Cellular effects of *Ferula Assafoetida* on breast cancer cells and inflammatory responses in cultured monocytes

by

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Abstract

In traditional medicine, *Ferula assafoetida (F. assafoetida),* has been used as an antiseptic, anti-diabetic, anti-inflammatory, and anti-cancer agent. In recent years its anti-cancer and anti-inflammatory activities have become a focus in drug research. We investigated the *in vitro* cytotoxicity and anti-inflammatory effects of ethanolic extracts of *F. assafoetida* and five known components (ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid) on a group of malignant and non-malignant breast cell lines and the THP-1 monocyte-like cell line. Our results showed that treatment with the ethanolic extract of *F. assafoetida,* and the components, had a significant effect on cell viability and apoptosis induction for the human MCF-7, MDA-MB-231, and murine 4T1 breast cancer cell lines compared to the non-malignant human HBL-100 breast cells. This research also showed that THP-1 peripheral blood monocytic leukemia cells, differentiated into macrophages, could be further polarized into the M1 inflammatory phenotype by treatment with extracts of *F. assafoetida* and the components. There was a significant increase in the expression of CD80, a marker associated with the M1 macrophage subtype, but no increase in expression of the M2 subtype marker, CD163, in treated cells. Further, this polarization of the THP-1-dependent macrophages showed an increased ability to damage MCF-7 or MDA-MB-231 cell monolayers in co-culture experiments. Therefore, treatment with *F. assafoetida* extracts can also indirectly cause the death of cancer cells via activation of immune cells. These results confirm that *F. assafoetida* is a potential source of anti-cancer and immune modulatory compounds and that further investigation is needed to reveal the mechanisms of *F. assafoetida*'s effects on apoptosis and immunomodulation.

Keywords: *Ferula assafoetida,* anti-cancer, pro-inflammation, cytotoxicity, apoptosis immunomodulation, macrophage polarization

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Abbreviations

Bcl- 2	B-cell lymphoma 2
BRCA1	Breast cancer type1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
ER	Estrogen receptor
APS	Ammonium persulfate
EtOH	Ethanol
FCS	Fetal calf serum
PAGE	Polyacrylamide gel electrophoresis
HBL100	Non-malignant human breast cells
BSA	Bovine serum albumin
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
g	Gram
MW	Molecular weight
HPLC	High-performance liquid chromatography
IFN	Interferon
IL	Interleukin
PMA	Phorbol-12-myristate-13-acetate

MTT	Methyl thiazole tetrazolium assay
SDS	Sodium dodecyl sulfate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nm	Nanometer
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
M1	Macrophage type 1
M2	Macrophage type 2
OD	Optical density
THP-1	Human acute monocytic cell line
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TdT	Terminal deoxynucleotidyl transferase
µg	Microgram
µl	Microliter

Chapter 1. introduction

1.1 Cancer

Cancer refers to malignant tumors and is defined as a group of diseases characterized by unchecked cell growth, cell division, and cell invasion. The uncontrolled cell multiplication leads to the spread of cancer to various parts of the body. Until a few hundred years ago, most people knew very little about cancer. However, our understanding of the disease has become more prominent in recent decades, in particular in western countries, and now almost everyone knows someone who has been impacted by cancer. At present, people from all around the world are affected by this disease and significant efforts are made to understand cancer and to treat patients diagnosed with cancer. To date, more than two hundred different types of cancers are known that affect people of all ages in every corner of the world. Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020 (1). In men, lung cancer, prostate cancer, and colorectal cancers are the most common cancer types, whereas, in women, breast cancer followed by gynecological malignancies such as ovarian, endometrial, uterine, and cervical cancers are the common cancer types.

1.2 The historical origin of cancer

Over the centuries, the diagnosis of cancer has been uneven. However, the prevalence of a diagnosis of this lethal disease has risen markedly over the years, and almost one out of every two individuals is likely to be diagnosed with this disease at some stage in life (2). Several factors, including living to a greater age, a change of lifestyle, food habits, and increased stress conditions, are some of the reasons for the increased incidence of the disease.

1.3 Significance of cancer study

Cancer incidence has increased significantly in many parts of the world over the last few decades, leading to high mortality in both men and women. Primary treatment methods such as surgery, chemotherapy, and radiation are performed to treat patients with different forms of cancer. However, effective control of this disease is still a long way off. The available treatment approaches have certain limitations. Surgical treatments can be highly effective for early-stage cancers but once the cancer cells have started to invade other tissues or metastasize it is of limited efficacy. Radiation therapy techniques have several side effects, including the possibility of nearby normal cells undergoing mutations and transforming into cancerous cells. Hence, it has been realized that it is challenging to eliminate cancer completely using radiation therapy. Moreover, although numerous chemotherapeutic drugs are available that have been approved by the Federal Drug and Administration (FDA) in the U.S. or similar agencies in other countries and are used to treat various types of cancer, success in certain types of cancer treatment is limited as cancer cell resistance to chemotherapies can become a significant clinical problem.

1.4 Biology of cancer

The human body is made up of trillions of cells. Cells cluster with one another to form tissues, while the groups of tissues join to form organs. This entire process of life's origin is very disciplined and controlled at every step. Minute mistakes in DNA replication can result in mutations, which can lead to altered cell or body functions. The term "cancer," in general, refers to an abnormal cellular growth caused by genetic changes in cells within the body. In normal human cells, cell division, differentiation, and death are strictly regulated by various

mechanisms including cell cycle checkpoints. Cancer cells, on the other hand, manage to escape many of the regulatory mechanisms regarding proliferation and differentiation (3). Hence, cancer cells form an entirely different lineage with distinct morphological and physiological properties compared to normal cells. Therefore, it is critical to identify any abnormality concerning uncontrolled cell division which results in a large group of abnormal cells, called a tumor.

Benign tumors, by definition, do not spread within a tissue or from one tissue to another through metastasis. They are neither invaders nor spreaders and are highly confined to a single location. Skin warts, for instance, are a type of benign tumor that can be easily removed with standard surgical procedures. Consequently, benign tumors are usually not fatal to the patient. In contrast, malignant tumors are capable of migrating out of the primary tumor site to adjacent tissues or to spread, via metastasis, to other organs. It is these malignant cells that possess the specific properties to make cancers so dangerous and life-threatening.

Cancers can also be divided into two categories based on the factors that cause them. Cancers caused by inherited gene mutation are called hereditary cancers where patients have a family history of genetic mutation in a specific gene related to a particular cancer type. Approximately 5-10% of breast cancer cases and 3-5% of colon cancer cases are attributed to inherited genes (4). Conversely, sporadic tumors are caused mostly by exposure to environmental factors such as cigarette smoke, pollutants, and ultraviolet (UV) radiation.

Harmful gene mutations pass from cell generation to cell generation, resulting in either homozygous or heterozygous conditions that can promote tumor development. Point or small deletion or insertion mutations in genes can alter the role of a functional gene and convert it into a mutagenic form via frameshift or other genetic alterations. Epigenetic changes can also

play an essential role in tumor development, primarily by altering the acetylation of histones and methylation of DNA in chromatin that affects chromatin conformation and therefore the efficiency of DNA transcription. Thus, DNA methylase/demethylases and histone acetylase/deacetylase (HAT/HDAT) and methylase/demethylases (HMT/HDMT) are critical in maintaining genomic stability and appropriate regulation of gene expression (5).

Tumor cells exhibit certain features that demonstrate their malignancy. The hallmarks of cancer are the fundamental characteristics shared by all cancer cells, regardless of their type or origin and researchers have highlighted six important characteristics that a cancer cell exhibits (6).

1. Rate of proliferation: The proliferation rate of cancerous cells varies significantly compared to that of a normal cell. Cancer cells proliferate in large numbers and divide at a much faster rate than normal cells. Generally, normal cells divide by the process of mitosis, which has four distinct sub-stages: gap phase 1 (G1), synthesis phase (S), gap phase 2 (G2), and mitosis. Cell cycle checkpoints exist in between the G1/S and G2/M phases to cross-check the previous cyclical event. In cancer, these cell cycle checkpoints may not function, resulting in a continuous division as the cells continue to cycle in an uncontrollable manner.
2. Activation of signaling mechanisms: The activation and regulation of signaling pathways is critical in maintaining the human body's homeostasis. In general, when a receptor molecule binds to a ligand, a change in receptor conformation takes place which recruits or activates downstream signaling molecules. In this way, the receptor-ligand (RL) interaction can trigger a specific pathway that can activate or inhibit the downstream genes. In normal cells, tumor suppressor genes (TSG) play a crucial role in preventing uncontrolled proliferative responses

by interfering with signal activation. Conversely, proto-oncogenes are generally activated in normal cells only when they are required to induce proliferation. Mutations in proto-oncogenes that cause inappropriate or constitutive activation of the protein product results in signals that always promote proliferation. The activation of these oncogenes occurs concurrently with the repression of tumor suppressors to cause uncontrolled proliferation (7). Thus, the appropriate activation and inactivation by tumor suppressor genes play a critical role in maintaining the body's homeostasis.

3. Insensitivity to growth inhibitors: Growth inhibitors, also known as cancer growth blockers, play a critical role in the regular maintenance of the cellular replication. For instance, cyclin-dependent kinase inhibitors (CDKI) are essential for maintaining the integrity of the cell cycle (8). Two different cyclin-dependent kinase inhibitors include the CDK4 (INK4) and the CIP/KIP family inhibitors. Cyclic kinase inhibitors have the potential to act as tumor suppressors. Their deletion, mutation, hyper methylation, and genetic alteration, such as transcriptional repression, can result in uncontrolled cell growth and division (9).

4. Invasion and metastasis: Uncontrolled proliferation of tumor cells is the primary indication of cancer development. Direct invasion and penetration into neighboring tissues is a fundamental part of tumor development. The growing tumor can physically breach the tissue of origin and continue to grow and invade adjacent tissue. The ability of cancer cells to further penetrate into the blood and lymphatic tissues and spread to other parts of the body is called metastasis. The tumor cells spread from the primary site of origin to the secondary site, distant from the first origin, by this phenomenon. Hence, this process establishes tumor development in naïve organs, making it difficult to treat the patients.

5. Sustained angiogenesis: Angiogenesis is a fundamental mechanism that occurs in both embryonic cells and tumor cells. Cancer cells divide unchecked at a particular site, resulting in nutrient and oxygen depletion. Moreover, the toxic levels of some metabolic products increase, making it difficult for the cells to maintain a healthy state. In order to escape the limiting nutrients in the environment and the overpopulation of cells, cancer cells promote angiogenesis by activating the vascular endothelial growth factor (VEGF) pathway that leads to the development of new blood vessels. VEGF overexpression is a common phenomenon in solid tumors. Cancerous cells trigger signals to neighboring blood vessels to activate the growth and invasion by endothelial cells to activate angiogenesis to ensure increased blood flow to maintain the long-term viability of the tumor.

6. Evasion of programmed cell death: Apoptosis, also known as programmed cell death, is essential to maintain homeostasis by eliminating unwanted and damaged cells. Alterations in apoptotic proteins are considered significant hallmarks for cancer development. Upregulation of antiapoptotic factors such as the B cell/lymphoma 2 (Bcl-2) family has been found to be significant in many cancer types (10). Increasing the antiapoptotic signals allows survival and proliferation of damaged cells to promote tumor growth.

1.5 Various types of cancer

Different types of cancer have been studied based on the site of origin as represented by the specific type of tissue where they are initiated. This classification allows scientists to focus on a particular type of cancer in relation to the properties of the cells of origin.

These types include:

1. **Carcinoma**: Initially, carcinomas were considered ulcers in a broader sense. This form of cancer primarily develops from epithelial cells, following an alteration in the genetic material (DNA). The damage in DNA can trigger mutations in the subsequent generations of daughter cells, resulting in faulty protein structure or activity. Moreover, the mutations in DNA are transmitted by progenitor cells and can be accumulated in successive generations that will no longer be like the parental generation. When these mutations result in the cells dividing uncontrollably, a tumor derived from these epithelial cells arises.

2. **Sarcoma**: Sarcomas are cancerous tumors of the connective tissues that arise from embryonic mesenchymal cells. Cancers of blood vessels, bone, nerves, muscles, cartilage, and fat cells all fall under this category. This type of cancer affects all age groups although the incidence of sarcomas increases with increased age.

3. **Leukemia**: Leukemias affect the white blood cells in the human hematopoietic system. The two most common types are lymphocytic leukemia and myelocytic leukemia. In leukemia, blood cells divide continously resulting in a malignancy. This condition often leads to other diseases such as anemia, bleeding, and infection as the malignant white cells compromise the bone marrow that is required to produce red blood cells and normal white cells (11). Leukemia is also classified as either chronic (progresses slowly) or acute (requires immediate care) depending on how quickly the cells replicate and the disease progresses (12)

4. **Lymphoma**: Lymphoma begins when healthy lymphocytes such as B, T, or NK cells in the lymphatic system transform and proliferate uncontrollably. This uncontrolled growth may form a tumor within the lymph nodes or lymphoid organs, involve other parts of the lymphatic system, or spread to distant parts of the body. Hodgkin's lymphoma is one of several types of cancer, derived from transformed B cells, that develops in the lymphatic system. It mostly

affects lymph nodes in the neck or the area between the lungs and behind the breastbone (13). Moreover, it can also involve groups of lymph nodes under an arm, in the groin, abdomen, or pelvis. Further, this disease can spread to the spleen, liver, bone marrow, or bones. Hodgkin lymphoma can also spread to other parts of the body, but this is unusual. In comparison, non-Hodgkin lymphoma (NHL) is a term that refers to a very large and diverse group of cancers of the lymphatic system derived from T, B, and NK cells. These cancers can have different signs and symptoms, as well as physical examination findings depending on the cell type, the relative stage of cellular differentiation, and the proliferation rate (13).

1.6 Chemotherapeutic strategies to combat cancer

Malignant tumors can invade nearby tissues and develop a secondary tumor. Chemotherapy is a traditional way to treat tumors using specific drugs. After some period of chemotherapy treatment, the treated patient may develop drug resistance, resulting in cancer recurrence. Multidrug resistance (MDR) in the cancer cells is caused by several factors, including upregulated expression of ATP-binding cassette (ABC) transport proteins, drug compartmentalization, inhibition of apoptotic pathways, alterations in targeted proteins and membrane lipids, and changes in tumor microenvironment development (14-17).

Overexpression of ABC transporters plays a significant role in the MDR mechanism. These transporters actively extrude several cytotoxic drugs out of the cell, reducing the efficacy of chemotherapeutic agents in cancer treatment. To continue this transport, energy is expended by adenosine triphosphate (ATP) hydrolysis. ABCC-1, also called multidrug resistance protein (MDRP), and ABCB-1, known as p-glycoprotein, play a crucial role in this phenomenon. Furthermore, breast cancer resistant protein (BCRP), also known as ABGC-2, is a primary

efflux pump protein that works by this mechanism (14,18). Recent studies have shown that maintaining hypoxic conditions in the tumor microenvironment can also lead to resistance to chemotherapeutic medicines.

1.7 Chemotherapy and breast cancer

Cancer metastasis occurs in more than 90% of patients with advanced stages of cancer and is, therefore, challenging to treat. Chemotherapy is a commonly used approach to manage and potentially cure metastasis, irrespective of cancer type. Taxanes and anthracyclines are highly recommended drugs for patients diagnosed at advanced stages, such as stage III and stage IV. However, using these drugs often results in patient resistance and disease recurrence in addition to multiple potentially debilitating side effects including nausea, gastrointestinal problems, and cardiac issues. Further, at advanced stages, metastatic cancer is often considered incurable using conventional methods such as chemotherapy and hormonal treatments and therefore more effective treatments are needed.

1.8 Role of taxanes in cancer treatment

Taxanes were first discovered in the bark of the tree, *Taxus brevifolia,* in a National Cancer Institute program to screen natural products for anti-cancer activity. Because of the scarcity of the natural taxol supply, the use of this drug was limited until it could be synthesized by chemical methods from renewable biological materials. Taxanes, such as paclitaxel, successfully completed clinical trials and were approved by FDA in 1992. In the early 1990s, paclitaxel, now made by semi-synthetic processes, were widely used as a treatment regimen for various cancers, including breast cancer. Platinum-based drugs, including cisplatin in

combination with paclitaxel, were commonly used to treat advanced ovarian cancer and non small cell lung cancers (NSCLC) (19). The taxanes are widely used and are commonly used to treat breast, prostate, and lung cancers, among others. For example, a clinical study has reported that 30% of patients with Stage III or IV ovarian cancer responded to Taxol treatment (20). Taxanes such as docetaxel and paclitaxel are mitotic inhibitors that are used to disrupt microtubule function (21). Microtubules play an important role in cell division, and taxanes bind to and stabilize microtubules causing cell cycle arrest and induced apoptosis.

1.9 Role of vinca alkaloids in cancer treatment

Vinca alkaloids were originally derived from the Madagascar periwinkle plant and are regarded as older anti-cancer drugs. Vinblastine, vinorelbine, and vincristine are the three alkaloids obtained from this plant that exhibit anti-cancer properties and are approved for use in the United States and many other countries (22). Vinblastine and vincristine, as well as other alkaloids, have the ability to block microtubule formation, which is required during the segregation of chromosomes during mitosis, thus resulting in cell cycle arrest. However, these alkaloids may not be able to differentiate between tumor and non-tumor cells, resulting in the blocking of mitotic spindle formation and induction of apoptosis in non-malignant cells. Vinblastine is widely used in treating carcinomas, Hodgkins and non-Hodgkin's lymphomas, breast cancers, and germ cell cancers. On the other hand, some side effects are also associated with vincristine usage, such as nervous system toxicity, peripheral neuropathy, bone marrow activity disfunction, nausea, and vomiting (22). These plant constituents also cause hypoglycemic and cytotoxic characteristics in treated patients.

1.10 Role of camptothecins

Camptothecin is a pentacyclic quinoline alkaloid derived from the tree *Camptotheca acuminata* and possesses high cytotoxic activity against a wide variety of cell lines. This compound can bind to topoisomerase I enzyme and inhibit DNA replication. This inhibition of topoisomerase results from a blockage of the re-ligation of DNA which also promotes DNA breaks and ultimately results in the induction of apoptosis of mitotic cells (23). The main drawback of using these alkaloids relates to their solubility in aqueous solutions. Therefore, several conjugate-based camptothecin drugs with increased solubility were developed for use in cancer treatment. Two of the derivatives, topotecan and irinotecan, were created by conjugating with hydrophilic compounds and are approved and available on the market. Irinotecan is used to treat patients with metastatic colorectal cancer whereas topotecan has been approved for treatment of ovarian cancer, cervical cancer and small cell lung cancer.

1.11 Role of anthracyclines

Anthracyclines are type II topoisomerase inhibitors that are widely used in treating breast cancer. One of the mechanisms by which anthracyclines induce cell death is by forming a complex with topoisomerase II, which blocks the re-ligation of the double-strand DNA breaks causing DNA damage which results in inhibition of cell division and apoptosis (24). Doxorubicin and daunorubicin are the first-generation anthracyclines that are widely used in chemotherapy. According to various studies, the use of these drugs has increased disease-free survival and the overall survival rate for patients undergoing chemotherapy (25). Second-generation anthracyclines, including epirubicin and idarubicin, have been improved in their capacity for enhancing disease-free survival. These drugs can also interfere with signal transduction mechanisms, bind to fas ligands, and trigger cell death (26,27). However, this

category of drugs exhibits severe side effects, including the generation of reactive oxygen species, DNA damage, and lipid peroxidation. Many investigations reported negative toxicological evidence with anthracycline usage resulting in cardiovascular diseases and hematological malignancy (26,27). Nonetheless, both the anthracyclines and taxanes are the most commonly considered agents in adjuvant therapy for breast cancer treatment.

1.12 Traditional and alternative medicine

Traditional medicines are primarily obtained from plant sources. Extracts from plants have long been used to treat various ailments, including a large number of deadly diseases. However, most traditional medical practises did not have an understanding of cancer and so current alternative medicine derived from traditional practise have focused on toxins or anti-inflammatory drugs. Plant parts, including leaves, flowers, stems, roots, and fruits, exhibit potential medicinal properties and are primarily used as anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, and anticarcinogenic agents (28,29).

Alternative medicine is a contemporary medical practise based on plant and herbal formulations which include a specific active component or combination of components in the treatment cocktail. These alternative medicines are often used to try and target the cell cycle mechanisms in order to slow down the process of cancer cell proliferation, although their mechanism of action is often unclear. Many alternative medicines have some preclinical data which allows them to be classified as anti-metabolites, alkylating agents, cell cycle inhibitors, topoisomerase inhibitors, mitotic inhibitors, and anthracyclines similar to approved pharmaceuticals. Most of these formulations are prescribed alone or in combination with other cancer drugs (30, 31).

The group of antimetabolites typically interfere with deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) replication and transcription mechanisms, slowing cancer cell division and eventually eliminating the cell. The drugs belonging to this category are often used to treat leukemia, breast, ovarian, and intestinal cancers.

1.13 Why are the new anticancer agents required?

Cancer is a highly complex disease that involves several biochemical pathways and intra-network signaling and metabolic connections within the human body. Cancer cells lack regulatory mechanisms and grow and spread in an uncontrolled manner. They also have several distinguishing characteristics that make them especially difficult to treat. For example, acquired resistance to chemotherapeutic drugs develops over time, which is a major challenge in treating patients with cancer. Drug resistance results in a significant number of casualties due to disease recurrence (13,15). Consequently, the development of novel drugs that can be used either alone or in combination with other FDA-approved medications has become critical to combat drug resistant tumours.

The completion of the human genome project and genomic sequencing revealed a large number of potential genes located downstream from critical proto-oncogenes and tumor suppressor genes which were candidates for anti-cancer drug development. Furthermore, the discovery of plant-based drugs containing high concentrations of active compounds including terpenes, anthracyclines, saponins, vinca alkaloids, taxanes, and campothecins, as well as several compounds whose chemical structures have not yet been studied at the molecular level, has led to the development of ethnomedicinal drugs that can inhibit these signaling molecules to cause tumor regression (30,31). The result has been an extraordinary complexity of bioactive

compounds which have been synthesized by the secondary metabolism in plants in an effort to improve plant survival aginst "predators" and parasites. Therefore, plant extracts can then be screened to isolate a variety of toxic compounds with properties than include high efficacy against cancer cells and which may have minimal side effects against the immune system. The success of this approach has piqued the interest of many pharmacologists regarding molecular insights of these phytochemical-based constituents and their reactivity and mode of action in combating cancer.

Since drug resistance has become an important issue in a subset of patients undergoing current treatment regimens the development of new therapeutics is critical. Resistance may develop for numerous reasons, the most important of which are related to tumor-supporting changes in the microenvironment and increased cancer stem cell generation (32). Therefore, new cancer drugs should have the capacity to shrink the tumor either in combination or as a single regimen by affecting the tumour microenvironment or cancer stem cell replication. This approach to treatment may be beneficial in terms of increasing survival rates and disease-free conditions for patients with cancer. To summarize, there is a need to develop novel drugs with high efficacy in controlling tumor progression and modern synthetic drugs specific to treatment procedures.

1.14 Apoptosis

The word apoptosis originated from two Greek words, "απο" and "πτωσιζ" envisaging "dropping off" which means falling of leaves from trees during the autumn season (33). Apoptosis is defined as "programmed cell death," and it generally occurs when a signal is induced to eliminate old and unhealthy cells from the body. This pathway was originally

described by Kerr et al., in the 1970s and is now regarded as one of the most investigated pathway in the field of biology (34). Numerous cell biologists have investigated the relationship between cancer and apoptosis. Both physiological and pathological conditions trigger this phenomenon. Apoptosis is, in fact, a two-edged sword when it comes to cancer, as both the low and high levels of apoptotic activation have outcomes related to the disease.

Some physiological events that utilize apoptosis include: normal cell destruction followed by their replacement by gut epithelial proliferation; programmed involution during the development of the fetus from the embryo to shape the tissues and organs; and, programmed involution during menstruation involves shredding of endometrium and regression of lactating breast tissue via apoptosis. Several pathological conditions also involve the activation of apoptosis including: induction of apoptosis by anticancer drugs which target tumor cells; tissue and graft rejections induced by cytotoxic T cells; the occurrence of progressive cell death in HIV-infected CD4+ helper T cells; triggering cell death in response to environmental factors such as radiation and thermal injuries and low oxygen levels (hypoxia); the initiation of cell death in certain heart failure conditions like myocardial infarctions; and, degenerative diseases like Alzheimer's and Parkinson's disease also trigger apoptosis (35,36).

Uncontrolled apoptosis lowers the rate of cancer while increasing the risk of neurodegenerative disorders. Apoptosis can be triggered by DNA damage, unchecked cell proliferation, cell stress, and certain toxins, and thus it plays an essential role in cellular homeostasis (37). Moreover, drug administration and hormone release may induce apoptosis. It is a well-defined process in which a cascade of cysteine proteases, known as caspases, are activated ultimately resulting in cell death (38).

1. Morphological hallmarks in apoptosis:

An apoptotic cell exhibits a similar set of nuclear and cytoplasmic morphologic alterations, irrespective of cell type and species (39). The major hallmarks of apoptosis include chromatin condensation, nuclear fragmentation followed by rounding of the cell, shrinkage of the cellular volume called pyknosis, and retraction of pseudopods resulting in nuclear fragmentation (Karyorrhexis). This phenomenon leads to cellular detachment from the surrounding tissue and cellular fragmentation, forming apoptotic bodies, which are phagocytosed by macrophages which is enhanced by the presence of phosphatidylserine in the cell membrane (40,41).

2. Biochemical process in apoptosis:

The major biochemical changes observed during apoptosis include activation of caspases, DNA and protein breakdown, and membrane alterations that lead to phagocytic cell recognition. The activation of the caspases plays a vital role in initiating the process. Inactive procaspases are present in the cell and are activated by proteolysis in a cascade where one caspase cleaves and activates the next in the series (42).

The series of biochemical events in apoptosis often occur as follows:

i. Expression of transglutaminase leads to DNA breakdown into large fragments followed by inter-nucleosomal cleavage (43), as a result of protein cross-linking (44).

ii. Membrane phosphatidylserine is enzymatically flipped from the inner leaflet to the outer layer of the cells' plasma membrane, where it enhances the early recognition of apoptotic cells or apoptotic bodies by the macrophage and increases phagocytosis and removal without the release of pro-inflammatory cellular components (45).

These biochemical events can be induced via both the extrinsic or intrinsic pathways of apoptosis. Activation of the intrinsic pathways also involves activation of signals in the mitochondria or the endoplasmic reticulum.

A. Extrinsic death receptor pathway:

As the name suggests, this pathway is triggered by external factors. Extracellular death ligands such as TNF and Fas ligand (FasL) bind to cell surface death receptors like TNF receptor (TNFR1) and a related protein called Fas (CD95), respectively (45). The intracellular death domain of these death receptors recruits adapter proteins like TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as the cysteine protease-like caspase-8 (46). The whole ligand-receptor-adaptor protein complex is known as the death-inducing signaling complex (DISC) (47). DISC then triggers a cascade of caspase activation by the assembly and activation of pro-caspase-8 to caspase-8 followed by the cleavage and activation of other downstream caspases.

B. Intrinsic mitochondrial pathway:

The intrisic pathway is triggered by intrinsic factors such as irreparable genetic damage, hypoxia, or a high concentration of cytosolic Ca^{2+}. Irrespective of these stimuli, increased permeability of the mitochondrial membrane results in the release of a proapoptotic factor named cytochrome C. Once in the cytoplasm, cytochrome C binds with Apaf-1 and caspase 9, forming a complex, called the "apoptosome," which initiates apoptosis (48). The intrinsic pathway is regulated by a group of proteins belonging to the Bcl-2 family, which are divided into two groups, *viz*. proapoptotic and antiapoptotic. The pro-apoptotic proteins (Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk) promote the release of cytochrome C from the mitochondria,

while anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Bfl-1, and Mcl-1) bind to and sequester the pro-apoptotic proteins to prevent the release of cytochrome C (49). There should be a balance in the expression of both types of Bcl-2 family members during the normal growth and development of cells. Certain proteins of the Bcl-2 family, like Noxa and Puma are involved in p53-mediated apoptosis (50).

C. Common pathway:

Apoptosis is initiated by activation of specific upstream caspases such as caspase-9 in the intrinsic pathway and caspase-8 in the extrinsic pathway. Both pathways converge on the activation of the executioner caspase, caspase-3, which cleaves the inhibitor of caspase-activated deoxyribonuclease, resulting in DNA fragmentation and nuclear apoptosis. Caspase-3 also causes the cleavage of protein kinases, cytoskeletal proteins, and DNA repair proteins, influencing the cell cycle, cytoskeleton, and signaling pathways that lead to the distinct morphological changes seen in apoptosis (51).

D. Intrinsic endoplasmic reticulum pathway:

All membrane and secretatory proteins synthesized by the cell are produced and modified in the endoplasmic reticulum (ER) to ensure their proper folding and translocation. When the ER is exposed to certain cellular stresses such as hypoxia, glucose starvation, or free radicals, it becomes injured and cannot perform its normal function resulting in an accumulation of improperly folded proteins trapped in the ER, a condition called the unfolded protein response (UPR). The UPR results in inhibition of cell growth and increased gene expression in order to restore protein homeostasis. The failure of UPR resolution results in apoptosis either through the extrinsic or intrinsic pathway. The accumulation of unfolded proteins and reduced protein synthesis in the cell leads to dissociation of an adaptor protein named TNF receptor-

associated factor 2 (TRAF2) from pro-caspase-12, thus triggering the activation of the proteins involved in the apoptosis pathway.

E. Apoptosis proteins:

The most important proteins that play a vital role in apoptosis are the caspases, the amyloid-B peptide, the Bcl-2 family of proteins, the p53 protein, and the heat shock proteins.

i. Caspases: The caspases are named after the fact that they are aspartate-dependent cysteine proteases (c-cysteine proteases; responsible for the cleavage at aspartic acid residues) (42). Caspases exist in the form of zymogens and are termed as procaspases which undergo appropriate cleavage for their activation to mature caspases. A caspase is made up of two identical large subunits and two identical small subunits forming a tetramer that may possess "death effector domains (DED)" or Caspase recruitment domains (CARD) which help in binding to other molecules either inside or outside the cell (52).

ii. Amyloid B peptide Abeta: These are chief apoptotic proteins derived from the amyloid–B precursor protein (APP) and play a crucial role in the apoptotic mechanisms the occur in the CNS in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Accumulation of Abeta proteins in the nerve cells eventually leads to cell death (52).

iii. Bcl-2 family: The Bcl-2 family of proteins consist of both pro-apoptotic and anti-apoptotic proteins, which either dependently or independently regulate internal death signals and external signals maintaining cellular homeostasis. The structural and functional subgroups of these proteins all contain a BH binding domain. When a cell receives a death signal, the Bcl-2 proteins, normally present in the outer mitochondrial membrane, undergo conformational changes and activate pro-apoptotic members to interact via their BH domains forming pores to increase mitochondrial membrane permeability while anti-apoptotic proteins are neutralized.

iv. p53: The p53 tumor suppressor protein is capable of inducing apoptosis and is expressed during hypoxia, heat shock, oncogene activation, etc. (52). It can arrest the cell cycle in both the G1 and G2 phases in response to irreparable DNA damage. P53 enhances the expression and activation of pro-apoptotic proteins and inhibits antiapoptotic proteins (53).

v. IAP: IAPs are an eight-membered caspase inhibitor family of proteins with a novel domain, the baculoviral IAP repeat (BIR). Apoptosis X-linked inhibitors (XIAP), IAP-like protein 2 (ILP-2), BIR-containing ubiquitin-conjugating enzyme (BRUCE)/(Apollon), livin, survivin, cellular IAP1 (cIAP1), cellular IAP 2 (cIAP2), and neuronal apoptosis inhibitory protein (NAIP) all belong to this family (54). IAPs directly inhibit some caspases, such as caspase-3, and provide protection from Fas/caspase-8-induced apoptosis via blockage of the proteolysis cascade by binding via their conserved BIR domain to the targeted caspase.

vi. Heat shock proteins (HSPs): HSPs such as HSP70 and HSP90 are basic apoptotic inhibitors that work in association with Apaf-1. They inhibit the release of cytochrome c from the mitochondria and thus prevent the release of pro-apoptotic factors (55).

1.15 Immune system responses in cancer

Immunity is the term given to the resistance acquired by the host system against a particular pathogen or disease. When a pathogen attacks the host body, the immune response in the host system is triggered and mediates a fight against the organism that expresses the detected foreign antigens. Specifically, the human body exhibits two types of immune systems, namely the innate and adaptive immune systems. The innate immune system is comprised of a group of receptors that recognize common pathogenic antigens that are constitutively expressed on macrophages and accessory immune cells. Pathogens bind to these receptors and activate

specific signaling pathways that result in increased free radical production and phagocytosis. The adaptive immune system mediates the reaction to antigens processed by the macrophages or other antigen processing cells and includes the humoral and cell-mediated immune responses. The humoral immune response produces antigen-specfic B-cells from bone marrow, whereas the cell-mediated immune system generates antigen-specific T-cells from the thymus gland. T helper cells also contribute to the differentiation of B cells into plasma cells that secrete antibodies and produce memory cells.

The tumor microenvironment plays a major role in cancer development. In cancer, various metabolic processes occur that are different from the normal cells in the body. The macro and microenvironments between normal tissues and tumour tissues also differ remarkably and contribute to defining the immune cells' response (56). The tumor microenvironment (TME) strictly maintains hypoxic conditions irrespective of oxygen availability when compared to normal tissue. This phenomenon is known as the Warburg effect. In brief, cancer cells oxidize glucose to lactate even in high oxygen conditions (57). This results in increased levels of reactive oxygen species (ROS), H+, hypoxia, and generation of metabolites, creating a toxic environment.

Metastasis is a significant factor in cancer development. Understanding the entire metastatic process is very important in terms of clinical practice. A solid, fully grown tumor undergoes metastasis through various steps, including: (1) tissue invasion; (2) vascular intravasation; (3) survival; (4) extravasation; and, (5) adaptability as well as proliferation in the new environment. In general, the host immune response has the capacity to target both the mutated and highly proliferating cells at any stage of this metastatic cascade. However, cancer cells exploit various mechanisms to evade elimination by the host's immune response.

The immune surveillance mechanism plays a significant role in the destruction of primary tumor cells by the immune response. Primary tumor cells possess tumor-specific antigens that are recognized as foreign antigens by the host's immune system (58). Besides this mechanism, cancerous cells, which exhibit mutant characteristics, generate altered proteins compared to normal cells. The production of altered proteins also triggers the immune response. However, the subsequent elimination mechanisms can also accelerate cancer cell metastasis. Metastases are generated from the primary tumor by hematologic or lymphatic spread and are considered secondary tumors.

Tumor cells tend to adapt to the local microenvironment or stroma, similar to non-malignant cells, and this combination of tumor cells and associated stroma is called the tumor stroma. Tumor stroma includes components like fibroblasts, nerve cells, endothelial cells, macrophages, and extracellular matrix. Macrophages in tumor cell stroma can multiply in response to angiogenic signals and can switch the mechanisms by which they interact with tumor cells in invasive breast carcinomas (59). Various subtypes of tumor-associated macrophages exist, which can act as both pro- and anti-tumor agents. The most common classification sytem is to characterize macrophages as one of two types: anti-tumor M1 macrophages and pro-tumor M2 macrophages (59, 60). Anti-tumor M1 macrophages induce interleukin (IL) -1, IL-6, TNF-α and other factors, triggering an inflammatory response by the immune system. This stimulation further generates excess nitrous oxide and some oxygen-derived free radicals, which eliminates the tumor cells (61). M2 tumor-associated macrophages secrete IL-10, TGF-β, and CCL-2 and inhibit several immune cells like natural killer (NK) cells, T-cells, and dendritic (DC) cells and enhances arginase expression which depletes arginine levels and nitrous oxide. Further, invasion of the extracellular matrix by tumor tisses can take

place due to the increased levels of matrix metalloproteases disrupting the extracellular matrix (or basement membrane of the epithelial tisses), which can enhance metastatic tumor shredding and spread through the vasculature (62).

The tumor cells are susceptible to fluid shear conditions, as well as to anoikis (apoptosis induced by lack of cell adherion), and to immune cell attack, as the potentially metastatic cells travel through the bloodstream. As a result of these processes, in particular the immune response, very few tumor cells survive with the capacity to generate a secondary tumor site, and most of the shed tumor cell population is destroyed or inactivated by the host's defense mechanism (63,64).

Some findings have shown that when a primary tumor successfully disseminates and travels to a distant site for secondary tumor formation, the tumor cells have already adopted some level of resistance against host immunity, thus showcasing immunological escape. One such mechanism involves the downregulation of major histocompatibility class I molecules (MHC I) on the target cell surface, which are crucial for recognition by cytotoxic T (CD8+) T-cells. This allows the tumour cells to escape immunological surveillance by CD8+ cells and can be critical for the survival of tumor cells (65).

1.16 Breast Cancer

Breast cancer is considered the most common form of cancer in women, irrespective of their age. According to the US breast cancer statistics reports, 276,480 new cases of invasive breast cancer were estimated in 2020. As of January 2020, more than 3.5 million breast cancer patients, including those who had completed treatment, were living in the US

(Breastcancer.org). Substantial improvements in the treatment of women with breast cancer has tremendously decreased the mortality rate in developed countries. Yet, it is still a major cause of death in many parts of the world. The American cancer society's estimates suggest that breast cancer is the second most common cause of cancer deaths, after lung cancer, in women out of all cancer types.

Advances in several scientific approaches and technological innovations have made tumor malignancies more manageable. Chemotherapy is one such major therapeutic domain, often prescribed to transform non-operable tumors into operable ones (66). Tumors may be subjected to chemotherapy both before and after the surgery, to shrink the tumor before surgery and to eliminate any residual tumor cells after surgery. However, the drugs involved in chemotherapy do not only kill neoplastic cells but also lead to collateral damage of normal cells in particular, fast growing gastrointestinal cells and hematopoetic cells (erythrocytes, platelets, and leukocytes). Practically, the efficacy of chemotherapy has been limited by several factors, including systemic toxicity in the case of less specific drugs, rapid drug metabolism, and drug resistance characterized by both intrinsic and extrinsic factors. In this era of targeted and tailored therapies, multidrug resistance remains a challenging and unpredictable aspect of chemotherapy (67).

1.17 Breast anatomy

Breasts are present in both males and females (68). They are largely made up of adipose tissues, a type of fatty tissues (69). Female breasts are comparatively more glandular (70). A typical female breast consists of 12-20 lobes that are further divided into lobules which are connected via milk ducts (71). The breast's adipose tissue is characterized by the presence of a

network of nerves, blood vessels, lymph vessels, and lymph nodes along with fibrous connective tissue and ligaments (72). Hormonal changes in the body can cause significant changes in the size and activity in the breasts, which are synchronous with the cyclic changes of the menstrual cycle (73).

Several apocrine and sebaceous sweat glands are present in the wrinkled and pigmented epidermis of the areola and nipple. Milk is carried to the nipples by 15-25 milk ducts, which expand and synthesize milk into the sinuses before terminating in cone-shaped ampullae. The big Montgomery's glands are modified sebaceous glands with milk ducts opening into Morgagni's tubercles in the epidermis of the areola. Several circular and radial smooth muscle fibers are present deep inside the dense connective tissue alongside the lactiferous duct that ends into the nipple. These muscle fibers are responsible for nipple erection, areola contraction, and milk sinus emptying (74).

1.18 Mechanisms of drug resistance

The first chemotherapeutic agents were developed to target the faster replication rate of tumor cells compared normal cells. Hence, drugs were designed to target cellular mechanisms involved in DNA replication and metabolism or chromatin segregation in daughter cells. However, these drugs also target the fast-dividing cells of bone marrow and gut, which can lead to significant, often life-threatening, side effects. This is the result of the drug targeting molecular changes in any dividing cells which meant these drugs were not specific for cancer cells as the target.

One of the major problems with chemotherapy treatment is the progression to drug resistance: many tumors change during treatment such that they are no longer inhibited by

chemotherapy and often this resistance is not only to the chemotherapy drug being used but may extend to other drugs. This is multidrug resistance. According to the observations of Johnstone et al. (2000) (75), drug pumps such as P-glycoprotein and intracellular detoxifiers like glutathione are overexpressed in tumor cells that develop drug resistance. Proteins that interfere with drug accumulation or its stability can also cause clinical drug resistance (76). The rational is that the drug pumps are able to maintain a low level of chemotherapy drug in the cytoplasm which decreases its affectiveness while detoxification enyzmes are able to repair or prevent the damage that is caused by chemotherapy. Some other changes may include increasing levels of enzymes that chemically degrade the chemotherapy drug to decrease its effective half-life or that may alter the target of the chemotherapy drug such that it is no longer effective.

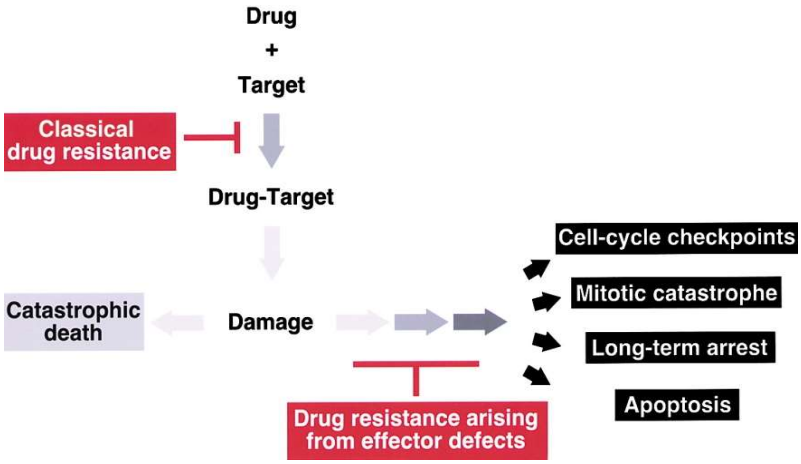

Figure 1.1: Mechanism of drug resistance. Source: Johnstone et al., 2002.

1.19 Histological and molecular markers as a criterion for breast cancer classification

Breast cancer is classified into several categories based on the specific cells affected. Carcinomas of the breast are a particular form of adenocarcinoma, resulting from epithelial cells, considered the most common type of breast cancer. Ductal carcinoma or *in-situ* carcinoma are another type that develops in milk ducts but does not spread to other regions. Moreover,

lobular breast cancer is formed in the breast region's lobules but cannot invade the surrounding areas. A type of cancer that can invade or spread to any other tissue or organ is considered an invasive breast cancer. Invasive cancer is further classified into another three types, namely invasive ductal breast cancer, inflammatory breast cancer, and metastatic breast cancer.

Breast cancers are also classified based on differences in specific hormone or growth factor receptor expression. The expression of hormonal epidermal receptor-2 (HER-2), estrogen receptor (ER), and progesterone receptor (PR) are important receptors that are involved in the development of breast cancer cells in the human body. The presence or absence of these hormone receptors are primarily used in immune histological studies to subtype breast cancers and to help decide the most effective mode of treatment and disease progression. Luminal A breast cancer cells express ER and/or PR but do not express HER (ER/PR+, HER2-), luminal B breast cancer cells express ER and PR and also HER-2 (ER+, PR+; HER-2+). An HER 2 enriched tumor type lacks ER and PR but has high HER levels (ER-; PR-; HER2+), whereas the basal form of breast cancer, also called triple-negative breast cancer does not have ER, PR, or HER expression, and is considered the most aggressive and difficult to treat. This classification of breast cancer has made cancer treatment type specific. Tumors that express ER are susceptivle to treatment wth Tamoxifen, an ER receptor modulator, that inhibits breast cancer growth. Anti-HER-2 antibodies, such as Herceptin or trastuzmab, have been used to treat breast (and other) cancer patients with HER2+ tumours since these antibodies block growth factor receptor function and target immune responses. Among all the types of breast cancer, triple-negative breast cancer (TNBC) is considered the most difficult to treat. Due to a lack of receptors, standard hormonal treatment methods and anti-HER2 antibodies are not used to treat patients of this type. On average, 10-15% of breast cancer cases are TNBC, for which

aggressive chemotherapy followed by hormonal medication are the most effective available treatments.

1.20 Serum tumor markers for breast cancer detection

In 2007, the American Society of Clinical Oncology recommended a few serum markers for to monitor patient progress following disease diagnosis. Mucin glycoproteins, including CA15-3, CA 27.29, and carcinoembryonic antigens, as well as plasminogen inhibitors and urokinase plasminogen activators, are highly endorsed in measuring tumour regression and detection in the treatment of patients.

1.21 Risk factors of breast cancer

Breast cancer risk factors include genetic and environmental factors, that includes increased exposure to endogenous or exogenous hormones, socioeconomic status, reproductive lifestyle, and behavioral factors that involve age, age of parity, age at first childbirth, body mass index (BMI), alcohol consumption, age at menarche, and hormonal imbalance (77). Exposure to polycyclic aromatic hydrocarbonss like coal burners, fuel, and cigarette smoke can also increase cancer risk. Risk factors are divided into two types, namely: preventable and non-preventable. Genetic factors, like mutations in the BRCA1 and BRCA2 genes play a significant role in nonpreventable cancer while cigarette smoking is an important preventative risk factor.

1.22 Family history and BRCA1 and BRCA2

It is extimated that ten percent of breast cancers in Western countries are caused by genetic predisposition. Breast cancer susceptibility can be inherited as an autosomal dominant

trait with limited penetrance. Mutation in the Breast cancer susceptibility (BRCA1/2) genes can lead to an inherited form of breast (and ovarian) cancer and mutations in these genes are linked with the increased incidence of breast cancer in most populations (78 - 80). The human BRCA1 gene is located on chromosome 17, while the BRCA2 gene is found on chromosome 13. BRCA1 and BRCA2 were the first breast cancer genes to be identified. BRCA1 is a human tumor suppressor gene that helps in the repair of damaged DNA. The wild type forms of these genes regulate the cell division cycle by preventing the cells from growing and dividing in an uncontrolled manner. Thus, they inhibit the growth of cells that line the milk ducts in the breast. They are involved in the repair of chromosomal damage. If BRCA1 and BRCA2 are mutated, the damaged DNA is not repaired correctly which leads to a higher risk of breast cancer (79).

1.23 Radiation

While exposure to high levels of ionizing radiation increases the risk of many cancers, the risks associated with low levels of radiation arising from mammograms and other x-rays are low (81).

1.24 Medicinal plants and cancer

Medicinal plant usage is a traditional technique still used in many parts of the world to treat various ailments. According to a survey conducted by the World Health Organization (WHO), 60-80% of the world's population receives medical treatment mainly from plants (28,82). Moreover, another recent survey showed that more than 40% of Americans prefer using ethnomedicine as a cancer treatment regimen because they believe it has higher efficacy and fewer side effects than conventional treatments. Plants and plant extracts have been used to treat a wide range of diseases because of the properties they exhibit. For centuries, certain plant

extracts have traditionally been used as a remedy to treat patients with conditions like cancer. In general, medicinal plants show anti-fungal, anti-bacterial, anti-viral, anti-inflammatory, and other promising characteristics. Therefore, a great deal of research has been conducted concerning medicinal herbs and their potential medicinal properties. A high percentage of chemical constituents in medicinal plants strengthens physiological and pharmacological efficacy in the ethnomedical treatment process. Consequently, botanical and pharmacological research is focusing on understanding the cellular, molecular, and biochemical properties of medicinal herbs.

Medicinal plants have long been used to treat and cure a variety of conditions and ailments. Arteether, a sesquiterpene derived from plants of the Asteraceae family, is used to treat malaria and is in clinical trials to demonstrate its effectiveness (30). Moreover, areether is also consumed as a traditional Chinese medicine. Another plant-based drug, derived from *Galanthus woronowii*, galantamine, is used to treat Alzheimer's disease. This drug is thought to work by inhibiting acetylcholinesterase. It also gradually slows down the aging process (30). Vinflunine, a derivative of vinblastine, derived from plant sources is currently used as a chemotherapeutic agent in combating cancer.

Medicinal plants have been the subject of extensive therapeutic research. For example, aloe vera has long been studied for its anti-viral, anti-bacterial, anti-cancer, immunoregulatory, and hepatoprotective properties (83). *Ocimum sanctum* L., also known as holy basil or tulsi (and many other Indian names) is predominantly used in folk and traditional medicine in Southeast Asia as a general tonic or as a treatment for disorders from eye problems to ring worm. Moreover, the leaves of *Mangifera indica* can be used to treat certain ailments, including dental diseases. Furthermore, *Centella asiatica,* commonly known as Adoni, has therapeutic properties

to treat cold/coughs. In addition, *Calotropis gigantean* can be utilized as a treatment for joint pain and rheumatism. The raw inner flesh of the *Ananas comosus* (anarosh) fruit is considered to be effective against fever and intestinal worms. Furthermore, regularly drinking *Terminalia arjuna's* bark-soaked water is used to help cure hypertension and heart diseases (83).

The whole plant of *Swertia chirata* has been traditionally used to treat diseases like gastric pain, diabetes, and liver diseases. Bel fruit, scientifically known as *Aegle marmelos*, treats heart stroke, ulcer, gastric pain, indigestion, and constipation. Additionally, the bark of *Alstonia scholaris,* commonly known as Chatim, is used to treat diabetes (84). Moreover, *Citrus aurantifolia*, traditionally called lemu, is utilized to relieve pyorrhea. *Mikania scandens* (commonly known as mayalota) has potential medicinal properties to cure dysentery. Its leaves are also employed to heal cuts and wounds and to treat diabetes. The ground paste of *Cassia alata* (Daad) leaves is employed as a remedy for skin diseases, such as scabies. Furthermore, *Cynodon dactylon, Tagetes erecta, Curcuma longa, Colocasia esculenta,* and *Piper betel* are used for their therapeutic properties to treat cuts and wounds (84).

Crocus sativus (belonging to the family Iridaceae and the superfamily Crocoideae), also known as saffron, is another important medicinal plant that is commonly used in traditional health care. It possesses several phytochemicals, including carotenoids, alpha-crocetin, and picrocrocin, which are responsible for its color and aromatic smell. *C. sativus* finds a wide range of applications for treating neurological disorders. It is also used as a stimulant, gingival sedative, herbal sedative, emmenagogue, and an expectorant (85).

According to numerous studies, the Banana pseudo stem is an excellent source of potassium and vitamin B, both of which aid in producing insulin and hemoglobin. Its inner part is edible and helps control high blood pressure (86). Similarly, the Banana stem is also

beneficial for treating kidney stones. Further, it is valuable in curing upset stomach and diabetes as it promotes insulin production. In addition, the banana flower is said to help cure diarrhea and dysentery (87). Likewise, the *Musa paradisiaca* flower is also known for its anti-cancerous properties (88).

1.25 Natural products in the treatment of breast cancer

A lot of research has been performed to find breast cancer treatments directed at different cellular mechanisms because of the highly variable mechanisms that can increase disease occurrence. Several studies have reported the significance of the tumor microenvironment in maintaining plasticity and cancer cell stemness and thus developing cancer recurrence. Chu et al., (89) explained the role of adipocytes in maintaining the tumor microenvironment. Obesity is a risk factor for breast cancer, with a higher incidence in urban areas compared to rural places. Obese women are more likely to be diagnosed with breast cancer than women with lower BMI, especially after menopause. Being overweight raises the risk of breast cancer in both post-menopausal and pre-menopausal women. Fat cells synthesize estrogen; thus, extra fat cells may lead to more estrogen in the body, facilitating the formation and expansion of hormone-receptor-positive breast cancer cells. Accumulation of high levels of free fatty acid, cholesterol, and glycerol in the form of droplets also increases tumor initiation, migration, and proliferation. They also activate the toll-like receptor (TLR)-4 to increase inflammation activation, thus increasing the risk of metastasis. These free fatty acids can also stimulate several signal transduction mechanisms, including PI3K and AKT, by activating GTP binding proteins and epidermal growth factor receptors which enhance cancer risk (89).

Quercetin is a flavonoid compound generally found in many fruits and vegetables, wine, tea, and coffee. Studies by Manouchehri et al., (90) have shown the anti-proliferative effect of this compound in the TRAIL-resistant breast cancer cell lines MCF-7 and BT-20. This flavonoid induces apoptosis by upregulating BAX and BAD expression. It has also been shown that quercetin initiates apoptosis by upregulating DR5 in both prostate and liver cancer cells. Moreover, quercetin has the capacity to downregulate cancer stem cell markers, including ALDH1A1 and epidermal-mesenchymal transition (EMT). Likewise, various studies reported the anti-cancer properties of the quinolone alkaloid, tetrandrine. This alkaloid blocks calcium channels and inhibits the activity of multidrug-resistant (MDR) proteins. Xu et al., (2011) studied the effects of this alkaloid using mammosphere cultures as surrogates of breast cancer tumor-initiating cells. Their results illustrated the anti-proliferative role of this compound against the SUM-149 and SUM-150 cell lines (91). Furthermore, flow cytometry studies have revealed a reduction in the ALDH+ cancer stem cell population after treating with a 1μM concentration of this tetrandrine, confirming its inhibitory activity against stem cell markers.

Garcinia is a medicinal plant that produces an active compound called garcinol. Active constituents from its extracts, including some phenolic compounds, exhibit several valuable anti-cancer, anti-bacterial, and anti-inflammatory characteristics. Studies conducted by Ahmed et al., (92) have shown the anti-cancer activity of garcinol in triple-negative breast cancer cell lines MDA-MB-231 and BT-549. Their research elucidated the molecular mechanism of this drug in TNBC treatment. Treating the cells with garcinol (25μM) reduced the mRNA expression level of EMT markers in these cell lines. Moreover, these studies also confirmed the role of microRNAs in switching from a mesenchymal to an epithelial state which is associated with a decrease in aggressive behaviour. Western blot data revealed decreased levels of P65, a

vital protein of the NF-kB pathway, which correlated with the downregulation of cancer progression following garcinol treatment.

Several reports have shown the role of curcumin in targeting the NF-kB pathway in breast cancer. Nevertheless, Aggarwal et al., (2005) demonstrated the suppression of metastatic breast cancer spread to lungs through increasing the paclitaxel-induced NF-KB pathway in nude mice. *In vitro* and *in vivo* studies confirmed the efficacy of paclitaxel and curcumin combination therapy in suppressing breast cancer metastasis (93).

1.26 *Ferula assafoetida* classification

Kingdom- Plantae

Division – Magnoliophyta

Class – Magnoliopsida

Subclass – Rosidae

Order - Apiales

Family – Apiaceae

Genus – *Ferula*

Species – *Ferula assafoetida*

(The information acquired from plant database of the United States, Department of Agriculture, and National Resources Conservation Service).

1.27 *Ferula assafoetida*

Ferula assafoetida is an herbaceous perineal plant that belongs to the umbelliferon family. The Gum Resin obtained from this plant has been used as a condiment in both India and

Iraqi food preparation. It has gained tremendous popularity as an endemic medicinal plant in Iran as well. In many countries around the world, including India, ancient texts refer to this plant as "hingu." Moreover, the resin, obtained from roots after rhizome incision, has been consumed as an Indian spice for centuries. The plant can grow up to a height of 1.5-2 meters. Ferula is a Latin word that means "vehicle" or "carrier." Hing or asafetida, in general, is pale yellow in color and exhibits a pungent odor and a bitter flavor. To this day, people use this spice as a flavor enhancer while cooking, especially in dishes including mushrooms and meat. Oil is used as a dissolving agent to improve the flavor. Assafoetida is also regarded as a popular household remedy and is used as a traditional medicine because of its potential healing properties. It occurs in two major forms, namely tears and masses. Masses are considered the most available form than tears regarding market availability (94).

1.28 Origin and distribution

Ferula assafoetida is widely distributed in central Asia, in particular West Afghanistan, Iraq, Turkey and Eastern Iran, Europe and North Africa. Afghanistan and Iran are the leading producers in central Asia (95). Although India is not a native producer of *Ferula assafoetida*, its gum resin is widely used both in cooking and in traditional medicine throughout the country. Another species of *Ferula assafoetida,* known as *Ferula narthex,* is also commonly used in India as a false assafoetida.

1.29 *Ferula Assafoetida* as a traditional medicine:

Assafoetida has been used as a traditional medicine for almost 1000 years. It was traditionally used to treat asthma, gastrointestinal disorders, intestinal parasites, epilepsy, and nervous disorders (96,97). *Ferula assafoetida* improves digestion, stimulates the nervous

system, and has also been used as a sedative in India (98). Its gum resin in combination with *Moringa pterygosperma* bark juice has been widely used to cure stomachaches (99). It also has diuretic properties and is used to treat influenza and weak digestion (100,101).

Ayurvedic medicine recommends the use of *Ferula assafoetida* to treat flatulence. Consuming assafoetida oleo gum resin roasted in ghee is used to alleviate many gastrointestinal ailments (102). Moreover, aqueous extracts of the dried gum have anti-helminthic properties (100, 103). South Pacific countries consume ferula oleo gum resin mixed with water to cure stomach upsets (100, 103). *Ferula assafoetida* root extracts are commonly used in Indian countries for its anti-bacterial, anti-spasmodic, diuretic, and laxative properties (104). Furthermore, Ferula root has also been consumed orally in Iran, and the United States, for its anti-spasmodic properties (103). Likewise, Iranian folk medicine studies claimed to use oleo-gum extracts as a mild laxative in older people (105).

1.30 Active constituents of *Ferula assafoetida*

The dried latex oleo gum from the underground root possesses many active compounds that are exceptionally useful in pharmacology and daily life. The color of the oleogum is grayish-white when fresh, but it eventually turns yellow, red, and brown as it ages. Many reports describe analysis of the chemical composition of the oleo gum resin of *Ferula assafoetida* using techniques such as GC-MS, HPLC, mass spectroscopy, and gas chromatography. Ferulic acid, umbelliferon, asaresinotannols, and farnesiferols A, B, and C, are the major components making up around 40-46% of the resinous material. Moreover, 25% of the gum is made up of several monosaccharides such as glucose, galactose, l-arabinose, glucuronic acid, and rhamnose. Additionally, 3-17% of the gum is volatile oil and contains

disulfides as prime components, including 2-butyl propenyl disulfide with alpha and beta-pinene monoterpenes, free ferulic acid, valeric acid, and traces of vanillin (106).

1.31 The pharmacological activity of *Ferula assafoetida*

Treatment with alpha pinene monoterpines exhibits sedative, anti-bacterial, anti-cancer, anti-viral, anti-inflammatory, and tranquilizer properties. Moreover, treatment with beta-pinene acts as a sedative. Di-allyl sulfide, derived from oleo extracts, has been used as an anti-cancer, anti-mutagenic, anti-HIV, immune stimulant, and anti-bacterial agent. Furthermore, luteolin possesses cytotoxic characteristics and has been used to induce apoptosis. Likewise, ferulic acid has been shown to exhibit anti-neoplastic, anti-inflammatory, anti-carcinogenic, and hepatoprotective properties (107).

1.32 *In vitro* studies of *Ferula assafoetida*

Ziai et al., (108) investigated the effect of a coumarin called umbelliprenin, which is present in extracts of *Ferula assafoetida* on chronic lymphocytic leukemia and showed dose-dependent inhibition of growth. Similarly, Abroudi et al., (109) evaluated the anti-proliferative activity of *Ferula assafoetida* in MCF-7 and PC-12 cell lines and observed that the ethanolic extracts of this plant had cytotoxic effects in a dose-dependent manner. Moreover, they also confirmed the apoptotic induction in PC-12 cell lines in a time-dependent manner. Bagheri et al., (110) confirmed the anti-proliferative activity of *Ferula assafoetida* essential oil on the MCF-7 breast cancer cell line in *in vivo* and *in vitro* studies. Biochemical analsysis of the *in vivo* experiments in Wistar rats showed that the treated group had higher levels of lactate

dehydrogenase than the control group but found no differences in hematological parameters between groups.

Verma et al., (111) showed an anti-proliferative effect of *Ferula assafoetida* on two liver cancer cell lines, HepG2 and SK-Hep1, and confirmed that treatment resulted in apoptotic induction. Likewise, the research group of Seyyed Majid Bagheri (112) carried out cytotoxic studies on 4T1 breast cancer cell lines using different concentrations of Oleo-gum resin (1-1000 ug/ml), ferulic acid (0.5-50 ug/ml), and essential oil (0.01-10 ug/ml). Oleo-gum-resin had no inhibitory effect on 4T1 cells after a 24 hour treatment period, yet at 48 and 72 hours post-treatment, there was significantly reduced cell viability. For example, treatment with 500 µg/ml ferulic acid reduced the 4T1 cell viability after 72 hours. However, at concentrations of 1 and 10 ug/ml, essential oil was shown to have a significant cytotoxic effect. Thus, essential oil illustrated a larger effect on destroying cell viability compared with oleo-gum resin and ferulic acid.

Tavassoli et al., (113) discovered that combining *Ferula assafoetida* and *Allium sativum* had a powerful anti-parasitic effect on equine gastrointestinal worms. The hydroalcoholic extracts of *Ferula assafoetida* killed around 90% of the larvae at 10, 50, and 100 µg/ml concentrations on day 1. Thus, it has been shown that *Ferula assafoetida* has anti-cancer activity as well as a strong anti-parasitic potential against larval forms. *In vitro* studies by Ramadan et al., (114) also demonstrated the chemoprotective activity of Assafoetida stem and root extracts against *Trichomonas vaginalis*. Hala et al., (115) used Ferula oleo gum powder and oil extracts to treat protozoans using metronidazole, an anti-protozoal drug as control, at concentrations of 2, 4, 8, 16, and 20 µg/ml for powdered extracts and 5, 10, 25, 40, and 50 mg/ml for oil extracts. The lowest concentration of both oil and powdered oleo extracts

inhibited the blastocystis infection by the protozoans. The highest concentration inhibited this species' multiplication, confirming it is a natural alternative for treating protozoan diseases. Furthermore, Farshad et al., (116) investigated the senescence reversal mechanism in human dermal fibroblasts *in vitro*. Their research found that oleo gum extracts reversed senescence in fibroblasts at 50-100 μg/ml concentrations.

1.33 *In vivo* studies

Saleem et al., (117) used Swiss albino mice in *in vivo* studies to confirm the anti-oxidant and anti-carcinogenic potential of *F. assafoetida*. The tumor was induced by topically applying phorbol ester to the skin region. Pretreating the animals with various concentrations of *F. assafoetida* confirmed the anti-oxidant nature of this plant extract. A significant difference was observed in anti-oxidant enzyme expression levels between pretreated and control groups illustrating the chemopreventive role of *F. assafoetida*. Moreover, Bagheri, (118) investigated the effect of *F. assafoetida* extracts on Alzheimer's disease prevention using active and passive avoidance tests. They concluded that these beneficial effects might be due to the presence of biologically active compounds in the extracts such as sulfur-containing and sesquiterpene coumarins. Furthermore, Bagheri et al., (119) induced demyelination by oral administration of 0.2% cuprizone through an intraperitoneal method of administration into 7-week-old C57BL/6 mice. Luxol fast blue histopathological studies on the animal brain sections confirmed that treatment *with F. assafoetida* caused the reduction of infiltrations which demonstrated the neuroprotective role of *F. assafetida* oleo gum extracts at 25 and 50 mg/kg body weights. Similarly, studies performed by Vijayalakshmi et al., (120) on male albino Wistar rats have

shown an improvement in learning and memory following treatment with *F. assafoetida* extracts at a dosage of 200 mg and 400 mg/kg body weight.

1.34 Macrophages

Macrophages are primarily considered innate immune cells that participate in various immune responses depending on their mode of generation. They are classified into two types, namely classical and alternative, which differ from one another in function. M1 macrophages are considered classical activators and are highly involved in inflammation and antitumor activity. Alternative activators, the M2 macrophages, are involved in angiogenesis, tumor cell activation, tissue remodeling, and anti-inflammatory mechanisms (121 - 123).

Many studies have reported the infiltration of macrophages and other leukocytes in establishing the tumor microenvironment. The origin of macrophages in the tumor microenvironment is still considered a mystery. There is a possibility that macrophages are incorporated in the tumor microenvironment via chemoattractants, including granulocyte macrophage-colony stimulating factor (GM-CSF), colony-stimulating factor-1 (CSF-1), and chemokine ligand 2. Several studies have found the presence of these chemotactic factors is important in creating a tumor microenvironment. As the tumor grows, a vasculature network forms, which promotes the differentiation of the spherical monocytes into tumor-induced macrophages (122).

1.35 Role of tumor-associated macrophages in cancer

Macrophages can be classification into the M1 and M2 subtypes based on different activities. Activation of classical M1 macrophages is derived from activation by T-helper 1 cells

to promote anti-tumor activities and secrete pro-inflammatory cytokines, including TNF and IL-1. In contrast, the M2 alternative macrophages mainly secrete pro-tumor factors and anti-inflammatory factors. Because of their high plasticity, their ability to differentiate into functionally different subtypes, and their presence in the tumor microenvironment, macrophages have become unique targets in oncogenic research (123).

1.36 Macrophage markers in cancer progression

Several scientific studies have implicated the involvement of macrophage markers in breast cancer progression (123). Infiltration of cells expressing these specific markers creates higher levels of tumor progression and recurrence through macrophage involvement. The involvement of cells expressing the CD68+ macrophage marker in the microenvironment promotes an increase in tumor progression that has been linked to poor prognosis (124). In contrast, some other studies have reported that the presence of CD68+ macrophages is unrelated to tumor progression and cancer proliferation. CD204 is another macrophage marker that can be used to confirm and target tumors with the worst disease prognostic conditions in breast cancer patients. The presence of macrophages expressing the CD163 (M2) marker has been reported in many TNBC and basal-like breast cancer patients and indicates a poor prognosis (125).

1.37 Macrophages and their role in TNBC disease progression

Triple-negative breast cancer cannot be cured with hormonal treatments due to the absence of ER, PR, and HER-2 (126). Tumor-associated macrophages linked to the PD-1/PD-L1 receptor/ligand self-recognition system can cause a severe disease progression state in breast

cancer. Wang et al., (126) suggested that chemokine ligand-5, in conjunction with the presence of M2-type TAM, are solely responsible for the worst levels of tumor progression in TNBC.

The role of macrophages in the tumor microenvironment suggests that it is critical to develop drugs that can target macrophage dissemination in order to impede the establishment of the pro-tumor microenvironment while also having an anti-metastatic effect against cancer progression. This approach might help patients overcome disease recurrence and increase the rates of disease-free survival.

1.38 Objectives and Experimental design

The aim of this study is to investigate the effects of *F. assafoetida extracts* on the growth and survival of different breast cancer cell lines *in vitro*. The cell viability will be assessed using a colormetric method, using the MTT cell viability assay, and the proliferative cell antigen Ki 67. Apoptotic events in the cancer cells will be detected using cell staining, western blotting, and the TUNEL assay.

This study will also examine the possible effects of *F. assafoetida* on innate immue cells. To do this, THP-1 cells, a human leukemia monocytic cell line will be used to study the effect of *F. assafoetida* on monocyte activity and macrophage differentiation activity by measuring cell viability and the expression of cell surface markers and cytokines which define the differentaion of macrophages into the M1 and M2 subtypes. THP-1 monocyte will be differentiated into macrophages using PMA (phorbol 12-myristate 13-acetate). In control experiments the THP-1 cells differentiated into macrophages (M0) will be stimulated with (IL-4) to obtain M2 polarized macrophages or with LPS in order to polarized toward the M1 subtype. THP-1 derived macrophages will also be incubated with *F. assafoetida* to determine whether *F. assafoetida* promotes differentiation of the macrophages into the M1 and/or M2 subtypes. The production of cytokines (TNF-α, IL-1 β, IL-6, IL-10 and TGF-β) will be measured using ELISA, and the cell surface markers (CD80, CD68 and CD163) will be analyzed using flow cytometry to evaluate macrophage differentiation.

This study also aimed to determine whether THP-1derived macrophages could cause damage to the cancer cell monolayers in co-culture experiments with MCF-7 and MDA-MB-231 cancer cells. To study the communication between the cancer cells and TAMs, THP-1 derived macrophages incubated with *F. assafoetida* will be co-cultured with cancer cells. In

order to assess the effect of co-culture on breast cancer cell behaviour, cell morphology and migratory assay will be performed.

The goal of the current study is also to develop and validate an HPLC (High-performance liquid chromatography) method to characterize the phenolic and flavonoid components present in *F. assafoetida* extracts.

The last aim of this research is to determine the cytotoxic effect of five known *F. assafoetida* compounds, ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid against breast cancer cell lines. Cytotoxicity will be accessed by MTT reduction and dual ethidium bromide and acridine orange staining.

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Chapter 2. Cytotoxic, anti-proliferative and apoptotic effects of ethanolic extract of *F. assafoetida* on human and murine breast cancer cell lines.

Abstract

In traditional medicine, *Ferula assafoetida (F. assafoetida),* has been shown to have a wide range of biological activities such as antiseptic, anti-diabetic, anti-inflammation, and anti-cancer activity (1). Among these, anti-cancer activity has become an important target for drug research. In the current study, we investigated the *in vitro* cytotoxicity of ethanolic extracts of *F. assafoetida* against the human, MCF-7, MDA-MB-231, and murine 4T1 breast cancer cell lines, and the non-malignant HBL-100 human breast cells. Cells were subjected to cytotoxicity and apoptosis assays including MTT viability assays, immunohistochemistry, and western blot analysis. Results showed that the ethanolic extract of *F. assafoetida,* at concentrations of 100 and 200 µg/ml, significantly decreased cell viability and promoted apoptosis induction in all of the breast cancer cell lines (MCF-7, MDA-MB-231, and 4T1) when compared to the non-malignant control. These results confirm that *F. assafoetida* is a potential source of anti-cancer compounds and that further scientific investigation is needed to reveal the mechanisms by which *F. assafoetida*'s extracts promote apoptosis induction.

Keywords: *F. assafoetida*, Apoptosis, Cytotoxicity, anticancer effects,

2 Background

Apoptosis, a process of programmed cell death, is activated when specific signaling pathways are activated. Apoptosis can be triggered via the intrinsic or the extrinsic pathways that lead to changes in cell morphology, chromatin condensation, DNA fragmentation, cell shrinkage and the generation of apoptotic bodies (2, 3). The extrinsic pathway is activated by the binding of extracellular ligands such as FasL (CD178) to a member of the tumor necrosis factor receptor super family such as CD120a, CD120b, or CD95/FAS (4) which ultimately activates a caspase cascade via activation of caspase-8. The abnormal expression of Fas/FasL has been reported to be related to the development of some common diseases, such as cancer (5). The intrinsic death pathway is mediated via mitochondria membrane permeabilization and is induced following the release of cytochrome C from the mitochondria via pores formed in the mitochondrial membrane following the activation of the pro-apoptotic Bcl-2 family members. The Bcl-2 family, which is involved in the regulation of apoptotic cell death, consists of anti-apoptotic and pro-apoptotic members which together are key regulators in maintaining the integrity of mitochondrial outer membrane (2). The anti-apoptotic members, such as Bcl-2 and Bcl-xl can block apoptosis by forming heterodimers with the pro-apoptotic family members and inhibiting the release of cytochrome C and apoptosis-inducing factor (AIF) into the cytoplasm of cells. The pro-apoptotic members of the Bcl-2 family initiate apoptosis by triggering the activation of pro-apoptotic Bax/Bak aggregates that form pores or channels allowing the release of cytochrome C in the inner-membrane space. This leads to activation of the caspase cascade via activation of caspase-9, which eventually leads to apoptosis (6).

Cancer is one of the leading causes of death in humans and more than seven million deaths occur each year, worldwide. Conventional cancer treatment, including surgery, chemotherapy, and radiotherapy, may not completely eliminate all malignant cells and prevent tumor recurrence (7). Therefore, searching for new and effective anti-cancer medicines is urgently needed. In recent years, there has been strong evidence that some herbal medicines are highly active in treating various diseases and disorders and are often much less toxic compared to standard chemotherapy drugs. Previous reports showed that more than 3000 plant species have been selected for studies on the treatment of cancer (8). Moreover, many studies have demonstrated that some natural products, when used in combination with chemotherapeutic agents, may enhance the efficacy of chemotherapy at lower doses and minimize the toxicity on normal cells (9). Some natural products demonstrate substantial efficacy by altering cancer initiation, development, and the progression of cancer, and can impact multiple mechanisms such as cell differentiation, cell proliferation, angiogenesis, metastasis as well as induce apoptosis in a variety of cancer cell types (10).

Ferula assafoetida is one of the related genera of medicinal plants that has been used as a traditional medicine for centuries. The biological activity of *Ferula* extracts may be due to the presence of multiple medicinal phytoconstituents such as terpenoids, sulfide derivatives, volatile oils, phenols, and minerals (11). Toxicity studies were performed to evaluate the safety of this extracts in Sprague Dawley rats and the results showed that short term use of *F. assafoetida* was safe at a dose of 250 mg/kg. The oleo-gum-resin obtained from the roots of *F. assafoetida* is known to possess pharmalogical activity (12). Recent pharmalogical and biological studies have pointed to the anti-oxidant, anti-microbial, anti-fungal, anti-cancer, and anti-diabetic properties of this plant. One of the important properties of this plant is its

anti-cancer effect. Previous studies have demonstrated beneficial effects from using extracts of a number of Ferula species as cancer chemopreventive agents (13 - 16). A previous study was conducted to determine whether oral administration of *F. assafoetida* inhibits mammary tumor progression in a mouse model. Briefly, 4T1 mammary carcinoma cells were implanted into the mammary fat pad of female BALB/c mice and the mice were then treated orally with a dose of 100 mg/kg *F. assafoetida* extract. This resulted in a decrease in the tumor volume and weight compared with the untreated group, and a decrease in tumour spreading to other regions of the body (16). Umbelliprenin (UMB) is an effective sesquiterpene component found in plants of the Ferula family which has been evaluated in *in vivo* and *in vitro* models for its anti-cancer effect. UMB can alter multiple activating cell signaling cascades, such as Wnt and NF-κB activation, leading to a block in cell cycle progression and suppression of cell invasion and migration (14). Ferulic acid is another effective component isolated from Ferula that has been shown to have anti-cancer effects on breast, colon and lung cancer cells. A study conducted by Zahang et al., showed that ferulic acid significantly reduced tumor volume and weight and induced apoptosis in human MDA-MB-231 breast cancer cell-derived tumours in a xenograft mouse model (17). Quercetin is another bioactive flavonoid component, present in Ferula extracts, that has been reported to have anti-cancer effects against various type of human cancer cells through supressing cell growth, inducing cell apoptosis, or improving antioxidant properties (18).

In this study, we further investigated the cytotoxic activity and the induction of apoptosis in breast cancer cells treated with *F. assafoetida* extracts. This chapter is designed to evaluate the anti-cancer effects of an ethanolic extract of this plant against malignant and

non-malignant breast cell lines. The data suggested a potential effectiveness of *F. assafoetida* for inducing cell death in breast cancer cells.

2.1 Materials and Methods

All procedures were performed in the laboratories at HSNRI and the Biology Department in Laurentian University of Sudbury in the period from September 2016 to June 2021.

2.1.1 Collection and extraction of Plant Materials

Samples of F. *assafoetida* were purchased from a well-known herbal medicine supplier (Almarwani, AL Madinah**,** Saudi Arabia) in August 2016. The shade dried herbs were stored in dried sterile containers at room temperature until use. For the preparation of ethanolic extracts, the dried plant product was powdered and extracted with 70% ethanol due to its higher polarity than pure ethanol. 10 g of *F*. *assafoetida* was suspended in 100 ml 70% ethanol and then boiled under reflux for 1 h. The mixture was allowed to cool to room temperature and was centrifuged at 400 x g for 10 min to remove insoluble material. The supernatant was then collected and filtered through a 0.22 μm syringe filter and then the filtrate stored in small aliquots in a -80 °C freezer.

2.1.2 Tissue culture

The 4T1 (murine mammary triple-negative carcinoma cell line), MCF-7 (human ER-positive breast cancer), MDA-MB-231 (human triple negative breast cancer), were obtained from ATCC [American Type Culture Collection, Manassas, VA] and the HBL-100 (human breast) cells were obtained from K. Yamada, [National Institutes of Health, Bethesda, USA]. The 4T1 cells were maintained in tissue culture plates (Sarstedt, Laval, QC) in Roswell Park Memorial Institute medium (RPMI 1640, Fisher-Hyclone, Toronto, ON) supplemented with 10% fetal bovine serum (FBS, Fisher-Hyclone), 100 μg/ml streptomycin, and 100 U/ml

penicillin (Invitrogen, Burlington, ON). The MCF-7, MDA-MB-231, and HBL-100 cells were maintained in tissue culture plates in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin. The cells were incubated in a humidified atmosphere at 5% CO2 and 37 °C in a tissue culture incubator. For experiments, the cells were treated with media or media including *F. assafoetida* extracts at different concentrations. All experiments were performed in independent triplicate experiments for each assay.

2.1.3 Cell Treatments

The MCF-7, MDA-MB-231, HBL-100, and 4T1 cells were cultured to confluent monolayers in media as described above. For all experiments, the cells were washed with PBS and harvested by incubation in 2 ml 0.25% trypsin, collected by centrifugation at 350 x g for 5 min, and resuspended in fresh medium at the desired concentration before plating. The selected concentrations were derived from optimization experiments, to a range between (1 µg/ml- 400 µg/ml). For plating to a 100 mm plate, 5–10 ml of cell suspension; 60 mm plate, 2 ml of cell suspension; or 100 µl of the cell suspensions onto each well of a 96 well culture plate (i.e., 2000 cells/well) was shown to work well. After incubating overnight, the cells were treated with culture media or different doses (25 µg/ml, 100 µg/ml, or 200 µg/ml) of ethanolic extract of F. *assafoetida* suspended in the appropriate culture media containing 10% FBS and incubated at 37°C in 5% CO2. The cells were treated with suspending media including the appropriate solvents at the same doses to generate negative controls.

2.1.4 MTT assay (Methyl Tetrazolium Blue)

The MTT assays were performed as previously described (27). Briefly, the cells were seeded in 96-well plates at a density of 2000 cells/well and allowed to attach overnight. Afterward, fresh media (DMEM or RPMI) or media containing various concentrations (25 µg/ml, 100 µg/ml, or 200 µg/ml) of ethanolic extract of *F. assafoetida* were added and the plates and incubated for the indicated period of exposure. Each day, for four days, one plate had 10 µl/ml of 0.4 µg/ml MTT in PBS, pH 7.4, added to each well and incubated for 4 h. After removal of the supernatant, 100 µL dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan product, and the absorbance (optical density (OD) value) was measured at 540 nm using a plate reader (SpectraMax 340 PC 389). Tests were performed in triplicate for at least three independent times, and the mean value was calculated to determine the relative number of viable cells and to estimate the differences in proliferative activity using an ANOVA using Graph Pad Prism Software. The inhibition percentage was calculated using this formula:

$$\%\ \text{inhibition} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100$$

2.1.5 Fluorescence Immunohistochemistry for Ki-67 protein expression

The level of the Ki-67 protein can be used as a nuclear proliferation marker for tumor cells, it is expressed in all proliferating cells in G1, S, G2, and M phases of the cell cycle, making it an excellent marker to assess whether the tumor cells are actively proliferating (26). 4T1 cells were seeded on sterile glass coverslips in 6-well plates in culture media overnight at 37◦C. (The assay was not conducted on MCF-7, MDA-MB-231, or HBL-100 cells.) The

cells on the coverslips were treated with suspending media or with media containing 25 µg/ml, 100 µg/ml, or 200 µg/ml of *F. assafoetida* extracts and incubated for 24 or 48 h. The treated cells were fixed by incubation with 4% formaldehyde in PBS, pH 7.4, for 5 min at room temperature. The cells were then washed three times with PBS, pH 7.4. Since Ki-67 is an intracellular protein, the cells were permeabilized by incubation in 0.1% Triton X-100 in PBS, pH 7.4, at 4°C for 5 min and washed with PBS three times. The cells were incubated in 10% FBS, 0.5% BSA in PBS, pH 7.4, to block nonspecific binding of the antibodies, overnight at 4°C. The cells were stained by incubation with 100 µl primary anti-Ki-67 mouse monoclonal antibody (Santa Cruz Biotech., Santa Cruz, CA; titre 1:20) in blocking buffer for 60 minutes at room temperature. After discarding the primary antibody, the coverslips were rinsed with PBS, pH 7.4, three times for 5 min and 100 µl of the secondary fluorescent antibody-FITC conjugate (green) (Santa Cruz Biotech, titre 1:100) was applied in blocking buffer for 60 min at room temperature. The coverslips were then washed three times for 5 min in PBS and mounted with a drop of 80% glycerol on a glass slide and sealed with nail polish to prevent drying and movement under the microscope. The slides then were ready for imaging using a fluorescence microscope (Zeiss Canada), or were stored in the fridge for a short period of time until use.

2.1.6 TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling)

The TUNEL assay was used to detect cells undergoing apoptosis. This assay relies on the enzyme, terminal deoxynucleotide transferase (TdT), which attaches deoxynucleotides to the 3'-hydroxyl terminus of DNA breaks (25). 4T1 and MCF-7 cells were seeded on sterile glass coverslips in 6-well plates in culture media overnight at 37◦C. (The assay was not

performed on MDA-MB-231 or HBL-100 cells.) The cells on the coverslips were treated with suspending media or with media containing 25 µg/ml, 100 µg/ml, or 200 µg/ml of *F. assafoetida* extracts and incubated for 24, 48, or 72 h. The treated cells were fixed by incubation with 4% formaldehyde in PBS, pH 7.4, for 5 min at room temperature, washed three times with PBS, pH 7.4., and then permeabilized by incubation in 0.1% Triton X-100 in PBS, pH 7.4, at 4°C for 5 min and washed with PBS three times. In each well, 50 µl of TUNEL reaction mixture (Roche, Laval, QB) was added and incubated for a period of 1 h at room temperature. The coverslips were then washed three times for 5 min in PBS and mounted with a drop of 80% glycerol on glass slides and sealed with nail polish to prevent drying and movement under the microscope. The slides then were ready for imaging using a fluorescence microscope (Zeiss Canada), or were stored in the fridge at 4°C for a short period of time until use.

2.1.7 Western blot analysis

The aim of this experiment was to detect changes in the levels of specific proteins in the cell lysates. Briefly, cells were cultured on 100 mm^2 plates, treated with suspending media or with media containing 25 µg/ml, 100 µg/ml, or 200 µg/ml of *F. assafoetida* extracts and incubated for 24, 48, or 72 h. The cells were collected in RIPA buffer (0.5% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 150 mM sodium chloride, and 25 mM Tris-HCl, pH 7.4) and lysed. Protein concentrations were determined using a BCA assay and 25 µg of cell lysate from each treatment group was separated by 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and then transferred to a nitrocellulose membrane (Whatman, Mandel Sci., Burlington, ON) using a BioRad semi-dry transfer machine for 60 min at 12 V. After transfer, the blots were stained with 0.5% Ponceau S in 1% acetic acid for 5 min, rinsed

with distilled water for 1–2 min, and photographed. Next, the membranes were blocked by incubation in blocking buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 0.05% Tween-20 (TBST) and 5% bovine serum albumin (BSA, Sigma), followed by incubation with primary antibody (against Caspase-9, Caspase-8, Bcl-2, or Bax, with GAPDH as a loading control (Santa Cruz Biotech., Santa Cruz, CA) overnight at 4º C. The blots were washed and then incubated with an appropriate secondary antibody (goat anti-mouse IgG-HRP or anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and then washed twice with TBST followed by one wash with TBS. The membrane was incubated in the ECL reagent (Enhanced Chemiluminescence, GE Health care) for 5 min and then exposed to X-ray film. The intensity of the bands was examined by densitometry and plotted as a fraction of the untreated control condition. The relative levels of each band were determined following normalization to the corresponding GAPDH loading control. The relative level of the cleaved caspase-9 band was also determined by normalization to the uncleaved caspase-9 band.

2.1.8 Cell Staining assay (Acridine Orange/Ethidium Bromide)

A cell staining experiment was performed to visualize any nuclear changes associated with apoptosis in response to treatment with *F. assafoetida*. The cells were cultured in 60 mm dishes and treated with 25 µg/ml, 100 µg/ml, or 200 µg/ml *F. assafoetida* extracts, The cells were treated with the compounds for 48 h and then stained with 10 µg/ml of acridine orange (Sigma-Aldrich) and 10 µg/ml of ethidium bromide (Sigma- Aldrich, St. Louis, MO) for 15 minutes. The fluorescently labelled cells were visualized on an LSM 510 fluorescence

microscope (Zeiss Canada, Mississauga, ON) and phase contrast (Ph), acridine orange (green) and ethidium bromide (red) images were captured.

2.1.9 Statistical analysis

The cell viability data were expressed as the mean ± standard deviation of three separate experiments and were analyzed using the Graph Pad Prism program and significant differences confirmed by ANOVA * ($P < 0.05$). Post-hoc analysis for differences between treatments was done using Tukey tests.

2.2 Results

2.2.1 Effect of *F. assafoetida* extracts on the viability and proliferation of malignant and non-malignant breast cell lines

An MTT assay was performed to test the *in vitro* effect of *F. assafoetida* against the viability of malignant (MCF-7, MDA-MB-231, or 4T1) and non-malignant (HBL-100) cell lines. In our investigation, we found that cells treated at a concentration as high as 200 µg/ml caused significant toxicity to MCF-7, MDA-MB-231, or 4T1 cancer cells and non-malignant, HBL-100 cell lines. However, a significant difference in effect was observed between the four cell lines ($p<0.05$) (Figure 2.1, Figure 2.2, Figure 2.3 and Figure 2.4). Moreover, a similar significant inhibitory effect was observed when MCF-7, MDA-MB-231, or 4T1 cells were treated with the medium dose at a concentration of 100 µg/ml, particularly at day three and four ($p<0.05$). However, this effect was not detected in the non-malignant HBL-100 cell line. In contrast, treatment of MCF-7, MDA-MB-231, or 4T1 cells and non-malignant HBL-100 cells with the low concentration of 25 µg/ml had no significant effect on cell viability compared to untreated control cells (Figure 2.1, Figure 2.2, Figure 2.3 and Figure 2.4).

In brief, the ethanolic extract of *F. assafoetida* was able to effectively decrease cell viability between different cell types. Additionally, the sensitivity to *F. assafoetida* varied from cell line to cell line with the non-malignant HBL-100 cells being the most resistant.

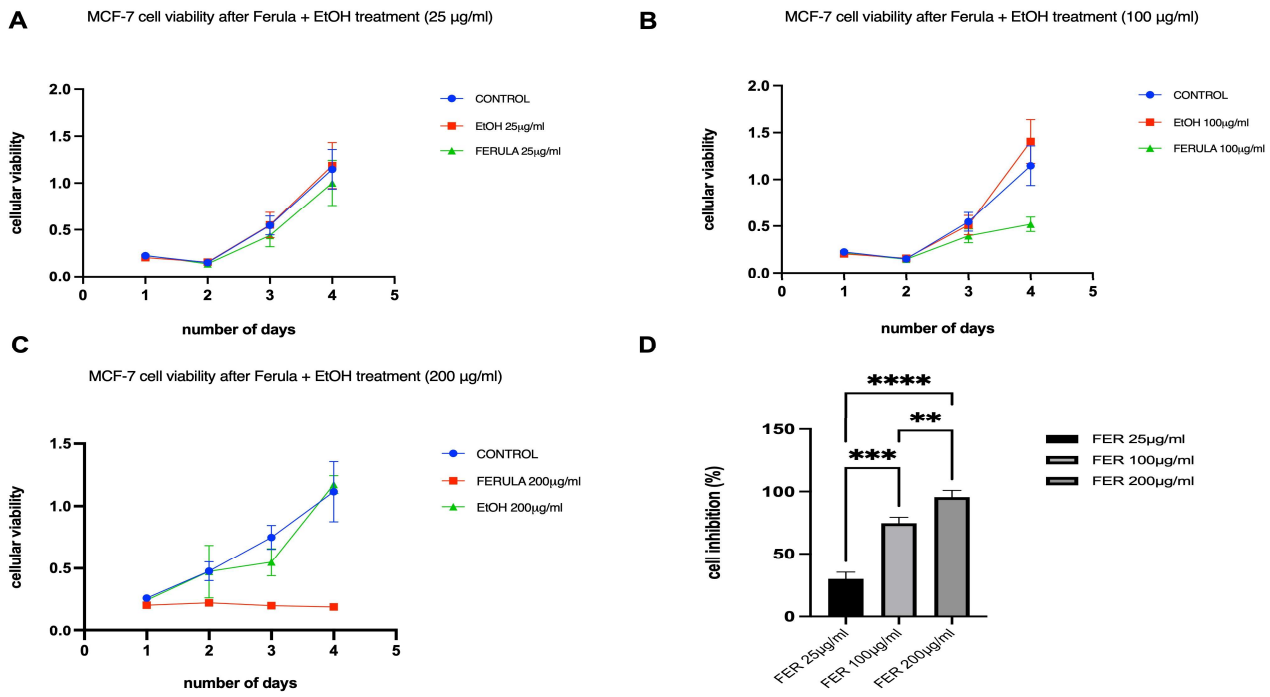

Figure 2.1: Viability Assays for MCF-7 Malignant Cell Line treated with *F. assafoetida*.

Viability assays were performed for the MCF-7 malignant cell line treated with (A) 25 µg/ml, (B) 100 µg/ml, or (C) 200 µg/ml of *F. assafoetida* extracted with 70% ethanol versus control media using the MTT assay. Panel (D) shows the relative viability of MCF-7 cells treated with *F. assafoetida* extracts (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the *F. assafoetida* extract for four days and absorbance was determined each day. Data were analyzed for at least three independent experiments and a p-value less than 0.05 (for n=3) is statistically significant.

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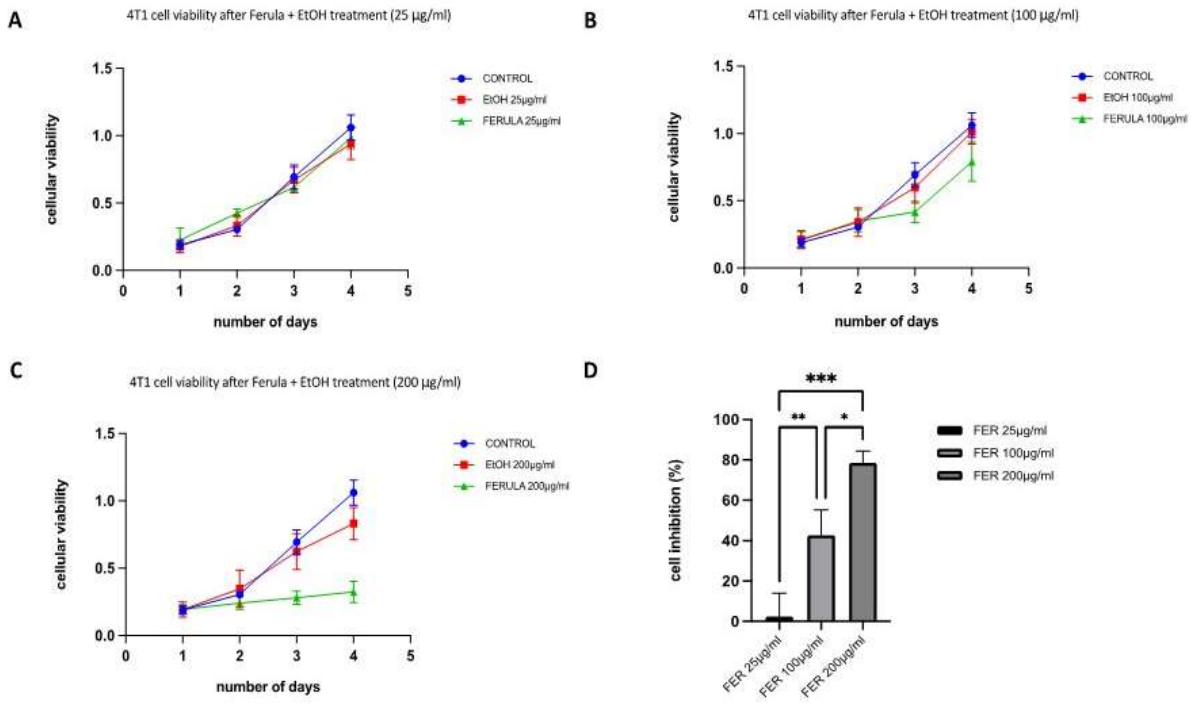

Figure 2.2: Viability Assays for 4T1 Malignant Cell Line treated with *F. assafoetida*.

Viability assays were performed for the 4T1 malignant cell line treated with (A) 25 µg/ml, (B) 100 µg/ml, or (C) 200 µg/ml of *F. assafoetida* extracted with 70% ethanol or control media using an MTT assay. Panel (D) shows the relative viability of 4T1 cells treated with *F. assafoetida* extracts (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the *F. assafoetida* extract for four days and the absorbance was determined each day. Data were analyzed for at least three independent experiments and a p-value less than 0.05 (for n=3) is statistically significant.

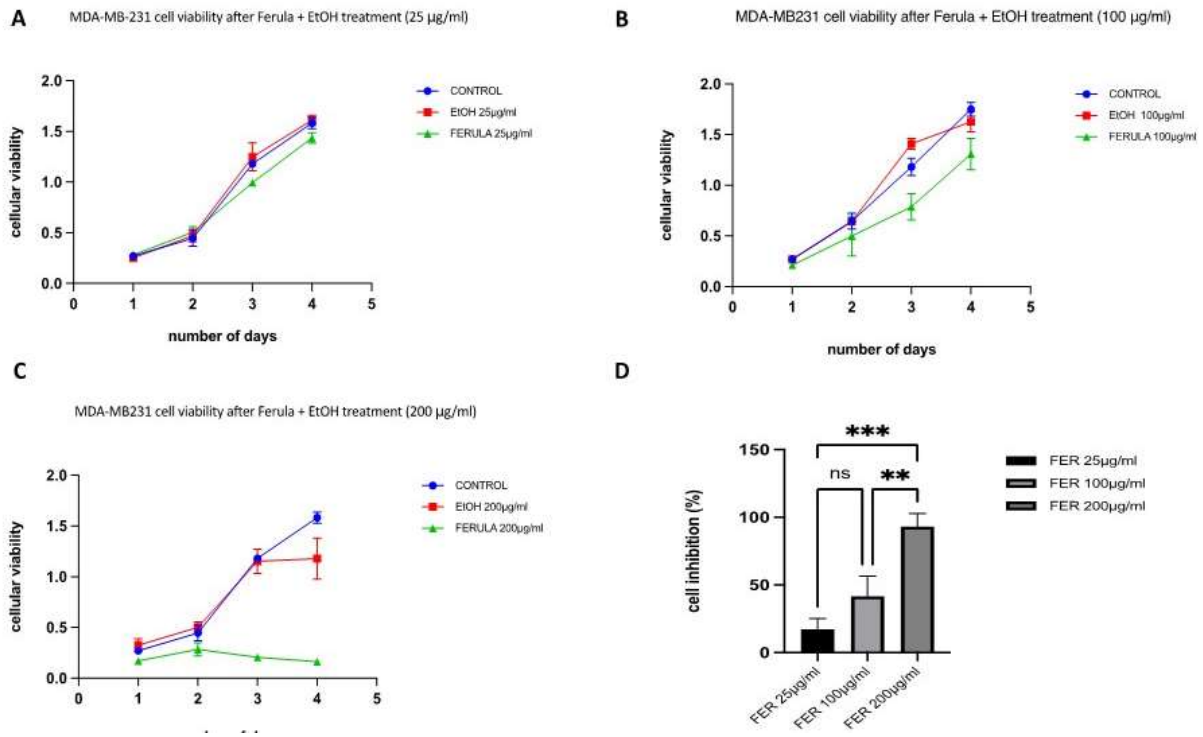

Figure 2.3: Viability Assays for the MDA-MB-231 Malignant Cell Line treated with *F. assafoetida*.

Viability assays were performed for the MDA-MB-231 malignant cell line treated with (A) 25 µg/ml, (B) 100 µg/ml, or (C) 200 µg/ml of *F. assafoetida* extracted with 70% ethanol or control media using the MTT assay. Panel (D) shows the relative viability of MDA-MB-231 cells treated with *F. assafoetida* extracts (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the *F. assafoetida* extract for four days and the absorbance was determined each day. Data were analyzed for at least three independent experiments and a p-value less than 0.05 (for n=3) is statistically significant.

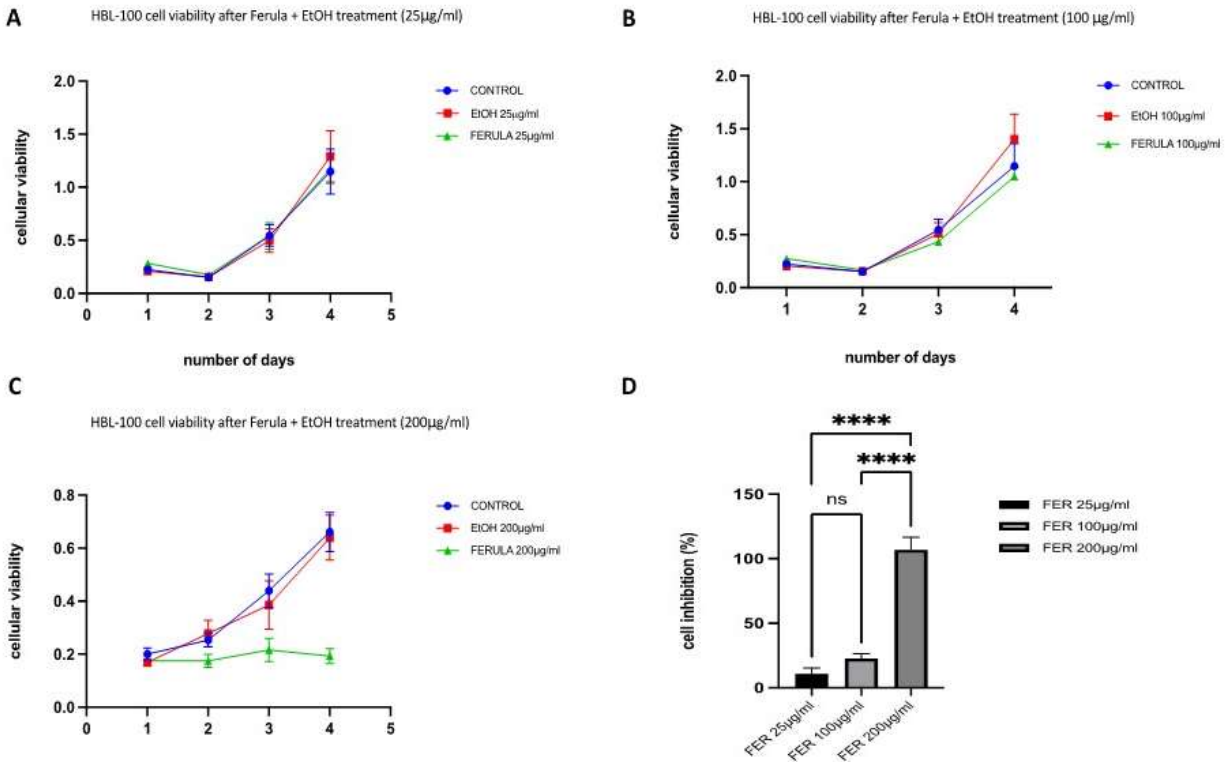

Figure 2.4: Viability Assays for the HBL-100 Non-Malignant Cell Line treated with *F. assafoetida*.

Viability assays were performed for the HBL-100 non-malignant cell line treated with (A) 25 µg/ml, (B) 100 µg/ml, or (C) 200 µg/ml of *F. assafoetida* extracted with 70% ethanol or control media using an MTT assay. Panel (D) shows the relative viability of HBL-100 cells treated with *F. assafoetida* extracts (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the *F. assafoetida* extract for four days and the absorbance was determined each day. Data were analyzed for at least three independent experiments and a p-value less than 0.05 (for n=3) is statistically significant.

2.2.2 Detection of apoptosis

As an additional test to show apoptosis, we performed an assessment of cell morphology following double, Acridine Orange (AO)/Ethidium Bromide (EtBr) fluorescent staining, imaged using a fluorescent microscope after exposure to 25 µg/ml, 100 µg/ml, and 200 µg/ml *F. assafoetida* extract for a 48 h incubation period. The AO/EtBr staining experiment is capable of differentiating between viable and apoptotic cells based on nuclear morphology and membrane integrity. If the cell is viable, the Acridine Orange (AO) binds to the DNA, making the cell nucleus stain green. However, if the cell is apoptotic, the Ethidium Bromide (EtBr) also binds to the DNA, making the cell nucleus stain red. Thus, apoptotic cells will be labelled bright green and red due to morphological changes, such as membrane blebbing chromatin condensation and nuclear conformation. Apoptotic cells exhibit fragmented and condensed green and red chromatin, while viable cells exhibit a regular green nucleus. The resulting images from the cell staining assay using AO/EtBr revealed that malignant cell lines treated with the low dose of 25 µg/ml *F. assafoetida* for 48 h showed some smaller changes in nuclear staining with a very low level of ethidium bromide staining (Figure 2.5, Figure 2.6, Figure 2.7, and Figure 2.8). Treatment with the middle dose of 100 µg/ml *F. assafoetida* showed bright green dots in the nuclei with increased level of ethidium bromide staining due to nuclear fragmentation and chromatin condensation. Malignant cell lines that were treated with the high dose of 200 µg/ml *F. assafoetida* showed significant changes in cell morphology such as membrane blebbing and complete damage of nuclear structure which occurs during the late stages of apoptosis (Figure 2.5, Figure 2.6, and Figure 2.7). The non-malignant cell line HBL-100 did not show changes in cell morphology when treated with the low and middle dose *of F. assafoetida*. However, when HBL-100 cells were

treated with the high dose it showed a significant change in cell morphology consistent with induction of apoptosis (Figure 2.8).

In order to detect DNA strand breaks and DNA fragmentation, which are key features of late-stage apoptosis, we further performed TUNEL assays. This procedure relies on the enzyme terminal deoxynucleotide transferase (TdT), which attaches deoxynucleotides to the 3'-hydroxyl terminus of DNA breaks. This assay was not performed on the HBL-100 and MDA-MB-231 cell lines since the original plan was to assess histological sections from murine tumors using the TUNEL assay and compare to the 4T1 in vitro results. Unfortunately, the animal work could not be completed as planned but that the TUNEL assay for the 4T1 cell line was performed in lieu of a full experiment with animal work. MCF-7 and 4T1 cells were cultured on coverslips and treated with 25 μg/ml, 100 μg/ml, or 200 μg/ml of *F. assafoetida* extracts for 24, 48, and 72 h to assess the effects of incubation time on the inhibition of cell viability, and then stained with the TUNEL reaction mixture. DNA fragmentation increased in a dose and time-dependent manner: at 24 h, cells showed a slight increase in TUNEL staining compared to the untreated cells while treatment for 48 and 72 h showed significant levels of TUNEL staining indicating that the cells are undergoing apoptosis (Figure 2.9 and Figure 2.10).

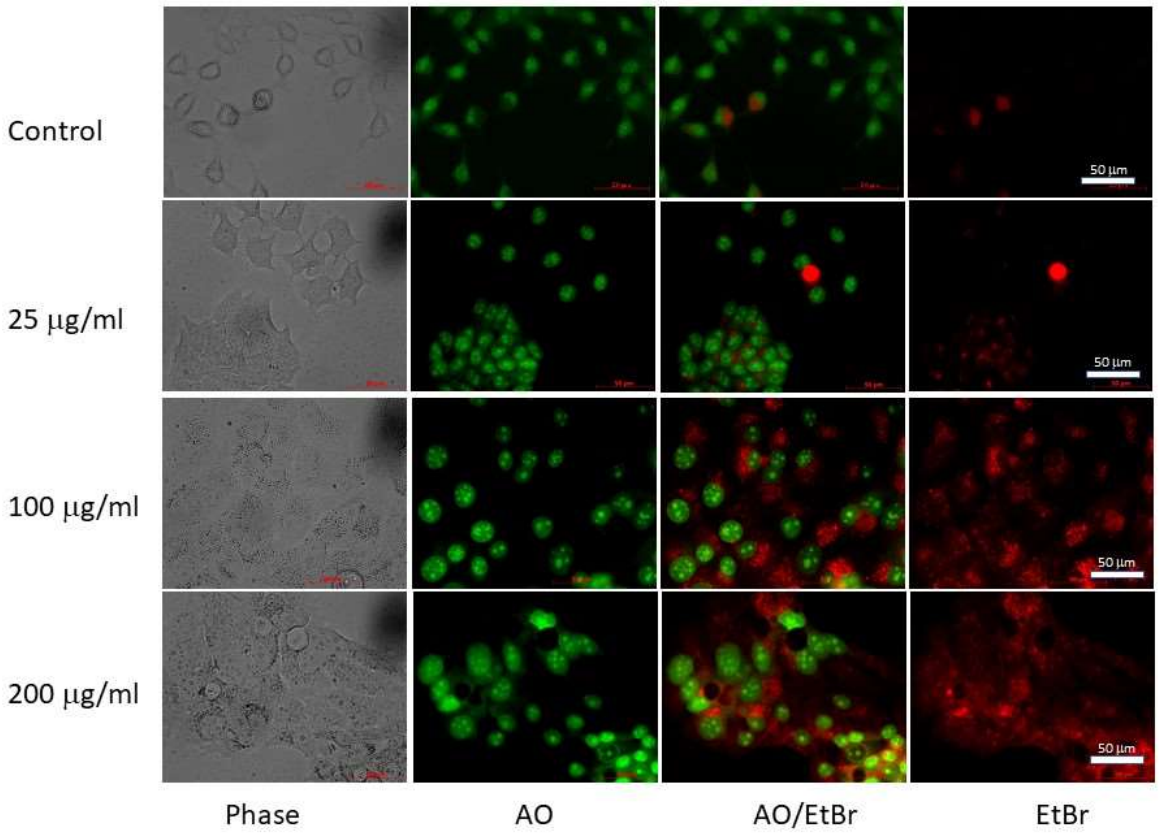

Figure 2.5: Apoptosis detected by AO/EtBr double staining of *F. assafoetida*-treated MCF-7 cells

Morphological study of MCF-7 cells cultured in 60 mm dishes and treated with different concentrations of *F. assafoetida* (25 µg/ml, 100 µg/ml, and 200 µg/ml) and analyzed after 48 h of treatment. The figure represents images viewed and captured using a Fluorescence microscope. Images were captured at different settings, Ph: Phase contrast, AO: Acridine Orange, EtBr: Acridine Orange and AO/ EtBr: Acridine Orange/Acridine Orange. Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells were red with highly condensed nuclei.

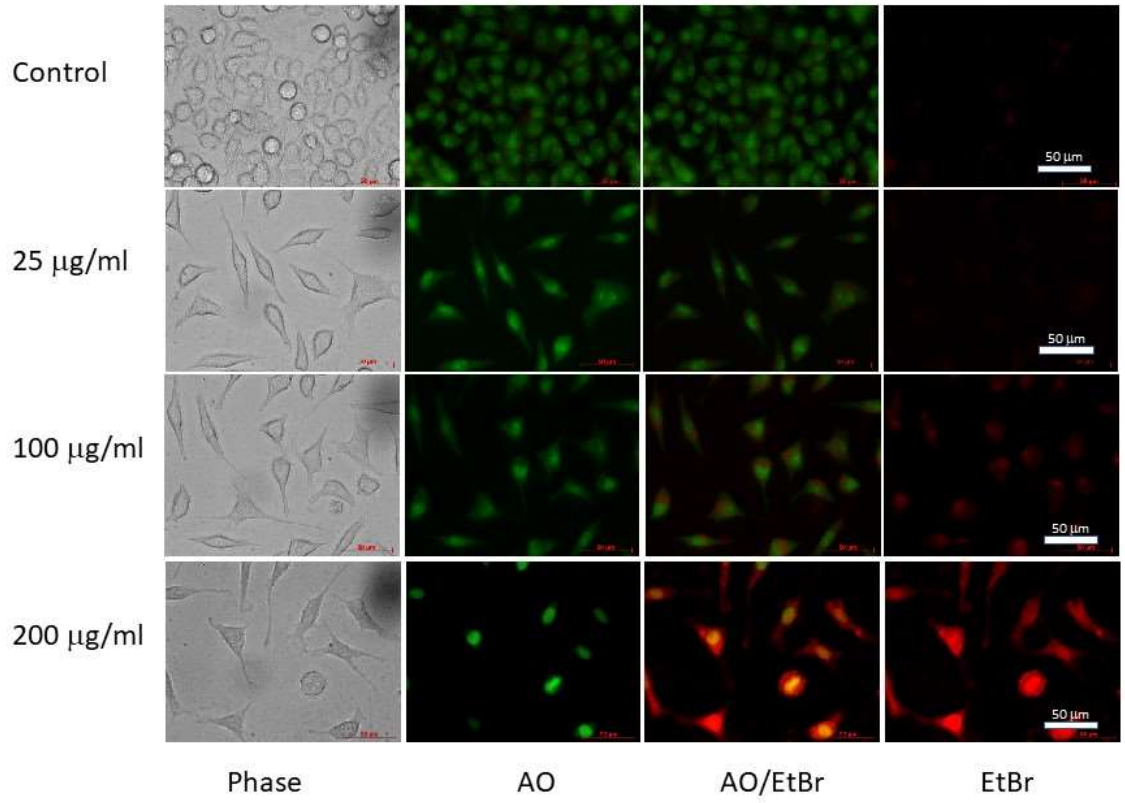

Figure 2.6: Apoptosis detected by AO/EtBr double staining of *F. assafoetida*-treated MDA-MB-231 cells

Morphological study of MDA-MB231 cells cultured in 60 mm dishes and treated with different concentrations of *F. assafoetida* (25 µg/ml, 100 µg/ml, and 200 µg/ml) and were analyzed after 48 h of treatment. The figure represents images viewed and captured by Fluorescence microscope. Images were captured at different settings, Ph: Phase contrast, AO: Acridine Orange, EtBr: Ethidium Bromide and AO/ EtBr: Acridine Orange/Acridine Orange. Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells were red with highly condensed nuclei.

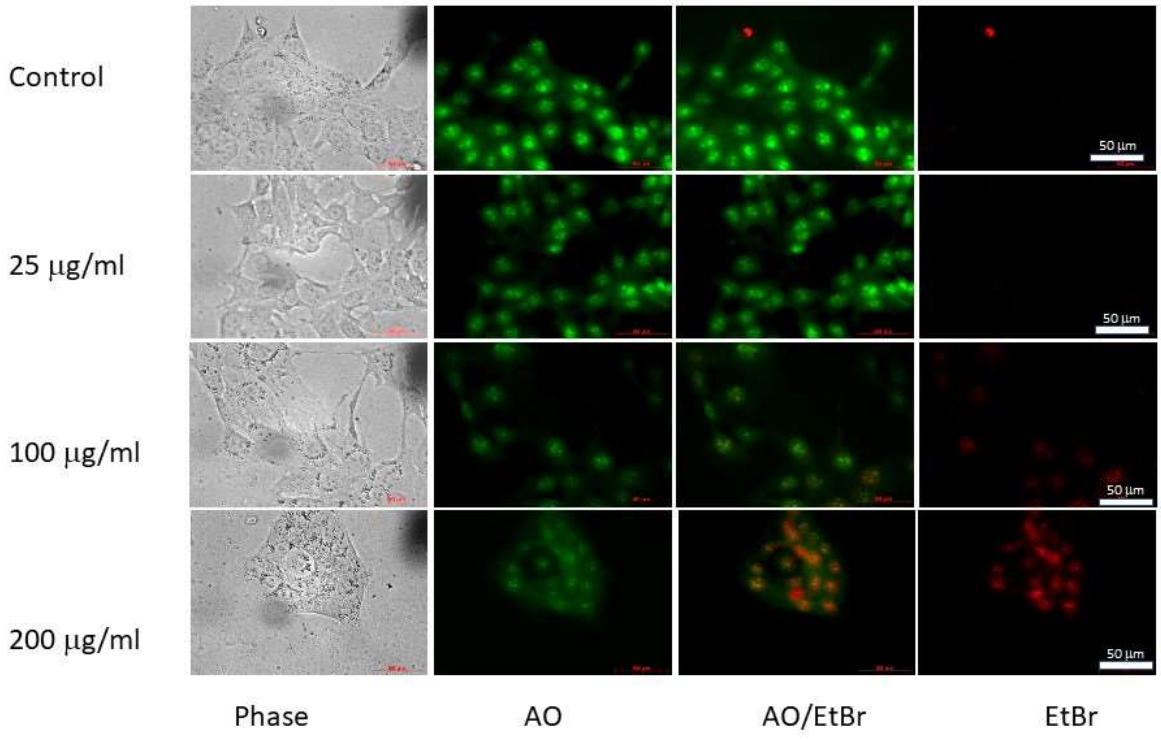

Figure 2.7: Apoptosis is detected by AO/EtBr double staining of *F. assafoetida*-treated 4T1 cells

Morphological study of 4T1 cells cultured in 60 mm dishes and treated with different concentrations of *F. assafoetida* (25 µg/ml, 100 µg/ml, and 200 µg/ml) and were analyzed after 48 h of treatment. The figure represents images viewed and captured by Fluorescence microscope. Images were captured at different settings, Ph: Phase contrast, AO: Acridine Orange, EtBr: Ethidium Bromide and AO/ EtBr: Acridine Orange/Acridine Orange. Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells were red with highly condensed nuclei.

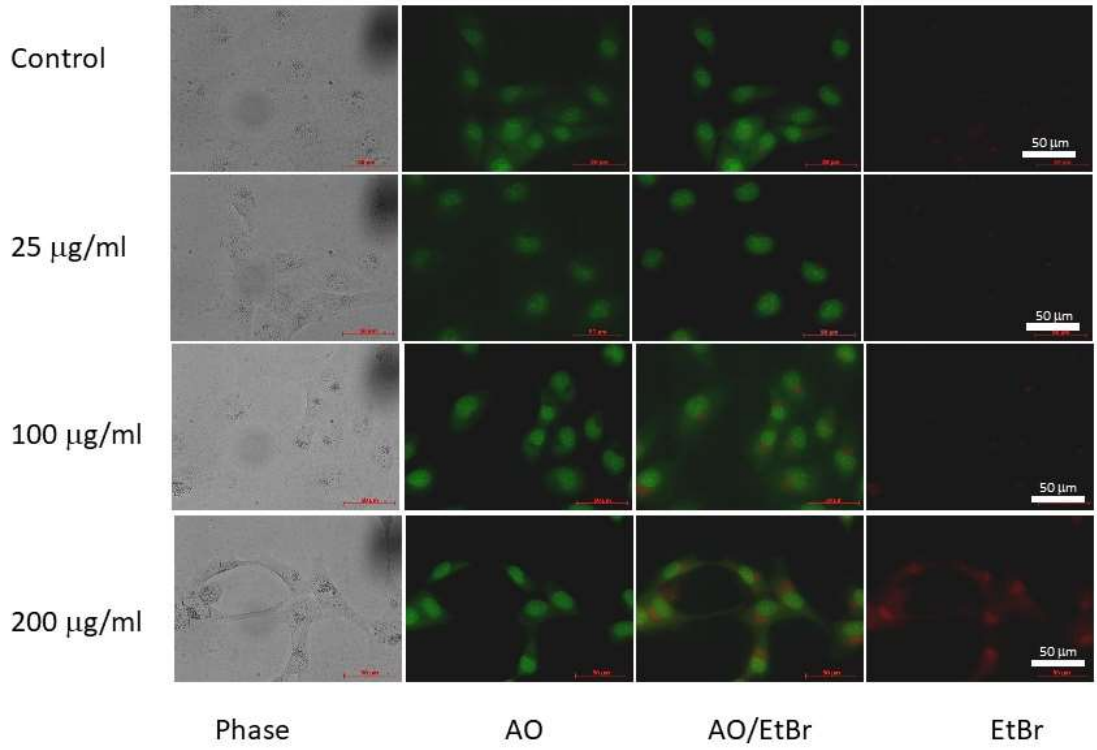

Figure 2.8: Apoptosis is detected by AO/EtBr double staining of *F. assafoetida*-treated HBL-100 cells

Morphological study of HBL-100 cells cultured in 60 mm dishes and treated with different concentrations of *F. assafoetida* (25 µg/ml, 100 µg/ml, and 200 µg/ml) and were analyzed after 48 h of treatment. The figure represents images viewed and captured by Fluorescence microscope. Images were captured at different settings, Ph: Phase contrast, AO: Acridine Orange, EtBr: Ethidium Bromide and AO/ EtBr: Acridine Orange/Acridine Orange. Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells were red with highly condensed nuclei.

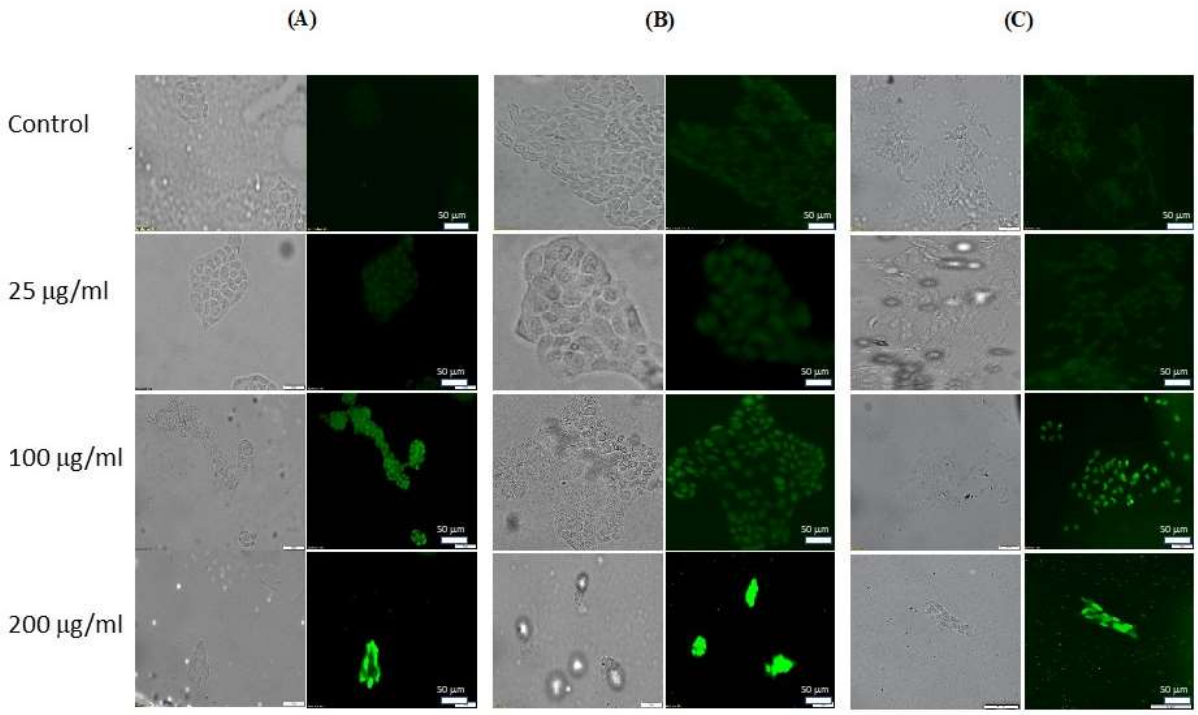

Figure 2.9: Identification of DNA fragmentation using a fluorescent TUNEL assay in MCF-7 cells treated with *F. assafoetida* extracts

MCF-7 cells were plated on glass coverslips and treated with different concentrations of *F. assafoetida* extracts (25 µg/ml, 100 µg/ml, and 200 µg/ml) and incubated for (A) 24, (B) 48, and (C) 72 h. The negative control represents treatment with only culture media. Cells were fixed and stained with TUNEL reaction mixture following the manufacturer's instructions and then analyzed using fluorescence microscope to detect labelled cells (green).

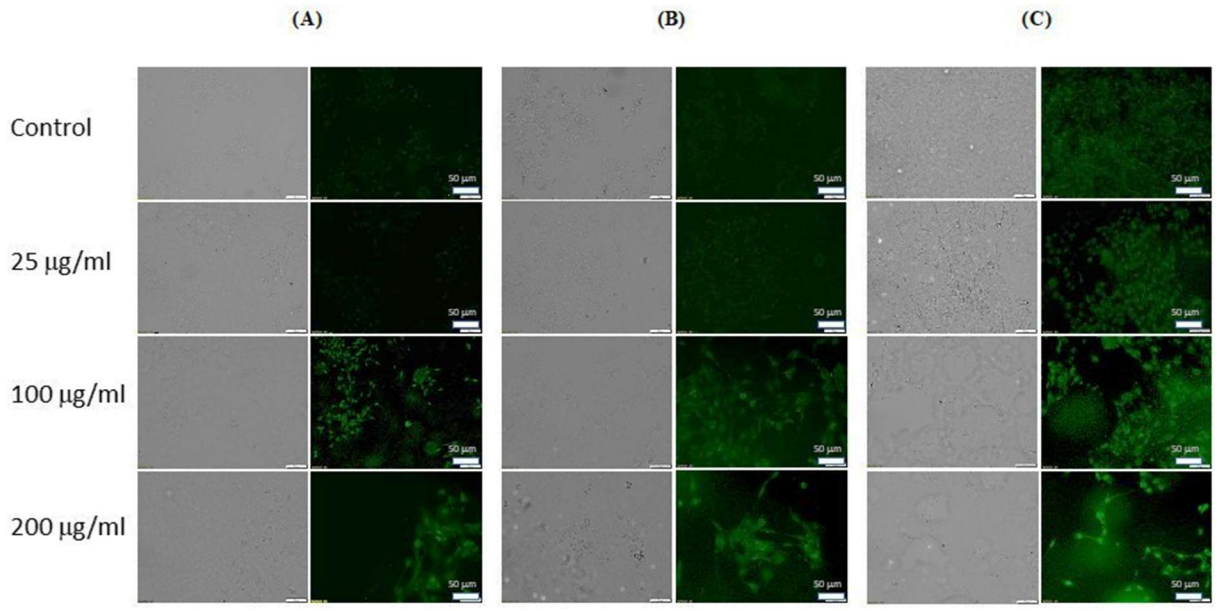

Figure 2.10: Identification of DNA fragmentation by fluorescent TUNEL assay in 4T1 cells treated with *F. assafoetida* extracts

4T1 cells were plated on glass coverslips and treated with different concentrations of *F. assafoetida* extracts (25 µg/ml, 100 µg/ml, and 200 µg/ml) and incubated for (A) 24, (B) 48, and (C) 72 h. The negative control represents treatment with only culture media. Cells were fixed and stained with TUNEL reaction mixture following the manufacturer's instructions and then analyzed using fluorescence microscope to detect labelled cells (green).

2.2.3 Inhibition of the proliferation of 4T1 cancer cells detected using Ki-67 protein expression

Inhibition of cell proliferation in breast cancer cell lines was also confirmed by detecting down regulation in the level of expression of Ki-67, an appropriate cellular marker for studying cell proliferation. It is expressed in the G1-, G2-, S-, and M-phases of the cell cycle and is absent in resting cells. The expression level of Ki-67 in normal breast is considered to be very low, whereas in breast cancer it is expressed at a much higher level, which has made it an excellent marker for determining tumor growth (19). Therefore, in this study we investigated the effect on the proliferation of 4T1 cell line after treatment with *F. assafoetida* extract for 24 h and 48 h. Our results demonstrated that the expression level of Ki-67 was decreased after 4T1 cells were treated with *F. assafoetida* extracts. In particular, treatment of 4T1 cells with the highest dose of *F. assafoetida* extract significantly reduced the Ki-67 expression level in cells treated with extract at both 24 h and 48 h. Cells treated with the medium dose of the *F. assafoetida* extract for 24 h caused a slight but not significant decrease in Ki-67 expression, whereas cells treated for 48 h showed a significant reduction in the expression level of Ki-67. However, a significant difference in staining at the low dose was not observed when compared with the untreated group. These findings correspond with the observations from the cell viability experiment (Figure 2.11).

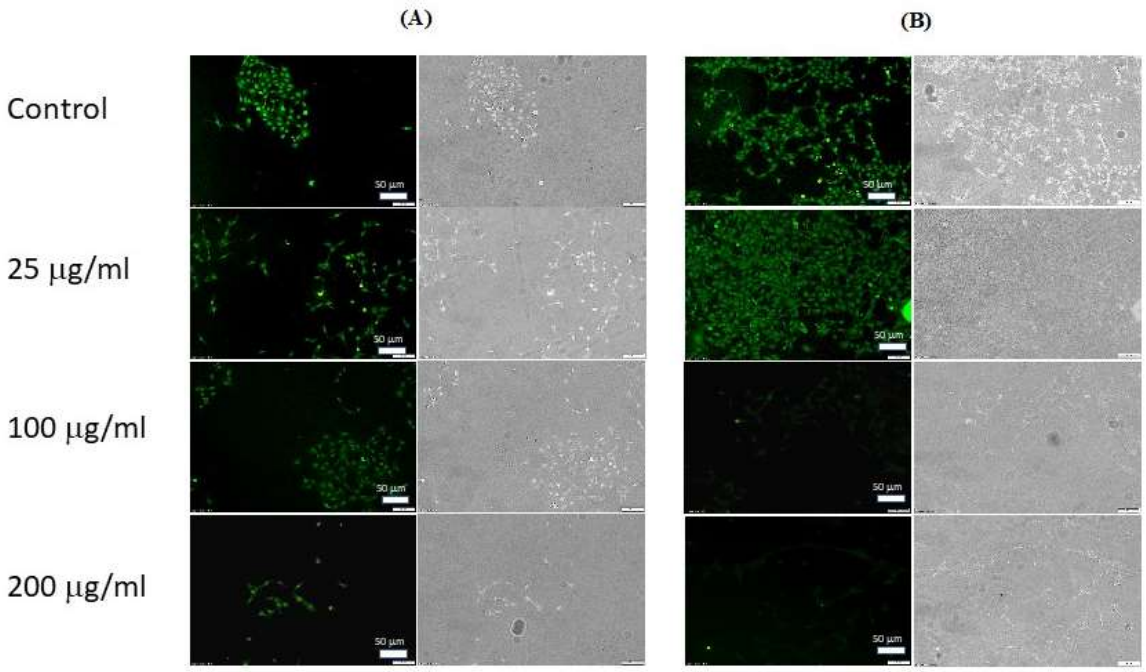

Figure 2.11: Inhibition of the proliferation of 4T1 cancer cells treated with *F. assafoetida* extracts detected using Ki-67 protein expression

4T1 cells were plated on glass coverslips and treated with different concentrations of *F. assafoetida* extracts (25 µg/ml, 100 µg/ml, and 200 µg/ml) and incubated for (A) 24 and (B) 48 h. The negative control represents treatment with only culture media. Cells were fixed and stained with primary anti-Ki-67 mouse monoclonal antibody and then analyzed using a fluorescence microscope to detect labelled cells (green).

2.2.4 *F. assafoetida* induced apoptosis through activation of the intrinsic pathway of apoptosis

Western blotting was used to determine the level of expression of specific proteins that play essential roles in programmed cell death. In particular, the expression levels and activation of caspase-9, and caspase-8, and the expression levels of Bax, and Bcl-2 were analyzed, after the cells were treated with different concentrations of *F. assafoetida* for 72 h. The total protein concentration of each sample was calculated using the BCA Assay kit and normalized. GAPDH was used as the control to ensure that a constant amount of protein was loaded into each lane of the gel. Figure 2.12 shows that the protein expression level of activated apoptotic proteins caspase-9, caspase-8, Bax, and Bcl-2 are changed after the MCF-7, MDA-MB-231, and 4T1 malignant breast cancer cells were treated with different concentrations of *F. assafoetida* for 72 h. These results indicated that the expression level of Bcl-2 protein was significantly lower in the treated groups than in the untreated group, whereas the expression level of Bax was increased. Western blot analysis also showed that there were significant expression levels of cleaved caspase-9 in the treated groups. Overall, western blotting analysis indicated that *F. assafoetida* may increase the expression levels of various activated forms of caspase-9 in a dose-dependent manner. To investigate the effect of *F. assafoetida* on extrinsic apoptosis, the expression level of caspase-8 was measured using western blotting, after treatment with various concentrations of *F. assafoetida* for 72 h. The result indicated that only pro-caspase-8, but not the cleaved caspase, was detected in the cells.

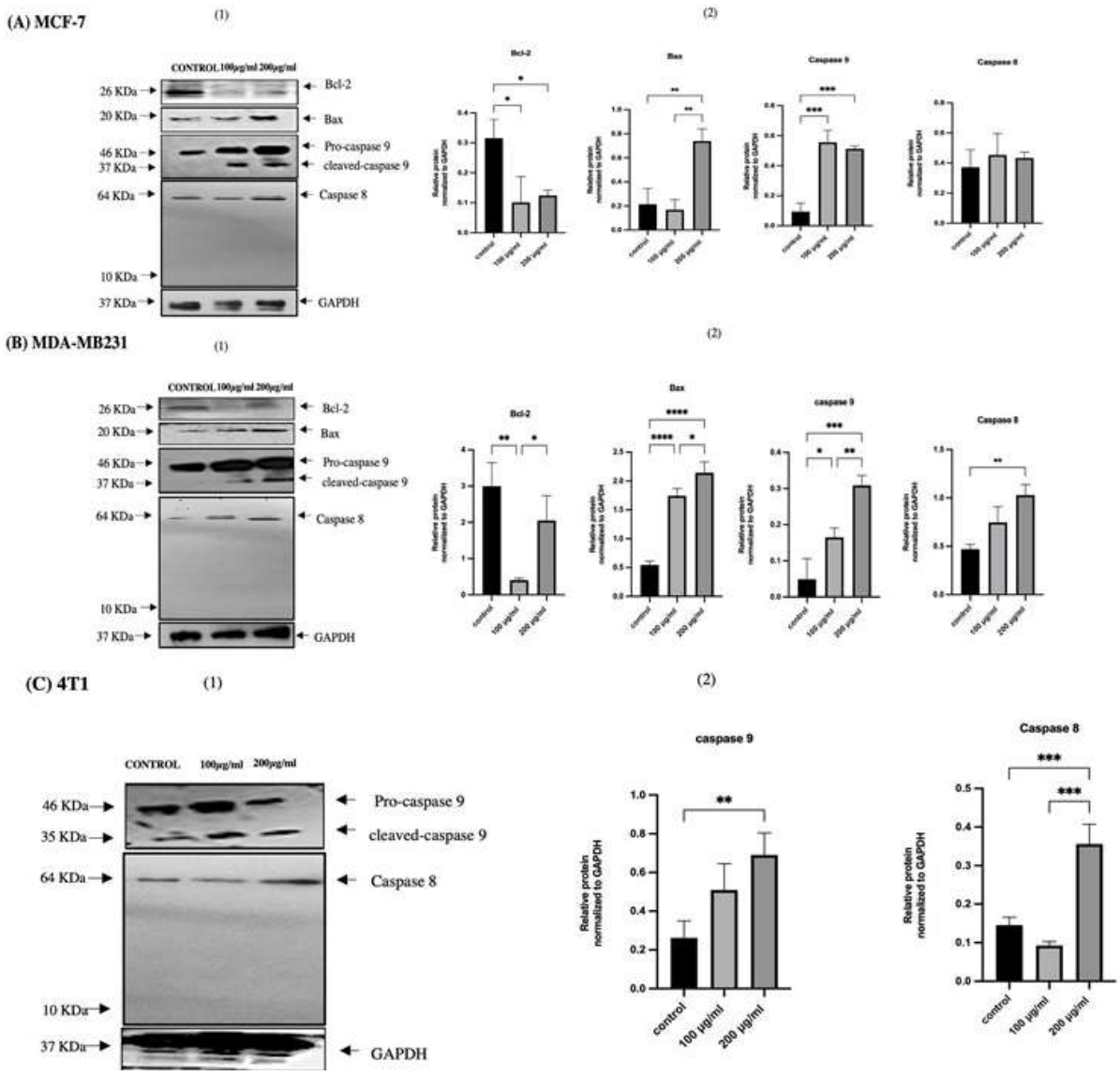

Figure 2.12: The effect of *F. assafoetida* on caspase-9, caspase-8, Bax, and Bcl-2 protein expression

MCF-7, MDA-MB-231, and 4T1 were treated with two different concentration of *F. assafoetida* (100 µg/ml and 200 µg/ml) for 72 h and then protein expression of caspase-9, caspase-8, Bax, and Bcl-2 were evaluated using western blot analysis. GAPDH was used as a loading control to confirm equal amount of protein in each lane (n=3). Graphs show results of densitometry for relative band intensity normalized for GAPDH with statistical differences indicated by * ($p<0.05$).

2.3 Discussion

Several studies have reported the cytotoxic and anti-cancer effects of treatment with extracts of different species of the *Ferula* genus. Nael Abutaha et al., demonstrated the cytotoxicity of the *Ferula hermonis* root hexane extract on colon and breast cancer cells (13). In another study, the anti-tumor activity of *Ferula sinkiangensis* extracts against HCT116, Caco-2, HepG2, and MFC cell lines were reported (24). In a different study, Bagheri et al., (20) evaluated the general cytotoxic effects of some Ferula species including *F. diversivittata, F. persia, F. ovena, F. brakema, and F. latisecta* on *Artemia salina* as a model. In another finding, Zhang et al., showed that ferulic acid (FA) significantly reduced tumor volume and weight and induced apoptosis of human MDA-MB-231 breast cancer cells in a xenograft mouse model (17).

In the current study, we have examined the anti-cancer activities of F. *assafoetida* against MCF-7, MDA-MB-231, or 4T1 cancer cells and the non-malignant HBL-100 cell line. Our results revealed that *F. assafoetida* could inhibit cell proliferation, in a dose and time dependent manner. According to the MTT assay, the viability of MCF-7, MDA-MB-231, or 4T1 cells was significantly inhibited after treatment with *F. assafoetida* extracted with 70% ethanol at concentrations of 100 and 200 μg/ml, whereas at a low concentration of 25 μg/ml it had a slight effect on cell viability. In addition, the growth of non-malignant HBL-100 cells was greatly decreased following treatment with the highest dose of 200 μg/ml of *F. assafoetida,* while the medium and low concentrations had no significant effect on cell viability. In all cell lines, we observed significant differences in staining that gradually increased with increasing exposure time. Ki-67 staining also was performed to investigate the anti-proliferative effect against the 4T1 murine cell line following treatment of *F. assafoetida*.

Our findings revealed that the expression level of Ki-67 was decreased after the 4T1 cells were treated with *F. assafoetida* extract. In particular, treatment of 4T1 cells with the highest dose of *F. assafoetida* extract significantly reduced the Ki-67 expression level after treatment for both 24 h and 48 h. Cells treated with the medium dose of the *F. assafoetida* extract for 24 h caused a slight but not significant decrease in Ki-67 expression level, whereas cells treated for 48 h showed a reduction in the expression level of Ki-67. However, differences in staining of cells treated at the low dose was not observed when compared with the untreated group. These results show that treatment with *F. assafoetida* extracts results in a decrease in cell viability, as measured by MTT assays, and a decrease in cell proliferation markers, such as Ki-67 (in the 4T1 cells). Further, the MTT assay and the AO/EtBr staining experiment show that the effects of *F. assafoetida* are more pronounced in the breast cancer cell lines compared to the non-malignant breast cell line. While these studies need to be expanded to a larger number of cells, it does suggest that *F. assafoetida* might have a bias towards cancer cells that would make it a better candidate for treatment of cancer patients since the weaker effects on non-cancerous tissues might be associated with less severe side effects. Corresponding with these findings, a previous study reported that galbanic acid, a major compound of *F. assafotida,* could inhibit Mcl-1 and induce apoptosis in H460 non-small carcinoma cells (22). Another study (23) also showed that galbanic acid isolated from *F. assafoetida* exerts anti-cancer, anti-angiogenic, and anti-proliferative effects *in vivo*. Treatment with galbanic acid was able to suppress VEGF-induced angiogenesis, decrease CD31 microvessel density index, and decrease staining with the Ki-67 proliferative marker in an immunohistochemistry assay. Overall, it could be proposed that *F. assafoetida* might be considered a potential toxic agent in anti-cancer studies

Apoptosis, a process of programmed cell death, happens when specific pro-apoptotic signaling pathways are activated. Apoptosis is characterised by morphological changes such as cell shrinkage, chromatin condensation, DNA fragmentation, and the generation of apoptotic bodies (2). Biochemical changes in cells includes the fragmentation of the genomic DNA during programmed cell death (3). In our present study, we sought to validate the mechanism of action of *F. assafoetida* and measure the apoptotic activity using different methods including dual acridine orange/ethidium bromide staining, TUNEL assays, and western blotting for apoptosis markers. Our results from dual acridine orange/ethidium bromide staining revealed that treatment with the middle dose of 100 µg/ml *F. assafoetida* showed bright green dots in the nuclei with an increased level of ethidium bromide staining due to nuclear fragmentation and chromatin condensation. Malignant cell lines that were treated with the high dose of 200 µg/ml *F. assafoetida* showed a significant change in cell morphology including membrane blebbing and complete damage of nuclear structure which occurs during the late stage of apoptosis. The non-malignant cell line HBL-100 did not show changes in cell morphology when treated with the low and middle doses of *F. assafoetida*. However, when the non-malignant cells were treated with the high dose it showed a significant change in cell morphology.

In order to detect DNA strand breaks and DNA fragmentation which are key features of late-stage apoptosis, we further performed (TUNEL) assays. The result showed that DNA fragmentation increased in a dose and time-dependent manner. At 24 h, MCF-7 and 4T1 cells showed a slight increase in TUNEL staining compared to the untreated cells while treatment for 48 and 72 h showed significant levels of TUNEL staining which indicate the cells are undergoing apoptosis.

An additional experiment was performed to detect whether *F. assafoetida* causes apoptosis via alterations in cellular signaling pathways using western blotting assays. Results showed that the expression level of the anti-apoptotic Bcl-2 protein was significantly lower in the treated groups than in the untreated group, whereas the expression level of pro-apoptotic Bax was increased. Western blot analysis also showed that there were significant expression levels of cleaved caspase-9 in the treated groups. Similar findings were observed in various previous studies, for example a study found that *F. hezarlalehzarica* was able to promote apoptosis by changing the expression of apoptosis genes such as Bcl-2 and Bax in Raji cells (21). We also investigated the effect of *F. assafoetida* on extrinsic apoptosis activation by measuring the expression level of cleaved caspase-8. The results indicated that only pro-caspase-8, but not the cleaved caspase, was detected in the cells which indicated that caspase-8 was not activated as would be expected if the extrinsic pathway were contributing to apoptosis.

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Chapter 3. Cytotoxic, anti-proliferative, and apoptotic effects of *F. assafoetida* components on human and murine breast cancer cell lines.

Abstract

Treatment of MCF-7 and MDA-MB-231 cells with ethanol extracts of *F. assafoetida* show decreased viability and increased apoptosis. Analysis of *F. assafoetida* ethanol extracts has identified several different components with potential biological activity. The relative cytotoxic effect of five of these known components, ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid, were investigated against human and murine breast cancer cell lines as an indicator of their potential anti-cancer activity. Cell viability was assessed using an MTT reduction assay and cellular apoptosis was assessed using dual ethidium bromide and acridine orange staining. Ellagic acid and quercetin showed a low level of cytotoxicity, whereas ferulic acid and vanillic acid did not show noticeable cytotoxic activity against the MCF-7 and MDA-MB-231 cell lines at the concentrations similar to that found in the *F. assafoetida* extracts. Our investigation also showed that treatment with trans-p-coumaric acid did not show significant inhibition of cell viability in the MDA-MB-231 and 4T1 cell lines. In conclusion, our results indicate that the five tested compounds seectively inhibit breast cancer viability and induces apoptosis, whereas they had a much weaker effect on the viability of a non-malignant cell line.

Keywords: Cytotoxicity; ferulic acid; vanillic acid; quercetin; ellagic acid; coumaric acid.

3. Background

F. assafoetida is one of the related genera of medicinal plants that has been used as traditional medicine for centuries due to the presence of multiple medicinal phytoconstituents such as terpenoids, sulfide derivatives, volatile oils, phenols, and minerals. Recent pharmalogical and biological studies have pointed to the anti-oxidant, anti-microbial, anti-fungal, anti-cancer, and anti-diabetic properties of this plant. One of the important properties of extracts of this plant is its anti-cancer effect. Previous studies have demonstrated beneficial effects of a number of Ferula species as cancer chemopreventive agents (1 - 4). One study showed that oral administration of *F. assafoetida* inhibited mammary tumor progression in a mouse model. Briefly, 4T1 mammary carcinoma cells were implanted into the mammary fat pad of female BALB/c mice. The mice were then treated orally with a dose of 100 mg/kg *F. assafoetida* extract which resulted in a decrease in the mouses's tumor volume and weight compared with an untreated group, and a decrease in the metastatic spread to other regions of the body (4). Umbelliprenin (UMB) is an effective sesquiterpene component, particularly found in plants of the Ferula family, that has been shown to have anti-cancer effects in both *in vivo* and *in vitro* models. UMB can alter activation of multiple cell signaling cascades, such as the Wnt and NF-κB pathways, which can interfere with cell cycle progression and can suppress cell invasion and migration (2). Ferulic acid is another effective component isolated from Ferula that has shown anti-cancer effects in various type of cancer including breast, colon and lung cancer. Zhang et al., showed that ferulic acid (FA) significantly reduced the tumor volume and weight and induced apoptosis of human breast cancer MDA-MB-231-derived tumours in a xenograft mouse model (5). Quercetin is another bioactive flavonoid component that has been reported to have anti-cancer effects against various types of human cancers

through suppression of cell growth, induction of cell apoptosis, or by improving anti-oxidant properties (6).

In the present study, we report the anti-cancer activity of ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid *in vitro*. Our results indicate that the five tested compounds selectively inhibit breast cancer viability and induces apoptosis, whereas they had a much weaker effect on the viability of a non-malignant cell line. These data show that ellagic acid and quercetin have the most potent effects *in vitro* and may be effective candidates for further studies on breast cancer intervention.

3.1 Materials and Methods

3.1.1 Plant Materials

The active ingredient including the phenolic compounds ferulic acid (#A1389014), vanillic acid (#A1207414), ellagic acid (#AC1177400), and p-coumaric acid (#A1516714) and the flavonoid compound quercetin (#A1580714) were purchased from Fisher Scientific (Mississauga, ON). For the preparation of the drug solutions, the powder product was dissolved in 70% ethanol and then stored in small aliquots in a -80 °C freezer.

3.1.2 Tissue culture

The 4T1 cell (murine mammary carcinoma cell line), MCF-7 (human ER-positive breast cancer), and MDA-MB-231 (human triple negative breast cancer), were obtained from ATCC [American Type Culture Collection, Manassas, VA] and the HBL-100 (human breast) cells were obtained from KM Yamada (NIH, Bethesda, MD). The 4T1 cells were maintained in tissue culture plates (Sarstedt, Laval, QC) in Roswell Park Memorial Institute medium (RPMI 1640, Fisher-Hyclone, Toronto, ON) supplemented with 10% fetal bovine serum (FBS, Fisher-Hyclone), 100 μg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Burlington, ON). The MCF-7, MDA-MB-231, and HBL-100 cells were maintained in tissue culture plates in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were incubated in a humidified atmosphere in a 5% CO_2 incubator at 37 °C. For experiments, the cells were treated with media including 70% ethanolic extract of *F. assafoetida* and the isolated compounds, that were dissolved in 70% ethanol, ferulic acid, vanillic acid, ellagic acid, p-coumaric acid, and

quercetin at the indicated concentrations. All experiments were performed in independent triplicate experiments for each assay.

3.1.3 Cell Treatments

The MCF-7, MDA-MB-231, HBL-100, and 4T1 cells were cultured in media as described above. For all experiments, the cells were washed with PBS and harvested by incubation in 2 ml 0.25% trypsin, centrifuged at 350 x g for 5 min and resuspended in fresh medium before plating. Cells were seeded at 15,000 cells/ml in 60 mm plates. After incubating overnight, the cells were treated with culture media, 100 µg/ml of 70% ethanolic extract of *F. assafoetida,* 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric, that were dissolved in 70% ethanol, suspended in the appropriate culture media containing 10% FBS and incubated at 37°C in 5% CO2. Selected concentrations of isolated compounds were derived from optimization experiments.

3.1.4 MTT assay (Methyl Tetrazolium Blue)

The MTT assays were performed as previously described (7). Briefly, the cells were seeded in 96-well plates at a density of 2000 cells/well and allowed to attach overnight. Afterward, fresh culture media (DMEM or RPMI) containing 100 µg/ml of *F. assafoetida,* 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, and 120 ng/ml trans-p-coumaric were added and duplicate plates were incubated for 1 to 4 days. Each day for four days, 10 µl/ml of 0.4 µg/ml MTT in PBS, pH 7.4, was added to each well and incubated for 4 h. After removal of the supernatant, 100 µl dimethyl sulfoxide (DMSO) was added in each well to solubilize the formazan product and the absorbance

(optical density (OD) value) was measured at 540 nm using a plate reader. Tests were performed in triplicate for at least three independent experiments, and the mean value was calculated to determine the relative number of viable cells. To estimate their difference in response to the different treatments an ANOVA using Graph Pad Prism Software was performed. The inhibition percentage was calculated using the formula:

$$\%\ \text{inhibition} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right) / A_{\text{control}}\right] \times 100$$

3.1.5 Cell Staining assay (Acridine Orange/Ethidium Bromide)

A cell staining experiment was performed to visualize any nuclear changes associated with apoptosis in response to treatment with *F. assafoetida* and the various isolated compounds. The cells were cultured in 60 mm dishes and treated with 100 µg/ml *F. assafoetida* extracts, as a positive control, or with the purified *F. assafoetida* compounds at concentrations corresponding to their relative concentration in a 100 µg/ml solution of the *F. assafoetida* extract (1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric). The cells were treated with the compounds for 48 h and then stained with 10 µg/ml of acridine orange (Sigma-Aldrich) and 10 µg/ml of ethidium bromide (Sigma- Aldrich, St. Louis, MO) for 15 minutes. The fluorescently labelled cells were visualized on an LSM 510 fluorescence microscope (Zeiss Canada, Mississauga, ON) and phase contrast (Ph), acridine orange (green) and ethidium bromide (red) images were captured.

3.2 Results

3.2.1 Cytotoxic effects of *F. assafoetida* compounds on the viability of malignant and non-malignant breast cell lines

An MTT assay was performed to test the *in vitro* cytotoxicity of *F. assafoetida* compounds against malignant (MCF-7, MDA-MB231 or 4T1) and non-malignant (HBL-100) breast cell lines. In our investigation, we found that cells treated with ferulic acid at a dose of 1460 ng/ml (corresponding to its concentration in 100 µg/ml of *F. assafoetida* extract) did not cause significant inhibition of MCF-7 or MDA-MB-231 cell line viability and a significantly different effect was not observed between the control and the treated groups ($p=0.12$) and ($p=0.33$, respectively) (Figure 3.1 and Figure 3.2 (A)). However, the decrease in viability value was higher when 4T1 cells were treated with ferulic acid, particularly at day four ($p<0.05$) of treatment. Cells that were treated with 194 ng/ml vanillic acid, 220 ng/ml quercetin, or 70 ng/ml ellagic acid caused a decrease in cell viability in all the malignant cell lines (Figure 3.1, Figure 3.2 and Figure 3.3(B), (C) and (D)). However, treatment with 120 ng/ml trans-p-coumaric acid did not effectively decrease cell viability in MDA-MB-231 and 4T1 cells and differences between the control and the treated group were not observed (Figure 3.2 and 3.3 (E)).

In addition, treatment of the non-malignant HBL-100 cell line with the five *F. assafoetida* compounds had no effect on cell viability compared to untreated control cells (Figure 3.4).

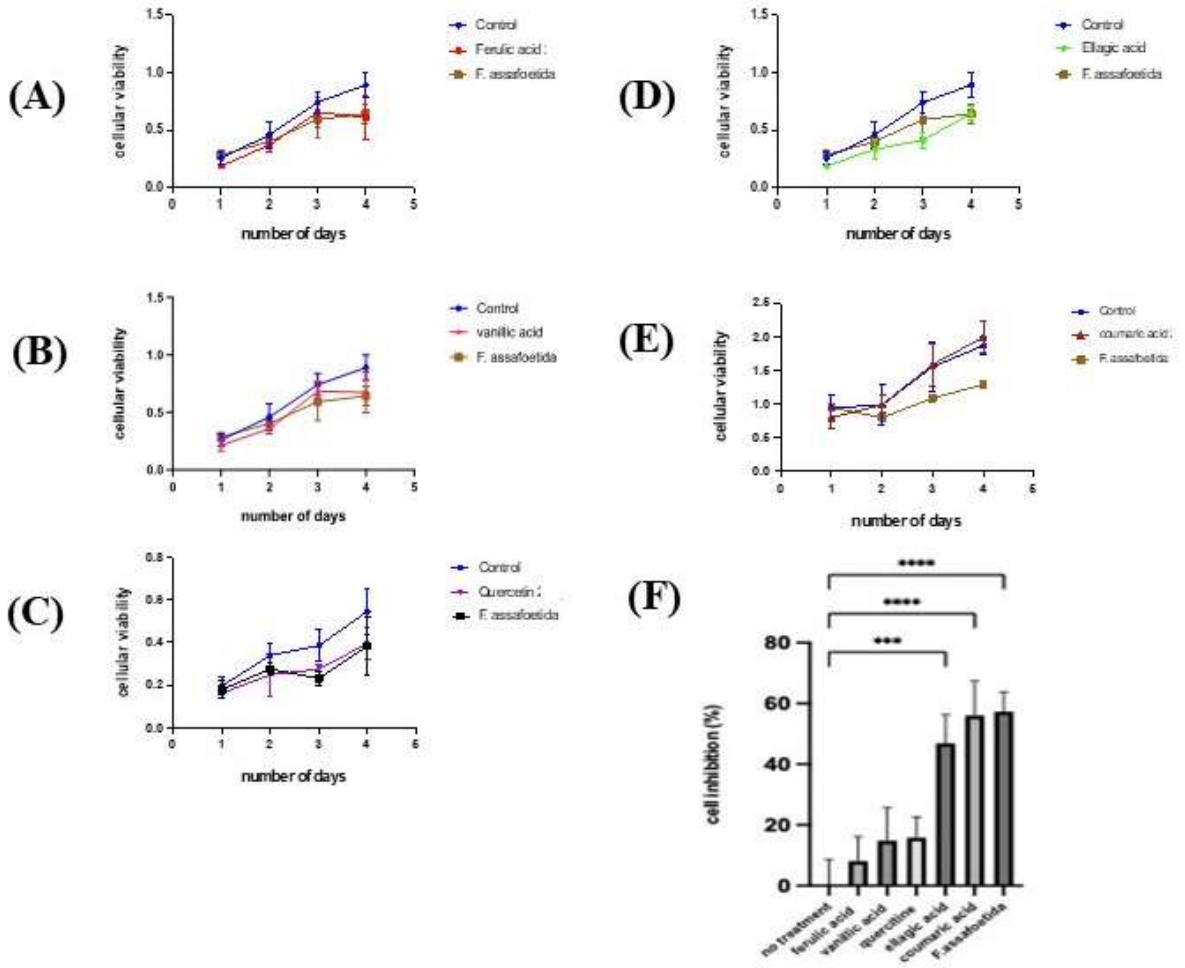

Figure 3.1: Cytotoxicity Assays for the MCF-7 Malignant Cell Line

Viability of the MCF-7 malignant cell line treated with (A) 1460 ng/ml ferulic acid, (B) 194 ng/ml vanillic acid, (C) 220 ng/ml quercetin, (D) 70 ng/ml ellagic acid, or (E) 120 ng/ml trans-p-coumaric acid (corresponding to their concentration in 100 µg/ml *F. assafoetida* extract), or with 100 µg/ml *F. assafoetida* (positive control) was measured using the MTT assay. Panel (F) shows the relative viability of MCF-7 cells treated with *F. assafoetida* extracts or the five compounds (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the compounds for four days and absorbance was determined each day. Data were analyzed for at least three independent experiments and a p-value less than 0.05 (for n=3) was statistically significant.

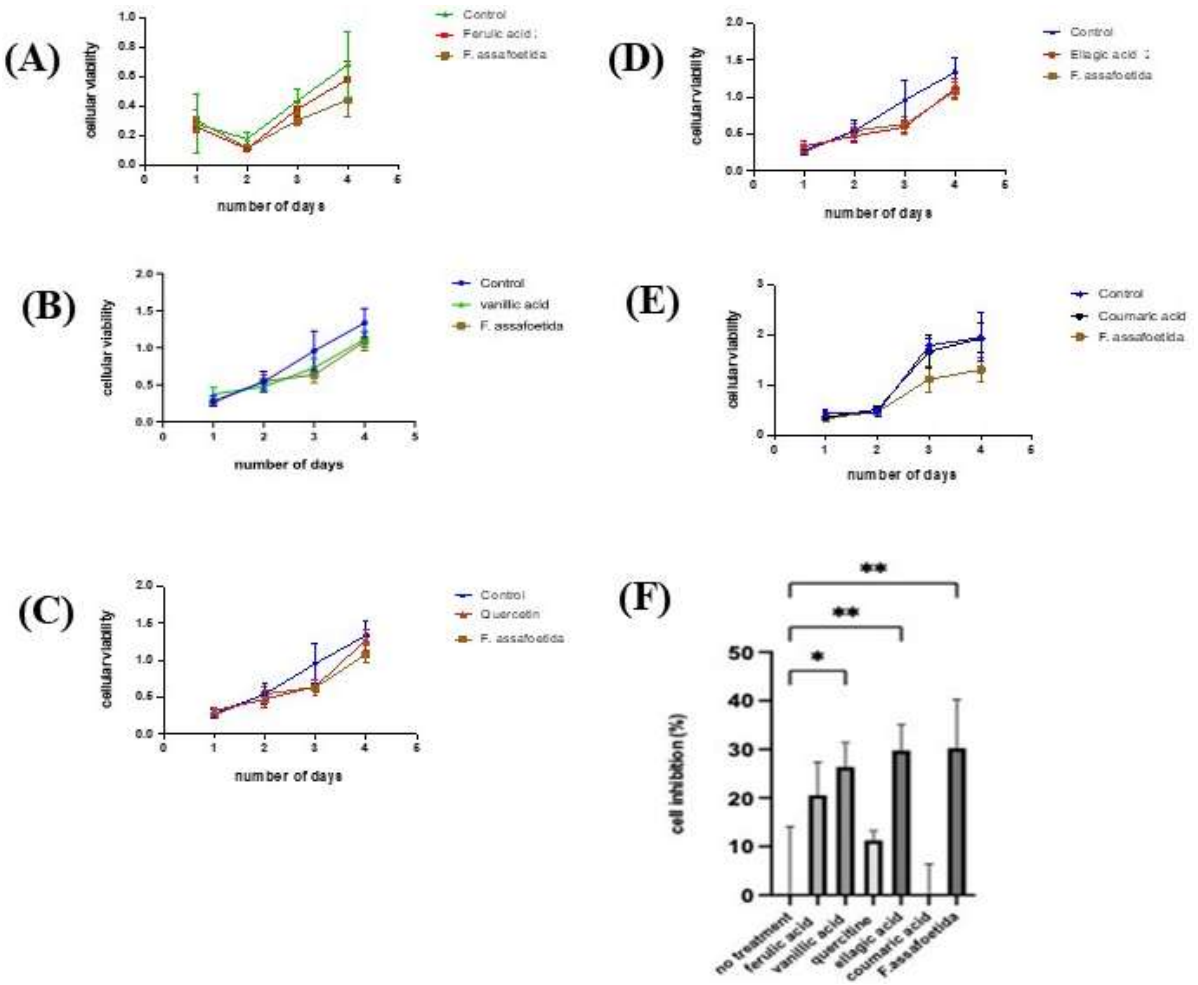

Figure 3.2: Cytotoxicity Assay for MDA-MB-231 Malignant Cell Line

Viability of the MDA-MB-231 malignant cell line treated with (A) 1460 ng/ml ferulic acid, (B) 194 ng/ml vanillic acid, (C) 220 ng/ml quercetin, (D) 70 ng/ml ellagic acid, or (E) 120 ng/ml trans-p-coumaric acid (corresponding to their concentration in 100 µg/ml *F. assafoetida* extract), or with 100 µg/ml *F. assafoetida* (positive control) was measured using the MTT assay. Panel (F) shows the relative viability of MDA-MB-231 cells treated with *F. assafoetida* extracts or the five compounds (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the compounds for four days and absorbance was determined each day. Data were analyzed at least three independent experiments and a p-value less than 0.05 (for n=3) was statistically significant.

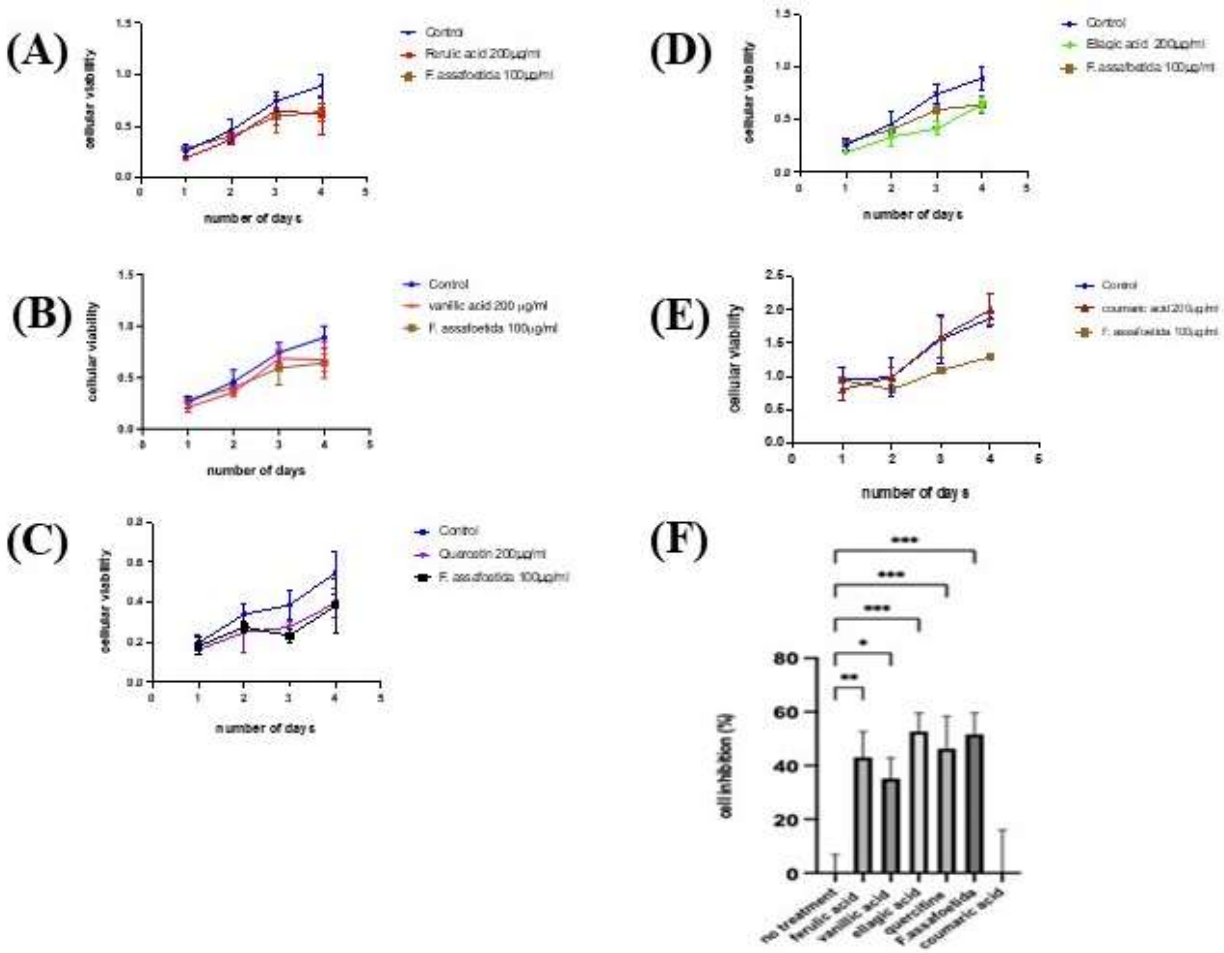

Figure 3.3: Cytotoxicity Assay for 4T1 Malignant Cell Line

Viability of the 4T1 malignant cell line treated with (A) 1460 ng/ml ferulic acid, (B) 194 ng/ml vanillic acid, (C) 220 ng/ml quercetin, (D) 70 ng/ml ellagic acid, or (E) 120 ng/ml trans-p-coumaric acid (corresponding to their concentration in 100 µg/ml *F. assafoetida* extract), or with 100 µg/ml *F. assafoetida* (positive control) was measured using the MTT assay. Panel (F) shows the relative viability of 4T1 cells treated with *F. assafoetida* extracts or the five compounds (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the compounds for four days and absorbance was determined each day. Data were analyzed for at least three independent experiments and a p-value less than 0.05 (for n=3) was statistically significant.

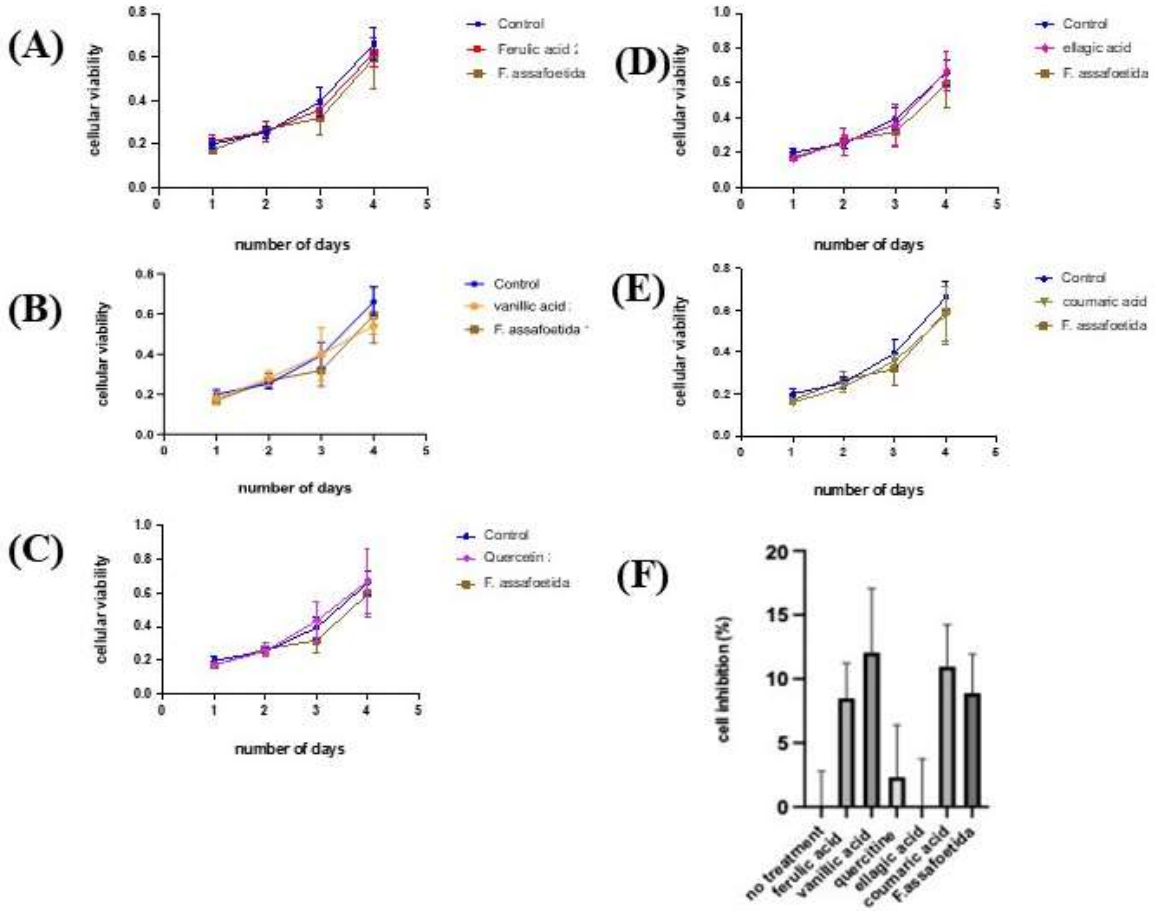

Figure 3.4: Cytotoxicity Assay for HBL-100 Non-Malignant Cell Line

Viability of the HBL-100 non-malignant cell line treated with (A) 1460 ng/ml ferulic acid, (B) 194 ng/ml vanillic acid, (C) 220 ng/ml quercetin, (D) 70 ng/ml ellagic acid, or (E) 120 ng/ml trans-p-coumaric acid (corresponding to their concentration in 100 µg/ml *F. assafoetida* extract), or with 100 µg/ml *F. assafoetida* (positive control) was measured using the MTT assay. Panel (F) shows the relative viability of HBL-100 cells treated with *F. assafoetida* extracts or the five compounds (day four) and the percent inhibition of cell viability was determined compared to media controls. The cells were incubated with the compounds for four days and absorbance was determined each day. Data were analyzed for three independent experiments and a p-value less than 0.05 (for n=3) was statistically significant.

3.2.2 Detection of apoptosis

To confirm the induction of apoptosis, we performed Acridine Orange/Ethidium Bromide (AO/EtBr) double fluorescent staining and imaging using a fluorescent microscope. The cells were treated with 100 µg/ml *F. assafoetida* extract, 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric for 48 h and then stained. The AO/EtBr staining experiment is capable of differentiating between viable and apoptotic cells based on membrane integrity. If the cell is viable, the acridine orange inserts into the DNA, making the cell stain green. However, if the cell is apoptotic and has a compromised plasma membrane, the ethidium bromide also inserts into the DNA, making the cell stain red. Thus, apoptotic cells will be labelled bright green and red due to morphological changes, such as membrane blebbing chromatin condensation and nuclear conformation. Apoptotic cells exhibit fragmented and condensed red chromatin, and viable cells exhibit a regular green nucleus. The resulting images from the cell staining assay using AO/EtBr revealed that the MCF-7 and MDA-MB-231 cell lines treated with ferulic acid for 48 h showed some smaller changes in nuclear staining with relatively low level of ethidium bromide staining when compared to the positive control, *F. assafoetida* (Figure 3.5 and Figure 3.6). Treatment with vanillic acid, quercetin, and ellagic acid showed bright green dots in the nuclei with an increased level of ethidium bromide staining in malignant cell lines due to nuclear fragmentation and chromatin condensation (Figure 3.5, Figure 3.6, and Figure 3.7). However, 4T1 and MDA-MB-231 cells treated with trans-p-coumaric acid showed only a small level of ethidium bromide staining compared to the positive control. Additionally, the non-malignant cell line HBL-100 did not show changes in cell morphology when treated with any of the five *F. assafoetida* compounds at the indicated concentrations (Figure 3.8). These

findings reconfirm previous results from the MTT assay, which showed the tested compounds caused a decrease in cell viability in the malignant cell lines but not in the non-malignant cell line. This suggests that the tested compounds that showed interesting results may be a suitable option to consider for developing drugs that can treat breast cancer.

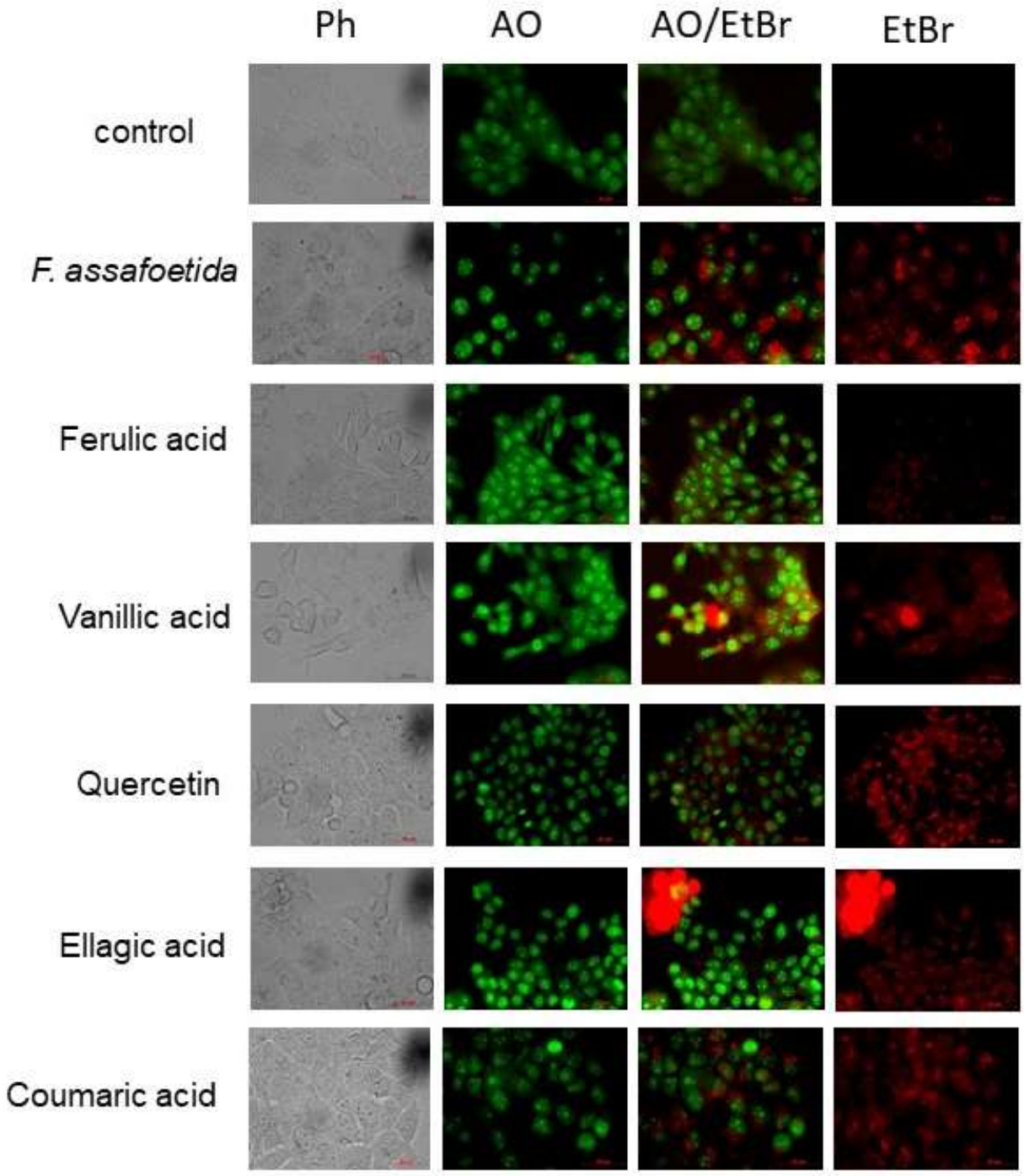
Ph
AO
AO/EtBr
EtBr
control
F. assafoetida
Ferulic acid
Vanillic acid
Quercetin
Ellagic acid
Coumaric acid

Figure 3.5: Apoptosis is detected by AO/EtBr double staining of MCF-7 cells

Morphological study of MCF-7 cells cultured in 60 mm dishes and treated with 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric acid (corresponding to the concentrations found in 100 µg/ml *F. assafoetida* extracts) or 100 µg/ml *F. assafoetida* extract (positive control). The figure represents images viewed using a Fluorescence microscope at using different optical settings including; Phase contrast (Ph), green for acridine orange (AO), and red for ethidium bromide (EtBr). Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells also stained red with highly condensed nuclei.

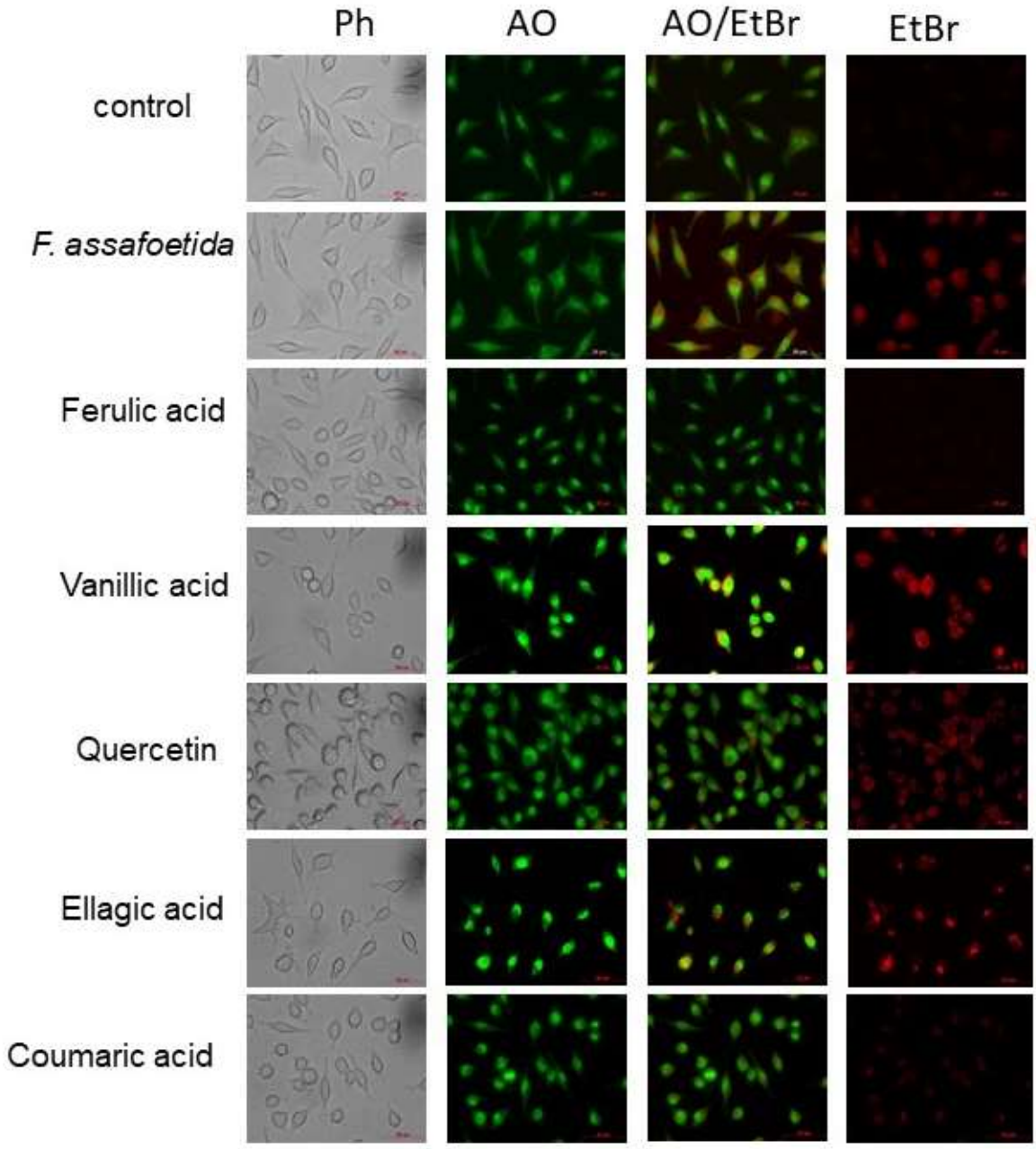
Ph
AO
AO/EtBr
EtBr
control
F. assafoetida
Ferulic acid
Vanillic acid
Quercetin
Ellagic acid
Coumaric acid

Figure 3.6: Apoptosis is detected by AO/EtBr double staining of MDA-MB-231 cells

Morphological study of MDA-MB-231 cells cultured in 60 mm dishes and treated with 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric acid (corresponding to the concentrations found in 100 µg/ml *F. assafoetida* extracts) or 100 µg/ml *F. assafoetida* extract (positive control). The figure represents images viewed using Fluorescence microscope at using different optical settings including; Phase contrast (Ph), green for acridine orange (AO) and red for ethidium bromide (EtBr). Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells also stained red with highly condensed nuclei.

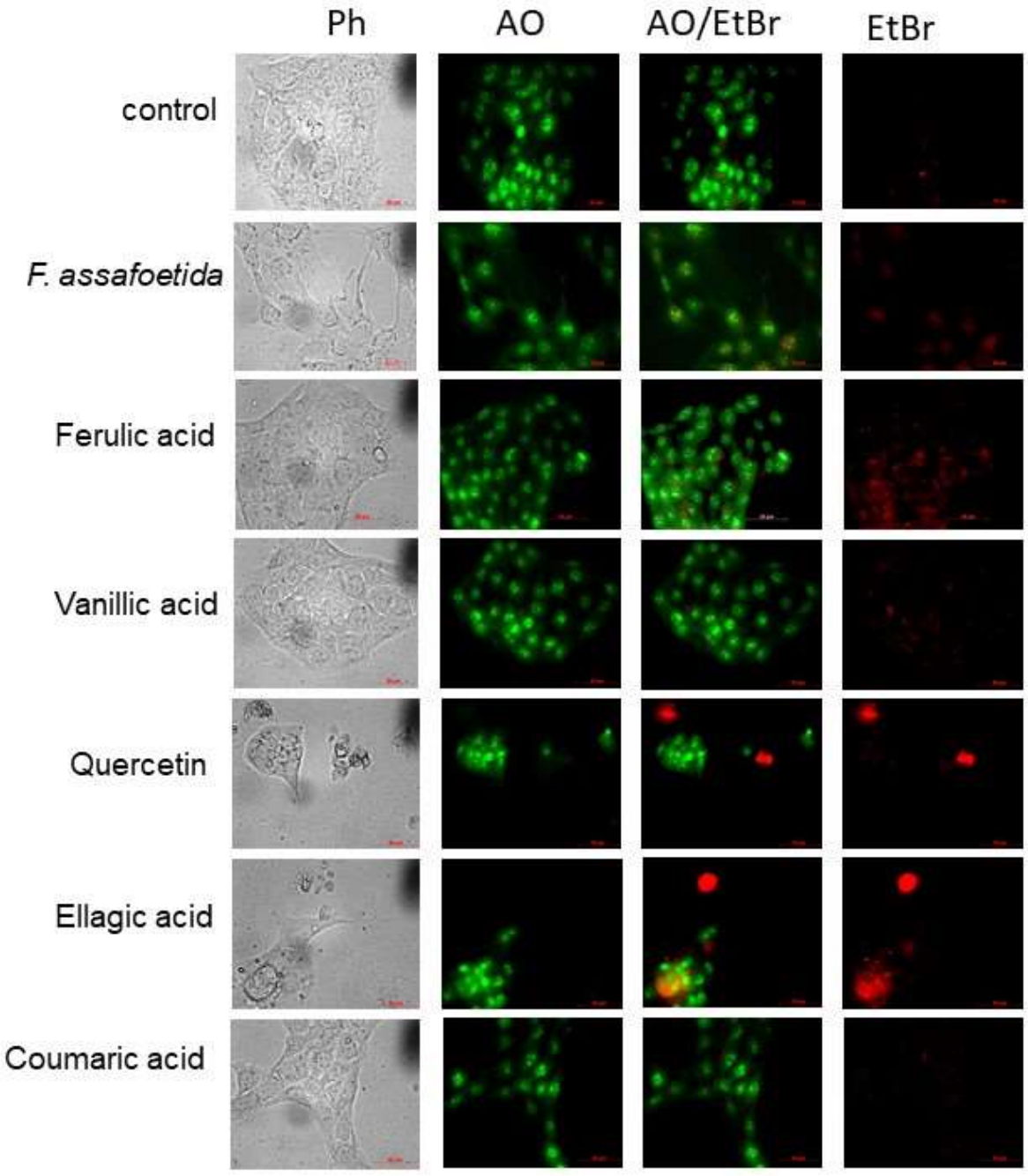
Ph
AO
AO/EtBr
EtBr
control
F. assafoetida
Ferulic acid
Vanillic acid
Quercetin
Ellagic acid
Coumaric acid

Figure 3.7: Apoptosis is detected by AO/EtBr double staining of 4T1 cells

Morphological study of 4T1 cells cultured in 60 mm dishes and treated with 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric acid (corresponding to the concentrations found in 100 µg/ml *F. assafoetida* extracts) or 100 µg/ml *F. assafoetida* extract (positive control). The figure represents images viewed using Fluorescence microscope at using different optical settings including; Phase contrast (Ph), green for acridine orange (AO), and red for ethidium bromide (EtBr). Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells also stained red with highly condensed nuclei.

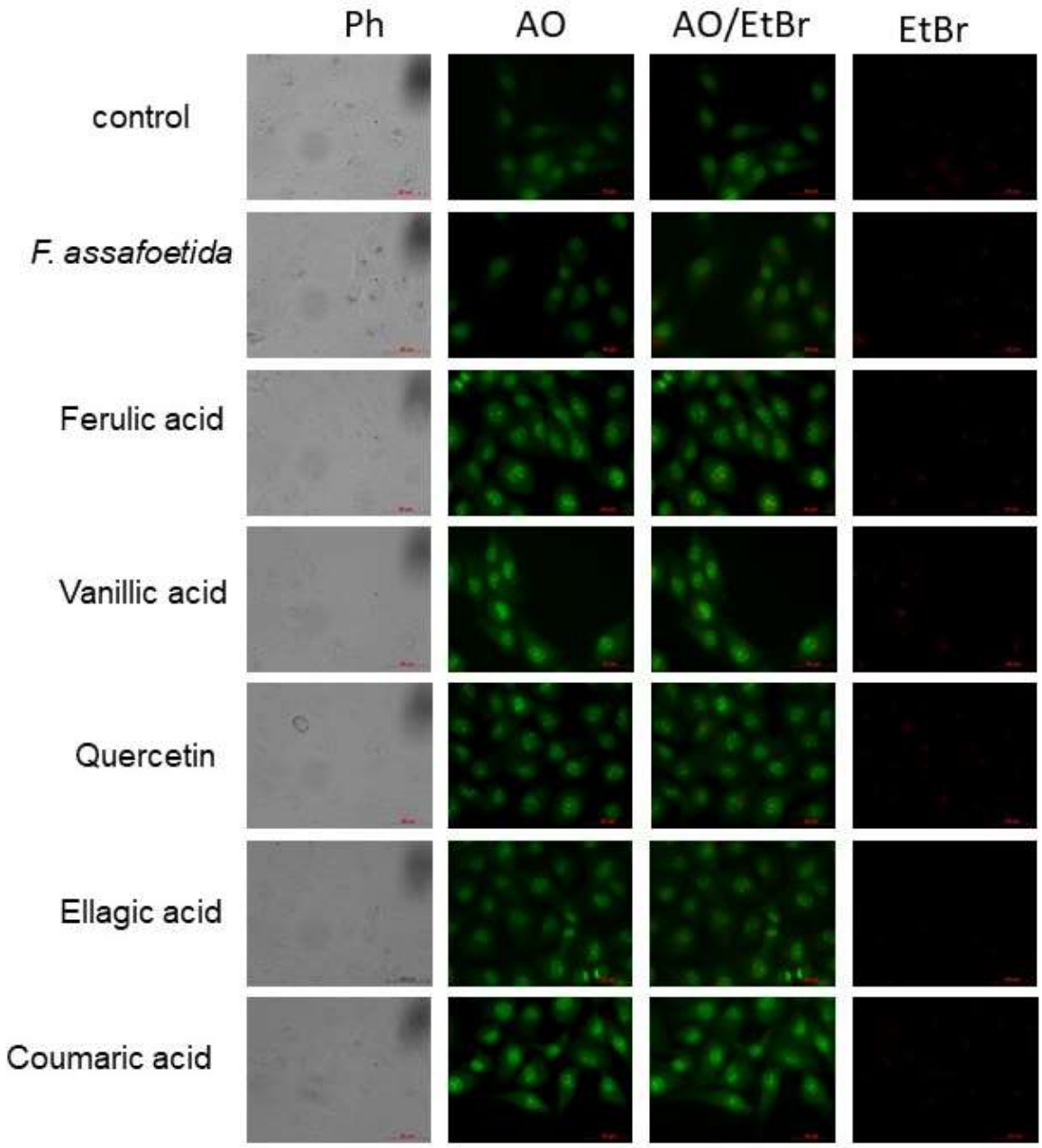
Ph
AO
AO/EtBr
EtBr
control
F. assafoetida
Ferulic acid
Vanillic acid
Quercetin
Ellagic acid
Coumaric acid

Figure 3.8: Apoptosis is detected by AO/EtBr double staining of HBL-100 cells

Morphological study of non-malignant HBL-100 cells cultured in 60 mm dishes and treated with 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric acid (corresponding to the concentrations found in 100 µg/ml *F. assafoetida* extracts) or 100 µg/ml *F. assafoetida* extract (positive control). The figure represents images viewed using Fluorescence microscope at using different optical settings including; Phase contrast (Ph), green for acridine orange (AO), and red for ethidium bromide (EtBr). Viable cells excluded ethidium bromide and their nuclei were bright green with intact structure, while apoptotic cells also stained red with highly condensed nuclei.

3.4 Discussion

F. assafoetida has been previously shown to be one of the most important potentially bioactive members of the umbelliprenin family (11). This plant is used traditionally for the treatment of various disorders including intestinal parasites, influenza, asthma, epilepsy, hysteria and neurological disorders (3, 4). A previous analysis of the *F. assafoetida* extract by HPLC has identified several components in the extract at detectable concentrations including ferulic acid, ellagic acid, quercetin, vanillic acid, and p-coumaric acid (Chapter 5, this thesis). The anti-proliferative effects of *F. assafoetida* and its bioactive compounds have been confirmed in many studies on different cell lines (4, 12). Previously, it has been reported that ellagic acid was able to inhibit breast cancer growth and metastasis *in vitro* and *in vivo* (8). Quercetin is another bioactive flavonoid component that has been previously reported to have anti-cancer effects against various types of human cancers through suppressing cell growth, inducing cell apoptosis, or improving antioxidant properties (6). Vanillic acid is another common bioactive phenolic compound present in *F. assafoetida* and in many other plants. It has been reported to have anti-proliferative effects and protect cells from DNA damage (9).

In these studies, the anti-proliferative effects of 5 different *F. assafoetida* extract components were investigated on malignant cell lines including MCF-7, MDA-MB-231, and 4T1 cells and the non-malignant cell line, HBL-100, using MTT viability assays and cell staining. The results of these studies showed that the five tested components and *F. assafoetida* extracts were able to decrease cell viability and promote apoptotic morphology in a dose- and time-dependent manner in some of the cell lines, suggesting that these components can at least partially explain the anti-cancer effects of *F. assafoetida* extracts on breast cancer cell lines. In particular, ellagic acid, vanillic acid, and quercetin showed strong inhibitory

effects on malignant cell lines (except vanillic acid in 4T1 cells), whereas they had very small effects on the proliferation of the non-malignant cell line indicating a selective anti-cancer activity for these compounds. The ability of these purified components, ellagic acid, vanillic acid, and quercetin, to inhibit proliferation and induce an apoptotic morphology is consistent with previous studies (6, 8, 9) although the concentrations used in the current study are much lower than those used in previous studies. We chose these concentrations based on the levels of the different components which were detected in the *F. assafoetida* ethanolic extracts so that our evaluation of their effect on the cells would be more relevant to their contribution in the whole extract (see Chapter 5 of this thesis). The observation that each of the individual components of the extract did not have as strong an impact on cellular proliferation and apoptosis as the complete *F. assafoetida* extract (except in 4T1 cells) suggests the possibility that additional active components are present in the extract or that the effects of the components are additive.

MCF-7 and MDA-MB-213 cells treated with ferulic acid showed very little effect on cell viability. This finding is similar to a previous study conducted by Maruyama et al., who investigated the effect of ferulic acid on a melanoma cell line which showed no effect on cell proliferation even at high concentration (9). Another study which also supports our findings showed that ferulic acid did not inhibit MDA-MB-231 cell proliferation and did not induce apoptosis, but cells were significantly affected when ferulic acid was combined with thymoquinone (10). This indicates that different bioactive compounds can have different anti-cancer activities when they are used in combination and that the total anti-cancer activity may be changed due to synergistic interactions. Our investigation also showed that treatment with trans-p-coumaric acid did not show significant inhibition of cell viability in the MDA-MB-

231 and 4T1 cell lines. The present study showed that *F. assafoetida* and some of the isolated components can act as inducers of apoptosis that inhibits the growth of cancer cells. However, more investigations are required to prove the mechanism of the *F. assafoetida* extract in inducing apoptosis and to determine how the various components of the extract can act together to affect cancer cell viability. Further, the previous HPLC analysis had identified only 5 separate components, but analysis of the chromatographs did indicate additional unidentified peaks and it is very likely that there were other components present in the extract that were below the level of detection for the wavelengths used in the analysis. In fact, other studies have reported additional components in *F. assafoetida* extracts, such as gallic acid, m-coumaric acid, sulfur-containing derivatives, sesquiterpenes, sesquiterpene lactones, and sesquiterpene coumarins. Therefore, it is likely that other components are present in *F. assafoetida* extracts that contribute to its anti-cancer activity and future studies to further characterize the extracts would be valuable.

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Chapter 4. The effect of *F. assafoetida* extracts on the polarization of THP-1-derived macrophages and on macrophage-mediated damage of MCF-7 or MDA-MB-231 cell monolayers.

Abstract

Plants of the Ferula genus, including *Ferula assafoetida*, are a traditional medicine that has been shown to have a variety of pharmacological properties relevant to inflammation (1). According to recent findings, extracts from plants of this genus are well documented as a good source of biologically active components such as sulfur-containing derivatives, coumarins, sesquiterpenes, sesquiterpene lactones, sesquiterpene coumarins, glucuronic acid, galactose, arabinose, rhamnose, and daucane esters (2). Some antiviral and antimicrobial compounds isolated from Ferula species have been reported to have strong inhibitory activity against viral infections such as AIDS and influenza H1N1(3). To test the effect of Ferula extracts on inflammatory processes, cultured monocyte-derived macrophages were treated with Ferula extracts and examined for the expression of macrophage polarization markers and the production of inflammatory cytokines. THP-1 monocytes were differentiated into macrophages using PMA (phorbol 12-myristate 13-acetate). Once differentiated (M0 macrophages), the control cells were further incubated with IL-4 in order to obtain M2 polarized macrophages or with LPS to obtain M1 polarized macrophages. The M0 macrophages were also treated with either *F. assafoetida* or with the isolated components to determine if the extracts were able to activate the M1 or M2 phenotypes. The surface markers of M1 and M2 macrophages were measured using flow cytometry, while the levels of cytokines secreted by M1 and M2 macrophages were detected using enzyme-linked

immunosorbent assay (ELISA). The results showed that *F. assafoetida* extracts and some of its components were able to promote the expression of the M1 markers, but not the M2 markers, supporting the idea that the *F. assafeotida* contains compounds that promote a pro-inflammatory phenotype in differentiated macrophages.

4 Background

The immune system plays a vital role in protecting the body against harmful bacteria, viruses and other pathogenic organisms. Macrophages are “plastic” cells that can switch from one type to another and are one of the most important cells types involved in the innate immune system (5). Macrophages have different functions including: phagocytosis of invading pathogens, debris or apoptotic cells; antigen presentation by displaying short peptides via major histocompatibility complex (MHC) class I and II molecules to be recognized by T cells; and, the release of appropriate cytokines and chemokines, such as pro-inflammatory cytokines tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β), and IL-6, or anti-inflammatory cytokines including IL-10 and TGF-β to alter the immune environment. Macrophages are produced through the differentiation of bone marrow-derived monocytes which circulate in blood, becoming macrophages which are present in many tissues (5)(6). Additionally, macrophages can be polarized depending on their function into two categories; classically activated (M1), and alternatively activated (M2) macrophages. In order for macrophages to be activated to the M1 type, they need to be stimulated by interferon (IFN-γ) or lipopolysaccharide (LPS), and then produce proinflammatory macrophage cytokines such as TNF-α, IL-1β, and IL-6. However, if M1 macrophages continue to over-produce the proinflammatory cytokines, it can lead to uncontrolled immune activation and result in tissue damage. In contrast the production of M2 polarized macrophages is stimulated by exposure to IL-4 or IL-13 and then they release high level of TGF-β, IL-10, and IL-6 cytokines which can suppress the inflammatory reaction, as well as contribute to tissue repair, maintain homeostasis and vasculogenesis (5 - 7).

Different studies have suggested that some natural compounds which exhibit anti-inflammatory activity also have the ability to regulate macrophage polarization. However, the molecular mechanisms by which natural compounds influence macrophage polarization are still not clear (8). Plants of the Ferula genus include naturally occurring plants that exhibit various pharmacological properties including anti-inflammatory effects (1). Plants from the Ferula genus are from the family Umbelliferae, which is comprised of about 180 species, are distributed widely throughout the Mediterranean area and central and southwest Asia. Members of this genus are well documented as a good source of biologically active components such as sulfur-containing derivatives, coumarins, and sesquiterpenes, and have been shown to exhibit anti-microbial activities (9). A previous study showed that some of the isolated compounds from *Ferula sumbul,* including coumarins, had anti-HIV activities (10). Another study also investigated the immunostimulatory activities of polysaccharides extracted from *F. gummosa* and found that the extract strongly improved the immune response by inducing macrophages to secret proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12 (11). Quercetin is one of the most active flavonoid compounds found in plants of the Ferula genus and has been previously shown to have immunostimulatory activities (12). Findings from previous studies also showed that ellagic acid, another component of Ferula extracts, was able to reduce markers of inflammation in Sprague-Dawley rats (13).

The interactions between cancerous cells and their surrounding tumor microenvironment (TME) can play an active role in tumor growth, invasion, and metastasis. The tumor microenvironment includes many cell types including fibroblasts, endothelial cells, lymphocytes, and macrophages. In this study study, we focused on the effects on macrophages. Tumor-associated macrophages (TAMs) with an M2 phenotype are known to

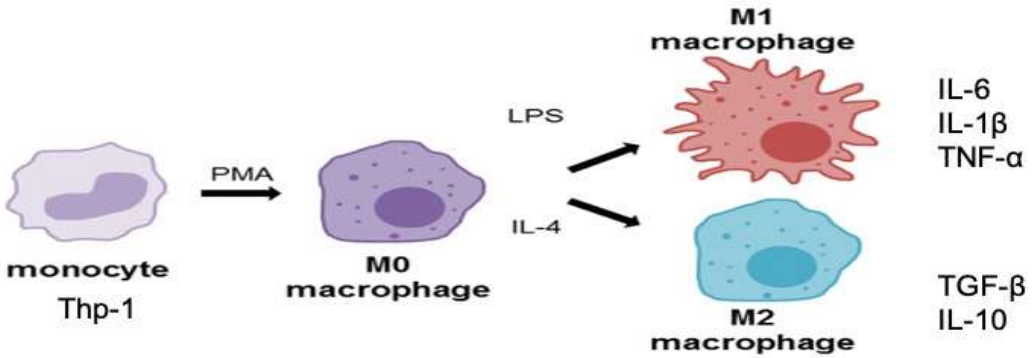
promote cancer cell proliferation and migration and to suppress adaptive immunity. Recently it has been shown that a higher tumor-associated macrophage (TAM) density is associated with a poor prognosis in human patients with breast cancer (14). This study is designed to show whether *F. assafoetida* extracts are able to influence macrophage polarization which might impact tumor growth.

4.1 Materials and methods

4.1.1 THP-1 cell differentiation and treatment

THP-1 cells were cultured in RPMI-1640 cell culture medium (Fisher-Hyclone, Toronto, ON) supplemented with 10% fetal bovine serum (FBS, Fisher-Hyclone) 100 µg/ml streptomycin, and 100 U/ml penicillin. The cells were sub-cultured, usually once a week, to maintain a concentration of less than 1×10^6 cells/ml and incubated in a humidified atmosphere in a 5% CO_2 incubator at 37 °C. THP-1 cells were plated (1 ml /tube) into a 15 ml tube (Fisher). To generate macrophage-like cells, THP-1 cells (1×10^6) were treated with 100 nM PMA (Sigma-Aldrich), or control media, and incubated for 3 days. On day 4, the media was removed, and the cells were collected by centrifugation and seeded back into the same tubes. To activate the cells to the M1 or M2 polarized phenotypes the cells were incubated with fresh medium containing 25µg/ml of *F. assafoetida* 70% ethanol extract, one of the five isolated compounds (dissolved in 70% ethanol) (70 ng/ml ellagic acid, 220 ng/ml quercetin, 1460 ng/ml ferulic acid, or 120 ng/ml trans-p-coumaric acid, or 194 ng/ml vanillic acid) and the cells were then incubated for 24 h. For control experiments the cells were incubated with 100 ng/ml LPS in RPMI to generate M1 polarized cells, or 100 ng/ml of IL-4 in RPMI to obtain M2 polarized macrophages. Afterward, the media were changed to serum-free media to prevent any significant background signals, and cells were then incubated for an additional 24 h to generate the conditioned media. In some of the posttreatment experiments, the cells were collected for flow cytometry and the conditioned media collected for ELISA (Figure 4.1).

A



B

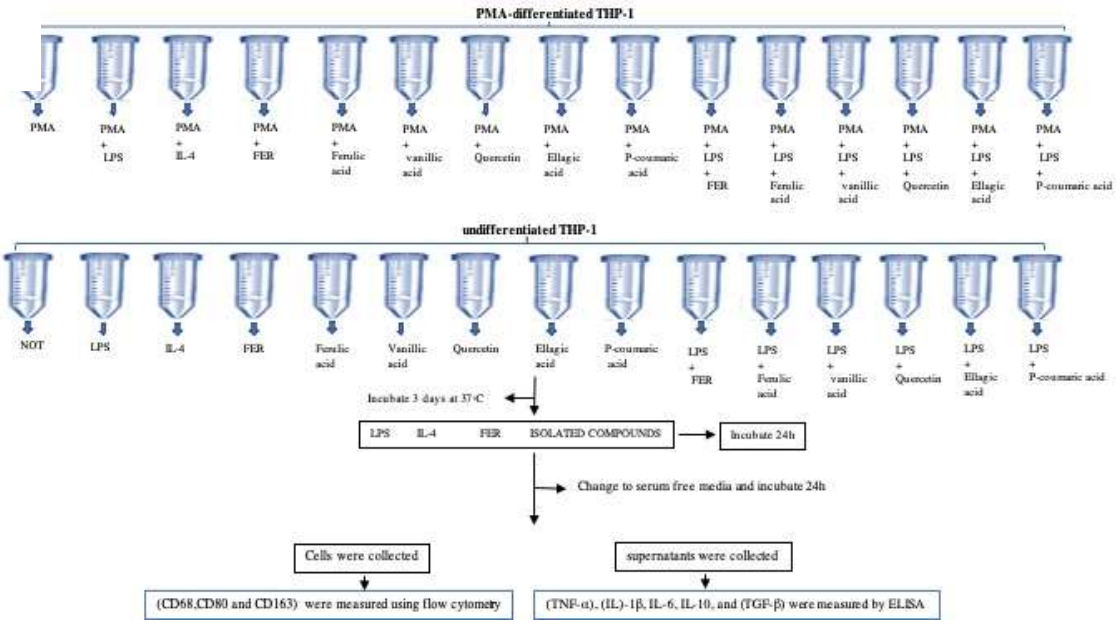


Figure 4.1: (A) Macrophage differentiation and polarization. Monocytes were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA). M0 macrophages were further polarized into two distinct types of macrophages, M1 and M2, by treatment with lipopolysaccharide (LPS) or interleukin 4 (IL-4). M1 macrophages (pro-inflammatory macrophages) are known to secrete many factors such as TNF-α, IL-1β, and IL-6. M2 macrophages (anti-inflammatory macrophages) are known to secrete factors such as IL-10, and TGF- β (15). (B) Summary of macrophage polarization protocol. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate, *F. assafoetida* and the isolated compounds were added in the presence or absence of lipopolysaccharide. Then cells were collected for flow cytometry and the culture supernatant collected for ELISA (16)

4.1.2 Cell proliferation assay.

Cell growth of THP-1 cells after treatment with *F. assafoetida* or the individual compounds was measured by determining the number of cells per high power microscope field. Briefly, THP-1 cells were plated at 4×10^3 cells per well in 24-well culture plates. After 24 h, cells were treated with of *F. assafoetida* 25 μg/ml or *F. assafoetida* compounds (1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, and 120 ng/ml trans-p-coumaric acid), for 1, 3, or 5 days. Each day, images from 5 different areas for each well were captured under the microscope and ImageJ software using the ImageJ plugin Cell Counter (NIH, Bethesda, MD, USA) was used to count the numbers of cells/microscope field and the mean number and standard deviation were calculated. The experiments were performed in triplicate.

4.1.3 MTT assay (Methyl Tetrazolium Blue)

The MTT assays were performed as previously described (17). Briefly, the cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and allowed to attach overnight. Afterward, fresh RPMI culture media containing *F. assafoetida* 25 μg/ml, 1460 ng/ml ferulic acid, 194 ng/ml vanillic acid, 220 ng/ml quercetin, 70 ng/ml ellagic acid, or 120 ng/ml trans-p-coumaric were added, and plates were incubated for 1, 3, or 5 days. Each day, 10 μl/well of 0.4 μg/ml MTT in PBS, pH 7.4, was added to each well and incubated for 4 h. After removal of the supernatant, 100 μL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and the absorbance (optical density (OD) value) was measured at 540 nm using a plate reader (SpectraMax 340 PC 389). Tests were performed in triplicate at least three times independently. The mean value was calculated to determine the relative number of

viable cells and an ANOVA using Graph Pad Prism Software was used to estimate differences in their proliferative activity.

4.1.4 Cytokine production in differentiated THP-1 cells as measured by ELISA.

F. assafoetida extracts and the identified components were analyzed for their ability to stimulate cytokine production from non-differentiated and differentiated THP-1 cells, in the presence or absence of LPS. The THP-1 cells were differentiated by incubation in 100 nM PMA for 3 days and then treated with 25 μg/ml ethanol extract of *F. assafoetida,* 70 ng/ml ellagic acid, 220 ng/ml quercetin, 1460 ng/ml ferulic acid, 120 ng/ml trans-p-coumaric acid, or 194 ng/ml vanillic acid in complete RPMI culture media and/or 100 ug/ml LPS and/or 100 ng/ml IL-4 for 48 h. Cell supernatants were collected, and the level of TNF-α, IL-1 β, IL-6, IL-10, and TGF-β in the THP-1 cell culture supernatants were quantified using commercially available ELISA kits (R&D Systems) according to the manufacturer's instruction. The assay was replicated 3 - 4 times.

To prepare the ELISA plates, (10 μg/mL) capture antibody (20 μl) was diluted in 10 ml of coating buffer and 100 μl of the capture antibody solution was added/well to the ELISA plates. The plates were sealed and incubated at 4° C overnight. After incubation, the plates were washed 5 times with PBS, pH 7.4, and 0.05% Tween-20 and then the wells were blocked by the addition of 100 μl/well PBS, pH 7.4, 0.05% Tween-20, and 1% BSA and incubated for 1 h at room temperature to minimize non-specific binding. The plates were washed 5 times with PBS, pH 7.4, and 0.05% Tween-20, air-dried, and stored at 4° C until used. The standards for each cytokine were prepared by dilution in PBS, pH 7.4, containing 1% (w/v) BSA and 0.05% Tween-20 and diluted to provide the standard curve as indicated by the manufacturer.

Samples of conditioned media from the different treated THP-1cells or samples of the standard were added to the wells (100 µl/well) in duplicate and incubated for 4 h in a darkened humidity chamber. The plates were washed 5 times with PBS, pH 7.4, 0.05% Tween-20 solution and then incubated with 100 µl/well of the secondary antibody-streptavidin conjugate (20 µl of the detection antibody were diluted in 10 ml PBS, pH 7.4 and 0.05% Tween-20) for 1 h at room temperature. The plates were washed 3 times with PBS, pH 7.4 and 0.05% Tween-20 and incubated with 100 µl/well of horseradishperoxidase-streptavidin conjugate (10 µl of the HRP-streptavidin was diluted in 10 ml PBS, pH 7.4 and 0.05% Tween-20) for 1 h at room temperature. The plates were washed 3 times with PBS, pH 7.4, and 0.05% Tween-20 and once with PBS, pH 7.4, and stained by incubation with 100 µl/well TMB (3,3', 5,5"-tetramethylbenzidine) substrate (prepared by mixing Reagent A and B in equal volumes, Fisher Scientific) for 30 min in the dark or until blue colour formation. The reaction between the TMB substrate and horseradish peroxidase (HRP), produces a measureable color change that absorbs at 450 nm. Then 50 µl of stop solution (2 M H_2SO_4) was added to each well. The absorbance of the samples was measured immediately using a plate reader at 450 nm. Microsoft Excel was used to calculate the concentration of cytokines in the supernatant samples using the standard curve from the known concentration.

4.1.5 Characterization of monocyte differentiation by flow cytometry.

Monocyte differentiation was characterized by measuring the expression of cell surface differentiation markers on THP-1 cells differentiated by treatment with PMA and then treated with LPS, IL-4 and/or *F. assafoetida* extracts or the different components. The THP-1 cells were aliquoted into 1 ml suspensions at 10^6 cells/ml in 15 ml conical tubes and treated with

100 nM PMA for 3 days. The cells were collected by centrifugation and resuspended in 1 ml of RPMI complete media. The cells were then treated with suspending media, 100 ng/ml LPS, 25 µg/ml ethanol extract of *F. assafoetida*, 70 ng/ml ellagic acid, 220 ng/ml quercetin, 1460 ng/ml ferulic acid, 120 ng/ml trans-p-coumaric acid, or 194 ng/ml vanillic acid in complete RPMI culture media for 48 h at 37° C. For analysis of the cells by flow cytometry, the cells were collected by centrifugation and resuspended in 500 µl of 1% paraformaldehyde in PBS, pH 7.4, and incubated for 10 min. The fixed cells were washed with PBS, pH 7.4 and incubated with BSA blocking reagent (Pierce Chemical Co) overnight and samples stored in PBS, pH 7.4 until analysis. On the day of analysis, the cells were incubated for 30 min with specific antibodies against cell specific cell surface CD markers at a titre of 1:1000 in blocking buffer including; anti-CD163-phycoerythrine (PE) (clone GH/61), anti-CD68-PER CP (clone KP1), or anti-CD80-fluorescein isothiocyanate (FITC) (clone F7) labelled anti-human monoclonal antibodies. Cells were also incubated with isotype matched, non-specific mouse IgG antibodies labelled with PE, PER-PC, or FITC at a titre of 1:1000 in block buffer to create the negative control histograms for each CD staining antibody. The cells were then analyzed by flow cytometery on an FC600 flow cytometer (Beckman). THP-1 cells were gated for analysis by a combination of forward scatter (FSC) and side scatter (SSC). Gated cells were analyzed for CD68 positivity, and CD68+ cells were then examined for CD80 (M1) and CD163 (M2) expression using samples stained with the isotype control antibodies (Santa Cruz Biotech). The percentage of cells positive for each of the CD68, CD80, and CD163 markers was determined by setting a cursor on the negative matched isotope or monocyte control to include 98–99% of the population. The histograms for the cells stained for CD68, CD80, and CD163 surface levels were compared using Beckman flow cytometry software. Microsoft® Excel®

2007 (Excel 2007) was used to organize output data of the percent positive values of each surface marker.

4.1.6 Monolayer damage assay

The THP-1 cells were treated with 100 nM phorbol ester for 3 days to differentiate them to macrophages and then treated with 100 ng/ml bacterial lipopolysaccharide (LPS), 25 µg/ml ethanol extract of *F. assafoetida*, 70 ng/ml ellagic acid, 220 ng/ml quercetin, 1460 ng/ml ferulic acid, 120 ng/ml trans-p-coumaric acid, or 194 ng/ml vanillic acid in complete RPMI culture media for 48 h at 37° C. The macrophages were then washed and diluted to 10^6 cells/ml in complete RPMI-1640 culture media. MCF-7 or MDA-MB-231 breast cancer cell monolayers were cultured to confluent monolayers in 24 well culture dishes in DMEM containing 10% FCS. The damage assay was performed in two different ways. In the first, 0.5 ml of the treated macrophages were added to a tissue culture insert with a semipermeable membrane (Fisher Scientific) which was then suspended above the MCF-7 or MDA-MB-231 monolayer and incubated for 24 h. In the second method, 0.5 ml of the treated macrophages were added directly to the MCF-7 or MDA-MB-231 monolayers and incubated for 24 h. At the end of the incubation, the monolayers were washed and fixed by incubation in 10% phosphate-buffered formalin. The monolayers were subsequently imaged using phase-contrast microscopy and photographed in quadruplicate. The confluence of the monolayers was determined from the photographs by overlaying a 12 x 10 grid and then measuring the percent of grid intersections that corresponded to exposed culture substrate.

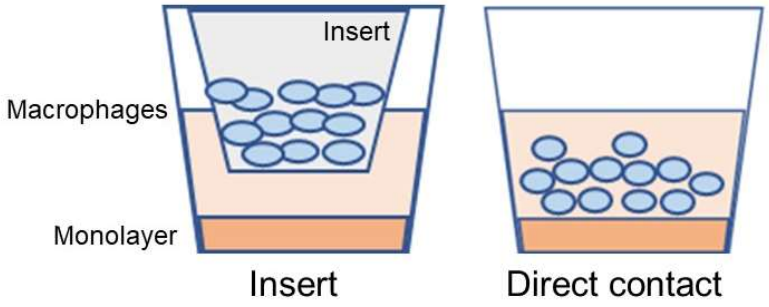


Figure 4.2: Diagrammatic representation of the cell damage assay. In one experiment, the macrophages were added to an insert with a semi-permeable membrane (3 μm holes) and incubated with the breast cancer cell monolayer. In the second experiment, the macrophages were added directly to the monolayer. (07 200 148 Corning; Costar)

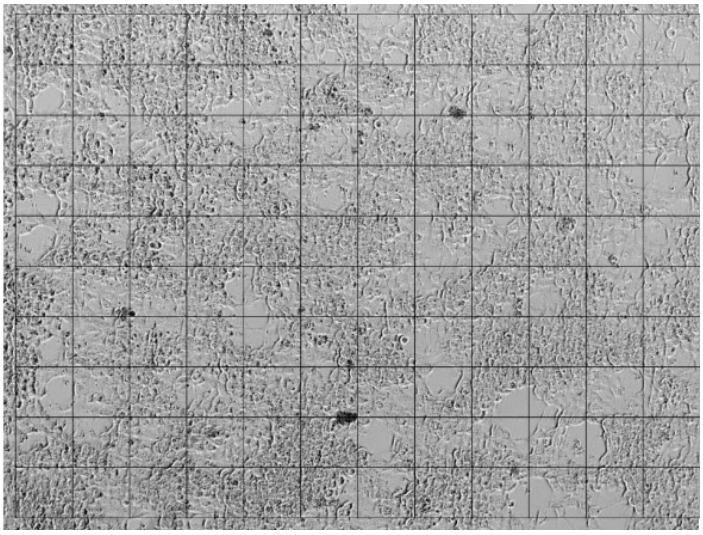


Figure 4.3: Cell damage scored using a 10 x 12 grid. The percent damage was determined by counting the percentage of grid intersections that overlaid exposed culture substrate.

4.1.7 Statistical analysis

Data was analyzed and graphed using Graph Pad (Prism) software and Microsoft Excel. Results were expressed as means ± SD, and the overall differences between group means were analyzed using one-way ANOVA. Multiple comparisons were carried out using Tukey's test and $P<0.05$ was considered to be statistically significant and asterisks were used to indicate different levels of significance (*$P<0.05$, **$P<0.01$).

4.2 Results

4.2.1 Cytotoxic effect of *F. assafotida* on THP-1 cells.

The impact of treatment with the *F. assafoetida* extract and the isolated compounds on THP-1 viability after 1, 3, or 5 days of exposure was investigated using the MTT assay in complete medium. Cells exposed to *F. assafoetida* or the isolated compounds showed reduced cell viability in a dose dependent manner. Cells treated with 100 or 50 µg/ml *F. assafoetida* showed a significant inhibition of cell viability, however, cells treated with 25µg/ml showed no inhibition in cell viability (Figure 4.4 and Figure 4.5). In addition, cells treated with 70 ng/ml ellagic acid, 220 ng/ml quercetin, 1460 ng/ml ferulic acid, 120 ng/ml trans-p-coumaric acid, or 194 ng/ml vanillic acid did not show any significant inhibition of cell viability (Figure 4.4 and Figure 4.5). Together, these results confirm that THP-1 cells that were treated with the lower concentration (25 µg/ml) are less likely to undergo cell death compared to cells treated with higher concentrations of *F. assafoetida* extract. As a result, the cells were treated with 25 µg/ml *F. assafoetida* extract or the isolated compounds at the indicated concentrations to avoid cell death for the following experiments to test macrophage activation.

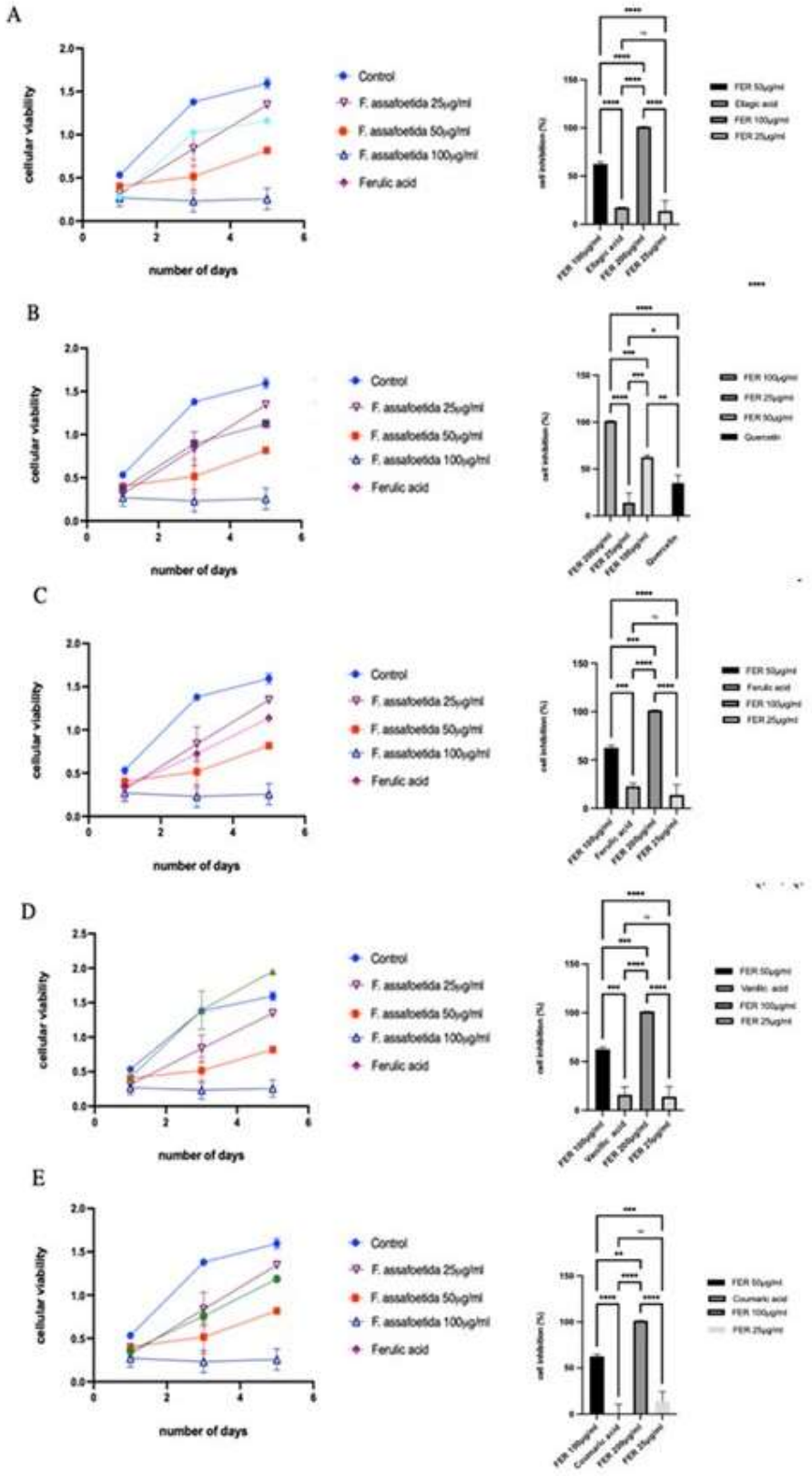

A
B
C
D
E
cellular viability
number of days
Control
F. assafoetida 25µg/ml
F. assafoetida 50µg/ml
F. assafoetida 100µg/ml
Ferulic acid
cell inhibition (%)
FER 50µg/ml
Ellagic acid
FER 100µg/ml
FER 25µg/ml
Quercetin
Vanillic acid
Coumaric acid

Figure 4.4: Cytotoxicity Assays for the THP-1 Cell Line

Viability of the THP-1 cell line treated with *F. assafoetida* (25, 50, or 100 µg/ml) or with (A) 70 ng/ml ellagic acid, (B) 220 ng/ml quercetin, (C) 1460 ng/ml ferulic acid, (D) 120 ng/ml trans-p-coumaric acid, or (E) 194 ng/ml vanillic acid was measured using the MTT assay. The cells were incubated with the compounds for 1-5 days and the absorbance resulting from the MTT assay was determined on day 1, 3, or 5. Each experiment was performed in triplicate and the results are from three independent experiments is shown in the graphs of the relative viability of THP-1 cells treated with *F. assafoetida* extracts or with the compounds and the percent inhibition of cell viability was determined compared to media controls. Data were analyzed for three independent experiments and a p-value less than 0.05 was statistically significant (*).

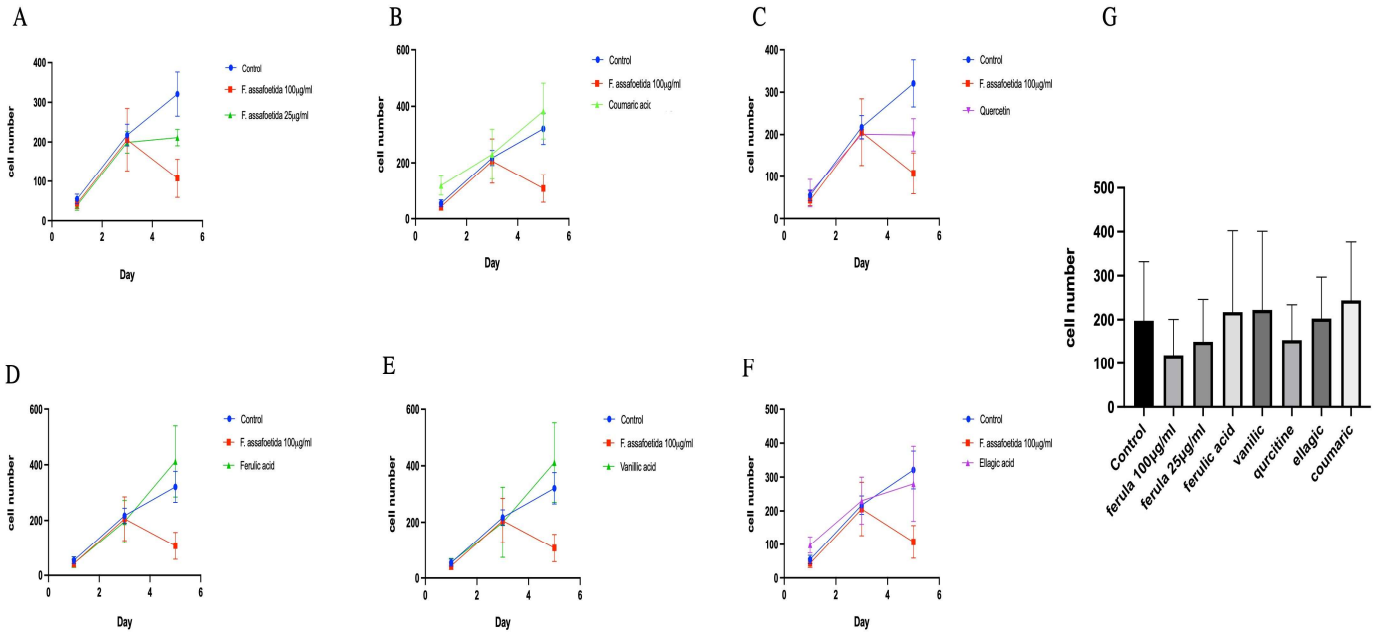


Figure 4.5: Cell viability and proliferation for the THP-1 Cell Line (ImageJ)

THP-1 cells cultured in 24 well plates were treated with (A) (25 µg/ml) *F. assafoetida* or *F. assafoetida* compounds (B) 120 ng/ml trans-p-coumaric acid, (C) 220 ng/ml quercetin, (D) 1460 ng/ml ferulic acid, (E) 194 ng/ml vanillic or (F) 70 ng/ml ellagic acid, for 1, 3, or 5 days. Each day, images were obtained from 5 different regions for each well using a phase contrast microscope and ImageJ software was used to count the numbers of cells in each of the images. (G) shows the relative viability of THP-1 cells treated with *F. assafoetida* extracts or with the compounds P value = 0.9129. The data was taken as the mean $\pm$ SD for three independent experiments.

4.2.2 Polarization of macrophages into different subtypes

Since treatment with Ferula extracts has been shown previously to have immunostimulatory activities, we next investigated the role of *F. assafoetida* on macrophage activation. PMA-differentiated THP-1 monocytes were treated with LPS to promote M1 polarization or with IL-4 to promote M2 polarization. Treatment of PMA-differentiated THP-1 cells with *F. assafoetida* extract or each of the five components (ellagic acid, quercetin, ferulic acid, trans-p-coumaric acid, or vanillic acid) was also tested for macrophage polarization and compared to the control conditions. THP-1 cells treated with PMA for 3 days had an extended, slender, spindle-shaped morphology while treatment with the ethanolic extract of *F. assafoetida* for an additional 24 h caused the cells to spread into a more "pancake" type morphology (Figure 4.6). The treated cells were tested for polarization state by examining the cells for surface expression of the CD68, CD80, and CD163 macrophage M0, M1, and M2 surface markers, respectively. Results obtained from flow cytometry showed that CD68 was expressed at high levels on the M0 cells which had been treated with PMA for 3 days compared to the levels on untreated negative controls (Figure 4.7 A). This supports the idea that the cell culture model used in this experiment is suitable and effective for differentiation of THP-1 cells into macrophages. Additionally, treatment of THP-1 derived macrophages with *F. assafoetida* or with the component compounds expressed higher levels of CD80 similar to that seen on the LPS-treated M1 control cells. However, it should be noted that the effects of treatment with ellagic acid were very weak and only a small proportion of treated cells were shown to express the CD80 cell surface marker. In addition, the level of cell surface CD80 in the vanillic acid- and p-coumaric acid-treated samples was not as high as the LPS positive control-, *F. assafoetida* extract-, or ferulic acid-treated samples. The expression of the CD80

marker is used as an indication of the pro-inflammatory, M1 phenotype and these results indicate that *F. assafoetida* and its components activate an inflammatory phenotype in treated macrophages. However, treatment with *F. assafoetida* or any of its components did not increase the expression of CD163, the M2 polarization marker to levels similar to IL-4 which suggests that the *F. assafoetida* extracts are not able to induce the immunosuppressive phenotype in differentiated THP-1 cells (Figure 4.7 A).

We further examined whether THP-1 cells could be differentiated into the M1 phenotype, as measured by cytokine secretion, by stimulation with *F. assafoetida* or each of the five compounds in the presence or absence of both LPS and PMA treatment. Interestingly, the expression level of IL-6 was significantly increased when PMA-differentiated THP-1 cells were treated with ellagic acid in the presence of LPS (Figure 4.8 A). IL-6 was also highly expressed when undifferentiated THP-1 cells were treated with both *F. assafoetida* extract and with LPS (Figure 4.9 A). Moreover, IL-1 β was also highly expressed when PMA-differentiated cells were treated with *F. assafoetida* extracts (Figure 4.8 B). When undifferentiated THP-1 cells were treated with vanillic acid or quercetin, with or without LPS, the cells showed a high production of IL-1 β (Figure 4.9 C). PMA-differentiated cells that were treated with ellagic acid, in the presence or absence of LPS, showed significant levels of TNF-α expression (Figure 4.8 C). Further, undifferentiated THP-1 cells treated with quercetin or ellagic acid in the absence of LPS increased the secretion of TNF-α (Figure 4.9 B).

We next tested whether THP-1 cells could differentiate into the M2 subtype following stimulation with *F. assafoetida,* or any of the five component compounds compared to the IL-4 control. Results obtained from the ELISA showed very weak expression of TGF- β and IL-

10 in both PMA-differentiated and undifferentiated THP-1 cells in the presence or absence of LPS (Figure 4.8 D and E, and Figure 4.9 D) (TGF-beta from undifferentiated THP-1 cells, did not show any interesting results and data are not shown) . However, treatment of the PMA-differentiated THP-1 cells with the positive control, IL-4, did increase IL-10 secretion as expected.

In conclusion, these data clearly showed that THP-1 cells possess the ability to differentiate into M1 polarized macrophages following stimulation with *F. assafoetida* or the isolated compounds with or without LPS. However, treatment with *F. assafoetida* did not have the ability to promote M2 polarization under these conditions.

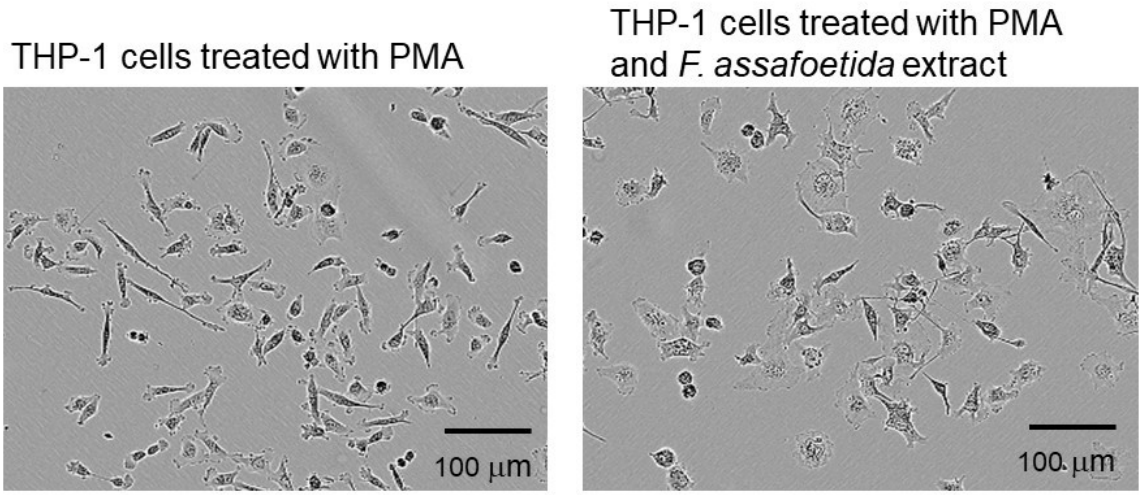


Figure 4.6. Morphology of THP-1 cells treated with phorbol ester or phorbol ester and *F. assafoetida* extracts. The THP-1 cells were treated with 10^{-6} M phorbol-13-myristrate for 3 days and then with (A) suspending media or (B) 25 μg/ml *F. assafoetida* extract for an additional 24 h. Phase contrast cell images were obtained and the bar shows 100 μm in length.

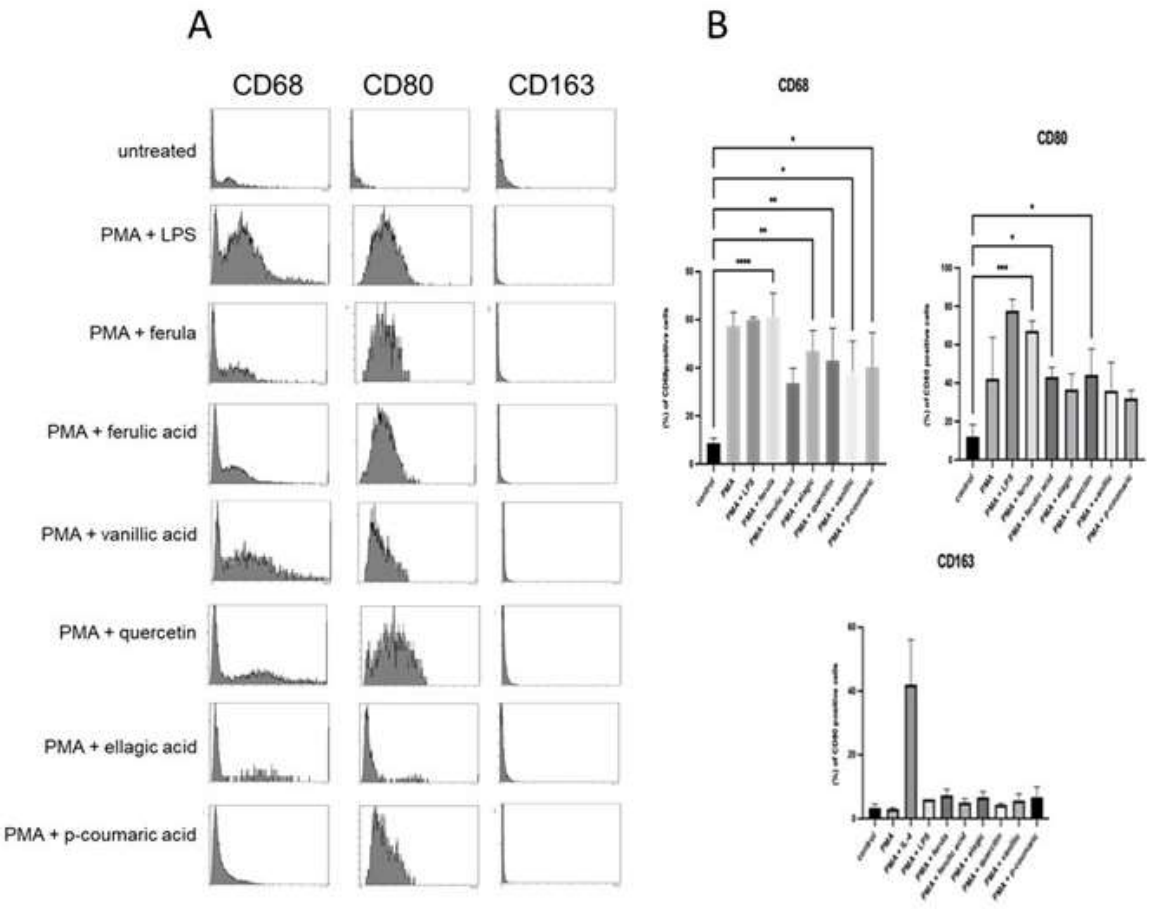


Figure 4.7: The effect of *F. assafoetida* extract on CD80, CD68 and CD163 protein expression

(A) The cell surface markers expression of CD86, CD80 and CD163 surface levels were compared by flow cytometry. A representative histogram is shown for each condition. (B) statistical analysis for the relative number of positive cells. Data are presented as the mean ± SD from three independent experiments. All results show the relative change compared to untreated THP-1 cells ($p < 0.05$).

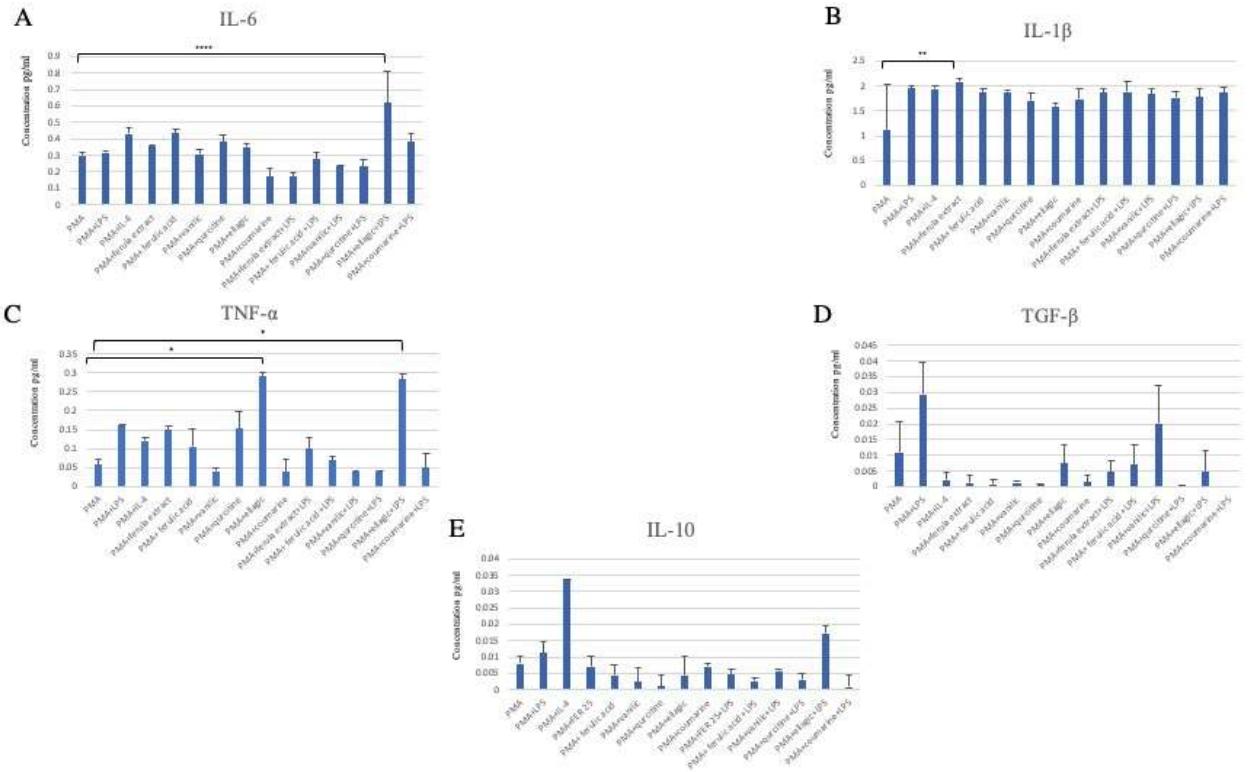


Figure 4.8: Expression of cytokine markers of M1 and M2 macrophages from PMA-differentiated THP-1 cells. THP-1 cells were treated with IL-4, LPS, *F. assafoetida* extract or one of the isolated compounds. (A) IL-6, (B) IL-1 β, (C) TNF- α, (D) TGF- β and (E) IL-10 cytokine levels were measured by ELISA and data presented as mean ± SD of three replicate experiments ($p < 0.05$).

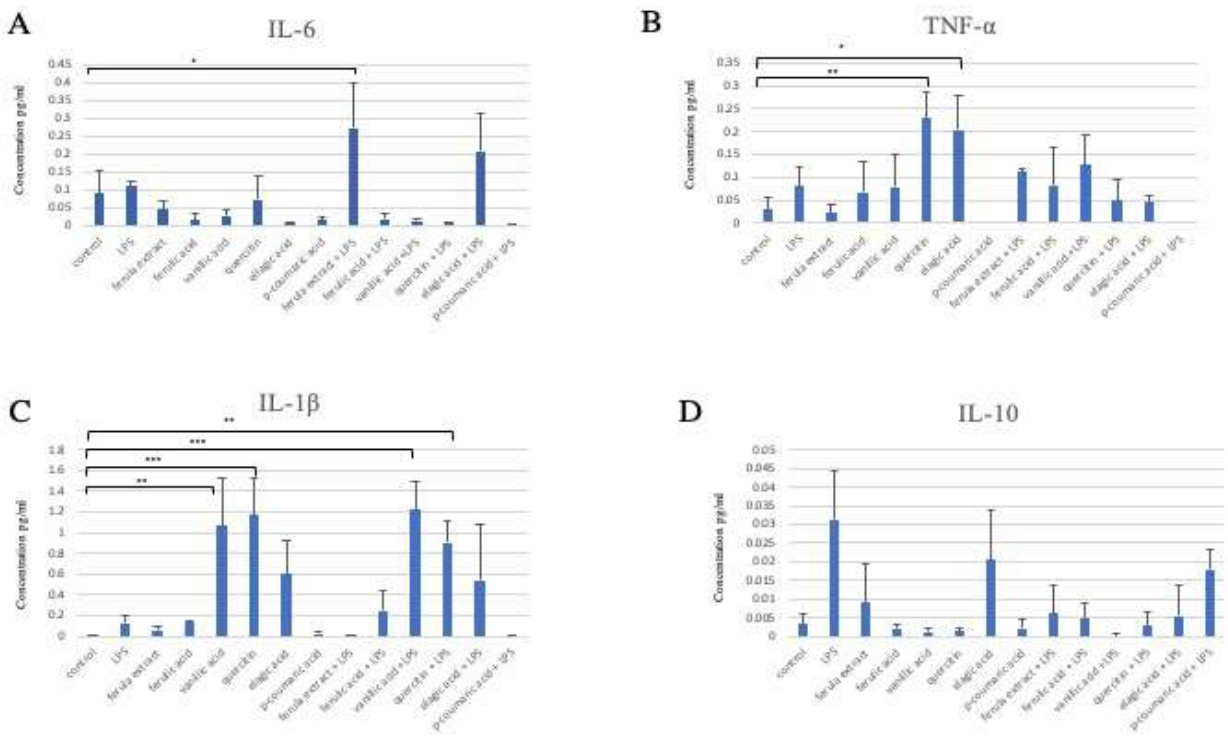


Figure 4.9: Expression of cytokine markers of M1 and M2 macrophages from undifferentiated THP-1 cells. THP-1 cells were treated with IL-4, LPS, *F. assafoetida* extract or one of the isolated compounds. (A) IL-6, (B) IL-1 β, (C) TNF- α, and (D) IL-10 cytokine levels were measured by ELISA and data presented as mean ± SD of three replicate experiments ($p < 0.05$).

4.2.3 Effect of M1 macrophage co-culture on cancer cell destruction

Because inflammation plays a crucial role in tumor progression, we further evaluated the inflammatory responses of cancer cells co-cultured with M1 differentiated macrophages. The data show that the LPS and/or *F. assafoetida*-treated macrophages (previously shown to be M1 polarized) were able to cause a higher level of damage to the MCF-7 (Figure 4.10) or MDA-MB-231 (Figure 4.11) monolayers compared to untreated, or ellagic acid-treated macrophages. In addition, the M1 differentiated macrophages incubated directly with the MCF-7 or MDA-MB-231 monolayers caused more damage than when the macrophages were present in the inserts and therefore did not make direct contact.

Macrophages treated with LPS, *F. assafoetida*, ferulic acid, vanillic acid, quercetin, and p-coumaric acid were able to cause a higher level of damage to both the MCF-7 (Figure 4.12) and MDA-MB-231 (Figure 4.13) monolayers than untreated, or ellagic acid-treated macrophages. In addition, the M1 differentiated macrophages incubated directly with both the MCF-7 and MDA-MB-231 monolayers caused more damage than when the macrophages were present in the inserts and therefore did not make direct contact. The data supports the idea that *F. assafoetida* extracts are able to indirectly cause the death of cancer cells *in vitro* via activation of immune cells.

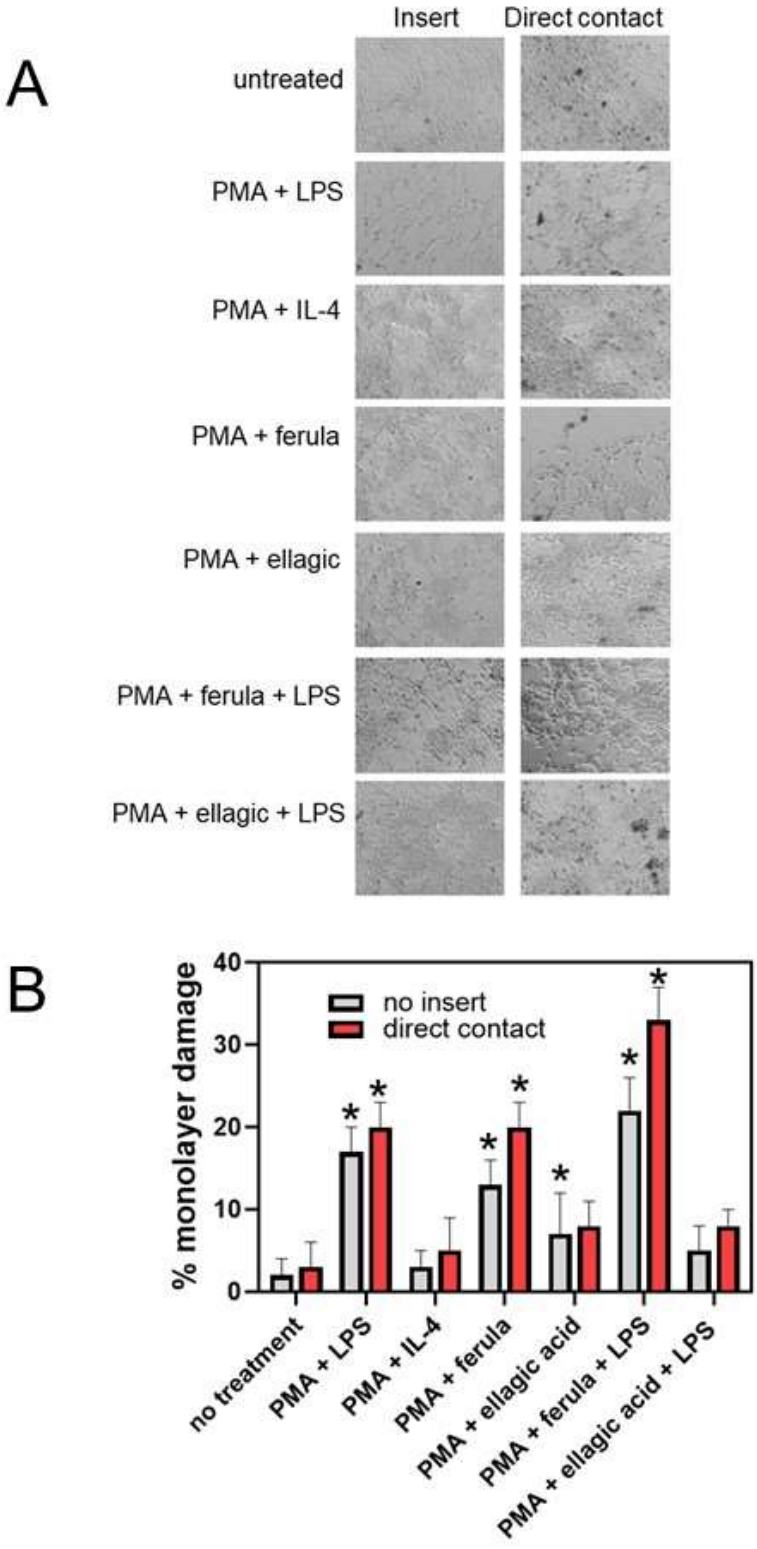

A
Insert
Direct contact
untreated
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ellagic
PMA + ferula + LPS
PMA + ellagic + LPS
B
% monolayer damage
40
30
20
10
0
no insert
direct contact
*
no treatment
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ellagic acid
PMA + ferula + LPS
PMA + ellagic acid + LPS

Figure 4.10: Treatment of THP-1-derived macrophages with *F. assafoetida* promotes damage to MCF-7 monolayers. THP-1 cells were treated with phorbol ester (PMA) for 3 days and then treated with bacterial lipopolysaccharide (LPS), *F. assafoetida* extract (ferula), ellagic acid (ellagic), or some combination for two days. The treated macrophages were incubated with confluent MCF-7 monolayers by adding to an insert or in direct contact for 24 h and phase contrast micrographs obtained (A). The percentage of exposed substrate (mean $\pm$ SD) was determined for quadruplicate measures (B). ANOVA followed by Tukey post-hoc test, (*) indicates $P<0.05$.

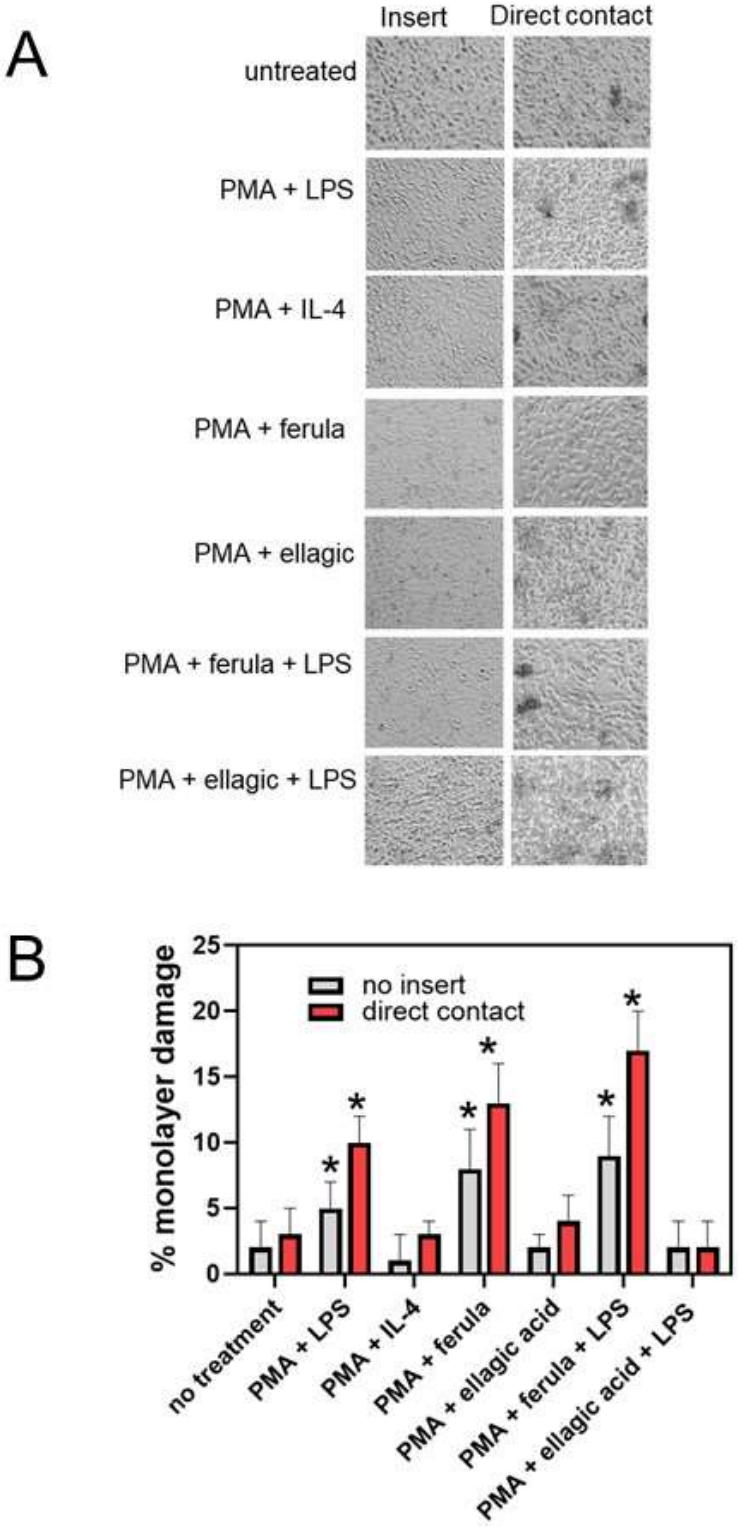

A
Insert
Direct contact
untreated
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ellagic
PMA + ferula + LPS
PMA + ellagic + LPS
B
% monolayer damage
25
20
15
10
5
0
no insert
direct contact
*
no treatment
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ellagic acid
PMA + ferula + LPS
PMA + ellagic acid + LPS

Figure 4.11: Treatment of THP-1-derived macrophages with *F. assafoetida* promotes damage to MDA-MB-231 monolayers. THP-1 cells were treated with phorbol ester (PMA) for 3 days and then treated with bacterial lipopolysaccharide (LPS), *F. assafoetida* extract (ferula), ellagic acid (ellagic), or some combination for two days. The treated macrophages were incubated with confluent MDA-MB-231 monolayers by adding to an insert or in direct contact with the monolayer for 24 h and phase contrast micrographs obtained (A). The percentage of exposed substrate (mean $\pm$ SD) was determined for quadruplicate measures (B). ANOVA followed by Tukey post-hoc test, (*) indicates $P<0.05$.

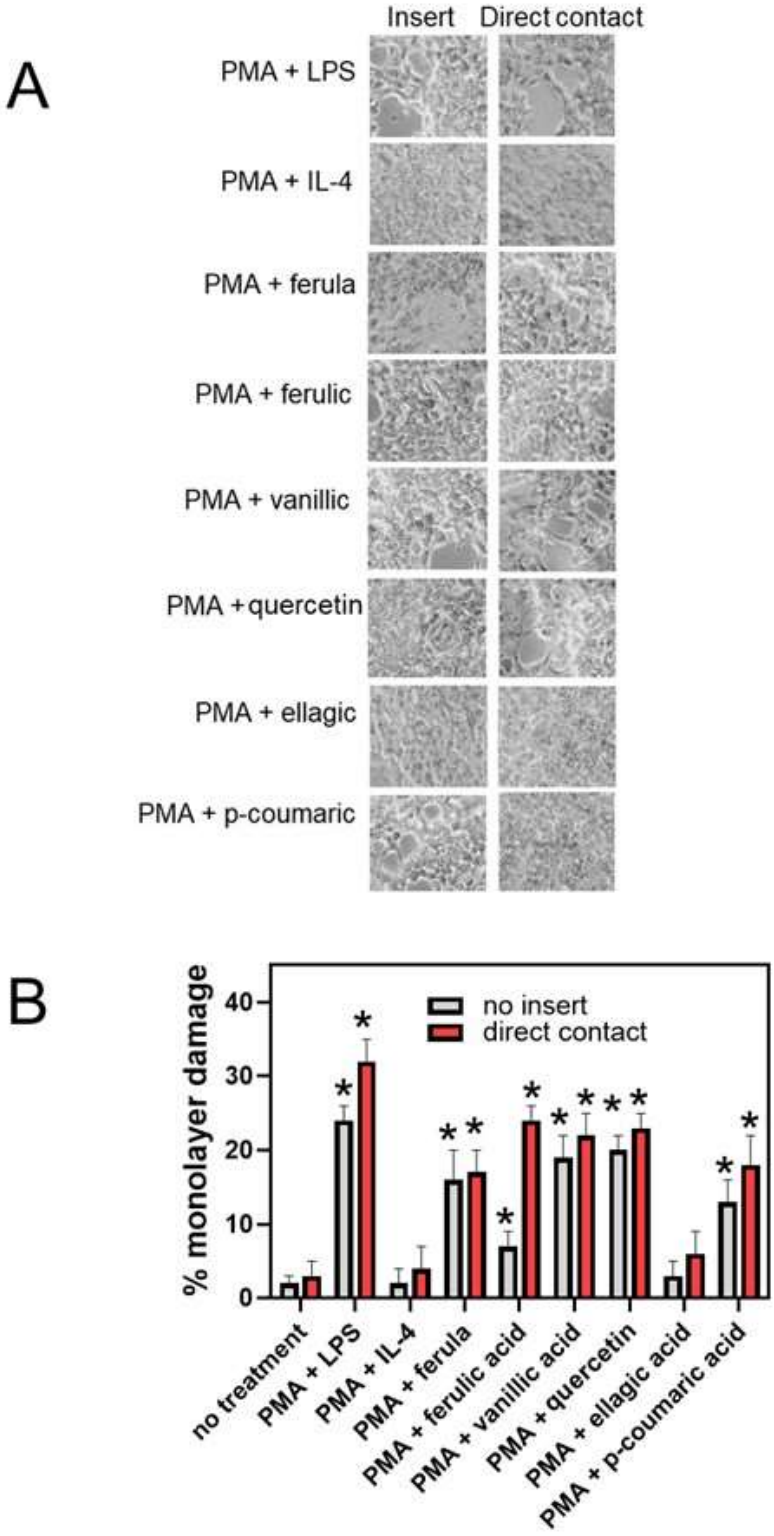
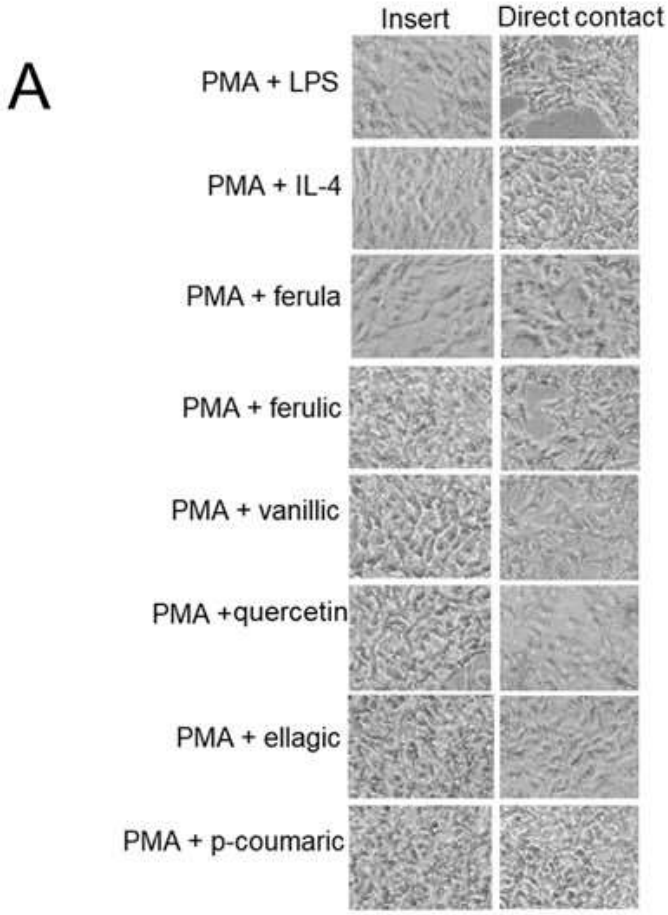
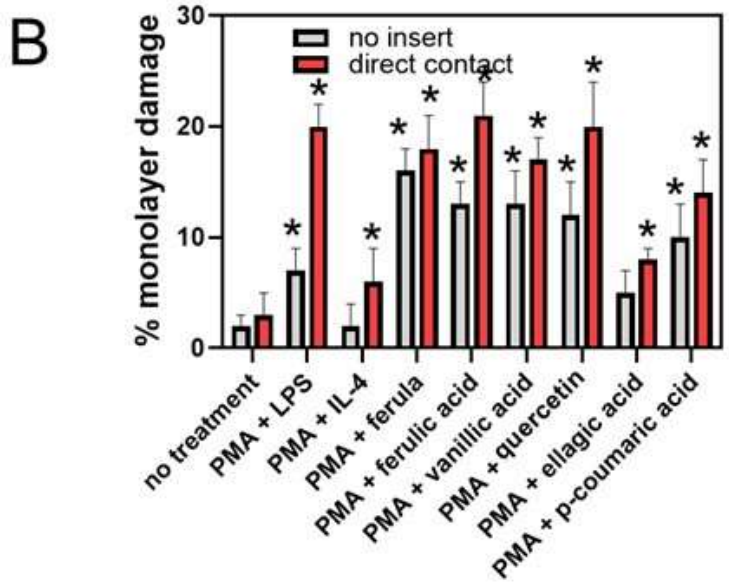

A
Insert
Direct contact
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ferulic
PMA + vanillic
PMA + quercetin
PMA + ellagic
PMA + p-coumaric
B
% monolayer damage
40
30
20
10
0
no insert
direct contact
no treatment
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ferulic acid
PMA + vanillic acid
PMA + quercetin
PMA + ellagic acid
PMA + p-coumaric acid

Figure 4.12: Treatment of THP-1-derived macrophages with *F. assafoetida* components promotes damage to MCF-7 monolayers. THP-1 cells were treated with phorbol ester (PMA) for 3 days and then treated with bacterial lipopolysaccharide (LPS), *F. assafoetida* extract (ferula), ferulic acid (ferulic), vanillic acid (vanillic), quercetin, ellagic acid (ellagic), or p-coumaric acid (p-coumarin) for two days. The treated macrophages were incubated with confluent MCF-7 monolayers by adding to an insert or in direct contact with the monolayer for 24 h and phase contrast micrographs obtained (A). The percentage of exposed substrate (mean $\pm$ SD) was determined for quadruplicate measures (B). ANOVA followed by Tukey post-hoc test, (*) indicates $P<0.05$.


A
Insert
Direct contact
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ferulic
PMA + vanillic
PMA +quercetin
PMA + ellagic
PMA + p-coumaric


B
% monolayer damage
30
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no insert
direct contact
no treatment
PMA + LPS
PMA + IL-4
PMA + ferula
PMA + ferulic acid
PMA + vanillic acid
PMA + quercetin
PMA + ellagic acid
PMA + p-coumaric acid

Figure 4.13: Treatment of THP-1-derived macrophages with *F. assafoetida* components promotes damage to MDA-MB-231 monolayers. THP-1 cells were treated with phorbol ester (PMA) for 3 days and then treated with bacterial lipopolysaccharide (LPS), *F. assafoetida* extract (ferula), ferulic acid (ferulic), vanillic acid (vanillic), quercetin, ellagic acid (ellagic), or p-coumaric acid (p-coumarin) for two days. The treated macrophages were incubated with confluent MDA-MB-231 monolayers by adding to an insert or in direct contact with the monolayer for 24 h and phase contrast micrographs obtained (A). The percentage of exposed substrate (mean $\pm$ SD) was determined for quadruplicate measures (B). ANOVA followed by Tukey post-hoc test, (*) indicates $P<0.05$.

4.3 Discussion

Macrophages are "plastic" cells that can switch from one phenotype to another. In order for macrophages to be polarized to the proinflammatory M1 type, they need to be stimulated by interferon (IFN-γ) or LPS. Once activated M1 macrophages produce proinflammatory macrophages cytokines such as TNF-α, IL-1β, and IL-6. Alternately, macrophages can be polarized to the M2 type by stimulation with IL-4 or IL-13. M2 polarized macrophages release high level of IL-10 and TGF-β cytokines which are involved in immunosuppression and tissue repair. In tumours, macrophages can play an important role in creating the environment that controls whether the cancer cells continue to grow or not. Tumour associated macrophages (TAMs) which are polarized to the M1 type promote an inflammatory environment that decreases cancer cell growth and promotes apoptosis. On the other hand, TAMs polarized to the M2 phenotype inhibit inflammation and promote tissue repair responses that can assist in cancer cell growth. In most tumours, both M1 and M2 macrophages are present and the ratio of M1 to M2 cells can be important in controlling tumour growth. Therefore, the ability to shift macrophage polarization could be an important component in cancer treatment.

This study was aimed to investigate the effect of treatment with *F. assafoetida* extracts on the polarization of macrophages and its effect on the anti-cancer activity of TAMs using the MCF-7 or MDA-MB-231 cell lines co-cultured with THP-1-derived macrophages. First, the effect of *F. assafoetida* extracts on THP-1 viability was tested to determine a concentration that did not kill the THP-1 cells but which could be used to modify their differentiation. The MTT test showed that treatment with 25 ug/ml of the *F. assafoetida* extract had no significant

effects on THP-1 cell viability. Similarly, treatment of the THP-1 cells with 70 ng/ml ellagic acid, 220 ng/ml quercetin, 1460 ng/ml ferulic acid, 120 ng/ml trans-p-coumaric acid, or 194 ng/ml vanillic acid did not have a significant effect on THP-1 cell viability. However, the number of dead cells increased in the THP-1 cell cultures with increasing concentration of *F. assafoetida* or the compounds.

THP-1 cells treated with PMA for 3 days were shown to differentiate into macrophage-like cells. The cells showed a difference in morphology and increased levels of cell surface CD68 expression corresponding with macrophages. Further, results obtained from ELISA and flow cytometry assays show that THP-1-derived macrophages treated with LPS were compatible with differentiation to an M1 phenotype while treatment with IL-4 were compatible with differentiation to an M2 phenotype. Treatment of the THP-1-derived macrophages with *F. assafoetida* extracts or the isolated compounds was shown to enhance the polarization of the THP1-derived macrophages towards the M1 subtype while preventing their polarization into the M2 phenotype. Specifically, treatment of THP-1-derived macrophages with *F. assafoetida* or with the compounds were shown to express higher level of the M1 cell surface marker, CD80, but did not affect the level of the M2 cell surface marker CD163. Additionally, the THP-1-derived M1 macrophages were also shown to express relatively high levels of the pro-inflammatory cytokines, TNF- α, IL-1β and IL-6 but did not express significant levels of the anti-inflammatory cytokines IL-10 and TGF- β. These results suggest that *F. assafoetida* and the isolated compounds may serve to suppress the expression and production of M2 (anti-inflammatory) cytokines that block anti-tumor immune responses and enhance the metastatic progression of cancer. A 2020 study investigating the immunomodulating effects of ellagic acid on THP-1 macrophage cells found that THP-1 cells

treated with ellagic acid significantly reduced the expression of the Toll-like receptor 4 which is a receptor for LPS that promotes an inflammatory response (18). Another study examined the impact of quercetin on macrophages and found that quercetin inhibited the polarization of M1 macrophages and significantly induced the expression of the M2 marker CD206 (19). Umbelliprenin, a natural occurring compound found in many Ferula species, also has been shown to increase the M1/M2 ratio significantly. The level of IL-10 production was reduced significantly in the cell culture of M1 and M2 cells after exposure to umbelliprenin, while a significant increase in IL-12 content was seen in the supernatant of M1 cells there was a decrease in the supernatant of the M2 cells.

Since the direct anti-tumour properties of Ferula have been studied on various types of cancer cells, we further investigated the anti-tumour effect of *F. assafoetida* on the TAMs ability to destroy cancer cell monolayers using the MCF-7 or MDA-MB-231 cell lines co-cultured with THP-1-derived M1-differentiated macrophages. In this experiment, THP-1 cells were treated with phorbol ester (PMA) for 3 days and then treated with bacterial LPS, *F. assafoetida* extract, ellagic acid, ferulic acid, vanillic acid, quercetin, or trans-p-coumaric acid for two days. The treated macrophages were then incubated with confluent MCF-7 or MDA-MB-231 monolayers by indirect contact using an insert, which allowed the exchange of soluble factors but not the trans-migration of cells, or by direct contact for 24 h. Our results demonstrated that when the macrophages were in direct contact with the co-cultured cancer cells, the cytotoxic effect of treated, M1 macrophages was higher than when the cells were in the tissue culture insert allowing only diffusion of soluble factors from the treated macrophages. The role of M1 macrophages is to produce pro-inflammatory cytokines, secrete free radicals, promote phagocytosis, and participate in antigen presentation and thus function

as an anti-tumor agent. Immunostimulatory activities of Ferula extracts have been previously documented in the literature. For example, in a 2021 paper, researchers investigated the effect of exposure of an adenocarcinoma gastric cell Line (AGS) to Umbelliprenin, an anti-tumor compound that is derived from Ferula species. They found that Umbelliprenin significantly altered the molecules secreted by macrophages and were acting in favor of an M1 phenotype. In their study, they also showed that the M1 co-cultured macrophages showed an increase in Nitric Oxide (NO) production (20). Nitric oxide plays a key role in the regulation of tumour progression and metastasis.

The results of this study show that macrophages treated with *F. assafoetida* extracts or with various components of the *F. assafoetida* extract promote their differentiation to an M1 phenotype. Further, these *F. assafoetida*-treated macrophages are able to promote the destruction of breast cancer cells in co-culture experiments suggesting that the ability of *F. assafoetida* treatment to activate the M1 phenotype promotes cancer cell destruction. Other expermeints have shown that treatment of breast cancer (and other cancer) cells with *F. assafoetida* extracts can directly promote their apoptosis suggesting its utility as an anti-cancer agent. The current studies show that treatment with *F. assafoetida* extracts can indirectly destroy breast cancer cells via activation of an immune mechanism thus providing additional evidence for its potential as an anticancer agent.

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Chapter 5. Characterization of the composition of *Ferula assafoetida* extracts by HPLC

Abstract

Ferula assafoetida gum resin is widely used as a food additive or traditional medicine. In order to gain more knowledge about *F. assafoetida* composition, analysis of both ethanol and aqueous extracts of the gum resin have been carried out using HPLC. Analysis on a C18 column using an isocratic mobile phase, based on previous studies, fractionated the extract into several peaks although this methodology did not efficiently differentiate some standard compounds. Analysis on the C18 column using a gradient mobile phase showed superior separation of the standard components and allowed separation and identification of ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid in ethanolic extracts of *F. assafoetida* based on retention time and relative absorbance. The results of this study are consistent with previous studies which looked at the composition of *F. assafoetida* samples, and include an improved method to separate some components in the extracts.

Keywords: Natural products, *Ferula assafoetida*, HPLC, phenolics.

5. Background

Ferula assafoetida is a plant indigenous to wide portions of the middle east from Afghanistan and Iran to Eastern Europe. Extracts of the plant have been used as a flavour enhancer in food preparation and as a traditional medicine for hundreds of years (1,2). A variety of recent studies have shown that extracts of *F. assafoetida* have anti-oxidant (3,4), anti-bacterial (5), anti-fungal (6), antiviral (7), anti-diabetic (8,9), anti-inflammatory (10), and anti-cancer (11-13) activities. The plant preparations can be broken down into three different fractions: the resin fraction, the gum fraction, and the essential oils (14,15). The resin fraction has been shown to contain ferulic acid, coumarins, and a variety of terpenoids while the gum fraction contains sugars such as glucose, galactose, arabinose, glucuronic acid, polysaccharides and glycoproteins and the essential oils include a variety of sulfur-containing components and volatile terpenoids (1,14).

The gum resin produced from the secretions of damaged roots is what is widely used as a flavour enhancer in Middle Eastern and Indian food preparation as well as a Traditional medicine for treatment of gastrointestinal and inflammatory disorders (16,17). *F. assafoetida* extracts are a complex mixture of sugars, amino acids, minerals, and biologically active components and the composition of *F. assafoetida* is dependent on its botanical and geographical origin as well as on the processing and storage conditions (18). Increased interest in its use as a potential therapeutic makes characterization and isolation of its components of particular value.

The composition of *F. assafoetida* is commonly analyzed using HPLC on C-18 columns and detected using either absorbance in the ultraviolet spectra or a refractive index detector. A variety of studies have used as single component, isocratic, mobile phase

consisting of acidified acetonitrile or methanol (4,9,17). In other experiments, improved resolution of some components has been measured using a gradient mobile phase changing from acidified water to acidified acetonitrile or methanol (16,19). *F. assafoetida* is rich in phenolic acids and flavonoids which are best known as natural anti-oxidants and which have been implicated as the active agents in the role of *F. assafoetida* as a benefit to human health, in particular in gastrointestinal health and anti-inflammatory activity (20). For characterization, analysis of the extracts with HPLC and detection using UV absorption can provide a good estimate of concentration of the individual components by comparison to a standard curve of a known sample subjected to the same chromatographic conditions (21-24).

In this study, we examined both the ethanolic and aqueous extracts of *F. assafoetida* gum-resin using HPLC on a C18 silica column and matched several of the component peaks to a group of common chemical constituents. We also compared an isocratic mobile phase consisting of acidified acetonitrile and water to a gradient mobile phase beginning with acidified water and changing to acidified acetonitrile to assess the efficiency of component resolution.

5.1 Material and Methods

5.1.1 Preparation of *Ferula assafoetida* extracts for analysis

The *Ferula assafoetida* root gum (purchased at a commercial market in Saudi Arabia) was crushed and then refluxed by boiling for 1 h in water, or 70% ethanol. The insoluble material was separated by centrifugation and the supernatant filtered through a 0.22 μm syringe filter. The purified standards including gallic acid (#AC4108600), chlorogenic acid (#AJ604573), caffeic acid (#AJ6045703), vanillic acid #A1207414), trans-p-coumaric acid (#A1516714), transferulic acid (#A1389124), ellagic acid (#AC1177400), and quercetin (#A1580714) [Fisher Scientific] were similarly dissolved in 70% ethanol to 1% (w/v) and filtered through a 0.22 μm filter.

5.1.2 Characterization of *Ferula assafoetida* extracts using isocratic mobile phase

The samples were subjected to HPLC on a Breeze 2 chromatography system (Waters Limited, Mississauga, ON) including a spectrophotometer, auto-injector, and column heater set to 30^{o} C fitted with a Sunfire C18 3.5 μm, 4.6 x 150 mm column and a Sunfire C18 3.5 μm, 4.6 x 20 mm guard column (Waters Limited). The samples (2 - 5 μl) were injected onto the column and the mobile phase (a solution of 90% acetonitrile, 5% acetic acid, and 5% water) was run in an isocratic manner at 0.5 ml/min for 20 min and peaks detected as absorbance at 280, 320, 370, and 425 nm. Two independent preparations of each solution were analyzed using different amounts of sample to ensure consistent separation and quantitation. The relative mobility of the different concentrations of the purified standards was determined and the corresponding peak in the *F. assafoetida* preparation was identified

using both the relative mobility of the peak and by comparison of the relative absorbance at each wavelength.

5.1.3 Characterization of *Ferula assafoetida* extracts using a gradient mobile phase

The different extracts were subjected to HPLC on a Breeze 2 chromatography system including a spectrophotometer and auto-injector, fitted with a Sunfire C18 3.5 μm, 4.6 x 100 mm column and a Sunfire C18 3.5 μm, 4.6 x 20 mm guard column (Waters Limited). The samples (2 - 5 μl) were injected onto the column and the mobile phase applied at 1 ml/min: the mobile phase was comprised of a gradient between Buffer A (5% acetic acid in water) and Buffer B (5% acetic acid in acetonitrile) which was set to change from 100% Buffer A to 50% Buffer A: 50% Buffer B after 40 min, to 100% Buffer B from 40 - 50 min, and then to 100% Buffer A from 50 - 60 min) (Almnayan, 2021). The absorbance of the eluent was measured at 280, 320, 370, and 425 nm. Two independent preparations of each extract were analyzed at least two separate times to ensure consistent separation and quantitation. The different individual peaks in the *F. assafoetida* extracts were identified by comparison to the relative mobility of the different purified standards and by comparison of the relative absorbance at each wavelength between extract and standard.

A standard curve of different concentrations of gallic acid, chlorogenic acid, caffeic acid, vanillic acid, trans-p-coumaric acid, transferulic acid, ellagic acid, and quercetin (Fisher Scientific) (24). Each standard was suspended at a known concentration and separately subjected to fractionation on HPLC (to identify the retention time) and then several standards were combined at equal concentrations and serially diluted before being fractionated on HPLC to create a standard curve based on using the area under the peak. The

relative amounts of each component in the *F. assafoetida* extract was then determined based on the area of the corresponding peaks in the chromatographs in comparison to the standard curve.

5.2 Results

5.2.1 Characterization of *Ferula assafoetida* using an isocratic mobile phase

Previous studies had shown significant differences between the ethanolic and aqueous extracts of *Ferula assafoetida* in terms of cell viability (11-13). Therefore, *F. assafoetida* root gum-resin was extracted by boiling in 70% ethanol or in water before being analyzed by HPLC. Previous studies had analyzed *F. assafoetida* extracts using an isocratic mobile phase of 90% acetonitrile, 5% acetic acid, and 5% water on a C18 silica HPLC column and measured at 280 and 320 nm (4). Alternately, 90% methanol and 5% acetic acid or phosphoric acid were used as the mobile phase (9,17). These papers generate a particular "fingerprint" chromatograph with large rapidly eluted peaks which diminish at later elution times with the early peak being characterized as ferulic acid. Our analysis of the *F. assafoetida* extracts on a C18 column with an isocratic mobile phase showed a very similar pattern of peaks, detected at wavelengths of 280, 320, and 370 nm, to the previously published chromatograms. The ethanol extracts were shown to include approximately 10 different peaks, as indicated by the retention time and the absorption characteristics, while a significantly lower amount of these compounds was present in the aqueous extract (Figure 5.1). The ethanol extracts were diluted 16-fold prior to examination by HPLC. The analysis of the area under each of the absorbance peaks shows that, on average, the ethanol extracts contained the compounds at about 20-fold higher concentration than each of these compounds in the aqueous extract. The molecular standards known to be present in *F. assafoetida,* or in other natural products, were also analyzed using the same isocratic mobile phase. Under these conditions, the standards showed relatively poor resolution. Several of the standards, including ferulic acid and p-coumaric acid, migrated with almost identical

retention times which was coincident with the first major peak in the chromatogram while vanillic acid and ellagic acid migrated coincident with the second major peak (Table 5.1) (Figure 5.2). These results suggested that this methodology was not suitable for identifying and quantifying specific components in the chromatogram.

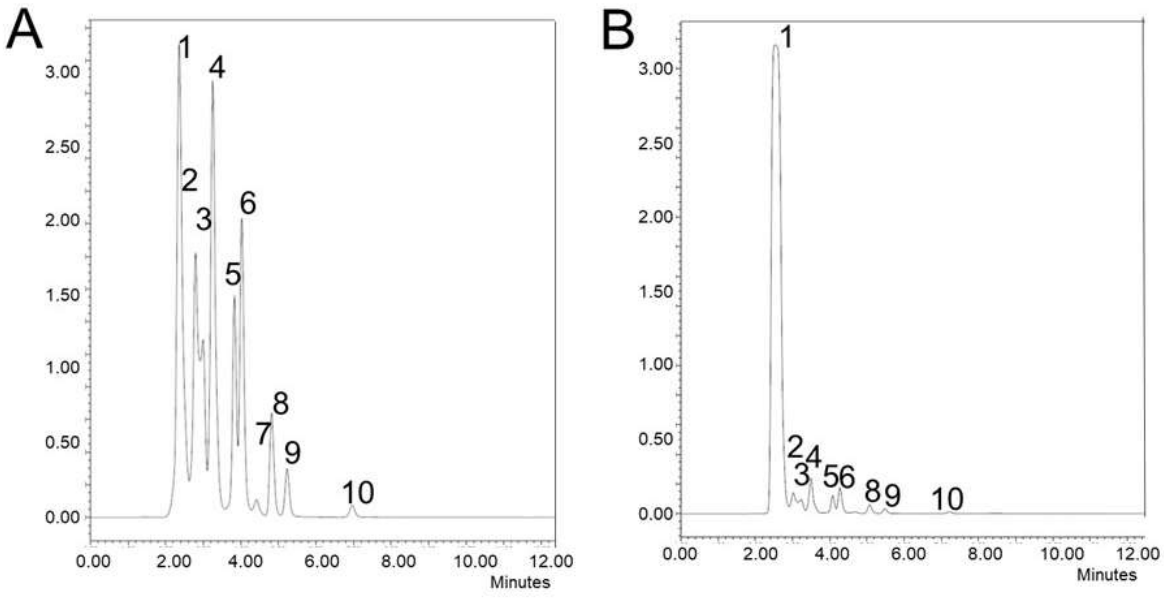

Figure 5.1: HPLC of *F. assafoetida* extracts using an isocratic mobile phase. HPLC of an ethanolic (A) or aqueous extract (B) of *F. assafoetida* gum-resin on a C18 silica column using a 90% acetonitrile 5% acetic acid mobile phase and measured at 320 nm.

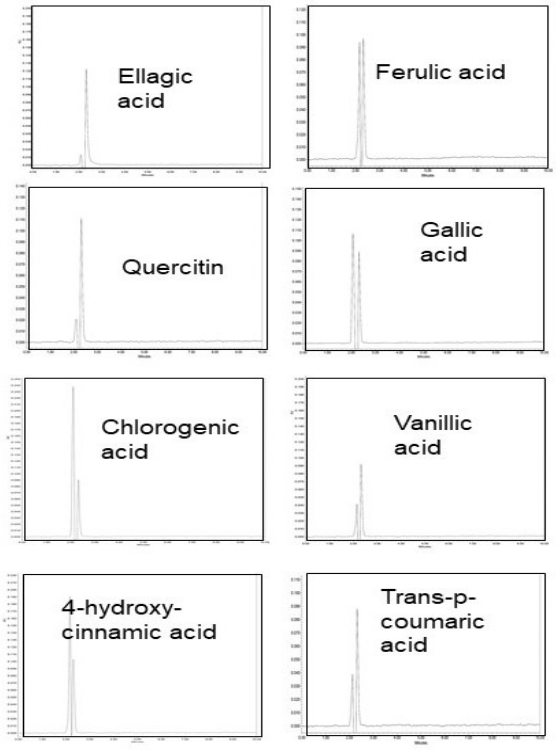

Figure 5.2: HPLC of standard compounds found in *F. assafoetida* extracts using an isocratic mobile phase. HPLC of the indicated purified compounds (1 μg/ml in 70% ethanol) on a C18 silica column using a 90% acetonitrile 5% acetic acid mobile phase and measured at 320 nm.

Table 5.1: Identification of individual peaks in an HPLC chromatogram of *F. assafoetida* extract by comparison to known standards.

	compound	Elution (min)
1	Trans-p-coumaric acid	2.155
1	Ferulic acid	2.184
2	Vanillic acid	2.312
2	Ellagic acid	2.273
3	Quercitin	2.712

5.2.2 Characterization of *Ferula assafoetida* using a gradient mobile phase

Previous analysis in our laboratory showed that the use of a gradient mobile phase was efficient at separating this set of standards into individual peaks for analysis of Sidr honey (25). Therefore, we used this methodology to analyze *F. assafoetida* extracts in order to identify the specific components.

Both the alcoholic extract of *F. assafoetida* and the aqueous extract of *F. assafoetida* were analyzed by fractionation on C18 HPLC columns with a gradient mobile phase (5% acetic acid in water to 5% acetic acid in acetonitrile) (Figure 5.3). The concentrations of the compounds extracted by 70% ethanol were found to be approximately 10 fold higher than the corresponding peaks in the aqueous extracts. In addition, the analysis of the ethanolic extract showed that the peaks were differentially detected at different analytical wavelengths with most peaks showing a maximal detection at 320 nm (Figure 5.4). Analysis of multiple phenolic and flavonoid standard compounds by fractionation on C18 HPLC columns with a gradient mobile phase (5% acetic acid in water to 5% acetic acid in acetonitrile) showed

good separation. The standards selected for this analysis were based on compounds that had been previously identified in natural products including gallic acid, chlorogenic acid, caffeic acid, vanillic acid, trans-p-coumaric acid, 4-hydroxy-3-methoxycinnamic acid, transferulic acid, ellagic acid, and quercetin. Each standard was separately subjected to fractionation on HPLC and its retention times identified (Figure 5.5). To confirm the retention times and to create standard curves for quantitation, two different combinations of standards, each containing 5 separate components, were created with each component at 100 μg/ml and then these mixes were serially diluted before being fractionated on HPLC (Figure 5.6). The areas under the peak for each standard component in the mixture was obtained for each of the known concentrations and these values were used to create a standard curve for each component (Figure 5.7, Figure 5.8). Individual peaks in the *F. assafoetida* chromatographs were identified based on comparison to the retention time of the standard as well as by comparing the change in peak area between chromatographs obtained at different wavelengths. The relative amount of each component was then determined based on the area of the corresponding peaks in the *F. assafoetida* chromatographs in comparison to the standard curve and then calculated based on the dilution of *F. assafoetida* required for the original chromatogram (Table 5.2) (Figure 5.8). From this analysis we were able to identify 5 of the peaks in the *F. assafoetida* extract, transferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid and determine their concentrations. Some of the peaks in the chromatogram were not identified by comparison to the standard compounds used and further efforts are required in order to identify these components.

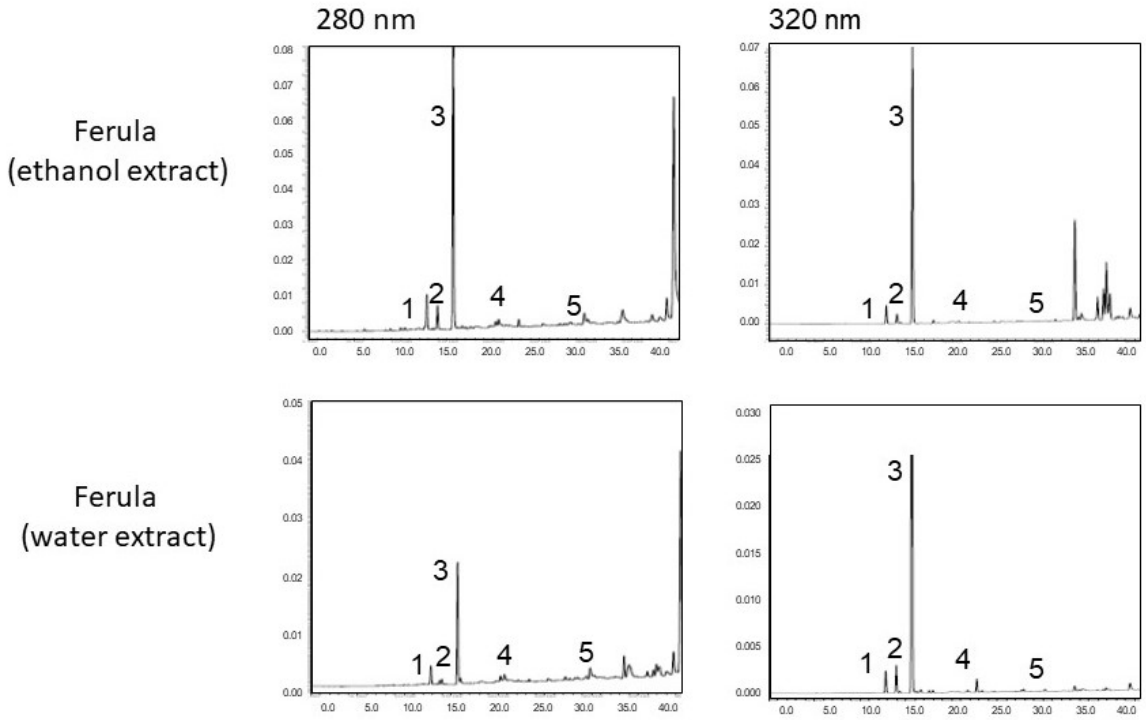

Figure 5.3: HPLC of *F. assafoetida* aqueous and ethanolic extracts using a gradient mobile phase with absorbance measured at 280 and 320 nm. HPLC of an ethanolic or aqueous extract of *F. assafoetida* gum-resin on a C18 silica column using a gradient mobile phase starting at 5% acetic acid in water to 50% acetonitrile and 5% acetic acid in water. Detection of the eluted peaks was measured at 280 and 320 nm. The numbered peaks correspond to: 1. vanillic acid; 2. trans-p-coumaric acid; 3. transferulic acid; 4. ellagic acid; and, 5. quercetin.

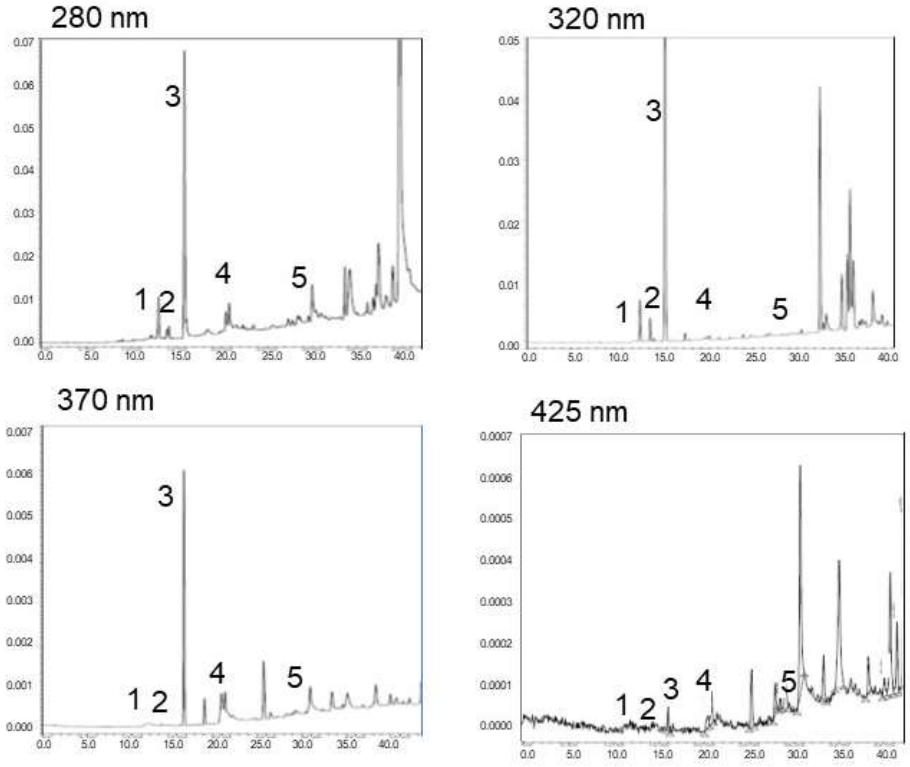

Figure 5.4: HPLC of *F. assafoetida* ethanolic extracts using a gradient mobile phase with absorbance measured at 280, 320, 370 and 425 nm. HPLC of an ethanolic extract of *F. assafoetida* gum-resin on a C18 silica column using a gradient mobile phase starting at 5% acetic acid in water to 50% acetonitrile and 5% acetic acid in water. Detection of the eluted peaks was measured at 280, 320, 370, and 425 nm. The numbered peaks correspond to: 1. vanillic acid; 2. trans-p-coumaric acid; 3. transferulic acid; 4. ellagic acid; and, 5. quercetin.

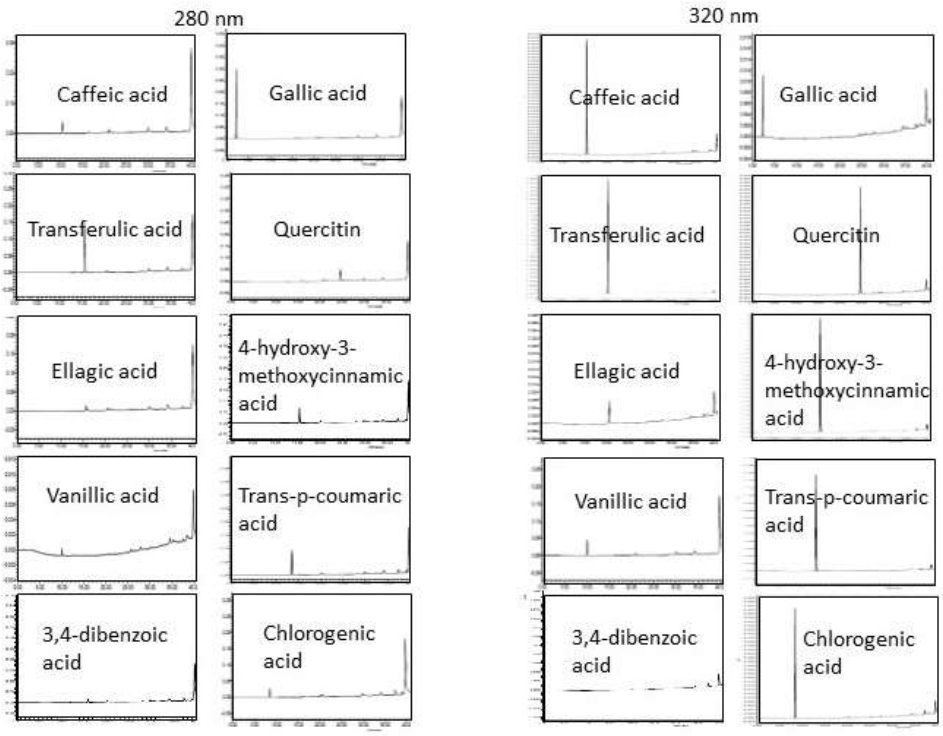

Figure 5.5: HPLC of purified reference compounds using a gradient mobile phase. HPLC of the indicated purified compounds (1 μg/ml in 70% ethanol) on a C18 silica column using a gradient mobile phase starting at 5% acetic acid in water to 50% acetonitrile and 5% acetic acid in water. Detection of the eluted peaks was measured at 280 and 320 nm.

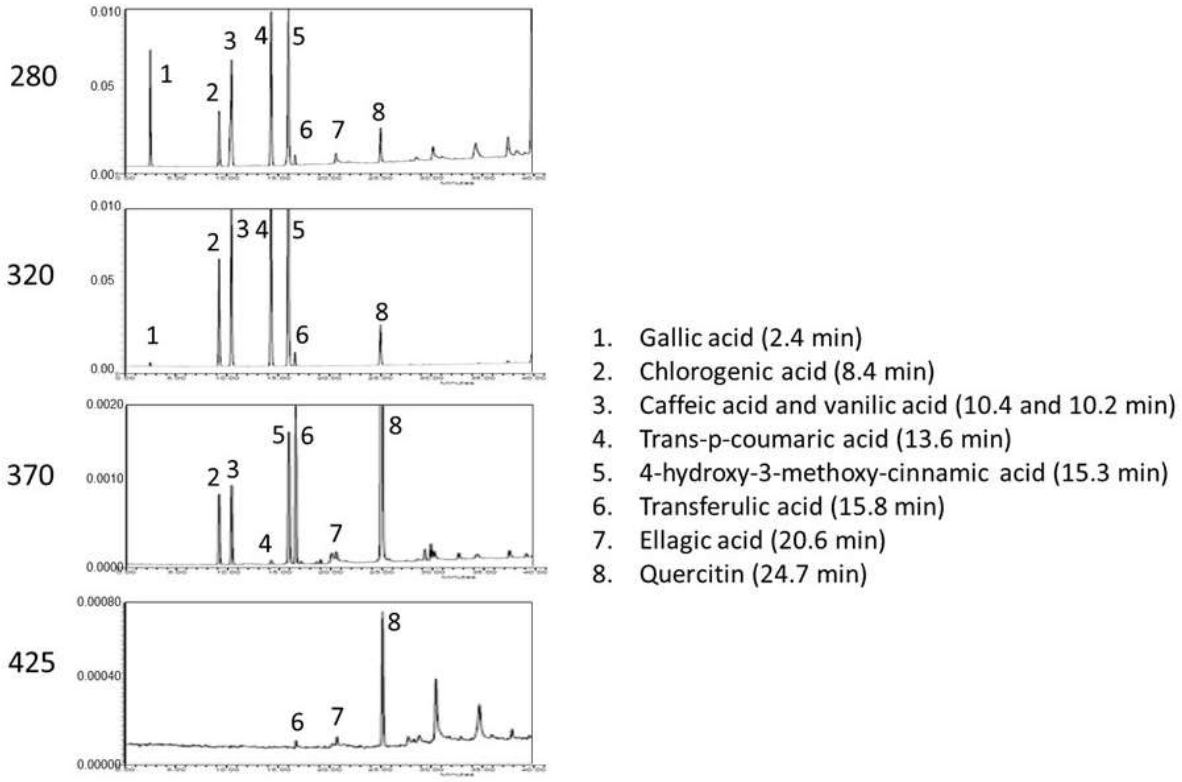

Figure 5.6: HPLC of purified reference compounds using a gradient mobile phase and detected at multiple wavelengths. HPLC of the mixture of the indicated purified compounds (each at 1 μg/ml in 70% ethanol) on a C18 silica column using a gradient mobile phase starting at 5% acetic acid in water to 50% acetonitrile and 5% acetic acid in water. Detection of the eluted peaks was measured at 280, 320, 370, and 425 nm.

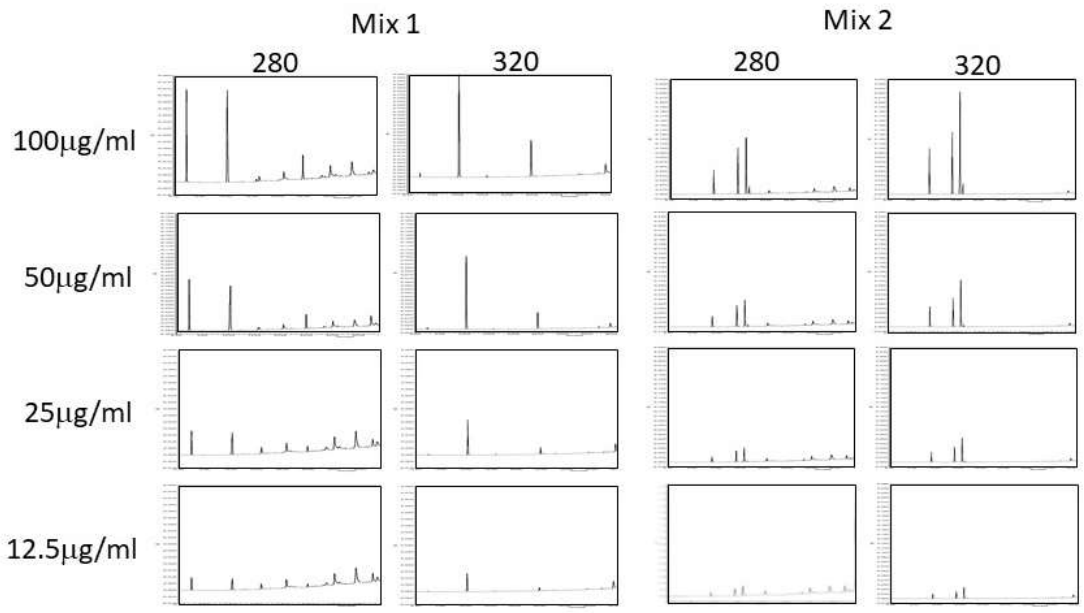

Figure 5.7: HPLC for standard curves of different concentrations of purified reference compounds using a gradient mobile phase. HPLC of the mixture of the indicated purified compounds at different concentrations on a C18 silica column using a gradient mobile phase starting at 5% acetic acid in water to 50% acetonitrile and 5% acetic acid in water. Detection of the eluted peaks was measured at 280 and 320 nm. Mix 1 included gallic acid, vanillic acid, ellagic acid, and quercetin and mix 2 included chlorogenic acid, trans-p-coumaric acid, hydroxy-3-cinnamic acid, and transferulic acid.

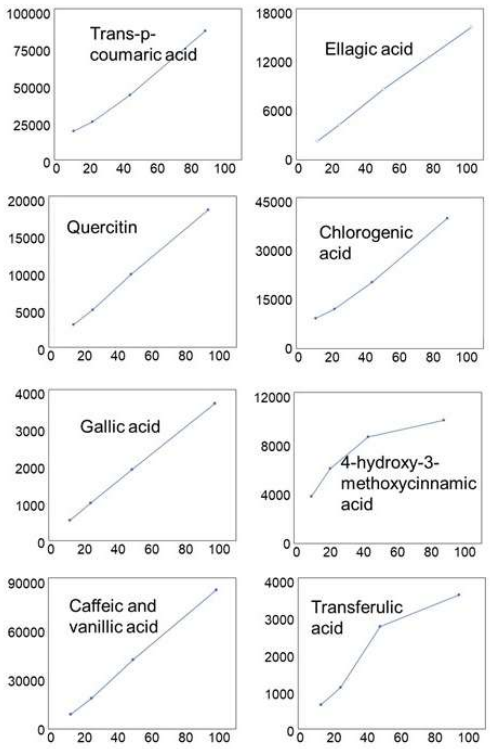

Figure 5.8: Standard curves following HPLC quantitation of reference compounds using a gradient mobile phase. The area under the curve for identified peaks measured following HPLC of the mixture of the standards at different concentrations on a C18 silica column using a gradient mobile phase starting at 5% acetic acid in water to 50% acetonitrile and 5% acetic acid in water.

Table 5.2: Identification and quantitation of individual peaks in an HPLC chromatogram of *F. assafoetida* extract by comparison to known standards.

	compound	280	320	370	425	Elution (min)	Conc. (ng/100 ug extract)
1	Vanillic acid	+	++	+	-	10.4	97
2	Trans-p-coumaric acid	++	++	+	-	13.6	60
3	Ferulic acid	+	+	++	-	15.8	730
4	Ellagic acid	+	-	+	+	20.6	35
5	Quercitin	+	+	++	++	29.7	110

5.3 Discussion

The results from this study showed that HPLC of *F. assafoetida* extracts could separate the extract into multiple peaks. In all of the analyses, the relative amount of the extracted compounds in the ethanol extract of *F. assafoetida* was significantly greater than the amount of the compounds extracted in the aqueous extracts as determined by the relative peak heights in the chromatograms. This indicates that ethanol is a superior solvent for these components as would be expected for the expected phenolic and terpenoid components which have been previously identified in Ferula extracts (1-3). Fractionation of both ethanol and aqueous extracts of *F. assafoetida* on a C18 column with an isocratic mobile phase of 90% acetonitrile and 5% acetic acid showed a chromatogram with approximately 10 major peaks. However, the absorbance corresponding to the peaks in the ethanolic extracts was at least 20-fold greater than for the peaks in the aqueous extracts. Fractionation on a C18 column with a gradient mobile phase starting at 5% acetic acid in water and ending with 50% acetonitrile and 5% acetic acid showed a chromatogram with at least 20 different peaks of absorbance. Similarly, the absorbance of the peaks derived from the ethanolic fraction were approximately 10-fold greater that for the peaks in the aqueous extracts. We used the same HPLC conditions to determine the migration of a group of standard compounds which have been previously shown to be present in extracts of various natural products including extracts of *F. assafoetida* in an attempt to identify some of the peaks in the chromatogram. HPLC analysis of the standard compounds using the isocratic mobile phase was not able to effectively resolve them. For example, ferulic acid and vanillic acid migrated with characteristics corresponding to the first peak in the extract while trans-p-coumaric acid and ellagic acid migrated with characteristics corresponding to the second peak and quercetin

corresponded to the third peak. Previous studies have indicated that ferulic acid is one of the first detectable compounds to elute using an acetonitrile isocratic mobile phase (20,21). Since the peaks from the *F. assafoetida* extract were quite broad is seems likely that each peak might comprise several components. In addition, our analysis was only able to identify constituents of the first three peaks while none of the standards we tested corresponded to peaks 4-10. HPLC analysis of Ferula extracts in other laboratories has identified a wide variety of sulfur-containing derivatives, coumarins, sesquiterpenes, sesquiterpene lactones, and sesquiterpene coumarins which were not included in our list of standard compounds and which may correspond to the unidentified peaks (1-3, 12, 14-16). Fractionation of the standards on C18 columns using the gradient mobile phase was better able to distinguish the selected standards into separate peaks. In fact, five of the standard peaks were well resolved and shown to correspond to five of the major separate peaks in the *F. assafoetida* extract chromatogram. The identity of the peaks in the *F. assafoetida* extracts samples were determined by comparison to the HPLC retention times for the standards as well as by comparison of the relative peak heights determined at the different wavelengths and corresponded to vanillic acid, transferulic acid, p-coumaric acid, ellagic acid, and quercetin. The quantities of the identified components were then calculated by comparing the areas under the peaks to the standards (24) and the amount of the component in the *F. assafoetida* extract determined by multiplying by the dilution that occurred during sample preparation. The relative amounts of the calculated standard components was similar to some of the published values for a few of the compounds, but combined these five components (97 ng/100 μg of extract for vanillic acid, 60 ng/100 μg of extract of p-coumaric acid, 730 ng/100 μg of extract for ferulic acid, 35 ng/100 μg extract for ellagic acid, and 110 ng/100 μg of

extract for quercetin) corresponded to approximately 1% of the total amount of the *F. assafoetida* extract.

The results of this study are consistent with previous studies that fractionated *F. assafoetida* and other Ferula extracts on HPLC and shows a good resolution of several phenolic compounds using a gradient mobile phase. We were able to identify 5 separate common secondary metabolites in the *F. assafoetida* extracts which correspond to compounds which have been previously shown to have some biochemical activities. However, since these identified components only correspond to about 1% of the total amount of material present in the extracts, further analysis of the *F. assafoetida* extracts should be performed.

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Chapter 6

6. General Conclusion

Cancer is one of the leading causes of death in humans and accounting for nearly 10 million deaths in 2020. Conventional cancer treatment, including surgery, chemotherapy, and radiotherapy, may not completely eliminate all malignant cells and prevent tumor recurrence. Therefore, the development of new and effective anti-cancer medicines which have minimal side effects are in increased demand. In recent years, there has been strong evidence that some herbal medicines are highly active in treating various diseases and disorders and are often less toxic compared to current standard chemotherapy drugs. Previous reports showed that more than 3000 plant species have been selected for studies on the treatment of cancer. Moreover, many studies have demonstrated that some natural products, when used in combination with chemotherapeutic agents, may enhance the efficacy of chemotherapy at lower doses and minimize the toxicity to normal cells. Some natural products demonstrate substantial efficacy by altering cancer initiation, development, and the progression of cancer, and can impact multiple mechanisms such as cell differentiation, cell proliferation, angiogenesis, metastasis as well as induce apoptosis in a variety of cancer cell types.

Ferula assafoetida is one of the related genera of medicinal plants that has been used as a traditional medicine for centuries. The biological activity of Ferula extracts may be due to the presence of multiple medicinal phytoconstituents such as terpenoids, sulfide derivatives, volatile oils, phenols, and minerals. The oleo-gum-resin obtained from the roots of *F. assafoetida* is known to possess pharmalogical activity. Recent pharmalogical and biological studies have pointed to the anti-oxidant, anti-microbial, anti-fungal, anti-cancer, and anti-diabetic properties of this plant. One of the important properties of this plant is its anti-cancer

effect. Previous studies have demonstrated beneficial effects from using extracts of a number of Ferula species as cancer chemopreventive agents. The aim of this study was to investigate the effects of *F. assafoetida* extracts and the effect of five known *F. assafoetida* compounds, ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid on the growth and survival of different breast cancer cell lines *in vitro.* Additionally, the possible effect of *F. assafoetida* extracts on monocyte activity and macrophage differentiation activity was examined. This study also aimed to determine whether THP-1-derived macrophages could cause damage to the cancer cell monolayers in co-culture experiments with MCF-7 and MDA-MB-231 cancer cells. The goal of the current study also includes experiments to develop and validate an HPLC (High-performance liquid chromatography) method to characterize the phenolic and flavonoid components present in *F. assafoetida* extracts.

These studies concluded that *F. assafoetida* extracts were effective in acting against the MCF-7, MDA-MB-231 and 4T1 mouse cancer cell lines. Results showed that the viability of MCF-7, MDA-MB-231, or 4T1 cells was inhibited after treatment with *F. assafoetida* or the five isolated compounds. Moreover, the morphological changes consistent with induction of apoptosis including, membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation and biochemical changes, such as down-regulation of anti-apoptotic Bcl-2, as well as the up-regulation of Bax and the activation of caspase-9, were shown following treatment of the breast cancer cells with *F. assafoetida* extracts. Apoptotic induction was also detectable using different methods including dual acridine orange/ethidium bromide staining, TUNEL assays, and western blotting. These data shown that treatment of breast cancer cells with *F. assafoetida* extracts as well as with different components of the extracts were shown to induce apoptosis in a dose- and time-dependent manner.

The results also showed that *F. assafoetida* extracts and some of its components were able to promote the expression of the M1 markers, but not the M2 markers, supporting the idea that the *F. assafeotida* contains compounds that promote a pro-inflammatory phenotype in differentiated macrophages. These results suggest that *F. assafoetida* and the isolated compounds may serve to suppress the expression and production of M2 (anti-inflammatory) cytokines that block anti-tumor immune responses and enhance the metastatic progression of cancer. Further, these *F. assafoetida*-treated macrophages were able to promote the destruction of breast cancer cells in co-culture experiments suggesting that the ability of *F. assafoetida* treatment to activate the M1 phenotype promotes cancer cell destruction. The current studies show that treatment with *F. assafoetida* extracts can indirectly destroy breast cancer cells via activation of an immune mechanism thus providing additional evidence for its potential as an anticancer agent.

In terms of HPLC, the ethanol and the aqueous extracts showed different amounts of the extracted compounds. In the ethanol extract of *F. assafoetida* there were significantly higher levels of several phenolic and flavonoid compounds, including ferulic acid, vanillic acid, p-coumaric acid, ellagic acid, and quercetin than in the aqueous extracts as determined by the relative peak heights in the chromatograms. This indicates that ethanol is a superior solvent for these components. Further, our experiments showed that the ethanolic extracts of *F. assafoetida* were more effective at inhibiting cancer cell proliferation than the aqueous extracts which indicates that these enriched compounds, including alkaloids, flavonoids, and phenolics, are more likely to be the bioactive components. This is supported by the experiments which showed that ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid had anti-proliferative activity. However, our characterization of the *F.*

assafoetida extracts only identified about 1% of the mass of the components in the extract and suggest additional compounds are likely to be important in mediating the bioactivity of *F. assafoetida*.

Taken together, these data show that *F. assafoetida* had some promising results as an anti-cancer agent *in vitro* and that further characterization and development might ultimately provide an additional effective agent which could be used in treating patients with cancer. Further analysis is needed to determine the proper mechanism of action and also to ensure that no side effects or complications can occur with this treatment.