DEVELOPMENT OF AN ISOLATION SCHEME FOR CYTOTOXIC CONSTITUENTS FROM ARTEMISIA DOUGLASIANA

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DEVELOPMENT OF AN ISOLATION SCHEME FOR CYTOTOXIC CONSTITUENTS FROM *ARTEMISIA DOUGLASIANA*

A Thesis

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Department of Chemistry
Abstract

of

DEVELOPMENT OF AN ISOLATION SCHEME FOR CYTOTOXIC CONSTITUENTS FROM ARTEMISIA DOUGLASIANA

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The leaves of California mugwort (Artemisia douglasiana) have been used by Native California tribes to treat colds, gastrointestinal problems, infections, dysmenorrhea, and premenstrual syndrome. Previous work in this lab has shown that ethanolic and hexane extracts of dried A. douglasiana leaves are cytotoxic towards estrogen receptor positive (ER+) and ER- breast cancer cells; and a cytotoxic compound was isolated but not characterized. The goal of this study is three-fold: to determine the effect of ethanolic and aqueous extracts on normal cells, to check the reproducibility of the previously developed isolation scheme using a new sample of leaves, and to evaluate and improve upon the previously developed isolation scheme. Results showed that both ethanolic and aqueous extracts are more cytotoxic towards normal human peripheral blood mononuclear cells than towards MDA-MB-231 breast cancer cells. Therefore, even when the cytotoxic constituents are isolated, they cannot be used as a cure for breast cancer. To assess reproducibility, separation techniques that were used in the previous study were repeated with a new sample of A. douglasiana leaves. Chromatograms of previous study extracts are different than current study’s extract, and the cytotoxic compound isolated in the previous study was not present in the new
extract. This indicates that this cytotoxic compound is degraded in the previous extract and that the chemical composition of mugwort leaves changes seasonally. The results of vacuum distillation showed that the cytotoxic constituents are not volatile, so the detection method was switched from gas chromatography/mass spectrometry to high performance liquid chromatography/charged aerosol detection (HPLC-CAD). A solid-liquid extraction step was added to the isolation scheme. The reproducibility of fractionation by flash column chromatography was improved. In the future, the cytotoxic constituents can be isolated from the extract from the current study.

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Mary McCarthy Hintz, Ph.D.

______________________________
Date
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Chapter 1
INTRODUCTION

1.1 Scope of the study

*Artemisia douglasiana* Besser, also known as California mugwort, has been used as an herbal medicine for colds, flu, fevers, headaches, premenstrual syndrome and dysmenorrhea by different native Californian tribes. Indeed, different species of mugwort have been used for these same maladies throughout the world (1, 2). Based on the use of *A. douglasiana* as a women’s medicine (2), it was hypothesized that *A. douglasiana* leaves contain chemical constituent(s) that kill or inhibit the proliferation of breast cancer cells. It has been demonstrated that *A. douglasiana* leaves contain one or more cytotoxic constituent(s) towards breast cancer cell lines MDA-MB-231 and BT-474 (3). Based on the results of previous work in this laboratory (3), the following aims were addressed in this project:

A) To determine the effect of mugwort leaf extracts on normal cells.

B) To determine if the results from the previously developed isolation scheme can be reproduced with a new mugwort leaf sample.

C) To evaluate and improve upon the previously-developed isolation scheme.

The isolation methodologies used in this study were similar to those developed in a previous thesis (3), including liquid-liquid extraction (LLE), thin layer chromatography (TLC), and flash column chromatography (FCC). High-performance liquid chromatography with a charged aerosol detector (HPLC-CAD) or an ultraviolet
spectrophotometer detector (HPLC-UVD) and gas chromatography – mass spectrometry (GC-MS) were used as detection methods. Biological efficacy was assessed by determining the EC₅₀ value (the concentration that kills 50% of the cells) in a mammalian cell assay using human breast cancer cell line and normal human mononuclear blood cells.

1.2 Breast Cancer

Of all cancer types, breast cancer is the most common cause of cancer, and the second most common cause of cancer death, in women in the U.S. (4). In U.S. women between the ages of 45 and 55, breast cancer is the most common cause of death (5). Although breast cancer is a common form of cancer in women, male breast cancer does occur and accounts for about 1% of all cancer deaths in men (6). The American Cancer Society's most recent (2010) estimates for breast cancer in the United States were that, this year, about 207,090 new cases of invasive breast cancer will be diagnosed in women, along with 54,010 new cases of non-invasive breast cancer (4). These estimates demonstrate that breast cancer is a huge problem among women in the U.S.

The human breast is made up of three main parts called the ducts, lobules and stroma (Figure 1). Lobules are the milk producing glands, whereas ducts are the tiny tubes that carry milk from the lobules to the nipple. The stroma is the fatty and connective tissue surrounding the glandular parts of the breasts. Breast cancer starts in the ductal or lobular tissues. (Cancer of the stromal tissues is extremely rare.) Thus, there are two types of breast cancers: ductal carcinoma and lobular carcinoma.
Breast cancer can be further categorized based on whether it is invasive or noninvasive. Invasive cancer has spread from the duct or lobule to other tissues, whereas noninvasive cancer has not yet moved to other tissues. Noninvasive breast cancer is called ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS). If DCIS is untreated for a long period, it may progress to invasive breast cancer (7).
Hormones, particularly estrogens, are one of the specific factors affecting the growth of breast cells. Estrogen and progesterone receptors are found in both healthy and cancerous breast cells and bind to estrogen and progesterone that travel in the bloodstream. When bound to its ligand, the estrogen receptor signals the cell to divide. Breast cancers that are sensitive to estrogen, meaning that normal levels of estrogen increase cell growth, are called estrogen receptor positive (ER+) breast cancer. ER+ breast cancer cells either have increased levels of estrogen receptors or have mutated estrogen receptors that are highly sensitive to estrogen. About 80% of breast cancers are (ER+), and of these, 65% are progesterone receptor positive (PR+). Only about 2% of breast cancers are ER- and PR- (8).

Similar to other cancers, breast cancer is caused by genetic abnormalities that are either inherited or that happen due to the aging process of cells. Only 5-10% of breast cancer cases are found to be inherited, whereas 90% of cases arise as a result of de novo mutations (9). Hence, these cancers develop due to genetic mutation in breast cells.

There are a number of genes responsible for inherited breast cancers. For instance, the BRCA1 and BRCA2 genes are related to hereditary breast cancer (10). HER-2/neu is another gene that helps cells to grow. Both hereditary and spontaneous breast cancer can be HER-2 positive, meaning there is an increase in the activity of the HER-2/neu gene (7). Cells that overexpress the HER-2/neu gene tend to grow faster and have a higher risk of spreading. About ~20% of breast tumors are associated with the HER-2/neu protein, and clinical studies show that alteration of this gene results in poor prognosis for breast cancer patients (11). Amplification of HER-2/neu is the most
frequently found oncogene (gene than has potential to cause cancers) mutation in breast cancers (12).

### 1.3 Treatments for Breast Cancer

Surgery, chemotherapy, radiotherapy, biological therapy, hormone therapy and targeted therapy are the currently used treatments for breast cancers. Although there are several treatments used to reduce the risk of breast cancer recurrence, the risk of recurrence differs from patient to patient, depending on the patient’s disease characteristics and hormone receptor status. For instance, hormone therapy is best for ER+ and PR+ breast cancers, because the drugs that are used in hormone therapy prevent the binding of hormone to the receptor sites of the cells. Tamoxifen, Anastrazole, Femara, Zoladex, Megace, and Halotstin are some drugs used in hormone therapy.

Tamoxifen is the main antiestrogen drug used in hormone therapy for breast cancers. It prevents estrogen from binding to cancer cells but allows some amount of estrogen to affect other parts of the body (13). Tamoxifen was the first selective estrogen receptor modulator (SERM) successfully tested on women having a high risk of breast cancer. Selective estrogen receptor modulators are ligands that act as estrogen in some tissues but block estrogen action in other tissues (14). Tamoxifen is currently approved by the US FDA to treat all types of ER+ breast cancers and to prevent breast cancer in women at high risk (15).
Femara is an orally active, potent selective aromatase inhibitor for advanced breast cancers. Aromatase is an enzyme that converts androgen to estrogen. Femara is used to treat postmenopausal women who have advanced breast cancer. Anastrazole is another orally active aromatase inhibitor that is used to treat breast cancers alone or with other treatments. Aromatase inhibitors are good drugs for ER+ breast cancer cells since they work by reducing the amount of estrogen produced in the body. They can stop or slow down the growth of ER+ cells that have metastasized (16).

Hormone therapy is not a viable treatment for hormone receptor negative breast cancers. Therefore, chemotherapy is still the most common treatment for patients having hormone receptor negative breast cancer, regardless of menopausal status (13). Clinical studies found that adjuvant chemotherapy, which uses a number of drugs together, is more effective than using a single drug. Therefore, to prevent the risk of recurrence of breast cancer, a patient should receive adjuvant treatments. Currently, breast cancer treatments focus more on synthetic drugs and natural products for chemotherapy and hormone therapy than on radiotherapy (17). Biological therapy and targeted therapy are coming into their own, but they are outside the scope of this project.

Treatment of breast cancer with chemotherapy is improving due to continual innovations in the pharmaceutical industry using natural products as well as organic synthesis methods. There are newer drugs for chemotherapy, such as taxane, vinorelbine, tomudex, and capecitabine. Taxanes are anticancer agents that bind to and stabilize microtubules, causing mitotic arrest and apoptosis (18). Taxanes are produced
from extracts of different species of the yew tree. For instance, paclitaxel, also known as taxol, was initially extracted from the bark of the Pacific yew, *Taxus brevifolia*, while docetaxel is synthesized from a compound extracted from the needles of the European yew, *Taxus baccata*. Both paclitaxel and docetaxel are similar to each other in their mechanism of action (19). Like taxane, many of the drugs for chemotherapy treatments for cancer, as well as for other diseases, were first isolated and/or synthesized from plant material. Likewise, many studies have focused on the isolation of bioactive compounds from *Artemisia* species towards many diseases. Although indigenous cultures and traditional medical systems did not know about the chemical compositions of *Artemisia* species when using them, they have been used as herbal medicines for millennia.

1.4 Mugwort as herbal medicine

Indigenous cultures (such as tribal African and Native American cultures) use herbs in their healing rituals, and Eastern medical systems (such as Ayurvedic and Traditional Chinese Medicine) use herbal therapies rather than synthetic drugs. Most Asian countries still use Ayurvedic medicine to treat numerous diseases, including different types of cancers. Many medical systems, such as Ayurvedic medicine, are complete healthcare systems that involve detoxification, diet, exercise, the use of herbs, and techniques to improve mental and emotional health. Herbs are nature’s gift for humans to live healthy lives.
Worldwide, mugworts have been used as herbal medicines for millennia. *Artemisia* (most likely *A. petrosa*) was well known in ancient Greece, where it was called "the mother of herbs". Mugwort species have been, and still are, used in Traditional Chinese Medicine, as well as in other traditional Asian medicine systems. Mugworts are also important sacred healing plants to Indians of South and North America (19).

Among the many herbs that are still used by California tribes to treat diseases, California mugwort (*Artemisia douglasiana* Besser) is special, as it used for many women’s ailments and for other common diseases (2). It is also considered a sacred plant by many California tribes.

*A. douglasiana* is taken over long periods of time in small dosages for improving appetite, digestive functions, and absorption of nutrients. It is also a tonic for the nervous system, reducing tension and nervousness, and it is believed to be helpful in cases of depression, especially when accompanied by loss of appetite. The essential oil extracted from *A. douglasiana* has been used for aromatherapy, inhaled for mental clarity and for easing mental distress (2).

Mugwort root contains a bitter principle that makes it a fine digestive stimulant and stomach tonic. In the Cuyo region of Argentina, *A. douglasiana* leaves are popularly known as “matico” and are widely used for the treatment of gastric ailments. It can be used to relieve pain in the stomach as well as for irritable bowel syndrome. As a digestive, it cools the digestive tract, stimulates the appetite, and eases nervous and sluggish digestion, dyspepsia, stomach acidity, travel sickness and indigestion (1).
Mugwort also can improve the functions of the liver and gallbladder by stimulating bile flow from the gallbladder through the bile ducts into the duodenum (20).

Chinese and Europeans have traditionally used mugwort (*A. annua* and *A. vulgaris*, respectively) for disorders and malfunctions in the reproductive system. Chinese practitioners use it to warm the body and to stop bleeding when the menstrual cycle is too long. But, in some cultures, mugwort can be used to induce menstruation. California mugwort was, and still is, used to treat premenstrual syndrome and dysmenorrhea by indigenous Californians. It is also said to relieve menstrual pains and cramps (2). In the first century A.D., Greek writings mentioned that passing soldiers used mugwort (probably *A. petrosa*) in their sandals to relieve the pain of sore feet (19). This indicates that mugwort might be used as an analgesic as well.

An *Artemisia* species has also been used as treatment for malaria. *A. annua*, a Traditional Chinese Medicinal plant, contains artimisinin and its derivatives, which effectively act as blood schistocidal antimalarials that have fewer adverse side effects than other antimalarial drugs (21). Artemisinin is currently the most widely used antimalarial in the world. Research has shown that dihydroartemisinin can also act against leukemia cells. These experimental results showed that *A. annua* contains cytotoxic compounds (22).

Although mugworts are used as treatments around the world for many diseases, excessive dosage can lead to poisoning. Previous studies showed that mugwort stimulates the nervous system, and it is used to stimulate the uterus to bring on menses. But an overdose of mugwort can lead to abortion (1). Therefore, it should not be used
by pregnant women, which is a good illustration that, before using an herbal medicine or any drug, it is essential to know about its side effects on healthy cells of the body.

1.5 Botany and Taxonomy

*Artemisia douglasiana* Besser (Figure 2) belongs to the family Asteraceae, the genus *Artemisia*, and the tribe Anthemideae. The genus *Artemisia* consists of about 500 species throughout the world (23). *A. douglasiana* Besser was named after Scottish botanist David Douglas (1798-1834), who made several trips to America and found and described plant species in California. *A. douglasiana* Besser (California mugwort) is a common perennial herb found throughout the western United States, especially northern California, Oregon, and Washington, at 1–800 m elevation, and preferring dry, partially shaded stream sides or riverbanks, on most types of geologic formations. It has a strong sage odor. The flowers are small and not particularly showy. Left to grow naturally, the plant grows three to four feet tall and spreads to form a colony. Well watered plants can grow even taller. *A. douglasiana* provides excellent cover and seeds for birds (1, 24, 25).
1.6 Chemical constituents of *Artemisia* species

Phytochemical studies on various *Artemisia* species show that members of this genus are rich in sesquiterpenes, monoterpenes, flavonoids and coumarins (26). Sesquiterpenes have the chemical formula $C_{15}H_{24}$ and are less volatile than monoterpenes, which have the general formula $C_{10}H_{16}$ (23). Due to their semi-volatility, sesquiterpenes can be extracted from their natural sources by steam distillation or extraction, and they can be purified using vacuum fraction distillation or column chromatography. Sesquiterpenes isolated from *Artemisia* species show many medicinal properties such as antidiarrheal (27) activity, anti-inflammatory activity (28)...
and antibacterial activity (29). For instance, dehydroleucodine (DhL), a sesquiterpene lactone isolated from *A. douglasiana*, shows antidiarrheal activity (27, 28).

*A. ludoviciana* also contains antidiarrheal compounds. *A. ludoviciana* var. *Mexicana*, commonly called Lusiana wormwood, cudweed, sagewort, gray sagewort, mugwort, wormwood, prairie sage, or white sagebrush “Silver King”, is native to the western United States east to Missouri (26). A study by Zavala-Sánchez *et al* showed the presence of monoterpenes like camphor and limonene, as well as sesquiterpenes and flavonoids in an antidiarrheal extract from *A. ludoviciana* leaves (29). Essential oil from the leaves was subjected to separation and the compound showing antidiarrheal activity was identified as nonanal (29).

*Artemisia suksdorfii* Piper, the “coastal worm wood,” is a native perennial of the coastal Pacific Northwest area of the United States. There is not much information on *A. suksdorfii* as a medicine, but there are studies that isolated and identified compounds from *A. suksdorfii* (30, 31). In the phytochemical analysis of aerial parts of *A. suksdorfii*, five novel polyol monoterpenses and several novel sesquiterpene lactones were isolated (31). Another two new α-pinene-type monoterpenes were isolated and identified as 7-hydroxymyrtol and 7-hydroxymyrtolen (Figure 3) by Ahmed *et al* (30).
Artemisia vulgaris, also known as mugwort, felon herb, chrysanthemum weed, and St. John’s herb, is native to Europe, Asia, northern Africa and North America. It has been used as a medicine for various diseases and is used as an infusion or herbal patch for abdominal pains and women’s diseases. *A. vulgaris*, prepared as a moxa, is used in Traditional Chinese Medicine to treat rheumatic arthritis (32). Many chemical constituents have been isolated from *A. vulgaris*, such as 1,8-cineole, α-thujone, camphor, isoborneol, elemol, limonene, α-eudesmol and spathulenol, which are all shown in Figure 4 (33).
Another commonly used *Artemisia* species is *A. annua*, also known as qinghao, sweet Annie, sweet sagewort, annual wormwood, or sweet wormwood. *A. annua* is native to Asia. For many centuries, the Chinese have used this plant to treat malaria, and the major antimalarial constituent, artemisinin, has become the most widely used antimalarial drug in the world (22). Artemisinin was first isolated in China from the aerial parts of *A. annua*. Since only *A. annua* contains artemisinin, this plant is very unique and important among other *Artemisia* species. As can be seen in Figure 5, artemisinin is an oxidized sesquiterpene lactone (34).
Studies by other research groups have found the following chemical constituents in *A. douglasiana*: hexanal, camphene, yomogi alcohol, 1,8-cineole, artemisia ketone, artemisia alcohol, α-thujone, camphor, myrtenal, vulgarone B, and DhL, which are all shown in Figure 6 (1). Most of these chemical compounds are found in the essential oil. Most previous studies on this species have been focused only on DhL (a sesquiterpene lactone guaianolide), one of the main constituents in California mugwort. However, a previous thesis project showed that DhL was not cytotoxic to breast cancer cells (3).

Another important sesquiterpene ketone isolated from *A. douglasiana* is vulgarone B (C_{15} H_{22} O; Figure 6), which is characterized as a gastropod repellent. Many species of *Artemisia* contain antifungal and allelopathic constituents in order to survive in the environment. Vulgarone B was isolated during the search for a natural product to solve a problem that arose in the commercial catfish industry because of the ram’s horn snail (*Pomacea canaliculata*). The isolated antifungal compound was identified as vulgarone B, which had previously been isolated and identified from *Chrysanthemum vulgare*,

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**Figure 5: Structure of artemisinin.** (https://scifinder-cas-org.proxy.lib.csus.edu/scifinder/view/scifinder/scifinderExplore.jsf)
another plant species (35). Several assays using reduced vulgarone B compounds found that antifungal activity was due only to compounds that have an α, β unsaturated carbonyl group (35). Assays carried out to measure the molluscicidal activity of vulgarone B on ram’s horn snails showed 100% mortality (35, 36).

Figure 6: Structures of chemical constituents isolated from leaves of *A. douglasiana*. (a) camphene, (b) yomogi alcohol, (c) artemisia ketone, (d) myrtenal, (e) hexanal, (f) dehydroleucodine (DhL), (g) vulgarone B. (https://scifinder-cas-org.proxy.lib.csus.edu/scifinder/view/scifinder/scifinderExplore.jsf)
1.7 Preparation of Plant Materials for Isolation and Analysis of Constituents

Plants contain a broad range of bioactive compounds such as pharmaceuticals, flavors, fragrances and pigments. These phytochemicals can be found with a wide range of polarities, from hydrophobic to water soluble. Phytochemicals can be grouped based on their polarity as follows (37): nonpolar (waxes, terpenoids), semi-polar (lipids, phenolic compounds, low-polar alkaloids), and highly polar (glycosides, most alkaloids, saccharides, peptides, proteins). Because any one plant contains a myriad of compounds of varying polarity, isolation of natural products is a complex process.

Before starting a purification process, plant materials need to be cleaned. Soil and contaminants can be removed from each plant sample by rinsing with deionized water, and, thereafter, more tightly bound particles can be removed by rinsing in liquids containing complexing agents such as ethylenediaminetetraacetic acid (38). In the preliminary preparation, plant materials are usually air-dried or lyophilized. Drying removes large amounts of water from the sample and allows the determination of the specific composition of the dry mass. Dried materials are extracted with a solvent or a mixture of solvents (37), then solids are removed using filtration, ultrafiltration or centrifugation before use in other purification techniques (39).

To get a sample enriched in a specific analyte, one needs to remove interfering compounds using several separation techniques. In every sample preparation procedure, especially for complex samples containing a large number of components, analyte enrichment and interference removal are essential. Most novel purification techniques minimize sample size, solvent usage and time. Therefore, much research
focuses on improving extraction techniques to get more effective and faster results in the purification of plant chemical constituents (37). Such is the focus of this study.

In the purification of desired compounds, unwanted compounds are removed using a number of separation techniques (36), including liquid/liquid extraction (LLE), selective adsorption, preparative liquid chromatography (PLC) with normal (NP-PLC) or reversed (RP-PLC) stationary phases, ion exchange chromatography (IEC), size-exclusion chromatography (SEC), solid-phase extraction (SPE), and solid phase micro-extraction (SPME), or solvent micro-extraction. In this project, the most commonly used separation tools are LLE, SPE and NP-PLC (flash column chromatography). Fractions prepared using those techniques are analyzed for purity using HPLC or GC. Finally, fractions prepared using those techniques are subjected to identification using nuclear magnetic resonance spectrometry (NMR), infrared spectroscopy (IR), and mass spectrometry (MS).

An important factor in analyzing plant constituents is that the sample analyzed be representative. It is difficult to collect representative samples due to variability amongst individuals of a species (37). For example, samples should be collected from a number of plants in the same area. Plant metabolites also vary seasonally and diurnally. For consistency, plants should be collected in the same season of the year and at the same time of day that previous collections have been done. In this project, *A. douglasiana* leaves were collected from the river bank of the American River, just north of the Guy West Bridge in Sacramento in the summer of 2009. In the previous study in this
laboratory, *A. douglasiana* leaves were collected from the same river bank and same site as leaves collected for this project, but a few months earlier in the year, in 2006.

1.8 Contribution of the Previous Study to the Current Study

The previous study in this lab tested aqueous, hexane and ethanol extracts of dried *A. douglasiana* leaves on two different breast cancer cell lines, MDA-MB-231 and BT-474. It was found that organic extracts were very cytotoxic towards breast cancer cells, whereas the aqueous extract showed very weak cytotoxicity. MDA-MB-231 is an estrogen-receptor negative breast cancer cell line, whereas BT-474 is an estrogen receptor positive breast cancer cell line. Because the previous study showed that extracts from dried leaves of *A. douglasiana* affected both cell lines approximately equally, it was concluded that cytotoxic constituent/s in these extracts do not have estrogenic effects. Additionally, it was shown that DhL, which is present in *A. douglasiana* leaves, is not cytotoxic, and that artemisinin, which is present in other *Artemisia* species, is not present in *A. douglasiana* leaves. The previous study also identified a candidate cytotoxic constituent, but there was not enough of this compound to characterize it. (Although this compound appeared to be purified to homogeneity as assessed by GC-MS, there is still a possibility that another compound co-purified with it, but was not detectable by GC-MS.) Therefore, the current study initially focused on the isolation and identification of this candidate cytotoxic constituent from the previous study. However, the initial goal was changed due to differences between the preliminary results from the current study and the results from the previous study.
1.8.1 Goal A: To Determine the Effect of Mugwort Leaf Extracts on Normal Cells

The first goal of this study was to determine the effect of mugwort leaf extracts on normal cells. The “half maximal effective concentration” (EC\textsubscript{50} value) is used to assess the efficacy of potential drugs. As shown in Figure 7, the EC\textsubscript{50} value is defined as the concentration of drug that provokes the desired or undesired response in half the individuals tested. This allows scientists to compare the dosage that elicits the desired effect (left-most curve in the figure), such as killing cancer cells, to an undesired effect (right-most curve), such as the death of normal cells or the death of the patient. For instance, if a drug kills normal cells at nearly the same concentration that it kills cancer cells, it will not be a good drug to use to treat cancer patients. Therefore, it is important to find the effect of drugs on normal cells as well as on cancer cells. In this study, the EC\textsubscript{50} value of ethanolic and aqueous extracts on normal and cancerous human cells was compared.
1.8.1 Goal B: To Evaluate the Reproducibility of the Previously Developed Isolation Scheme

The second goal was to determine whether the isolation scheme developed in the previous study was reproducible with a new extract of *A. douglasiana* leaves. Since, in the current study, plant leaves were collected at a different time of the year than those used in the previous study, it is necessary to determine if the same compounds were present in the same proportions in the plant leaves. Previous studies on *A. douglasiana* found that the chemical composition of plant materials change from gathering place to gathering place and also seasonally. For instance, *A. douglasiana* from Argentina
produces eudesmanolides in the spring and guainolides in the autumn (41), whereas neither compound was found in *A. douglasiana* from California (42, 43).

1.8.2 Goal C: To Evaluate and Improve the Previously-Developed Isolation Scheme

Although the previous study provided fundamentals like type of solvent to be used for the initial extraction and polarity of the cytotoxic constituent for this study, many factors remained to be investigated. For instance, even though the previous study indicated that a compound eluting at 10.5 min in GC-MS is a cytotoxic constituent, it may be that a cytotoxic constituent co-eluted with that compound and may not be volatile enough to be detected by GC-MS analysis. Therefore, before proceeding with a detection method like GC-MS or HPLC, it is necessary to determine whether the cytotoxic constituent/s is/are volatile or not. Hence, in this study, vacuum distillation was performed to determine the volatility of the cytotoxic constituent/s.

Although the previous study established fairly good solvent systems for flash chromatographic separation of the crude ethanolic extract, more refinement of the separation scheme was required to find the best eluents to separate cytotoxic constituent/s from other components. To improve the isolation scheme that was developed in the previous study, it was necessary to run columns multiple times to collect enough mass for instrument analysis, cytotoxicity assays, and subsequent
purification. Furthermore, multiple chromatographic runs using the same column also examined the reproducibility of the separation.

1.8 Contribution of the Current Study

The current study extends the knowledge of the nature of the cytotoxic constituents present in *A. douglasiana* leaves, which ultimately will lead to a reproducible isolation scheme for these compounds. By knowing the cytotoxic constituents present in *Artemisia* species, they can be used as standards for analysis of herbal medicines. Isolating and identifying cytotoxic constituents from this important medicinal plant will also allow assessment of their safety and efficacy to determine whether it can be used as an anti-cancer drug to treat breast cancer. Apoptosis and genetic alterations of cancer cells by the drug can be further studied before use as an anticancer drug.
Chapter 2

EXPERIMENTAL

2.1 Summary of Isolation Steps

This is a summary of the extraction and isolation processes done in this study. A flow chart of the process is presented in Figure 8. Because many of the steps used similar protocols, detailed protocols are described later in this chapter.

Dried *Artemisia douglasiana* leaves were extracted with ethanol or deionized water for 48 hours to obtain an ethanol extract and an aqueous extract. EC\textsubscript{50} values were determined for both extracts on human peripheral blood mononuclear cells (PBMC). An EC\textsubscript{50} value was determined for the ethanol extract on MDA-MB-231 breast cancer cells.

A portion of the ethanol extract was rotary evaporated to dryness, and, from the dried extract, a hexane extract was made by SLE. The hexane extract and ethanol extracts were tested on breast cancer cells and analyzed using GC-MS in order to compare to the previous study.

The ethanol extract was treated with lead tetraacetate and partitioned with chloroform using LLE to remove chlorophyll. Chloroform fraction 1 obtained from LLE was further isolated using column chromatography (Column 1). Another, identical column was performed using the ethanol extract as the sample loaded (Column 2). Eluent fractions were analyzed by GC-MS.
Another portion of the ethanol extract was rotary evaporated to dryness, and, from the dried extract, a diethyl ether extract was made by SLE. A cytotoxicity assay was performed with the ether extract, the ethanol extract and the residue left after extraction with ether. Columns 3, 4 and 5 were run on the ether extract, and collected fractions were analyzed using GC-MS, HPLC (Columns 4 and 5 only) and a cytotoxicity assay. Column 4 was repeated 4 times and Column 5 was repeated twice in order to scale up the isolation process and test reproducibility.
Figure 8: Summary of the Isolation Process.

Dried Leaves → Ethanol Extraction → Ethanol Extract → Rotary Evaporation → Dried Ethanol Extract → Hexane Extraction → Hexane Extract → Residue

A. Lead Tetraacetate - Ethanol
B. Chloroform Extraction

Chloroform Fraction 1

Column 1

Chloroform Fraction 2

Column 2

Chloroform Fraction 3

Column 3

Diethyl Ether Extraction → Ether Extract → Residue

Dried Ethanol Extract → Rotary Evaporation → Chloroform Fraction 2 → Column 2

Chloroform Fraction 3 → Column 3

Residue → Column 4

Column 5

Water Extraction → Aqueous → Ethanol Extraction → Ethanol Extract → EC50
2.2 Chemicals

Ethanol (95%, ACS/USP grade) was obtained from Pharmco Products, Inc. Diethyl ether (ethylether anhydrous, ACS grade) was purchased from EMD Millipore. All the other solvents used were HPLC-UV grade (Pharmco Products Inc). Nanopure water was obtained by filtration through an EASYpure LF Compact Ultrapure Water System (Barnstead). Media for cells consisted of Improved Minimum Essential Media, Zinc Option, 1X, containing L-glutamine, 2 mg/L L-proline, and 50 μg/μL gentamicin sulfate (IMEM, Invitrogen), 10% heat-inactivated fetal bovine serum (Axenia Biologix), 50 IU/mL each penicillin/streptomycin (Cellgro by Mediatech, Inc.), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma-Aldrich). Trypsin (0.25% with 0.1% EDTA in Hank's Balanced Salt Solution without calcium, magnesium or sodium bicarbonate; Cellgro) was purchased from Mediatech Inc. Fico/lite was purchased from Atlanta Biologicals. Phosphate Buffered Saline (PBS; pH = 7.4) consisted of sodium chloride (8 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.44 g), monopotassium phosphate (0.24 g) in 1L of distilled water. Trypan Blue (0.4% in phosphate buffered saline), was purchased from Mediatech, Inc. CellTiter 96Aqueous One Solution was purchased from Promega, Inc. Column chromatography was performed using silica gel 60 with 0.063-0.200 mm beads (EM Science, now EMD Chemicals; 200-400 mesh).
2.3 Materials

All glassware was soaked in a sodium hydroxide/isopropanol bath (400 ml of 10% aqueous sodium hydroxide and 3.6 L isopropanol) for at least 6 h and then rinsed with distilled water 10 times followed by 3 rinses with ethanol and 3 rinses with acetone prior to use. Round bottom flasks (2 L, 1 L, 500 ml, 100 ml, 50 ml, 25 ml: Chemglass) were used to rotary evaporate initial extracts and in vacuum distillation apparatuses. Liquid -liquid extractions were performed using a 30 ml separation funnel. MDA-MB-231 cell line (American Type Culture Corporation) and peripheral blood mononuclear cells (anonymous donor) were grown in T-25 and T-75 flasks (VWR scientific), and 15-ml and 50-ml Falcon tubes and 1.5-ml microcentrifuge tubes (USA Scientific) were utilized for cell centrifugation. Sterile filtration of samples was done using 25 mm diameter, 0.45 µm and 0.25 µm (Fisherbrand) pore sterile syringe filters. Cytotoxicity assays were carried out in individual wells of a Microtest tissue culture 96-well plate with flat bottom wells and a low evaporation lid (Becton Dickinson Labware). Thin Layer Chromatography analysis was performed on aluminum plates pre-coated with 0.25 mm silica gel 60 UV254 (Whatman).

2.4 Instruments and Equipment

All cell culture procedures were performed in Level 2A biosafety cabinet (LABCONCO). Centrifugation was done using a Sorvall GLC-2B General Laboratory Centrifuge (Du Pont Instruments). Mixing of cell pellets and test samples was done with a vortexer from Fisher Scientific. A water bath manufactured by Chicago Surgical
& Electrical Co. was used to warm up solutions used in cell culture procedures. Cryogenic vials (Nunc) were used to freeze down cells and store cells in cryogenic chamber. Falcon tubes (15ml, 50 ml) purchased from Fisher Scientific were used in all culture procedures. Parafilm (VWR Scientific), hemacytometer (Fisher Scientific) and sterile 96-well plate (Becton Dickinson Labware) were used in the procedure of cell plating. All the cell cultures were incubated in an incubator (IR Auto Flow purchased from Nuaire). In the cytotoxicity assay, a microtiter plate reader (BioRad Model 680) was used to read cells treated with Celltiter (Promega).

Flash column chromatography was performed using glass columns (250 x 16 mm, 220 x 18 mm, and 240 x 18 mm) purchased from Kontes Glass Co. Gas chromatography-mass spectrometry (GC-MS) was done using a gas chromatograph (7890A GC system from Agilent Technologies) coupled with a mass spectrometer (5975C inert XL EI/CI MSD from Agilent Technologies), with a silica capillary column (stationary phase HP-5MS, size 30 m X 0.25 mm X 0.25 μm, Agilent J&W GC). High Performance Liquid Chromatography (HPLC) was performed on an Agilent 1100 Series chromatograph equipped with a variable wavelength UV detector (VWD Agilent) and a custom built charged aerosol detector (CAD; Thermal System Inc). An Agilent RP-C18 column (4.6 x 150 mm, 5 μm) with a guard cartridge (7.5 x 4.6 mm Benson Carbohydrare BC-100, Alltech) was used in HPLC.
2.5 Initial Extraction

*A. douglasiana* leaves were collected from the eastern bank of the American River, just north of the Guy West Bridge across from the California State University, Sacramento. The leaves were air dried for 3-4 days. The dried leaves (200 g) were diced and extracted with 5000 ml of 95% ethanol for 2 days at room temperature, with stirring. This "ethanol extract" was filtered using a large Buchner funnel, and the filtrate was evaporated to dryness using a rotary evaporator (Yamato Scientific Company). The residue was dissolved in 95% ethanol for further experiments. Five milliliters of ethanol extract were evaporated to dryness using a rotary evaporator and then extracted with hexane three times, 5ml at a time. The three volumes of hexane were combined to make the "hexane extract". Another 5 ml of ethanol extract was evaporated to dryness and extracted with 1 ml diethyl ether ("ether extract").

2.6 Cytotoxicity Assays

2.6.2 Cell culture conditions

A Level 2A biosafety cabinet was irradiated with ultraviolet light for at least 30 minutes before use. All cell culture solutions were thawed or warmed in a 37 °C water bath, and all equipment and culture flasks were wiped down with 70% ethanol before being placed in the hood.
2.6.3 Starting Culture of Cancer Cell Line

Aliquots of the human breast cancer cell line MDA-MB-231 were stored in growth media with 7% dimethylsulfoxide in cryogenic vials under liquid nitrogen in a cryogenic chamber. A vial containing MDA-MB-231 cells was retrieved from the cryogenic chamber and was thawed in a 37°C water bath for 1-2 min. Four milliliters growth media were added to a 15-ml sterile Falcon tube. Thawed cells were transferred to this tube and were then centrifuged for 5 min at 3000 rpm in a clinical centrifuge. After centrifugation, the media was aspirated, and the cell pellet was resuspended in another 5 mL of growth media. Again the cell suspension was centrifuged and resuspended in another 5 ml of media. Cells were transferred to a labeled T-25 flask and kept in an incubator at 37°C in 5% CO₂ and 100% humidity in an incubator.

2.6.4 Maintaining Cell Cultures

Cultures were maintained in growth media in T-25 or T-75 cell culture flasks. Cells were cultured at 37 °C in 5% CO₂ and 100% humidity in an incubator. Cells were split 1 to 5 or 1 to 10 when they reached approximately 90% confluency, as follows. When cells were ready to split, media was aspirated, and 1.0 ml or 1.5 ml of trypsin was added to the T-25 flask or T-75 flask, respectively. Cells were then incubated for 15 min in an incubator at 37 °C in 5% CO₂ and 100% humidity. When cells started to float and separate from each other, 5 ml of media was added and the cell suspension was then transferred to a sterile 15-ml Falcon tube and centrifuged for 5 minutes at 3000 rpm in a clinical centrifuge. After centrifugation, the supernatant was discarded and the cell
pellet was resuspended in another 5 mL growth media. One ml of cell suspension was added to a T-25 or T-75 flask, followed by 4 ml or 14 ml of media, respectively. Then cells were incubated at 37 °C in 5% CO$_2$ and 100% humidity in an incubator.

2.6.5 Cell Proliferation Assay for Cancer Cells

Media was aspirated from the cell culture flask, and 1 ml trypsin was added to detach the cells from the flask and each other. The flask was then incubated for 10 minutes at 37 °C in 5% CO$_2$ and 100% humidity. Upon trypsination, 5 mL fresh growth media were added into the flask to inactivate the trypsin, and the cell suspension was then transferred to a 15-ml sterile Falcon tube and centrifuged for 5 minutes at 3000 rpm. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in another 5 mL growth media. Ten μL of cell suspension was added to 10 μL of Trypan Blue on Parafilm and mixed thoroughly, then 10 μL of this mixture was placed into one side of a hemacytometer. This procedure was repeated to load the other side of the hemacytometer. The cells in each of the four quadrants on both side of the hemacytometer were then counted under a Swift compound microscope. The average of the number of cells from the eight (1 X 10$^{-4}$ ml) quadrants was calculated, and this number was multiplied by 2 to account for the 1:2 dilutions with trypan blue. This value was then multiplied by 10,000 to get cells/ml, and $C_1V_1 = C_2V_2$ was used to calculate the cell suspension volume required to get a final concentration of 5 x 10$^4$ cells/ml. One hundred μL of cell suspension was placed in each of the middle 60 wells of a sterile 96-well plate using micropipette channel. (Cells in outer wells grow well
than cells in inner wells, which was shown by a study in this laboratory.) The plate was incubated in an incubator at 37 °C for 48 h in 5% CO₂ and 100% humidity.

2.6.6 Preparation of Test Media

Prior to mixture preparation, samples of interest were sterile-filtered using 0.2 μm syringe filters. Test media were prepared by mixing 1987 μL growth media and 13 μL of the sample of interest (in ethanol). A negative control using 13 μL of 95% ethanol was also prepared. Then the media was aspirated from each of the middle 60 wells of the 96-well plate using a sterile pasteur pipette. Each set of 3, 6, or 10 wells was inoculated with a different test medium, with 100 μL of test medium pipetted into each well. (The number of wells per set varied depending on the volume of sample available and the number of fractions to be tested.) The plate was then incubated for another 48 h at 37 °C in 5% CO₂ and 100% humidity.

2.6.7 Determination of Relative Cell Number for Cancer Cells

After 48 h, the media was aspirated from each of the wells using a fresh sterile pasteur pipette for each row and 100 μL fresh medium added. Fresh growth medium was also added to the first row, to serve as blanks for the microtiter plate reader. Cell Titer 96Aqueous One Solution (20 μL) was then added into each well that contained media. The plate was incubated at 37 °C for 1-2 h and placed in a microtiter plate reader to measure the absorbance at 490 nm. The standard deviation for each sample was calculated from 2, 6, or 10 individual tests, using the following formula:
Standard deviation = \[\sqrt{\frac{(Y - \bar{Y})^2}{n-1}}\]

\(Y\) = Absorbance of the particular well

\(\bar{Y}\) = Mean of absorbance for n number of wells

\(n\) = Number of wells

In the determination of activity, the mean absorbance for each sample set was divided by the mean absorbance for the control set, and then multiplied by 100%, so that the number of viable cells was expressed as percent of control.

### 2.6.8 Determination of EC\(_{50}\)

All the sample preparation was performed in a biosafety hood. The sample to be tested was made to 10 mg/ml (stock), then was sterile filtered. For each sample, a series of six concentrations was prepared in sterile 15-ml Falcon tubes. One hundred microliters of stock was added to the first tube. For the second tube, 80 \(\mu\)l stock extract and 20 \(\mu\)l sterile ethanol were added. The third solution was made by adding 60 \(\mu\)l stock extract and 40 \(\mu\)l sterile ethanol. To the fourth tube, 40 \(\mu\)l stock extract and 60 \(\mu\)l sterile ethanol were added. The final dilution was made with 20 \(\mu\)l stock extract and 80 \(\mu\)l sterile ethanol. One hundred \(\mu\)l sterile ethanol was used as the control. To each tube, 9.9 mL of growth media were added. Test media were tested on cells using the cytotoxicity assay as described above. In the determination of EC\(_{50}\), the number of viable cells (percentage of control) was plotted against the concentration of the sample.
2.6.9 Preparation of Normal Cells

Human blood was donated by an anonymous student at California State University, Sacramento. Blood was drawn by a licensed phlebotomist at the CSUS Health Center or at the UC Davis Center for Regenerative Medicine, according to the protocol that was approved by the CSUS Human Subjects Institutional Review Board (Appendix A). Informed consent was obtained and is on file in the office of Mary McCarthy Hintz of the CSUS Chemistry Department.

Twenty milliliters of blood were added to 20 ml of PBS buffer in a 50-ml Falcon tube. Twenty milliliters of blood-PBS mixture were carefully layered onto 20 ml of Fico/lite solution in each of two 50 ml Falcon tubes. Then each tube was centrifuged in a clinical centrifuge at 1800 rpm at room temperature for 30 min, with the brake off. After centrifugation, the upper (plasma) layer was carefully aspirated and discarded. Then the mononuclear white blood cell layer ("buffy coat") was carefully withdrawn from atop the Fico/lite (bottom) layer. White blood cells were transferred into one new 50-ml Falcon tube, and PBS was added until the solution became milky (~20 ml). After adding PBS, the tube was centrifuged at 1800 rpm for 5 min, with the brake on. The supernatant was discarded, and cells were resuspended in 5 ml media. Plating of normal cells was carried out following the same protocol for cancer cells but using 50 µL cell suspension (1 X 10⁶ cells per ml). The EC₅₀ was determined following the same protocol described for cancer cells, except that, instead of aspirating the media and adding 100 ml test sample, 50 µL test sample was added directly to each well because
normal cells do not attach to surfaces of wells and therefore the media cannot be aspirated before the samples were added.

### 2.6.10 Determination of Relative Cell Number for Normal Cells

After 48 h, 100 μL fresh growth media were added to the first row, to serve as blanks. Cell Titer 96Aqueous One Solution (20 μL) was then added into each well that contained media. The plate was incubated at 37 °C for 4-5 h and placed in a micro-plate reader to measure the absorbance at 490 nm.

### 2.7 Isolation of Cytotoxic Constituent(s)

#### 2.7.1 Lead Tetra-acetate Preparation

Thirty grams of lead tetra-acetate (Pb₃O₄) were dried at 200 °C and stored in a dessicator. The dried Pb₃O₄ was slowly added to a three-neck round bottom flask containing 55 ml acetic acid and 17 ml acetic anhydride, while maintaining the temperature of the solution at 40 °C. The solution was filtered through a Buchner funnel with filter paper (Whatman 1mm) covered with celite. The supernatant was discarded, and the precipitate was washed with glacial acetic acid.

#### 2.7.2 Removal of chlorophyll

To 10 ml of ethanol extract, 10 ml of 4% lead tetra-acetate were added. Then the resultant solution was partitioned 3 times with 10 ml of chloroform using a 30 ml
separatory funnel. The three chloroform fractions were collected separately. Each fraction was analyzed by GC-MS.

2.7.3 Flash Column Chromatography

Flash column chromatography was performed using silica gel columns and different elution gradients. Samples from 1 ml to 5 ml were chromatographed on columns with increasing polarity of solvent mixtures. The eluting systems are indicated in Table 1.

2.7.4 Vacuum Distillation

Standard vacuum distillation apparatuses were used. One gram of ethanol extract of *A. douglasiana* leaves was placed in a round bottom flask (100 ml) with 25 ml of water. In the first trial, the distillate was collected in 5 ml of ethanol while maintaining the temperature at about 0 °C. The vapor started distilling at 42°C, and the temperature increased to 57°C and then started dropping rapidly. The distillate was stored in the freezer for future use.

Since ethanol is not very volatile, most of the volatile compounds may inadvertently be removed from the distillate when using a rotary evaporator to dry the sample. Therefore, another vacuum distillation was run using the dried ethanol extract (1.0 g), and the distillate was collected into a 100 ml pre-weighed empty round bottom flask. The vapor started distilling at 55.5°C (pressure at 111.2 Torr) and then again started boiling at 55.5°C (at a pressure of 120.0 Torr), then the temperature started
dropping rapidly. Both the distillate and the left over aqueous suspension were extracted 3 times with an equal volume of diethyl ether and dried using a rotary evaporator.

2.7.5 Gas Chromatography-Mass Spectrometry Analysis

Ethanol and hexane extracts were evaporated using a rotary evaporator and the residue was dissolved in 2-3 drops (from a pasteur pipette) of dichloromethane, then the samples were analyzed by injection into a gas chromatograph coupled with a mass spectrometer (GC-MS). This step was repeated after every fractionation step. Samples that were in ether were directly analyzed by GC-MS. All fractions obtained from columns were collected into pre-weighed vials (1 dram) and evaporated to dryness using a rotary evaporator. Column fractions that were in ethanol, ethyl acetate, or hexane were evaporated to dryness and dissolved in dichloromethane prior to GC-MS analysis. The extracted compounds were separated on a 5 % phenylsilicone stationary phase on a fused silica capillary column. The carrier gas was helium (Praxair) at a flow rate of 1.0 ml/min. A 2.0 μl volume of the sample was injected using the splitless mode for more dilute samples or the split mode for concentrated samples. The column temperature program was 40 °C for 1 min, 20 °C/min to 150°, 30°C/min to 280°C, hold 2 minutes. Each run required 13 to 17 min.
Table 1: Conditions of flash chromatography columns

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<th>3</th>
<th>4</th>
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</table>
2.7.6 High Performance Liquid Chromatography (HPLC) Analysis

An Agilent Liquid Chromatography Series 1100 (HPLC) equipped with a charged aerosol detector (CAD) and a UV detector (UVD) was used to determine the purity of each fraction collected from Silica gel flash columns. All samples were filtered and dissolved in 95% ethanol to a concentration of 1 mg/ml prior to each run. An RP-C18 Agilent column was used (4.6 x 150 mm, 5 μm) with a guard cartridge (7.5 x 4.6 mm). The mobile phase consisted of nanopure water (solvent A) and acetonitrile (solvent B) run with an oven temperature of 30°C. The injection volume was 4 μl. The flow rate was 1 ml/min under ambient temperature and maximum pressure of 250 bar. Each run required 26 minutes. Detection was by UV at 210 nm and by CAD. The sample was run following the program listed below, with linear solvent gradients between each event time. The solvent gradient was water: acetonitrile, 90:10) for the first 5 min, then a linear gradient, 90:10 to 0:100, over next 5 min, then 0:100 for the next 10 min, and, finally, a gradient back to 90:10 over the next 6 min (Table 2).

Table 2: HPLC gradient table

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Eluent B(ACN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>26</td>
<td>10</td>
</tr>
</tbody>
</table>
Chapter 3
RESULTS AND DISCUSSION

3.1 Safety and Efficacy

3.1.1 Determination of EC$_{50}$ of Ethanol Extract towards Cancerous Cells and Normal Cells

In theory, the EC$_{50}$ value, or the half maximal concentration, is the concentration of a drug, antibody or toxicant that affects half of the individuals in the sample. If the EC$_{50}$ value of a cytotoxic compound or extract is the same or less for normal cells as for cancer cells, then there will be an effect on normal cells in the body, and the cytotoxic compound cannot be used as a cure for cancer. Therefore, the EC$_{50}$ value of a cytotoxic compound or extract on normal cells should be at least 100 times higher than for cancer cells to safely use it to treat cancers.

To assess the relative cytotoxicity of an ethanolic extract of *A. douglasiana* leaves towards breast cancer cells and normal cells, EC$_{50}$ values were established using the protocols described in Chapter 2. This assay measures the number of live, growing cells in a sample. Therefore, it cannot distinguish between cytotoxicity and growth inhibition (although growth would have to be severely inhibited to give a reading of 0% live cells). In keeping with the literature in this field, all cytotoxicity assay graphs are presented with number of live cells as percent of control.

The EC$_{50}$ values of the ethanol extract were found to be 39 ± 4 μg/ml for the breast cancer cell line MDA-MB- 231 and 21 ± 3 μg/ml for PBMC (Figure 9). These results
suggest that organic extracts of dried *A. douglasiana* leaves are more likely to affect normal cells than cancer cells in the human body. Cytotoxicity towards cultured normal cells may be due either to compounds that are only cytotoxic to normal cells or to compounds that are cytotoxic to normal cells as well as cancer cells. Hence, in order to determine if any of the cytotoxic constituents could be used to treat breast cancer, constituents that are cytotoxic towards normal cells as well as those that are cytotoxic towards breast cancer cells must be isolated and identified.

### 3.1.2 Determination of EC$_{50}$ of Aqueous Extract towards Normal Cells

*A. douglasiana* is still used as a tea by California tribes to relieve premenstrual pains (2) and for colds and allergies (Vince La Peña, personal communication). To assess the cytotoxicity of an aqueous extract of *A. douglasiana* leaves towards normal cells, the EC$_{50}$ was established on PBMC using the protocol described in Chapter 2. The EC$_{50}$ of the aqueous extract on normal cells was found to be 75 µg/ml (Figure 10), compared to >200 µg/ml on cancerous cells, as had previously been determined in this laboratory. Although the EC$_{50}$ of the aqueous extract on normal cells is higher than that of the ethanol extract, 79 ± 21 µg/ml is still a low value. These results strongly support the traditional wisdom that mugwort tea should only be taken under the supervision of a trained healer.
Figure 9: EC$_{50}$ determination for ethanol extract of *A. douglasiana* leaves. Cytotoxicity assay on MDA-MB-231 breast cancer cells and normal human peripheral blood mononuclear cells. Error bars denote one standard deviation from the average of ten samples.

Figure 10: EC$_{50}$ determination for aqueous extracts of *A. douglasiana* on normal human cells. Error bars denote one standard deviation from the average of ten samples.
3.2 Reproducibility of Previously-Developed Isolation Scheme

3.2.1 Initial Extraction

A previous study on *A. douglasiana* used ethanol and hexane as its initial extractants (12). Both hexane and ethanol extracted cytotoxic compounds. Although both hexane and ethanol extracts of air-dried leaves showed strong cytotoxic activity, ethanol was chosen as the solvent for the initial extraction based on three main factors: First, the initial extraction needed approximately 5 L of solvent, and, as ethanol is much less expensive than hexane, it is more cost-effective for large-scale extractions. Second, hexane is highly volatile and difficult to use and store in sample preparations. Third, mammalian cells can survive in medium with small amounts of ethanol (up to 1%), whereas hexane is toxic to cells even at less than 1% in the medium. Therefore, for this study the initial extraction was done using ethanol. Twenty milliliters of ethanol extract was rotary evaporated to dryness and partitioned with 10 ml of *n*-hexane three times, which were combined to make the “hexane extract.”

3.2.2 Comparison of Cytotoxicity of Ethanol and Hexane Extracts

Both the ethanolic and hexane extracts were evaporated to dryness and reconstituted in 95% ethanol at a concentration of 40 mg/ml in order to test on cells. After concentration and filter sterilization, both extracts were diluted to 400 μg/ml in growth media and tested on the ER⁺ human breast cancer cell line MDA-MB-231 (Figure 11). Both extracts showed strong cytotoxicity on cancer cells, which confirmed the cytotoxic effect of *A. douglasiana* leaves that was demonstrated by previous studies.
in this laboratory. The cytotoxicity of the two extracts were not statistically different from each other (p = 0.5306), but both the extracts were significantly more cytotoxic than the control (p = 2.71x10^{-10} and p = 4.14x10^{-11} for hexane and ethanol, respectively). Although both extracts showed similar cytotoxicity, ethanol extract was used for further separations.

Figure 11: Cytotoxicity assay of 95% ethanol and n-hexane extracts of *A. douglasiana* leaves (400 μg/ml). Error bars denote one standard deviation from the average of ten samples. * indicates samples are significantly different from the control (p<0.001).
3.2.3 Analysis of the Ethanol and Hexane Extracts by GC-MS.

To compare the results of the previous study with those from this study, GC-MS analysis was performed on the ethanol extracts (Figures 12A and Figure 13A) and hexane extracts (Figure 14A and 15A) from both the previous study and the current study. Prior to GC-MS analysis, both the ethanol and hexane extracts were rotary evaporated and reconstituted in dichloromethane. Since the study by Gary Lai used a different GC-MS instrument than was used in the current study, ethanol and hexane extracts from both studies were analyzed using a newly acquired GC-MS instrument, which was then used throughout this project (Figure 12A, 13A, 14A, and 15A). Although the same solvents (ethanol and hexane) were used in both studies, the composition of the extracts were different based on the total ion chromatograms. The compound identified as a candidate cytotoxic constituent in the previous study eluted at 10.5 min in GC-MS analysis (using the original instrument). A peak at 10.5 min or any peak related to the mass spectrum of candidate compound (Figure 16) did not appear in any of the chromatograms of extracts from the previous study (Figures 12A and 14A).

As stated above, even though the same extraction solvents were used in the previous study and the current study, the GC-MS results were different, i.e., the chromatograms shown in Figures 12A and 14A do not match those shown in Figures 13A and 15A. For instance, the chromatogram of the ethanol extract from the previous study had its largest peak at 10.99 min (Figure 12A); while in the current study, the largest peak is at 5.18 min (Figure 13A). In addition, in the current study, both the ethanol and hexane extracts contain more compounds (Figure 13A and 15A) than the
corresponding extracts from the previous study (Figure 12A and 14A, respectively). These differences may be due to three reasons: first, use of different models of GC-MS in the previous and current study; second, degradation of the cytotoxic constituent identified in the previous study during storage; and, third, seasonal changes in the chemical constituents of the plant. Each of these possibilities is addressed below.

To determine if the differences between chromatograms of the same extract were because a new GC-MS instrument was being used, the hexane extract from the previous study was re-analyzed on the old GC-MS instrument. As can be seen by comparing Figures 14A and 14B, the chromatograms differ depending on which instrument was used, even though the same conditions were used on both instruments. Additionally, a peak at 10.5 min appeared on the chromatogram from the old GC-MS instrument (Figure 14B). However, the mass spectrum of this peak did not match the mass spectrum of the cytotoxic constituent isolated in the previous study (Figures 14E and Figure 16). These results indicate that the compound eluting at 10.5 min in the previous study degraded in the extracts from previous research, and the degradation product eluted at a different time.

For the previous study, plant leaves were collected in April 2006, while, for the current study, the leaves were collected in June 2009. Environmental changes throughout the year may change the chemical composition of the plant. This is supported by studies on *A. douglasiana* collected from different places in the world, which showed variation of the chemical compositions according to season and location (41, 42, 43).
Figure 12: GC-MS analysis of ethanol extract from the previous study analyzed by the new GC-MS instruments.

A) Total ion chromatogram

B) Mass spectrum of peak eluting at 7.70 min
C) Mass spectrum of peak eluting at 10.99 min.
Figure 13: GC-MS analysis of ethanol extract from the current study using new model of GC-MS.

A) Total ion chromatogram

B) Mass spectrum of peak eluting at 5.18 min.
Figure 14: GC-MS analysis of hexane extract from the previous study analyzed by the new (A, C, D) and old (B, E, F) GC-MS instruments

A) Total ion chromatogram of hexane extract from previous study using new model of GC-MS.

B) Total ion chromatogram of hexane extract from previous study using old model of GC-MS.
C) Mass spectrum of peak eluting at 5.18 min.

D) Mass spectrum of peak eluting at 10.99 min.

E) Mass spectrum of peak eluting at 10.5 min.

F) Mass spectrum of peak eluting at 13.4 min.
Figure 15: GC-MS analysis of the hexane extract from current study analyzed by the new GC-MS instruments.

A) Total ion chromatogram.

B) Mass spectrum of peak eluting at 10.90 min.
3.2.4 Removal of Chlorophyll

Plant leaves contain chlorophyll, which might affect the purification process. At the very least, since chlorophyll is so abundant, its presence may drastically increase the EC$_{50}$ of the extract simply because it contributes so greatly to the mass of the sample that more sample is needed to effect a response. Additionally, chlorophyll may modulate the effect of cytotoxic compounds; it has been shown to affect the cytotoxic and hyperproliferative effects of heme from red meat on rat colon, although this was an in vivo study (44). Therefore, lead tetraacetate was used to remove chlorophyll from the extract, as described in the literature (3). The filtered extract was partitioned three times with 10 ml of chloroform, and all three chloroform fractions were tested separately.
using a cytotoxicity assay (Figure 17) as well as GC-MS (Figures 18-20), as described under Methods and Materials.

Cytotoxic activity was found only in the first two chloroform fractions (Figure 17). The first and second chloroform fractions showed strong cytotoxicity ($p = 1.52 \times 10^{-4}$ and $p = 8.73 \times 10^{-4}$ compared to the control). The cytotoxicity of chloroform fraction 2 was significantly less than that of chloroform fraction one ($p = 1.48 \times 10^{-3}$). Chloroform fraction 1 is significantly different from the blank ($p = 0.00317$), whereas chloroform fraction 2 is not ($p = 0.0758$). Chloroform fraction 3 did not show cytotoxicity. (Even though the $p$ value is less than 0.05 compared to the control ($p = 2.98 \times 10^{-3}$), Figure 17 shows that the number of cells in the samples treated with chloroform fraction 3 are higher than the samples treated with the control.) The GC-MS chromatograms of the first and second fractions (Figure 18) contain a unique signal at 7.70 min. Even though the area of the peak at 7.70 min is much smaller for the second chloroform fraction (Figure 19), it still shows cytotoxicity (Table 3). Chloroform fraction 3 is not cytotoxic, and it does not have the signal at 7.70 min (Figure 20). These results indicate that the compound eluting at 7.70 min may correlate with cytotoxicity. Therefore, chloroform fraction 1 was used in the first flash column for further isolation.
Figure 17: Cytotoxicity assay of chloroform layers (400 μg/ml) from LLE of the ethanol extract previously treated with 4 % lead tetraacetate. Error bars denote one standard deviation from the average of ten samples. * indicates samples are significantly different from each other and from the control (p < 0.05). ** indicates samples are significantly different from the blank, the control, and the other samples (p < 0.05).

Figure 18: Total ion chromatogram of the first chloroform wash from LLE.
Figure 19: Total ion chromatogram of the second chloroform wash from LLE.

Figure 20: Total ion chromatogram of the third chloroform wash from LLE.
Table 3: Analysis of chloroform washes. Percentage of total peak area for the compound eluting at 7.7 min compared to the percentage of cells killed by each chloroform fraction collected from LLE of the ethanol extract treated with 4% lead tetraacetate.

<table>
<thead>
<tr>
<th>Chloroform Wash Number</th>
<th>Total Percentage of Peak Area</th>
<th>Percentage of Cells Killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.862</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>2</td>
<td>0.081</td>
<td>91.7±11</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>&lt; 0</td>
</tr>
</tbody>
</table>

3.2.5 Replicate Last Column from Previous Study

The original goal was to isolate the compound that eluted at 10.5 minutes in GC-MS in the previous study and to check whether the isolation scheme introduced in the previous study is reproducible with a new sample of leaves. Thus, flash column chromatography of the ethanolic extract was performed using silica gel columns with different volumes of solvents and solvent mixtures and fractions from the first few columns were analyzed by GC-MS without performing cytotoxicity assays.

As the first chloroform fraction from LLE of the lead tetraacetate-treated extract showed strong cytotoxicity, it was used for further purification by silica gel column chromatography. As in the previous study, the first column used ethyl acetate and ethanol as eluents (Table 1, Column 1). After analyzing all the eluted fractions by GC-MS, the retention times and the mass spectra (MS) of the resultant peaks were
compared to those obtained in the previous study. Neither the compound eluting at 10.5 min nor any compound showing a similar mass spectrum as the peak that was isolated in the previous study appeared in the chromatograms of any of the fractions obtained from column 1 (Appendix A). (Note: Although all the GC-MS runs were 20 min long, since no signals appeared after 13 min, all the GC chromatograms show only up to 13 min.) As stated before in this chapter, the 10.5 min signal did not appear in either the ethanol or hexane extracts. Therefore, it is not surprising that the peak eluting at 10.5 min is not present in any of the fractions. But all the signals were analyzed to find any peak that might have a mass spectrum similar to that of the previously isolated cytotoxic constituent. No fraction showed any mass spectrum that was similar to the previously isolated cytotoxic constituent. Thus another column was performed using the ethanol extract (Table 1, Column 2).

3.3 To Evaluate and Improve Upon the Previously Developed Isolation Scheme

3.3.1 Separation of Ethanol Extract Constituents by Flash Column Chromatography

As mentioned above, to remove chlorophyll, the ethanol extract was treated with lead tetraacetate followed by LLE with chloroform. Since lead tetraacetate is an oxidizing agent, it may cause other effects besides just the removal of chlorophyll, such as oxidation of cytotoxic compounds. Therefore, it was deemed prudent to discontinue the use of lead tetraacetate and, instead, to use the ethanol extract directly for further purification. Hence, Column 1 was repeated using 1 ml of ethanol extract instead of

Figure 21: Total ion GC-MS chromatograms of fractions collected from Column 2. One milliliter of ethanol extract was loaded onto a 220 x 16 mm silica gel column and eluted with ethyl acetate and then ethanol.

A) Fraction 1

B) Fraction 2
chloroform fraction 1. (This is referred to as “Column 2”.) Although the same solvent system was used as in Column 1, only three fractions were collected. Each fraction was rotary evaporated and concentrated in dichloromethane in order to analyze by GC-MS (Figure 21). GC-MS analysis (Figures 21A, B and C) showed that most of the compounds eluted in the first fraction. Again, although the previous study suggested that a compound eluting at 10.5 min in GC-MS is a cytotoxic compound, this peak or a mass spectrum related to this peak did not show up in any of the fractions in Column 2. Since a cytotoxicity assay was not performed on any of the fractions collected from Column 2, it could not be determined from Column 2 fractions whether any other peak correlated with cytotoxicity. Therefore, cytotoxicity assays were performed along with GC-MS analysis for all fractions collected from future columns.
3.3.2 Solid-Liquid Extraction with Diethyl Ether

Results obtained up to this point showed that cytotoxic constituent isolated in the previous study may not be present in the new sample of *A. douglasina* leaves. Therefore, this project changed its focus to isolating cytotoxic constituent/s by comparing chromatograms with cytotoxicity assays rather than comparing chromatograms to GC-MS data from the previous study. However, based on the results of the first two columns, if this constituent was present (in small quantities), it might have remained on the columns and, therefore, might be less polar than ethyl acetate. Diethyl ether has a polarity between hexane and ethanol. Therefore, a new extract was made by solid-liquid extraction of the dried ethanol extract (5 ml) with 5 ml diethyl ether and analyzed by GC-MS (Figure 22). It is very clear that later eluting compounds are more abundant in the ether extract (Figure 22) than the ethanol extract (Figure 12A). For instance, a compound that eluted at 5.18 min is highly abundant in the ethanol extract, whereas compounds eluting at 7.70 min and 10.99 min were highly abundant in the ether extract.

A cytotoxicity assay of the ethanol and ether extracts and of the residue left after SLE (Figure 23) shows that the ether extract is more cytotoxic towards MDA-MB-231 than the ethanol extract (*p* = 1.51 x 10^{-3} compared to each other). Therefore, the ether extract was used for further isolation. Statistical analysis showed that the cytotoxicity assay results (absorbance) of both the ether and ethanol extracts were significantly different from the absorbance of the blank (*p* = 6.28 x 10^{-6} and *p* = 2.55 x 10^{-3}, respectively).
Figure 22: Total ion GC-MS chromatogram of diethyl ether extract made from SLE (Solid-Liquid Extraction).

The residue from the ethanol extract was partially soluble in diethyl ether. Compounds that dissolved in diethyl ether are less polar than compounds remaining in the residue. A cytotoxicity assay was performed with the diethyl ether extract, the ethanol extract, and the residue after SLE. As shown in Figure 23, the ether extract killed around 98% percent of the cells, whereas ethanol killed 95% of the cells. This difference, which was statistically significant ($p = 4.90 \times 10^{-8}$) indicates that the ether extract is more concentrated with cytotoxic constituent/s than the ethanol extract is. The residue (after extraction with diethyl ether) showed weak cytotoxicity. Therefore, these results showed that the cytotoxic constituent/s is/are intermediate in polarity.
between ethanol and diethyl ether. The total ion chromatogram of the ether extract (Figure 22) is similar to the total ion chromatogram of the ethanol extract (Figure 13A), but not exactly same. There are some compounds that are more abundant in the diethyl ether extract than in ethanol extract (for instance, the compound eluting at 7.71 min).

Figure 23: Cytotoxicity assay of the ethanol-soluble and ether-soluble fractions (assay concentration of 40 μg/ml) of *A. douglasiana* leaves. All samples are presented with error bars that denote one standard deviation from the average of ten samples. * indicates samples are significantly different from each other and from the control (p < 0.05).
3.3.3 Separation of Ether Extract Constituents by Flash Column Chromatography

Two milliliters of ether extract were chromatographed on silica gel using 50 ml of diethyl ether as the eluent. Eluted fractions were collected and analyzed by GC-MS. No compounds were detected in fractions 1-3, and fraction 7 was accidentally spilled while preparing samples. Therefore, only fractions 4, 5, 6, and 8 were assayed for cytotoxicity (Figure 26). Fraction 6, which had the compound eluting at 7.71 min as its most abundant compound (Figure 24C), was highly cytotoxic, whereas fraction number 5, which had the 10.99 min peak as its most abundant peak, but still a significant peak at 7.71 min, showed less cytotoxicity (Figure 24B). To determine if the abundance of this compound correlates with cytotoxicity, the area of the peak at 7.71 min in each fraction was compared to the number of cells killed (rather than the number of live cells, as is shown in other cytotoxicity profiles) as shown in Figure 27. Neither fraction 4 nor fraction 8 showed cytotoxicity. Fraction 4 has a signal at 7.656 min (Figure 25A), but it has a different mass spectrum than the signal at 7.71 min in fraction 5 and 6 (Figure 25B and 25C). Therefore, it is clear that the compound eluting at 7.71 min is absent in fraction 4. From these results in can be concluded that the compound eluting at 7.71 min in GC (in fractions 5 and 6 from Column 3) correlates with cytotoxicity and may be cytotoxic. At the very least, this compound co-elutes from flash column chromatography with a cytotoxic compound.
Figure 24: GC-MS chromatograms of fractions collected from Column 3. Two milliliters of diethyl ether extract were loaded onto a 250 x 16 mm silica gel column and eluted with 50 ml of ether. Arrows point to the peak at 7.70/7.71 min.
Abundance

TIC: ETHERCOLFRC6.D\data.ms

D) Fraction 6

TIC: ETHERCOLFRC8.D\data.ms

E) Fraction 8
Figure 25: Mass spectra of peaks eluting at 7.66 min and 7.70/7.71 min from Column 3 fractions. Two milliliters of diethyl ether extract were loaded onto a 220 x 16 mm silica gel column and eluted with 50 ml of ether.

A) Mass spectrum of peak eluting at 7.65 min from fraction 4.

B) Mass spectrum of peak eluting at 7.70 min from fraction 5.
C) Mass spectrum of peak eluting at 7.71 min from fraction 6.

Figure 26: Cytotoxicity of fractions collected from ether Column 3 (400 μg/ml). Two milliliters of ether extract were loaded onto a 250 x16 mm silica gel column and eluted with 50 ml of ether. All points are presented with error bars that denote one standard deviation from the average of ten samples.
3.3.4 Scale-up of Flash Column Chromatography

Only fraction 6 from Column 3 showed strong cytotoxicity, indicating that these column conditions were suitable for separation of cytotoxic constituents. Therefore, another column (Column 4, Table 1) was performed, collecting smaller fractions (6.5 ml). The same size column as for column 3 was used and eluted with 100 ml of diethyl ether; 15 fractions were collected. All the fractions were rotary evaporated and reconstituted to 40 mg/ml in 95% ethanol to assay for cytotoxicity (final concentration in media of 400 μg/ml). As shown in Figure 28, fractions 6, 7, 8, and 9 and fractions 12, 13, 14, and 15 were cytotoxic. This result indicates that one (or more) relatively nonpolar cytotoxic constituent/s eluted early from the column, whereas another more...
polar cytotoxic constituent/s eluted later from the column. This result leads to the conclusion that *A. douglasiana* leaves contain more than one cytotoxic constituent. After performing a cytotoxicity assay on fractions from this column, there was not enough of each sample to perform GC-MS analysis.

Figure 28: Cytotoxicity assay of fractions (5 ml) collected from diethyl ether (100ml) Column 4. The concentration of each fraction in the assay was 400 µg/ml. All points are presented with error bars that denote one standard deviation from the average of five samples.

3.3.5 Determination of Appropriate Analytical Technique

Although a compound eluting at 7.70 min in GC-MS correlated with cytotoxicity, another compound may have co-eluted with this compound from flash chromatography but cannot be detected by GC-MS. The previous study did not determine the volatility of the cytotoxic constituent/s. Therefore, before further analyzing fractions with GC-
MS, it should be determined whether GC-MS is a suitable detection method; it must be determined whether or not the cytotoxic compounds are volatile. If they are volatile, purity analysis can be performed with GC-MS, but if they are not volatile, the analytical technique should be switched to HPLC.

To determine the volatility of the cytotoxic constituent(s), vacuum distillation was performed using 1.0 g of fresh *A. douglasiana* leaves in distilled water, and the distillate was collected in ethanol and assayed for cytotoxicity without drying the sample (assay concentration of 31 μg/ml; Figure 29). The results showed that the distillate has a slight but significant cytotoxic effect on cells, indicating that at least some of the cytotoxic compounds are volatile.

Another cytotoxicity assay was performed after drying the distillate and reconstituting it to 5 mg/ml (assay concentration of 50 μg/ml; Figure 29). Distillate that was dried and reconstituted to 5.0 mg/ml was more cytotoxic than the distillate that was not dried. This indicates that the cytotoxic compound(s) is/are not extremely volatile, since at least some of it was not removed by drying. According to the results of the cytotoxicity assay shown in Figure 29, some or all of the cytotoxic constituents are semi-volatile, since the distillate from fresh leaves showed weak cytotoxicity, but the cytotoxicity persisted after ethanol was evaporated from the sample. Since the residue in the boiling flask was not tested on cancer cells, it cannot be concluded that distillate has nonvolatile compounds.

It is easy to distill and collect volatile compounds from the ethanol extract, which already has compounds extracted from leaf tissues. Therefore, another vacuum
distillation was performed using 1.0 g of ethanol extract, rather than starting with fresh or dried leaves. The distillate was collected into a pre-weighed empty round bottom flask. After distillation, the volume of the collected distillate and the residue in the boiling flask were measured (3.3 ml and 22 ml, respectively), and both were extracted with ether three times using a volume equal to that of the sample. The distillate and residue extracts were evaporated to dryness using a rotary evaporator and reconstituted in 95% ethanol to 5.0 mg/ml. A cytotoxicity assay was performed on both extracts at 50 μg/ml (Figure 30). The distillate showed no activity on the cancer cells, whereas the residue showed strong cytotoxicity.

The results obtained from the cytotoxicity assay for both distillations shown in figure 29 and 30 gave conflicts results since distillate from fresh leaves showed weak cytotoxicity whereas distillate from ethanol extract showed no cytotoxicity. However, it may be that the residue from distillation of fresh leaves is more cytotoxic than distillate. Although the residue from first distillation was not tested on cells, the cytotoxicity assay for distillates and residues of the second vacuum distillations indicate that cytotoxic constituent/s is/are not very volatile, so GC-MS is not the appropriate analytical technique. Therefore, based on vacuum distillation results all further analysis was performed using HPLC.
Figure 29: Cytotoxicity assay of the distillate collected from vacuum distillation of fresh *A. douglasiana* leaves. Distillate collected in ethanol from vacuum distillation of 1.0 g of fresh *A. douglasiana* leaves in water. (Distillate without drying and distillate reconstituted up to 5 mg/ml). All points are presented with error bars that denote one standard deviation from the average of ten samples.

Figure 30: Cytotoxicity assay of the distillate collected from vacuum distillation of ethanol extract of dried *A. douglasiana* leaves. Distillate and residue from vacuum distillation of 1.0 g of ethanol extract of *A. douglasiana* dried leaves in water. All points are presented with error bars that denote one standard deviation from the average of ten samples.
3.3.6 HPLC Analysis of Extracts

Once vacuum distillation results confirmed that cytotoxic constituents are not volatile, reverse-phase HPLC with charged aerosol detection (CAD) was used for further purity analysis. Both the original ethanol and ether extracts were concentrated to 1 mg/ml and analyzed by HPLC-CAD (Figures 33 and 34, respectively). When comparing the HPLC chromatograms of the ethanol extract and the ether extract (Figure 33 and 34), it is seen that a compound eluting at 1.5 min is highly abundant in the ethanol extract, whereas it is apparently nonexistent in the ether extract. Since reverse phase HPLC (a C18 column) was used in this study, compounds eluting first are more polar than compounds eluting later from the column. Therefore, the compound eluting at 1.5 min is highly polar, as it eluted early from the HPLC column and it is more prevalent in the ethanol extract (Figure 33). It can be concluded that this compound was not extracted by ether and, because the ether extract is more cytotoxic than the ethanol extract, the compound eluting at 1.5 min is likely not cytotoxic (Figure 34). In should be noted that another compound elutes at 0.90 min, establishing that the peak at 1.5 min does not represent compounds eluting at the void volume of the instrument.

A compound eluting at 10.4 min is highly abundant in the ether extract. Also, the chromatograms of both the ethanol and ether extracts showed most of the compounds eluting between 10 and 20 min, but these compounds are in higher abundance in the ether extract than in the ethanol extract (Figure 33). Further purification and analysis were done using the ether extract, since cytotoxic constituents should be one or more of the compounds represented in the HPLC chromatogram of the ether extract.
3.3.7 HPLC and Cytotoxicity Analysis of Flash Column Chromatography Fractions

3.3.7.1 Replication of Column 4

Column 4 was scaled up by (1) running it four times and (2) increasing the diameter of the column. (Table 1, Column 4A to 4D). Two milliliters of ether extract (6 mg/ml) were loaded onto each column. All twenty-two eluate fractions (5 ml) from each
column were analyzed by HPLC and tested for cytotoxicity on MDA-MB-231 at a concentration of 50 µg/ml (Figure 33).

As shown in Figure 33, for Column 4A, fractions 17, 19, 20 and 21 showed strong cytotoxicity; for Column 4B, fractions 12 to 22 showed strong activity, except for fraction 21; for Column 4C, fractions 10 to 22 showed strong cytotoxicity, except for fraction 19; and for Column 4D, fractions 11 to 22 showed strong activity on cancer cells, except for fraction 21.

Even though the same conditions were used in all four of these columns, the results of the cytotoxicity assay of fractions from Column 4A are different than those of the other three columns (Columns 4B, 4C, and 4D). The differences between Column 4A and the other 3 columns might be due to differences in packing the columns, because Column 4A was the first column packed out of the four columns. Although Column 4A is a lot different from other three columns, columns 4B to 4D exhibited good reproducibility. The assay results of columns 4B through 4D were very similar, as all showed similar cytotoxicity patterns in fractions 12 through 22. Column 4C showed weak cytotoxicity at fraction 19, whereas in Column 4B and 4D showed weak cytotoxicity at faction 21 (Figure 33B, C, D). This may be because collection of fractions in Column 4C started one or two fractions earlier than Column 4B and 4D. Nonetheless, from the result of the cytotoxicity assays from Column 4A to the other three columns, it can be concluded that there is more than one cytotoxic constituent and that cytotoxic constituents may still remain in the column.
Figure 33: Cytotoxicity assays of fractions collected from diethyl ether Column 4A, 4B, 4C and 4D. Fractions were dried and reconstituted in 95% ethanol to 5 mg/ml and then tested on cells at 50 μg/ml in media. All points are presented with error bars that denote one standard deviation from the average of five samples.

A) Column 4A

B) Column 4B
C) Column 4C

D) Column 4D
All the fractions from diethyl ether columns 4A through 4D were made up to a concentration of 1 mg/ml and were analysed by HPLC, as described in Chapter 2. These chromatograms are shown in Appendices B-E. The chromatograms were compared to the cytotoxicity assays for those particular column fractions. There were no discernable peaks that correlated with cytotoxicity. For all fractions collected from Column 4A through 4D, only HPLC data, not GC-MS data, were analyzed and compared to cytotoxicity data.

All the HPLC-CAD chromatograms of fractions obtained from Column 4A through 4D were analyzed using Agilent Chemstation software. Even though the cytotoxicity results from Columns 4A through 4D are not identical, they can still be compared to the HPLC data. For instance, Figures 34 and 35 shows the chromatograms and analysis of fractions 18 through 22 from Column 4A, comparing a constituent eluting at 14.67 min to cytotoxicity. At first glance, it appears that there is no correlation between the area of this peak and cytotoxicity. Figure 35 shows that, although the 14.67 min peak is small and has about the same area in both fraction 19 and fraction 22, fraction 19 was cytotoxic, but fraction 22 was not. But this does not mean that the compound eluting at 14.67 min is not responsible for the cytotoxicity of fractions 20 and 21, which have much larger amounts of this compound. Instead, it is possible that this compound is cytotoxic, but that another cytotoxic compound is also present in fraction 19. For instance, the compound eluting at 22.58 min is only present in fraction 19 (Figure 34B). This suggests that the compound eluting at 22.58 min might be responsible for the cytotoxicity of fraction 19, while the compound eluting at 14.67 min might be
responsible for the cytotoxicity of fractions 20 and 21. All fractions from Columns 4A-4D were analyzed in this manner, comparing all peak areas with cytotoxicity. From this analysis, it can be concluded that multiple compounds appear to be responsible for cytotoxicity (Table 4).

Figure 34: HPLC-CAD chromatograms of fractions 10 to 22 collected from Column 4A. Two milliliters of diethyl ether extract were loaded onto a 240 x 16 mm silica gel column and eluted with 100 ml of ether. (Integration lines shown were only used to determine the retention times.)
C) Fraction 20

D) Fraction 21

E) Fraction 22
3.3.7.2.1 Elution of Nonpolar and Polar Cytotoxic Constituents from Flash Chromatography Column

Most of the dried fractions from the diethyl ether columns did not dissolve completely in ethanol because compounds eluted with diethyl ether are relatively nonpolar. When considering cytotoxicity assays from column 4B - 4D, strong cytotoxicity was seen in fraction 22, the last fraction collected (Figure 33 B - D). This indicates that there might be polar cytotoxic compounds that never eluted from the column. Therefore, another column (Table 1, Column 5) was performed using a step gradient of 80 ml of diethyl ether, 40 ml ether:ethanol (1:1), and 80 ml of ethanol, in order elute both nonpolar and polar compounds. This column was run in duplicate to
check for reproducibility (Columns 5A and 5B). Thirty-eight and thirty-nine fractions were collected from Columns 5A and 5B, respectively.

While running the columns, two bands appeared as green and yellow-brown in color. Fractions 1 to 24 were dried using nitrogen gas, and fractions 25 to 39 were dried using a rotary evaporator. As fractions 1 to 24 took much less time to dry compared to the rest of the fractions, it was clear that ethanol eluted from the column starting at fraction number 24/25. This was also confirmed by the yellow-brown color of fraction 24/25.

All the fractions were reconstituted to 4 mg/ml in 95% ethanol, and a cytotoxicity assay was performed at 40 μg/ml (Figure 36A and 36B). Fractions 13 to 29 from Column 5A showed cytotoxicity against breast cancer cells (Figure 36A). Fractions 12 to 28 from column 5B showed strong cytotoxicity, except for fraction 23 (Figure 36B). After the cytotoxicity assay, fractions were diluted to 1 mg/ml in ethanol and analyzed by HPLC.

Although Column 5A and Column 5B were supposed to be identical to each other, there are a few very notable differences in the cytotoxicity assays: First, it can be seen that the fraction in which cytotoxic constituent/s first eluted is different by one fraction in the two columns. This slight shift of cytotoxicity (Figure 36) in the fractions from Columns 5A and 5B could cause by a millimeter or two difference between the heights of the two columns. Alternatively, this may be due to starting fraction collection at slightly different times. This is borne out by the slight cytotoxicity of fraction 29 from Column 5A, whereas, in Column 5B, fraction 29 is not cytotoxic.
Another very obvious difference in the cytotoxicity assay of the two columns is that fraction 23 of Column 5B showed no cytotoxicity whereas fraction 23 of Column 5A is cytotoxic. Again, this may also be due to a millimeter or two difference in the height of the columns, allowing separation of two cytotoxic compounds in Column 5B. Or it may be due to starting fraction collection at slightly different times, resulting in two cytotoxic compounds eluting overlapping 22 and 23 fractions in Column 5A, whereas they did not overlap in Column 5B, fraction 22, 23 or 23, 24. Although the cytotoxicity assays look different, the lack of activity in fraction 23 of Column 5B is more informative and also suggests that there is more than one cytotoxic constituent (Figure 36).

When comparing the results of the cytotoxicity assays of fractions from Column 5A and 5B to the HPLC chromatograms of each fraction, it was found that more than one compound is responsible for cytotoxicity. Comparing HPLC chromatograms of cytotoxic fractions to those of noncytotoxic fractions gives information about compounds that can be correlated with cytotoxicity. Analysis was done by overlapping chromatograms of each fraction obtained from Columns 5A and 5B with nearby fractions. A couple of examples of the type of comparison performed are discussed below, and one example is shown in Figure 41. The results of this analysis are shown in Table 4.
Figure 36: Cytotoxicity assay for fractions collected from ether/ethanol Columns 5A and 5B. Three and half milliliters of ether extract were loaded on to 240 x 18 mm silica gel column. All points are presented with error bars that denote one standard deviation from the average of five samples.

A) Column 5A

B) Column 5B

A number of compounds (e.g., those eluting at 1.8, 9.9, 10.2, 10.9, 14.1, 15.7, 18.1, 23.1, 24.7 min) are found in the ether blank (collected from the flash column; Figure 37) as well as in all the inactive and active fractions from both columns. Therefore,
compounds eluting at those retention times do not exist in the original ether extract.

Fractions 1 to 12 from Column 5A and fractions 1 to 11 from Column 5B showed no cytotoxicity (Figure 38A and 38B respectively). Most of the signals appearing in the HPLC chromatogram of the initial inactive fractions in Column 5A and Column 5B also appeared in the blank sample (Appendix F and Appendix G respectively).

Figure 37: HPLC-CAD chromatogram of ether blank, obtained from Column 5A. Blank was obtained before loading sample onto the 240 x18 mm silica gel column. (Integration lines shown are only used to determine the retention times.)

Fractions 13 to 30 from Column 5A showed strong cytotoxicity (Figure 36A). In Column 5B, fractions 12 to 29 showed strong cytotoxicity, except for fraction 23 (Figure 36B). Chromatograms of fractions 13, 14 and 15 from Column 5A and fractions 12, 13, and 14 from Column 5B have prominent peaks at 13.04, 13.34, and 13.73 min (Figure 38A-D and Figure 41A-D, respectively). The amount of the compound eluting at 13.83 min increases from fractions 13 to 15 in Column 5A and from fractions 12 to 14 of
Column 5B, whereas amount of the compound eluting at 13.11 min and 13.42 min decreases with the fraction number (Figure 42). However, the compounds eluting at 13.11 min and 13.42 min cannot be ruled out as being candidate cytotoxic compounds, because another cytotoxic compound might elute in the same fractions, while these compounds decrease in abundance. This analysis indicates that any or all of the compounds eluting at 13.11, 13.42 and 13.83 min might be responsible for cytotoxicity.

But when comparing the HPLC chromatograms of fractions collected from Columns 4A through 4D and 5A and 5B, the constituent eluting at 13.83 min (13.66 and 13.73 in Column 4A and 5B, respectively) was found to be highly abundant in a noncytotoxic fraction from Column 4A (Table 4). Therefore, it can be concluded that the compound eluting at 13.83 min is not cytotoxic.

Another example is that, although fraction 15 from Column 5A showed strong cytotoxicity (Figure 36A), there is no prominent peak other than the one at 13.11 min that could represent a cytotoxic compound (Figure 38D). This data suggests that this might be a cytotoxic compound or that a cytotoxic compound might coelute with this compound and may not be detected by the Charged Aerosol Detector. The same reasoning can be applied to other cytotoxic fractions that do not show any prominent peak that could be a cytotoxic compound.
Figure 38: HPLC-CAD chromatograms of fractions collected from column 5A. Three and half milliliters of diethyl ether extract were loaded onto a 240 x 18 mm silica gel column and eluted with ether (80 ml), ether:ethanol 1:1 (40 ml) and ethanol (80 ml). (Integration lines shown are only used to determine the retention times.)
Figure 39: HPLC-CAD chromatograms of fractions collected from column 5B. Three and half milliliters of diethyl ether extract were loaded onto a 240 x18 mm silica gel column and eluted with ether (80 ml), ether:ethanol 1:1 (40 ml) and ethanol (80 ml). (Integration lines shown are only used to determine the retention times.)

A) Fraction 11

B) Fraction 12

C) Fraction 13

D) Fraction 14
HPLC-CAD analysis of column fractions did not result in the clear identification of specific compounds that correlated with cytotoxicity. However, based on the analysis summarized in Table 4, since the compound eluting at 14.66 min in HPLC-CAD is found only in active fractions from all four columns, it is a good candidate for the cytotoxic effect on cancer cells. Additionally, Table 4 shows that several compounds eluting at different retention times can be excluded as candidates, since those compounds are found in at least one non-cytotoxic fraction (for instance, the peak eluting at 1.5 min). Other compounds are inconclusive as to whether they are cytotoxic or not. Additionally, some cytotoxic fractions had no discernible signal that correlated
with cytotoxicity. This may be because that particular cytotoxic compound might not be detected by the CAD. It is possible that another type of detector, such as MS, might detect additional compounds.

Table 4: Analysis of Column 4A, 4C, 4D, 5A and 5B. Retention times of peaks that can be responsible for cytotoxic in fractions obtained from Column 4 and 5.

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CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

The aims of this study were (1) to determine the effect of mugwort leaf extracts on normal cells, (2) to test the reproducibility of the previously developed isolation scheme with a new sample of *A. douglasiana* leaves, and (3) to evaluate and improve the previously-developed isolation scheme.

The first aim was to determine whether ethanolic and aqueous extracts of *A. douglasiana* leaves affect normal human cells. From the results obtained from EC$_{50}$ determination on cancer cells and normal cells, the following conclusions can be made:

1) The ethanol extract has one or more cytotoxic constituents that kill normal cells as well as or better than cancer cells. Therefore, although this cytotoxic compound will eventually be isolated, it is unlikely that it will be useful as a cure for breast cancer. This does not eliminate the possibility that other constituents might only be toxic towards cancer cells.

2) The aqueous extract has a significant cytotoxic effect on normal cells.

The second goal was to check the reproducibility of the previous isolation scheme with a new sample of *A. douglasiana* leaves. From the experimental results, the following conclusions can be made:

1) The previously developed isolation scheme could not be reproduced with a new sample of leaves.
2) A compound eluting at 10.5 min, which correlated with cytotoxicity in the previous study, did not appear in any of the extracts or fractions in the current study. Therefore, this compound might not be responsible for cytotoxicity.

3) The cytotoxic constituent isolated from the previous study may degrade in the extract.

4) The chemical composition of the leaves may change seasonally.

Because the previously developed isolation scheme was not reproducible with a new sample of leaves, a third goal was pursued: The isolation scheme was improved. The following parts of the scheme were evaluated: solid-liquid extraction of the dried ethanolic extract (ether extraction), appropriateness of the analytical method, and solvents used as eluents in flash column chromatography. From evaluating the previously developed isolation scheme and developing a new isolation scheme, the following conclusions can be made:

1) There is more than one cytotoxic constituent in *A. douglasiana* leaves.

2) The cytotoxic constituents are intermediate in polarity between hexane and ethanol.

3) Ether is a good solvent to use for SLE from the dried ethanol extract, since it does not extract non-cytotoxic polar compounds (e.g., the compound eluting at 1.5 min in HPLC).

4) The cytotoxic constituents are not very volatile, so HPLC should be used for purity analysis.
5) FCC using a silica gel column eluted first with diethyl ether, then with ethanol, as in Column 5, resulted in fairly good, but not complete separation of chemical constituents.

5.2 Future Directions

For future studies, a number of recommendations can be made from the results obtained in this study.

1) For further purification of the cytotoxic constituents, the ethanol extract from the current study should be used.

2) Since most of the cytotoxic candidates have intermediate polarity, an ether extraction should be the first step in further separation of cytotoxic constituent/s from the dried ethanol extract.

3) Of special note is the compound eluting as a very broad peak at 20.67 min (in HPLC) in fraction 17 from Column 4A, since this compound is the only one that appeared in this active fraction and not in nearby inactive fractions. In order to investigate this region, the HPLC gradient should be modified so that the area from 19 min to 21 min in the HPLC chromatogram can be expanded.

4) The isolation method should be scaled up by running a much larger (wider) column using the same conditions as in Column 5, or by running Column 5 at least 5 times, so that more of the active fractions can be collected.

5) Pooled fractions from Column 5 should be run through another flash column using a shallower step gradient with increasing polarity.
6) Column fractions showing activity should be analyzed again by HPLC using a different detector like MS. LC-MS may be able to detect compounds that CAD did not. Additionally, MS will give more information about the chemical nature of candidate compounds, which might help when determining the next step that should be taken in the purification scheme.
APPENDIX A

GC-MS Chromatograms of Column 1

Total ion chromatograms of fractions collected from Column 1 using ethylacetate and ethanol as eluents. A half milliliter of the first chloroform wash from LLE was loaded onto a 220 x 16 mm silica gel column and eluted with ethylacetate and then ethanol.

A) Fraction 1

B) Fraction 2
Abundance

TIC: ETOACPUREFRAC3.D\data.m

Time--> Abundance

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11.108 11.192 11.212
11.222

Time--> Abundance

TIC: ETOACPUREFRAC4.D\data.m

Time--> Abundance

C) Fraction 3

D) Fraction 4
E) Fraction 5

F) Fraction 6
G) Fraction 7

H) Fraction 8
I) Fraction 9

J) Fraction 10
K) Fraction 11
APPENDIX B

HPLC Chromatograms of Column 4A

HPLC-CAD chromatograms of Column 4A. Two milliliters of diethyl ether extract were loaded onto a 220 x16 mm silica gel column and eluted with 100 ml of ether. (Integration lines shown were only used to determine the retention times.)
APPENDIX C

HPLC Chromatograms of Column 4B

HPLC-CAD chromatograms of fractions 10 to 22 collected from Column 4B. Diethyl ether extract (2 ml) was loaded onto a 240 x16 mm silica gel column and eluted with 100 ml of ether. (Integration lines were only used to determine the retention times.)
APPENDIX D

HPLC Chromatograms of Column 4C

HPLC-CAD chromatograms of fractions 10 to 22 collected from column 4C. Two milliliters of diethyl ether extract were loaded onto a 240 x 16 mm silica gel column and eluted with 100 ml of ether. (Integration lines shown were only used to determine the retention times.)

a) Fraction 10

b) Fraction 11
c) Fraction 12
d) Fraction 13

e) Fraction 14
f) Fraction 15
g) Fraction 16
h) Fraction 17
i) Fraction 19  
j) Fraction 21  
k) Fraction 20
APPENDIX E

HPLC Chromatograms of Column 4D

HPLC-CAD chromatograms of fractions 10 to 22 collected from Column 4D. Two milliliters of diethyl ether extract were loaded onto a 240 x 16 mm silica gel column and eluted with 100 ml of ether. (Integration lines shown were only used to determine the retention times.)
i) Fraction 18

j) Fraction 19

k) Fraction 20

l) Fraction 21
APPENDIX F

HPLC Chromatograms of Column 5A

HPLC-CAD chromatograms of fractions collected from column 5A. Three and half milliliters of diethyl ether extract were loaded onto a 240 x 18 mm silica gel column and eluted with 100 ml of ether. (Integration lines shown are only used to determine the retention times.)
APPENDIX G

HPLC Chromatograms of Column 5B

HPLC-CAD chromatograms of fractions collected from column 5B. Three and half milliliters of diethyl ether extract were loaded onto a 240 x18 mm silica gel column and eluted with ether and ethanol. (Integration lines shown are only used to determine the retention times.)

1) Blank ethanol

2) Fraction 1

3) Fraction 2

4) Fraction 3

5) Fraction 4

6) Fraction 5

7) Fraction 6

8) Fraction 7
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