the positive controls (FIGURE 3.1.2.2).

Likewise, hibiscus caused a dose-dependent slowing of cell proliferation rates in both MCF-7 and MDA-MB-468 cells, although none of the doses used were as effective as either positive controls FIGURE 3.1.2.3).
FIGURE 3.1.2.1. Lemongrass extract caused a dose-dependent slowing/halting of the proliferation rates of both the ER+ and triple negative cell lines. MCF-7, MDA-MB-231, and MDA-MB-468 cells were each plated overnight in four 12-well plates at a density of 80000, 100000, and 60000 cells per/well, respectively, as these cell densities gave a 70% confluency by the time the cells were treated with various doses of ethanolic lemongrass extract. Following treatment, a trypan blue cell exclusion assay was implemented to quantify the number of viable cells at each timepoint post treatment. Results are shown as mean ± SD from three independent experiments, except for MDA-MB-231.
FIGURE 3.1.2.2. *Eleuthero ginseng* extract caused a dose-dependent slowing/halting of the proliferation rates of both the ER+ and triple negative cell lines. MCF-7, MDA-MB-231, and MDA-MB-468 cells were each plated overnight in four 12-well plates at a density of 80000, 100000, and 60000 cells per/well, respectively, as these cell densities gave a 70% confluency by the time the cells were treated with various doses of ethanolic ginseng extract. Following treatment, a trypan blue cell exclusion assay was implemented to quantify the number of viable cells at each timepoint post treatment. Results are shown as mean ± SD from three independent experiments, except for MDA-MB-231.
FIGURE 3.1.2.3. Hibiscus extract slowed the proliferation rates of both the ER+ and triple negative cell lines. MCF-7 and MDA-MB-468 cells were each plated overnight in four 12-well plates at a density of 80,000 and 60,000 cells per/well, respectively, as these cell densities gave a 70% confluency by the time the cells were treated with various doses of aqueous water extract. Following treatment, a trypan blue cell exclusion assay was implemented to quantify the number of viable cells at each timepoint post treatment. Results are shown as mean ± SD from three independent experiments.
3.1.3 – Programmed Cell Death Induction (AVPI Assay):

Since previous results found each NHP extract to decrease metabolic viability and cell proliferation rate of human breast cancer cell lines, the next objective was to assess if lemongrass, eleuthero ginseng and/or hibiscus cause cell death. Apoptosis is a well-known form of programmed cell death. An early marker of the onset of apoptosis is the reorganization of the cell’s membrane phospholipids, specifically, externalization of phosphatidylserine from the inner leaflet of the cell membrane to the outer leaflet (Bratton et al., 1997). This early marker of apoptosis was observed with an Annexin-V binding assay. Following treatment of MCF-7 cells for 48hrs (also observed at 24 and 96hrs), all three extracts were shown to cause a small increase in green fluorescence compared to the untreated control cells. In addition, there was an increase in red fluorescence, although this increase from hibiscus was very small/negligible (FIGURE 3.1.3.1A). These green and red markers are indicative of early and late stages of apoptosis, respectively. Subsequent quantification of Annexin-V-positive and Propidium Iodide-positive cells confirmed the small increase in cell death induced by each NHP extract. The efficacy of lemongrass and eleuthero ginseng on MCF-7 cells was shown to be dose-dependent, whereas hibiscus was not (FIGURE 3.1.3.1B).

Furthermore, NHP-induced cell death was observed in MDA-MB-231 cells with similar (or greater in the case of lemongrass) efficacy than in the MCF-7 cells (FIGURE 3.1.3.2). Both doses of each NHP extract caused a significant, moderate and minor reduction in the proportion of viable cells from lemongrass, ginseng and hibiscus, respectively. Both doses of lemongrass extract were shown to be more effective at
inducing cell death compared to Staurosporine, a positive control for cell death, whereas both doses used for ginseng and hibiscus was less effective compared to Staurosporine.

For both MCF-7 (FIGURE 3.1.3.3) and MDA-MB-231 (FIGURE 3.1.3.4) cells, some combinations of extracts were shown to be more effective at inducing cell death compared to either extract alone. Other combinations of extracts were tested as well, but did not show any enhanced effect on cell death compared to either extract alone. These other combinations include (ethanolic) ginseng and (hot water) hibiscus for MCF-7 cells, and (hot water) lemongrass and (hot water) hibiscus for MDA-MB-231 cells.

Moreover, treatment of MCF-7 cells with tamoxifen an hour before eleuthero ginseng may enhance the amount of cells undergoing cell death (FIGURE 3.1.3.5). Similarly, treatment of MDA-MB-231 cells with 4-OH-tamoxifen an hour after hibiscus may enhance the proportion of cells undergoing cell death (FIGURE 3.1.3.6).
FIGURE 3.1.3.1. **NHP extracts caused the induction of cell death in MCF-7 breast cancer cells.** MCF-7 cells were treated with increasing doses of lemongrass, eleuthero ginseng and hibiscus extracts and analyzed for the induction of cell death. A, Hoechst, Annexin-V, and propidium iodide staining of MCF-7 cells 48hrs after treatment. Similar results were seen at both 24 and 96hrs post-treatment. Magnification at 400X. Scale bar = 25µm. B, Quantification of Annexin-V, Propidium iodide or both for MCF-7 cells at both 24 and 48hrs post-treatment with an NHP extract. Values are expressed as mean ± SD from three independent experiments. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
FIGURE 3.1.3.2. **NHP extracts caused the induction of cell death in MDA-MB-231 breast cancer cells.** MDA-MB-231 cells were treated with increasing doses of lemongrass, eleuthero ginseng and hibiscus extracts and analyzed for the induction of cell death. At both 24 and 48hrs post-treatment, cells were stained with Annexin-V and Propidium Iodide and the cells were quantified using an Invitrogen Countess II FL Automated Cell Counter. Values are expressed as mean ± SD from three independent experiments. * p < 0.05 vs control, ** p < 0.01 vs control, *** p < 0.001 vs control, **** p < 0.0001 vs control.
FIGURE 3.1.3.3. Some combinations of extracts enhanced the induction of cell death in MCF-7 cells compared to either extract alone. MCF-7 cells were treated with single or combinations of extracts at low doses and analyzed for the induction of cell death. At 48hrs post-treatment, cells were stained with Annexin-V and Propidium Iodide and the cells were quantified using an Invitrogen Countess II FL Automated Cell Counter. Values are expressed as mean ± SD from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
FIGURE 3.1.3.4. Some combinations of extracts enhanced the induction of cell death in MDA-MB-231 cells compared to either extract alone. MDA-MB-231 cells were treated with single or combinations of extracts at low doses and analyzed for the induction of cell death. At 48hrs post-treatment, cells were stained with Annexin-V and Propidium Iodide and the cells were quantified using an Invitrogen Countess II FL Automated Cell Counter. Values are expressed as mean ± SD from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
FIGURE 3.1.3.5. Pre-treatment with tamoxifen enhanced the induction of cell death in MCF-7 cells by eleuthero ginseng extract. MCF-7 cells were treated with an eleuthero ginseng extract an hour before or after treatment with Tamoxifen or 4-OH-Tamoxifen to see if the cells can be sensitized. Following treatment, the cells were analyzed for the induction of cell death. At 48hrs post-treatment, cells were stained with Annexin-V and Propidium Iodide and the cells were quantified using an Invitrogen Countess II FL Automated Cell Counter. Values are expressed as mean ± SD from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
FIGURE 3.1.3.6. Treatment of MDA-MB-231 cells with 4-OH-tamoxifen an hour after hibiscus extract enhanced the induction of cell death. MDA-MB-231 cells were treated with a cold water hibiscus extract an hour before or after treatment with Tamoxifen or 4-OH-Tamoxifen to see if the cells can be sensitized. Following treatment, the cells were analyzed for the induction of cell death. At 48hrs post-treatment, cells were stained with Annexin-V and Propidium Iodide and the cells were quantified using an Invitrogen Countess II FL Automated Cell Counter. Values are expressed as mean ± SD from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
3.2 – INVESTIGATING POTENTIAL MECHANISM(S) OF ACTION

3.2.1 – Mitochondrial Membrane Potential (TMRM Assay):

Since the mitochondria are directly or indirectly linked to many cell death pathways (including both extrinsic and intrinsic apoptosis), the effect of lemongrass, eleuthero ginseng and hibiscus extracts on the integrity and function of mitochondria was analyzed. The mitochondrial membrane potential ($\Delta \Psi_m$) was evaluated by staining with TMRM, a dye that fluoresces red when localized to the mitochondrial matrix with an intact membrane potential. Treatment of both MCF-7 and MDA-MB-231 cells with lemongrass or eleuthero ginseng extracts for 24hrs destabilized the membrane potential in a dose-dependent manner, which was observed by the loss of red fluorescence (FIGURE 3.2.1.1 and FIGURE 3.2.1.2). Lemongrass was shown to be more potent at disrupting the mitochondrial potential compared to ginseng. Hibiscus was also analyzed for its ability to destabilize the mitochondria, but failed to have any effect compared to the control cells, and therefore, these results were not shown. Permeabilization of the mitochondrial membrane potential is a characteristic of apoptosis, and thus this event may play a key role in the mechanism of cell death induced by both lemongrass and eleuthero ginseng extracts, but not for hibiscus.
FIGURE 3.2.1.1. Lemongrass extract disrupted the mitochondrial membrane potential ($\Delta \Psi_m$) in a time- and dose-dependent manner for both ER+ and triple negative breast cancer cells. Following treatment with lemongrass extract, MCF-7 and MDA-MB-231 cells were incubated with TMRM dye to detect the loss of mitochondrial membrane potential. Red fluorescence indicates intact mitochondria with a normal membrane potential ($\Delta \Psi_m$), while blue fluorescence from Hoechst was used as a counterstain. Staurosporine (STS) was used as a positive control for the depolarization of the mitochondrial membrane potential. A, Fluorescence spectroscopy of lemongrass-treated MCF-7 and MDA-MB-231 cells at 24hrs after treatment. Magnification at 400X. Scale bar = 25µm. B, Quantification of TMRM (RFP)-positive mitochondria for both MCF-7 and MDA-MB-231 cells at various timepoints post-treatment.
FIGURE 3.2.1.2. Eleuthero ginseng extract disrupted the mitochondrial membrane potential ($\Delta \Psi_m$) in a time- and dose-dependent manner for both ER+ and triple negative breast cancer cells. Following treatment with ginseng extract, MCF-7 and MDA-MB-231 cells were incubated with TMRM dye to detect the loss of mitochondrial membrane potential. Red fluorescence indicates intact mitochondria with a normal membrane potential ($\Delta \Psi_m$), while blue fluorescence from Hoechst was used as a counterstain. Staurosporine (STS) was used as a positive control for the depolarization of the mitochondrial membrane potential. A, Fluorescence spectroscopy of ginseng-treated MCF-7 and MDA-MB-231 cells at 24hrs after treatment. Magnification at 400X. Scale bar = 25µm. B, Quantification of TMRM (RFP)-positive cells for both MCF-7 and MDA-MB-231 cells at various timepoints post-treatment. Eleuthero ginseng extract reduces the number of mitochondria with an intact membrane potential in a dose-dependent manner, and appears to have similar efficacy to the control Staurosporine.
3.2.2 – Induction of Autophagy (MDC Assay):

Autophagy is involved in the maintenance of cellular homeostasis that is activated in response to stressful physiological conditions (He et al., 2013). Although usually considered a pro-survival process, autophagy can also be used as a mode of programmed cell death (Denton et al., 2015). Cells undergoing autophagy contain autophagolysosomes that can be stained and visualized with monodansylcadaverine (MDC), giving a ‘punctate’ staining appearance. After treating MCF-7 cells with an NHP extract for 48hrs, the cells were stained with MDC, as well as counterstained with Propidium Iodide (PI) to label dead cells, to be able to conclude if any autophagy present is pro-death or pro-survival. Tamoxifen was used as a positive control for the induction of autophagy. Compared to the controls, only eleuthero ginseng caused an increase in blue fluorescence in a dose-dependent manner, but lemongrass and hibiscus extracts did not (FIGURE 3.2.2). Thus, autophagy may play a role in the induction of cell death by eleuthero ginseng, but not lemongrass and hibiscus. In order to confirm these results and better conclude if autophagy is induced, these results have to be quantified in future experiments.
FIGURE 3.2.2. Activation of autophagy may not play a role in the induction of cell death by lemongrass and hibiscus extracts, but may be activated by eleuthero ginseng. MCF-7 cells were treated with lemongrass, ginseng and hibiscus extracts for 48hrs and analyzed for the induction of autophagy. Tamoxifen (TAM) was used as a positive control for the induction of autophagy. Following treatment, cells were stained with monodansylcadaverine (MDC) for detection of autophagic vacuoles and counterstained with PI to detect cell death. Images were captured using fluorescence microscopy. Magnification at 400X. Scale bar = 25µm. Since no punctate staining or increased blue fluorescence was observed in the lemongrass- and hibiscus-treated cells, autophagy may not be involved in the mechanism of cell death.
3.2.3 – Measuring the Levels of Reactive Oxygen Species (H₂DCFDA Assay):

Under normal physiological conditions, mitochondria are major producers of reactive oxygen species (ROS). However, levels of ROS increase dramatically under pathological conditions such as in a cancer cell. Since cancer cells are so heavily dependent on glycolysis for ATP production, in order to continue glycolysis, regeneration of NAD⁺ is dependent on reduction of pyruvate to lactate by LDHA. High levels of lactic acid lower the intracellular pH of a cancer cell, and thus cancer cells are susceptible to mitochondrial dysfunction (Koppenol et al., 2011). Furthermore, ROS production is known to have a dual role; it may be the cause or effect of apoptosis induction. One such role is ROS’ ability to activate certain death receptors in the extrinsic apoptosis pathway, such as TNFα or Fas (Simon et al., 2000). Thus, mitochondria are implicated in the initiation and/or execution of apoptosis. On the other hand, ROS may promote tumor progression by causing DNA damage (mutations) to tumor suppressor genes, or to the activation of proto-oncogenes. Cells react rapidly to a redox imbalance (either increased or decreased) with many biological responses including cell growth arrest, altered gene expression of antioxidant enzymes, initiation of signal transduction pathways, or repair of damaged DNA. These early events of responding to a redox imbalance likely determine whether a cell will undergo cell death via apoptosis, necrosis, senesce, or survive and proliferate (Limoli et al., 1998).

To further assess the mechanism of each NHP extract inducing cell death in human breast cancer cells, the production of ROS was studied. MCF-7 and MDA-MB-231 cells were treated, and after 3 and 6hrs of treatment, whole-cells were incubated with H₂DCFDA analyzed for ROS levels directly proportional to green fluorescence. Paraquat
(PQ) was used as a positive control for the induction of oxidative stress. In both cell lines, lemongrass treatment reduced levels of ROS (per 10,000 cells) compared to the untreated control cells (FIGURE 3.2.3), although the reduction was not statistically significant. The reduction of ROS at such early timepoints (before any significant cell death occurs) indicated that ROS is not likely the cause of the induction of cell death by lemongrass extract.

In contrast, hibiscus treatment increased the level of ROS in both cell lines compared to the untreated control cells (FIGURE 3.2.3), although the increase was not statistically significant at both 3 and 6hrs. The increase of ROS at such early timepoints (before any significant cell death occurs) indicates that ROS may be an early event that leads to the induction of cell death by hibiscus extract.

Moreover, eleuthero ginseng did not alter the level of ROS in both cell lines compared to the untreated control cells (FIGURE 3.2.3). Since there were similar levels of ROS at such early timepoints, ROS is not likely the cause of the induction of cell death by eleuthero ginseng extract.
FIGURE 3.2.3. Lemongrass extract had antioxidant properties, and thus oxidative stress is not an early event that leads to the induction of cell death by lemongrass extract. MCF-7 and MDA-MB-231 cells were treated with lemongrass extract for 3 and 6hrs and analyzed for whole-cell levels of ROS. Paraquat (PQ) was used as a positive control for the induction of oxidative stress. Following treatment, cells were incubated with H$_2$DCFDA for detection of oxidative stress levels directly proportional to green fluorescence. RFU was measured by fluorescence spectroscopy, and the concentration of live cells was measured by trypan blue cell exclusion assay. Values are expressed as mean ± SD from at least three independent experiments, except for 500µM PQ, which was only done once. For both cell lines, the reduction of ROS between control and lemongrass (0.25mg/mL) was not statistically significant at both 3 and 6hrs.
3.2.4 – RT² Profiler PCR Array (Human Cancer PathwayFinder):

Potentially the best method of deciphering the mechanism for how each NHP extract causes cell death in human breast cancer cells, PCR was performed using the RT² Profiler PCR Array (Human Cancer PathwayFinder), which contained gene-specific primer sets for 84 relevant genes representative of 9 different biological pathways involved in transformation and tumorigenesis. Compared to control MDA-MB-231 cells, treatment with lemongrass extract (0.25mg/mL) caused (FIGURE 3.2.4.1) an upregulation of two pro-apoptotic genes, a downregulation of two antiapoptotic genes, as well as an upregulation of genes known to have DNA repair effects, tumor suppression, growth arrest and/or cell cycle control (Johnen et al., 2013). Furthermore, genes involved in the cell cycle and metabolism were found to be downregulated. These results further confirm previous results such as the reduction in metabolic viability (FIGURE 3.1.1.1) and cell proliferation rates (FIGURE 3.1.2.1) and the induction of cell death (FIGURE 3.1.3.2).

Likewise, treatment with eleuthero ginseng extract (0.25mg/mL) caused a change in the gene expression of similar genes, for the most part (FIGURE 3.2.4.2). Specifically; an upregulation of two (possibly three) pro-apoptotic genes, downregulation of one anti-apoptotic gene, as well as an upregulation of genes known to have DNA repair effects, tumor suppression, growth arrest and/or cell cycle control (Johnen et al., 2013). Furthermore, two genes involved in the cell cycle were found to be downregulated, while genes involved with telomeres and telomerase were upregulated. These results further confirm previous results such as the reduction in cell proliferation rate (FIGURE 3.1.2.2) and the induction of cell death (FIGURE 3.1.3.2).
In contrast to lemongrass and eleuthero ginseng, hibiscus was found to have very different effects on the expression levels of the genes of interest (FIGURE 3.2.4.3). Treatment with Hibiscus extract (0.25mg/mL) caused a small reduction in the expression of most genes in these pathways, with only three genes found to be statistically different from basal levels of expression; reduced expression of FASLG, HMOX1, and DDB2 genes. Although, the reduced expression of NOL3 was trending towards significance (P = 0.0711). The slightly reduced expression of half the metabolism and cell cycle genes may compliment previous results such as the modest reduction in metabolic viability (FIGURE 3.1.1.3) and cell proliferation rates (FIGURE 3.1.2.3).
FIGURE 3.2.4.1. Treatment of MDA-MB-231 breast cancer cells with lemongrass extract changed the gene expression profile of important genes involved in transformation and tumorigenesis, ultimately promoting cell death. The effect of lemongrass extract on gene expression of MDA-MB-231 cells was evaluated using the RT2 profiler PCR assay. Following treatment with 0.25mg/mL lemongrass extract for 24hrs, RNA was isolated, converted into cDNA and then incubated with oligo-dT primers. Amplification of specific gene products was detected using the SYBR Green PCR mastermix and the real time amplification data was gathered using the ABI 7900HT software. All gene expression levels in the figure are relative to baseline expression levels of each gene in untreated (control) cells, which were set to 1. Values are expressed as mean ± SD from three independent experiments.
FIGURE 3.2.4.2. Treatment of MDA-MB-231 breast cancer cells with eleuthero ginseng extract changed the gene expression profile of important genes involved in transformation and tumorigenesis, ultimately promoting cell death. The effect of ginseng extract on gene expression of MDA-MB-231 cells was evaluated using the RT2 profiler PCR assay. Following treatment with 0.25mg/mL eleuthero ginseng extract for 24hrs, RNA was isolated, converted into cDNA and then incubated with oligo-dT primers. Amplification of specific gene products was detected and the real time amplification data was gathered. All gene expression levels in the figure are relative to baseline expression levels of each gene in untreated (control) cells, which were set to 1. Values are expressed as mean ± SD from three independent experiments.
FIGURE 3.2.4.3. Treatment of MDA-MB-231 cells with hibiscus extract did not significantly change the expression level of most genes of interest. The effect of hibiscus extract on gene expression of MDA-MB-231 cells was evaluated using the RT2 profiler PCR assay. Following treatment with 0.25mg/mL hibiscus cold water extract for 48hrs, RNA was isolated, converted into cDNA and then incubated with oligo-dT primers. Amplification of specific gene products was detected using the SYBR Green PCR mastermix and the real time amplification data was gathered using the ABI 7900HT software. All gene expression levels in the figure are relative to baseline expression levels of each gene in untreated (control) cells, which were set to 1. Values are expressed as mean ± SD from three independent experiments.
3.3 – EFFECTS OF NHP EXTRACTS *IN VIVO*

3.3.1 – Efficacy in Tumor Xenografts of Immunocompromised Mice:

In order to scientifically evaluate and validate the efficacy of NHP extracts as a form of cancer treatment, a mice study was performed for two independent trials. Six-week-old female CD-1 nu/nu immunocompromised mice were subcutaneously injected with two human breast cancer cell lines (MDA-MB-231 on left flank, and SUM149 on right flank) and matrigel and the cells were allowed to establish a tumor for approximately 10 days. Once established, for a period of approximately 10 weeks (each trial) these mice were orally treated (by gavage) with lemongrass, eleuthero ginseng, hibiscus, or a combination of ginseng and hibiscus extracts at a dose of 25mg/kg three times per week (Mon, Weds, Fri), while control mice were gavaged with PBS. Each time the mice were treated, they were observed for signs of toxicity, and mice weights and tumor volumes were recorded. For the sensitization mice group, there were also treated with tamoxifen 5mg/kg (dissolved in 10% EtOH, and 90% peanut oil) on Tuesdays and Thursdays.

Based on the tumor volume data obtained from the mice studies, lemongrass extract did not appear to be an effective treatment for breast cancer cells *in vivo*. For the MDA-MB-231 cells, the average trend line for lemongrass-treated tumors approximately followed the same trend as the control tumors until day 50, but then slowed the rate of tumor growth compared to the controls, although the difference is not significant. However, for the SUM149 tumors, it appeared lemongrass treatment slightly enhanced the growth rate of the cancer compared to control (FIGURE 3.3.1.1A). With regards to
mice weights, no significant difference was observed between the lemongrass-treated group and the control group (FIGURE 3.3.1.1B), which suggested a lack of toxicity.

Eleuthero ginseng extract may slow the rate of breast cancer tumor growth in vivo, although the difference between the control and ginseng tumor volumes was not significant, even past day 50 (FIGURE 3.3.1.1A). The average mice weight in the ginseng group was slightly higher than the control group, especially the second half of the studies. However, both groups followed a similar trend over time, and there was no significant changes observed between the two groups (FIGURE 3.3.1.1B), which suggested a lack of toxicity.

Depending on the specific breast cancer cell line, hibiscus extract may be an effective treatment in vivo. For the MDA-MB-231 tumors, the trend line for average tumor volume for hibiscus-treated tumors was much lower than the control tumors, which indicates hibiscus effectively slowed/halted the growth of the MDA-MB-231 tumors. However, for the SUM149 tumors, treatment with hibiscus had very mixed results within each trial and between each trial (FIGURE 3.3.1.1A). Like lemongrass and ginseng, there was no significant difference in mice weights observed between the hibiscus-treated group and the control group (FIGURE 3.3.1.1B), which suggests a lack of toxicity.

Although each individual extract may have slowed the rate of tumor growth, combining hibiscus and eleuthero ginseng extracts was not an effective treatment for breast cancer cells in vivo. For the MDA-MB-231 tumors, the combination NHP extract had no effect on slowing the rate of tumor growth (FIGURE 3.3.1.2A). However, for the SUM149 tumors, it appeared the combination treatment slightly enhanced the growth rate of the cancer compared to control (FIGURE 3.3.1.2B). The average mice weight in the
NHP combination group was slightly higher than the control group for the majority of the study. However, both groups followed a similar trend over time, and there was no significant changes observed between the two groups, which suggested a lack of toxicity.

Treatment of SUM149 cells with hibiscus, followed by tamoxifen a day later did not slow the growth of the tumors any more than tamoxifen on its own did (FIGURE 3.3.1.3). However, the MDA-MB-231 tumors did not establish for any of the mice being treated with hibiscus and tamoxifen. There was no significant difference in mice weights observed between the two groups (FIGURE 3.3.1.3B), which suggests a lack of toxicity.
FIGURE 3.3.1.1. Some NHP extracts slowed the rate of tumor growth *in vivo*, but it depends on the particular cancer cell line being treated. Immunocompromised CD-1 nu/nu female mice were subcutaneously injected with human breast cancer cells (MDA-MB-231 on left flank, SUM149 on right flank) and matrigel. After allowing the tumors to establish, the mice were randomly separated into treatment groups. The mice were treated three times a week (Monday, Wednesday, Friday) for the duration of the study. Values are expressed as mean ± SD from two independent mice studies. 

*A*, Each time the mice were treated, tumor volumes were measured using a standard caliper and were calculated by multiplying the recorded length, width and height measurements. 

*B*, Along with measuring tumor volumes every time the mice were treated, mice weights was recorded.
Combining eleuthero ginseng and hibiscus extracts was not effective in treating both MDA-MB-231 and SUM149 cells \textit{in vivo}. Immunocompromised CD-1 nu/nu female mice were subcutaneously injected with human breast cancer cells (MDA-MB-231 on left flank, SUM149 on right flank) and matrigel. Values are expressed as mean ± SD from two independent mice studies. \textit{A}, Each time the mice were treated, tumor volumes were measured using a standard caliper and were calculated by multiplying the recorded length, width and height measurements. \textit{B}, Along with measuring tumor volumes every time the mice were treated, mice weights was recorded.

FIGURE 3.3.1.2. Combining eleuthero ginseng and hibiscus extracts was not effective in treating both MDA-MB-231 and SUM149 cells \textit{in vivo}. Immunocompromised CD-1 nu/nu female mice were subcutaneously injected with human breast cancer cells (MDA-MB-231 on left flank, SUM149 on right flank) and matrigel. Values are expressed as mean ± SD from two independent mice studies. \textit{A}, Each time the mice were treated, tumor volumes were measured using a standard caliper and were calculated by multiplying the recorded length, width and height measurements. \textit{B}, Along with measuring tumor volumes every time the mice were treated, mice weights was recorded.
FIGURE 3.3.1.3. **Pre-treatment with hibiscus extract did not slow the rate of SUM149 tumor growth in vivo compared to tamoxifen alone.** Immunocompromised CD-1 nu/nu female mice were subcutaneously injected with human breast cancer cells (MDA-MB-231 on left flank, SUM149 on right flank) and matrigel. After allowing the tumors to establish, the mice were randomly separated into treatment groups. For the duration of the study, the mice were treated with hibiscus three times a week along with tamoxifen twice a week on alternate days. Mean values are shown for the one mice study. 

*A*, Each time the mice were treated, tumor volumes were measured using a standard caliper and were calculated by multiplying the recorded length, width and height measurements. 

*B*, Along with measuring tumor volumes every time the mice were treated, mice weights was recorded.
3.3.2 – Assessing Toxicity by Hematoxylin & Eosin (H&E) Staining:

Following the completion of the mice study, mice were sacrificed and their organs and tissues (kidneys, liver, heart and tumors) were obtained and fixed in 10% formaldehyde for histopathological analysis. Mice organs were cryosectioned into 10-micron sections and stained with Hematoxylin & Eosin.

Results appear to show no gross morphological differences in the liver, kidneys and heart of the lemongrass, ginseng and hibiscus-treated groups compared to the control mice treated with PBS. However, there does appear to be fewer nuclei in the SUM149 tumors of the lemongrass treated group (FIGURE 3.3.2B), potentially indicating some minor toxicity to the cancer cells. These results indicate that oral treatment of lemongrass extract may not be toxic to normal tissues, but may have some toxicity to SUM149 breast cancer cells. Aside from this, lemongrass, ginseng, hibiscus and the combination extracts was well-tolerated by all tissues (FIGURE 3.3.2). However, a pathologist will need to analyze the results in order to confirm the conclusions.
FIGURE 3.3.2. Lemongrass extract caused minor toxicity to SUM149 tumors, but overall the NHP extracts was well-tolerated by normal tissues. Following completion of the xenograft mice studies, the mice were sacrificed and their tissues harvested. After slicing the tissues, they were stained with Hematoxylin & Eosin for histopathological studies. Images were obtained using a brightfield microscope. A, H&E staining of tissues at 100x magnification. Scale bar = 200µm. B, H&E staining of tissues at 400x magnification. Scale bar = 50µm.
CHAPTER 4 – GENERAL DISCUSSION

The main issue surrounding current treatment options for patients with breast cancer, or cancer in general, is that many of these treatment options are genotoxic to all cells of the body, including normal cells. Specifically, chemotherapy and radiation are notorious for inducing DNA damage and inducing cell death to all cells, but especially rapidly dividing cells, such as cancer or cells of the gastrointestinal tract (Chabner et al., 2006). Although chemotherapy and radiation are highly effective for inducing cell death in cancer cells, damage and subsequent cell death to the normal cells is responsible for causing a multitude of side effects associated with current cancer treatments, including but no limited to: nausea, vomiting, hair loss, loss of appetite, lethargy, as well as the most common and most serious side effect of bone marrow suppression (Canadian Cancer Society, 2015). In particular, bone marrow suppression is a condition in which one or more of the main blood cell types is reduced. This may include a reduction in red blood cells, white blood cells, or platelets, which can result in fatigue, decreased immunity, and increased risk of internal bruising and/or bleeding, respectively (Wang Y, Probin V, Zhou D, 2006). These side effects may be so severe that they can greatly affect the physical and mental well-being of the patient. The lack of efficacious, non-toxic cancer therapies encourages further investigation and research into other potentially efficacious forms of treatment. Natural health products (NHPs) are one form of potential treatment options, as many NHPs have been used for centuries in traditional medicines (such as in Traditional Chinese Medicine, and Ayurvedic Medicine). NHPs are usually considered to have low potential risk to a patient due to their long history of human use in traditional medicine, although this is only based on anecdotal evidence. To date, there are
only anecdotal evidences and little scientific research to validate the therapeutic uses, efficacies and lack of toxicity for most NHPs. Although there is some research to support the claims of potential anticancer effects of some natural health products such as Lemongrass, Hibiscus and Eleuthero Ginseng (Halabi & Sheikh, 2014; Wong YH et al., 2014; Wong AS et al., 2014), these studies did not investigate the anticancer effects on breast cancer, nor did they give much insight to the anticancer mechanism of action.

The main reasoning for investigating NHPs as a potential cancer treatment is that they are in the category of food and are considered to be safe when consumed based on anecdotal evidence. These complex polychemical mixtures may contain up to hundreds or thousands of different compounds, many of which may be pharmacologically active and target multiple signaling pathways within a cell where the cancer may be vulnerable (Ovadje P. et al., 2015). Out of all the anticancer drugs approved since the 1940s, over 75% of them have come from natural sources or are semi-synthetic derivatives of natural products (Mann J., 2002). This highlights the potential for finding new and efficacious anticancer compounds that have yet to be identified, and therefore necessitates the need for investigating NHPs as a potential treatment option.

The main objective of the study was to investigate the anticancer potential of lemongrass, eleuthero ginseng, and hibiscus extracts as a form of breast cancer treatment since previous research showed these extracts may possess some anticancer effects. These three extracts were chosen from a list of 28 NHPs our lab has previously screened for their ability to reduce metabolic viability in leukemia and lymphoma cells. All three extracts showed activity on leukemia and lymphoma cells, but the goal of this project was
to assess their anticancer potential in specifically breast cancer cells, as that had not been investigated yet.

Each extract was prepared using two different solvents, at different temperatures and filtration processes, and the effect of each extract on metabolic viability of breast cancer cells was assessed by WST-1 assay and compared to one another. Viability was shown to be reduced by the unfiltered ethanolic extracts of lemongrass (Figure 3.1.1.1) and eleuthero ginseng (Figure 3.1.1.2), whereas for hibiscus, hot and cold water extracts were found to have equivalent activity (Figure 3.1.1.3). Since these extracts had the most potent activity for each NHP, they were used for all subsequent experiments.

We observed that following treatment with lemongrass or eleuthero ginseng for 48hrs, cell viability increased at 96hrs, which suggests that the cells may be able to metabolize and excrete the drugs and therefore reduced the effectiveness of the treatment over time. Thus, we wanted to assess the effect of multiple treatments on cell viability. Treatment of cells with lemongrass and ginseng multiple times was more effective at reducing viability at 96hrs than single treatments (Figure 3.1.1.4), which suggests these extracts might be degraded by the cells over time. The other possibility is that the extracts were not degraded by the cells, but rather treating a second time increased the total dose of extract and thereby potentiated the effects in a dose-dependent manner. On the other hand, treatment of cells with hibiscus extract a second time actually increased the viability compared to single treatment. This may be the result of a false-positive reading if the hibiscus extract itself may absorb some of the light at 450nm, and thus increasing the total dose of extract (assuming that the extract is not degraded by the cells) by treating a second time may account for the increased viability.
Following the assessment of metabolic viability, the effect on cell proliferation rates was observed using a trypan blue cell exclusion assay, following treatment with the aforementioned extracts. Lemongrass, hibiscus and eleuthero ginseng were all shown to slow the rate of cell proliferation in a dose-dependent manner. Lemongrass had the most potent activity (Figure 3.1.2.1), since a dose of 0.1mg/mL caused a 90% reduction in growth rates of the cancer cells. Eleuthero ginseng (Figure 3.1.2.2) had the second most potent activity, since a dose of 0.25mg/mL caused an average reduction of 50-60% in growth rates. Lastly, hibiscus had the least potent activity (Figure 3.1.2.3), since a dose of 0.25mg/mL only slowed the growth rates approximately 20-30%. These results were observed in ER+ MCF-7 cells and triple negative MDA-MB-231 and MDA-MB-468 cells.

The WST-1 assay measured the effect on metabolic viability, whereas the trypan blue cell exclusion assay measured the rate of cell proliferation. Neither assay was able to conclude whether or not cell death was induced by an NHP extract. Thus, the next objective was to assess for cell death induction via Annexin-V binding assay, a marker for early apoptosis, counterstained with propidium iodide to indicate the later stages of cell death.

Based on the qualitative and quantitative results (Figure 3.1.3.1), lemongrass was shown to cause a small increase (~10%) in the percentage of MCF-7 cells marked with green (Annexin-V) and red (PI) fluorescence, which indicated the induction of cell death. Furthermore, lemongrass extract doses of 0.1 and 0.25mg/mL effectively induced cell death in MDA-MB-231 cells at both 24 and 48hrs in a dose- and time-dependent manner (Figure 3.1.3.2). The different receptor profiles of the three cell lines may account for the
differing amounts of cell death induction (Galluzzi L. et al., 2007). Although there appeared to be a slight increase (~5-10%) in the percentage of Annexin-V-positive cells in the triple negative cells treated with lemongrass extract, it was not a statistically significant increase compared to the control (untreated) cells. Rather, there was a significant decrease (at 24hrs: 33% and 47% for 0.1 and 0.25mg/mL, respectively) in the proportion of viable cells after treatment with lemongrass extract, and thus it can be concluded that lemongrass induces programmed cell death (PCD) in breast cancer cells.

Similar to lemongrass, eleuthero ginseng was also found to induce cell death in a small percentage (~16% at 24hrs, and ~40% at 48hrs post-treatment) breast cancer cells, although the ginseng doses used were slightly higher (0.25 and 0.5mg/mL; Figure 3.1.3.1 and 3.1.3.2). However, ginseng was found to have similar efficacy for inducing cell death in both cell lines (MCF-7 and MDA-MB-231) as opposed to lemongrass, which had weak efficacy on MCF-7 cells and strong efficacy on MDA-MB-231 cells. Like lemongrass, the small increase (~5-10%) in the amount of green fluorescence (in cells treated with ginseng) was not statistically different from the control/untreated cells, but there was a significant difference in the proportion of viable cells (at least at 24hrs; Figure 3.1.3.1 and 3.1.3.2), which indicated cell death.

Hibiscus was found to be less effective compared to lemongrass and eleuthero ginseng for inducing cell death in cell culture. Qualitative and quantitative data (Figure 3.1.3.1) showed a small increase (~5%) in the percentage of MCF-7 cells with green fluorescence, but the difference was not significant. Like eleuthero ginseng, the effect of hibiscus on the two breast cancer cell lines was fairly consistent. Hibiscus caused a dose-independent decrease in the proportion of viable cells (i.e. induced cell death), but
compared to the control/untreated cells the reduction was not substantial. On average, the untreated cells were 92% viable, but after treatment with any dose of hibiscus (0.1, 0.25 or 0.5mg/mL) the percentage of viable cells was reduced to around 70-80% (Figure 3.1.3.1 and 3.1.3.2), which is not a large reduction in viable cells.

Now that lemongrass, eleuthero ginseng, and hibiscus were shown to have an effect on breast cancer cells (both ER+ and triple negative) by reducing metabolic viability, slowing/halting cell proliferation, as well as inducing cell death (to varying degrees depending on the particular cell line and dose used), the next objective was to investigate the mechanism(s) for how these effects occur. Since the mitochondria are directly or indirectly linked to many cell death pathways, the effect of each NHP extract on the integrity and function of mitochondria was analyzed via TMRM assay. However, since hibiscus may only cause a small amount of cell death in all three breast cancer cell lines (Figure 3.1.3.1 and 3.1.3.2), hibiscus’ mechanism of action was not investigated until later in the study when hibiscus was found to enhance the effect of another NHP extract when used in combination (Figure 3.1.3.3 and 3.1.3.4). Other potential mechanisms may be involved in cell death including autophagy and oxidative stress. These mechanisms/pathways were analyzed by monodansylcadaverine (MDC) and 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) assays, respectively. Additionally, PCR was performed to assess the gene expression levels of 84 genes from 9 different biological pathways involved in transformation and tumorigenesis. Although PCR measures the expression levels of mRNA and not actual protein levels, this method was found to give a lot of information on the potential mechanisms for how each NHP extract may cause cell death in breast cancer.
Treatment of MCF-7 and MDA-MB-231 cells with lemongrass extract was found to destabilize the mitochondrial membrane potential ($\Delta \Psi_m$) of both cell lines in a time- and dose-dependent manner (Figure 3.2.1.1), which was observed as a loss of red fluorescence. The percent of TMRM-positive (Red fluorescence) indicates the proportion of mitochondria (in all cells) that have an intact mitochondrial potential. Therefore, a reduction in TMRM/red fluorescence indicates the average percentage of mitochondria lost within a cell. In normal cells, this loss of mitochondrial integrity would normally indicate a significant reduction in cellular energy production, however, cancer cells already have dysfunctional mitochondria and thus are dependent on glycolysis for energy rather than mitochondria, this is known as the ‘warburg effect’ (Pelicano H., et al, 2006). This means that loss of TMRM fluorescence in cancer cells does not correlate with a large reduction in cellular energy, but rather may implicate the mitochondria in the mechanism of cell death since destabilization of the mitochondria causes the release of pro-apoptotic proteins is known to cause cell death (Cande et al., 2002). In breast cancer cells, loss of TMRM (red) fluorescence occurred early (6hrs post-treatment with lemongrass extract) and the proportion of mitochondria in each cell undergoing this event increased over time (Figure 3.2.1.1). In addition to reduced ATP production, collapse of the electrochemical proton gradient ($\Delta \Psi_m$), causes the mitochondrial matrix to begin to swell, causing osmotic shock to the organelle and results in rupture and subsequent release of pro-apoptotic proteins, which can lead to the induction of caspase-dependent apoptosis via the intrinsic pathway (Cande et al., 2002). For this reason, depolarization of the mitochondrial membrane at early time points (before any significant cell death occurs) may play a key role in the induction of cell death in breast cancer cells by
lemongrass extract. Furthermore, mitochondrial depolarization does not necessarily lead to the induction of apoptosis via cytochrome-C release, since mitochondrial permeability transition has been identified as a key event that can lead to the induction of apoptosis or necrosis (Kim, He, Lemasters, 2003). Factors that can promote the mitochondrial permeability transition include the following: pH > 7, pyridine nucleotide oxidation, increased mitochondrial Ca\(^{2+}\) levels, oxidative stress, depletion of glutathione (by oxidation), increased mitochondrial inorganic phosphate levels, and mitochondrial depolarization (Lemasters et al., 1998). Of these factors, only oxidative stress mitochondrial depolarization was looked into during the study. Thus, in order to further investigate how lemongrass extract depolarized the mitochondria, these other factors should be tested/measured.

Another mechanism that can lead to programmed cell death is autophagy, and thus following treatment of MCF-7 cells with lemongrass extract, levels of autophagosomes were observed using MDC and fluorescence microscopy. Following staining with MDC, lemongrass treatment for 48hrs did not cause an increase in blue fluorescence intensity compared to the untreated control cells (Figure 3.2.2). The absence of punctate staining (unlike that of the tamoxifen-treated cells) suggested that autophagy may not be induced by lemongrass. However, autophagy cannot be ruled out as a potential mechanism for lemongrass-induced cell death, since autophagy was not quantified. In future studies, a biochemical method that can be used to quantify autophagy is western blotting for autophagy proteins, specifically conversion of LC3-I to LC3-II (Kabeya et al., 2000). Based on the preliminary data obtained from the MDC
assay, activation of autophagy does not appear to be required for the induction of cell death by lemongrass extract.

Cancer cells are susceptible to increased oxidative stress due to the acidic cellular pH, thanks to their dependence on glycolysis and subsequent lactate production in order to regenerate NAD+ to continue glycolysis (Koppenol et al., 2011). Since increased levels of reactive oxygen species (ROS) can play a role in mitochondrial permeability transition and subsequent cell death induction, a \( \text{H}_2\text{DCFDA} \) assay was used to study the production of ROS in breast cancer cells, following treatment with lemongrass extract. In order to further investigate the mechanism of lemongrass’ role in cell death induction, both MCF-7 and MDA-MB-231 cells were treated for 3 and 6hrs time points with lemongrass extract (0.25mg/mL), which reduced levels of ROS (Figure 3.2.3). This result agrees with literature in that lemongrass is known to have antioxidant properties (Cheel et al., 2005). Therefore, the reduction of ROS at early time points (before any substantial cell death occurs) indicated that oxidative stress (which is characterized as increased levels ROS above baseline) is not likely an early event that leads to induction of cell death via mitochondrial permeability transition.

Our results indicate that lemongrass extract has been shown to induce cell death (to varying degrees depending on the cell line), and this may be caused by destabilization of the mitochondrial membrane potential, but autophagy and oxidative stress (i.e. increased levels of ROS) do not appear to be involved.

To further investigate the mechanism for how lemongrass causes cell death, RT² Profiler array was performed on MDA-MB-231 cells. PCR is one example of a method that can provide some valuable information in regards to which proteins might be up- or
down-regulated as a result of NHP extract treatment. Other examples include Northern blotting and Western blotting, although those methods measure expression levels of mRNA and protein, respectively. Compared to the untreated control cells (Figure 3.2.4.1), lemongrass (0.25mg/mL) upregulated the expression of two pro-apoptotic proteins, BCL2L11 and FASLG, although this upregulation was not statistically significant. However, all three trials showed an increase in expression of both of these genes, just with large variance of increased fold expression. FASLG can activate the FAS receptor (FASR) on adjacent cells to induce extrinsic apoptosis. Increased FASLG expression within a cancer cell does not necessarily mean increased FAS-induced cell death of that cancer cell, unless the FASR is activated by the increased expression of FASLG. In a future study, expression or activation of the FASR and/or TNFR should be measured to better conclude if extrinsic apoptosis is induced by lemongrass extract.

Furthermore, the anti-apoptotic proteins NOL3 and BIRC3 were down-regulated. NOL3 is a protein that inhibits both intrinsic and extrinsic apoptosis by multiple mechanisms, including blocking activation of caspase-2 and caspase-8 (extrinsic pathway), as well as binding to and inhibiting Bax translocation (intrinsic pathway) (Wu L. et al., 2010). Like NOL3, BIRC3 can inhibit both extrinsic and intrinsic apoptosis by binding and inhibiting TNF receptor-associated factors 1 and 2 (TRAF1, TRAF2), and by inhibiting activation of caspase-3 and -9, respectively (Saleem et al., 2013). Overall, the changes in gene expression levels of BCL2L11, FASLG, NOL3 and BIRC3 following treatment with lemongrass extract appears to promote apoptosis in breast cancer cells.

Furthermore, DDIT3, GADD45G and PPP1R15A expression levels were increased following treatment with lemongrass extract. According to the literature,
DDIT3 is activated by endoplasmic reticulum (ER) stress, and promotes cell cycle arrest and apoptosis (Jauhiainen et al., 2012). Additionally, it increases expression of a few key proteins including: TNFRSF10B/DR5, PPP1R15A/GADD34, and BCL2L11/BIM, among others (Lurlaro and Muñoz-Pinedo, 2016). All three of these genes can promote cell death. PPP1R15A can be induced in response to genotoxic stress to promote apoptosis like BAX, however, the PPP1R15A response to stress occurs independent of p53 status (Hollander et al., 1997). TNFRSF10B is a death receptor for TNF ligand (TRAIL) and causes apoptosis (Liu G., et al., 2012). GADD45G is another protein that is upregulated in response to cellular stress and is involved in DNA repair, cell cycle, and apoptosis (Esaki Tamura et al., 2012). Since lemongrass extract was found to increase the expression of BCL2L11, DDIT3, GADD45G and PPP1R15A (Figure 3.2.4.1), these genes may indicate some kind of cellular stress that causes the induction of cell death, whether that be DNA damage, ER stress, or hypoxia. In order to identify what form of cellular stress causes the cell death, further experiments need to be performed. For example, TUNEL staining can be used to detect DNA damage, measuring mRNA levels of unfolded protein response (UPR) target genes to detect ER stress, and measuring HIF-1α levels by western blotting to detect hypoxic conditions in cell culture.

Based on the gene expression results, two other genes of interest are ADM and HMOX1, both of which showed a significant increase in expression. These two proteins are upregulated downstream of HIF-1α during hypoxic conditions to help the cancer cells adapt to the stressful environment (Krieg et al, 2009). Since the expression levels of all the genes of interest are relative to the basal expression levels in untreated control MDA-MB-231 cells, the hypoxic conditions that caused the upregulation of ADM and HMOX1
appear to be caused by treatment with lemongrass extract. However, we did not specifically test for hypoxia, and therefore cannot conclude if lemongrass extract actually mimics hypoxic conditions to breast cancer cells *in vitro*. In order to determine if hypoxia occurs in cell culture, western blotting should be performed to measure levels of HIF-1α, as this protein is stabilized during conditions of hypoxia (Wu and Yotnda, 2011).

Interestingly, a study published in 2007 showed that in HCT116 colon cancer cells, hypoxia induced p53 protein levels and p53-dependent apoptosis. During this same study, this group showed that most classical p53 target genes (such as p21, MDM2 and Bax) are not upregulated by hypoxia (Liu T. et al., 2007). This indicated that activation of p53 in response to hypoxia leads to a different gene expression pattern from that induced by DNA damage for irradiation or UV light. The different genes that were upregulated in response to hypoxia-induced activation of p53 were: DDIT3, ANXA1, SEL1L and SMURF1 (Liu T. et al., 2007). This group also showed that blocking Fas/CD95 signaling with an antibody or a caspase-8 inhibitor abolished the p53-dependent apoptosis in response to hypoxia. Although MDA-MB-231 cells have a mutant p53 (only one conservative mutation at position 280: Arg → Lys [Huovinen M., et al., 2011]), the activity of p53 may be similar and therefore the results from the HCT116 study may help explain the mechanism for how lemongrass extract induced cell death (at least in MDA-MB-231 cells).

A proposed mechanism for how lemongrass can cause cell death in MDA-MB-231 cells (and possibly similar in other breast cancer cell lines such as MDA-MB-468 which has a similar conservative mutation at position 273: Arg → His [Huovinen M., et al., 2011]) is as follows: treatment with lemongrass extract may mimic hypoxic
conditions as indicated by the increased expression of ADM and HMOX1. These hypoxic conditions activate p53, which in turn upregulates DDIT3 (among other genes). DDIT3 upregulates the expression of TNFRSF10B, PPP1R15A, and BCL2L11. Upregulation of these genes may induce extrinsic apoptosis via TNFRSF10B or FAS signaling. Evidence to support this includes increased expression of FASLG and decreased expression of NOL3 and BIRC3 shown in the PCR results (Figure 3.2.4.1). Since NOL3 and BIRC3 can no longer inhibit the activation of caspase-8 via a death receptor (either TNFRSF10B and/or FASR), caspase-8 will activate caspase-3 to execute extrinsic apoptosis as well as cleaving BID to tBID, allowing for activation of Bax and subsequent release of cytochrome C. Ultimately, extrinsic and intrinsic apoptotic pathways may both be involved in lemongrass-induced cell death.

Previous results compliment this proposed pathway, such as upregulation of DDIT3, GADD45G and PPP1R15A can arrest the cell cycle, and therefore explains why lemongrass extract was shown to slow/halt cell proliferation (Figure 3.1.2.1). Additionally, decreased expression of a few metabolism genes (such as ACSL4, CPT2, GPD2, PFKL and UQCRFS1) compliments previous results that showed lemongrass extract decreases metabolic viability (Figure 3.1.1.1). Furthermore, lemongrass was found to have antioxidant properties and did not induce autophagy, which compliments the proposed mechanism in that oxidative stress and autophagy are not involved in causing the cell death. Lastly, caspase-8-induced cleavage of BID to tBID and subsequent release of cytochrome C compliments the mitochondrial destabilization seen in previous results, as indicated by the loss of TMRM (Figure 3.2.1.1). In summary, lemongrass extract has been shown to possess cell death-inducing activity. While this may be an
advantage for the treatment of breast cancer, this NHP extract may also cause cell death in normal cells, and therefore, it cannot be concluded if lemongrass acts selectively against cancer cells, or against all cells in general.

Eleuthero ginseng extract was found to have many of the same effects on breast cancer cells as treatment with lemongrass extract, with a few key differences. Like lemongrass, eleuthero ginseng reduced metabolic viability (Figure 3.1.1.2), cell proliferation rates (Figure 3.2.1.2), and induced cell death (Figure 3.1.3.1 and 3.1.3.2) in a dose- and time-dependent manner, although these effects required higher doses to achieve the same effect that lemongrass had. Furthermore, eleuthero ginseng was also found to destabilize the mitochondrial membrane potential of both MCF-7 and MDA-MB-231 cells like lemongrass, although not nearly as effectively, even at higher doses (Figure 3.2.1.2).

However, in contrast to lemongrass, eleuthero ginseng may induce autophagy as part of its mechanism for inducing cell death. This was observed (qualitatively) as an increase in blue fluorescence in MCF-7 cells following treatment with ginseng (Figure 3.2.2). Although there was not necessarily ‘punctate’ staining like that of the tamoxifen-treated group, MDC fluorescence increased in a dose-dependent manner, as both 0.25 and 0.5mg/mL treatment groups had much more fluorescence intensity than the untreated control cells. This indicates that autophagy may play a role in cell death induced by eleuthero ginseng, but this cannot be concluded until autophagy is quantified by another method such as western blotting (for LC3-I to LC3-II conversion).
In both MCF-7 and MDA-MB-231 cells, eleuthero ginseng extract (0.25mg/mL) did not alter levels of ROS (Figure 3.2.3). Therefore, no change in the levels of ROS at early timepoints (3 and 6hrs post-treatment, before any substantial cell death occurs) indicated that oxidative stress is not likely an early event that leads to induction of cell death induced by ginseng, although measuring levels of ROS at later timepoints like 12 and 18hrs post-treatment will give a better indication if oxidative stress occurs to play a role in cell death induction.

So far eleuthero ginseng extract has been shown to induce cell death (to varying degrees depending on the cell line), and this may involve destabilization of the mitochondrial membrane and possibly induction of autophagy, but oxidative stress does not appear to be involved. In order to further investigate the mechanism for how eleuthero ginseng causes cell death, PCR was performed on MDA-MB-231 cells. Compared to the untreated control cells (Figure 3.2.4.2), treatment with eleuthero ginseng extract (0.25mg/mL) increased the expression of two pro-apoptotic proteins, BCL2L11 and CASP7, although BCL2L11 upregulation was not statistically significant. FASLG mRNA levels were measured as well, but the three trials showed inconsistent results, including both upregulation and downregulation of the gene depending on the trial. Like lemongrass, eleuthero ginseng caused a moderate (and statistically significant) reduction in BIRC3 expression. BIRC3 can inhibit both extrinsic and intrinsic apoptosis (Saleem et al., 2013), and therefore reducing its expression can promote apoptosis. However, the antiapoptotic protein XIAP was found to be upregulated by a small amount, which may inhibit activation of apoptosis via the extrinsic pathway. Overall, the gene expression
levels of BCL2L11, CASP7 and BIRC3 following treatment with eleuthero ginseng extract appear to promote apoptosis in breast cancer cells.

Furthermore, eleuthero ginseng was found to upregulate DDIT3, GADD45G and PPP1R15A (Figure 4.10) with the same ratio of increased fold expression between those three genes that lemongrass caused. DDIT3 is activated by endoplasmic reticulum (ER) stress, and promotes cell cycle arrest and apoptosis (Jauhiainen et al., 2012). Furthermore, it increases expression of a few key proteins including: TNFRSF10B/DR5, PPP1R15A/GADD34, and BCL2L11/BIM, among others (Lurlaro and Muñoz-Pinedo, 2016). Upregulation of DDIT3 may explain why BCL2L11 and PPP1R15A were also upregulated. Overall, the increased expression of all of these genes may promote apoptosis and/or cell cycle arrest, which compliments previous data showing a slowing/halting of cell proliferation and the induction of cell death. Like lemongrass, eleuthero ginseng extract was found to increase the expression of BCL2L11, DDIT3, GADD45G and PPP1R15A (Figure 3.2.4.2). Just like what was observed with lemongrass extract treatment, increased expression of these genes by eleuthero ginseng may indicate some kind of cellular stress that causes the induction of cell death, whether that be DNA damage, ER stress, or hypoxia. Further experiments need to be performed in order to identify what form of cellular stress causes the cell death induced by ginseng.

Additionally, eleuthero ginseng was found to cause a significant increase in the expression of HMOX1 and ADM, just like lemongrass did. Since the treatment (NHP extract and PBS) was the only difference between the two groups, the hypoxic conditions that caused the upregulation of ADM and HMOX1 appear to be caused by treatment with eleuthero ginseng extract.
Aside from the possibility that eleuthero ginseng may induce autophagy as part of its mechanism (although this cannot be concluded without quantification data), it appears that eleuthero ginseng may cause the induction of cell death very similarly to how lemongrass was proposed: treatment with ginseng extract may cause hypoxic conditions as indicated by the increased expression of ADM and HMOX1, since these two proteins are upregulated downstream of HIF-1α during hypoxic conditions to help the cancer cells adapt to the stressful environment (Krieg et al, 2009). However, an experiment that tests for hypoxia in vitro must be performed in order to confirm eleuthero ginseng-induced hypoxia. One such experiment is the use of western blotting to measure levels of HIF-1α during (Wu and Yotnda, 2011). Furthermore, hypoxia is also known to induce autophagy (via HIF-1α) as a cell survival response in order to control ROS production and DNA damage (Mazure and Pouysségur, 2010). Based on the results obtained from this study, this further implicates hypoxia playing a role in cell death by eleuthero ginseng since autophagy appeared to be induced by ginseng (Figure 3.2.2), and the levels of ROS were not altered following treatment (Figure 3.2.3). These hypoxic conditions can activate p53, which in turn upregulates DDIT3 (among other genes). DDIT3 upregulates the expression of TNFRSF10B, PPP1R15A, and BCL2L11. Upregulation of these genes may induce extrinsic apoptosis via TNFRSF10B or FAS signaling. Although it was not clear if FASLG was up- or down-regulated in response to ginseng, evidence to support the proposed mechanism includes decreased expression of BIRC3, as well as increased expression of BCL2L11, PPP1R15A and GADD45G shown in the gene expression results (Figure 3.2.4.2). Since BIRC3 can no longer inhibit the activation of caspase-8 via death receptor (either TNFRSF10B and/or FASR), caspase-8 can activate caspase-3 to
execute extrinsic apoptosis as well as cleaving BID to tBID, allowing for activation of Bax and subsequent release of cytochrome C. Ultimately, extrinsic and intrinsic apoptotic pathways may both be involved in eleuthero ginseng-induced cell death.

However, may also play a role in the cell death induced by eleuthero ginseng. For instance, two other genes of interest that were shown to double in expression in response to eleuthero ginseng were TNKS and TNKS2 (Figure 3.2.4.2). These two genes are known to have poly(ADP-ribose) polymerase (PARP) activity, which causes the depletion of its substrate NAD+ in response to excessive DNA damage. Resynthesis of NAD+ depletes ATP, and with enough DNA damage, the sudden loss of energy can lead to oncosis (Walisse and Thies, 1999). Although DNA damage was not measured using TUNEL staining (an experiment that should be done in a future experiment), upregulation of TNKS and TNKS2 may indicate that there was some DNA damage caused by eleuthero ginseng, and the possibility that this lead to induction of oncosis. However, since the mitochondria were not destabilized to a significant degree 24hrs post-treatment (Figure 3.2.1.2), and the fact that metabolic viability did not suddenly drop off over time (Figure 3.1.1.2), oncosis is not likely induced by eleuthero ginseng.

Hibiscus extract was found to have some of the same effects on breast cancer cells as lemongrass and eleuthero ginseng extracts, with a few key differences. Like lemongrass and eleuthero ginseng, hibiscus reduced metabolic viability (Figure 3.1.1.3), cell proliferation rates (Figure 3.1.2.3) in a dose- and time-independent manner, although these effects required higher doses to achieve the same effect that ginseng had. However, hibiscus only had a minor amount of cell death induction (on average, around 10%
reduction in viable cells) (Figure 3.1.3.1 and 3.1.3.2), and it did not destabilize the mitochondrial membrane potential (not shown), hibiscus was not looking very effective/useful until it was found that hibiscus may enhance the effects of another NHP extract when combined with it (Figure 3.1.1.5 and 3.1.1.6).

Like lemongrass, hibiscus does not appear to cause the induction of autophagy. Following staining with MDC, hibiscus treatment for 48hrs did not cause an increase in blue fluorescence intensity compared to the untreated control cells (Figure 3.2.2). The absence of punctate staining (unlike that of the tamoxifen-treated cells) indicated that autophagy may not be induced by hibiscus. However, autophagy cannot be ruled out since the amount of autophagosomes was not quantified. Based on the preliminary data obtained from the MDC assay, activation of autophagy does not appear to be induced by hibiscus.

Furthermore, hibiscus extract (0.25mg/mL) was shown to increase levels of ROS in both MCF-7 and MDA-MB-231 cells (Figure 3.2.3), although the increase at both 3 and 6hrs post treatment was not significantly different than control levels. Therefore, this small increase in the levels of ROS at early timepoints (before any substantial cell death occurs) indicated that oxidative stress may play a role in the sensitizing effects of hibiscus extract to combinations either other extracts and/or sensitizing breast cancer cells to hormonal therapy. However, measuring levels of ROS at later timepoints like 12 and 18hrs post-treatment will give a better indication if oxidative stress occurs to play a role in cell death induction.

So far, hibiscus has been shown to cause a reduction in metabolic viability (Figure 3.1.1.3), and slow the rate of cell proliferation (Figure 3.1.2.3) of breast cancer cells.
Hibiscus may cause a small amount of cell death in breast cancer cells (Figure 3.1.3.1 and 3.1.3.2), but destabilization of the mitochondrial membrane and autophagy do not appear to be involved. On the other hand, oxidative stress may play a role in causing or enhancing the cell death induced by another NHP extract. In order to further investigate the mechanism for how hibiscus affects MDA-MB-231 cells, PCR was performed.

Interestingly, treatment of MDA-MB-231 cells with hibiscus extract did not significantly alter the expression of most genes of interest (Figure 3.2.4.3). If anything, the expression of most genes of interest was slightly decreased. This may indicate the possibility of hibiscus extract hindering RNA polymerase, which would explain why basically all genes were uniformly reduced by a small amount. However, no literature supports this theory. The other possibility is that by the time the cells were collected and lysed for RNA extraction (48hrs post-treatment), most of the hibiscus extract has already been metabolized by then and thus most of the gene expression was similar to basal levels before hibiscus treatment. Based on the WST-1 results, hibiscus reduced metabolic viability to a greater extent at 48hrs compared to 96hrs (Figure 3.1.1.3), and this may be because hibiscus extract was metabolized relatively quickly. However, this second possibility does not really make sense since AVPI data showed that hibiscus extract (at 0.1 and 0.25mg/mL) caused a similar reduction in the proportion of viable MDA-MB-231 cells (by approximately 10-12%) at both 24 and 48hrs compared to the untreated control cells (Figure 3.1.3.1 and 3.1.3.2). Thus, collecting the MDA-MB-231 cells 24hrs post-treatment instead of 48hrs may not have showed any changes in expression levels of the genes of interest.
Interestingly, hibiscus extract caused a significant reduction in the expression of FASLG, as well as HMOX1 (Figure 3.2.4.3). This is in stark contrast to the increased expression of these two genes in response to lemongrass (and ginseng, at least for HMOX1). Additionally, the expression of the anti-apoptotic protein NOL3 was reduced. Although this reduction was not significant, it was trending towards significance (P = 0.0711). Based on this PCR data, it makes sense that hibiscus has been shown to induce much less cell death in breast cancer cells than lemongrass or eleuthero ginseng, since no genes that promote apoptosis were upregulated.

Based on the data obtained from all these *in vitro* experiments, lemongrass, eleuthero ginseng and hibiscus extracts have been shown to affect metabolism, cell proliferation and induce cell death to varying degrees. Lemongrass was shown to be the most effective of these three NHP extracts *in vitro*, followed by eleuthero ginseng, and then hibiscus. But the real question was whether or not these NHP extracts would be an effective treatment against breast cancer growth *in vivo*. After completing three independent trials (each of about 10 weeks) and measuring the tumor volumes and mice weights during each of the three times a week the mice were treated by gavage, there was some pretty different results than what was seen *in vitro*. Lemongrass and eleuthero ginseng extracts (at a dose of 25mg/kg) had different efficacies between the two xenograft cell lines, but overall both lemongrass and eleuthero ginseng did not appear to be able to slow the rate of tumor growth *in vivo*. Both lemongrass and ginseng slightly slowed the growth rate of MDA-MB-231 tumors during the second half of the study (Figure 3.3.1.1A), but the error bars of both the control group (error bars not shown) and the NHP group had such large variance that overlapped between NHP and control groups,
the slowed growth rates from NHP extracts were not significantly less than the control
groups. Lemongrass somewhat enhanced the growth rate of SUM149 tumors compared to
the control group, and eleuthero ginseng somewhat slowed the growth rate, but again the
variances were so large that there was no significant difference. These error bars became
so large closer to the end of the study because of the 4 mice in each group that started,
some groups may have had one (or two in rare cases) mice that had to be put down as a
result of becoming sick or stopped eating, but was not because of the NHP extracts
themselves. In some cases, there may have only been 2 or 3 mice left in the group at the
end of the study, and depending on how the cancer cells were injected and how/where the
tumors formed (for example, the surrounding microenvironment), there was some large
variances in efficacies within the same treatment group. That was the only major issue
with this xenograft experiment; depending on how deep the cancer cells were injected in
each mouse (on each flank), and how close in proximity to blood supplies, the NHP
extracts may have not had the same access to the tumors to exert an effect. In the future, a
better method of measuring the efficacies in vivo would be to use a mouse model that can
spontaneously develop a breast cancer tumor. That would better model how cancers are
treated in patients.

On the other hand, hibiscus extract alone (25mg/kg) may be an effective treatment
for breast cancer in vivo, depending on the particular cancer cell line (Figure 3.3.1.1A).
For MDA-MB-231 xenograft tumors, hibiscus extract was efficacious in slowing/halting
growth of the tumors. However, the difference between hibiscus and control MDA-MB-
231 tumors was not statistically significant since there was such large variance in the
control tumors (especially during the second half of the study). However, hibiscus extract
did not slow the growth of SUM149 tumors compared to the control tumors. The fact that hibiscus slowed the rate of MDA-MB-231 tumors was unexpected, given the fact that hibiscus only caused a moderate reduction in cell proliferation rates and very minimal induction of cell death in MDA-MB-231 cells in vitro.

Since lemongrass, eleuthero ginseng and hibiscus extracts showed very different activity in vitro compared in vivo, this may be explained because of the different pharmacokinetics between each extract. Although lemongrass and eleuthero ginseng showed pretty good activity in vitro, these extracts may have had poor absorption into the mice gastrointestinal tracts, or the other possibility is that the active compounds within each of these NHP extracts may have been metabolized into metabolites with weaker or reduced activity. Same reasoning for hibiscus extract; hibiscus may have had better absorption, and/or metabolized into metabolites with stronger activity, which may explain the improved efficacy in vivo.

During the studies, mice toxicity was assessed by measuring the weight of each mouse each time the mice were treated and had their tumor volume(s) measured. Furthermore, after completion of each trial, mice were sacrificed and their organs and tissues (liver, kidneys, heart and tumors) were collected and stored in formaldehyde. These tissues were sliced and stained with H&E to assess for toxicity. However, these were preliminary methods of assessing for NHP extract-induced toxicity. In order to confirm whether or not there was some toxicity, the tissues need to be sent to a pathologist for analysis.

Based on the mice weight data collected during the studies, lemongrass did not cause any fluctuations in weight compared to the controls (Figure 3.3.1.1B), and thus did
not appear to cause toxicity because if it was toxic to the mice, it would affect their appetite and there would have been some sudden changes in mice weights. Furthermore, the average mice weight in the eleuthero ginseng and hibiscus groups was slightly higher than the control groups, especially during the second half of the studies (Figure 3.3.1B). However, both NHP and control groups followed a similar trend of slowing increasing weight over time, and there was no significant changes observed, which suggested a lack of toxicity. Moreover, based on the H&E data, lemongrass, eleuthero ginseng and hibiscus did not cause any gross morphological differences compared to the controls (Figure 3.3.2), and therefore, further suggested a lack of toxicity induced by each NHP extract. The tissue legions were considered mild and not of a type or frequency indicative of toxicity to the mice tissues. The one exception to this is that lemongrass may have caused some minor toxicity to the SUM149 tumors, as indicated by a reduction in purple nuclei staining. However, in order to confirm that these NHP extracts do not cause toxicity to normal tissues, the tissues should be sent to a pathologist.

Another main objective of this research was to see if combinations of extracts can enhance or have synergistic effects on breast cancer cells. Based on the WST-1 combination data (Figure 3.1.1.5 and 3.1.1.6), certain combinations of extracts may enhance the reduction of metabolic viability in both MCF-7 and MDA-MB-231 cells. Although there was not a synergistic or additive effect, there was a statistically significant difference when comparing the effects of individual NHP treatments, and the NHP combination treatments. Although most combinations of extracts reduced metabolic viability, only a few of these combinations were found to enhance the induction of cell death (Figure 3.1.3.3 and 3.1.3.4). For the MCF-7 cells, there was such large variance in
the amount of viable cells in all treatment groups, including the untreated control cells (Figure 3.1.3.3). Due to this large variance, it was difficult to determine how much more beneficial it was to combine extracts, but there did appear to be an enhanced reduction in the amount of viable cells 48hrs after treatment. For the MDA-MB-231 cells, there was clearly an enhanced induction of cell death when ethanolic eleuthero ginseng 0.1mg/mL was combined with 0.1mg/mL of either hot water hibiscus or hot water lemongrass extract (Figure 3.1.3.4). Both of these combinations had similar efficacy in reducing the amount of viable cells. This enhancement was not substantial, but was statistically different from the effects of either extract alone. Other combinations of extracts were tested for their effect on cell death, but did not show any enhanced effect, so those results were not shown.

Of the two combinations mentioned directly above, the combination of ethanolic eleuthero ginseng and hot water hibiscus was assessed for its effect on breast cancer tumor growth in vivo. Compared to the effect of eleuthero ginseng and hibiscus extracts on both MDA-MB-231 and SUM149 (Figure 3.3.1.1A) on their own, combining ginseng and hibiscus did not slow the rate of tumor growth. In the case of SUM149 tumors, the combination of eleuthero ginseng and hibiscus increased the growth of the tumors. Therefore, although some combinations of extracts may have an enhanced effect on breast cancer cells in vitro, that may not necessarily translate into an enhanced effect in vivo, at least for combining hibiscus and eleuthero ginseng.

Finally, the last objective of this research was to determine if treating with an NHP extract an hour before or after hormonal therapy (such as tamoxifen and 4-OH-tamoxifen) can cause an enhanced or synergistic effect on breast cancer cells. Based on
the WST-1 sensitization data (Figure 3.1.1.7 and 3.1.1.8), there was only two treatments that enhanced the reduction of metabolic viability in breast cancer cells. For the MCF-7 cells, treatment with tamoxifen or 4-OH-tamoxifen an hour after eleuthero ginseng caused an enhanced reduction of metabolic viability. As for the MDA-MB-231 cells, treating with hibiscus an hour before or after treatment with tamoxifen (or 4-OH-tamoxifen) had an enhanced effect. However, the enhanced effect in both cases was not statistically different compared to individual treatments. Compared to the effect of eleuthero ginseng alone on the proportion of viable cells, pretreating with tamoxifen an hour before ginseng enhanced the number of dead MCF-7 cells (Figure 3.1.3.5). Additionally, treating with 4-OH-tamoxifen an hour after hibiscus enhanced the number of dead MDA-MB-231 cells (Figure 3.1.3.6). In both cases, the treatment with both NHP extract and hormonal therapy caused a statistically different effect than NHP extract alone.

Of the two treatments mentioned directly above, treatment with hibiscus and tamoxifen was assessed for its effect on SUM149 tumor growth in vivo. However, rather than treating an hour before or after hibiscus treatment, the mice were treated with hibiscus and tamoxifen on separate days. Compared to the effect of hibiscus extract alone on SUM149 tumors (Figure 3.3.1.1A), treating with hibiscus followed by tamoxifen a day later did not slow the rate of tumor growth. Tamoxifen on its own somewhat slowed the rate of tumor growth (at the end of the study) and showed similar activity to the hibiscus and tamoxifen group. However, both the tamoxifen-only group and the hibiscus and tamoxifen group were only completed for one trial. The reduction of tumor volumes compared to the control groups was not significant, and in order to confirm if there is any
benefit to treating with hibiscus and tamoxifen on separate days, at least one more trial should be performed. Therefore, based on this preliminary data, sensitizing breast cancer cells with an NHP extract before or after hormonal therapy treatment does not appear to have any enhanced/beneficial effect.

CONCLUSIONS AND FUTURE DIRECTIONS

The results presented in this thesis offer some scientific validation for the use of NHPs, such as lemongrass, eleuthero ginseng and hibiscus extracts, in the treatment of breast cancer. These extracts may provide an alternative treatment option for patients, but in the hopes of eventually seeing NHP use as a mainstream cancer treatment, future studies need to directly compare the efficacy of each NHP extract to current chemotherapy used to treat breast cancer (such as Paclitaxel). Therefore, much more scientific research is required.

Future work on this project should include further investigating the potential mechanism(s) of action to better understand how these NHP extracts work, as well as confirming results found in this research using other methods. For instance, confirm the changes in gene expression induced by each NHP extract using Western Blotting. The results presented in this thesis provide some insight into the mechanisms, but there may be other signaling pathways involved. By understanding the mechanism of action for each of these NHP extracts on breast cancer, we will have a better understanding of when or for what types of breast cancer these extracts may be useful in treating patients. Furthermore, it will be beneficial to identify all of the bioactive compounds within each NHP extract, as it will allow for better extraction procedures to be used to extract larger
amounts of the pharmacologically active compounds. In addition to identifying active compounds, selectivity to breast cancer versus normal breast epithelial cells should be investigated, since the whole point of using NHPs as a treatment against cancer is to provide an alternative treatment option that effectively targets cancer cells, while leaving normal cells unharmed, thereby causing less side-effects to patients and ultimately improve their quality of life.
References


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