Uncovering the therapeutic potential of *Uncaria tomentosa* using B16-BL6 mouse melanoma and 4T1 mouse breast cancer cells

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Abstract

Uncaria tomentosa (Rubiaceae), a medicinal plant native to Peru, has been traditionally used for centuries as a treatment for a wide variety of diseases, as well as to maintain health. It grows primarily in the Amazon rainforest and throughout South and Central America. *U. tomentosa* extracts have been demonstrated to have anti-oxidant, anti-apoptotic, anti-inflammatory and anti-cancer properties. The goal of this study was to examine *Uncaria tomentosa* in both *in vitro* and *in vivo* models in order to evaluate its potential anti-cancer activity using the B16-BL6 mouse melanoma and 4T1 mouse breast cancer cell lines. Both ethanol and PBS extracts of *U. tomentosa* were prepared and used to measure the effects on cell growth and survival using several different methodologies. Treatment of cells with ethanol extracts was much more effective at inhibiting cancer cell growth than treatment with PBS extracts *in vitro*, but no significant differences in the cancer inhibitory effects were observed *in vivo*. The *in vitro* experiments showed that treatment with the *U. tomentosa* extract significantly inhibited the growth of both B16-BL6 and 4T1 cell lines. It also inhibited the expression of the Ki-67 proliferation marker and promoted cell death as measured by increased DNA fragmentation using TUNEL assays in cancer cells. Treatment with the ethanol extract of *U. tomentosa* caused a significant increase in the fraction of apoptotic cells in flow cytometry (i.e. sub G1 peaks). Furthermore, two animal experiments were performed in order to evaluate the effect of *U. tomentosa* treatment on B16-BL6 cells in C57BL/6 mice. The results of the *in vivo* experiments concluded that treatment with *U. tomentosa* reduced tumour weight and tumour size. Histochemical analysis of the B16-BL6 tumours showed a strong reduction in the Ki-67 cell proliferation marker and a small but not significant increase in DNA

fragmentation in *U. tomentosa*-treated mice compared to the control. Further, *U. tomentosa* extracts reduced staining for Factor VIII, a marker for endothelial cells, indicating a decrease in angiogenesis in treated mice. Since *U. tomentosa* has been shown to affect immune system function, the infiltration of several different immune cells into the tumour was examined. No significant differences in the number of infiltrating T cells (including T helper and cytotoxic T cells), B cells, or platelets were found between the treated groups and the control. Collectively, the results in this study concluded that *U. tomentosa* has potent anticancer activity that significantly inhibited cancer cell growth both *in vitro* and *in vivo*. The discovery of new medicinal plants that are effective against cancer cells may provide a strategy to develop cancer therapy and requires more attention.

Keywords:

Uncaria tomentosa extracts, 4T1 breast cancer cells, B16-BL6 mouse melanoma cells, anticancer, apoptosis.

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Abbreviations

ATCC	American Type Culture Collection
Bcl- 2	B-cell lymphoma 2
BHT	butylated hydroxytoluene
BHA	butylated hydroxylanisole
BRCA1	Breast cancer type1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BCT	Breast conservation therapy
BSA	bovine serum albumin
^{o}C	degrees Celsius
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid FBS fetal bovine serum
g	gram
HPLC	high-performance liquid chromatography
IFN	interferon
IL	interleukin
MTT	Methyl thiazol tetrazolium assay
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
nm	nanometer
PBS	phosphate-buffered saline
ROS	reactive oxygen species

SDS	sodium dodecyl sulfate
TNF-α	tumour necrosis factor-α
TdT	terminal deoxynucleotidyl transferase
μg	microgram
μl	microliter
U/ml	units per ml

Chapter 1. General introduction

1.1 Cancer

Cancer is a deadly disease that is considered one of the most prevalent diseases worldwide. Cancer is defined as an abnormal growth of cells in the body due to alterations in the expression of several genes, such as the inactivation of tumour suppressor genes, the activation of oncogenes, and changes in epigenetic regulation that eventually lead to tumour formation (Vogelstein and Kinzler, 2004; Anand *et al*., 2008). The number of cancer deaths in 2007 is estimated at 7.6 million deaths worldwide, and is expected to rise to 17 million by 2030 (American Cancer Society, 2007; International Agency for Research on Cancer, 2008). In Canada, 206,200 people were diagnosed with cancer and 80,800 patients died of cancer in 2017 (Canadian Cancer Society, 2017a).

Various intrinsic and acquired factors can increase the risk of cancer development, such as inherited mutations, stress, alcohol use, unhealthy diet, obesity, hormones, infectious diseases, inflammation, harmful chemical use, a lack of physical activity, and infection with certain viruses (e.g. papillomaviruses or hepatitis B). In addition, cancer can occur following exposure to many forms of DNA-damaging radiation, including ultraviolet light and ionizing radiation. Statistics indicate that 5–10% of all cancer cases can be caused by genetic disorders, while 90–95% of cancer cases occur through lifestyle choices and exposure to environmental agents. Specifically, it is estimated that about 25-30% of all cancer-related deaths are caused by tobacco use, while 30–35% of cancer deaths are associated with an unhealthy diet. It has also been shown

that 15–20% of cancer deaths are in response to various infections and the remainder occurred through the involvement of a large number of other factors (Anand *et al*., 2008).

Cancer is a complex disease that can affect people of all ages (Jemal *et al*., 2007). According to the National Cancer Institute (NCI) classification, cancer can be classified into many different types based on the tissue and cell type involved, including carcinomas, sarcomas, leukemias, lymphomas, and central nervous system cancers (Jena, 2012). Carcinomas develop from epithelial cells and make up about 90% of human cancers, while sarcomas are considered rare in humans and arise from cells in connective tissues, such as muscle, bone, and fibrous tissue. Leukemias and lymphomas constitute about 8% of all human malignancies. Leukemias and lymphomas arise from cells of the immune system and the blood forming tissues of bone marrow and disseminate through the circulation or lymphoid organs, respectively. Lastly, cancers of the central nervous system arise from the cells in the brain or spinal cord (Cooper, 2000).

Over 100 different types of cancer have been discovered that greatly differ in their behavior and in their response to treatment. Some types of cancer are commonly diagnosed in men such as liver, colorectal, stomach, lung, and prostate cancer, while breast, lung, colorectal, and cervical cancer are the most commonly found types in women. Furthermore, cancers can also differ in the number and type of mutations present. Mutations in a number of genes that usually regulate the cell division processes, which is a balance between stimulators and inhibitors, are frequently involved in cancer development. Cancer cells do not respond normally to the signals that regulate cellular growth and death because of multiple mutations in these genes and this can eventually lead to a loss of regulatory cell growth resulting in the unregulated growth into a tumour

(Cooper, 2000, Yankeelov *et al*., 2011). According to various research findings from the Cancer Genome Project, the majority of cancer cells accumulate more than 60 mutations (Vogelstein and Kinzler, 2004).

There are two types of tumours: benign and malignant. Benign tumours do not spread or invade other parts of the body. As well, their cells are often more differentiated and grow more slowly compared to malignant tumours. Examples of benign tumours are uterine fibroids and moles. Benign tumours can cause some damages such as nerve damage, tissue death, and a decrease in blood flow because of the compression caused by the impact of tumours on tissues or organs; however, benign tumours do not usually cause fatal disease. On the other hand, malignant tumours have the ability to invade and migrate from the primary site to organs that reside further from the original site via the lymphatic system or blood vessels, in a process called metastasis. In addition, malignant cells have a higher rate of proliferation, disorganized pattern of growth, avoid differentiation, are resistant to apoptosis, and are genetically unstable. Further, malignant tumours often contribute to fatal disease (Cooper, 2000).

The incidence of malignant tumour cases can increase with age, and age is one of the most important risk factors of malignancy (Anisimov, 1983). Scientists have several hypotheses to explain the direct correlation between age and cancer. Some researchers believe that individuals that are exposed to carcinogens for a long period of time may be more likely to develop cancer (Peto *et al*., 1985). Other studies suggest that age can provide a suitable environment for malignant cells. For instance, increased proliferative senescence can contribute to prevention of apoptosis in malignant cells and enhance oncogenic mutations (Anisimov, 1987; Campisi, 2005). The third hypothesis shows that

cancer can occur through the interaction of several factors, such as a decrease in the effectiveness of the immune system or the accumulation of DNA damage that are enhanced, particularly in old age (DePinho, 2000).

1.2 Breast cancer

Breast cancer is a serious public health problem. Breast cancer is considered the most common type of cancer among women worldwide and although it can develop in men but at a lower rate. Breast cancer occurs rarely in men, about one in every 100 breast cancer cases; however, affected men are less likely to survive the disease than women because the majority of breast cancer cases in men are diagnosed at advanced stages (Peter and Bernard, 2008; Lakhani *et al*., 2012).

A breast consists of three major types of structures: lobules, ducts, and connective tissue. Breast cancer can occur in all of the different tissues of the breast but the majority of breast cancer cases arise from ducts or lobules. Breast cancer can be associated with severe disease or death especially in individuals with acute medical conditions and/or in the elderly population. Among women, the second-most frequent cause of cancer-related deaths is breast cancer (Russo, 2000). According to the Canadian Cancer Society, 26,300 women and 230 men were diagnosed with breast cancer in 2017. The number of deaths associated with breast cancer is estimated at 5000 women and 60 men in 2017 (Canadian Cancer Society, 2017b).

Breast cancer is diagnosed based on has several morphological characteristics, various immunohistochemical profiles, and histopathological subtypes. Multiple distinct types of breast cancer have been identified that differ in their ability and to spread to

other locations in the body. These types include noninvasive, inflammatory, invasive ductal carcinoma, invasive lobular carcinoma, and Paget's disease of the nipple (Sharma *et al*., 2010). Specifically, invasive ductal carcinoma (IDC), also called infiltrating ductal carcinoma, is the most frequent type of breast cancer and constitutes approximately 80% of all breast cancer cases. This tumour begins to grow in a milk duct that carries breast milk to the nipple and can spread to the surrounding tissues, as well as possibly to other places in the body, such as the lymph nodes, bones, and liver (Albrektsen *et al*., 2010; Makki, 2015).

Invasive lobular carcinoma (ILC) is the second most frequent type of breast cancer and constitutes about 10% of all breast cancer cases, although its incidence is increasing, particularly among elderly patients. Invasive lobular carcinoma starts from epithelial cells in the milk-generating glands (lobules) and over time can metastasize to other places of the body (Li *et al*., 2003). Invasive ductal carcinomas and invasive lobular carcinoma are significantly different in terms of clinicopathological features and in their response to systemic therapy (Barroso-Sousa and Metzger-Filho, 2016).

Additionally, *in situ* ductal and lobular breast cancers are not able to spread to other areas of the body, however, if the patients are not appropriately treated these tumours can show an increased risk of developing into an invasive breast cancer. Many other types of invasive carcinomas have been identified, such medullary and tubular breast cancer which make up 5% and 2% of breast cancer cases, respectively, as well as less frequent tumours such as adenoid cystic, mucinous, papillary, and low-grade adenosquamous carcinoma (Winer *et al*., 2001; Sharma *et al*., 2010; Makki, 2015).

1.3 Breast cancer risk factors

Several environmental and hereditary factors can increase the risk of developing breast cancer, including being a woman, old age, genetic mutations, hormone therapy, a family history of breast cancer, not being active, and excessive consumption of alcohol (Mettlin, 1999; Russo, 2000). In addition, there are other factors that are thought to increase the risk of developing breast cancer but are still under study, such as smoking, sleep patterns, and exposure to environmental toxins (Gram *et al*., 2016; Lu *et al*., 2017). Evidence shows that inherited genetic mutations can be found in some cases, which are present in approximately 5-9% of all breast cancer patients. However, the majority of breast cancer cases are linked to somatic mutations that occur in response to various environmental factors (Ford and Easton, 1995; Godet and Gilkes, 2017).

Specifically, age has a significant role in predicting the incidence of breast cancer since the majority of breast cancers are diagnosed in women at age 50 - 69 years (Reeder and Vogel, 2008). In Canada, statistics indicate that in 2016, about 83% of all breast cancer cases appeared in women aged 50 and older. Additionally, having a family history of breast cancer can increase the risk of developing the disease. Risk can be higher if one or more first-degree relatives, such as parent or sibling, were diagnosed with breast cancer. However, the increased risk will be lower if only second-degree relatives, such as an aunt or grandmother, were diagnosed with breast cancer (Claus *et al*., 1990; Colditz *et al*., 1993).

Inherited mutations in specific genes can increase the risk of developing breast cancer at both early and later ages. For example, the BRCA1 and BRCA2 tumour suppressor genes that regulate the growth of cells and repair damaged DNA, are the best-

known genes related to breast cancer and ovarian cancer risk. When mutations that impair normal function occur in either of these genes the cells are able to replicate at an uncontrollable rate. Researchers have found that the BRCA1 and BRCA2 mutations are associated with certain ethnic groups, such as people of Ashkenazi (European) Jewish descent. Statistics indicate that about 55 – 65% of females with the harmful BRCA1 mutation can be affected by breast cancer before age 70. Furthermore, 45% of females with the harmful BRCA2 mutation can be affected by breast cancer before age 70 (Couch *et al*., 1996; Greene, 1997). There are other gene mutations that can also lead to hereditary breast cancer, such as mutations in ATM, CDH1, CHEK2, PALB2, PTEN, STK11, and TP53, but the presence of inherited mutations in these genes are less common (De Jong *et al*., 2002).

Normally, individuals are exposed to ionizing radiation from natural and medical sources, such as radioactive radon gas, radioactive elements (e.g. potassium, uranium, and thorium), cosmic rays, medical x-rays, and occupational radiation. However, growing evidence shows that exposure to high doses of ionizing radiation or examination by x-ray can enhance the risk of developing breast cancer. Furthermore, the carcinogenic effect of radiation can be increased as a result of an interaction between radiation and various factors, including a woman's age at exposure, estrogen levels, and genetic profile. Women who are exposed to radiation therapy for either malignant or non-malignant diseases directed to specific parts of the body, such as the chest, neck, and armpit, show a significantly sensitivity to the carcinogenic effects of radiation, especially when the patient is treated at a younger age (Preston *et al*., 2002; Golubicic *et al*., 2008). For

example, the use of radiation therapy to treat Hodgkin lymphoma, particularly in youth, can significantly increase the risk of a future breast cancer (van Leeuwen *et al*., 2003).

Most epidemiologic studies during the past two decades have indicated that consuming alcoholic drinks can increase the risk of developing breast cancer (Singletary and Gapstur, 2001). According to the International Agency for Research on Cancer (2007), alcoholic beverages are considered a Group 1 carcinogen and can increase the risk of breast cancer in women although the mechanisms of increased risk for breast cancer by alcohol are not fully understood. Researchers have suggested that alcohol could increase the levels of estrogen and androgen which could be a significant mechanism underlying the association of alcohol with breast cancer. It has also been suggested that alcohol may increase the susceptibility of mammary gland tissue to carcinogens. Studies also showed that alcohol could decrease the levels of some essential nutrients that protect the cells from damage, including vitamin A and vitamin C (Singletary and Gapstur, 2001).

Other factors can be involved, directly or indirectly, in the risk of developing breast cancer and many of them are still under study, for example smoking and exposure to second-hand smoke (International Agency for Research on Cancer, 2004; Gaudet *et al*., 2013). Although it is not possible to control several risk factors such as being female and old age, some environmental factors can be controlled or avoided by changing strategic lifestyle, which might lower the risk of breast cancer. Death rates from breast cancer have been reduced by 44 % since the peak in 1986 because of increased awareness, screening, early detection, and improved treatments. Therefore, early

diagnosis of this disease can provide more treatment options and potentially extend the patients' survival for a longer period (Canadian Cancer Statistics, 2015).

1.4 Treatment of breast cancer

Treatment plans for patients with breast cancer are designed to increase the life expectancy of cancer patients or result in a complete cure. The treatment plan for patients with breast cancer depends on the subtype of the tumour as determined by screening various specific biomarkers such as hormone receptor and HER2 status. Choosing the appropriate therapy for patients with breast cancer also depends on different physical and biological characteristics of the disease, such as staging, which reflects the tumour size, and the extent of cancer spreading. As well, the age of patient, overall health, dosing plan, and adverse drug reactions should be considered before the identification of treatment. Treatments are divided into local and systemic therapies, including surgery, chemotherapy, radiotherapy, and hormonal therapy (McDonald *et al*., 2016).

Surgical treatment is the first option that can be used to treat breast cancer. Two fundamental types of breast surgery have been identified include breast conserving surgery (BCS) and mastectomy. Various factors are taken into consideration before the surgical management of breast cancer, such as tumour size, tumour location, the shape of the patient's breast, and patient preference. Breast cancer surgery can also include the removal of one or more lymph nodes from the armpit in order to determine if the cancer has spread to auxillary lymph nodes. Breast cancer surgery can also include breast reconstruction. In advanced cancer, surgery may be performed to relieve symptoms that

result when the tumour pressed down on other organs or tissues causing complications or pain (McDonald *et al*., 2016).

Breast-conserving surgery (lumpectomy) alone is used to treat stage 0 ductal carcinoma *in situ* whose area is estimated at < 0.5 cm in diameter. In this surgery, tumour tissues and nearby margins that might be cancerous are removed. Larger tumours require adjuvant radiation therapy in addition to a lumpectomy. On the other hand, mastectomy, to remove the entire breast including all of the breast tissue and nearby tissues, in combination with adjuvant tamoxifen therapy is commonly employed in order to treat extensive ductal carcinoma *in situ* (Fisher *et al*., 1998). Furthermore, auxiliary lymph node status should be considered in patients with stage I or II breast cancer followed by lumpectomy and radiation therapy (Arriagada *et al*., 1996).

Radiation therapy (also known radiotherapy) is one of the more common treatment modalities prescribed for breast cancer patients. External beam radiation is the most common form of radiotherapy used for patients with breast cancer. External radiation uses a machine to direct a beam of X-rays to destroy the patient's tumor and can be applied in different ways such as hypofractionated radiation therapy, 3D-conformal radiotherapy, and intraoperative radiation therapy (Mondal and Sharma, 2016). Radiation therapy can be used at various times during the course of breast cancer treatment, including following surgery to reduce the risk of local and regional recurrences, before surgery to reduce the tumour size, or to mitigate pain or symptoms of advanced disease. Some patients with stage 0 ductal carcinoma *in situ* and the majority of patients with stage 1 or higher invasive cancers receive radiation therapy as a part of their treatment plan (Poortmans, 2007; McDonald *et al*., 2016). Studies showed that including

radiotherapy for treatment of breast cancer was able to lengthen survival time and decrease the mortality rate (Vinh-Hung and Verschraegen, 2004).

Chemotherapy is used as a treatment option for various types of breast cancer and can be administered intravenously, given orally, and sometimes injected into the spinal fluid. The majority of chemotherapy is used as a systemic therapy, which means drugs flow throughout the body to kill the cancer cells or inhibits their growth and division. It can be used as a primary treatment (neoadjuvant) before surgery or radiation treatment, especially for individuals that have locally advanced breast cancer. Neoadjuvant chemotherapy can shrink the size of a large tumour prior to surgery and can also be used with some types of breast cancer, such as inflammatory breast cancer (Bonev *et al*., 2014). Adjuvant therapy, this which is used after surgery but before radiation therapy, is often used in order to prevent recurrence of the disease (Anampa *et al*., 2015).

Chemotherapy can be used as the first therapy or in combination with other therapies for the treatment of metastatic breast cancer cases or to relieve symptoms. Using a combination of chemotherapy drugs is more effective than using just a single agent and treatments usually involve combinations of two or three drugs (Carrick *et al.*, 2009). The common types of chemotherapy drugs are classified as anthracyclines, taxanes, carboplatin, and cyclophosphamide. Other treatment approaches can include hormonal treatment and targeted therapies, which have been used for some types of breast cancer (Pegram *et al*., 2004).

1.5 Side effects of some treatments

The various methods used to treat cancer can also have adverse side effects and remarkable toxicities associated with breast cancer therapies have been identified. The side effects resulting from external radiation therapy, include swelling in the breast, skin peeling, secondary malignancy, fatigue, difficulty in breastfeeding later on for some women, lymphedema, and cardiac and pulmonary toxicity (Brown *et al*., 2015). While the majority of these side effects are temporary, there may be permanent or long-term effects in some cases including permanent changes in cardiac and pulmonary function. For example, many studies have shown an increase in the death rate by cardiac disease for individuals who received radiation for left-sided breast cancer (Clarke *et al*., 2005; Darby *et al*., 2005). Other studies have reported that nerves in the arm could be damaged by radiation, which can lead to pain, shoulder weakness, and numbness (McCredie *et al*., 2001).

Chemotherapy drugs can also cause several side effects, which can adversely impact the quality of life for individuals in the short and long term. These adverse effects include vomiting, weight changes, hair loss, neuropathy, hormonal shifts and early menopause, loss of fertility, mouth sores, diarrhea, etc. In breast cancer survivors, fatigue is observed to a greater extent in patients who had received chemotherapy compared to patients who were treated with other options (Goedendorp *et al*., 2012).

Furthermore, researchers have shown that chemotherapy treatments can accelerate menopause in young females, leading to loss of fertility and various physiological symptoms, such as hot flashes and night sweats (Rosenberg and Partridge, 2013). Studies show that chemotherapy can increase the risk of opportunistic microbial infections by

affecting the immune system. It can also affect the self-renewal of epithelial cells, which can disrupt barriers that protect the host from invasion by microorganisms (Khan and Wingard, 2001; Elting *et al*., 2003).

1.6 Importance of medicinal plants

Since ancient times humans have widely used natural products for the treatment and prevention of disease. The natural products used today were often identified from traditional use due to their pharmacological properties and the belief that herbal therapies have a relative lack of toxicity. Natural products traditionally were the basis of the majority of early medicines; several communities, such as Native American, European, Egyptian, Indian, Greek, and Chinese communities, have all used herbs as a primary source of healthcare. For example, herbs have been used to decrease stress, support the immune system, increase relaxation, and improve disease symptoms. These herbs were given orally to individuals or pasted on the diseased area to mitigate pain, injuries, and maintain wellness (Pal and Shukla, 2003; Hong-Fang *et al*., 2009).

Natural products fell out of use in western medicine following the industrial revolution and the discovery of modern drugs, but recently, there has been a renewed interest in the use of natural products (Pal and Shukla, 2003; Koehn and Carter, 2005). Evidence shows that many developed countries, such as Canada, United Kingdom, Europe countries, and Australia, have recently increased the use of complementary and alternative medicine (CAM) to serve as potentially safe and effective therapeutic agents (Calapai, 2008; Braun *et al*., 2010; Anquez-Traxler, 2011). In some parts of the world, such as China, India, and South America, natural products have always been used for

treatment of disease. Statistics indicate that about four billion people worldwide rely on medicinal herbs to maintain health and promote safety. For example the proportion of people who were consumers of CAM in the United States increased from 34% to 42% during the period of 1990-1997 (Eisenberg *et al*., 1993; Eisenberg *et al*., 1998).

Studies have demonstrated that a large number of medicinal herbs contain antioxidant, anticancer, anti-inflammatory, immunomodulatory, and anti-microbial activity. Multiple plant extracts have been tested *in vitro* and *in vivo* against various kinds of diseases, including many types of cancer (e.g. lung cancer, skin cancer, prostate cancer, breast cancer, etc.), Alzheimer's disease, lung dysfunction, respiratory infections, and cardiovascular diseases using modern techniques. It should be noted that the efficacy of these medicinal herbs, their biological properties, composition, and their potential to treat various diseases have been analyzed in many studies (Koehn and Carter, 2005; Saklani and Kutty, 2008; Aravindaram and Yang, 2010).

Several pharmacological and chemical studies have shown that extracts from the stems, roots, bark, leaves, fruits, and seeds of some plants have the ability to provide very specific biological activities. The extracts have been shown to contain a large diversity of chemicals with unique molecular structures and a greater complexity than can be present in small molecule synthetic drugs (Gonzales and Valerio, 2006). In addition, some herbal medicines can contain a number of natural components that can affect many disorders at the same time by targeting several different mechanisms. Various studies have shown that more than 119 different chemical components have been obtained from extracts of various types of plants and developed into new drugs (Farnsworth *et al*., 1985).

Ecological conditions and cultivation technologies can affect the ability of the plant to produce different chemical components, which determines the effectiveness of the extract against disease. These include differences in the type of ground (e.g. climate, soil quality, etc.), time of harvest, manufacturing process, length of storage, and geographical location. One of the important examples is the soil the plant is grown in. Soil contains several nutritional elements (e.g. potassium, nitrates, phosphorous, etc.), which play a primary role in plant growth, the creation the structures of plant tissues, and the metabolic activity of the plant. Consequently, the medicinal quality of the plant extracts represents and reflects the environment that the plant is grown in (Barlóg, 2002; Liu *et al*., 2007).

Recent studies exploring the applications of traditional medicine has shown that patients have become more accepting of treatments based on CAM (Frass *et al*., 2012). The reasons for this acceptance include the high cost of conventional medicine, especially in some developed countries, as well as the acute side effects resulting from the use of some synthetic drugs (Fraenkel *et al.*, 2004; Pagan and Pauly 2005). In the sphere of anti-cancer therapy, researchers showed that CAM can play a significant positive role in enhancing the quality of life of some cancer patients in terms of the physical, emotional, psychosocial, and social effects. Although there is no evidence to indicate that CAM can replace conventional treatment, an increasing number of cancer patients will eschew conventional chemotherapy / radiation treatment in favor of “alternative medicine”. Having the ability to bridge “alternative medicine” and conventional modern cancer therapies allows for an expanded range of treatments that may help divert reluctant cancer patients into evidence-based treatment programs (Adams and Jewell, 2007).

Statistics indicate that 60% of drugs approved by the food and drug administration (FDA) during the period of 1984 to 1994 were originally obtained from plants (Kumar *et al*., 2012). Furthermore, of the 121 anticancer drugs that have been approved it was shown that 90 anticancer drugs were derived from medicinal herbs. Some examples of anticancer drugs derived from natural source origins include; topotecan, etoposide, podophyllotoxin, paclitaxel, vincristine, and camptothecin (Mukherjee *et al*., 2002; Cragg *et al*., 1997). Extensive research findings have shown that natural products can play a huge role in the development of new drugs. Further, the use of natural products may become more popular as the therapeutic active ingredients are identified and their efficacy demonstrated to be as good as some conventional treatments, as well as being more cost-effective (Rajesh *et al*., 2015).

1.7 Side effects of some herbs

Although an enormous number of plants may have medical value and promising potential, a large proportion of plants have still not been tested for clinical value; they have either been poorly monitored or have not yet been tested at all. Unfortunately, a large number of plant extracts that are currently promoted for use by patients have also not been properly investigated. These untested plants need to proceed to animal-based preclinical experiments and then to human clinical trials before it is possible to assess the complete range of side effects and other potential complications from treatment with these extracts. It should be noted that a number of plants contain components that have high toxicities and can cause serious adverse effects, such as hepatotoxicity, cardiotoxicity, and nephrotoxicity (Ekor, 2013). For instance, plants used in Traditional

Chinese Medicine that contain aristolochic acid can cause kidney failure and are considered a strong carcinogen (Yang *et al*., 2014).

Studies show that some herbs have high toxicities due to the presence of non-organic pollutants in their products such as lead, mercury, and arsenic. In addition, allergic reactions can be caused by several components isolated from plants, such as salicylic glycosides and lactonic sesquiterpenes (Bent and Ko, 2004; Firenzuoli and Gori, 2007). Therefore, plants must be adequately tested before they should be recommended as new strategies to improve anticancer response rates and response to side effects, and to potentially extend patients' survival. As well, relevant regulatory authorities should be involved to inform and sensitize the general public about the rational and safe use of herbal medicines (Ekor, 2013).

1.8 Apoptosis

One of the major tests to determine the potential anti-cancer potential of treatment with a natural product or other therapeutic is its ability to induce apoptosis in treated cells. Apoptosis, or programmed cell death, is a physiological process that has a significant role in the development and homeostasis in normal tissues. It normally acts as a defense mechanism by removing the damaged and injured cells caused by various diseases, such as ischemic damage and cancer. Apoptosis also plays a major role in a number of processes such as normal cell turnover, embryonic development, and the immune system response. Several morphological features have been identified for programmed cell death, including reduction in cellular volume, chromatin condensation, DNA fragmentation, and membrane blebbing. Ultimately, cell components are packaged

into apoptosomes that are degraded via macrophages. Together, apoptosis is considered a type of cell death that has a programmed sequence of events leading to cell degradation without causing harmful effects in the surrounding area (Elmore, 2007).

Additionally, there are a large number of stimuli that lead to apoptosis and cells can die differently depending on the cell type involved as well as physiological and pathological conditions. Although several apoptotic proteins have been identified, researchers are still learning more about the molecular mechanisms of action of these proteins in order to provide a better understanding of how apoptosis is activated. Two pathways, called the extrinsic or, "death receptor," pathway and the intrinsic or "mitochondrial" pathway regulate apoptosis. In the extrinsic pathway, extracellular ligands bind to death receptors in the plasma membrane due to the activation of specific signals generated from other cells. For example, specific Tumor necrosis factor-like cytokines, such as TRAIL or Fas Ligand, released by neighboring cells, bind to and activate specific TNF receptor-like death receptors, such as TNF-R1 and Fas, activating downstream apoptosis activators. This process results in the activation of proteases called caspases (in particular caspases 6 and 8) that cleave a variety of important target proteins leading to apoptosis (Wong, 2011).

Under cell stress, the intrinsic pathway is activated via intracellular signals to increase the permeability of the mitochondria. Several internal stimuli can initiate this pathway, including hypoxia and severe oxidative stress (Karp, 2008). The process is summarized in the release of cytochrome c from the mitochondria into the cytoplasm, leading to the activation of caspases (including caspase 9 and 3), which then degrades important molecules in the cell (Danial and Korsmeyer, 2004). Many factors have been

identified to promote apoptosis (pro-apoptotic proteins), such as Bax, Bak, Bid, Bcl-Xs, and Bok/Mtd, which form permeability pores in the mitochondrial membrane to enhance the release of cytochrome c while other members of the Bcl family inhibit apoptosis (anti-apoptotic proteins) by preventing formation of permeability pores, such as Bcl-2, Bcl-xL, Mcl-1, XIAP and Bcl-B/Bcl2L10 (Reed, 1997; Elmore, 2007). The process of apoptosis from the initiation of cell death to the final cellular fragmentation normally takes several hours to days and the duration relies on many factors, such as the stimulus and the type of cell (Ziegler and Groscurth, 2004).

Apoptosis has been implicated in the pathological conditions underlying serious diseases such as cancer, degenerative diseases, autoimmunity, and atrophy, which may result from excessive or insufficient apoptosis. In terms of cancer, the mechanism of programmed cell death is complicated and is highly regulated at many levels. The existence of defects in any of these levels may lead to a loss of balance between cell division and cell death, eventually causing cancer. Furthermore, decreased caspase function and impaired death receptor signaling can occur because of the reduction in apoptosis (Wong, 2011). Studies show that defective apoptosis contributes to and enhances tumor progression. One of the examples in promoting carcinogenesis is an alteration in the expression of members of the Bcl-2 family, which regulate the intrinsic apoptotic pathway. For example, increased levels of Bcl-2 play a significant role in blocking apoptosis and promoting cell survival (Vaux *et al*., 1988) and can promote it to act as an oncogene as in human leukemia and follicular lymphoma (Tsujimoto *et al*., 1984; Tsujimoto *et al*., 1985).

The downregulation of p53 can also promote carcinogenesis; p53 is a tumor suppressor encoded by the tumour suppressor gene TP53 that has a strong link to apoptosis. Mutations in p53 are found in most human tumours (about 50%) and are linked to advanced tumour stage (Wallace-Brodeur and Lowe, 1999; Bai and Zhu, 2006). p53 also has a critical role in development, differentiation, gene amplification, cell cycle regulation, and DNA recombination (Lane, 1992). Yonish-Rouach *et al*. (1991) indicated that p53 can enhance apoptosis; however, in a myeloid leukemia cell line overexpression of p53 was able to promote cell survival and reduce apoptosis. Together, apoptosis can play a significant role in both normal physiological development and in pathological conditions.

Several components extracted from different plants have been used as inducers of apoptosis in cancer treatment. One example of a plant extract that can promote apoptosis is curcumin. Curcumin is a phenol component of the spice tumeric and is promoted as an effective treatment for several types of cancer such as gastric, prostate, colorectal, and pancreatic cancer (Taraphdar *et al*., 2001; Qi *et al*., 2010). Curcumin can suppress the NF-Kβ signaling pathway in order to increase the response of tumor cells to doxorubicin. It also has the ability to promote other anticancer factors to enhance the success of treatment of multi-drug resistance (Hemaiswarya and Doble, 2006). It should be noted that curcumin did not show toxicity in human patients treated in clinical trials at a dose of 8000 mg/day (Ramachandran *et al.*, 2005). Another example of a plant extract that can promote apoptosis is genistein, which is a phytosterol from the flavonoids family of compounds. Genistein can significantly stimulate apoptosis in human pro-myelocytic HL-60 leukaemic cells (Qi *et al*., 2010). Genistein can also block proliferating cells

during the cell cycle in the G2/M phase, inhibit tyrosine kinases, and block angiogenesis (Wheat and Currie, 2008).

1.9 Immune system

The immune response can be categorized as two types of immune responses: innate immunity and adaptive immunity. Innate immunity refers to a nonspecific defense mechanism that acts very quickly against invading foreign bodies, such as bacteria or viruses, to mediate a primary response in inflammation. The innate immune system includes physical barriers to infection as well as a system of signaling receptors for structural components of bacteria and/or viruses that activate changes in gene expression and protein secretion in response to an infection. These receptor systems are highly expressed on neutrophils, monocyte/macrophages, and dendritic cells, which can both physically destroy pathogens via production of free radicals and phagocytosis and activate other components of an immune response by the production and secretion of cytokines and chemokines. However, the innate immune system does not change in response to infection and does not show a “memory” based on reinfection with the same pathogen. In contrast, adaptive immunity shows changes in responsiveness to an infectious agent such that the responses are accelerated and more intense following reinfection with the same pathogen. Adaptive immune responses involve stimulation of clones of T and/or B lymphoctes in an antigen-specific manner which can destroy infected cells, produce antibodies against the foreign antigen, or release cytokines and chemokines to recruit macrophages to destroy the foreign antigen. Further, the adaptive immune system can develop very specific responses to a specific antigen on the target and can “remember” those specific antigens for extended durations even in the absence of

reinfection. Immune cells such as macrophages, dendritic cells, natural killer (NK) cells, NK T cells, and γδ T cells can act as a bridge between innate and adaptive immunity (Chien *et al*., 1996; Murphy *et al*., 2008; Waldhauer and Steinle, 2008; Sun and Lanier, 2009).

Natural killer cells and phagocytes, such as neutrophils, monocytes, and macrophages, have a major role in cell-mediated innate immune responses. NK cells are a subpopulation of lymphocytes of the innate immune system, which have been shown to control many types of tumours and cells with viral and microbial infections. NK cells have the ability to recognize abnormal cells that express low levels of "self-antigens" in response to multiple pathological challenges and can respond quickly to kill the abnormal cell by releasing perforin and granzyme to induce apoptosis. In this way, cells infected with viruses or bacteria are killed before they are able to produce new infective agents. On the other hand, phagocytes can also kill a wide range of tumour cells and microorganisms by production of free radicals and by phagocytosis. Phagocytes can also ingest and engulf dead cells and debris that result from tissue injury. The process of phagocytosis involves enveloping the pathogen within a vesicle formed by involution of the cell membrane, called the phagosome, which then fuses with lysosomes to destroy microorganisms (Sun and Lanier, 2009). Additionally, several cells such as eosinophils, basophils, and mast cells can secrete inflammatory mediators, such as proteolytic enzymes, vasoconstrictors, and cytokines, to participate in cellular innate immunity (Murphy *et al*., 2008).

Cells of the adaptive immune system are extremely specialized defender cells, called lymphocytes, including B cells and T cells. Both B cells and T cells arise from

multipotent hematopoietic stem cells in the bone marrow (Murphy *et al*., 2008). T cells then mature in the thymus to generate αβ T cells and γδ T cells, which are two kinds of T cells characterized by distinct T-cell receptors (TCR) expressed on their surface. Specifically, a small group of T cells are classified as γδ T cells, while the majority of T cells are αβ T cells, which can be further divided into two subsets known as CD4+ T helper cells and CD8+ cytotoxic T cells (Chien *et al*., 1996; Koretzky, 2010; Luckheeram *et al*., 2012).

T cells are selected in the thymus for the ability to respond to a specific antigen before being released into circulation. The initial activation of T cells occurs in peripheral lymphoid organs, when the T cell receptor (TCR) on either a CD4+ helper T cell or a CD8+ cytotoxic T cell binds to a peptide derived from the specific "foreign" antigen held within in the major histocompatibility complex (MHC) expressed on the surface of the antigen- presenting cell (APC), such as a dendritic cell or macrophage. The interaction between the T cell receptor and the peptide-MHC complex must be accompanied by a secondary signal for activation of helper T cells and cytotoxic T cells. Secondary signals include interactions of the co-receptor protein CD28 on the surface of the T cell with the B7 proteins (CD80 and CD86) expressed on the surface of the APC, which leads to T-cell proliferation (Smith-Garvin *et al*., 2009).

There are three common classes of effector T cells in circulation, including T helper cells, regulatory T cells (Treg), and cytotoxic T cells. Two types of helper T cells have been identified; TH1 and TH2 cells. TH1 cells secrete IFN-γ and interleukin-2 (IL-2) that are essential for cell-mediated immunity. TH2 cells secrete interleukin-4, (IL-4) IL-5, IL-10, IL-13, and IL-31 in order to regulate the immune responses to extracellular

pathogens. Moreover, TH1 cells activate macrophages, B cells, and cytotoxic T cells, while TH2 cells stimulate the differentiation of B cells into antibody-secreting plasma cells (Gutcher and Becher, 2007). Treg play an important role in regulating different aspects of immune reactions. They can secrete transforming growth factor-beta (TGF-β), IL-35, and IL-10 to decrease inflammation (Workman *et al*., 2009). Cytotoxic T cells are activated through many cytokines to directly destroy the targeted invaders via release of perforin and granzyme (Andersen *et al*., 2006).

B cells are a primary component of the humoral immune response of the adaptive immune system. B cells differentiate into plasma cells that are responsible for generating antibodies, and can produce immunological memory, antigen presentation, and regulatory cytokine production. B cells are activated in the secondary lymphoid organs, such as the spleen and lymph nodes. Activation of B cells occurs through antigen recognition by the B-cell receptors (BCRs) and signals produced by helper T cell receptors such as CD40 and by T cell cytokines. This activation leads to proliferation of the B cells and their differentiation into either memory cells or antibody-secreting effector cells (Hoffman *et al*., 2016).

1.10 Immune system responses in cancer

Recently, a wide range of studies has focused on the role of the immune system in cancer. Evidence indicates that the interactions between tumour cells and different components of the immune system are very complicated and can continuously change. This means immune responses can potentially play a significant role in anti-tumour immunity or conversely can enhance tumour growth. Further, the interaction between

tumours and the immune system can alter the tumour cells in a process called immunoediting. Three phases in the cancer immunoediting process have been determined, including elimination, equilibrium, and escape. In the elimination phase, the innate and adaptive immune systems are able to protect the host by recognizing and inhibiting tumour cell proliferation and survival (Kim *et al*., 2007).

If the tumour cells are not fully rejected during the elimination phase, they enter the equilibrium phase. The equilibrium is the longest period in the immunoediting process and is characterized as a balance between the progression of cancer and cancer elimination by the immune system. During this phase, the immune system is still active against the tumour and can control tumor growth and metastasis but does not fully eliminate the tumour. Over time however, the cancer may proceed to the escape phase by developing many mechanisms to avoid immune surveillance and stimulate tumour tolerance (Kim *et al*., 2007). Mechanisms activated during the escape phase include downregulation of expression of classical MHC class I expression, increased expression of PD-L1, upregulation of suppressive T cells, and orchestration of an immunosuppressive microenvironment. Researchers have speculated that the appearance of clinical symptoms of cancer are linked to the escape phase (Dunn *et al*., 2004, 2006; Schreiber *et al*., 2011; Mellman *et al*., 2011; Chen and Mellman, 2013).

Generally, during the development of cancer many components of the innate and acquired immune systems are activated in order to prevent tumour growth and progression. In terms of the innate immune response, inflammatory cytokines can be released by, or in response to, the growing tumour cells and stromal cells in the surrounding area. These inflammatory cytokines can activate various innate immune

cells, such as NK, NK-T, γδ T cells, macrophages, and DCs in the area of the tumour. In particular, NK cells are the most significant components of the innate immune system that combats the cancer cells in the early stages of carcinogenesis (Dunn *et al*., 2002). NK cells have the ability to detect some transformed cells, since tumour cells frequently downregulate MHC class I antigen, which leads to the destruction of these transformed cells and removal of their fragments via macrophages and dendritic cells. NK cells use various mechanisms to inhibit their targets such as secretion of IFN-γ for activation and maturation of antigen presenting cells, or by production of TNF-α, as well as by induction of apoptosis, which relies on the secretion of perforin, and granzyme to introduce pores into the cancer cell membranes. NK cells can also express Fc receptors for IgG (CD16) on their cell surface which can kill infected cells that are coated with IgG by a process called antibody-dependent cell-mediated cytotoxicity (Waldhauer and Steinle, 2008).

NK-T cells can also play a significant role in regulating immune responses to tumour cells. NK-T cells can release IFN-γ in order to activate the effector functions of both NK cells and CD8+ T cells which in turn can induce apoptosis in the tumour cells. For example, an interaction between NK-T cells and dendritic cells through CD40 ligand-CD40 signaling leads to the release of IL-12, which is then able to activate the NK or CD8+ T, cells to inhibit cancer progression (Terabe and Berzofsky, 2008). Other cells, such as γδ T cells, can stimulate cell death in malignant cells. γδ T cells can also recognize tumour antigens via CD16 (Fc receptor) in order to mediate antibody-dependent complement cytotoxicity (Gogoi and Chiplunkar, 2013).

Proteins produced due to mutations in the tumour cells can be recognized by the adaptive immune system. Research has shown that both T cells and B cells can recognize

tumor-specific antigens that are expressed following specific mutations. For example, CD4+ T cells in melanoma have been shown to recognize a tumor-specific antigen containing a non-synonymous point mutation, which is located in the gene coding for triosephosphate isomerase (Wang *et al*., 1999). Also in melanoma, CD4+ T cells can recognize a tumor-specific antigen formed by a chromosomal rearrangement that leads to the fusion of a low-density lipid receptor gene with a fucosyltransferase gene (Wang *et al*., 1999). In another study, CD8+ T cells were shown to recognize p16INK4a with a point mutation in a melanoma patient (Wolfel *et al*., 1995). Moreover, many CD8+ T cell epitopes were generated by the presence point mutations in proteins from patients with non-small cell lung cancer (Echchakir *et al*., 2001; Karanikas *et al*., 2001).

The expression of cell surface markers on the surface of the cancer cells can be changed in response to genetic and epigenetic alterations (Pinho and Reis, 2015). For example, the expression of the MHC class I protein on the surface of the cells is considered a marker of all nucleated cells. In the cancer cells, reduction in the expression of the MHC class I protein is observed. This reduction can allow the NK cells to induce apoptosis in the cancer cells in response to binding NKG2D receptors located on the NK cell surface, to MICA/B located on the tumour cells. Normal expression of MHC class I can inhibit NK cells by activating inhibitory receptors, which eventually leads to apoptosis resistance (Waldhauer and Steinle, 2008).

Additionally, mutation in genes associated MHC class I antigen presentation have been identified. Studies showed that mutations in these genes can lead to a loss of function in CD8+ T cells, which can recognize cancer cells (Garrido *et al*., 1997). In particular, mutations in the β2-microglobulin gene, which has a primary role in MHC

class I molecule expression on the cell surface, have been reported (D'Urso *et al.*, 1991; Perez *et al.,* 1999). Due to the downregulation of surface MHC class I molecules and appearance of immunosuppressive factors, cancer cells can become resistant to CD8+ T cells and NK T cells and can escape from immune recognition (Garrido *et al*., 1997).

Another mechanism cancer cells use to evade the immune response is to release an abnormally high amount of transforming growth factor β (TGF-β) and IL-10, which are classified as immunosuppressive cytokines that can enhance cancer progression. TGF-β is both a tumor suppressor and a tumor promoter. TGF-β can be a strong inducer of apoptosis in early stages of cancer. In other cases, TGF-β signaling works in a contrary manner and enhances tumor growth (Principe *et al*., 2014; Lippitz, 2013).

Innate immunity can play a significant role in the development of cancer by increasing secretion of proteases such as neutrophil elastase, which exists in neutrophil granules, and supporting the growth of cancer cells. Other proteases can cleave extracellular matrix proteins, which can enhance metastasis. Moreover, neutrophils express NADPH oxidase that generates reactive oxygen species (ROS). ROS has the ability to stimulate cancer invasion through genetic modifications (Gregory and Houghton, 2011). Cancer cells can produce thymic stromal lymphopoietin, which can upregulate OX40 ligand expression on dendritic cells, and other APCs (Palucka and Banchereau, 2012). Expression of OX40 on T cells can interact with the OX40 ligand on APCs to work as a secondary co-stimulatory signal to CD28 signaling which can activate T cells and differentiate them into the TH2 T cell subset (Sharpe, 2009).

Many regulatory factors have been identified that function as immune checkpoints in the adaptive immune responses. Research has shown that tumour cells can hijack the

immune checkpoints in order to promote the inhibition of immune surveillance. In the first interaction between antigen/MHC class II receptors on APCs and T cell receptors, a second co-stimulatory signal is also required, such as CD28 and ICOS (Sharpe, 2009). An interaction between the ICOS ligand on APCs and the ICOS receptor on T cells occurs, while an interaction between CD28 on T cells and CD80 (B7.1)/CD86 (B7.2) on APCs can promote activation of the naïve T cells (Podojil and Miller, 2009; Sharpe, 2009). Dysregulation of costimulatory signals can disable the function of the T cell and block activation of adaptive immunity, promoting the progression of cancer (Crespo *et al*., 2013).

T cells can upregulate two co-inhibitory molecules; cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programed death 1 (PD-1) on the cell surface. Normally, these co-inhibitory molecules control the excess of T cell activation. In the case of cancer, increased expression of CTLA4 has been observed in various types of cancer cells. As well, expression of PD-L1 has been found in several types of cancer, which significantly contribute to the escape phase. In addition, researchers have shown that an interaction between CTLA4 on T cells and the CD80/CD86 proteins on APCs enhances immune tolerance. Taken together, the innate and adaptive immune responses can participate in preventing tumour growth and cancer progression (Podojil and Miller, 2009; Sharpe, 2009).

1.11 *Uncaria tomentosa*

Uncaria tomentosa is distributed in various places, including the Amazon rainforest and throughout South and Central America. Commonly known as Cat's Claw,

U. tomentosa is a large woody vine in the Rubiaceae family and Cinchonoideae subfamily. This species is found in the Tropical rainforest, which grows up to 30 metres in height and has leaves that contain a smooth edge that grows in two opposite whorls. *U. tomentosa* has been historically used in Peru as a traditional medicine to treat several disorders. Currently, *U. tomentosa* is provided commercially as a dietary supplement and is considered one of the best-selling herbs in industrialized countries such as the United States and in Europe (Keplinger *et al*., 1999; Zhang *et al*., 2015).

In many places in the world such as Peru, Germany, Austria, England, Japan, Canada, and the United States, multiple studies have been conducted on *U. tomentosa* extracts (Honório *et al*., 2016). These studies showed that the plant has activity in the treatment of a number of diseases, such as cancer, AIDS, gastrointestinal disorders, diabetes, respiratory infections, rheumatoid arthritis, herpes, epidemic diseases, and multiple sclerosis (Zhang *et al*., 2015). Analytical characterization of the plant extracts showed that cat's claw contains various active components, such as alkaloids, quinovic acid glycosides, polyhydroxylated triterpenes, catechins, sterols, and flavonoids that can have significant antioxidant, anti-inflammatory, and anti-neoplastic properties (Laus *et al*., 1997; Heitzman *et al*., 2005; Luna-Palencia *et al*., 2013).

Since 1985, many studies have been conducted on the alkaloids obtained from *U. tomentosa* to test the effectiveness of this plant and identify its active components. Most these studies showed that pentacyclic oxindole alkaloids are a rich resource for drug discovery and can be responsible for many of the biological activities in this plant (Wagner *et al*., 1985; Reinhard, 1999; Bacher *et al*., 2006; Paniagua-Pérez *et al*., 2009). However, some other studies showed that different components such as triterpenes and

quinovic acid glycosides might play a significant role in the useful outcomes that are produced by *U. tomentosa* (Aquino *et al*., 1990, 1991). In addition, a group of scientists believed that these various components work together synergistically to produce pharmacological effects (Rizzi *et al*., 1993; Reinhard, 1999).

Researchers have assessed the efficacy of many compounds derived from *U. tomentosa* extracts against several cancer cells *in vitro* and *in vivo*. They found that cat's claw had effective actions in cancer therapy by suppressing the growth of these cancer cells, including human neuroblastoma, glioma, MCF-7 breast cancer, and HL60 promyelocytic leukaemia cells (Sheng *et al*., 1998; Riva *et al*., 2001; Pilarski *et al*., 2007).

Kośmider *et al*. (2017) examined the cytotoxic effects of the tetracyclic alkaloid-free aqueous extract of *U. tomentosa* against both normal (NHDF) and cancer (HepG2) cells. The *U. tomentosa* extracts showed cytotoxic effects against HepG2 cells. However, the plant was not cytotoxic to the NHDF cells. In HepG2 cells, *U. tomentosa* extracts increased ROS production and reduced GSH levels. It caused apoptosis in the HepG2 cells by activation of caspase-3 and caspase-7. The plant extract also decreased the activity of NF-κB in cancer cells. In contrast, ROS production, GSH level, and NF-κB activity were not affected in the non-malignant cells (Kośmider *et al*., 2017).

A study by Rinner *et al*. (2009) used fractions derived from *U. tomentosa* to treat MTC cells. They found that *U. tomentosa* extracts inhibited the growth of MTC cells and the enzymatic activity of mitochondrial dehydrogenase. In addition, the expression of caspase-3 and -7 and poly (ADP-ribose) polymerase (PARP) fraction was increased. However, bcl-2 expression was not changed. Specifically, MTC cells that were treated

with the alkaloids isopterpodine and pteropodine isolated from *U. tomentosa* showed remarkable pro-apoptotic properties; while treatment with the alkaloid-poor fraction reduced cell proliferation but did not induce pro-apoptotic effects (Rinner *et al*., 2009).

Different types of oxindole alkaloids, including isopteropodine, pteropodine, isomitraphylline, uncarine F, and mitraphylline were extracted from *U. tomentosa* and then tested against human lymphoblastic leukaemia T cells (CCRF-CEM-C7H2). Four of them prevented the proliferation of lymphoblastic leukaemia cells. Furthermore, pteropodine and uncarine F showed significant pro-apoptotic effects on leukaemic lymphoblasts (Bacher *et al*, 2006).

Due to its ability to promote DNA repair and reduce harmful side effects, pain, and mutations caused by chemotherapy, *U. tomentosa* has been used as an adjunct treatment for breast cancer patients undergoing treatment (Araújo *et al*., 2012). Clinical trial data supports the ability of treatment with extracts of this plant to mitigate weight loss, nausea, secondary infections, and hair loss in patients treated with chemotherapy. Moreover, studies have demonstrated that *U. tomentosa* increases the number of myeloid progenitors (Sheng *et al*., 2000; Riva *et al*., 2001; Araújo *et al*., 2012). The immune system response, such as the proliferation of normal T and B lymphocytes, are also regulated in response to *U. tomentosa* (Wurm *et al*., 1998). Cytokines, such as IL-1, IL-6, and TNF-α, are also regulated via treatment with extracts of this plant (Allen-Hall *et al*., 2007).

Continuing research has found that *U. tomentosa* extracts prevented the LPS-dependent activation of specific NF-κB and AP-1 transcription factors in immune cells (Allen-Hall *et al*., 2010). Allen *et al*. (2017) has documented that THP-1 cells treated

with both *U. tomentosa* and 5–9 Gy ionizing radiation showed a strong reduction in cell proliferation and promoted caspase cleavage and DNA fragmentation in the cells. Furthermore, *U. tomentosa* extracts reduced the expression of Cyclin E and Cyclin B and reduced p38, ERK, and SAP/JNK kinase activation (Allen *et al*., 2017). Gurrola-Díaz *et al*. (2011) showed that aqueous *U. tomentosa* extracts enriched in alkaloids prevented Wnt-signaling activity in three different cancer cell lines including HeLa, HCT116, and SW480 cells, by decreasing the expression of the Wnt-target gene, c-Myc. They also found that treatment with extracts of *U. tomentosa* extracts enriched in alkaloids were more efficient at inhibiting Wnt-signaling activity than treatment with aqueous extracts (Gurrola-Díaz *et al*, 2011).

Fazio *et al*. (2008) examined the effect of a hydroethanolic *U. tomentosa* extract against several cancer cell lines *in vitro*, including B16-BL6 (murine melanoma), K1735 (amelanotic murine melanoma), HT29 (human colon carcinoma), A549 (human lung carcinoma), WEHI 164 (mouse fibrosarcoma), and LEC (mouse liver endothelial) cell lines. As well, the study tested the effect of the plant extracts on B16-BL6 melanoma cell growth and metastasis in the C57BL/6 mouse. Researchers showed that *U. tomentosa* did not observe a strong cytotoxic effect in the laboratory; however, it prevented tumor growth and metastasis *in vivo*. In addition, the plant suppressed tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), and Nuclear factor kappa B (NF-κB) activity *in vitro* (Fazio *et al*., 2008).

Based on the previous studies conducted on *U. tomentosa* extracts *in vitro* and *in vivo*, this plant has been demonstrated to have extensive biological activities, including anti-oxidant, anti-cancer, anti-microbial, immunomodulatory, and anti-inflammatory

activities (Heitzman *et al*., 2005; Luna-Palencia *et al*., 2013). These features support the idea that this plant is an important molecular tool with significant therapeutic potential. Specifically, previous results *in vitro* showed that *U. tomentosa* extracts significantly inhibited cell proliferation and induced morphological changes in a variety of cancer cell lines, such as breast cancer cells MCF7, HL60, EBV-transformed B lymphoma cells, and human leukemia cells K562 (Sheng *et al*., 1998; Riva *et al*., 2001; Cheng *et al*., 2007).

There are few studies showed that *U. tomentosa* extracts significantly blocked the proliferation of neoplastic cells *in vivo* (Fazio *et al*, 2008; Pilarski *et al*., 2010). However, there are insufficient data in the previous studies to describe the mechanism of *U. tomentosa* action on cancer cells. Therefore, *U. tomentosa* extracts will be tested against cancer cells in the current research using modern techniques *in vitro* and *in vivo* in order to investigate their effectiveness as possible pharmaceutical sources that could potentially generate different cancer therapeutic approaches. As well, these results are expected to provide valuable information concerning the molecular mechanisms by which this plant works against cancer cells.

1.12 Objectives and Experimental Summary

The current study will be examined the ability of extracts of *U. tomentosa* to inhibit the growth and survival of cancer cells *in vitro*. Both 70% ethanol and PBS, pH 7.4, extracts of *U. tomentosa* will be tested in order to evaluate their potential anticancer activity. Two cancer cell lines will be used *in vitro,* including the B16-BL6 mouse melanoma and 4T1 mouse breast cancer cells. In terms of molecular techniques, HPLC will be performed to characterize the alkaloids present in the *U. tomentosa* extracts and in various fractions. In addition, the ability of *U. tomentosa* to inhibit cell proliferation *in vitro* will be assessed using MTT cell proliferation assays (Methyl-Tetrazolium), and histochemistry using Ki-67 antigen detection. The ability of *U. tomentosa* to induce apoptosis in the cancer cells will be assessed using cell morphology, flow cytometery, and histochemistry with the TUNEL reagent.

This study will also examine the effect of *U. tomentosa* on the growth and survival of cancer cells using the isogenic tumour transplantation model. The B16-BL6 mouse melanoma cell line will also be used in the animal models. Specifically, tumours will be formed by subcutaneous injection of B16-BL6 cancer cell in the right flank of mice. The ability of *U. tomentosa* extracts to inhibit tumour growth *in vivo* will be tested by injecting extracts either intraperioneally or into the tumour and assessed by weighing and measuring tumour growth. Histological analysis of the tissues will be performed in order to evaluate the anti-cancer effects of *U. tomentosa* extracts in treated mice by measuring the level of the Ki-67 proliferation antigen and the amount of TUNEL-specific DNA fragmentation. Furthermore, changes in immune cell infiltration will be examined

using immunohistochemistry for various immune cell markers including CD3, CD4, CD8, ICAM-1, E - selectin, PECAM -1, Integrin αIIb, CD19, and CD45 in order to determine if the *U. tomentosa* extracts can affect the activation and tumour infiltration of immune cells.

Together, these data are expected to elucidate the effects of extracts of this plant on the B16-BL6 and 4T1 cell death pathway, and to determine if pro-apoptotic mechanisms are activated in response to treatment with extracts of *U. tomentosa in vitro* and *in vivo*. In conclusion, the goal of this research is to determine if *U. tomentosa* can be developed into a reliable and effective treatment for cancer that may ultimately be suitable for human patients.

Chapter 2: *Uncaria tomentosa* extracts were examined *in vitro* to measure the growth and survival of cancer cells (B16-BL6 and 4T1)

2.1 Introduction

U. tomentosa, commonly known as Cat's Claw, has been used historically by the Asháninkas indigenous people for the treatment of a set of pathologies, including inflammations of the urinary tract and bone pain (Keplinger *et al*., 1999). Recently, *U. tomentosa* extracts have been tested for a variety of pharmacological applications, such as the treatment of Alzheimer's, rheumatoid arthritis, respiratory infections, and several types of cancer (Zhang *et al*., 2015). *U. tomentosa* has been shown to have anticancer activity against a number of different cancer cell lines in the laboratory, such as breast cancer, leukemia, cervical carcinoma, and osteosarcoma (De Martino *et al*., 2006; Cheng *et al*., 2007). Aqueous extracts of *U. tomentosa* were also shown to suppress the growth of human neuroblastoma, glioma, MCF-7 breast cancer, and HL60 promyelocytic leukaemia cells (Sheng et al., 1998; Riva et al., 2001; Pilarski et al., 2007).

Researchers have shown that different compounds derived from *U. tomentosa* extracts have activity against several cancer cells *in vitro* and *in vivo*. Different oxindole alkaloids, including isopteropodine, pteropodine, isomitraphylline, uncarine F, and mitraphylline isolated from ethanolic extracts of *U. tomentosa* were shown to inhibit the growth of several cancer cell lines including CCRF-CEM-C7H2 (human lymphoblastic leukaemia T cells) (Bacher et al., 2006), B16-BL6 (murine melanoma), K1735 (amelanotic murine melanoma), HT29 (human colon carcinoma), A549 (human lung

carcinoma), WEHI 164 (mouse fibrosarcoma), and LEC (mouse liver endothelial) cell lines (Fazio et al., 2008). MTC cells treated with isopterpodine and pteropodine isolated from *U. tomentosa* showed remarkable pro-apoptotic properties; while treatment with alkaloid-poor fractions reduced cell proliferation but did not induce apoptosis (Rinner et al., 2009). The tetracyclic alkaloid-free aqueous extract of *U. tomentosa* showed cytotoxic effects against HepG2 cells but not non-malignant NHDF cells (Kośmider et al., 2017). Treatment with this extract promoted apoptosis, increased ROS production, and decreased NF-kB activation in HepG2 cells.

Several different active compounds have been found in this plant, including alkaloids, polyphenols, and triterpene derivatives (De Martino *et al*., 2006; Keplinger *et al*., 1999). These bioactive compounds can be complementary or synergic in their actions to produce the effects of *U. tomentosa* extracts (Wirth and Wagner, 1997). Several studies showed that *U. tomentosa* extracts significantly reduced the production of cytokines, such as TNF-α and IL-6, from immune cells. As well, NF-κB activity was blocked by *U. tomentosa* extracts (Caballero *et al*., 2005; Fazio *et al*., 2008; Dreifuss *et al*., 2010). Further studies are required to describe underlying molecular mechanism of *U. tomentosa.*

Generally, the overarching goal of this research is to shed light on the use of *U. tomentosa* as a new effective approach in the treatment of cancer. The present study assesses the efficacy of *U. tomentosa* extracts to block the growth and promote apoptosis in B16-BL6 and 4T1 breast cancer cells *in vitro*. Various methods were performed to characterize the anti-cancer ability of this plant. These methods included MTT assays,

immunohistochemistry, flow cytometry, and high-performance liquid chromatography (HPLC).

Specifically, the MTT cell proliferation assay was performed to measure the changes in cell viability and proliferation rate in cells treated with *U. tomentosa* extracts. Immunohistochemistry was utilized to detect specific target antigens, in particular the Ki-67 protein and TUNEL assay. The proliferation of cancer cells was examined following the treatment with *U. tomentosa* extracts using Ki67 proliferation marker which has been shown to label those cells that are actively proliferating. The TUNEL assay was used to detect DNA fragmentation in cancer cells treated with *U. tomentosa* extracts. Flow cytometry was also used to evaluate the presence of apoptotic cells in sub-G1 peaks for propidium iodide stained cells indicating the presence of subcellular fragmentation into apoptosomes. Finally, HPLC was performed to characterize the alkaloids found in the *U. tomentosa* extracts and also in different fractions used to treat the cancer cells. Together, these different methods were used in the laboratory in order to determine the effects of *U. tomentosa* extracts and their possible therapeutic applications that might be a potential source for the development of cancer drugs.

2.2 Materials and Methods

2.2.1 Tissue culture

The 4T1 cell line and the B16-BL6 cell line were obtained from ATCC [American Type Culture Collection, Manassas, VA] to be used in this research. The 4T1 cells were maintained in tissue culture plates (Sarstedt, Laval, QC) in Roswell Park Memorial Institute medium (RPMI, 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 B16-BL6 cells were maintained in tissue culture plates in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS and 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 and subcultured 1:5 approximately every 2-3 days for the 4T1 cells and every 3-4 days for the B16-BL6 cells. For experiments, the cells were treated with media including *U. tomentosa* extracts at various doses. All experiments were performed in triplicate for each assay.

2.2.2 *Uncaria tomentosa* extracts

U. tomentosa was obtained from Rosario Rojas (Lima, Peru) as a dried powder prepared from the bark or was purchased as a natural product supplement (Cat's Claw extract, Now Foods, Bloomington, IL. code 84618). The dried plant product was extracted with either PBS, pH7.4, or 70% ethanol and the soluble fraction used in the current research. For the preparation of the ethanolic extracts, 20 g of *U. tomentosa* powder was suspended in 200 ml 70% ethanol and heated to a slow boil with refluxing

for 1 hour. For the preparation of PBS extracts, 20 g of *U. tomentosa* powder was suspended in 180 ml water and 20 ml 10xPBS buffer and heated to a slow boil with refluxing for 1 hour. The mixture was allowed to cool and the bark debris was removed by centrifugation of the suspension at 2000 x g for 10 min. The supernatant was removed and filtered using a 0.22 μm syringe filter and then the extract was stored in aliquots at -80 °C. For both the animal experiments and the chemical fractionation experiments, the *U. tomentosa* extract supernatants were frozen and dried by lyophilization into a powder. The powder was weighed and resuspended in water immediately prior to use for the experiments.

The 70% ethanol extract of *U. tomentosa* was fractionated over an ethanol gradient. For this experiment, 100 ml of 70% ethanol extract was freeze-dried and resuspended in 100 ml water. The resuspended *U. tomentosa* extract was applied to a polyvinylpolypyrollidone (PVPP) column and fractionated into multiple 100 ml fractions by increasing ethanol washes as follows; a water fraction, 20% ethanol fraction, 40% ethanol fraction, 60% ethanol fraction, 80% ethanol fraction, and 95% ethanol fraction. Each of the fractions was freeze-dried and resuspended in 70% ethanol at 10 mg/ml of dried powder. The PVPP fractions were analyzed by HPLC and examined for effects against the B16-BL6 and 4T1 cancer cell lines to evaluate cell growth using MTT assays and compared to both (ethanol and PBS) whole *U. tomentosa* extracts.

2.2.3 Cell Treatments

Both the B16-BL6 melanoma and 4T1 breast cancer cells were cultured in media

as described. For experiments, the cells were harvested by incubation in 0.25% trypsin, collected by centrifugation at 400 x g for 10 min, and resuspended in culture media at $2x10^4$ cells/ml. Then, the cells were plated on different culture substrates depending on the experiments: 100 μl of the cell suspensions were plated onto each well of a 96 well culture plate (i.e. 2000 cells/well); 1 ml of cell suspension was plated into each well of a 24 well plate; or 2 ml of the cell suspension was plated on a 60 mm culture dish (i.e. $4x10^4$ cells/plate) and cultured overnight. On day 0 of the experiment, the cells were treated with culture media or various doses of either the alcoholic or PBS extracts of *U. tomentosa* suspended in the appropriate culture media containing 10% FBS and incubated at 37°C in 5% CO_2. For the *in vitro* experiments, the cells were treated with 4 μg/ml, 20 μg/ml, 40 μg/ml or 200 μg/ml of the *U. tomentosa* extract based on the weight of the original powdered root preparation prior to extraction. (The observation that the inhibitory effects of treatment with the various doses of the extract were highly reproducible suggests that the extraction process had a similar efficiency at extracting the active agents between multiple preparations.) The cells were treated with suspending media including the appropriate solvents at the same doses to generate negative controls.

2.2.4 MTT assay (Methyl Tetrazolium Blue)

The methyl thiazol tetrazolium (MTT) assay was performed to measure cell viability and proliferation rate in cells treated with the *U. tomentosa* extracts over the course of multiple days. The B16-BL6 or 4T1 cells were plated onto replicate 96-well plates, with one plate per day of experiment, and incubated overnight with 5% CO_2 at 37°C. The cells were treated in media containing FBS and various concentrations of *U.*

tomentosa extracts (control, 4 μg/ml, 20 μg/ml, 40 μg/ml or 200 μg/ml) on day 0. Each column of cells on the 96 well plate (n = 8) was treated with same conditions. The cells were incubated in the continued presence of the extracts over the course of 4 - 5 days without a media change. On each day of the experiment, one of the replica plates was treated by adding 10 μl of MTT solution in water into each well, at a final concentration of 0.25 mg/ml, and incubated for 4 hours. Following the incubation, the media was removed and 100 μl/well of dimethyl sulfoxide (DMSO) was added to solubilize the converted formazan crystals. The absorbance of each well was read at 540 nm using a (Spectramax 340PC 389) plate reader. Statistical analysis for the absorbance corresponding to each experimental condition for each day of the experiments was performed using an ANOVA using Graph Pad Prism Software.

2.2.5 Cell Morphology

This experiment examined the structure, shape, and appearance of both cancer cell lines. B16-BL6 melanoma cells or 4T1 breast cancer cells were plated on 24-well plates in culture media and incubated at 37°C for 24 hours. The cells were then treated with different doses of *U. tomentosa* extracted with 70% ethanol or PBS (control, 4 μg/ml, 40 μg/ml, and 200 μg/ml). Micrographs of the cells were captured 24 hours, 48 hours, and 72 hours after the treatment to evaluate the morphological responses to treatment using an Axiovert 100 Microscope and phase contrast optics (Zeiss Canada).

2.2.6 Fluorescence Immunohistochemistry for Ki-67 protein expression

Ki-67 was used as a proliferation marker to assess whether the cancer cells were actively proliferating. B16-BL6 or 4T1 cells were plated on sterile glass coverslips in 6-well plates in culture media overnight at 37◦C. These sterile glass cover slips were pretreated with HCl (2 N) for 1 hour and then washed with PBS, pH 7.4, to increase cell adhesion. The cells on the coverslips were treated with suspending media or with media containing 4 μg/ml, 20 μg/ml, 40 μg/ml or 200 μg/ml of *U. tomentosa* extracts and incubated for 24 hours, 48 hours, or 72 hours. Some cells were treated with 6 μg/ml of camptothecin for 24 hours, 48 hours, and 72 hours as a positive control for inhibition of cell proliferation. The treated cells were fixed by incubation with 4% formaldehyde (freshly prepared) in PBS, pH 7.4, for 5 minutes. The treated cells were then washed with PBS, pH 7.4, and permeabilized by incubation in 0.1% Triton X-100 in PBS, pH 7.4, at 4°C for 5 min. The cells were blocked by incubation in 10% FBS, 0.5% BSA in PBS, pH 7.4, 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. The primary antibody was removed and the coverslips were washed three times with PBS, pH 7.4. Then, the cells were incubated with 100 μl goat anti-mouse IgG, secondary antibody-FITC conjugate (green) (Santa Cruz Biotech.; titre 1:100) in blocking buffer for 60 minutes at room temperature. The secondary antibody- FITC was removed and the coverslips were washed three times with PBS, pH 7.4. Lastly, the coverslips were mounted on glass slides in 80% glycerol, sealed with nail polish, and images were captured using a fluorescence microscope (Zeiss Canada) for further analysis.

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

The TUNEL assay was performed to detect DNA fragmentation by labeling the terminal end of nucleic acids through terminal deoxynucleotidyl transferase (TdT). This assay identifies individual cells undergoing apoptosis. B16-BL6 or 4T1 cells were grown on glass coverslips in culture media at 37°C overnight to allow sufficient time for the cells to adhere. These cells were then treated with suspending media or with 4 μg/ml, 40 μg/ml, or 200 μg/ml of *U. tomentosa* extracts and incubated at 37°C for 24 hours, 48 hours, or 72 hours. As a positive control for apoptosis, cells were treated with 6 mg/ml camptothecin for 24 hours, 48 hours, or 72 hours. The treated cells were fixed by incubation with 4% formaldehyde in PBS, pH 7.5 (freshly prepared) for 5 minutes and then permeabilized by incubation with 0.1% TritonX-100 in 0.1% sodium citrate (freshly prepared) for 5 min on ice. The cells were washed with PBS and treated with 50 μl/well TUNEL reaction mixture (Roche, Laval, QB) for a period of 60 minutes at room temperature. The coverslips were washed with PBS, pH 7.4, and mounted on glass slides in 80% glycerol. Microscopic images were captured using an LSM510 fluorescence microscope (Zeiss Canada) for further analysis.

2.2.8 Flow Cytometry

Flow cytometry was performed to measure the DNA content in treated cell lines using the propidium iodide dye. The relative proportion of cells in the Sub-G1 (apoptosis peak), G1, S (DNA replication), and G2/M (cell checkpoint and mitotic phases) under the influence of *U. tomentosa* extracts was determined. 4T1 cells were grown in 60 x 15 mm

tissue culture plates in culture media containing FBS and incubated overnight at 37 °C. The cells were treated with *U. tomentosa* extract at concentrations of 4 μg/ml, 40 μg/ml or 200 μg/ml for a period of 24 hours and 48 hours. The 4T1 cells were also treated with 6 μg/ml of camptothecin as a positive control for apoptosis. Cells were washed with PBS, pH 7.4, and harvested by incubation in 0.25% (w/v) trypsin-EDTA. Following that, the cells were collected by centrifugation at 400 x g for 10 min. Treated cells were fixed by incubation in 70% ethanol at -20ºC and stored for up to 2 weeks. On the day of staining, the cells were collected via centrifugation at 400 x g for 10 min and washed with PBS, pH 7.4. These cell pellets were suspended in 500 μl of propidium iodide (PI) staining solution (150 mM NaCl, 0.1% Triton 100, 5.0 mg/ml RNase A, and 20 μg/ml of propidium iodide) and incubated for at least 30 min. Cell cycle analysis was performed using a Beckman Coulter LS600 flow cytometer and cell profiles created. The experiment was replicated for three different experimental days.

2.2.9 High-performance liquid chromatography (HPLC)

U. tomentosa root powder (1 g/10 ml) was extracted in 70% ethanol or PBS, pH 7.4, by boiling for an hour. The supernatant was filtered through a 0.22 μm syringe filter and then analyzed by HPLC on a Breeze 2 chromatography system (Waters Inc., Toronto, ON). In another experiment, the 70% ethanol extract of *U. tomentosa* was fractionated over an ethanol gradient and each fraction analyzed by HPLC. For this experiment, 100 ml of 70% ethanol extract was freeze-dried and resuspended in 100 ml water. The resuspended *U. tomentosa* extract was applied to a polyvinylpolypyrollidone (PVPP) column and fractionated into multiple 100 ml fractions by increasing ethanol washes as

follows; a water fraction, 20% ethanol fraction, 40% ethanol fraction, 60% ethanol fraction, 80% ethanol fraction, and 95% ethanol fraction. Each of the fractions was freeze-dried and resuspended in 70% ethanol at 10 mg/ml of dried powder. The composition of each fraction was identified through HPLC.

HPLC was performed on a Sunfire C18 column 3.5μm resin 4.6x100 mm. A gradient mobile phase was pumped onto the column at a flow rate of 1 ml/min. Specifically, the linear gradient was formed by mixing the solvents starting with 100% buffer A (60 volumes 10 mM phosphate buffer, pH 6.6, 20 volumes acetonitrile, and 20 volumes methanol) and finishing with 100% B (30 volumes 10 mM phosphate buffer, pH 6.6, 35 volumes acetonitrile, and 25 volumes methanol) over a period of 40 min. Then, 100% buffer B were pumped onto the column for 10 min and lastly a gradient starting with 100% B and finishing with 100% A were pumped through the column for 5 min. The column was injected with a 5 μl aliquot of each sample at the beginning of each run. Finally, a wavelength of 245 nm was used to detect the components in the *U. tomentosa* extracts and peak area calculated to determine the relative amount of each component. Various peaks were identified by comparison to a series of standards, which had been previously shown to be present in *U. tomentosa* extracts following analysis of 1 mg/ml of each standard using the same conditions for HPLC characterization. These standards included uncarine D, mitraphylline, uncarine C, isomitraphyline, rhynophyline, and uncarine E (ChromaDex, Invine, CA).

2.3 Results

2.3.1 The effect of *U. tomentosa* extracts on the proliferation of 4T1 breast cancer and B16-BL6 melanoma cancer cells

An MTT assay was performed to evaluate the effect of *U. tomentosa* extracts on the growth of 4T1 cells and B16-BL6 cells *in vitro*. Two different extracts of *U. tomentosa* were prepared, one in 70% ethanol and one in PBS, pH 7.4, and used to treat the cells over a period of four days. The results of these experiments indicated that cell viability decreased in a concentration-dependent manner when treated with *U. tomentosa* extracts.

Treatment with *U. tomentosa* extracted with 70% ethanol showed no decrease in the growth of 4T1 cells at a low dose of 4 μg/ml, and a small effect when treated at the medium dose of 40 μg/ml. However, a complete inhibition in the growth of 4T1 cells was detected when treated with the highest dose of 200 μg/ml (Figure 2.1). In contrast, treatment of 4T1 cells with a PBS-extract of *U. tomentosa* was much less effective. Treatment with the low and medium doses of *U. tomentosa* extracted with PBS did not show any significant effect on 4T1 cell growth. Treatment with the high dose of *U. tomentosa*, 200 μg/ml, showed a significant inhibition of cell growth of approximately 35% by day 4 of treatment (Figure 2.2).

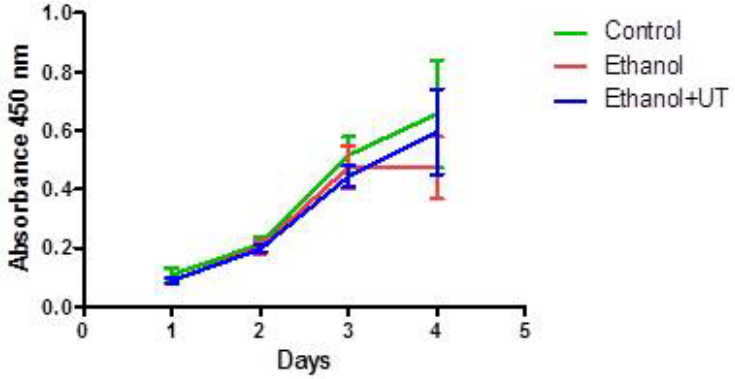
The B16-BL6 melanoma cells appeared to be somewhat more sensitive to *U. tomentosa* extracts than 4T1 cells. Treatment of B16-BL6 cells with the 70% ethanol extract for 4 days showed an approximately 10% decrease in cell number at the lowest concentration of 4 μg/ml and an approximately 80% decrease in cell number at the

medium dose of 40 μg/ml. Treatment with *U. tomentosa* extracted with ethanol at the highest dose of 200 μg/ml was able to effectively kill all of the B16-BL6 cells (Figure 2.3). However, treatment with *U. tomentosa* extracted with PBS at the low and medium doses did not significantly inhibit the growth of B16-BL6 cells although treatment with the high dose greatly suppressed B16-BL6 cells by greater than 80% (Figure 2.4). These results showed that there were some differences in the sensitivity of the different cell lines (4T1 and B16-BL6) to *U. tomentosa* extracts in a dose-dependent manner. Further, treatment with *U. tomentosa* extracted with 70% ethanol had much stronger effects on both 4T1 cells and B16-BL6 cells compared to treatment with *U. tomentosa* extracted with PBS.

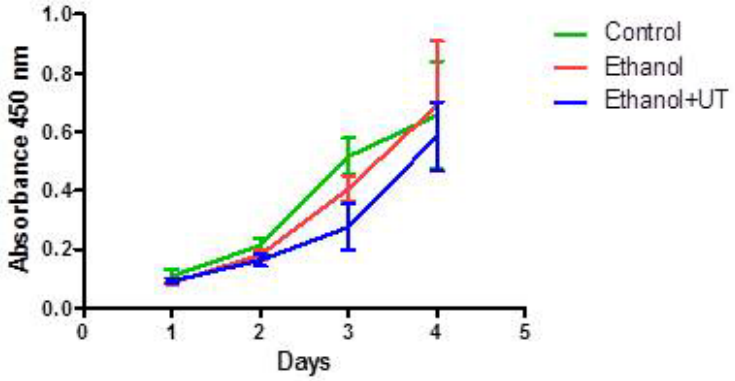
Figure 2.1 Effect of *U. tomentosa* extracted with ethanol on the growth of the 4T1 cell line

4T1 breast cancer cells were treated with (A) 4 μg/ml, (B) 40 μg/ml, or (C) 200 μg/ml of *U. tomentosa* extracted with 70% ethanol or control media on day 0 of treatment and cell number determined every day over a period of 4 days in order to measure cell growth. These treated cells were compared to control (untreated cells suspended in media) and cells treated with ethanol suspended in RPMI media. Relative cell number was determined for replica plates using the MTT assay and absorbance was determined every day at 450 nm. Data were analyzed for three independent experiments and significant differences confirmed by ANOVA * ($P < 0.05$). Graph pad prism was used for analysis.

A


Absorbance 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
Ethanol
Ethanol+UT

B


Absorbance 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
Ethanol
Ethanol+UT

C

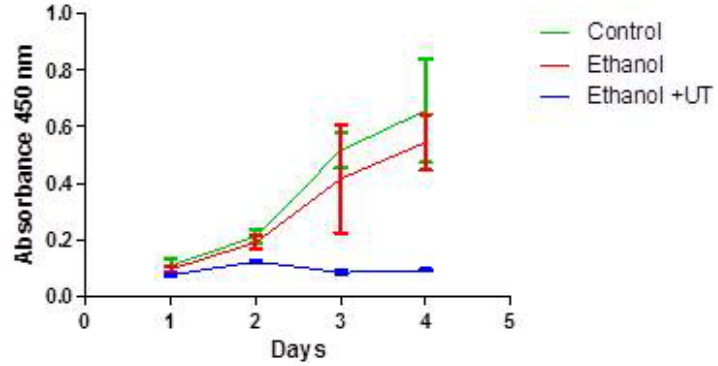

Absorbance 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
Ethanol
Ethanol +UT

Figure 2.2 Effect of *U. tomentosa* extracted with PBS on the growth of the 4T1 cell line

4T1 breast cancer cells were treated with (A) 4 μg/ml, (B) 40 μg/ml, or (C) 200 μg/ml of *U. tomentosa* extracted with phosphate-buffered saline, pH7.4, or control media on day 0 of treatment and cell number determined every day over a period of 4 days in order to measure cell growth. These treated cells were compared to control (untreated cells suspended in media) and cells treated with PBS, pH 7.4, suspended in RPMI media. Relative cell number was determined for replica plates using the MTT assay and absorbance was determined every day at 450 nm. Data were analyzed for three independent experiments and significant differences confirmed by ANOVA * ($P < 0.05$). Graph pad prism was used for analysis.

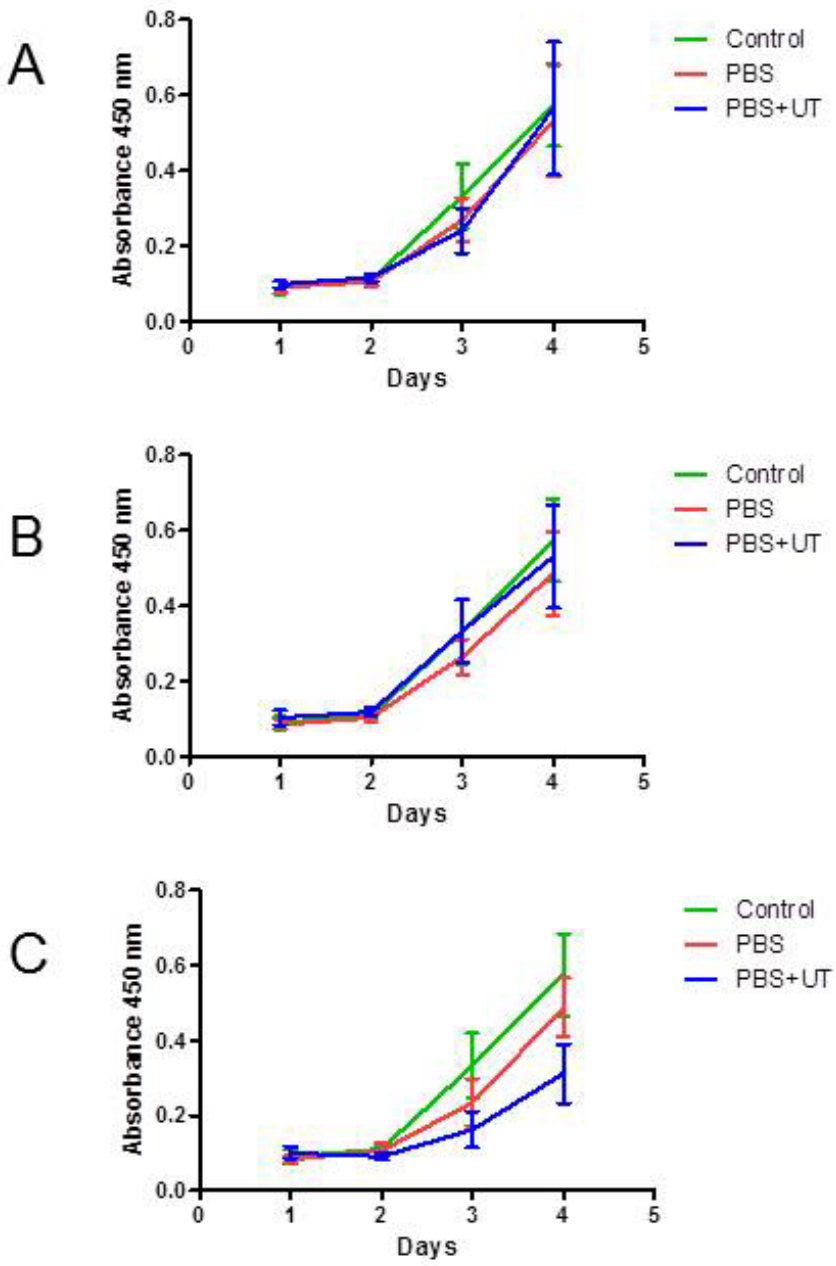
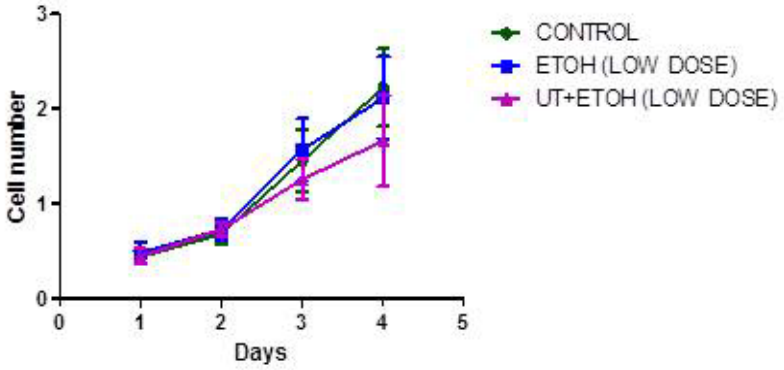

A
B
C
Absorbance 450 nm
Days
Control
PBS
PBS+UT
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
5

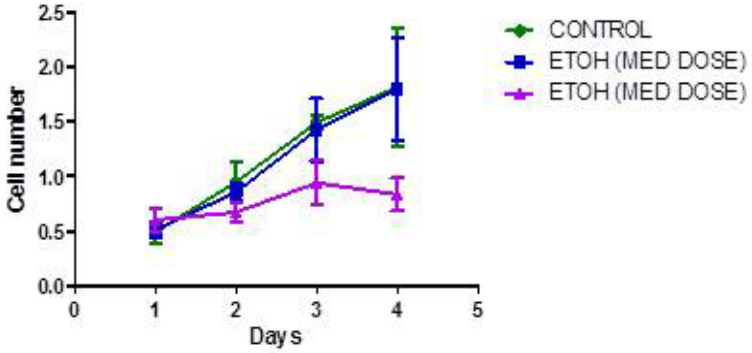
Figure 2.3 Inhibition of B16- BL6 cell proliferation treated with *U. tomentosa* extracted with ethanol

B16-BL6 melanoma cells were treated with (A) 4 μg/ml, (B) 40 μg/ml, or (C) 200 μg/ml) of *U. tomentosa* extracted with 70% ethanol or control media on day 0 of treatment and cell number determined every day over a period of 4 days. The treated cells were compared to control (untreated cells suspended in media) and cells treated with ethanol suspended in DMEM culture media. Relative cell number was determined for replica plates using the MTT assay and absorbance was determined every day at 450 nm. Data were analyzed for three independent experiments and differences were considered significant when $p \leq 0.05$.

A


Cell number
Days
CONTROL
ETOH (LOW DOSE)
UT+ETOH (LOW DOSE)

B


Cell number
Days
CONTROL
ETOH (MED DOSE)
ETOH (MED DOSE)

C

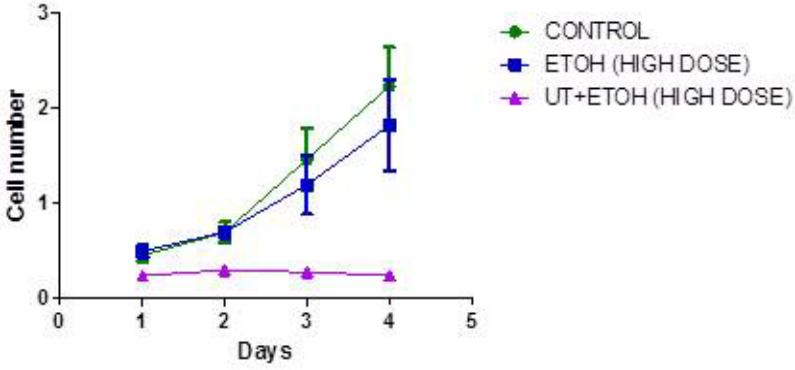
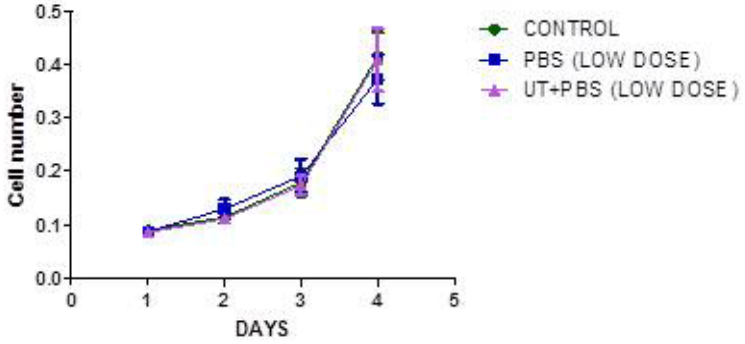

Cell number
Days
CONTROL
ETOH (HIGH DOSE)
UT+ETOH (HIGH DOSE)

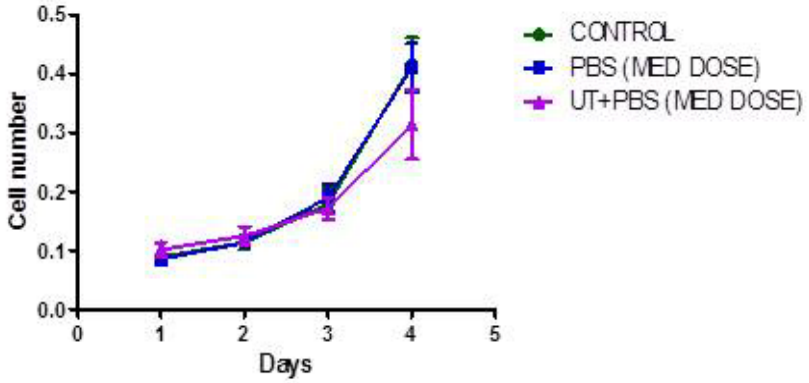
Figure 2.4 Inhibition of B16- BL6 cell proliferation treated with *U. tomentosa* extracted with PBS

B16-BL6 melanoma cells were treated with (A) 4 μg/ml, (B) 40 μg/ml, or (C) 200 μg/ml) of *U. tomentosa* extracted with phosphate-buffered saline, pH 7.4, or control media on day 0 of treatment and cell number determined every day over a period of 4 days. The treated cells were compared to control (untreated cells suspended in media) and cells treated with PBS, pH 7,4, suspended in DMEM culture media. Relative cell number was determined for replica plates using the MTT assay and absorbance was determined every day at 450 nm. Data were analyzed for three independent experiments and differences were considered significant when $p \leq 0.05$.

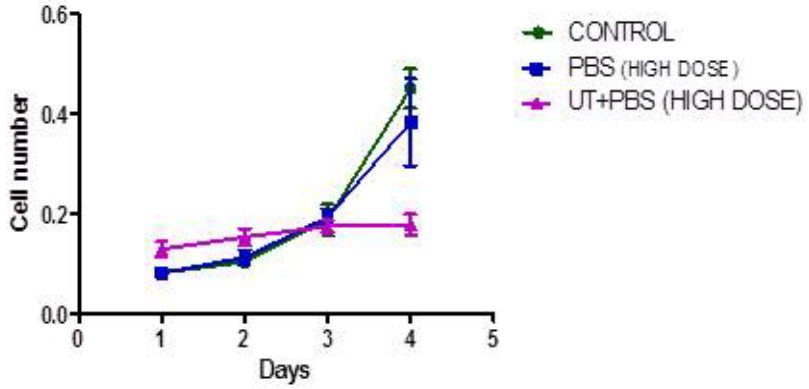
A


Cell number
0.0
0.1
0.2
0.3
0.4
0.5
0
1
2
3
4
5
DAYS
CONTROL
PBS (LOW DOSE)
UT+PBS (LOW DOSE)

B


Cell number
0.0
0.1
0.2
0.3
0.4
0.5
0
1
2
3
4
5
Days
CONTROL
PBS (MED DOSE)
UT+PBS (MED DOSE)

C


Cell number
0.0
0.2
0.4
0.6
0
1
2
3
4
5
Days
CONTROL
PBS (HIGH DOSE)
UT+PBS (HIGH DOSE)

2.3.2 Cell morphology

The effect of treatment with *U. tomentosa* extracts on cell morphology was tested using phase contrast microscopy. 4T1 breast cancer cells were treated with *U. tomentosa* extracted with ethanol or PBS to evaluate the cell morphology over a period of two days. Cells treated with the lowest dose of the ethanol extract (4 μg/ml) did not significantly change in size or shape. Treatment of the 4T1 cells with the medium dose (40 μg/ml) of the *U. tomentosa* ethanol extract showed an increase in the number of rounded cells and a lower cell density of cells compared to control cells, in particular after two days of treatment. 4T1 cells treated with the highest dose of the *U. tomentosa* ethanol extract (200 μg/ml) showed a significant increase in the number of rounded and atrophied cells, as well as a huge reduction in the total number of surviving 4T1 cells (Figure 2.5). The morphological changes in these cells included changes in number, shape, and structure of the cells. On the other hand, cells were treated with *U. tomentosa* extracted with PBS showed much less obvious morphological changes even when the cells were treated with the highest dose of the extract for 2 days. In contrast to the high dose, cells treated with low doses of the PBS extracts showed a relatively low frequency of rounded or atrophied cells compared to control (Figure 2.6).

B16-BL6 cells were treated with different concentrations of *U. tomentosa* extracts and changes in cell morphology were examined over the course of four days using phase contrast microscopy. Treatment of B16-BL6 cells with the ethanol extract of *U. tomentosa* showed dose- and time-dependent changes in cell morphology. Cells treated with the low dose showed a decreased cell density over the course of the four days of treatment although the shape and size of the cells were similar to media-treated controls.

An increase in the frequency of atrophied cells was observed following the treatment with the medium dose of the *U. tomentosa* ethanol after four days and a complete atrophy of the cells was found when they were treated with the highest dose (Figure 2.7). Treatment with the highest dose of the ethanol extract of *U. tomentosa* for 3-4 days completely destroyed the cells as determined by phase contrast microscopy. Furthermore, B16-BL6 cells treated with PBS extract of *U. tomentosa* also showed significant dose- and time-dependent changes in cell morphology (Figure 2.8). Cells treated with the low and medium doses of the extract showed some increase in cell rounding but very little change in cell density. However, cells treated with the highest dose of the PBS extract of *U. tomentosa* for 3-4 days showed significant decreases in cell number and increased atrophied cells. Taken together, these data show that treatment with the ethanol extract at high doses caused a marked atrophy in both the 4T1 cells and B16-BL6 cell lines and decreased the number of cells. However, treatment with the PBS extract, promoted significant atrophy in the B16-BL6 cells treated with the highest dose, but relatively little change was recorded in the 4T1 cells.

Figure 2.5 Effect of *U. tomentosa* ethanol extract on the morphology of the 4T1 cell line

4T1 cells were plated on 6 well plates and treated with culture media (control) or 4 μg/ml (low dose), 40 μg/ml (medium dose), or 200 μg/ml (high dose) of *U. tomentosa* extracted with 70% for 24 hours or 48 hours. Micrographs of random fields were obtaining using phase contrast optics using an Axiovert 100 microscope and Northern Eclipse Software and representative images shown.

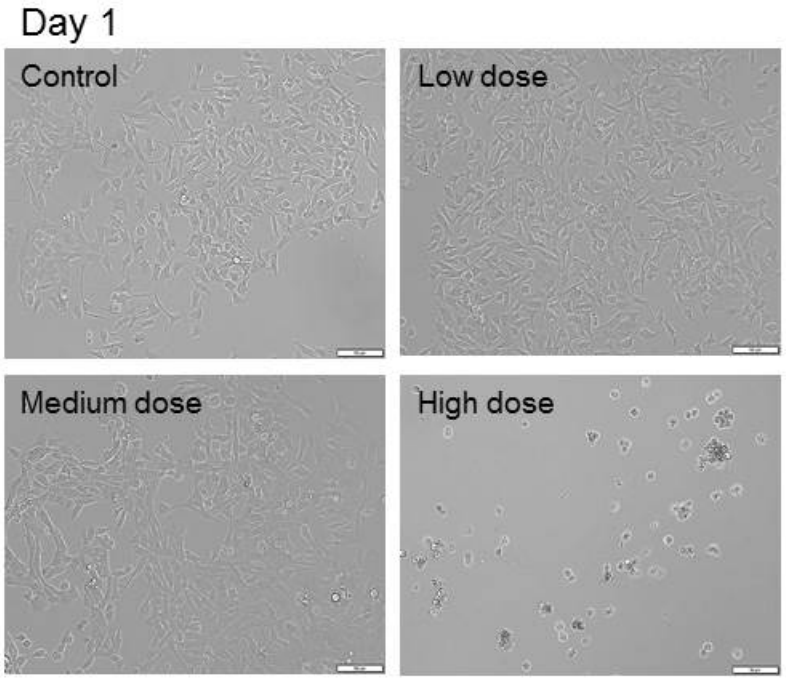

Day 1
Control
Low dose
Medium dose
High dose
Day 2
Control
Low dose
Medium dose
High dose

Figure 2.6 Effect of *U. tomentosa* PBS extract on the morphology of the 4T1 cell line

4T1 cells were plated on 6 well plates and treated with culture media (control) or various doses 4 μg/ml (low dose), 40 μg/ml (medium dose), or 200 μg/ml (high dose) of *U. tomentosa* extracted with phosphate-buffered saline for 24 hours or 48 hours. Micrographs of random fields were obtaining using phase contrast optics using an Axiovert 100 microscope and Northern Eclipse Software and representative images shown.

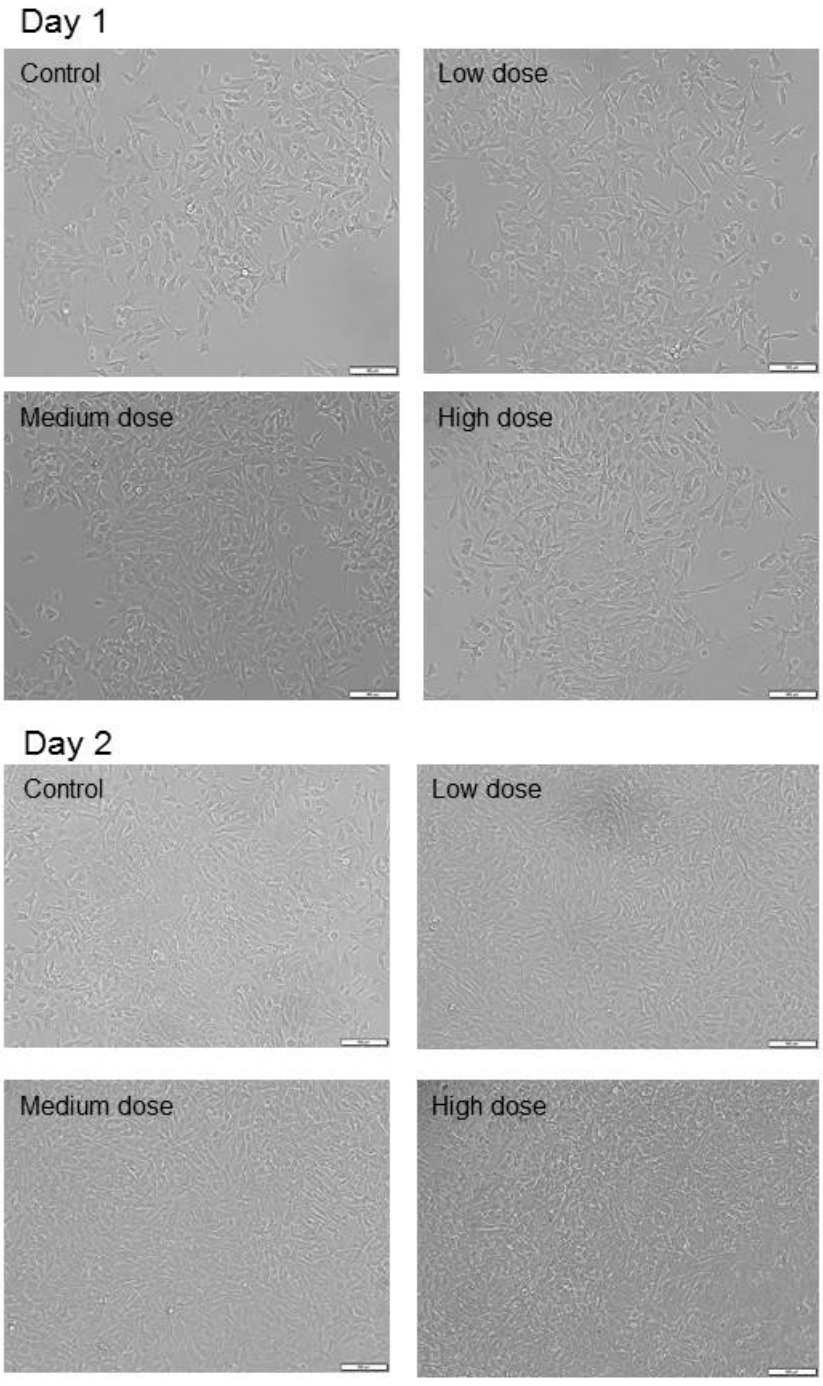
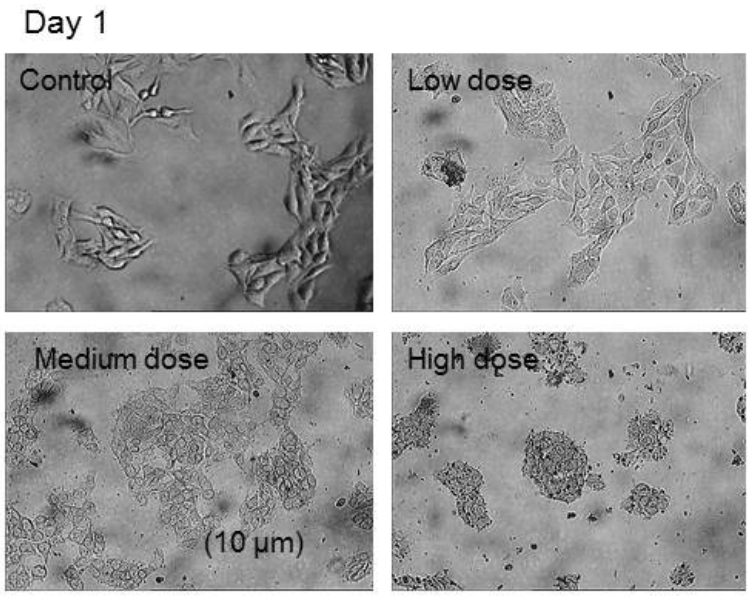
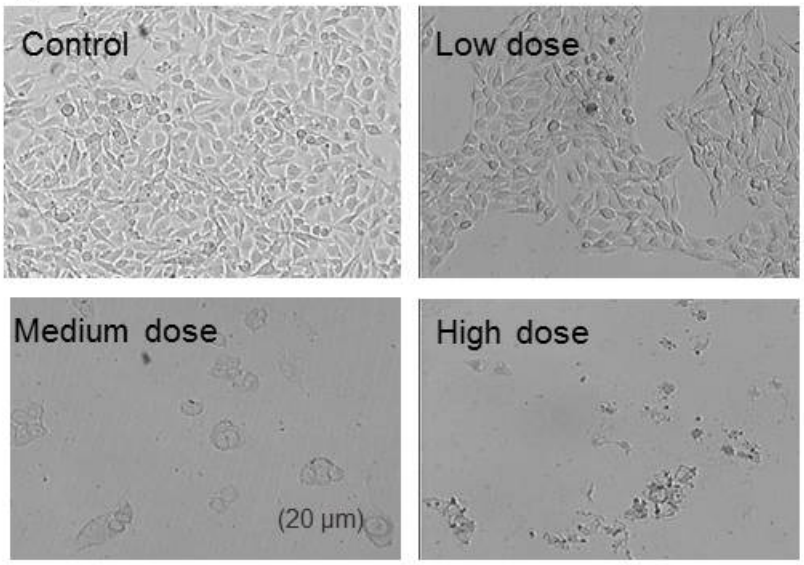

Day 1
Control
Low dose
Medium dose
High dose
Day 2
Control
Low dose
Medium dose
High dose

Figure 2.7 Effect of *U. tomentosa* ethanol extract on the morphology of the B16-BL6 cell line

B16-BL6 cells were plated on 6 well plates and treated with culture media (control) or various doses 4 μg/ml (low dose), 40 μg/ml (medium dose), or 200 μg/ml (high dose) of *U. tomentosa* extracted with 70% ethanol for 1, 2, 3, and 4 days. Micrographs of random fields were obtaining using phase contrast optics using an Axiovert 100 microscope and Northern Eclipse Software and representative images from three independent experiments is shown.


Day 1
Control
Low dose
Medium dose
High dose
(10 μm)
Day 2
Control
Low dose
Medium dose
High dose
(20 μm)

Day 3


Control
Low dose
Medium dose
(20 μm)
High dose

Day 4

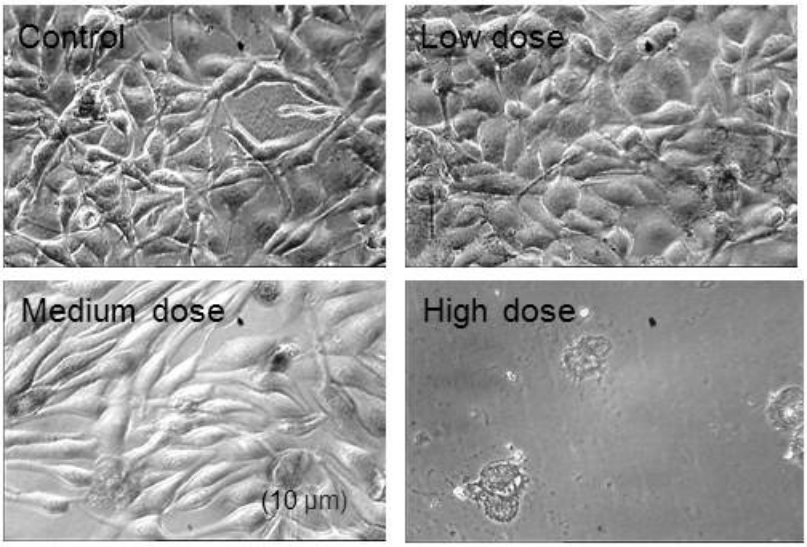
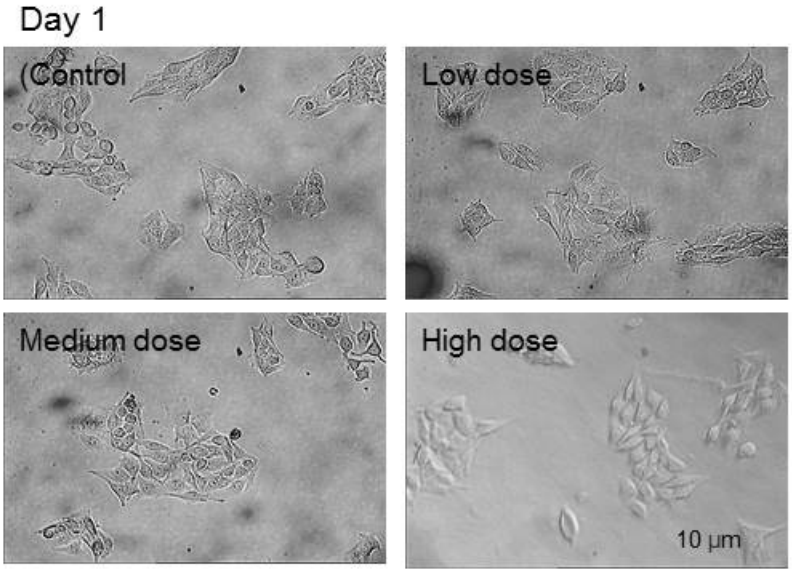
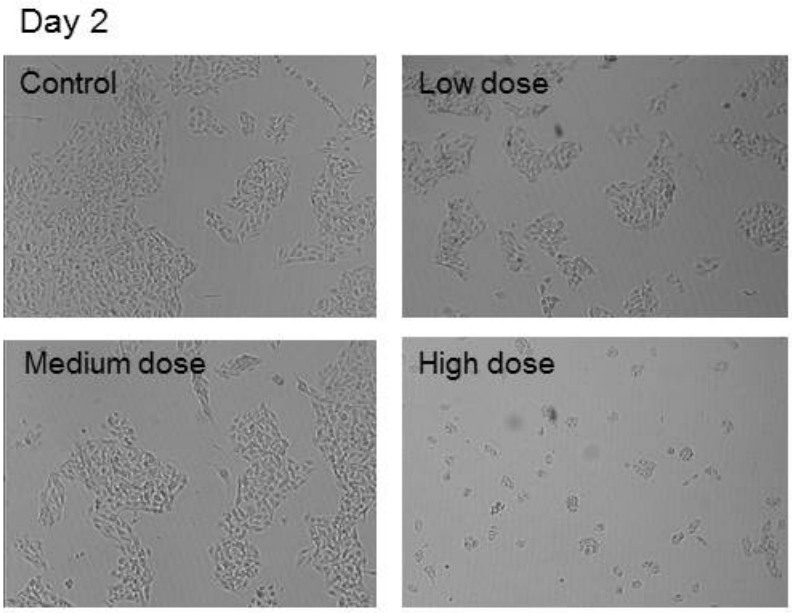
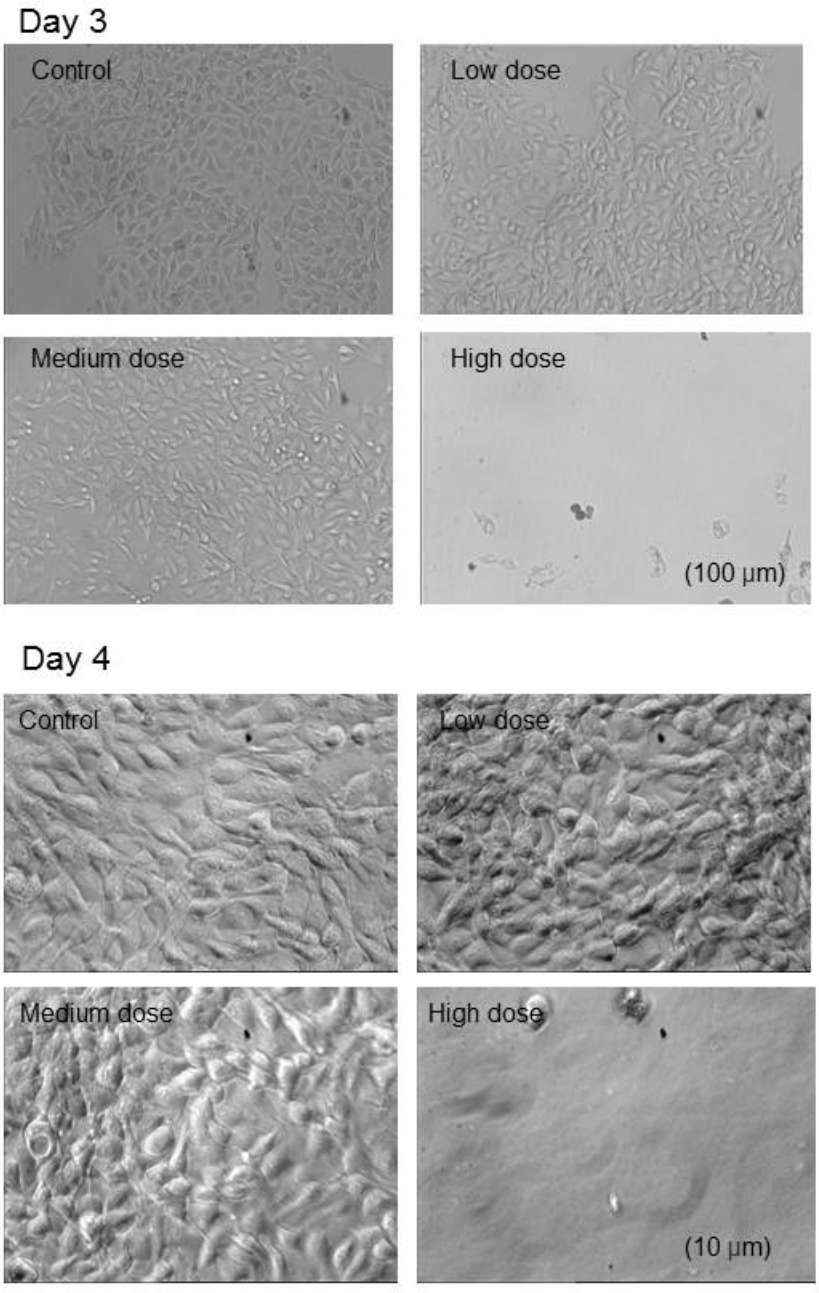

Control
Low dose
Medium dose
(10 μm)
High dose

Figure 2.8 Effect of *U. tomentosa* PBS extract on the morphology of the B16-BL6 cell line

B16-BL6 cells were plated on 6 well plates and treated with culture media (control) or various doses 4 μg/ml (low dose), 40 μg/ml (med dose), or 200 μg/ml (high dose) of *U. tomentosa* extracted with PBS, pH7.4, for 1, 2, 3, and 4 days. Micrographs of random fields were obtaining using phase contrast optics using an Axiovert 100 microscope and Northern Eclipse Software and representative images from three independent experiments is shown.


Day 1
(Control
Low dose
Medium dose
High dose
10 µm


Day 2
Control
Low dose
Medium dose
High dose


Day 3
Control
Low dose
Medium dose
High dose
(100 µm)
Day 4
Control
Low dose
Medium dose
High dose
(10 µm)

2.3.3 Inhibition of the proliferation of cancer cells (4T1 and B16-BL6) using Ki-67 protein expression

The expression levels of the Ki-67 proliferation nuclear antigen in cultured 4T1 cells was evaluated after treatment of the cells with suspending media or *U. tomentosa* extracted with ethanol or PBS for 24 hours and 48 hours. The expression of the Ki-67 antigen in the nuclei of cells is associated with active cell proliferation both *in vitro* and *in vivo*. Therefore, staining with the primary antibody was used to determine the growth fraction of an Uncaria-treated cell population. For these experiments, an FITC-labeled secondary antibody was used for detection. The stained cell cultures were examined using a fluorescence microscope and each experiment was performed three times on 3 separate days. Images of camptothecin-treated cells were after 48 h of treatment as a control for the lack of cell proliferation. The results of these studies showed treatment with *U tomentosa* extracts were able to reduce the expression of Ki-67 in a dose-, time- and extract-dependent manner. Specifically, treatment of 4T1 cells with the highest dose of *U. tomentosa* extracted with ethanol significantly blocked the expression Ki-67 at both 24 and 48 h post-treatment (Figure 2.9 and 2.10). Treatment of 4T1 cells with the medium dose of the *U. tomentosa* ethanol extract for 24 hours showed a slight inhibition in the expression Ki-67 and treatment for 48 hours showed a more significant reduction in the expression of Ki-67.

On the other hand, 4T1 cells treated with the highest dose of *U. tomentosa* extracted with PBS for 24 h did not show a significant amount of inhibition in the expression Ki-67 (Figure 2.11) but treatment with the extract for 48 hours did show a small decrease in Ki-67 staining (Figure 2.12).

The ability of treatment with *U. tomentosa* extracts to inhibit Ki-67 staining in a dose- time-, and extract-dependent manner was also seen for B16-BL6 mouse melanoma cells. Figures 2.13 and 2.14 shows the results of B16-BL6 cancer cells treated with *U. tomentosa* extracts for 72 hours. The expression of Ki-67 was significantly inhibited in B16-BL6 cells after treatment with high doses of *U. tomentosa* extracted with ethanol (Figure 2.13). Treatment with the medium dose of the ethanol extract of *U. tomentosa* partially inhibited the number of cells that stained with the Ki-67 antibody while low doses of the ethanol extract did not significantly inhibit Ki-67 expression. Treatment of B16-BL6 cells with the highest dose of *U. tomentosa* extracted into PBS resulted in a small reduction in the staining with Ki67 while treatment with both the medium and low doses did not significantly decrease Ki-67 staining (Figure 2.14). These results are consistent with the results from the cell viability experiments. Overall, treatment with *U. tomentosa* extracted with ethanol showed the strongest reduction in the number of Ki-67-positive cells in both cell lines (4T1 cells and B16-BL6 cells), consistent with the decreases in cell number. Further, treatment of the cells with *U. tomentosa* extracted with PBS had a much weaker effect on inhibiting the number of Ki-67-positive cells corresponding to a weaker effect on inhibiting cell proliferation.

Figure 2.9 Effect of *U. tomentosa* extracted with ethanol on 4T1 cell proliferation

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with ethanol for 24 hours. To measure the cell proliferation index, treated 4T1 cells were stained by incubation with the anti-Ki-67 primary antibody and then detected by staining with an FITC-labeled secondary antibody. The monolayers were analyzed using a fluorescence microscope and representative photos taken.

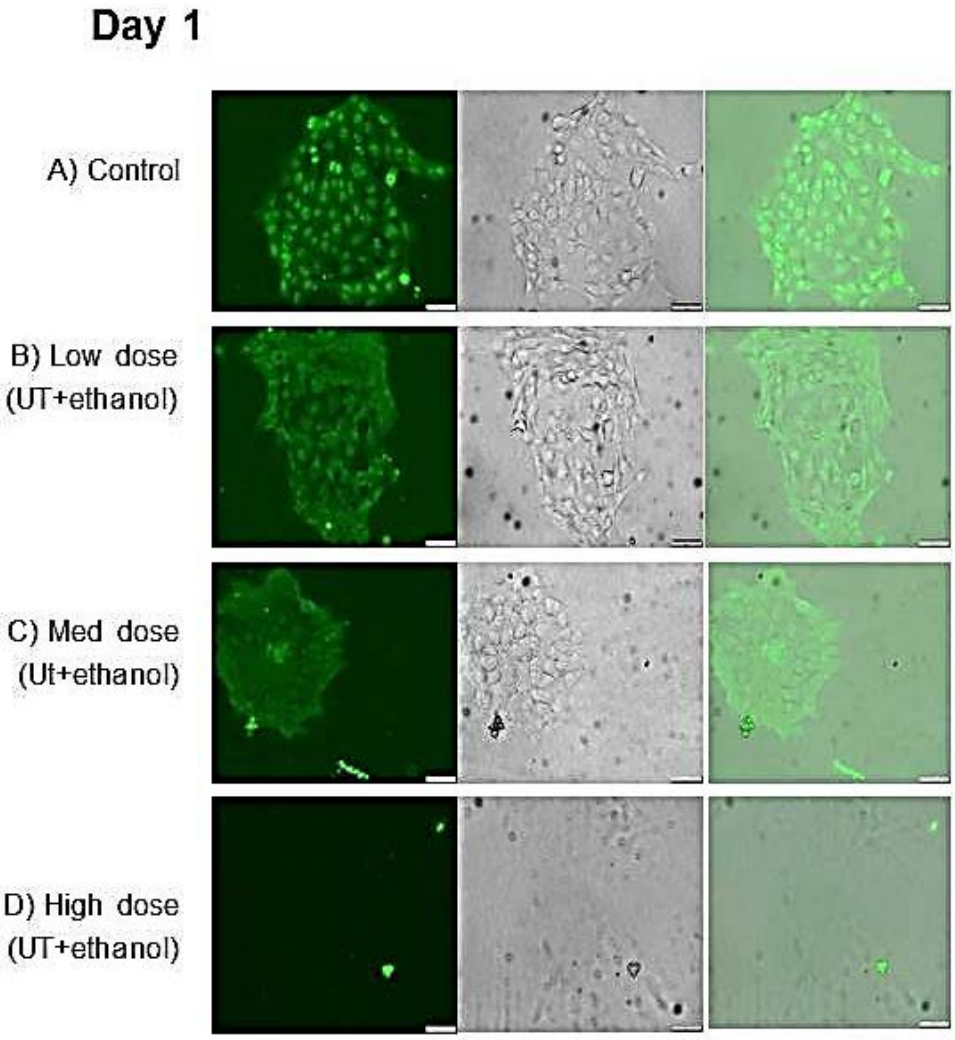

Day 1
A) Control
B) Low dose (UT+ethanol)
C) Med dose (Ut+ethanol)
D) High dose (UT+ethanol)

Figure 2.10 Effect of *U. tomentosa* extracted with ethanol on 4T1 cell proliferation

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with ethanol for 48 hours. The cells were also treated with 6 μg/ml of camptothecin (E) as a positive control for cell death. To measure the cell proliferation index, treated 4T1 cells were stained by incubation with the anti-Ki-67 primary antibody and then detected by staining with an FITC-labeled secondary antibody. The monolayers were analyzed using a fluorescence microscope and representative photos taken

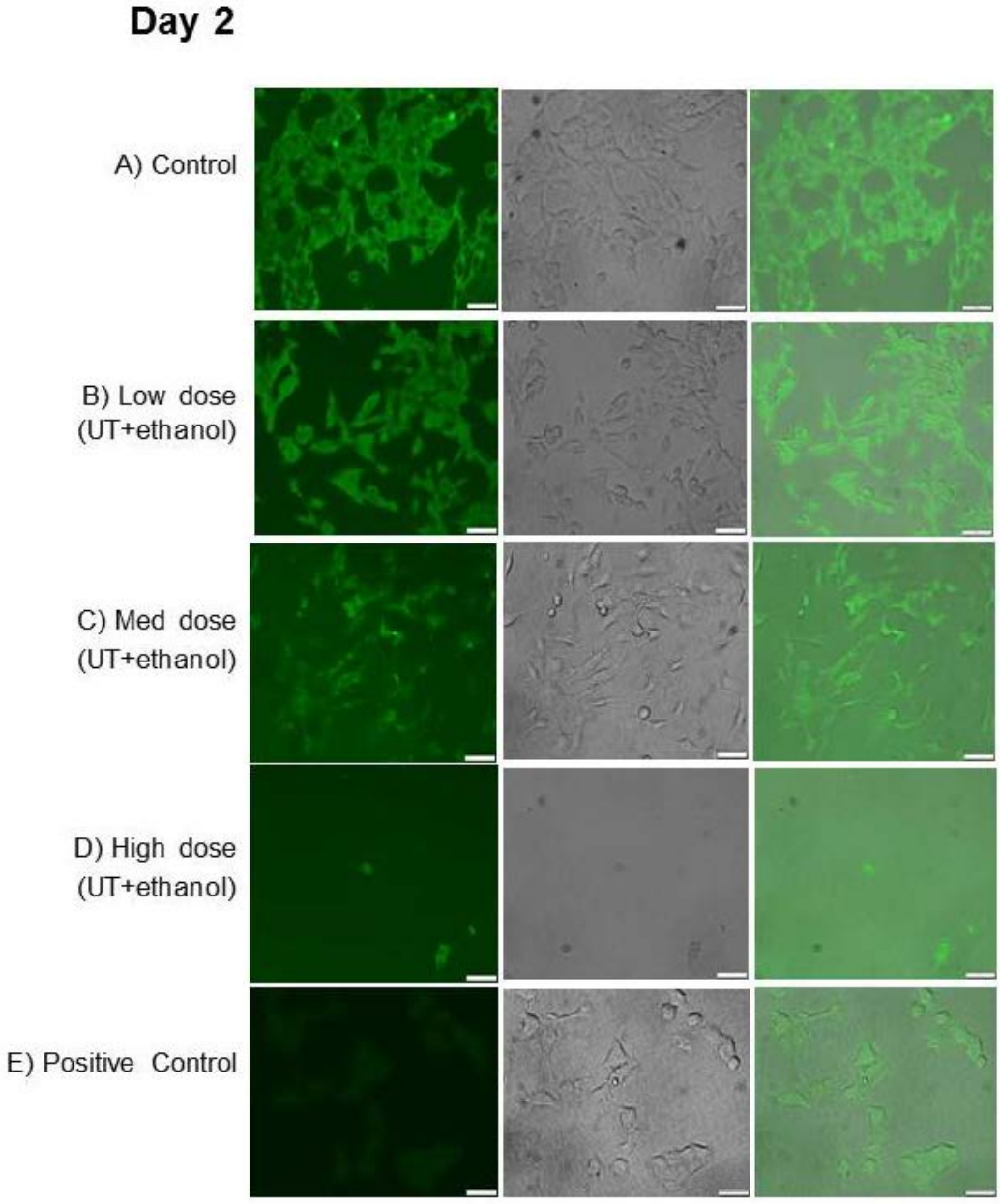

Day 2
A) Control
B) Low dose (UT+ethanol)
C) Med dose (UT+ethanol)
D) High dose (UT+ethanol)
E) Positive Control

Figure 2.11 Effect of *U. tomentosa* extracted with PBS on 4T1 cell proliferation

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml doses of *U. tomentosa* extracted with PBS for 24 hours. Also, cells were treated with culture media as a negative control. To measure the cell proliferation index in treated 4T1 cells, the coverslips were stained by incubation with the anti-Ki-67 primary antibody and then detected by staining with an FITC-labeled secondary antibody. The monolayers were analyzed using a fluorescence microscope and representative photos taken.

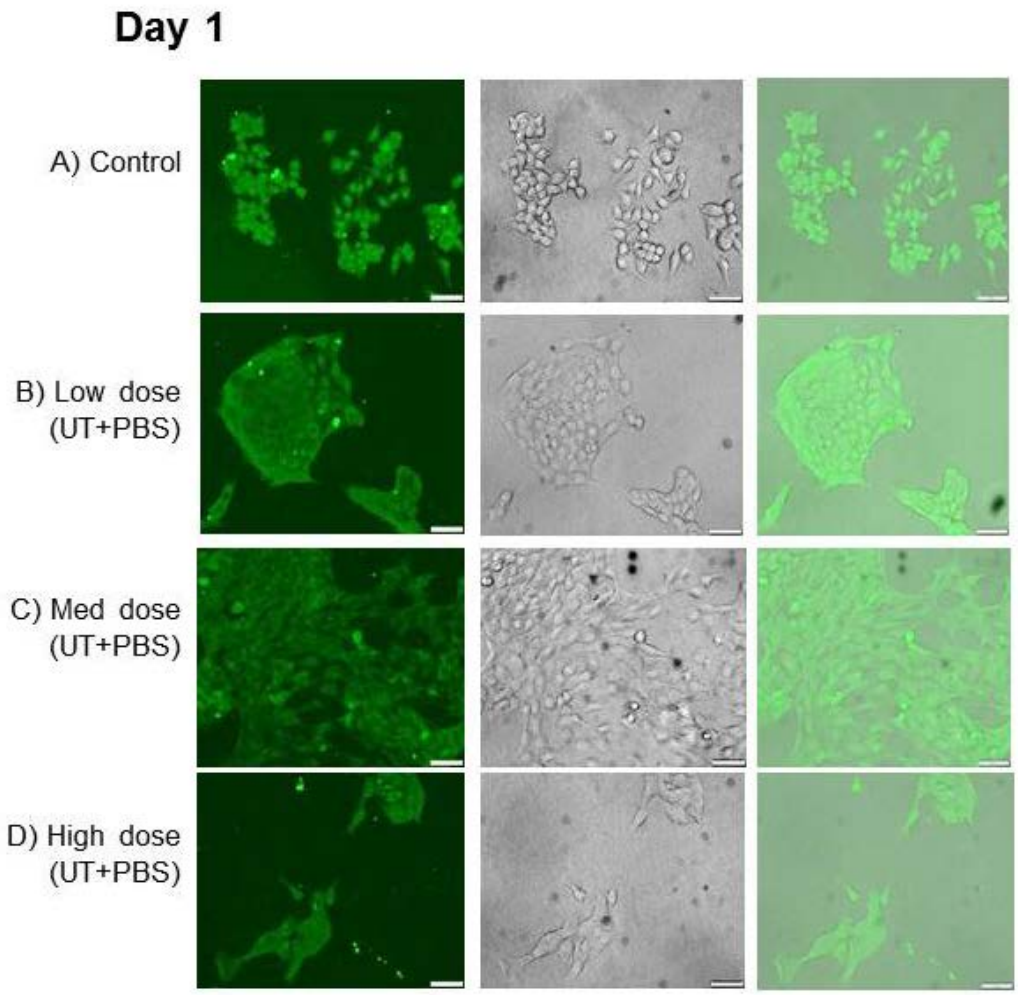

Day 1
A) Control
B) Low dose (UT+PBS)
C) Med dose (UT+PBS)
D) High dose (UT+PBS)

Figure 2.12 Effect of *U. tomentosa* extracted with PBS on 4T1 cell proliferation

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml doses of *U. tomentosa* extracted with PBS for 48 hours. The cells were also treated with 6 μg/ml of camptothecin (E) as a positive control for cell death. To measure the cell proliferation index in treated 4T1 cells, the coverslips were stained by incubation with the anti-Ki-67 primary antibody and then detected by staining with an FITC-labeled secondary antibody. The monolayers were analyzed using a fluorescence microscope and representative photos taken.

Day 2

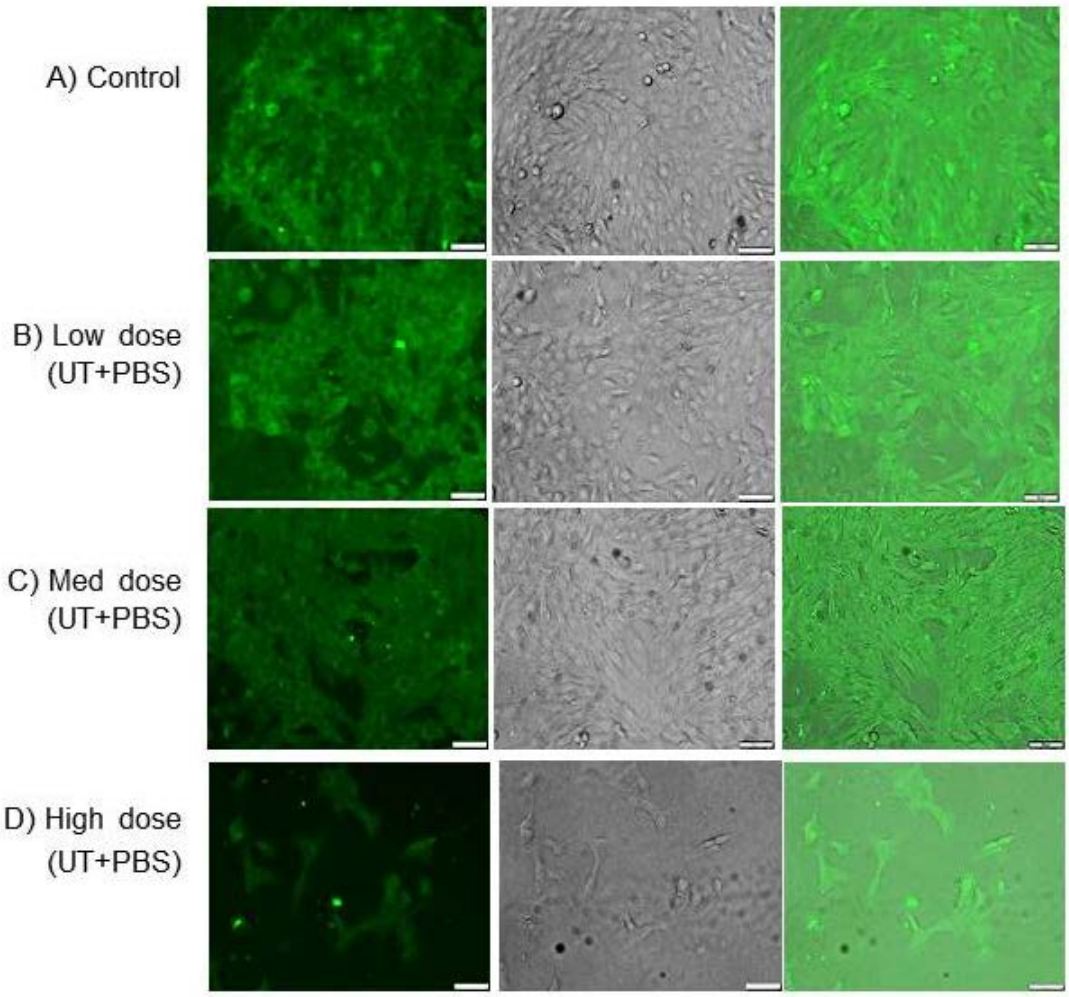


Figure 2.13 Effect of *U. tomentosa* extracted with ethanol on B16-BL6 cell proliferation

B16-BL6 cells were plated on tissue culture plates and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with 70% ethanol for 72 hours. To measure the cell proliferation index in treated B16-BL6 cells, the cells were stained by incubation with the anti-Ki-67 primary antibody and then detected by staining with an FITC-labeled secondary antibody. The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

Day 3

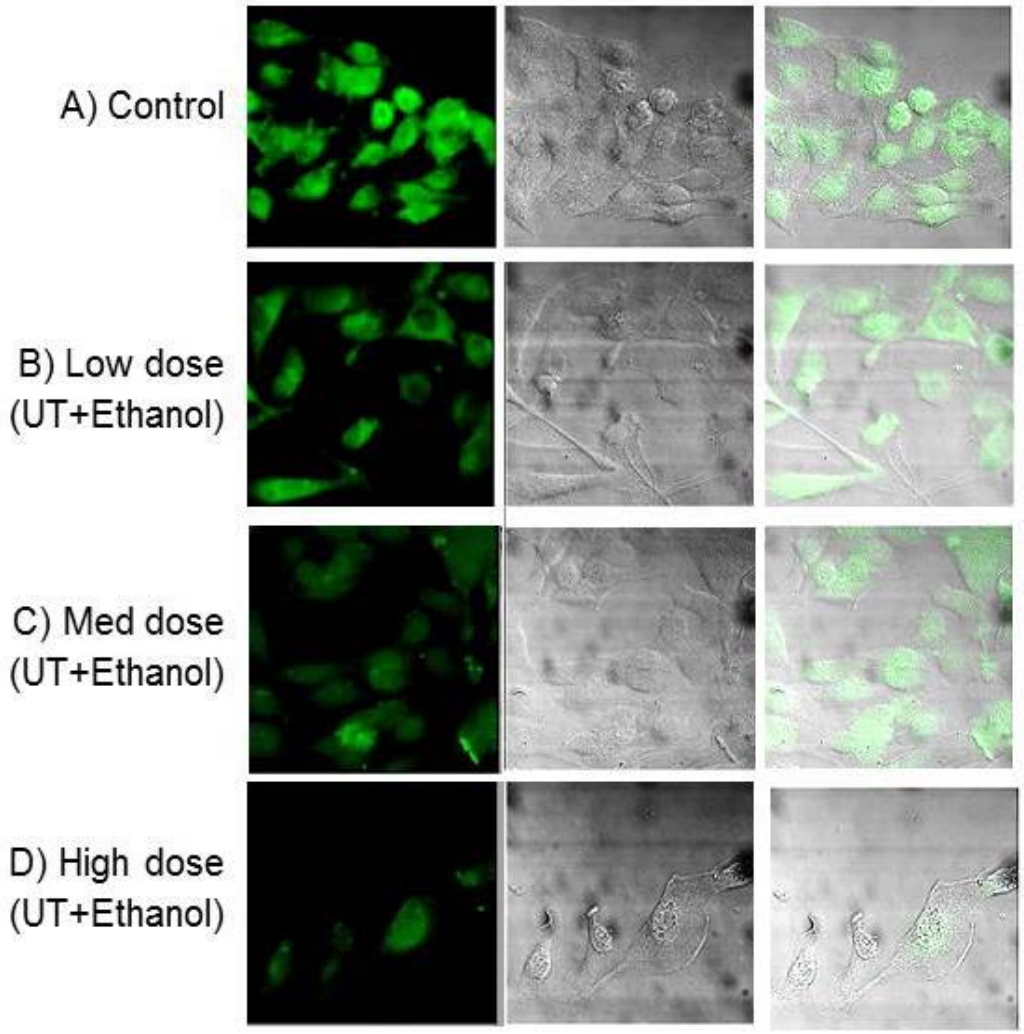

A) Control
B) Low dose (UT+Ethanol)
C) Med dose (UT+Ethanol)
D) High dose (UT+Ethanol)

Figure 2.14 Effect of *U. tomentosa* extracted with PBS on B16-BL6 cell proliferation

B16-BL6 cells were plated on tissue culture plates and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with PBS, pH 7.4, for 72 hours. To measure the cell proliferation index in treated B16-BL6 cells, the cells were stained by incubation with the anti-Ki-67 primary antibody and then detected by staining with an FITC-labeled secondary antibody. The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

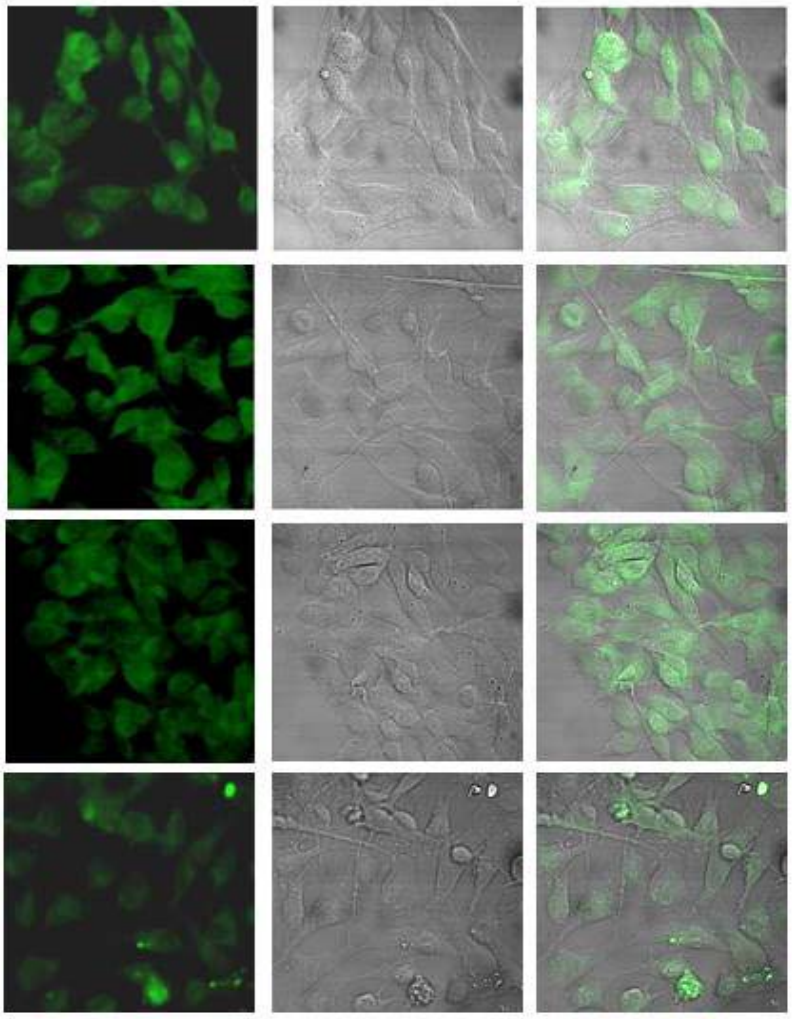
Day 3

A) Control

B) Low dose (UT+PBS)

C) Med dose (UT+PBS)

D) High dose (UT+PBS)



2.3.4 Detection of DNA fragmentation in 4T1 cells and B16-BL6 cells treated with *U. tomentosa* extracts

A TUNEL assay was performed to detect DNA fragmentation in cell cultures treated with *U. tomentosa* extracts. DNA fragmentation is considered a critical hallmark of apoptosis. 4T1 cells were treated with various doses of either the ethanol or PBS extracts of *U. tomentosa* for a period of 24 hours and 48 hours and then analyzed using a TUNEL reagent kit based on the ability of terminal transferase to add a fluorescently labelled oligonucleotide to free DNA ends. Fluorescence microscopy (Olympus IX3) was used to measure the level of fluorescence corresponding to nucleotide incorporation and experiments were performed three times. Images of camptothecin-treated cells were taken during the treatment of 48 hours as a positive control for increased apoptosis. Treatment of 4T1 cells with the highest dose of *U. tomentosa* ethanol extract for 24 hours and 48 hours clearly showed TUNEL staining in the majority of the cells that remained alive (Figure 2.15, and 2.16). Cells treated with the medium dose of *U. tomentosa* ethanolic extract for 24 hours showed a low level of TUNEL staining associated with cell death (Figure 2.15) but treatment for 48 hours, caused a significant increase in DNA fragmentation (Figure 2.16). On the other hand, no significant staining for the TUNEL reagent was observed in 4T1 cells treated with *U. tomentosa* extracted with PBS at the highest dose for 24 hours (Figure 2.17). However, TUNEL staining was observed in some cells when the treatment period was extended to 48 hours (Figure 2.18). Treatment with the PBS extract of *U. tomentosa* at the low and medium doses did not promote any significant TUNEL staining in cells treated for either 24 hours or 48 hours.

TUNEL staining of the B16-BL6 cells treated with *U. tomentosa* extract showed similar results to the 4T1 cells. Specifically, the level of TUNEL staining in B16-BL6

cells was assayed after 24 hours and 72 hours of treatment with *U. tomentosa* extracts. A significant increase in the proportion of apoptotic cells was observed after treatment with the high dose of *U. tomentosa* extracted with ethanol, at both the 24 hour and 72 hour treatments (Figure 2.19 and 2.20). Treatment of B16-BL6 cells with the low dose of *U. tomentosa* extracted with ethanol did not show any increase in TUNEL staining after 24 h but almost all of the cells were labeled after treatment for 72 hours. However, the level of TUNEL staining that was detected after treatment with the high dose of *U. tomentosa* extracted with PBS for 24 hours was not detected while treatment for 72 hours promoted staining in some of the cells (Figure 2.21, and 2.22). Treatment with the low dose of *U. tomentosa* extracted with PBS, pH 7.4, did not promote TUNEL staining. Overall, treatment of *U. tomentosa* extracted with ethanol significantly induces apoptosis in both cell lines (4T1 cells and B16-BL6 cells) to a greater extent than treatment with *U. tomentosa* extracted with PBS correlating with the cell proliferation results.

Figure 2.15 Detection of DNA fragmentation in 4T1 cells treated with *U. tomentosa* extracted with ethanol using the TUNEL assay for 24 hours

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with ethanol for 24 hours. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

Day 1

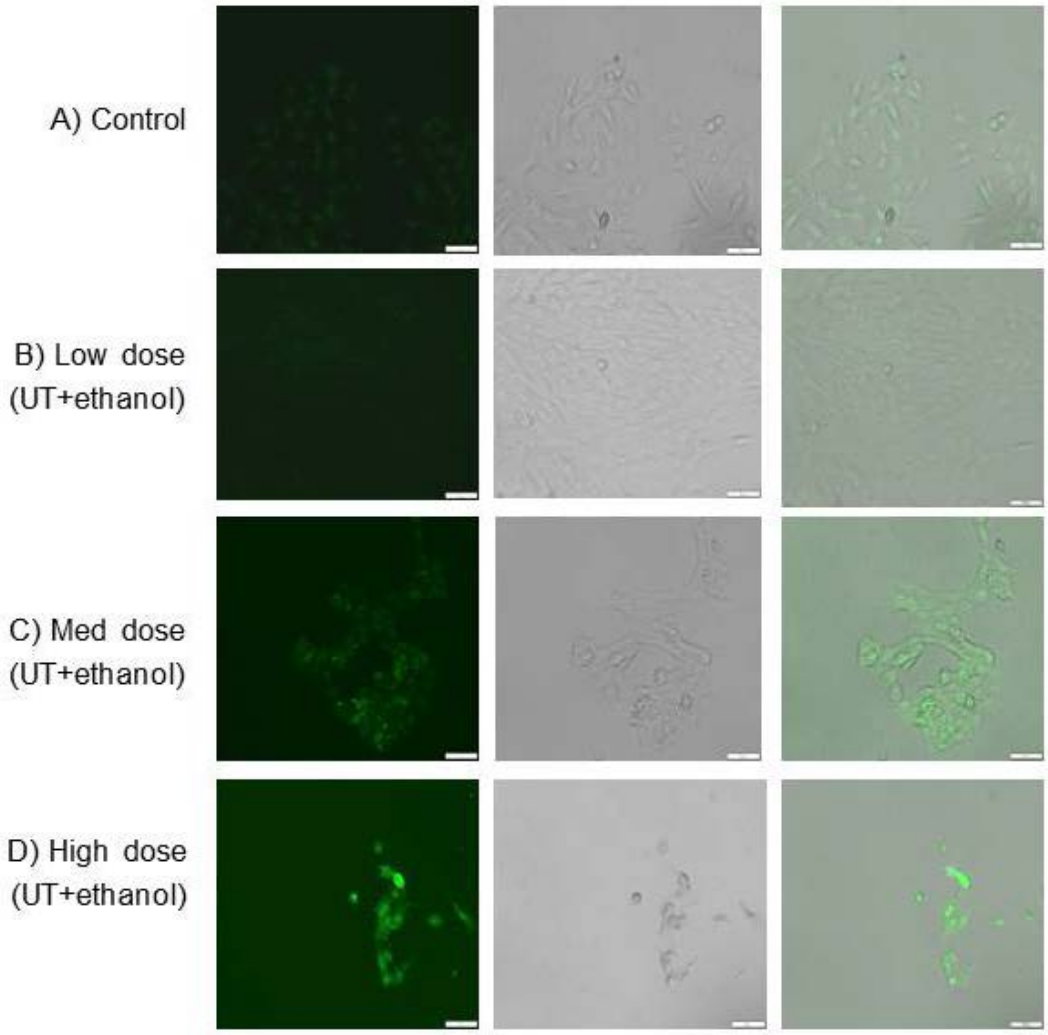

A) Control
B) Low dose (UT+ethanol)
C) Med dose (UT+ethanol)
D) High dose (UT+ethanol)

Figure 2.16 Detection of DNA fragmentation in 4T1 cells treated with *U. tomentosa* extracted with ethanol using the TUNEL assay for 48 hours

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with 70% ethanol for 48 hours. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

Day 2

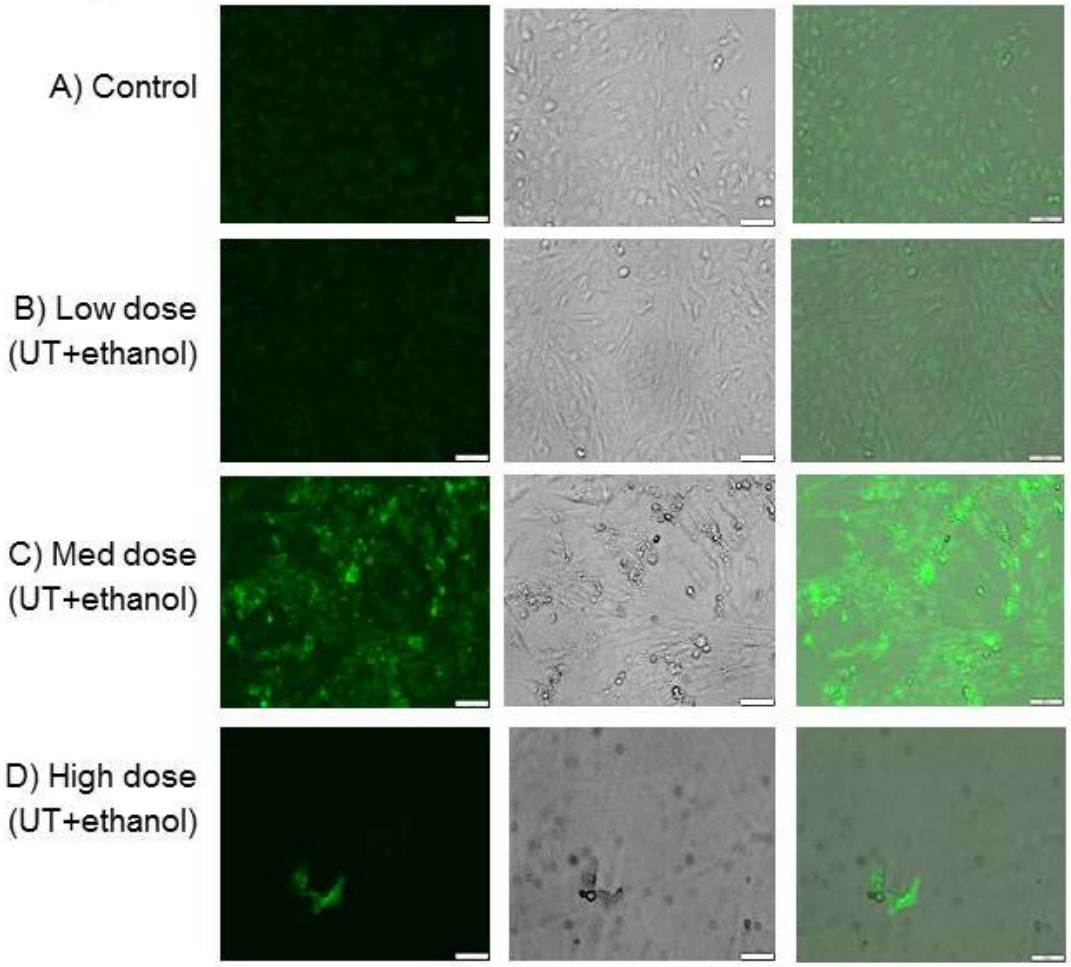

A) Control
B) Low dose (UT+ethanol)
C) Med dose (UT+ethanol)
D) High dose (UT+ethanol)

Figure 2.17 Detection of DNA fragmentation in 4T1 cells treated with *U. tomentosa* extracted with PBS using the TUNEL assay for 24 hours

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with PBS, pH 7.4, for 24 hours. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

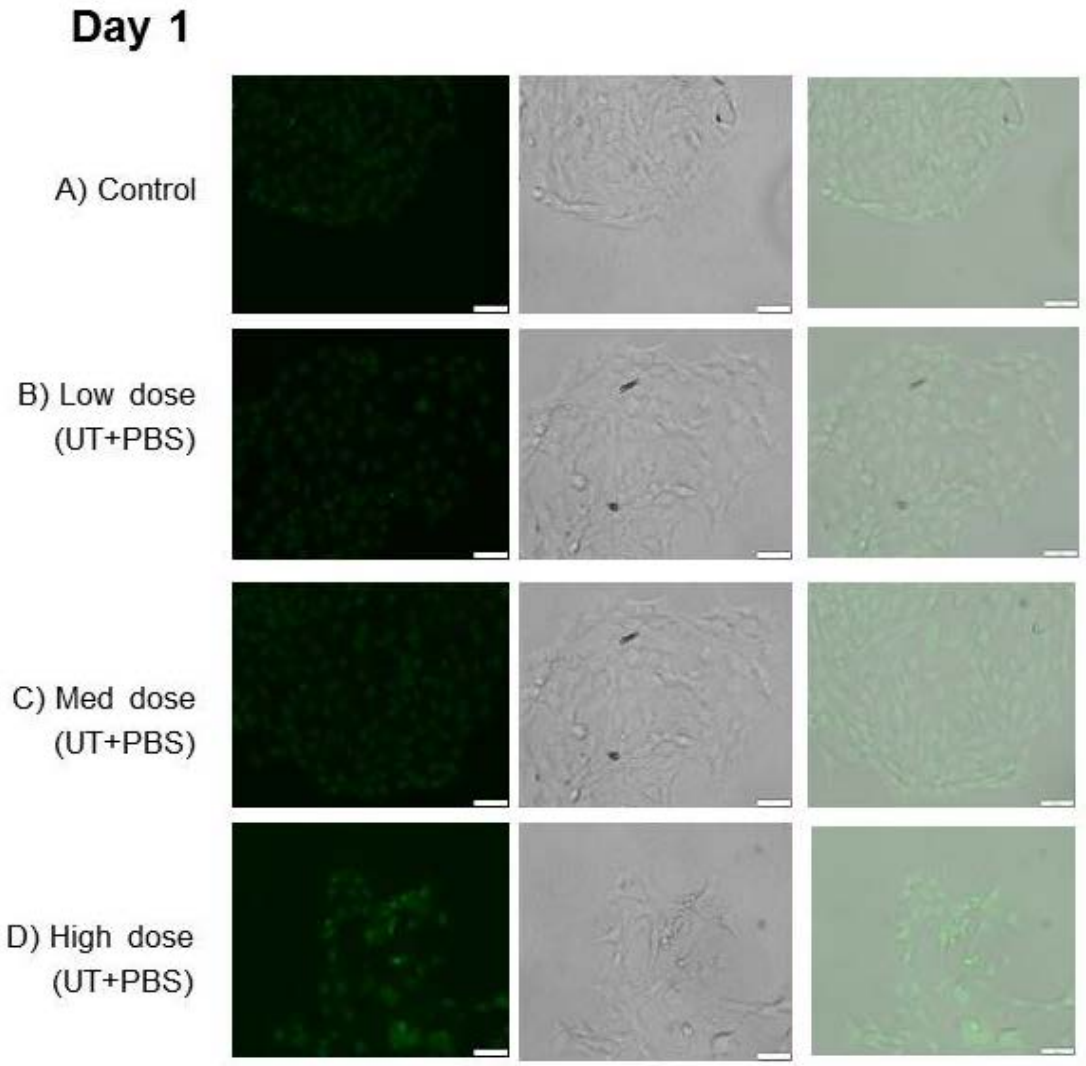

Day 1
A) Control
B) Low dose (UT+PBS)
C) Med dose (UT+PBS)
D) High dose (UT+PBS)

Figure 2.18 Detection of DNA fragmentation in 4T1 cells treated with *U. tomentosa* extracted with PBS using the TUNAL assay for 48 hours

4T1 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), medium (C, 40 μg/ml) and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with PBS, pH 7.4, for 48 hours. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

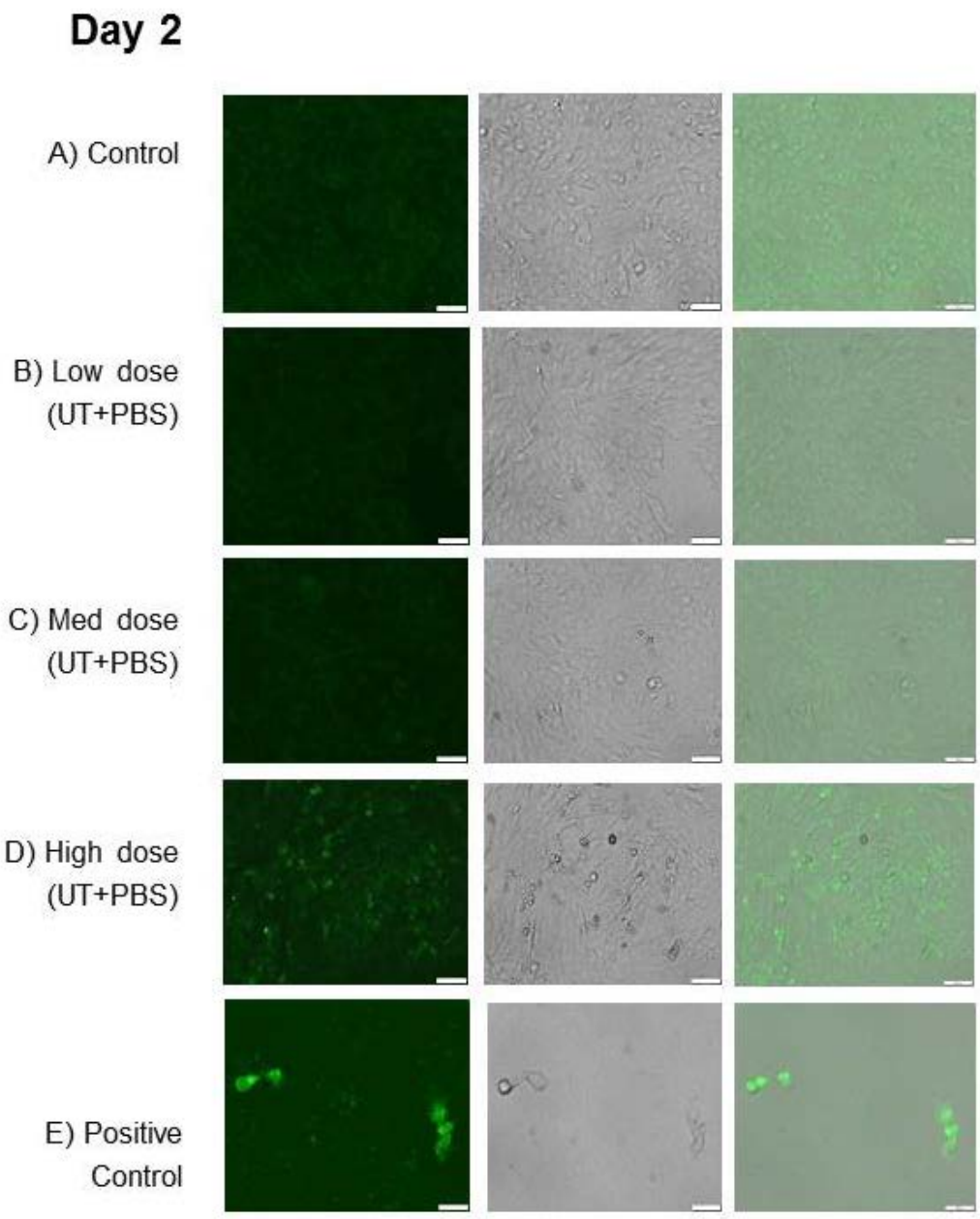

Day 2
A) Control
B) Low dose (UT+PBS)
C) Med dose (UT+PBS)
D) High dose (UT+PBS)
E) Positive Control

Figure 2.19 Detection of cell death in B16-BL6 cells treated with *U. tomentosa* extracted with ethanol using the TUNEL assay for 24 hours

B16-BL6 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), and high (D, 200 μg/ml) doses of *U. tomentosa* extracted with 70% ethanol for 24 hours. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

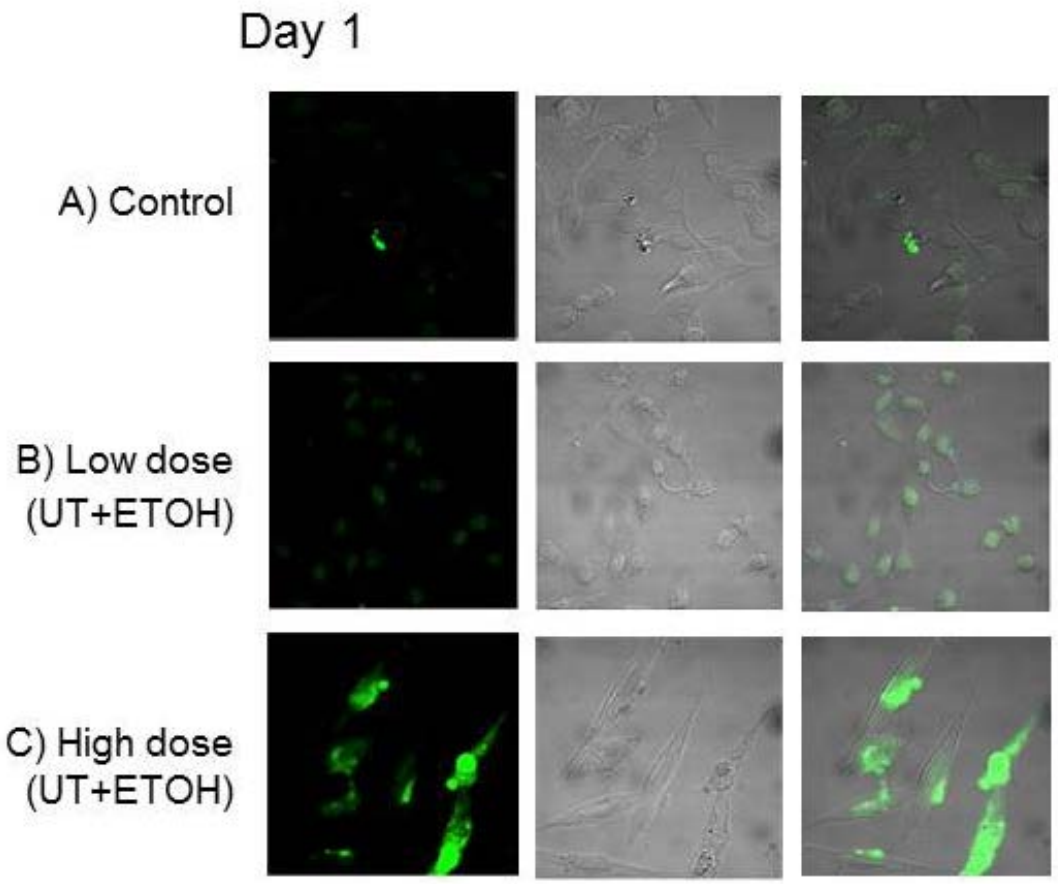

Day 1
A) Control
B) Low dose
(UT+ETOH)
C) High dose
(UT+ETOH)

Figure 2.20 Detection of cell death in B16-BL6 cells treated with *U. tomentosa* extracted with ethanol using the TUNAL assay for 72 hours

B16-BL6 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), and high (C, 200 μg/ml) doses of *U. tomentosa* extracted with 70% ethanol for 72 hours. Cells treated with 6 mg/ml camptothecin for 72 h (D) were used as a positive control for apoptosis. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

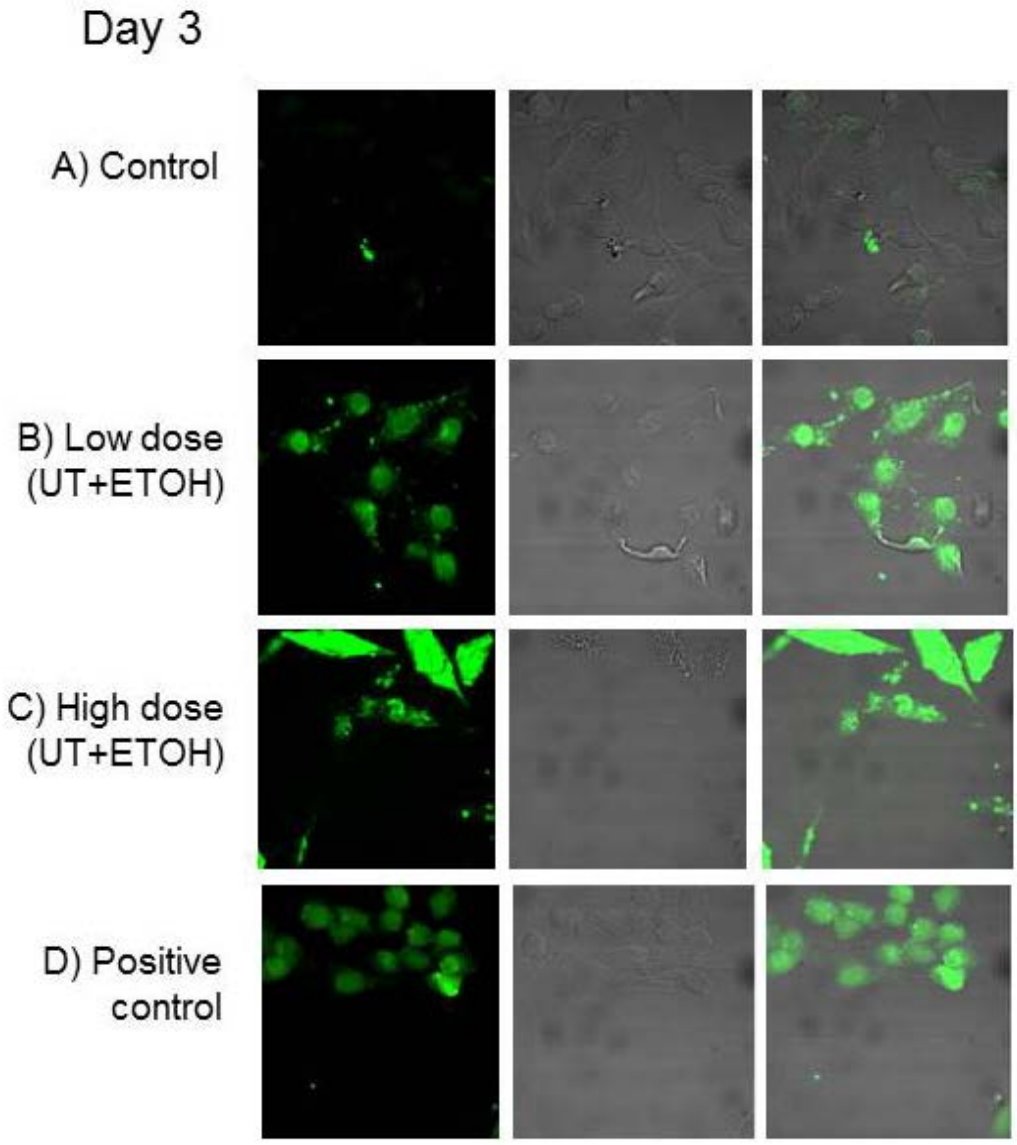

Day 3
A) Control
B) Low dose (UT+ETOH)
C) High dose (UT+ETOH)
D) Positive control

Figure 2.21 Detection of cell death in B16-BL6 cells treated with *U. tomentosa* extracted with PBS using the TUNEL assay for 24 hours

B16-BL6 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), and high (C, 200 μg/ml) doses of *U. tomentosa* extracted with PBS, pH 7.4, for 24 hours. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.

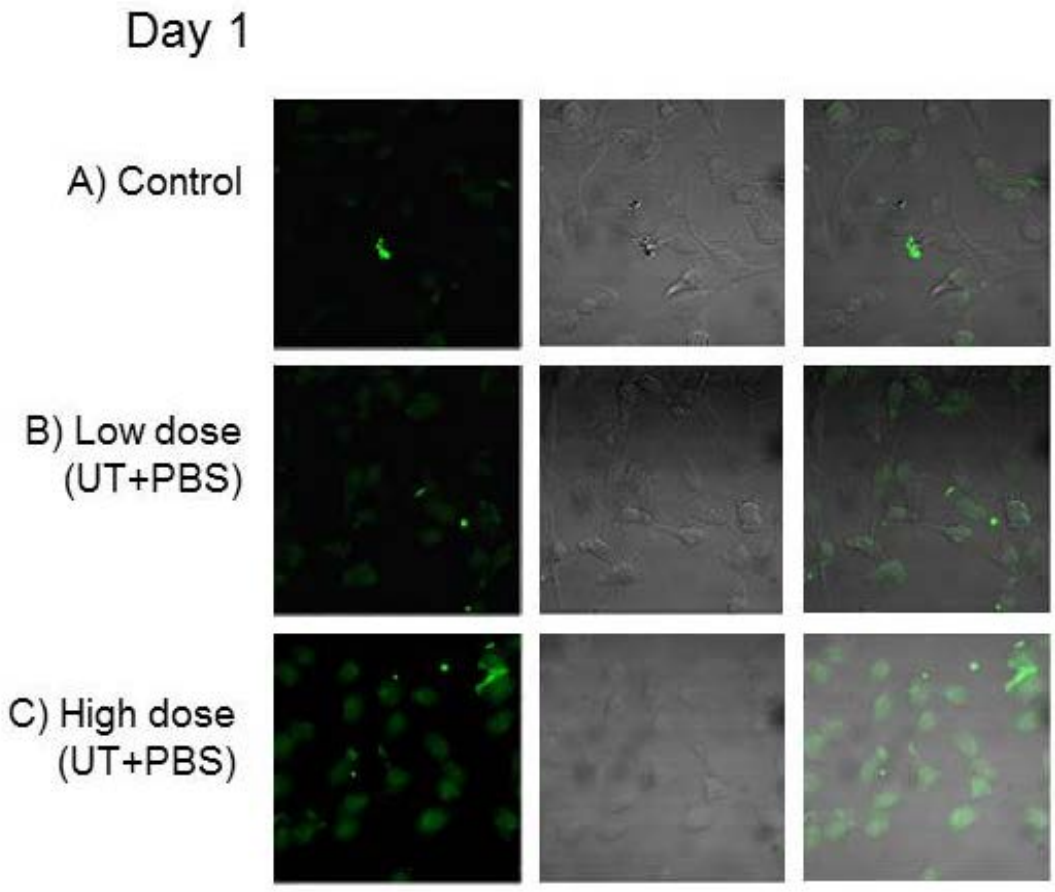
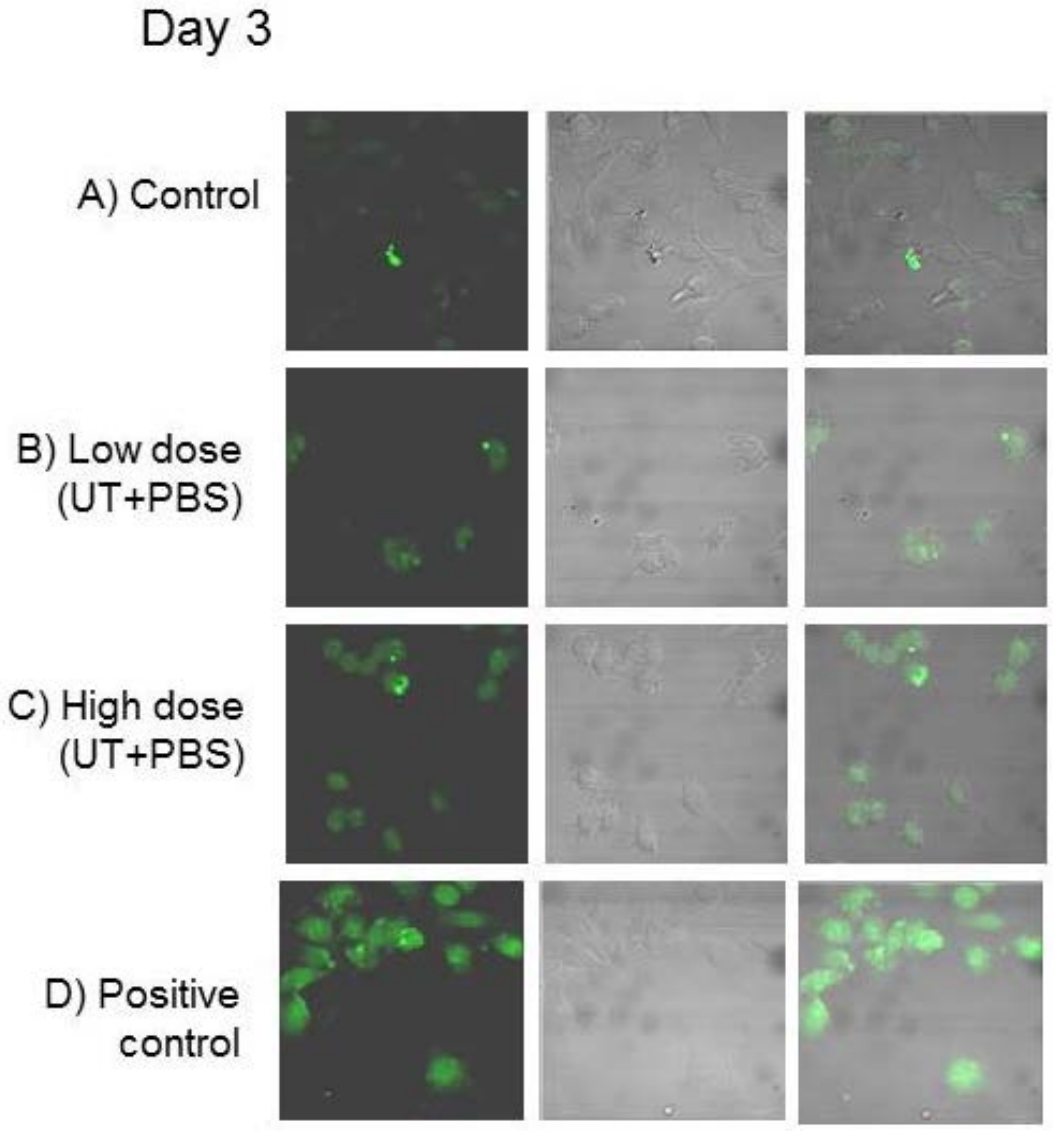

Day 1
A) Control
B) Low dose (UT+PBS)
C) High dose (UT+PBS)

Figure 2.22 Detection of cell death in B16-BL6 cells treated with *U. tomentosa* extracted with PBS using the TUNEL assay for 72 hours

B16-BL6 cells were plated on glass coverslips and treated with suspending media (A, control) and low (B, 4 μg/ml), and high (C, 200 μg/ml) doses of *U. tomentosa* extracted with PBS, pH 7.4, for 72 hours. Cells treated with 6 μg/ml camptothecin for 72 h (D) were used as a positive control for apoptosis. Cells were stained by incubation with 50 μl/well TUNEL reaction mixture to detect free DNA ends (DNA fragmentation). The monolayers were analyzed using a fluorescence microscope and representative photos taken. Images show the fluorescence channel (excitation at 488 nm and emission at 520 nm), phase contrast channel, and the combination.


Day 3
A) Control
B) Low dose (UT+PBS)
C) High dose (UT+PBS)
D) Positive control

2.3.5 Detection of 4T1 cell accumulation in sub-G1 using flow cytometry

Flow cytometry was conducted to investigate the effect of treatment with *U. tomentosa* extracts on 4T1 cell cycle distribution and the presence of sub-G1 apoptotic cells. In these experiments, cells were treated with low (4 μg/ml), medium (40 μg/ml) and high (200 μg/ml) doses of *U. tomentosa* extracted with either ethanol or PBS for 24 hours and 48 hours. These cells were stained with propidium iodide (PI) dye to measure the amount of DNA in each cell. Treatment of the 4T1 cells with the high dose of the ethanol extract of *U. tomentosa* for 24 hours and 48 hours induced a large increase in the percentage of the cells in the sub-G1 peak indicating a large increase in apoptosis. In particular, the treatment during 48 hours at the highest dose showed a huge increase in sub-G1 cells, which increased to 78.4 % while the treatment during 24 hours increased the sub-G1 population to 61.9 % (Figure 2.23). This is similar to the effect of treatment with camptothecin, which was used as a positive control to promote formation of apoptotic cells and showed an increase to 74.9% of the cells in the sub-G1 peak (Figure 2.23). Treatment with the medium dose of the ethanol extract of *U. tomentosa* for 24 hours did not have a large affect the number of cells in different phases of the cell cycle although there was a small increase in the number of cells in the sub-G1 peak. After treatment for 48 hours, treatment with the medium dose significantly increased the number of cells in the sub-G1 peak and decreased the number of cells in G2/M. Treatment with the low dose of the ethanol extract of *U. tomentosa* for 24 or 48 hours did not significantly alter the number of cells in the different phases of the cell cycle and did not increase the number of cells in the sub-G1 peak.

On the other hand, the treatment of 4T1 cells with *U. tomentosa* extracted with PBS, pH 7.4, was less active. Treatment of 4T1 cells with the high dose of *U. tomentosa* extracted with PBS, pH 7.4, promoted a small increase in the number of cells in the sub-G1 phase from 5.0% to 11.9 % after 24 hours, and to 13.8% after treatment for 48 hours (Figure 2.24). Treatment with the low and medium doses of *U. tomentosa* extracted with PBS, pH 7.4, did not significantly alter the cell cycle pattern from the untreated control. Together, the results of the flow cytometry confirmed the findings of previous experiments that *U. tomentosa* extracted with ethanol can significantly induce apoptosis in 4T1 cells more effectively than *U. tomentosa* extracted with PBS.

Figure 2.23 Effect of *U. tomentosa* extracted with ethanol on cell cycle profiles (sub-G1 peaks) in 4T1 cells

To confirm apoptosis in 4T1 cells treated with *U. tomentosa*, flow cytometry analysis was applied. Cells were grown in 60 x 15 mm tissue culture plates and treated with suspending media (control), and low (4 μg/ml), medium (40 μg/ml), and high (200 μg/ml) doses of *U. tomentosa* extracted with ethanol over a period of 24 hours and 48 hours. Camptothecin was used as a positive control during the treatment for 48 hours. The cells were incubated with propidium iodide and analysis was performed using a Beckman Coulter LS600 flow cytometer. The experiments were run in triplicate. The histogram displayed the percentage of cells in the Sub-G1 (apoptotic cells) and other phases of the cells cycle and the table of percentages in each phase are the means for the 3 replicates.

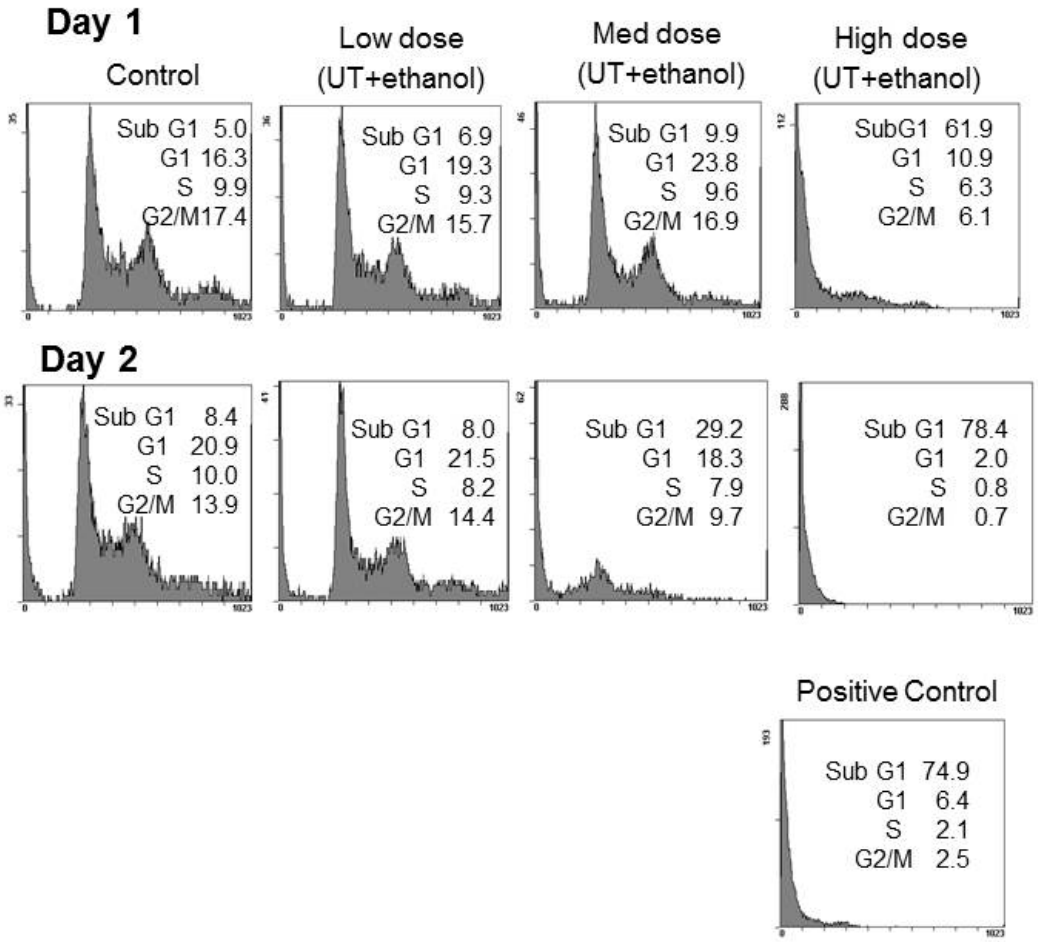
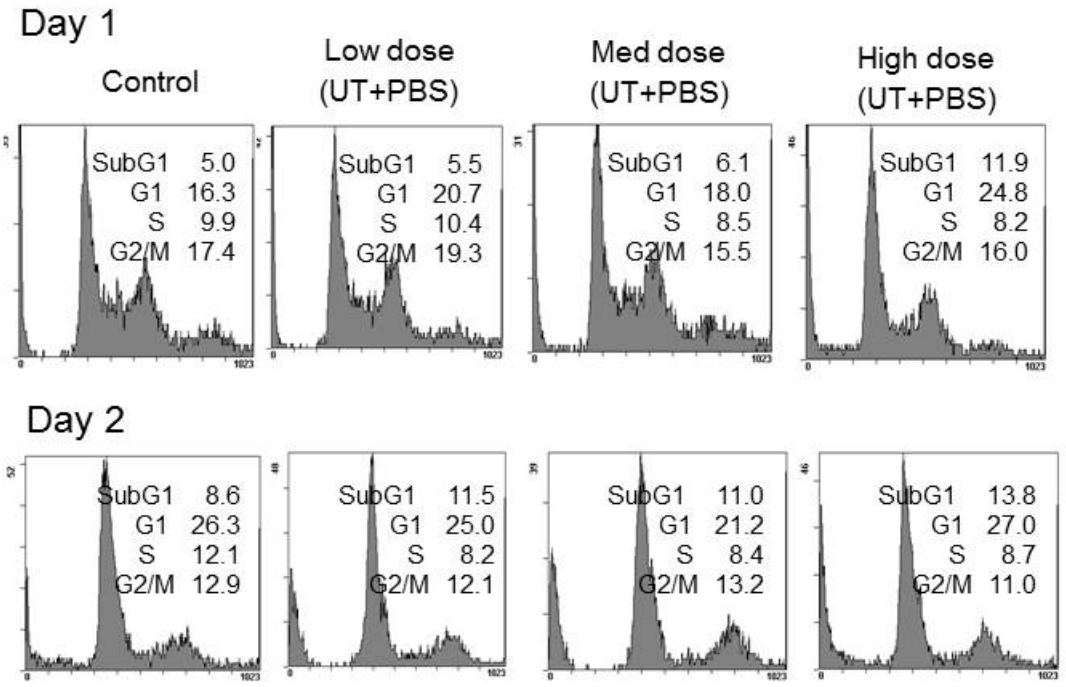

Day 1
Control
Sub G1 5.0
G1 16.3
S 9.9
G2/M17.4
Low dose
(UT+ethanol)
Sub G1 6.9
G1 19.3
S 9.3
G2/M 15.7
Med dose
(UT+ethanol)
Sub G1 9.9
G1 23.8
S 9.6
G2/M 16.9
High dose
(UT+ethanol)
SubG1 61.9
G1 10.9
S 6.3
G2/M 6.1
Day 2
Sub G1 8.4
G1 20.9
S 10.0
G2/M 13.9
Sub G1 8.0
G1 21.5
S 8.2
G2/M 14.4
Sub G1 29.2
G1 18.3
S 7.9
G2/M 9.7
Sub G1 78.4
G1 2.0
S 0.8
G2/M 0.7
Positive Control
Sub G1 74.9
G1 6.4
S 2.1
G2/M 2.5

Figure 2.24 Effect of *U. tomentosa* extracted with PBS on cell cycle profiles (sub-G1 peaks) in 4T1 cells

To confirm apoptosis in 4T1 cells treated with *U. tomentosa*, flow cytometry analysis was applied. Cells were grown in 60 x 15 mm tissue culture plates and treated with suspending media (control), and low (4 μg/ml), medium (40 μg/ml) and high (200 μg/ml) doses of *U. tomentosa* extracted with PBS, pH7 .4, over a period of 24 hours and 48 hours. The cells were incubated with propidium iodide and analysis was performed using a Beckman Coulter LS600 flow cytometer. The experiments were run in triplicate. The histogram displayed the percentage of cells in the Sub-G1 (apoptotic cells) and other phases of the cells cycle and the table of percentages in each phase are the means for all 3 replicates.


Day 1
Control
SubG1 5.0
G1 16.3
S 9.9
G2/M 17.4
Low dose
(UT+PBS)
SubG1 5.5
G1 20.7
S 10.4
G2/M 19.3
Med dose
(UT+PBS)
SubG1 6.1
G1 18.0
S 8.5
G2/M 15.5
High dose
(UT+PBS)
SubG1 11.9
G1 24.8
S 8.2
G2/M 16.0
Day 2
SubG1 8.6
G1 26.3
S 12.1
G2/M 12.9
SubG1 11.5
G1 25.0
S 8.2
G2/M 12.1
SubG1 11.0
G1 21.2
S 8.4
G2/M 13.2
SubG1 13.8
G1 27.0
S 8.7
G2/M 11.0

2.3.6 HPLC analysis of *U. tomentosa* extracts and fractions and their effect on cell growth

U. tomentosa was extracted in 70% ethanol or PBS, pH 7.4. Ground bark was suspended in 70% ethanol or PBS, pH 7.4, at a concentration of 0.1 mg/ml and boiled with refluxing for a period of one hour. Bark residue was removed by centrifugation and the supernatant filtered through 0.22 μm filters for use in experiments. This filtrate was also subjected to analysis by HPLC on a C18 column using a gradient mobile phase comprised of 10 mM phosphate buffer, pH 6, acetonitrile, methanol buffer (60/20/20 to 20/35/25 over 40 min) at 1 ml/min. Peaks corresponding to components of the *U. tomentosa* were detected at a wavelength of 245 nm as indicated in Figure 2.25. Molecular standards corresponding to the known components of the *U. tomentosa* extracts including the alkaloids, uncarine D, mitraphylline, uncarine C, isomitraphyline, rhynophyline, and uncarine F, were subjected to analysis using the same conditions on the same day and were used to identify the peaks present in the extracts. Results indicated the presence of a significant amount of these alkaloids in the ethanol extract of *U. tomentosa*, while a lower amount of these alkaloids was present in the PBS extract. Analysis of the area under each of the absorbance peaks suggests that, on average, there is about tenfold less of each of the alkaloids in the PBS extract compared to the alcohol extract.

The *U. tomentosa* extracts were fractionated into different components in order to enrich for the potential active molecules and then tested for activity by measuring their effect on the proliferation of both 4T1 and B16-BL6 cells. In this experiment, 200 ml of a 70% ethanol extract of *U tomentosa* was freeze-dried and resuspended in 20 ml water.

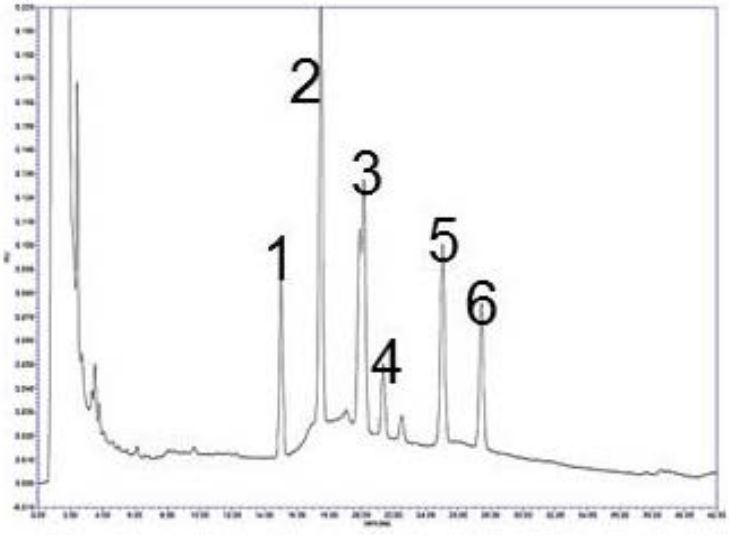
The resuspended *U. tomentosa* ethanol extract was then applied to a 50 ml polyvinylpyrrollidone (PPVP) column equilibrated in water. The extract was eluted from the column using 4 volumes each of water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 95% ethanol. Each of these fractions was lyophilized, combined for each elution, and resuspended in 70% ethanol at 10 mg/ml final concentration. The composition of each of the fractions was analyzed using HPLC as indicated in Figure 2.26. This analysis shows that the level of the various indolalkaloids was different in each of the PVPP fractions of *U. tomentosa*. The highest concentrations of the alkaloids were in the 40% ethanol fraction.

The effect of these, five different fractions on cell growth was examined against both the 4T1 and B16-BL6 cell lines, using MTT assays (Figure 2.27). Cells were treated with 1 μg/ml of the reconstituted fraction in culture media on day 0 and cell numbers were determined over 3 to 4 days in the continued presence of the extract. Interestingly, the results were somewhat different between the B16-BL6 and 4T1 cells. For the 4T1 cells, treatment with the 80% ethanol fraction showed the strongest inhibition of cell growth while treatment with the 60% ethanol fraction significantly inhibited cell proliferation. For the B16-BL6 cells the results showed that the 60% ethanol fraction had the strongest inhibitory effect while the 80% ethanol fraction was able to significantly inhibit cell proliferation using the MTT assay.

Figure 2.25. Analysis of *U. tomentosa* extracts using HPLC

U. tomentosa was extracted in 70% ethanol or PBS followed by boiling over a period of one hour. HPLC was performed for the filtrate on a C18 column. For detection, a wavelength of 245 nm was used. The peaks were identified by comparison to the elution of standards for *U. tomentosa* under the same conditions on the same day: (1) uncarine D, (2) mitraphylline, (3) uncarine C, (4) isomitraphyline, (5) rhynophyline, and (6) uncarine E.

70% ethanol extract



PBS extract

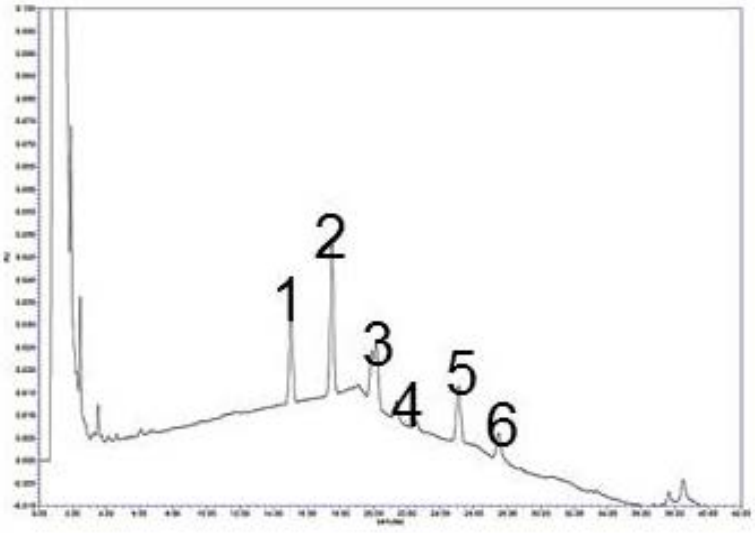


Figure 2.26. Analysis of *U. tomentosa* extracts fractionated by PVPP column chromatography using HPLC

Another experiment was performed by drying 70% ethanol extract and suspended in water. Then, *U. tomentosa* extracted with 70% ethanol was fractionated by PPVP column with increasing ethanol washes and analyzed through HPLC. The peaks were identified by comparison to the elution of standards for *U. tomentosa* under the same conditions on the same day: (1) uncarine D, (2) mitraphylline, (3) uncarine C, (4) isomitraphyline, (5) rhynophyline, and (6) uncarine E.

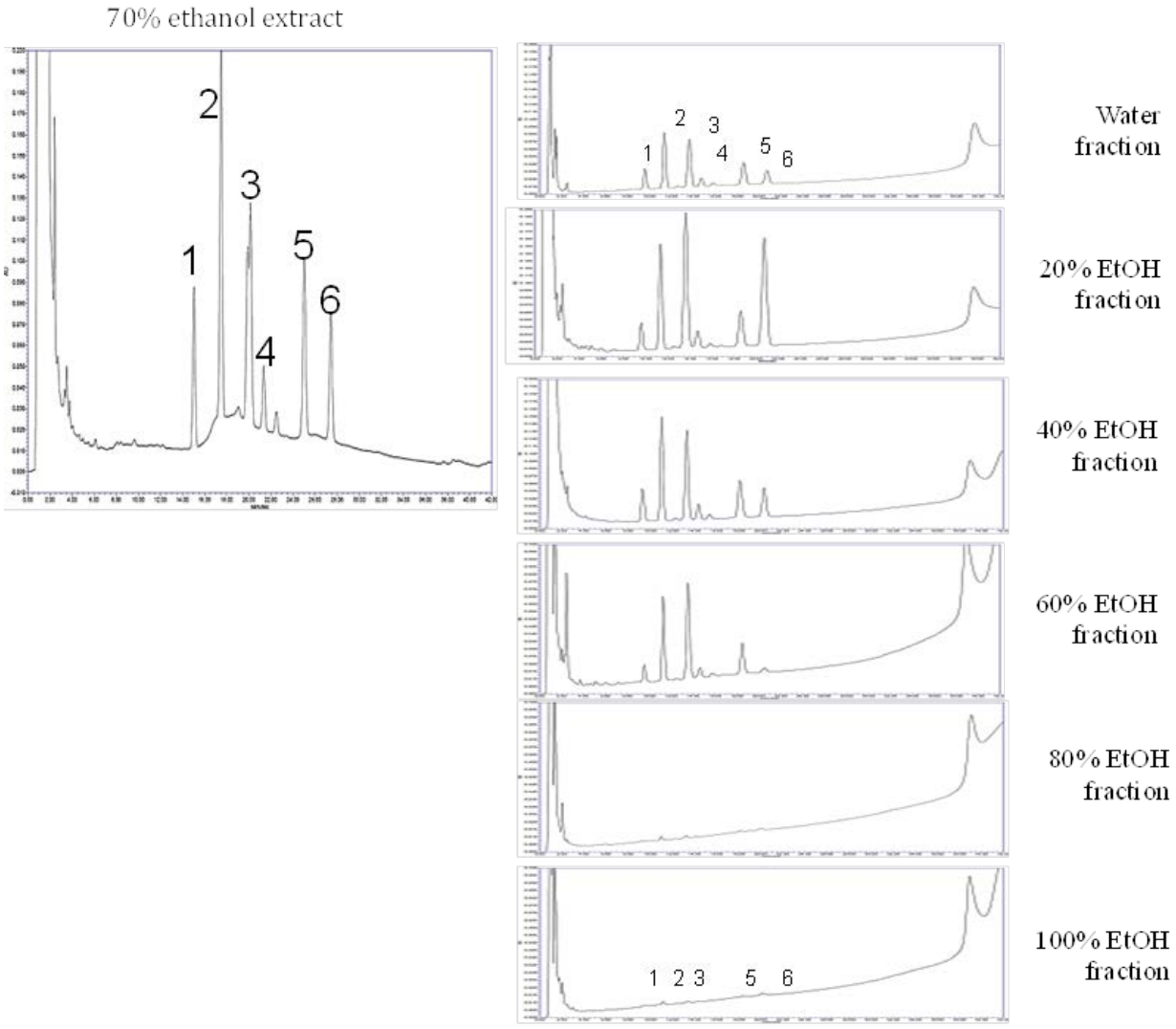
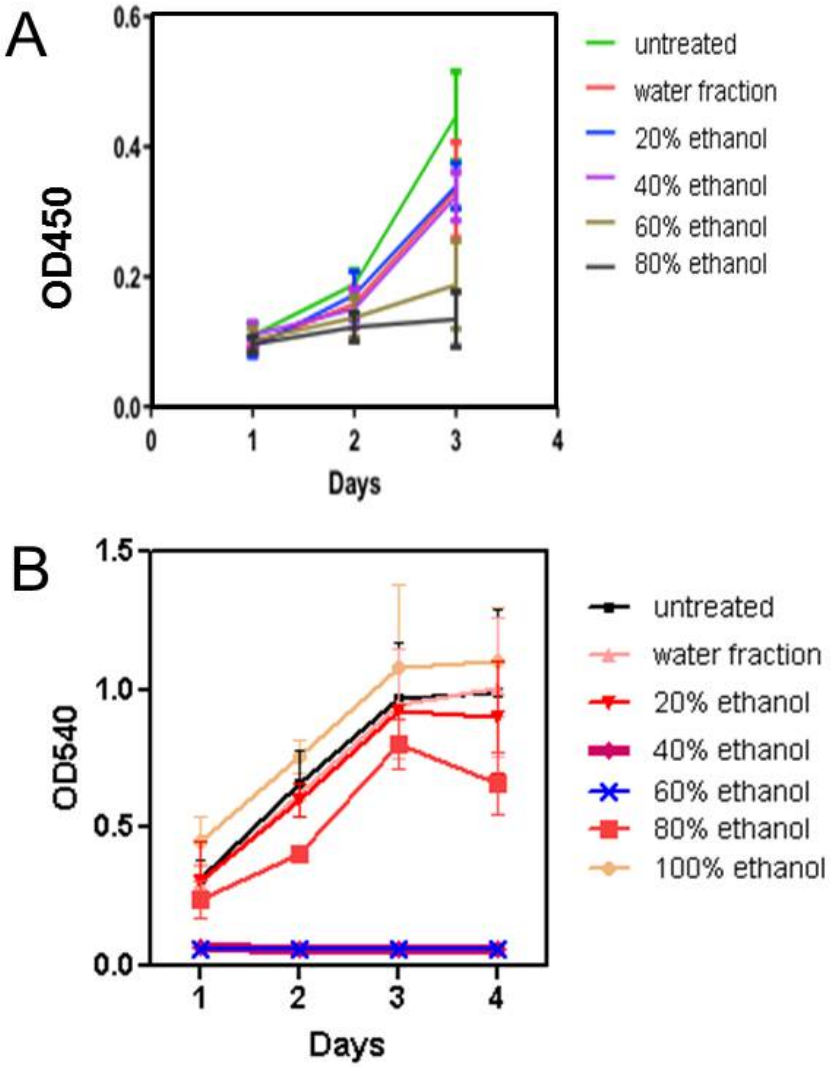

70% ethanol extract
1
2
3
4
5
6
Water fraction
20% EtOH fraction
40% EtOH fraction
60% EtOH fraction
80% EtOH fraction
100% EtOH fraction
1 2 3 5 6

Figure 2.27. Effect of *U. tomentosa* fractions on cell proliferation

MTT assay was performed *in vitro* experiments to evaluate the effect of various fractions of *U. tomentosa* on the growth of A. 4T1 cells or B. B16-BL6 cells. These fractions (water fraction, 20% ethanol fraction, 40% ethanol fraction, 60% ethanol fraction, and 80% ethanol fraction) were individually tested against 4T1 breast cancer cells or B16-BL6 cells at a dose (10 ul) over a period of 3 to 4 days. Then, treated cells were compared to the control (untreated cells suspended in media). Data were analyzed for three independent experiments and significant differences confirmed by ANOVA * ($P < 0.05$). Graph pad prism was used for analysis.


A
OD450
0.6
0.4
0.2
0.0
0
1
2
3
4
Days
untreated
water fraction
20% ethanol
40% ethanol
60% ethanol
80% ethanol
B
OD540
1.5
1.0
0.5
0.0
1
2
3
4
Days
untreated
water fraction
20% ethanol
40% ethanol
60% ethanol
80% ethanol
100% ethanol

2.4 Discussion

2.4.1 Identification of *Uncaria tomentosa*

U. tomentosa has a significant potential as a medicinal natural product because it contains several key active components, such as alkaloids, triterpenes, and polyphenols (Zhang *et al*., 2015, Navarro-Hoyos *et al*., 2015, 2018). There are approximately 50 compounds that have been separated and characterized from extracts of this species. It is noteworthy that a number of these components, about 30 chemical markers, appear to be unique to this species of plants (Navarro-Hoyos *et al*., 2015, 2018). Additionally, the importance of this plant is supported by the fact that several studies have already revealed a broad range of biological activities, like antioxidant, anti-inflammatory, immunomodulatory, antidiabetic, and anticancer properties (Heitzman *et al*., 2005; Zhang *et al*., 2015).

Although many studies have suggested that a group of alkaloids are responsible for the health effects of *U. tomentosa*, some studies imply that these health effects might be accredited to a synergistic interaction between multiple ingredients found in the extracts (Falkiewicz and Lukasiak, 2001). Furthermore, studies have shown that the polyphenol fractions have strong effects on many molecular mechanisms. For example, it has been demonstrated that polyphenols are able to modulate pathways connected to chronic inflammation and energy metabolism (Funes *et al*., 2010; Hernandez-Aguilera *et al*, 2013).

Navarro-Hoyos *et al*. (2018) have studied the polyphenolic constituents found in both the aqueous and ethanolic extracts of *U. tomentosa* bark and leaves. High amounts

of the total phenolics and proanthocyanidins were found in the leaves and were shown to contain antioxidant activity. Researchers identified 32 ingredients among the phenolics and proanthocyanidins, including hydroxycinnamic acids, hydroxybenzoic acids, flavan-3-ol monomers, procyanidin dimers and trimers, flavalignans–cinchonains, and propelargonidin dimers (Navarro-Hoyos, *et al*., 2015, 2018). One of the main bioactive compounds of *U. tomentosa* is the pentacyclic oxindole alkaloid, mitraphylline. It has been shown that mitraphylline has potent anticancer activity against human sarcoma and breast cancer cells (García Giménez *et al*., 2010). Treatment of mice with mitraphylline results in changes in cytokine modulation corresponding to a decrease in inflammatory signals (Rojas-Duran *et al*., 2012).

In the current research, HPLC analysis was performed on the *U. tomentosa* extracts to characterize the potential bioactive components. A high level of alkaloids was observed in ethanol extract, while a much lower level of the alkaloids was detected in PBS extract. This indicates that there is a similarity with other studies, showing that the *U. tomentosa* extracts contain a significant amount of alkaloids. Many of the peaks that were identified were shown to correspond to the known standard alkaloid components of *U. tomentosa*, including uncarine D, mitraphylline, uncarine C, isomitraphyline, rhynophyline, and uncarine E. The observation that our ethanol extracts had much stronger anti-proliferative effects than did the PBS extracts supports the idea that the alkaloid fraction contributes to the biological activity of *U. tomentosa*.

The synergistic action of alkaloids in combination with non-polar compounds is proposed to lead to the antitumor activities of *U. tomentosa* extracts (Pilarski *et al*., 2010). The compounds, which appear to be important, include uncarine F,

isomitraphylline, mitraphylline, isopteropodine, and pteropodine. These compounds also have been shown to display anti-apoptotic activities against lymphoblastic leukemia cells (Bors *et al*., 2012). Furthermore, studies have proved that extracts of *U. tomentosa* have potent antioxidant properties. It has been shown that extracts of the plant were able to scavenge oxygen-derived radicals like hydroxyl radicals and superoxide anions, as well as avert lipid membrane oxidation (Goncalves *et al*., 2005). Collectively, these data provide compelling evidence that the *U. tomentosa* extract contains strong anticancer bioactive compounds that require further isolation and additional characterization.

2.4.2 Anti-proliferation activity of *U. tomentosa* extracts

The changes in proliferation rates for B16-BL6 cells and 4T1 cells treated with various doses of *U. tomentosa* extracts were determined using the MTT assay. The MTT assay converts yellow tetrazolium MTT to purple formazan crystals based on the activity of NAD(P)H-dependent cellular oxidoreductase enzymes. Therefore, the amount of formazan that formed reflected the number of metabolically active viable cells present in the culture (van Meerloo *et al*., 2011). In the present research, the growth of 4T1 cell cultures was significantly inhibited following treatment with *U. tomentosa* extracted with 70% ethanol at concentrations of 40 and 200 μg/ml (the medium and high doses). Similarly, the growth of B16-BL6 cell cultures was greatly inhibited following treatment with 40 and 200 mg/ml of the ethanol extracts of *U. tomentosa*. In both cell lines, there was no significant difference in cell number until

at least day 2 or 3 of treatment. This supports the idea that treatment with the *U. tomentosa* extracts does not simply induce necrosis in the cell cultures but rather induces a process that requires a few days (or a few cell divisions) for the differences to be detected.

Treatment of 4T1 and B16-BL6 cells with *U. tomentosa* extracted with PBS at 200 μg/ml (high dose) was able to partially inhibit cell growth although not to the same extent as treatment with the ethanol extract. Treatment of B16-BL6 cells with 200 μg/ml of the PBS extract of *U. tomentosa* almost completely inhibited cell growth while similar treatment of the 4T1 cells only blocked cell growth by approximately 50%. Treatment of both B16-BL6 and 4T1 cells with 40 μg/ml of the PBS extract of *U. tomentosa* did not significantly inhibit cell growth. The findings from this research indicated that the B16-BL6 cell line was more sensitive to *U. tomentosa* treatment, while the 4T1 cell line was more resistant. In addition, treatment with the ethanol extract of *U. tomentosa* was more effective as an anti-cancer agent than the PBS extract of *U. tomentosa in vitro*.

This result is similar to other studies where *U. tomentosa* extracted with water (C-Med-100) was tested against two human leukemic cell lines (K562 and HL60) and one human EBV-transformed B lymphoma cell line (Raji) using a clonogenic assay. The results showed that the treatment significantly inhibited HL60 and Raji cells, but K562 was more resistant to the *U. tomentosa* treatment (Sheng *et al*., 1998). Other results from our laboratory (Alfarteesh MSc thesis 2014; Aljehani MSc thesis 2015) have also shown that treatment of a number of human cancer and non-malignant cell lines, including MCF-7, MDA-MBA-231, HBL-100 breast cells, HeLa cervical cancer

cells, HEK293 embryonic kidney cells, and HSG epithelial cells with *U. tomentosa* extracts can significantly inhibit cell growth. Further, these data all showed that the ethanol extract of *U. tomentosa* had a stronger inhibitory effect on cell proliferation than the PBS extract. These results are consistent with our finding that *U. tomentosa* has the ability to inhibit the proliferation of both the B16-BL6 and 4T1 murine cancer cell lines.

The effect of various ethanol fractions derived from the ethanol extract of *U. tomentosa* was tested for antiproliferative effects on B16-BL6 and 4T1 cells. These fractions were shown to have different levels of the alkaloid components using HPLC. The 40% ethanol fraction was shown to have the highest amount of the total alkaloids, which included uncarine D, mitraphylline, uncarine C, isomitraphyline, rhynophyline, and uncarine E. The 20% fraction had lower levels of total alkaloids but had a higher relative percentage of rhynophyline, and uncarine E. The 60% fraction had a higher relative percentage of uncarine D and mitraphylline. Both the water and 80% ethanol fractions had very low levels of total alkaloids. Treatment of both B16-BL6 and 4T1 cells with the 60% ethanol fraction promoted a significant inhibition of cell growth. However, treatment with the 80% ethanol fraction had the most potent effect on 4T1 cell growth but still had a strong effect on B16-BL6 cells. Treatment with the 40% ethanol fraction had a relatively small effect on cell proliferation. This suggests that the alkaloids present in *U. tomentosa* are not sufficient to promote the changes in cell proliferation; the alkaloids may be required in combination with other components that were not detected by the HPLC analysis. Further, B16-BL6 and 4T1 cells did not show identical responses to the different *U. tomentosa* fractions suggesting the potential for cell-specific differences in

response to components of *U. tomentosa*. However, it should be noted that the purified, lyophilized fractions had been stored for several months between testing on the B16-BL6 and 4T1 cells and some potentially bioactive components might have been degraded during that period which could explain the different results.

2.4.3 Anti-proliferation activity of *U. tomentosa* extracts using Ki-67 protein expression

The expression of the Ki-67 antigen is linked to tumour cell proliferation. Ki-67 staining is commonly used in pathology labs and cancer diagnosis as a proliferation marker (Li *et al*., 2015). In this research, Ki-67 staining was performed to test for the effect of treatment with *U. tomentosa* on the proliferation of B16-BL6 and 4T1 cells. Both B16-BL6 and 4T1 cells treated with the ethanol extract of *U. tomentosa* showed a dose-dependent reduction in Ki-67 staining. Cells treated with the low dose of *U. tomentosa* (4 μg/ml) for 2 days showed a small reduction in Ki-67 staining while cells treated with the high dose of the ethanol extract of *U. tomentosa* (200 μg/ml) showed a high reduction of Ki-67 staining. Treatment of both B16-BL6 and 4T1 cells with the PBS extract of *U. tomentosa* showed a lower reduction of Ki-67 staining compared to the ethanol extract. Treatment with the low dose of the PBS extract of *U. tomentosa* for 2 days did not significantly inhibit Ki-67 staining while treatment with the high dose for 2 days was able to partially inhibit Ki-67 staining. This confirms the relative effectiveness of the ethanol extract of *U. tomentosa* against cancer cells.

Similarly, Zhu *et al*. (2014) showed that Artemisinin, a component isolated from a Chinese medicinal plant, has strong anticancer activities against several types

of human tumours. Researchers examined the effect of treating neuroblastoma cells with either DMSO (control) or 300 μM artemisinin over a period of 72 hours. Then, cells were stained with an antibody against Ki-67 and the percentage of Ki-67-positive cells was measured using a microscope. The results have shown that treatment with the plant extract significantly inhibited the proliferation of neuroblastoma cells as measured by a decrease in Ki-67 staining (Zhu *et al*., 2014). Collectively, the results from Zhu and colleagues (2014) support the idea that extracts from medical plants are effectively able to inhibit Ki-67 staining in different cancer cell lines.

2.4.4 DNA fragmentation caused by treatment with the *U. tomentosa* extracts

A TUNEL assay was used to detect DNA fragmentation that is considered an essential feature of apoptotic cell death. The TUNEL assay depends on the enzyme terminal deoxynucleotidyl transferase (TdT), which adds labelled deoxynucleotides to the 3'-hydroxyl terminus of the free ends of DNA that are significantly increased when the DNA is fragmented by caspase or nuclease activity (Nagata, 2000). In the present research, the TUNEL technique was performed to investigate whether treatment with the *U. tomentosa* extract induces apoptosis in the B16-BL6 and 4T1 cell lines. Treatment of both B16-BL6 and 4T1 cells with the high dose of the ethanol extract of *U. tomentosa* (200 μg/ml) showed the presence of apoptotic cells after 24 h which were significantly increased at longer treatment times. Treatment of cells with both the low (4 mg/ml) and medium (40 mg/ml) doses of the ethanol extract was shown to promote apoptosis in some cells at longer treatment times.

On the other hand, treatment with high doses of the PBS extract of *U. tomentosa* for 2 or 3 days showed a lower level of cell death in both B16-BL6 and 4T1 cell lines when compared to the ethanol extract. However, no apoptotic cells were detected when the cells were treated with the low doses of the PBS extract. This indicates that the treatment with the ethanol extract was more effective at inducing apoptosis than the treatment with the PBS extract.

Similar studies from our laboratory have examined the effectiveness of *U. tomentosa* on the growth and survival of B16-BL6 cells. Results indicated that *U. tomentosa* extracted with ethanol was able to increase DNA fragmentation in the B16-BL6 cells using the TUNEL assay (Alfarteesh, 2014). An *in vitro* study has previously used the *U. tomentosa* as a treatment against MCF 7 cancer cells in *vitro*. The study showed that treatment with *U. tomentosa* extracted with ethanol significantly increased the apoptotic cells in MCF 7 cells. However, treatment with *U. tomentosa* extracted with PBS demonstrated a lower effect (Aljehani, 2015). These results are consistent with our finding that *U. tomentosa* extracted with ethanol clearly increased DNA fragmentation in both cell lines, especially when treated at the highest dose. It is also interesting that in all of these studies, treatment of the cells with *U. tomentosa* required at least 1 or 2 days before there were a significant number of apoptotic cells. This is consistent with the observation that the differences in cell culture growth, as measured by MTT assays, required at least 3 days to be significant. This further suggests that the mechanism by which treatment with *U. tomentosa* extracts inhibits cell growth is by the induction of apoptosis.

2.4.5 Apoptosis induction by *U. tomentosa* extract detected using flow cytometry

In order to confirm that *U. tomentosa* extract induces apoptosis in 4T1 cancer cells, flow cytometry was performed using propidium iodide staining. In this research, 4T1 cells were treated with various doses of *U. tomentosa* extracts over two different time periods (24 hours and 48 hours). At the highest dose (200 μg/ml), the results showed that the *U. tomentosa* extracted with ethanol induced a significant accumulation of cells in the sub-G1 peak after 48 hours, which increased to over 74% of apoptotic cells. However, the treatment with *U. tomentosa* extracted with PBS was less active and recorded around 13% of the apoptotic cells after 48 hours. Treatment of cells with the low dose of *U. tomentosa* extracts did not significantly increase the percentage of apoptotic cells in the pre-G1 peak compared to controls. It is interesting to note that treatment with *U. tomentosa* extracts did not affect the cell cycle distribution of cells between the G1, S, and G2/M phases of the cell cycle. This suggests that treatment with *U. tomentosa* extracts did not inhibit cell culture growth by inhibiting cell proliferation, but rather promoted its effects primarily by inducing apoptosis.

Similar data was obtained through other studies that used the *U. tomentosa* extract against cancer cells *in vitro*. Rinner *et al*. (2009) showed that the alkaloids isopterpodine and pteropodine isolated from *U. tomentosa* had a remarkable pro-apoptotic effect on Medullary thyroid carcinoma (MTC). In this study, flow cytometry was used to examine the effect of the plant extracts on MTC cell cycle distribution.

Researchers concluded that the number of cells in the sub-G1 peak was increased following treatment with the *U. tomentosa* extract for 48 h (Rinner *et al*., 2009).

In another study from our laboratory, the effect of *U. tomentosa* extract on B16-BL6 cells was investigated using flow cytometry. Briefly, cells were treated with multiple doses of *U. tomentosa* extracted with ethanol or PBS and then incubated with PI staining solution and analyzed using a Beckman Coulter LS600 flow cytometer. The study found that treatment with *U. tomentosa* extracted with ethanol caused a great increase in sub-G1 cells that increased to over 70% of the cells after 3 days of treatment (Alfarteesh, 2014). Taken together, these results are strongly consistent with our finding that treatment with *U. tomentosa* extract significantly induced apoptosis in 4T1 cancer cells.

Chapter 3. *Uncaria tomentosa* extracts were examined *in vivo* to measure the growth and survival of B16-BL6 cancer cells

3.1 Introduction

U. tomentosa has a long history of use in traditional medicine for over 2000 years by indigenous cultures in South America (Keplinger *et al*., 1999). Several studies have shown that this plant has immunostimulant, anti-inflammatory, anti-oxidant, and anti-cancer properties (Heitzman *et al*., 2005; Zhang *et al*., 2015). However, there is relatively little *in vivo* data to show how *U. tomentosa* might inhibit tumour growth. Various studies have shown *U. tomentosa* can affect both the response of the immune system and the growth of the cancer cells themselves. For example, the antitumor and antioxidant activities of *U. tomentosa* hydroalcoholic extract were examined *in vivo*. Researchers found that animals treated with *U. tomentosa* extracts showed a reduction in the tumor growth of Walker-256 cancer cells and a reduction in the activity of aspartate aminotransferase (AST). In addition, tumoural activity of catalase (CAT) was reduced in tumour tissue, while in the liver CAT activity was increased. In the liver and in the tumour, SOD activity was significantly decreased (Dreifuss *et al*., 2010).

Another study showed that a pentacyclic oxindole alkaloid extract from *U. tomentosa* bark had the ability to treat T-helper 1 immune-mediated disorders without showing immunotoxicity or cytotoxic on murine splenocytes (Domingues *et al*., 2011). Since tumour growth is usually considered to be a balance between immune system escape and tumour cell growth, the experiments were designed to show the impact of *U.*

tomentosa on immune responses against the tumour and the effects of *U. tomentosa* directly on the tumour cells.

In this study, an isogenic tumour transplantation model was performed to determine the anti-cancer activities of *U. tomentosa* extracts in C57BL/6 mice. This animal model was selected because the B16-BL6 cells are syngeneic with the C57BL/6 mice allowing experiments to be performed in an immuno-competent host. Tumours were formed by subcutaneous injection of B16-BL6 cancer cell in the right flank of mice. Two types of injections were used for treatment with *U. tomentosa* extracts, including intraperitoneal (IP) and intratumour (IT). Subsequently, animals were sacrificed and tumour tissues were collected in order to measure tumour size and weight.

Histochemical analysis of the B16-BL6 tumours was also performed to examine the anti-cancer activities of *U. tomentosa* extracts on cell growth (Ki67 proliferation marker), cell death (TUNEL staining), and angiogenesis (Factor VIII). As well, alterations in immune cell infiltration caused by *U. tomentosa* extracts were evaluated using immunohistochemistry for a number of immune cell markers including CD3, CD4, CD8, ICAM - 1, E - selectin, PECAM -1, Integrin αIIb, CD19, and CD45. Together, these animal experiments were performed in order to evaluate the suitable composition, significant medicinal activity, as well as the safety and risk of the plant extracts.

3.2 Materials and Methods

3.2.1 *Uncaria tomentosa* extracts

Uncaria tomentosa was obtained from Rosario Rojas (Lima, Peru) as a dried powder prepared from the bark or was purchased as a natural product supplement (Cat's Claw extract, Now Foods, Bloomington, IL. code 84618). The dried plant product was extracted with either PBS, pH7.4, or 70% ethanol and the soluble fraction used in the current research. For the preparation of the ethanolic extracts, 20 g of *U. tomentosa* powder was suspended in 200 ml 70% ethanol and heated to a slow boil with refluxing for 1 hour. For the preparation of PBS extracts, 20 g of *U. tomentosa* powder was suspended in 180 ml water and 20 ml 10xPBS buffer and heated to a slow boil with refluxing for 1 hour. The mixture was allowed to cool and the bark debris was removed by centrifugation of the suspension at 2000 x g for 10 min. The supernatant was removed and filtered using a 0.22 μm syringe filter and then the extract was stored in aliquots at -80 °C. For both the animal experiments and the chemical fractionation experiments, the *U. tomentosa* extract supernatants were frozen and dried by lyophilization into a powder. The powder was weighed and resuspended in water immediately prior to use for the experiments.

3.2.2 Animal Experiments

The B16-BL6 melanoma/C57bl/6 isogenic tumour transplantation model was performed to determine the anticancer activities of *U. tomentosa* extracts in mice. A total of 50 syngeneic male C57BL/6 mice (8 weeks old) were used for two experiments

(Charles River, Pointe-Claire, QB, Canada). In particular, 25 male mice were used for each experiment (experiment 1 and experiment 2) over a period of 21-24 days or when the tumor size reached the endpoint. Animals were housed in disposable plastic shoebox cages with 5 individuals per cage and acclimated in their cages over a period of 3 weeks. Each cage had a high density and actively vented system with a complete cage sanitization weekly during the experiment period. In addition, the room was maintained on a 12:12-h light: dark cycle; average room temperature was between 20°C and 21°C. The mice received a standard pellet diet and water providing *ad libitum*.

Tumours were induced by injection of B16-BL6 cancer cells in the right flank of mice subcutaneously. The mice were injected with 2×10^5 cells, suspended in 100 μl of PBS. All of the animals were injected with tumour cells and were then randomly assigned into one of five equal groups (5 individuals in each group). The first group was the vehicle control (mice were received just PBS to determine whether the vehicle alone causes any effects), the second group consisted of mice that were treated with 100 μl of 200 μg/ml ethanol extract of *U. tomentosa* using intraperitoneal injection, the third group of mice were treated with 100 μl of 200 μg/ml ethanol extract of *U. tomentosa* using intratumour injection, the fourth group of mice were treated with 100 μl of 200 μg/ml PBS extract of *U. tomentosa* using intraperitoneal injection, and the fifth group consisted of mice that had been treated with 100 μl of 200 μg/ml PBS extract of *U. tomentosa* using intratumour injection. For animals receiving intraperitoneal injections, the first injection was performed 3 days after injection of the tumour cells and then performed two times per week for 2 weeks (4 injections in total). For animals receiving intratumour injections, the animals in experiment 1 received injections only after the tumours were palpable (day

11) while the animals in experiment 2 received injections beginning 3 days after injection of the tumour cells. Therefore, the animals in experiment 1 received two intratumour injections while the animals in experiment 2 received 4 intratumour injections.

Physiological and behavioral data were assessed daily during the experiment, including general appearance, foraging or feeding, muscle tone, body tone, sleeping, and aggressive or defensive behavior. Also, animals were monitored for any signs of morbidity or mortality and regular weight measurements were recorded every two days by individually weighing each animal. At experimental endpoints, mice were euthanized via CO_2 exposure, in compliance with the Canadian Council for Animal Care recommendations.

Post treatment, all tumours were fully collected from the mice and their diameter and weight were measured. For additional analysis, the tumours were fixed in EFA (720 ml ethanol (100%), 180 ml distilled water, 50 ml glacial acetic acid and 50 ml formaldehyde). Tumour weight and size data were tested by two-way analysis of variance (ANOVA) and post-hoc Tukey HSD tests (SPSS for windows). Differences were considered significant at P values of 0.05 or less. All animal experiments were approved by the Laurentian University Animal Care Committee, protocol 2013-12-01, in accordance with guidelines established by the Canadian Council for Animal Care.

3.2.3 Histochemical analysis of tissue sections

Histochemical analysis of the isolated tumours was performed in the current research. Several immune, cell growth, and cell death markers were used to examine the

impact of treatment on B16-BL6 tumours. Specifically, 29 samples were sent to University of Ottawa, Department of Pathology and Laboratory Medicine, Histology Core Facility (HCF) for processing, embedding, and sectioning. These 29 samples included 10 tumours treated with just PBS and used as vehicle control, 10 tumours treated with ethanol extract of *U. tomentosa* using intraperitoneal (IP) injection, and 9 tumours treated with PBS extract of *U. tomentosa* using intraperitoneal (IP) injection.

Immunohistochemistry staining was performed in the lab for Ki-67 and factor VIII staining using ABC immunohistochemistry kits (Santa Cruz Biotech). The slides were deparaffinized by submerging in the first container of xylene for 2 min, the second container of xylene for 2 min, the third container of xylene for 2 min, and then rehydrated using an ethanol series of 100% ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 3 min, 40% ethanol for 3 min, 20% ethanol for 3 min, and distilled water for 1 min. Then, slides were submerged in sodium citrate buffer (10 nM sodium citrate, 0.05% Tween 20, pH 6.0) at 95°C for 30 min for antigen retrieval. Blocking serum (Santa Cruz Biotech) was incubated with the slides for 1 hour and then the slides were incubated with anti-Ki-67 (Santa Cruz Biotech) or anti-Factor VIII (Santa Cruz Biotech) antibodies at a titre of 1:100 in binding buffer in a humidified chamber overnight. The following day, the slides of tumours were washed 3 times using PBS, pH 7.4, for 5 min each. Biotinylated secondary antibody was incubated with the sections for 30 min at room temperature. Slides were washed 3 times with PBS for 5 min. The AB enzyme reagent was used to treat the sections for 30 min and then slides were washed 3 times using PBS. Peroxidase Substrate was added for 20 min and then samples were washed with distilled water for 5 min. Samples were counterstained by incubating in Haematoxyline for 6 min. Slides were

rinsed with lukewarm running water and then 1% acid alcohol solution (70% ethanol and 1% HCl) was used (3 dips). The samples were rinsed with tap water and then with saturated lithium carbonate (approximately 0.2 M lithium carbonate) for 3 dips to develop the haematoxyline stain. The slides were then rinsed with tap water for 10 min. The stained sections were then dehydrated in an ethanol series of 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, and then placed in the first container of xylene for 2 min, the second container of xylene for 2 min, and the third container of xylene for 2 min. Coverslips were mounted on glass slides in Permount and then analyzed using an Olympus IX3 microscope.

For immune markers, the slides were deparaffinized and hydrated by submerging in the first container of xylene for 2 min, the second container of xylene for 2 min, the third container of xylene for 2 min, and then in the ethanol series composed of 100% ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 3 min, 40% ethanol for 3 min, 20% ethanol for 3 min, and distilled water for 1 min. The slides were also submerged in sodium citrate buffer (10 nM sodium citrate, 0.05% Tween 20, pH 6.0) at 95°C for 30 min for antigen retrieval. Then, the slides were incubated with the blocking serum for 1 hour. The slides were then incubated with a combination of 3 fluorescently-tagged primary antibodies labeled with Alexa-488, Alexa-546, or Alexa-633. In the first section on the slide, three control antibodies were combined (normal rat IgG2a-Alexa-633, normal mouse IgG2b-Alexa-546, normal mouse IgG1-Alexa-488) in a total of 90 μl (30 μl each) suspended in 1.5 ml of block buffer. Each slide section was stained by incubation with 50 μl of this solution overnight at room temperature in a humidified chamber. In the second section of the slide, cells were stained with antibodies that

recognize T cells. Anti-CD3-Alexa-546, anti-CD4-Alexa-488 and anti-CD8-Alexa-633 antibodies were combined in a total of 90 μl (30 μl each) and suspended in 1.5 ml of block buffer. The sample was stained with 50 μl of this solution overnight at room temperature in a humidified chamber. In a third tissue section, the slides were incubated with antibodies against the leukocyte markers, anti-Integrin αIIB-Alexa-633, anti-CD19 and anti-CD45 were combined in a total of 90 μl (30 μl each) and suspended in 1.5 ml of block buffer. The sample was stained with 50 μl of this solution overnight at room temperature.

Markers corresponding to endothelial cells and to activated endothelial cells were also used. In the fourth tissue section, anti-ICAM-1-Alexa-633, anti-E-selectin-Alexa-546, and anti-PECAM-1-Alexa-488 were combined in a total of 90 μl (30 μl each) and suspended in 1.5 ml of block buffer. The sample was stained with 50 μl of this solution overnight at room temperature. All slides were analyzed using an Olympus IX3 fluorescence microscope through different channels (green, red, and far red) with control experiments showing minimal overlap between the channels.

3.2.4 TUNEL staining of histochemical sections

For TUNEL staining of histochemical sections, the slides were deparaffinized and hydrated by submerging in the first container of xylene for 2 min, the second container of xylene for 2 min, the third container of xylene for 2 min, 100% ethanol for 5 min, 95% ethanol for 5 min, 70% ethanol for 3 min, 40% ethanol for 3 min, 20% ethanol for 3 min, and distilled water for 1 min. Following that, slides were submerged in 10 nM sodium

citrate buffer and treated in the microwave oven for 1 min. Samples were washed three times with PBS, pH 7.4. The slides were then immersed in a block buffer containing 10 ml of 100 mM Tris-HCl, pH 7.5, 20% FBS, and 3% BSA for 30 minutes. Samples were washed twice with PBS, pH 7.4. Each section was stained by incubation with 50 μl of TUNEL reaction mixture (Roche) for 60 min at 37°C in a humidified chamber in the dark. Images were captured using an Olympus IX3 fluorescence microscope for analysis.

3.3 Results

3.3.1 Examining the anticancer activities and protective effects of *U. tomentosa* extracts on male C57BL/6 mice by measuring body weight, tumour weight, and tumour size

A total of 50 male C57BL/6 mice were used for two experiments. Animals were divided randomly into five equal groups (5 mice in each group) in each experiment. Tumours were initiated by injection of approximately 10^5 B16-BL6 cells in the right flank of mice subcutaneously. For these experiments, *U. tomentosa* was extracted into 70% ethanol or PBS, pH 7.4, and the supernatant lyophilized to produce a powder. This powder was then resuspended in PBS, pH 7.4 for injection to avoid injecting ethanol into the mice. These *U. tomentosa* extracts resuspended in PBS, pH 7.4, were injected into mice intraperitoneally or directly into the tumor. In the first experiment, the mice received a total of 4 intraperitoneal injections of the extracts; the first injection was 3 days after injection of the tumour cells and then injections were made twice a week. However, in experiment 1 the mice received only 2 injections into the tumour starting only during week 2 (Tables 3.1 – 3.3). This was done to allow time for palpable tumours to form before injections. In the second experiment, all of the treated groups, both intraperitoneal injection and injection into the tumour were performed 4 times over a period of 14 days. The injections into the tumour for week two, before there was a palpable tumour, were made into the approximate site of tumour injection (Tables 3.4 – 3.6). Animal weight was measured and the tumour size was estimated using calipers every second day. The animals were sacrificed on around day 18 and all tumours were excised and collected. The weights of each mouse and the weight and average diameter of

each tumour were recorded. The tumours were then fixed by incubation in EFA (ethanol formalin, acetic acid fixative) for histochemical analysis.

For statistical analysis, two-way analysis of variance was applied by combining the results for both experiments (Tables 3.7 – 3.9). In terms of body weight, no significant differences between the five groups were found (Figure 3.28). Also, no significant effects of experiment or group by experiment interaction on body weight were recorded. Significant effects of group ($P < 0.001$) on tumour weight were found in mice treated with *U. tomentosa* extracted with ethanol or PBS (Table 3.8, Figure 3.29). Furthermore, there was a significant group by experiment interaction ($P < 0.05$). However, no significant effects of experiment on tumour weight were found. Tukey HSD tests were used for additional analysis. In these tests, significant differences between each of the four treated groups and the vehicle control ($P < 0.05$) were recorded. However, no significant differences between the four treated groups were shown.

Additionally, tumour size was less affected than tumour weight in mice treated with *U. tomentosa* extracted with ethanol or PBS. In particular, significant effects of group ($P < 0.01$) on tumour size were found using two-way ANOVA (Table 3.9, Figure 3.30), but no significant effects of experiment or group by experiment interaction on tumour size were shown. In Tukey HSD tests, results indicated no significant differences among the four treated groups. However, the vehicle control was significantly different from each of the four groups ($P < 0.05$).

A strong relationship between tumour size and tumour weight ($P < 0.001$) was recorded (Table 3.10, Figure 3.31). The tumour size is strongly correlated to the tumour

weight ($r = 0.85$, $P < 0.001$). Furthermore, results indicated that all treated groups decreased tumour weight and tumour size in the same manner. Specifically, no significant major effects or interactions between the levels of treatment (injection type: IP vs IT and extract type: ETOH vs PBS) were found using two-way ANOVA.

Table 3.1 The weight of the mice at sacrifice for experiment 1.

Weight of individual mice (g)				
Groups	N	Mean	Standard deviation	Standard Error
Control	5	27.04	0.79	0.35
ETOH IP	5	26.04	2.09	0.93
PBS IP	5	25.96	1.63	0.73
PBS IT	5	25.66	2.46	1.10
ETOH IT	4	25.83	2.21	1.11

Table 3.2 The weight of the B16-BL6 tumour at sacrifice for experiment 1.

Weight of tumour (g)				
Groups	N	Mean	Standard deviation	Standard Error
Control	5	2.62	0.98	0.44
ETOH IP	5	1.66	0.29	0.13
PBS IP	5	0.98	0.51	0.23
PBS IT	5	1.50	1.02	0.46
ETOH IT	4	1.68	0.62	0.31

Table 3.3 The average diameter of the B16-BL6 tumour at sacrifice for experiment 1.

Tumour diameter (cm)				
Groups	N	Mean	Standard deviation	Standard Error
Control	5	2.98	0.77	0.34
ETOH IP	5	1.96	0.57	0.25
PBS IP	5	1.74	0.21	0.09
PBS IT	5	1.66	0.64	0.29
ETOH IT	4	2.23	0.29	0.14

Table 3.4 The weight of the mice at sacrifice for experiment 2.

Weight of individual mice (g)				
Groups	N	Mean	Standard deviation	Standard Error
Control	5	26.94	2.51	1.12
ETOH IP	5	24.48	1.94	0.87
PBS IP	4	26.68	2.45	1.22
PBS IT	3	24.43	1.53	0.88
ETOH IT	4	22.73	1.11	0.55

Table 3.5 The weight of the B16-BL6 tumour at sacrifice for experiment 2.

Weight of tumour (g)				
Groups	N	Mean	Standard deviation	Standard Error
Control	5	3.10	0.22	0.10
ETOH IP	5	0.72	0.26	0.12
PBS IP	4	1.53	0.96	0.48
PBS IT	3	2.13	0.55	0.32
ETOH IT	4	0.88	0.32	0.16

Table 3.6 The average diameter of the B16-BL6 tumour at sacrifice for experiment 2.

Tumour diameter (cm)				
Groups	N	Mean	Standard deviation	Standard Error
Control	5	2.74	0.61	0.27
ETOH IP	5	1.54	0.57	0.26
PBS IP	4	1.68	0.71	0.35
PBS IT	3	2.17	0.32	0.19
ETOH IT	4	1.50	0.48	0.24

Table 3.7 The weight of the mice at sacrifice for combined experiments 1 and 2.

Weight of individual mice (g)						
Group	Count	Mean	Minimum	Maximum	Standard Deviation	Standard Error
Control	10	27.0	22.6	28.8	1.8	0.6
ETOH IP	10	25.3	22.9	28.6	2.1	0.7
PBS IP	9	26.3	24.0	29.1	1.9	0.6
PBS IT	8	25.2	22.7	29.5	2.1	0.8
ETOH IT	8	24.3	21.1	28.2	2.3	0.8

Table 3.8 The weight of the B16-BL6 tumours at sacrifice for combined experiments 1 and 2.

Weight of tumour (g)						
Groups	Count	Mean	Minimum	Maximum	Standard Deviation	Standard Error
Control	10	2.9	1.5	4.2	0.7	0.2
ETOH IP	10	1.2***	0.3	2.0	0.6	0.2
PBS IP	9	1.2***	0.3	2.5	0.7	0.2
PBS IT	8	1.7**	0.3	2.6	0.9	0.3
ETOH IT	8	1.3***	0.4	2.6	0.6	0.2

Table 3.9 The average diameter of the B16-BL6 tumours at sacrifice for combined experiments 1 and 2.

Tumour diameter (cm)						
Groups	Count	Mean	Minimum	Maximum	Standard Deviation	Standard Error
Control	10	2.9	1.8	4.3	0.7	0.2
ETOH IP	10	1.8**	0.8	2.5	0.6	0.2
PBS IP	9	1.7**	0.8	2.3	0.5	0.2
PBS IT	8	1.9**	0.6	2.4	0.6	0.2
ETOH IT	8	1.9**	0.8	2.6	0.5	0.2

Significantly different from the control (**P < 0.01 and ***P < 0.001).

Table 3.10 Regression statistics for tumour size (cm) as a function of tumour weight (g) (N=45).

Model Summary

Model	R	R Square	Adjusted R square	Standard Error of the Estimate
1	0.852[a]	0.726	0.720	0.375

ANOVA[b]

Model	Sum of Squares	Df	Mean Square	F	Sig
1 Regression	16.060	1	16.060	114.177	P <.001[a]
Residual	6.048	43	0.141		
Total	22.108	44			

Model	Unstandardized Coefficients		Standardized Coefficients	T	Sig.
	B	Std. Error	Beta		
Constant	0.958	0.115		8.366	P <0.001
Tumour weight	0.636	0.060	0.852	10.685	P <0.001

a. Predictors: (constant). tweight

b. Dependent Variable: tsize

Figure 3.28 Body weight was evaluated following the use of *U. tomentosa* treatment

In this research, all animals were injected with B16-BL6 tumour cells and then received intraperitoneal (IP) injections or injections directly into the tumour (IT) with *U. tomentosa* extracted with 70% ethanol (ETOH) or PBS, pH 7.4, (PBS) in accordance with guidelines provided by the Animal Care Committee at Laurentian University. At the endpoint of the experiment, the mice were euthanized by CO_2 asphyxiation and tumours were surgically removed to determine the tumour diameters, weights, and body weights. The average of the total of body weight is shown in grams for the animals in each of the five groups ($\pm$ standard error, N= 9-10). Results demonstrated that there were no significant differences in total body weight between the five groups.

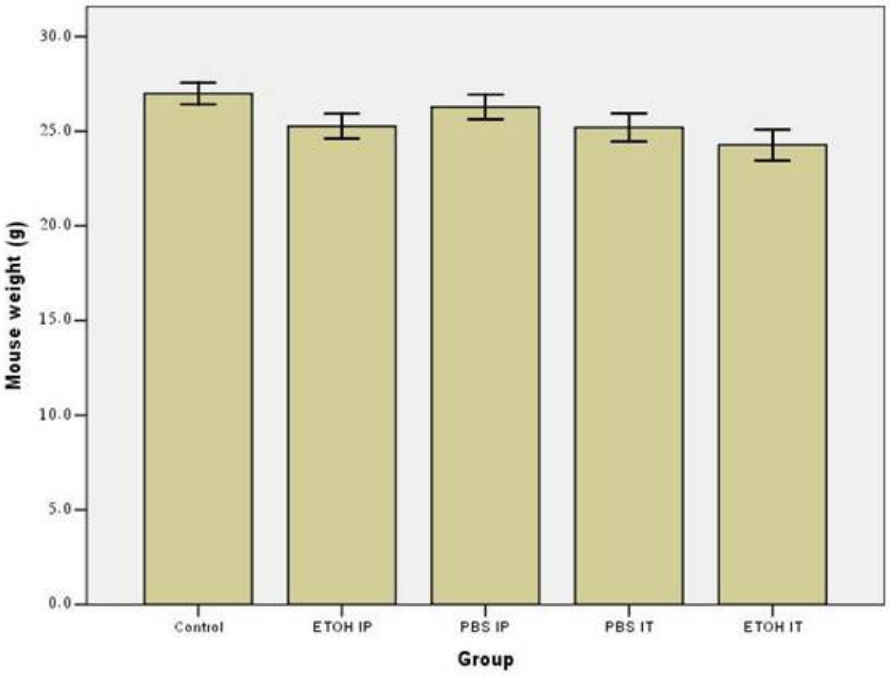

Mouse weight (g)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
ETOH IP
PBS IP
PBS IT
ETOH IT
Group

Figure 3.29 Tumour weight was evaluated following the use of *U. tomentosa* treatment

In this research, all animals were injected with B16-BL6 tumour cells and then received intraperitoneal (IP) injections or injections directly into the tumour (IT) with *U. tomentosa* extracted with 70% ethanol (ETOH) or PBS, pH 7.4, (PBS) in accordance with guidelines provided by the Animal Care Committee at Laurentian University. At the endpoint of the experiment, the mice were euthanized by CO_2 asphyxiation and tumours were surgically removed to determine the tumour diameters, weights, and body weights. The average tumour weight in grams for each of the five treatment groups ($\pm$ standard error, N= 9-10) is shown. Results demonstrated that there were significant differences between each of the four treated groups compared to the control.

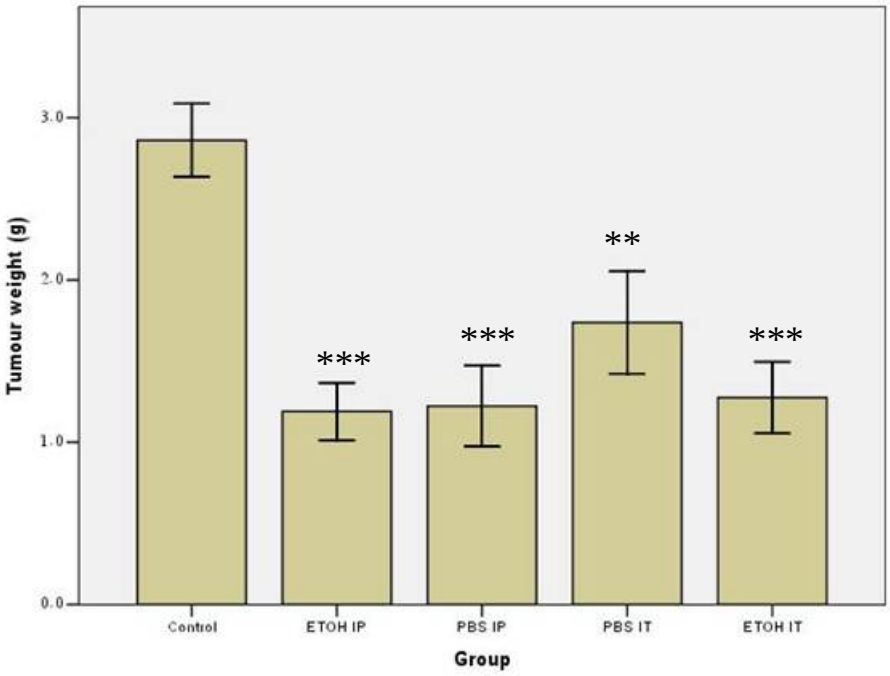

Tumour weight (g)
3.0
2.0
1.0
0.0
***
***
**
***
Control
ETOH IP
PBS IP
PBS IT
ETOH IT
Group

Figure 3.30 Tumour size was evaluated following the use of *U. tomentosa* treatment

In this research, all animals were injected with B16-BL6 tumour cells and then received intraperitoneal (IP) injections or injections directly into the tumour (IT) with *U. tomentosa* extracted with 70% ethanol (ETOH) or PBS, pH 7.4, (PBS) in accordance with guidelines provided by the Animal Care Committee at Laurentian University. At the endpoint of the experiment, the mice were euthanized by CO_2 asphyxiation and tumours were surgically removed to determine the tumour diameters, weights, and body weights. The average tumour diameter in cm for the animals in each of the five groups ($\pm$standard error, N= 9-10) is shown. Results demonstrated that there were significant differences between each of the four treated groups compared to the control.

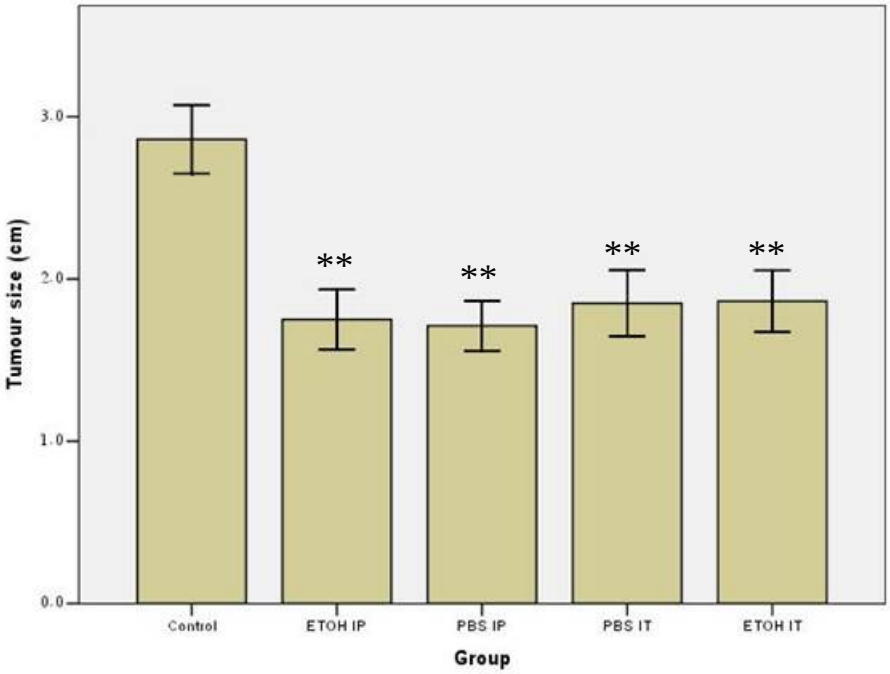
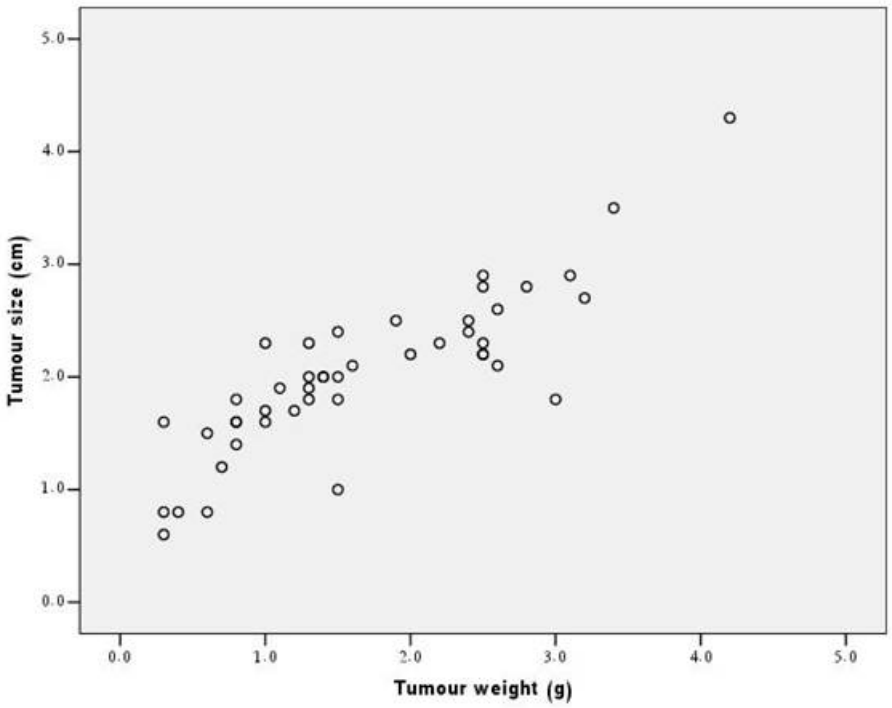

Tumour size (cm)
3.0
2.0
1.0
0.0
**
**
**
**
Control
ETOH IP
PBS IP
PBS IT
ETOH IT
Group

Figure 3.31 The correlation between tumour weight and tumour size in C57BL/6 mice

The *in vivo* study showed that when the tumour size increased, the tumour diameter also increased. This means there was a direct positive proportional relationship between tumour weight and tumour size, as indicated in the Figure 3.30.


Tumour size (cm)
5.0
4.0
3.0
2.0
1.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
Tumour weight (g)

3.3.2 Histochemical analysis was performed for B16-BL6 tumours

The B16-BL6 tumours isolated from the mice were subjected to histochemical analysis to measure the impact of treatment with *U. tomentosa* on immune, cell growth, and cell death markers. The tumours were resected and fixed by incubation in EFA fixative and then subjected to processing, embedding, and sectioning in collaboration with the University of Ottawa Core Histochemistry facility. Twenty-nine tumour samples including ten tumours taken from mice treated with just PBS and used as vehicle control, ten tumours from mice treated with the ethanol extract of *U. tomentosa* using intraperitoneal injection, and nine tumours from mice treated with the PBS, pH 7.4, extract of *U. tomentosa* using intraperitoneal injection. Different staining protocols were applied to the tissues depending on the marker used for analysis as described in detail in the materials and methods. The level of staining was assessed using an ordinal intensity staining scale where a value of 0 is assigned to sections that showed no staining and value of 5 was assigned to a section that stained very strongly. For each marker, the cumulative staining intensity was determined for 3 random fields taken from the center of the tumour and 5 random fields from the periphery of the tumour for each mouse. The staining intensity value for each mouse was then averaged for all mice in the same group and used for analysis. One-way ANOVA was used for statistical analysis and also LSD tests were performed for further analysis.

The level of staining with the Ki-67 antibody was determined in each of the tumour sections and scored for the average for each mouse (Figure 3.32). The Ki-67 antigen has been shown to be expressed in the nuclei of cells that are actively proliferating. Examination of the sections at high magnification confirmed that the

positive staining was associated with the nucleus in many of the cells. Staining with the Ki-67 antibody was detected in a number of different cells in the majority of the sections. The staining intensity for Ki-67 in tumours isolated from mice that had been treated with only PBS, pH, showed a significantly higher staining score (25.2±1.4, SEM) than did tumours from mice treated with the ethanol extract of *U. tomentosa* (17.1±1.2) or the PBS extract of *U. tomentosa* (17.1±1.6) (Table 3.11, Figure 3.33). There was no difference in Ki-67 staining intensity between mice treated with the ethanol extract compared to the PBS extract of *U. tomentosa*.

Staining for factor VIII was conducted as a marker of endothelial cells in the tumour sections as a marker of angiogenesis. Factor VIII staining was frequently associated with the presence of vessels in the tumour cross-section but in some sections staining was more diffuse in the tissue, which may indicate weakly organized vascular beds within the growing tumour. The average staining intensity for the factor VIII was greater in the tumours from mice treated with only PBS, pH 7.4 (24.3±1.3, SEM) compared to tumours from mice treated with the ethanol (17.4±1.6) or PBS (18.2±2.0) extract of *U. tomentosa* (Table 3.12, Figure 3.34).

The level of the TUNEL staining reaction was examined as a measure of cellular apoptosis within the tumour tissue. The TUNEL reaction measures the amount of free DNA ends as an indicator of DNA fragmentation during apoptosis. Examination of the sections at high magnification indicated that positive staining was consistent with localization in the nucleus. The average staining intensity for TUNEL positive cells showed that the level of staining was lowest for the tumours from animals injected with only PBS, pH7.4, however, this was not significantly different from the level of staining

in the *U. tomentosa* extracted with ethanol or PBS (Table 3.13, Figure 3.35). It is interesting to note that the variability in TUNEL staining (as indicated by the standard deviation in scores) was significantly greater for the *U. tomentosa*-treated groups compared to the control group.

Table 3.11 The mean and variation in Ki-67 staining intensity for tumours from mice treated with *U. tomentosa* extracts

Relative staining intensity for Ki-67						
Groups	N	Mean	Standard deviation	Standard Error	Minimum	Maximum
Control	10	25.2	4.44	1.40	17	33
Ethanol extract	10	17.2	3.79	1.20	12	23
PBS extract	9	17.1	4.67	1.56	8	25
Total	29	19.9	5.68	1.06	8	33

Table 3.12 The mean and variation in Factpr VIII staining intensity for tumours from mice treated with *U. tomentosa* extracts

Relative staining intensity for Factor VIII						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	24.3	4.19	1.33	18	30
Ethanol extract	10	17.4	4.99	1.58	10	25
PBS extract	9	18.2	5.91	1.97	6	25
Total	29	20.0	5.80	1.08	6	30

Table 3.13 The mean and variation in TUNEL staining intensity for tumours from mice treated with *U. tomentosa* extracts

Relative staining intensity for TUNEL staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	27.7	4.62	1.46	11	28
Ethanol extract	10	27.9	8.09	2.56	10	38
PBS extract	9	26.8	9.26	3.09	9	38
Total	29	25.8	7.60	1.41	9	38

Figure 3.32 The presence of Ki-67-positive cells was detected in histochemical sections from mice treated with *U. tomentosa* extracts

Twenty-nine tumours were used for histochemical analysis. Ki-67-staining was performed in order to measure the growth fraction of B16-BL6 tumours *in vivo*. The relative intensity of proliferating neoplastic cells was determined directly by light microscopy. The intensity of Ki-67-positive staining was determined in 8 random fields per slide: scores varied between fields based on the relative number of positive cells. Six scores were given to the photos: a score of 5 was given to a section with a high level of Ki-67-positive cells, and a score of 1 was assigned to a section with a low level of Ki-67-positive cells while a section that did not have Ki-67-positive cells was scored as 0. The figure shows a typical microscopic filed corresponding to each of the assigned scores from 0 – 5.

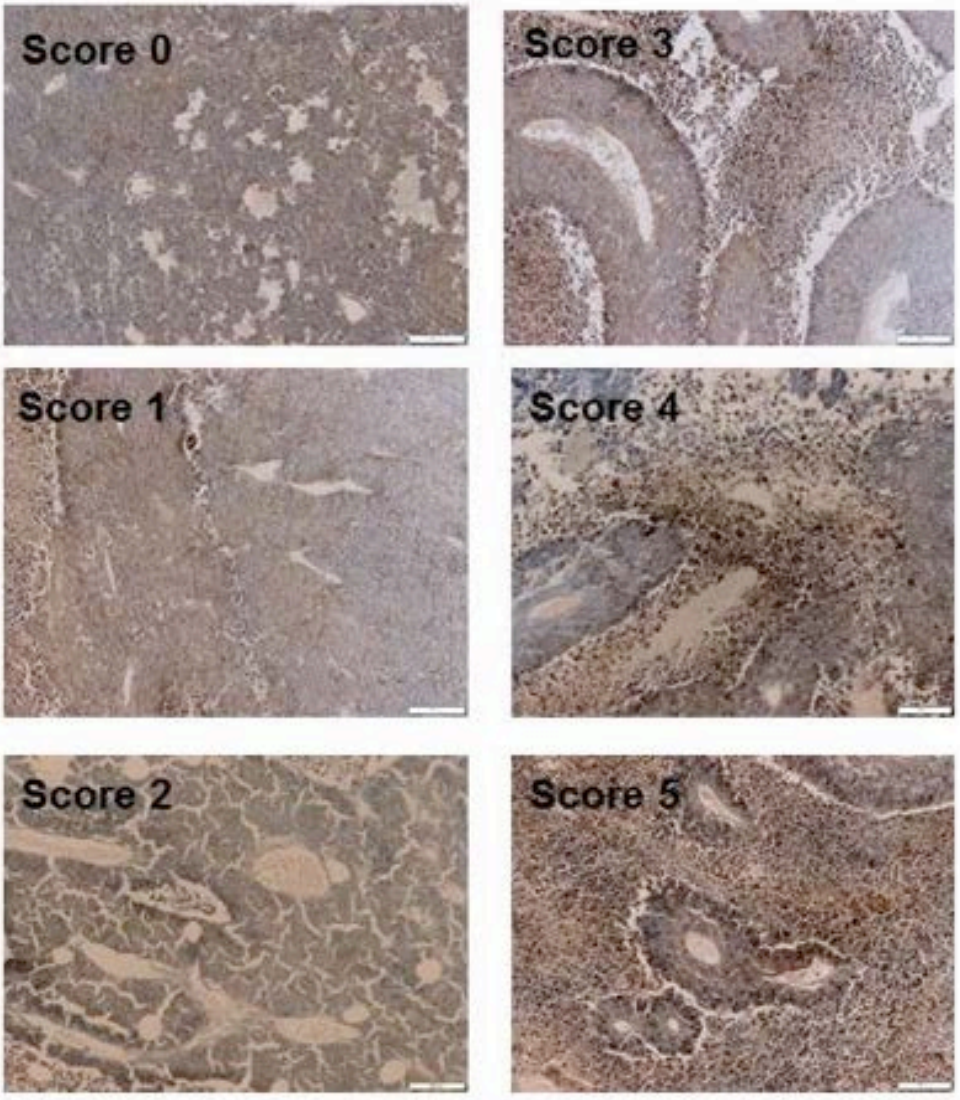
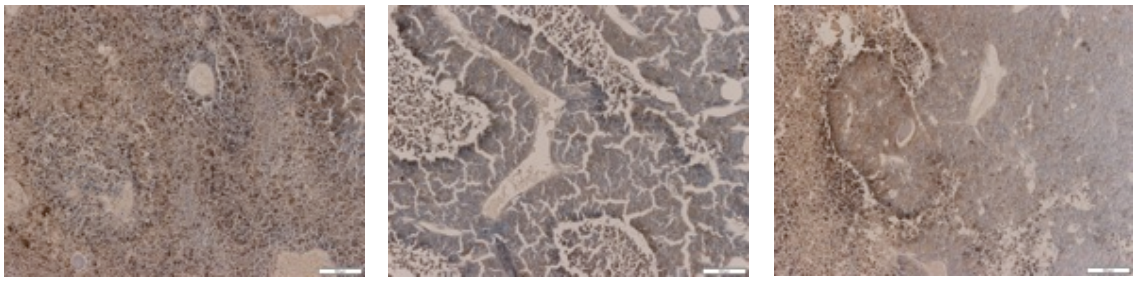

Score 0
Score 3
Score 1
Score 4
Score 2
Score 5

Figure 3.33 The relative level of Ki-67-positive cells was decreased in histochemical sections from mice treated with *U. tomentosa* extracts

Twenty-nine tumours were used for histochemical analysis using Ki-67-staining to measure the growth fraction of B16-BL6 tumours *in vivo*. The relative intensity of proliferating neoplastic cells was determined directly by light microscopy for 8 random fields per slide. The level of staining was assigned a score ranging from 5, which corresponded to a large proportion of the tissue stained positive for Ki-67, to 0, where no staining was present in the section. The cumulative score was determined for each mouse and then the average was determined for all mice in the same treatment group. A. This figure shows representative images corresponding to the average staining intensity for Ki-67 for each treatment group. B. The bar graph shows the relative staining intensity for each group. One-way ANOVA showed significant differences ($P < 0.0001$) between the three groups. Further analysis with LSD tests showed significant differences between tumours treated with *U. tomentosa* extracted with ethanol and control tumours ($P < 0.0001$), as well as, tumours treated with *U. tomentosa* extracted with PBS and the control tumours ($P < 0.0001$).

A

Control Ethanol extract PBS extract



B

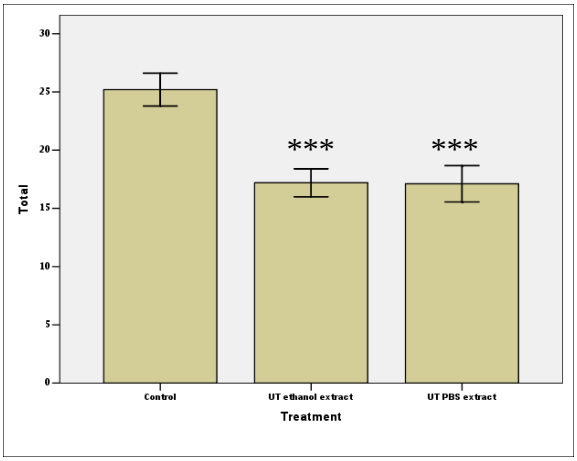
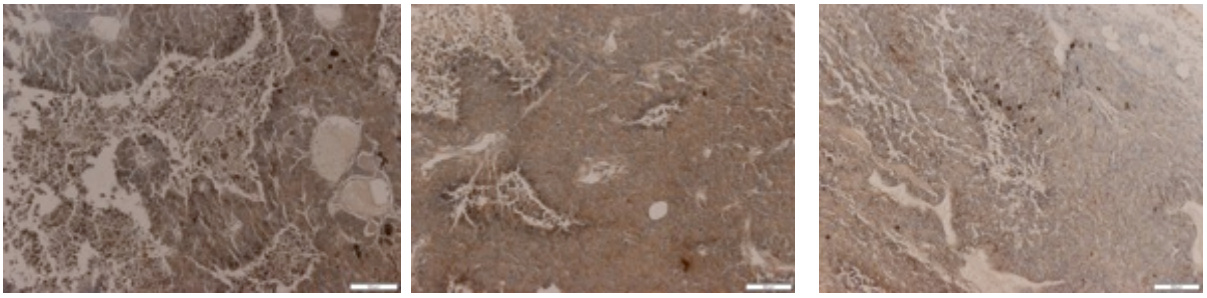


Figure 3.34 The relative level of Factor VIII-positive cells was significantly decreased in histochemical sections from mice treated with *U. tomentosa* extracts

Histochemical analysis using staining with an antibody against Factor VIII in the B16-BL6 tumours *in vivo* was performed and the relative intensity for each histochemical section was determined directly by light microscopy for 8 random fields per slide. The level of staining was assigned a score ranging from 5, which corresponded to a large proportion of the tissue stained positive for Factor VIII, to 0, where no staining was present in the section. The cumulative score was determined for each mouse and then the average was determined for all mice in the same treatment group. A. This figure shows representative images corresponding to the average staining intensity for Factor VIII for each treatment group. B. The bar graph shows the relative staining intensity for each group. One-way ANOVA showed significant differences ($P < 0.05$) between the three groups. Further analysis with LSD tests showed significant differences between tumours treated with *U. tomentosa* extracted with ethanol and control tumours ($P < 0.01$), as well as, tumours treated with *U. tomentosa* extracted with PBS and the control tumours ($P < 0.05$).

A

Control Ethanol extract PBS extract



B

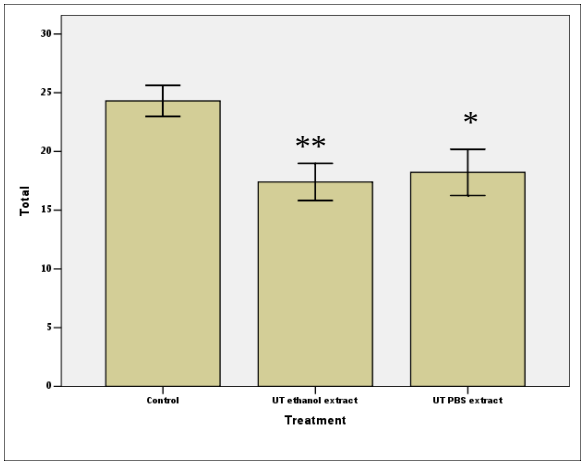
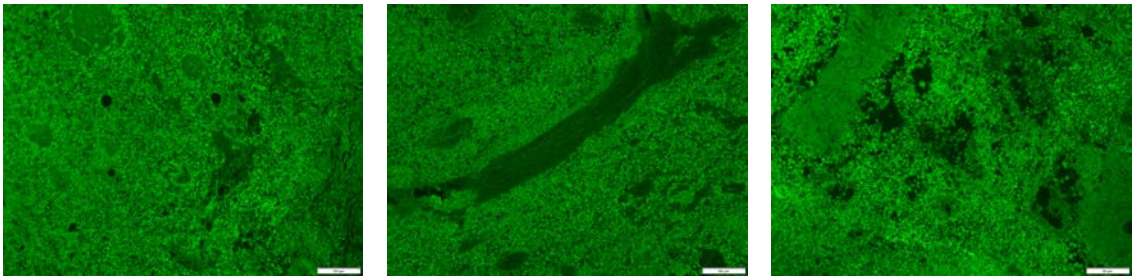


Figure 3.35 The relative level of TUNEL-positive stained cells in histochemical sections from mice treated with *U. tomentosa* extracts was not significantly altered

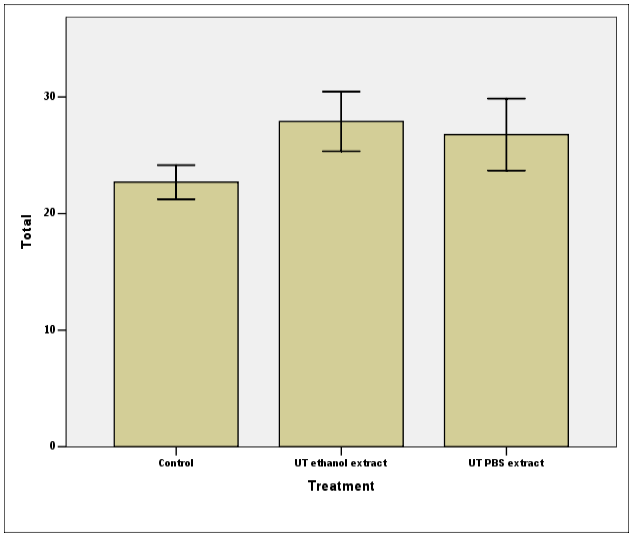
Histofluorescence analysis using a TUNEL stain to detect the free ends of DNA in the B16-BL6 tumours was performed and the relative intensity for each section was determined directly by fluorescence microscopy for 8 random fields per slide. The assay is based on the incorporation of a fluorescent nucleotide at free DNA ends and increased fluorescence intensity correlates with DNA fragmentation. The level of staining was assigned a score ranging from 5, which corresponded to a large proportion of the tissue stained positive for the TUNEL reaction, to 0, where no staining was present in the section. The cumulative score was determined for each mouse and then the average was determined for all mice in the same treatment group. A. This figure shows representative images corresponding to the average TUNEL staining intensity for each treatment group. B. The bar graph shows the relative staining intensity for each group. One-way ANOVA did not show significant differences between the three groups.

A

Control Ethanol extract PBS extract



B



3.3.3 Immune cell infiltration in B16-Bl6 tumours was studied using several immune markers

Since *U. tomentosa* has been shown to have an effect on immune cell function, the effect of *U. tomentosa* injections on immune infiltration into the B16-BL6 tumours was examined. Tumours were isolated and prepared for processing, embedding, and sectioning from animals treated with PBS, pH 7.4 (n = 10), or *U. tomentosa* extracted with either 70% ethanol (n = 10) or PBS (n = 9). The sections were stained for various immune cell markers to determine if the *U. tomentosa* extracts can affect and potentially enhance the immune response against cancer as measured by immune cell infiltration into the tumour. Different fields from the middle and periphery of the tumours were imaged using an Olympus IX3 fluorescence microscope to detect labeled cells. For each immune marker, the relative number of positive cells was determined for 8 randomly chosen fields for each tumour samples (n=29). The scores varied between fields using an ordinal scale based on the relative number of stained cells. The photographs from each field was given a score from 0 – 5: a score of 5 was given to a section with a high level of stained cells, and a score of 1 was assigned to a section with a low level of stained cells while a section that did not have stained cells was scored as 0 (examples shown in Figure 3.36).

Sections were stained for the presence of T cells (CD3, CD4, and CD8 positive cells), activated endothelial cells (PECAM-1, E-selectin, and ICAM-1 positive cells), total leukocytes (CD45 marker positive), B cells (CD19 positive cells) and platelets (integrin αIIB positive cells). Each section was simultaneously stained with three different primary antibodies each labelled with a different fluorochrome (Alexa-488 green, Alexa-546 red, Alexa-633 far red). This allowed for direct comparison of different

subsets of cell within each section. For example, the relative level of CD4 and CD8 T cells infiltrating the tumour could be directly compared. Preliminary results showed relatively little overlap in staining between the different fluorochromes and images show differences in cells labelled with the different antibodies. Staining with control, non-specific IgG showed a relatively low level of staining of the sections since exposure durations required to capture images were much longer for the control sections compared to all of the antibody-specific tissues (Figure 3.37). Further, analysis of the level of staining for the control sections showed no significant differences between the tumours from animals treated with the different experimental conditions (Table 3.14 – 3.16). For statistical analysis, none of the immune markers had significant differences among the three groups using one-way ANOVA.

The tissue sections were stained with antibodies against the T cell markers CD3, CD4, and CD8 (Figure 3.38). CD3 is a multimeric protein complex that complexes with the T-cell receptor (TCR) and the ζ-chain (zeta-chain) to activate T lymphocytes (Kuhns *et al*., 2006) and is a marker common to all T cells (including both CD4 and CD8 T cells). For CD3 staining, a red channel was used for analysis. This showed a significant level of T cell infiltration into the majority of the tumour sections, which was present throughout the tumour tissue. Results indicated that the relative intensity of the tissue sections stained with the anti-CD3 antibody was not significantly altered between the three treatment groups (Table 3.18). CD4 is the common marker to identify helper T cells, although it can be found at low concentration on macrophages, monocytes, and dendritic cells. CD4 is considered a biomarker particular for MHC class II-mediated antigen presentation and “helps” the T cell receptor (TCR) to communicate with antigen-

presenting cells (Zhu *et al*., 2010). CD4 cells were also present at significant levels in all of the tissue sections indicating a high level of infiltration into the tumours. In this study, the relative intensity of CD4-positive cells, in the green channel, showed no significant differences ($P > 0.05$) between the three treatment groups using one-way ANOVA (Table 3.17). The CD8 co-receptor is associated with the T cell receptor that recognizes antigen in coordination with the MHC class I system (O'Rourkea and Mescher, 1993). CD8 is the common marker for Cytotoxic T lymphocytes. The tissue sections also showed significant staining for CD8 positive cells indicating significant infiltration of CD8+ T cells into the tumour tissue. One-way ANOVA showed no significant differences ($P > 0.05$) between the three treatment groups in the relative amount of CD8-positive cells measured in the far red panel (Table 3.19).

PECAM -1 is a glycoprotein used as a marker for the presence of endothelial cells but is also expressed on the cell surfaces of monocytes, platelets, neutrophils, and subpopulation of T cells (Ganjei-Azar and Nadji, 2007). PECAM -1 staining was detected in the green channel (Figure 3.39) and was present at high levels in the tumour sections. In this research, the relative number of PECAM -1-positive cells showed no significant differences ($P > 0.05$) between the three groups using one-way ANOVA (Table 3.20). E-selectin is expressed on cytokine-stimulated endothelial cells and has a significant role in inflammation (Collins *et al*., 1991). E-selectin is a common marker of vascular inflammation and was present at significant levels in all of the tissue sections examined. Results indicated that the relative intensity of cells stained with E-selectin, in the red channel, was not greatly altered between the three groups (Table 3.21). The intercellular adhesion molecule-1 (ICAM-1) is an integral membrane protein of the

immunoglobulin superfamily. It plays an important role in immune responses and inflammation and is also a marker of vascular inflammation (Marlin and Rothlein, 1998). ICAM-1 was expressed in the majority of tissue sections. One-way ANOVA showed no significant differences ($P > 0.05$) between the three groups in the relative intensity of ICAM-1-positive cells (3.22).

Histochemical sections were stained with antibodies that recognize CD45, CD19, and integrin αIIb (Figure 3.40), CD45 is a transmembrane glycoprotein that is expressed on nucleated cells of hematopoietic origin acting as a common leukocyte marker. It has a significant role for T- and B-lymphocyte activation (Altin and Sloan, 1997). In this study, high levels of CD45 staining were detected in all of the tissue sections supporting the idea that there was a significant level of immune cell infiltration into the tumours. The relative intensity of CD45-positive cell staining showed no significant differences ($P > 0.05$) between the three treatment groups using one-way ANOVA (Table 3.23). The human CD19 antigen is considered a transmembrane glycoprotein that expressed in all B lineage cells. It has a significant importance in human B cells by reducing the threshold for B cell receptor signaling pathways through its work in the CD19/CD21 complex. CD19 is also considered an important biomarker for B lymphocyte development because of its existence on all B cells (Scheuermann and Racila, 1995). The number of CD19+ cells indicated that there was significant infiltration of B cells into the B16-BL6 tumours. Results indicated that the percentage of cells stained with CD19 was not greatly altered between the three treatment groups (Table 3.24).

Integrins are considered heterodimeric integral membrane proteins that contain an alpha chain and a beta chain which are expressed in a cell-type specific manner. They can

generate more than 20 various receptors that can bind ligands in the extracellular matrix such as collagen and fibronectin or mediate cell-cell adhesion. Signals transduced via integrins can regulate cell proliferation, activation, migration and homeostasis (Anderson *et al*., 2014). The integrin aIIb is expressed on platelets and can be used to mark blood clots in histologic sections. In this study, one-way ANOVA showed no significant differences ($P > 0.05$) between the three treatment groups in the amount of positive cells (Table 3.25).

Figure 3.36 Scoring relative staining intensity of immune cell infiltration for the three fluorochromes used for analysis

Three different fluorochromes were used for analysis, including green, red, and far red fluorescence. Various scores were given to the photos based on the relative amount of stained cells. For some immune markers, CD4, PECAM -1, CD45, and the control IgG1 antibody the Alexa-488 dye was used in the green channel. For other immune markers, CD3, E-selectin, CD19, and IgG2b the Alexa546 red channel was used for analysis. Finally, the Alexa-633 dye in the far red channel (but printed in blue) was used for the rest of immune markers, including CD8, ICAM- 1, Integrin αllb, and the IgG2a control. Panel A shows examples corresponding to the scoring system for different photos stained with Alexa-488 (green). Panel B shows examples of the scores for different photos stained with Alexa-546 (red). Panel C shows examples of the scoring system for photos from sections stained with Alexa-633 (blue).

A B C

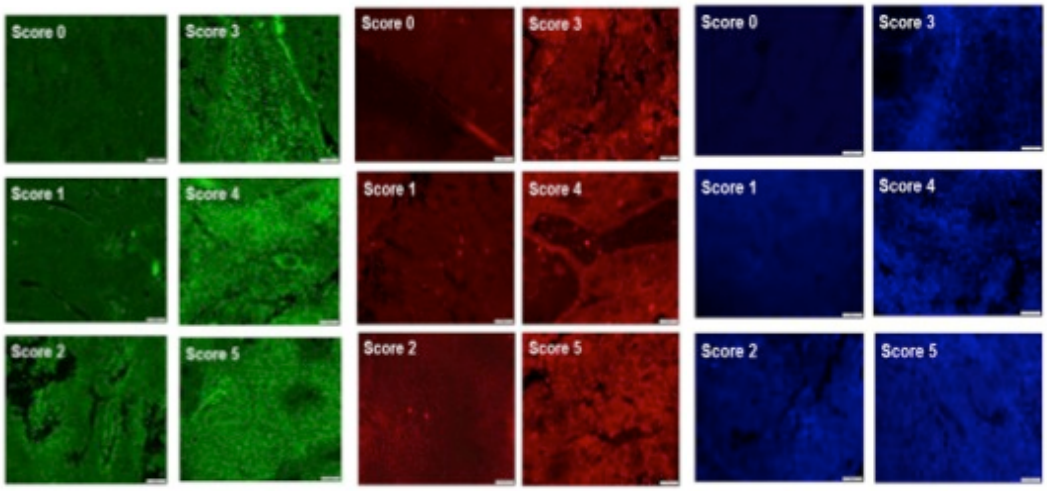

Score 0
Score 3
Score 1
Score 4
Score 2
Score 5
Score 0
Score 3
Score 1
Score 4
Score 2
Score 5
Score 0
Score 3
Score 1
Score 4
Score 2
Score 5

Figure 3.37 B16-Bl6 tumours were stained with control normal mouse IgG1, normal mouse IgG2b, and normal rat IgG2a

Control immunoglobulins including normal mouse IgG1, normal mouse IgG2b, and normal rat IgG2a were used to determine the level of background staining in the sections. Eight fields were imaged (5 peripheral and 3 central) using an Olympus IX3 fluorescence microscope set with a long exposure time to maximize detection of signal. The sections were stained with a normal mouse IgG1-Alexa-488 conjugate (detected in the green channel) a normal mouse IgG2b-Alexa-46 conjugate (detected in the red channel) and a normal mouse IgG2a-Alexa-633 conjugate (detected in the far red channel, but coloured blue) and a representative section is shown for each condition in Panel A (the Bar represents 100 μm). The relative intensity for the sections stained with the IgG1 and IgG2 control antibodies was determined and a mean and standard deviation determined for each condition and showed in Panel B. These results showed no significant differences ($P > 0.05$) between the three treatment groups using one-way ANOVA for each of the control antibodies.

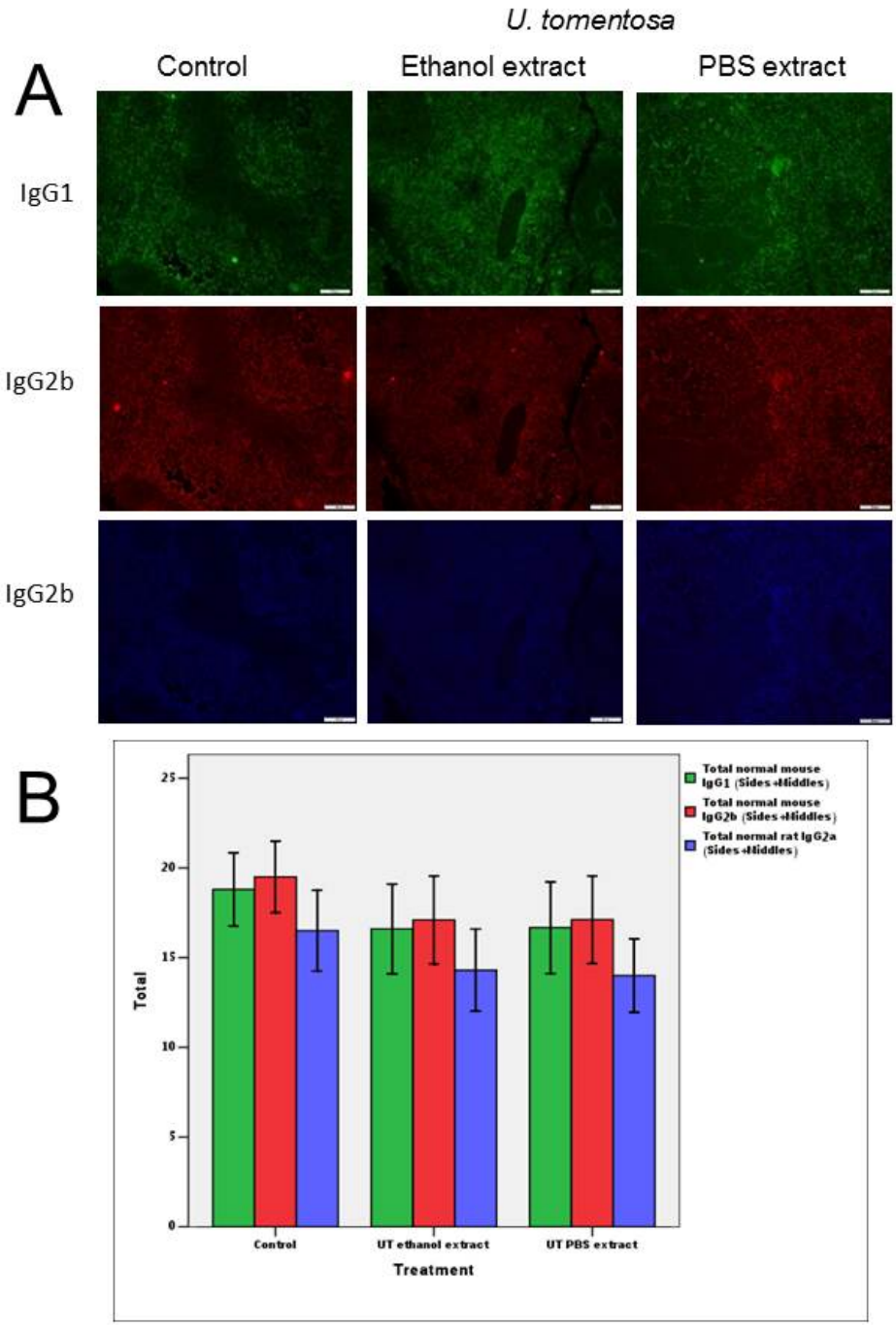

A
U. tomentosa
Control
Ethanol extract
PBS extract
IgG1
IgG2b
IgG2b
B
Total normal mouse IgG1 (Sides+Middles)
Total normal mouse IgG2b (Sides+Middles)
Total normal rat IgG2a (Sides+Middles)
Total
25
20
15
10
5
0
Control
UT ethanol extract
UT PBS extract
Treatment

Table 3.14 Comparison of relative staining intensity for control IgG1-Alexa-488.

Relative staining for control IgG1						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	18.5	6.43	2.03	8	31
Ethanol extract	10	16.6	7.92	2.50	7	27
PBS extract	9	16.7	7.65	2.55	4	30
Total	29	17.4	7.16	1.33	4	31

Table 3.15 Comparison of relative staining intensity for control IgG2-Alexa-546.

Relative staining for control IgG2						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	19.5	6.31	2.00	10	33
Ethanol extract	10	17.1	7.77	2.46	8	28
PBS extract	9	17.1	7.36	2.45	8	30
Total	29	17.9	7.00	1.30	8	33

Table 3.16 Comparison of relative staining intensity for control IgG2-Alexa-633.

Relative staining for control IgG2						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	16.5	7.09	2.24	8	32
Ethanol extract	10	14.3	7.24	2.29	6	26
PBS extract	9	14.0	6.12	2.04	6	25
Total	29	15.0	6.71	1.25	6	32

Figure 3.38 B16-Bl6 tumours were stained for T cell markers CD4, CD3, and CD8

Tissue sections were stained for T cell immune markers including CD4, CD3, and CD8. Eight fields were imaged (5 peripheral and 3 central) using an Olympus IX3 fluorescence microscope to detect stained cells. The sections were stained with an anti-CD4-Alexa-488 conjugate (detected in the green channel), an anti-CD3-Alexa-546 conjugate (detected in the red channel), and an anti-CD8-Alexa-633 conjugate (detected in the far red channel, but coloured blue) and a representative section is shown for each condition in Panel A. The relative intensity for the sections stained with the anti-CD4, anti-CD3, and anti-CD8 antibodies was determined and a mean and standard deviation determined for each condition and is shown in Panel B. These results showed no significant differences ($P > 0.05$) between the three treatment groups using one-way ANOVA for each of the control antibodies.

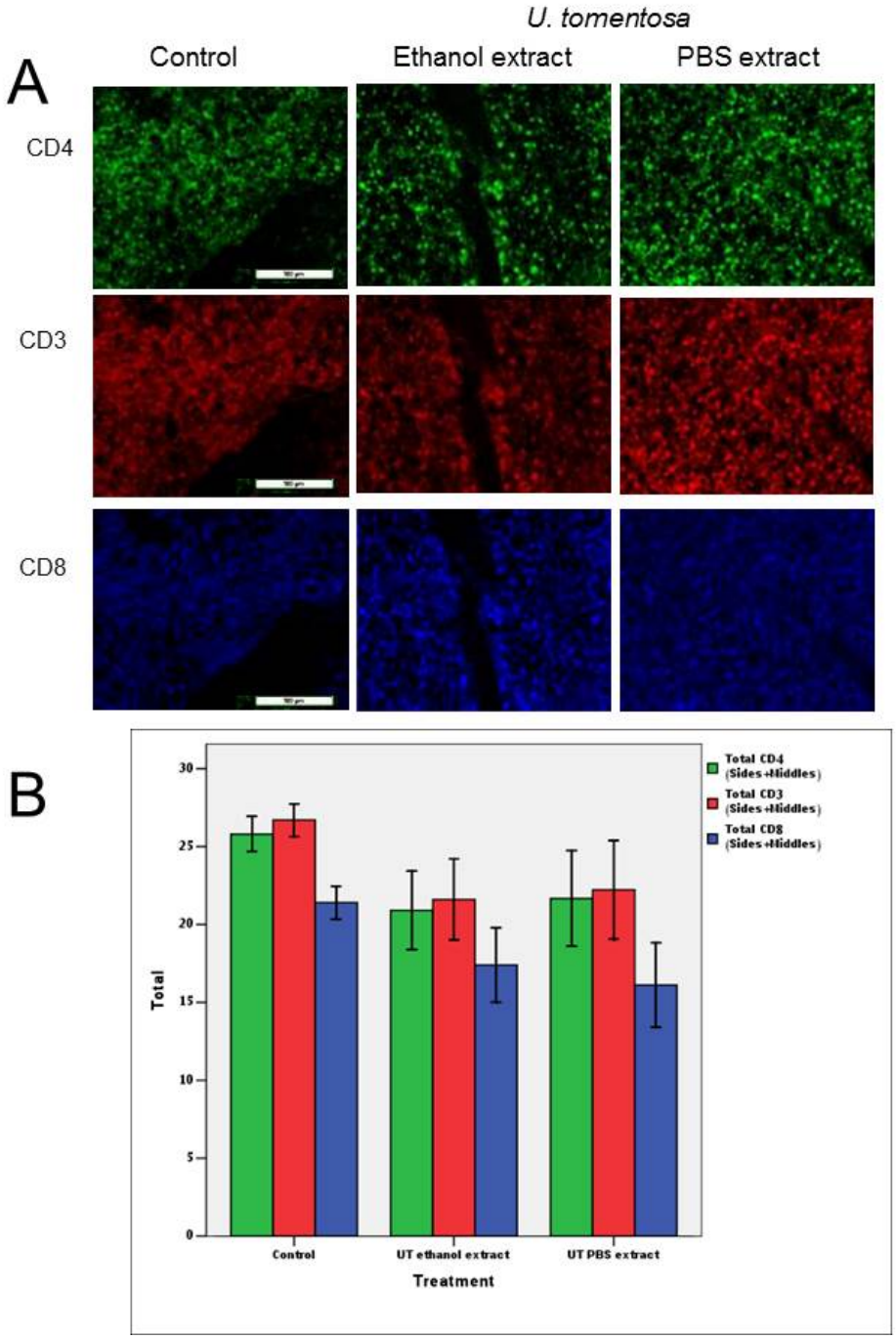

A
U. tomentosa
Control
Ethanol extract
PBS extract
CD4
CD3
CD8
B
Total CD4 (Sides+Middles)
Total CD3 (Sides+Middles)
Total CD8 (Sides+Middles)
Total
30
25
20
15
10
5
0
Control
UT ethanol extract
UT PBS extract
Treatment

Table 3.17 Comparison of relative staining intensity for anti-CD4-Alexa-488.

Relative staining intensity for anti-CD4 staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	25.8	3.58	1.13	22	32
Ethanol extract	10	20.9	7.98	2.52	10	32
PBS extract	9	21.7	9.21	3.07	7	34
Total	29	22.8	7.33	1.36	7	34

Table 3.18 Comparison of relative staining intensity for anti-CD3-Alexa-546.

Relative staining intensity for anti-CD3 staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	26.7	3.34	1.00	23	32
Ethanol extract	10	21.6	8.21	2.60	10	32
PBS extract	9	22.2	9.50	3.17	7	34
Total	29	23.6	7.52	1.39	7	34

Table 3.19 Comparison of relative staining intensity for anti-CD8-Alexa-633.

Relative staining intensity for anti-CD8 staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	21.4	3.34	1.05	17	27
Ethanol extract	10	17.4	7.56	2.39	7	27
PBS extract	9	16.1	8.13	2.71	4	30
Total	29	18.4	6.79	1.26	4	30

Figure 3.39 B16-Bl6 tumours were evaluated for PECAM-1, E-selectin, and ICAM-1 expression

Endothelial cell markers such as PECAM-1, E-selectin, and ICAM-1 were used for histochemical analysis of the mouse tissues. Eight fields were imaged (5 peripheral and 3 central) using an Olympus IX3 fluorescence microscope to detect stained cells. The sections were stained with an anti-PECAM-1-Alexa-488 conjugate (detected in the green channel), an anti-E-selectin-Alexa-546 conjugate (detected in the red channel), and an anti-ICAM-1-Alexa-633 conjugate (detected in the far red channel, but coloured blue) and a representative section is shown for each condition in Panel A. The relative intensity for the sections stained with the anti-PECAM-1, anti-E-selectin, and anti-ICAM-1 antibodies was determined and a mean and standard deviation determined for each condition and is shown in Panel B. These results showed no significant differences ($P > 0.05$) between the three treatment groups using one-way ANOVA for each of the control antibodies.

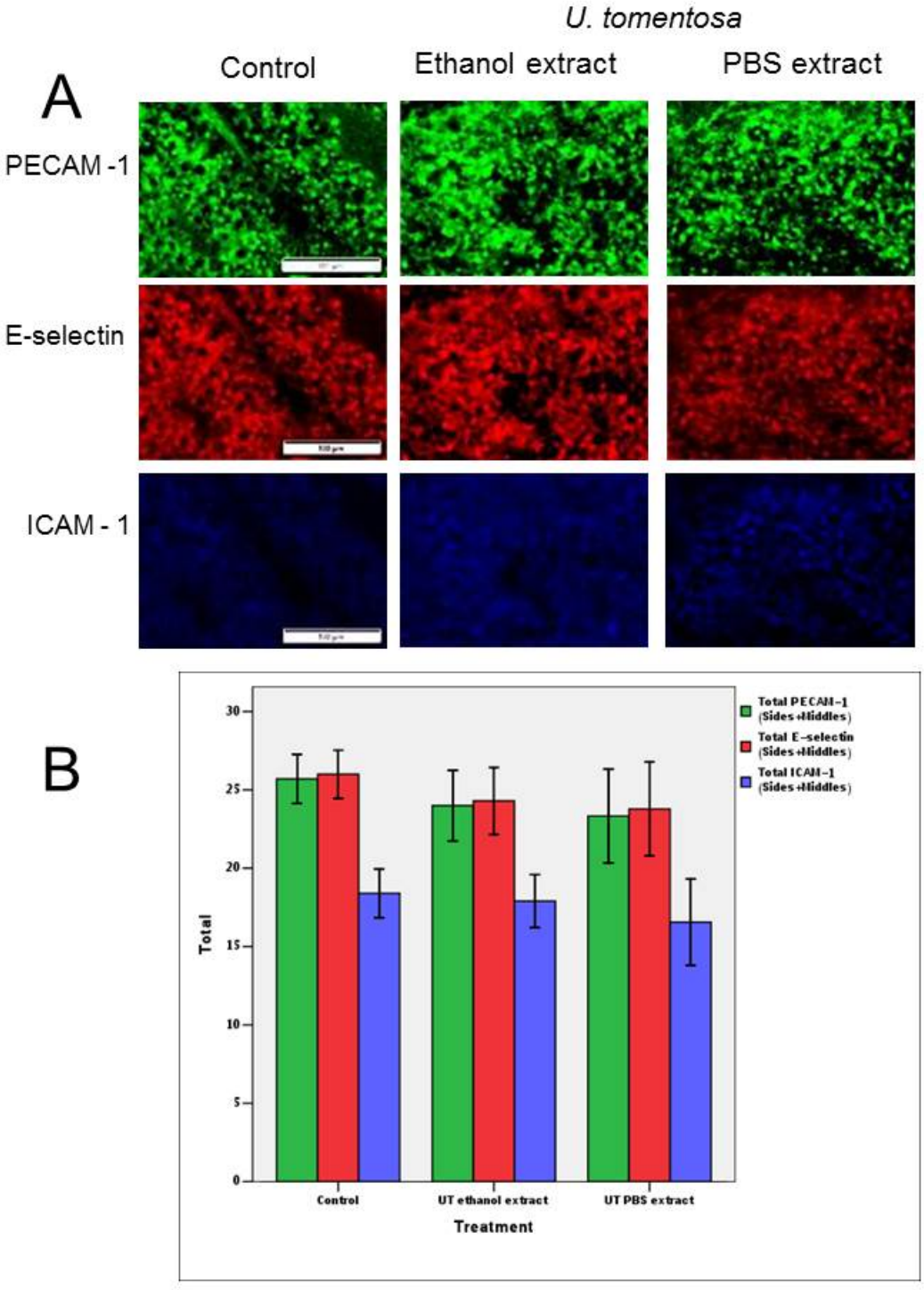

A
U. tomentosa
Control
Ethanol extract
PBS extract
PECAM -1
E-selectin
ICAM - 1
B
Total PECAM-1 (Sides+Middles)
Total E-selectin (Sides+Middles)
Total ICAM-1 (Sides+Middles)
Total
30
25
20
15
10
5
0
Control
UT ethanol extract
UT PBS extract
Treatment

Table 3.20 Comparison of relative staining intensity for anti-PECAM-1-Alexa-488.

Relative staining intensity for anti-PECAM-1 staining						
Group	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	25.7	4.99	1.58	19	35
Ethanol extract	10	24.0	7.10	2.25	14	33
PBS extract	9	23.3	8.96	2.99	9	35
Total	29	24.4	6.94	1.29	9	35

Table 3.21 Comparison of relative staining intensity for anti-E-selectin-Alexa-546.

Relative staining intensity for anti-E-selectin staining						
Group	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	26.0	4.85	1.54	20	35
Ethanol extract	10	24.3	6.77	2.14	16	33
PBS extract	9	23.8	8.98	2.99	10	35
Total	29	24.7	6.80	1.26	10	35

Table 3.22 Comparison of relative staining intensity for anti-ICAM-1-Alexa-633.

Relative staining intensity for anti-ICAM-1 staining						
Group	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	18.4	4.95	1.56	10	27
Ethanol extract	10	17.9	5.36	1.69	10	24
PBS extract	9	16.6	8.23	2.74	7	32
Total	29	17.7	6.09	1.13	7	32

Figure 3.40 B16-Bl6 tumours were evaluated through CD45, CD19, and Integrin αllb

Immune markers such as the common leukocyte marker CD45, the B cell marker CD19, and the platelet marker Integrin αllb were used for histochemical analysis of the mouse tissues. Eight fields were imaged (5 peripheral and 3 central) using an Olympus IX3 fluorescence microscope to detect stained cells. The sections were stained with an anti-CD45-Alexa-488 conjugate (detected in the green channel), an anti-CD19-Alexa-546 conjugate (detected in the red channel), and an anti-integrin αIIb-Alexa-633 conjugate (detected in the far red channel, but coloured blue) and a representative section is shown for each condition in Panel A. The relative intensity for the sections stained with the anti-CD45, anti-CD19, and anti- integrin αIIb antibodies was determined and a mean and standard deviation determined for each condition and is shown in Panel B. These results showed no significant differences ($P > 0.05$) between the three treatment groups using one-way ANOVA for each of the control antibodies.

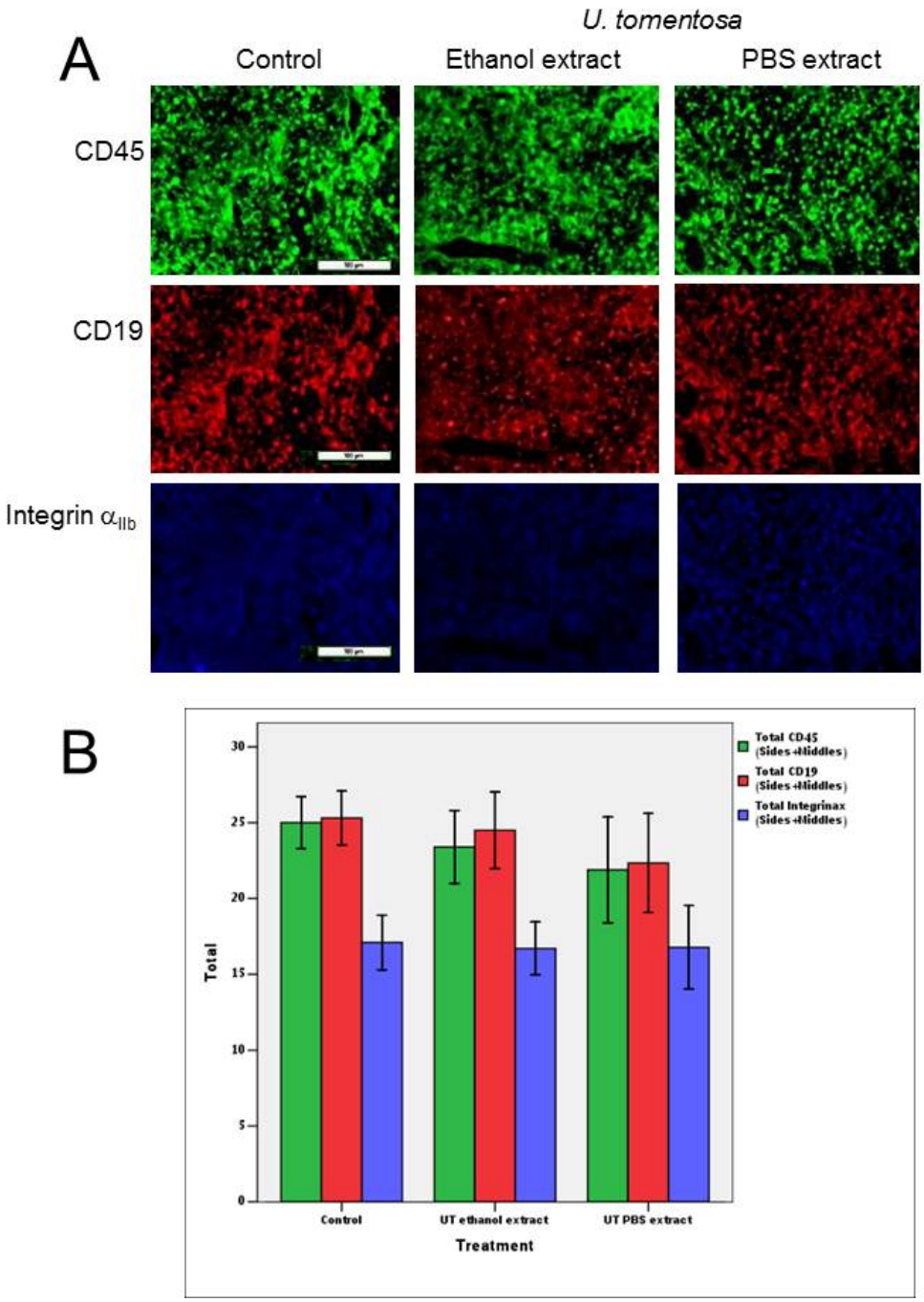

A
U. tomentosa
Control
Ethanol extract
PBS extract
CD45
CD19
Integrin α_{IIb}
B
Total CD45 (Sides+Middles)
Total CD19 (Sides+Middles)
Total Integrinax (Sides+Middles)
Total
30
25
20
15
10
5
0
Control
UT ethanol extract
UT PBS extract
Treatment

Table 3.23 Comparison of relative staining intensity for anti-CD45-Alexa-488.

Relative staining intensity for anti-CD45 staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	25.0	5.48	1.73	18	34
Ethanol extract	10	23.4	7.62	2.41	9	38
PBS extract	9	21.9	10.44	3.48	5	34
Total	29	23.5	7.82	1.45	5	38

Table 3.24 Comparison of relative staining intensity for anti-CD19-Alexa-546.

Relative staining intensity for anti-CD19 staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	25.3	5.64	1.78	18	35
Ethanol extract	10	24.5	8.00	2.53	9	39
PBS extract	9	22.3	9.81	3.27	8	34
Total	29	24.1	7.74	1.44	8	39

Table 3.25 Comparison of relative staining intensity for anti-integrin αIIb-Alexa-633.

Relative staining intensity for anti-integrin αx staining						
Groups	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Control	10	17.1	5.68	1.80	9	27
Ethanol extract	10	16.7	5.52	1.75	9	25
PBS extract	9	16.8	8.26	2.75	6	27
Total	29	16.6	6.30	1.17	6	27

3.4 Discussion

3.4.1 The effect of *U. tomentosa* extract on tumour weight and size in animal models

The ability of *U. tomentosa* extracts to inhibit the growth of tumours *in vivo* was tested by measuring the impact on tumours formed by B16/BL6 cells injected into C57BL/6 mice. This experimental model was chosen because the B16-BL6 cells are syngeneic with the C57BL/6 mice, which allows tumour growth to be measured in the presence of a normal immune system. This was considered to be important because treatment with *U. tomentosa* extracts has been shown to modulate the immune system, which might be important for modulating tumour growth.

Two animal experiments were performed using a total of 50 male C57BL/6 mice all of which had been injected with 10^5 B16-BL6 cells to establish tumours. For each experiment, the mice were divided into 5 groups, each containing 5 mice. The 5 groups included: the control group that were not treated with *U. tomentosa*; a group treated with the ethanol extract of *U. tomentosa* (resuspended in PBS, pH 7.4) by intra-peritoneal injection; a group treated with the ethanol extract of *U. tomentosa* by direct injection into the tumour; a group treated with the PBS extract of *U. tomentosa* by intra-peritoneal injection; and, a group treated with the PBS extract by direct injection into the tumour. For treatments, individual mice were each injected with 100 µl of 200 µg/ml ethanol extract or PBS extract twice per week, starting 3 or 4 days after injection of the tumour cells, using intraperitoneal or intratumour injections. The mice were sacrificed approximately 18 days after injection of the tumour cells and the tumours excised and measured for weight and diameter.

Results showed that treatment with *U. tomentosa* extract caused a significant decrease in the tumour weight and tumour size although effect on the tumour size was less impacted. In some control tumours, there appeared to be more blood vessels apparent and more significant bleeding during necropsy, which could potentially be associated with tumour invasion and migration from the primary. However, no overt metastases were found during the post-mortem examination of the control or *U. tomentosa*-treated mice. All *U. tomentosa* treatment groups showed a significant decrease in tumor weight and tumour size compared to control-treated animals. However, there were no significant differences in tumour weight or size between the different treatment groups which means there was no difference in the effectiveness of the treatment *in vivo* that depended on injection type (i.e. intra-peritoneal versus directly into the tumour) or the type of the extract (i.e. ethanol versus PBS extracts). This is somewhat different from the *in vitro* results which showed that the ethanol extract of *U. tomentosa* was significantly more effective than the PBS extract. The reason for this difference is not clear but could be related to potential differences in mechanism between *in vitro* and *in vivo* effectiveness of *U. tomentosa*. It is also possible that the difference might be related to the resuspension of lyophilized powder derived from the ethanol extract in PBS, pH 7.4, prior to injection into the mice. Since it was not advisable to inject relatively large volumes of ethanol into the mice, the extracts had to be resuspended in an aqueous buffer, which may not have been able to solubilize all of the bioactive components prior to injection.

Similar results have been obtained in other studies that used *U. tomentosa* extract as an anticancer treatment in animal models. Pilarski *et al*. (2010) examined the

effect of *U. tomentosa* extract on mice injected with Lewis lung carcinoma cells. In this study, IC50 values were used in order to prepare different quantitative and qualitative oxindole alkaloid compositions. One of the preparations produced by these researchers was to extract the bark in water at 37 ° C. They found that animals treated with this preparation had a significant reduction in tumor growth *in vivo (*Pilarski *et al*., 2010).

Fazio *et al*. (2008) examined the effect of a hydroethanolic extract of *U. tomentosa* against B16/BL6 cells in female C57BL/6 mice. They documented that individual mice treated with the *U. tomentosa* extract had a significant reduction in tumor growth and metastasis (Fazio *et al*., 2008). Taken together, these results are strongly consistent with our findings that *U. tomentosa* extract significantly reduced the tumour weight and size *in vivo*.

3.4.2 B16-BL6 tumours were analyzed using histochemical detection of Ki-67, Factor VIII, and TUNEL markers

In the present research, tumour samples (n=29) were analyzed for proliferation (Ki-67 staining), angiogenesis (factor VIII staining), and apoptosis (TUNEL staining) markers. Specifically, the expression of the cell proliferation nuclear marker, the Ki-67 antigen, was measured in tumours isolated from control mice or mice treated by intra-peritoneal injection with *U. tomentosa* extracted with ethanol or PBS. The relative level of Ki-67 antigen staining was calculated from 8 fields per each tumour slide and then the average staining for animals within each group were determined. Results showed that the Ki-67 staining levels were significantly reduced in the *U. tomentosa*-

treated groups compared to the control. This means that *U. tomentosa* caused a significant reduction in the proliferation index of B16-BL6 *in vivo*. It is interesting that there was no difference in tumour staining that depended on whether PBS or ethanol extracts of *U. tomentosa* were injected which is different from the *in vitro* results.

Furthermore, factor VIII staining was used in this study to evaluate the presence of angiogenesis in B16-BL6 tumours. Angiogenesis is the creation of new blood vessels arising from pre-existing blood vessels, which is required to meet the metabolic requirements of the rapidly growing tumour. For example, it is responsible for supplying oxygen and nutrients to the cells, as well as removing the metabolic waste (Rajabi and Mousa, 2017). The results demonstrated that the level of factor VIII-positive cells were significantly greater in tumours isolated from animals in the control group compared to tumours isolated from the *U. tomentosa* extract-treated groups. This suggests that *U. tomentosa* caused a significant reduction in angiogenesis and metastasis *in vivo*. There was no difference in the anti-angiogenic impact of *U. tomentosa* depending on whether the ethanol or PBS extract was used. It would be interesting to see if *in vitro* experiments would show that the ethanol and PBS extracts of *U. tomentosa* would have the same effect on the growth and migration of endothelial cell cultures. If the two extracts had the same effect, this would support the idea that *U. tomentosa*-specific effects on tumour growth *in vivo* was dependent on more than the direct effects of *U. tomentosa* on the viability of cancer cells *in vivo*.

Additionally, the histological tumour sections were stained with the TUNEL assay in order to evaluate cell death. The number of TUNEL-positive cells was calculated on 8 randomly selected fields for each tumour section via the fluorescence

microscope. The present study indicated that the number of TUNEL-positive cells in the treated tumours was not significantly higher than the control tumours. This means a large number of TUNEL stained cells were found in all three groups. The duration of the experiment might be the reason for showing a large number of apoptotic cells, even in the control group. For example, large tumours formed by the fast growing B16-BL6 tumours usually show relatively high levels of apoptosis due to the lack of oxygen or metabolites even in the absence of any treatment. In addition, the tumours were isolated at the end of the experiment when any treatment-dependent changes in cellular apoptosis might be less significant. For example, changes in the level of apoptosis could be more significant at earlier times during tumour growth when the tumours were small and when cell death in the untreated tumours would be expected to be lower allowing any effect from *U. tomentosa* treatment to be detected. Therefore, different results might have obtained if the level of apoptotic cells in the tumours was examined earlier in the life of the tumour.

3.4.3 Immune infiltration into B16-BL6 tumours were studied through immune markers

Extracts isolated from a wide range of plants has shown significant anticancer activities through different biological pathways, including immune system modulation. Varied types of immune cells are frequently involved in this process, particularly cytotoxic T cells, natural killer cells, and monocyte/macrophages. It has been documented that a number of medicinal plants, such as *Uncaria tomentosa, Glycyrrhiza glabra, Camellia sinensis, Panax ginseng, Prunus armenaica, Arctium*

lappa, Allium sativum, and *Curcuma longa* have the ability to both alter immune responses and treat breast cancer. Based on the results of various studies, these plants have been shown to have bioactive immunomodulatory compounds, such as ginsan, glabridin, quinic acid, ajoene, arctigenin, β-carotene, and epigallocatechin-3-gallate (Baraya *et al*., 2017).

In the current research, several immune markers were used in order to determine if the *U. tomentosa* extracts were able to affect the immune response against B16-Bl6 cells as measured by immune infiltration into the tumours. These immune markers include, general leukocyte markers such as CD45, T cell markers including CD3, CD4, and CD8, the B cell marker CD19, monocyte markers including integrin αIIB and endothelial activation markers including PECAM -1, E-selectin, and ICAM -1. Results indicated that all immune markers did not show significant differences in the relative levels of these markers between the control group and the *U. tomentosa*-treated groups. This indicates that the level of infiltrating immune cells is not significantly different, independent of *U. tomentosa* treatment. Since *U. tomentosa* has been shown to have effects on immune cell activation in some experiments it is not clear why no differences were detected in these tumor samples. Some possible explanations for this lack of effect could be related to the observation that B16-BL6 cell tumours are able to strongly activate immune infiltration which can overcome any treatment effects and that the tumours are examined very late in their development where the levels of immune infiltrates have stabilized to similar levels independent of treatment. However, it should be noted that there were differences in the relative staining of some of the markers, in particular the T cell markers, which indicate that there are small decreases (15-20%) in

the mean relative staining levels in tumours from *U. tomentosa*-treated mice (although the variation in staining levels among animals was relatively large and therefore these differences were not significant). Therefore, further studies and investigation is required to determine the mechanism of inhibiting B16-BL6 tumours *in vivo* by *U. tomentosa* extracts.

Chapter 4. General Conclusion

4.1 Cancer and natural products

Cancer is a collective term for diseases distinguished by the growth of abnormal cells with the possibility of invading or spreading to other organs (WHO, 2018a; NCI, 2018). Cancer is the second leading cause of death in the world and statistics show that cancer caused 8.8 million deaths in 2015, which means that one in six deaths worldwide are caused by cancer. Cancer can have multiple anatomic and molecular subtypes that each need particular management plans (WHO, 2018a). The common treatments for cancer include surgery, chemotherapy, radiation therapy, hormone therapy, and targeted therapy. Each treatment is used according to the type of cancer and the stage of the cancer. Some cancer patients may only receive a single type of treatment. However, most patients receive a combination of treatments (NCI, 2017). These treatments often aim to cure the patient of cancer but are always directed at prolonging life and improving the patients' quality of life (WHO, 2018a).

Breast cancer is a frequent cancer among women. Every year, over 1.5 million women are affected by breast cancer. Furthermore, statistics indicated that approximately 570,000 women died of breast cancer in 2015, which is about 15% of all cancer deaths for women (WHO, 2018b). The rate of deaths due to breast cancer has decreased over the last several years as earlier diagnosis and improved treatment protocols have been developed. However, many of the chemotherapy treatments recommended for women with breast cancer can be quite toxic and are associated with significant side effects including anemia, thrombocytopenia, neutropenia, immune dysfunction, gastrointestinal

complications, fatigue, nausea, and hair loss (Ewertz and Jensen, 2011; Narod *et al*, 2015). The serious side effects associated with conventional treatments as well as their limitations in efficacy (Lonning *et al*., 2004; Carpenter and Miller, 2005; Ewertz and Jensen, 2011) have motivated researchers to examine the anticancer properties of natural products, including medicinal plants. Hence, studies on medicinal plants have gained more interest among the public since they are believed to be less toxic and more "natural". It has also been found that a number of plant-derived drugs have pharmacological properties and several of these have been demonstrated to be effective agents to treat cancer and some have been shown to reduce the side effects of conventional cancer treatments (Araújo *et al*., 2012). Further, many of the current chemotherapy drugs, such as paclitaxel, doxorubicin, vinblastine, etoposide, and camptothecin have been derived from natural products (Cragg *et al*., 1997).

4.2 *Uncaria tomentosa* extracts

U. tomentosa (Rubiaceae) is a typical tropical rain forest plant, and is naturally dispersed throughout South and Central America, chiefly in Peru and Brazil. It has been traditionally used as a medicinal plant by South American indigenous people who have used it for a very long time in the treatment of a wide range of different ailments. In the search for new therapeutics, researchers have cataloged a large number of Amazonian plants in an attempt to find natural compounds with low toxicity and high efficacy. With this aim in mind, the history of *U. tomentosa* use is notable because it is has been used in traditional medicine to treat a variety of inflammation-related different diseases (Heitzman *et al*., 2005; Zhang *et al*., 2015). In addition, Araújo *et al*. (2012) have shown

that *U. tomentosa* can be used as an efficient adjuvant therapy for breast cancer. Furthermore, it has been demonstrated that treatment with extracts of *U. tomentosa* can decrease the side effects of chemotherapy in cancer patients; it decreases the neutropenia caused by chemotherapy and is also capable of restoring cellular DNA damage (Araújo *et al*., 2012), further supporting the idea that this plant has clinical therapeutic potential.

4.3 Comparing the effects of *U. tomentosa* extracts between *in vitro* results and *in vivo* results

In this research, we showed that treatment with *U. tomentosa* extracts can greatly inhibit the growth of the B16-BL6 and 4T1 cell lines, especially when the cells were treated with the 70% ethanol extract at the highest dose of 200 μg/ml using an MTT assay. In addition, the expression of the Ki-67 antigen was significantly decreased when cancer cells were treated with *U. tomentosa* extracted with 70% ethanol at 200 μg/ml *in vitro*. However, the treatment with *U. tomentosa* extracted with PBS showed a lower effect. On the other hand, the *in vivo* results indicated that treatment with *U. tomentosa* extracted with ethanol and PBS significantly reduced tumour weight and size in the treated groups compared to the control in the same manner. This means there were no significant effects or interactions between the types of extract used for treatment *in vivo*.

Since *U. tomentosa* extracts significantly blocked Ki-67 expression in cell cultures, the level of Ki-67 staining was also determined in B16-BL6 tumours isolated from animals using histochemical analysis. The Ki-67 staining intensity showed a significant reduction in the tumours from animals that treated with *U. tomentosa* compared to the control animals *in vivo*. Interestingly, no significant difference was

found between the two treated groups (ethanol and PBS) in terms of the Ki-67 staining intensity. This is somewhat different from the *in vitro* results which showed that the ethanol extract of *U. tomentosa* was significantly more effective than the PBS extract. The reason for this difference is not clear but could be related to potential differences in mechanism between *in vitro* and *in vivo* effectiveness of *U. tomentosa*. It is also possible that the difference might be related to the resuspension of lyophilized powder derived from the ethanol extract in PBS, pH 7.4, prior to injection into the mice. Since it was not advisable to inject relatively large volumes of ethanol into the mice, the extracts had to be resuspended in an aqueous buffer, which may not have been able to solubilize all of the bioactive components prior to injection.

In the laboratory, *U. tomentosa* extracted with ethanol greatly promoted cell death in both cancer cell lines at the highest dose of 200 μg/ml using the TUNEL assay. However, a lower effect was observed when cells were treated with *U. tomentosa* extracted with PBS. Similarity, a huge increase in the percentage of the apoptotic cells present in the sub-G1 peak was found after the treatment with *U. tomentosa* extracted with ethanol using flow cytometry. However, the treatment of 4T1 cells with *U. tomentosa* extracted with PBS showed a smaller increase in the number of apoptotic cells in the sub-G1.

Since *U. tomentosa* extracts induced significant DNA fragmentation in cell cultures, the level of TUNEL staining was determined in B16-BL6 tumours using histochemical analysis *in vivo*. The *in vivo* results showed that there were small differences between the treated groups and the control. However, these differences were not statistically significant. These results are somewhat different from the *in vitro* results.

The duration of the experiment might be the reason for showing a large number of apoptotic cells, even in the control group. For example, the fast growing B16-BL6 tumours usually show relatively high levels of apoptosis due to the lack of oxygen or metabolites even in the absence of any treatment. In addition, the tumours were isolated at the end of the experiment when any treatment-dependent changes in cellular apoptosis might be less significant. For example, changes in the level of apoptosis could be more significant at earlier times during tumour growth when cell death in the untreated tumours would be expected to be lower allowing any effect from *U. tomentosa* treatment to be detected. Therefore, different results might have obtained if the level of apoptotic cells in the tumours was examined earlier in the life of the tumour.

In terms of HPLC, ethanol and PBS extracts showed different amounts of alkaloids. Our results suggest that these differences correlate with cell apoptosis induction *in vitro*. However, the *in vivo* data suggest the possibility that other components of *U. tomentosa* might be involved in tumour growth *in vivo*. Further experiments are required to test the possibility.

4.4 Conclusion

Cancer is a serious public health problem that is considered a major cause of death worldwide. Medicinal plants have been used by several communities over thousands of years to improve health and treat many disorders. Recently, there has been considerable interest in examining the properties of various medicinal plants in order to learn more about their possible pharmacological properties for the development of new anti-cancer drugs with a relative lack of non-specific toxicity. These studies concluded that *U. tomentosa* extracts were an effective treatment against the B16-BL6 and 4T1 mouse cancer cell lines. The ethanol extract of *U. tomentosa* had a much higher inhibitory effect than the PBS extract *in vitro*. The results showed that *U. tomentosa* extracted with ethanol prevented the proliferation of both cell lines and caused significant morphological changes. The extract also has the ability to induce cell death in cancer cells; in particular, DNA fragmentation was significant in treated cancer cells. Furthermore, the flow cytometry showed a large percentage of apoptotic cells in the sub-G1 peak.

On the other hand, the *in vivo* results documented that *U. tomentosa* successfully reduced both the tumour weight and tumor size. However, biweekly injection of *U. tomentosa* extracts did not have a detectable negative impact on mouse health. There were no significant differences in mouse weight, in the treated groups compared to the control and no changes in mouse behaviour were noted. Interestingly, there were no significant differences in the anti-tumour effects between the ethanol and PBS extracts of *U. tomentosa* or between intraperitoneal or intratumour injection *in vivo*. Histochemical

analysis of the B16-BL6 tumour tissues excised from the mice, showed that treatment with the plant extract inhibited the proliferation of B16-BL6 *in vivo* using ki67 staining. Cell death as identified by TUNEL staining *in vivo* showed very small differences between the treated groups and the control. All immune markers did not show significant differences among the treated groups and the control. Collectively, *U. tomentosa* showed promising results as anticancer agents both *in vitro* and *in vivo* that might ultimately be effective in treating patients with cancer. Further analysis is required to understand the mechanism of action and also to evaluate the potential complications from this treatment.

References

Adams, M., and Jewell, A. (2007) The use of complementary and alternative medicine by cancer patients, International Seminars in Surgical Oncology, 4:10.

Altin, J.G., and Sloan, E.K. (1997) The role of CD45 and CD45-associated molecules in T cell activation, Immunology and Cell Biology, 75(5): 430-45.

Allen-Hall, L., Cano, P., Arnason, J.T, Rojas, R., Lock, O., and Lafrenie R.M. (2007) Treatment of THP-1 cells with *Uncaria tomentosa* extracts differentially regulates the expression if IL-1β and TNF-α, Journal of Ethnopharmacology, 109(2): 312–317.

Allen-Hall, L., Arnason, J. T., Cano, P., and Lafrenie, R. M. (2010) *Uncaria tomentosa* acts as a potent TNF-α inhibitor through NF-κB, Journal of Ethnopharmacology, 127(3): 685-93.

Alfarteesh, H. (2014) The Effect *of Uncaria tomentosa* on the Murine Melanoma Cell Line, B16-BL6 (M.Sc. dissertation, Laurentian University of Sudbury).

Aljehani, A. (2015) Treatment with Extracts of *Uncaria tomentosa* Promotes Apoptosis in the Human Breast Cancer Cell Line, MCF7 (M.Sc. dissertation, Laurentian University of Sudbury).

Allen, L., Buckner, A., Buckner, C., Cano, P., and Lafrenie, R. (2017) *Uncaria tomentosa* (Willd. ex Schult.) DC (Rubiaceae) Sensitizes THP-1 Cells to Radiation-induced Cell Death, Pharmacognosy Research, 9(3): 221–229.

Albrektsen, G., Heuch, I., and Thoresen, S. (2010) Histological type and grade of breast cancer tumors by parity, age at birth, and time since birth: a register-based study in Norway, BMC Cancer, 10: 226.

American Cancer Society. Global Cancer Facts and Figures 2007. Atlanta, GA: American Cancer Society; 2007.

Andersen, M.H., Schrama, D., Thor P., and Becker J.C. (2006) Cytotoxic T cells, Journal of Investigative Dermatology, 126(1): 32-41.

Anisimov, V.N; and Petrov, N.N. (1987) Carcinogenesis and Aging, CRC Press Inc; Boca Raton, FL (USA).

Anquez-Traxler, C. (2011) The legal and regulatory framework of herbal medicinal products in the European Union: a focus on the traditional herbal medicines category, Drug information journal, 45(1): 15-23.

Anderson, L., Owens, T., and Naylor, M. (2014) Structural and mechanical functions of integrins, Biophysical Reviews, 6(2): 203–213.

Anisimov, V.N. (1983) Carcinogenesis and aging, Advances in Cancer Research, 40: 365-424.

Anampa, J., Makower, D., and Sparano, J. (2015) Progress in adjuvant chemotherapy for breast cancer: an overview, BMC Medicine, 13: 195.

Anand, P., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., Sung, B., and Aggarwal, B.B. (2008) Cancer is a preventable disease that requires major lifestyle changes, Pharmaceutical research, 25(9), 2097-2116.

Aquino, R., De Simone, F., Vincieri, F.F, and Pizza, C. (1990) New polyhydroxylated triterpenes from *Uncaria tomentosa*, Journal of Natural Products, 53: 559–564.

Aquino, R., De Feo, V., De Simone, F., Pizza, C., and Cirino G. (1991) Plant metabolites. New compounds and anti-inflammatory activity of *Uncaria tomentosa*, Journal of Natural Products, 54: 453–459.

Araújo, M.D., Farias, I.L., Gutierres, J., Dalmora, S.L., Flores, N., Farias, J., et al. (2012) *Uncaria tomentosa*—Adjuvant Treatment for Breast Cancer: Clinical Trial, Evidence-Based Complementary and Alternative Medicine, 2012, 1-8.

Arriagada, R., Lê, M.G., Rochard, F., and Contesso, G. (1996) Conservative treatment versus mastectomy in early breast cancer: patterns of failure with 15 years of follow-up

data. Institute Gustave-Roussy Breast Cancer Group, Journal of Clinical Oncology, 14(5): 1558-1564.

Aravindaram, K., and Yang, N. (2010) Anti-Inflammatory Plant Natural Products for Cancer Therapy, Planta Medica, 76(11): 1103-17.

Baraya, Y.S., Wong, K. K., and Yaacob, N.S. (2017) The immunomodulatory potential of selected bioactive plant-based compounds in breast cancer: a review, Anti-Cancer Agents in Medicinal Chemistry, 17(6), 770-783.

Bai, L., and Zhu, W.G. (2006) p53: structure, function and therapeutic applications, J Cancer Mol, 2(4): 141–153.

Barroso-Sousa, R., and Metzger-Filho, O. (2016) Differences between invasive lobular and invasive ductal carcinoma of the breast: results and therapeutic implications, Therapeutic Advances in Medical Oncology, 8(4): 261–266.

Bacher, N., Tiefenthaler, M., Sturm, S., Stuppner, H., Ausserlechner, M.J., Kofler, R., and Konwalinka, G. (2006) Oxindole alkaloids from *Uncaria tomentosa* induce apoptosis in proliferating, G0/G1-arrested and bcl-2-expressing acute lymphoblastic leukaemia cells, British Journal of Haematology, 132(5): 615-22.

Barlóg P. K. (2002) Effect of magnesium and nitrogenous fertilisers on the growth and alkaloid content in Lupinus Aangustifolius L, Australian Journal of Agricultural Research, 53(6): 671-676

Bent, S., and Ko, R. (2004) Commonly used herbal medicines in the United States: a review, The American Journal of Medicine Home Page,116(7): 478–85.

Bonev, V., Evangelista, M., Chen, J.H., et al. (2014) Long-term follow-up of breast-conserving therapy in patients with inflammatory breast cancer treated with neoadjuvant chemotherapy, American Journal of Surgery, 80(10): 940-3.

Bors, M., Michałowicz, J., Pilarski, R., Sicińska, P., Gulewicz, K., and Bukowska, B. (2012) Studies of biological properties of *Uncaria tomentosa* extracts on human blood mononuclear cells, Journal of ethnopharmacology, 142(3), 669-678.

Braun, L., A., Tiralongo, E., Wilkinson, J., M., Spitzer, O., Bailey, M., Poole S., et al. (2010) Perceptions, use and attitudes of pharmacy customers on complementary medicines and pharmacy practice, BMC Complementary and Alternative Medicine, 10:38.

Brown, L., Mutter, R., and Halyard, M. (2015) Benefits, risks, and safety of external beam radiation therapy for breast cancer, International Journal of Women's Health, 7: 449–458.

Canadian Cancer Statistics, (2015). Government of Canada, Canadian Cancer Society. http://www.cancer.ca/~/media/cancer.ca/CW/cancer%20information/cancer%20101/Canadian%20cancer%20statistics/Canadian-Cancer-Statistics-2015-EN.pdf?la=en

Canadian Cancer Society. (2017a), Available from: http://www.cancer.ca/en/cancer-information/cancer-101/cancer-statistics-at-a-glance/?region=ab

Canadian Cancer Society. (2017b), Available from: http://www.cancer.ca/en/cancer-information/cancer-type/breast/statistics/?region=bc

Calapai, G. (2008) European legislation on herbal medicines: a look into the future, Drug Safety, 31 428–431.

Campisi, J. (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors, Cell, 120: 513-522.

Carpenter, R., and Miller, W. R. (2005) Role of aromatase inhibitors in breast cancer, British journal of cancer, 93(Suppl 1): S1–S5.

Carrick, S., Parker, S., Thornton, C.E., Ghersi, D., Simes, J., and Wilcken N. (2009) Single agent versus combination chemotherapy for metastatic breast cancer, The Cochrane Database of Systematic Reviews, 15;(2): CD003372.

Chien, Y.H., Jores, R., and Crowley, M. P. (1996) Recognition by γ/δ T cells, Annual Review of Immunology, 14: 511–532.

Chen, D.S., and Mellman I. (2013) Oncology meets immunology: the cancer-immunity cycle, Immunity, 39(1): 1–10.

Cheng, A.C., Jian, C.B., Huang, Y.T., Lai, C.S., Hsu, P.C., and Pan, M.H. (2007) Induction of apoptosis by Uncaria tomentosa through reactive oxygen species production, cytochrom c release, and caspases activation in human leukemia cells. Food and Chemical Toxicology ;45:2206–2218.

Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., et al. (2005) Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials, Lancet, 366(9503): 2087-106.

Claus, E.B., Risch, N.J., and Thompson, W.D. (1990) Age at onset as an indicator of familial risk of breast cancer, American Journal of Epidemiology,131: 961–972.

Cooper, G., and Hausman, R.E. (2000). The cell; a molecular approach, 2nd edition. Oxford: Sinauer Associates.

Couch, F.J., Kant, J., Calzone, K., DeShano, M., Peng, Y., Chen, K., Garber, J.E., and Weber, B.L. (1996) Genetic heterogeneity in hereditary breast cancer: role of BRCA1 and BRCA2, American Journal of Human Genetics, 59:547–53.

Collins, T., Williams, A., Johnston, G.I., Kim, J., Eddy, R., Shows, T., Gimbrone, M.A., and Bevilacqua, M.P. (1991) Structure and chromosomal location of the gene for endothelial-leukocyte adhesion molecule 1, The Journal of Biological Chemistry, 266 (4): 2466–73.

Colditz, G.A., Willett, W.C., Hunter, D.J., Stampfer, M.J., Manson, J.E, Hennekens, C.H, et al. (1993) Family history, age, and risk of breast cancer, Prospective data from the Nurses' Health Study, JAMA, 270(3): 338-43.

Cragg, G. M., Newman, D. J., Weiss, R. B. (1997) Coral reefs, forests, and thermal vents: the worldwide exploration of nature for novel antitumor agents, Seminars in Oncology, 24:156-163.

Crespo, J., Sun, H., Welling, T.H., Tian, Z., and Zou, W. (2013) T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment, Current Opinion in Immunology, 25(2): 214–221.

Darby, S.C., McGale, P., Taylor, C.W., and Peto, R. (2005) Long-term mortality from heart disease and lung cancer after radiotherapy for early breast cancer: prospective cohort study of about 300,000 women in US SEER cancer registries, The Lancet Oncology, 6:557–565.

Danial, N.N., and Korsmeyer, S.J. (2004) Cell death: critical control points, Cell, 116(2): 205–219.

De Jong, M., Nolte, I., et al. (2002) Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility, Journal of Medical Genetics, 39(4): 225–242.

De Martino, L., Martinot, J.L., Franceschelli, S., Leone, A., Pizza, C., and De Feo, V. (2006) Proapoptotic effect of Uncaria tomentosa extracts. Journal of Ethnopharmacology. 107, 91–94.

DePinho, R.A. (2000) The age of cancer, Nature, 408: 248-254.

Domingues, A., Sartori, A., Valente, L., Golim, M., Siani, A., Viero, R. (2011) Uncaria tomentosa Aqueous-ethanol Extract Triggers an Immunomodulation toward a Th2 Cytokine Profile. Phytotherapy Research ;25:1229–1235.

Dreifuss, A.A., Bastos-Pereira, A.L., Ávila, T.V., Soley, B.D., Rivero, A.J., Aguilar, J. L., and Acco, A. (2010) Antitumoral and antioxidant effects of a hydroalcoholic extract

of cats claw (*Uncaria tomentosa*) (Willd. Ex Roem. & Schult) in an *in vivo* carcinosarcoma model, Journal of Ethnopharmacology, 130(1), 127-133.

D'Urso, C.M., Wang, Z.G., Cao, Y., Tatake, R., Zeff, R.A., and Ferrone, S. (1991) Lack of HLA class I antigen expression by cultured melanoma cells FO-1 due to a defect in B2m gene expression, Journal of Clinical Investigation, 87(1): 284-92.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., and Schreiber, R.D. (2002) Cancer immunoediting: from immunosurveillance to tumor escape, Nature Immunology, 3(11): 991-8.

Dunn, G.P., Old, L.J., and Schreiber, R.D. (2004) The three Es of cancer immunoediting, Annual Review of Immunology, 22: 329–360.

Dunn G.P., Koebel C.M., and Schreiber R.D. (2006) Interferons, immunity and cancer immunoediting, Nature Reviews Immunology, 6(11): 836–848.

Echchakir, H., Mami-Chouaib, F., Vergnon, I., Baurain, J.F., Karanikas, V., Chouaib, S., et al. (2001) A point mutation in the alpha-actinin-4 gene generates an antigenic peptide recognized by autologous cytolytic T lymphocytes on a human lung carcinoma, Cancer Research, 61: 4078–83.

Eisenberg, D.M., Kessler, R.C., Foster, C., Norlock, F.E., Calkins, D.R., and Delbanco T.L. (1993) Unconventional medicine in the United States: prevalence, costs, and patterns of use, The New England Journal of Medicine, 328: 246-52.

Eisenberg, D.M., Davis, R.B., Ettner, S.L., Appel, S., Wilkey, S., et al. (1998) Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey, JAMA, 1280(18): 1569-75.

Ekor, M. (2013) The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety, Frontiers in Pharmacology, 4: 177.

Elmore, S. (2007) Apoptosis: A Review of Programmed Cell Death, Toxicologic Pathology, 35(4): 495–516.

Elting, L.S., Cooksley, C., Chambers, M., Cantor, S.B., Manzullo, E., and Rubenstein, E.B. (2003) The burdens of cancer therapy. Clinical and economic outcomes of chemotherapy-induced mucositis, Cancer, 98: 1531–1539.

Ewertz, M., and Jensen, A.B. (2011) Late effects of breast cancer treatment and potentials for rehabilitation, Acta Oncologica, 50(2), 187-193.

Farnsworth, N. R., and D. D. Soejarto. (1985) Potential consequences of plant extinction in the United States on the current and future availability of prescription drugs, Economic Botany, 39(3): 231–240.

Fazio, A., Ballen, D., Cesari, IM., Abad, M., Arsenak, M., and Taylor, P. (2008) An ethanolic extract of *Uncaria tomentosa* reduces inflammation and B16-BL6 melanoma growth in C57BL/6 mice, Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 7 (5): 217 – 224.

Falkiewicz, B., and Lukasiak, J. (2001) Vilcacora (*Uncaria tomentosa* (Willd.) DC. and *Uncaria guianensis* (Aublet) Gmell.)—A review of published scientific literature, Case Reports and Clinical Practice Review, 2(4), 305-316.

Firenzuoli, F., and Gori, L. (2007) Herbal medicine today: clinical and research issues, Evidence-Based Complementary and Alternative Medicine, 4(Suppl 1): 37–40.

Fisher, B., Costantino, J.P., Wickerham, D.L., Redmond, C.K., Kavanah, M., et al. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study, Journal of the National Cancer Institute, 90(18): 1371-1388.

Ford, D., and Easton, D.F. (1995) The genetics of breast and ovarian cancer, British Journal of Cancer, 72: 805–12.

Frass, M., Strassl, R., Friehs, H., et al. (2012) Use and Acceptance of Complementary and Alternative Medicine Among the General Population and Medical Personnel: A Systematic Review, Ochsner Journal, 12(1): 45–56.

Fraenkel, l., Bogardus J.r., Concato, J., and Wittink, D.R. (2004) Treatment Options in knee Osteoarthritis: The Patient's Perspective, Archives of Internal Medicine, 164(12): 1299–304.

Funes, L., Laporta, O., Cerdán-Calero, M., and Micol, V. (2010) Effects of verbascoside, a phenylpropanoid glycoside from lemon verbena, on phospholipid model membranes, Chemistry and physics of lipids, 163(2), 190-199.

Gaudet, M.M., Gapstur, S.M., Sun, J., Diver, W.R., Hannan, L.M., and Thun, M.J. (2013) Active smoking and breast cancer risk: Original cohort data and meta-analysis, Journal of the National Cancer Institute, 105(8): 515–525.

García Giménez, D., García Prado, E., Sáenz Rodríguez, T., et al. (2010) Cytotoxic effect of the pentacyclic oxindole alkaloid mitraphylline isolated from *Uncaria tomentosa* bark on human Ewing's sarcoma and breast cancer cell lines, Planta Medica, 76(2): 133-6.

Ganjei-Azar, P., and Nadji, M. (2007) Color Atlas of Immunocytochemistry in Diagnostic Cytology 2007th Edition, Springer, LLC. p. 47.

Garrido, F., Ruiz-Cabello, F., Cabrera, T., Perez-Villar, J.J., Lopez-Botet, M., Duggan-Keen, M., et al. (1997) Implications for immunosurveillance of altered HLA class I phenotypes in human tumours, Immunology Today, 18:89–9510.

Godet, I., and Gilkes, D.M. (2017) BRCA1 and BRCA2 mutations and treatment strategies for breast cancer, Integrative Cancer Science and Therapeutics, 4(1).

Goedendorp, M., Andrykowski, M., Donovan, K., et al. (2012) Prolonged Impact of Chemotherapy on Fatigue in Breast Cancer Survivors, Cancer, 118(15): 3833–3841.

Gonzales, G. F., and Valerio, L. G. (2006) Medicinal Plants from Peru: A Review of Plants as Potential Agents Against Cancer, Anti-cancer Agents in Medicinal Chemistry, 6(5): 429-44.

Gonçalves, C., Dinis, T., and Batista, M. T. (2005) Antioxidant properties of proanthocyanidins of *Uncaria tomentosa* bark decoction: a mechanism for anti-inflammatory activity, Phytochemistry, 66(1), 89-98.

Gogoi, D., and Chiplunkar, S.V. (2013) Targeting gamma delta T cells for cancer immunotherapy: bench to bedside, The Indian Journal of Medical Research, 138:755–761.

Golubicic, I., Borojevic, N., and Pavlovic, T. (2008) Risk factors for breast cancer: is ionizing radiation among them? Journal of the Balkan Union of Oncology, 13(4): 487-94.

Gram, I., Little, M., Lund, E., and Braaten, T. (2016) The fraction of breast cancer attributable to smoking: The Norwegian women and cancer study 1991–2012, British Journal of Cancer, 115(5): 616–623.

Greene, M.H. (1997) Genetics of breast cancer, Mayo Clinic Proceedings, 72:54–65.

Gregory, A.D., and Houghton, A.M. (2011) Tumor-associated neutrophils: new targets for cancer therapy, Cancer Research, 71(7): 2411–2416.

Gutcher, I., and Becher, B. (2007) APC-derived cytokines and T cell polarization in autoimmune inflammation, Journal of Clinical Investigation, 117 (5): 1119–27.

Gurrola-Díaz, C. M., García-López, P. M., Gulewicz, K., Pilarski, R., and Dihlmann, S. (2011) Inhibitory mechanisms of two *Uncaria tomentosa* extracts affecting the Wnt-signaling pathway, Phytomedicine, 18(8-9), 683-690.

Hemaiswarya, S., and Doble, M. (2006) Potential synergism of natural products in the treatment of cancer, Phytotherapy Research, 20(4): 239–49.

Heitzman, M.E., Neto, C.C., Winiarz, E., Vaisberg, A.J., and Hammond, G.B. (2005) Ethnobotany, phytochemistry and pharmacology of Uncaria (Rubiaceae), Phytochemistry, 66(1): 5-29.

Hernández-Aguilera, A., Rull, A., Rodríguez-Gallego, E., Riera-Borrull, M., Luciano-Mateo, F., Camps, J., et al. (2013) Mitochondrial dysfunction: a basic mechanism in inflammation-related non-communicable diseases and therapeutic opportunities, Mediators of inflammation.

Hoffman, W., Lakkis, F., and Chalasani, G. (2016) B Cells, Antibodies, and More, Clinical Journal of the American Society of Nephrology, 11(1): 137–154.

Hong-Fang, J. i., Xue-Juan, L. i., and Hong-Yu, Zhang. (2009) Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? The European Molecular Biology Organization, 10(3): 194-200.

Honório, I., Bertoni, B., and Pereira, A. (2016) Uncaria tomentosa and Uncaria guianensis an agronomic history to be written. Ciência Rural.

International Agency for Research on Cancer. (2004) IARC Monographs on Evaluation of Carcinogenic Risks to Humans: Tobacco Smoke and Involuntary Smoking. Lyon, France.

International Agency for Research on Cancer. Lyon: IARC Press; 2008. World Cancer Report, 2008. Edited by Boyle, P. and Levin, B.E.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., and Thun, M.J. (2007) Cancer statistics, 2007, A Cancer Journal for Clinicians, 57:43–66.

Jena, J., Ranjan, R., Ranjan, P., and Sarangi, M. (2012) A Study on Natural Anticancer Plants, Inrernational Journal of Pharmaceutical and Chemical Sinences, 1(1): 365–8.

Karp, G. (2008) Cell and molecular biology: Concepts and experiments. 5. John New Jersey: Wiley and Sons, pp. 653–657.

Karanikas, V., Colau, D., Baurain, J.F., Chiari, R., Thonnard, J., Gutierrez-Roelens, I., et al. (2001) High frequency of cytolytic T lymphocytes directed against a tumor-specific mutated antigen detectable with HLA tetramers in the blood of a lung carcinoma patient with long survival, Cancer Research, 61: 3718–24.

Keplinger, K., Laus, G., Wurm, M., Dierich, M.P., and Teppner, H. (1999) *Uncaria tomentosa* (Willd.) DC. ethnomedicinal use and new pharmacological, toxicological and botanical results, Journal of Ethnopharmacology, 64(1): 23–24.

Khan, S.A., and Wingard, J.R. (2001) Infection and mucosal injury in cancer treatment, National Cancer Institute monograph, (29): 31-6.

Kim, D., Emi, M., Tanabe, K. (2007) Cancer immunoediting from immune surveillance to immune escape, Immunology, 121(1): 1–14.

Kośmider, A., Czepielewska, E., Kuraś, M., Gulewicz, K., Pietrzak, W., Nowak, R., and Nowicka, G. (2017) *Uncaria tomentosa* Leaves Decoction Modulates Differently ROS Production in Cancer and Normal Cells, and Effects Cisplatin Cytotoxicity, Molecules, *22*(4), 620.

Koehn, F.E, and Carter, G.T. (2005) The evolving role of natural products in drug discovery, Nature Reviews Drug Discovery, 4(3): 206–20.

Koretzky, G.A. (2010) Multiple roles of CD4 and CD8 in T cell activation, Journal of Immunology,185(5): 2643–2644.

Kumar, N., George, C., Suresh, P., and Kumar, A. (2012) Cytotoxicity, apoptosis induction and anti-metastatic potential of Oroxylum indicum in human breast cancer cells, Asian Pacific Journal of Cancer Prevention, 13(6): 2729–34.

Kuhns, M.S., Davis, M.M., and Garcia, K.C. (2006) Deconstructing the form and function of the TCR/CD3 complex, Immunity, 24(2): 133-9.

Lane, D.P. (1992) p53, guardian of the genome, Nature, 358: 15–16.

Laus, G., Brössner, D., and Keplinger, K. (1997) Alkaloids of Peruvian *Uncaria tomentosa*, Phytochemistry, 45: 855–860.

Lakhani, S.R., Ellis, I.O., Schnitt, S.J., Tan, P.H., and Van de Vijver, M.J. (2012) WHO Classification of Tumours of the Breast. Fourth Edition, IARC; Lyon. ISBN.13.

Li, C.I., Anderson, B.O., Daling, J.R., and Moe R.E. (2003) Trends in incidence rates of invasive lobular and ductal breast carcinoma, The Journal of the American Medical Association, 289(11): 1421-4.

Li, L.T., Jiang, G., Chen, Q., and Zheng J.N. (2015) Ki67 is a promising molecular target in the diagnosis of cancer (review), Molecular Medicine Reports, (3):1566-72.

Lippitz, B.E. (2013) Cytokine patterns in patients with cancer: a systematic review, Lancet Oncology, 14(6): e218-28.

Liu, Y., Zhang, Z. S., He, Y, L., Zhang, B. G. and Li, X. E. (2007) Quality of crude traditional Chinese drugs and ecological environment. Modernization of Traditional Chinese Medicine and Materia Materia-World Science and Technology 9, 65–69.

Lonning, P.E., Geisler, J., Krag, L.E., Ottestad, L., Bremnes, Y., Hagen, A. I., et al. (2004) Effect of exemestane on bone: a randomized placebo controlled study in postmenopausal women with early breast cancer at low risk, Journal of Clinical Oncology, 22(14_suppl), 518-518.

Luna-Palencia, G.R., Huerta-Heredia, A.A., Cerda-García-Rojas, C.M., and Ramos-Valdivia, A.C. (2013) Differential alkaloid profile in *Uncaria tomentosa* micropropagated plantlets and root cultures, Biotechnology Letters, 35: 791–797.

Luckheeram, R.V., Zhou, R., Verma, A.D., and Xia, B. (2012) CD4 +T cells: differentiation and functions, Clinical and Developmental Immunology, 2012: 925135.

Lu, C., Sun, H., Huang, J., et al. (2017) Long-Term Sleep Duration as a Risk Factor for Breast Cancer: Evidence from a Systematic Review and Dose-Response Meta-Analysis, BioMed Research International.

Makki, J. (2015) Diversity of Breast Carcinoma: Histological Subtypes and Clinical Relevance, Clinical Medicine Insights Pathology, 8: 23–31.

Marlin, S., and Rothlein, R. (1998) Intercellular Adhesion Molecules: ICAM-1, ICAM-2 And ICAM-3, Encyclopedia of Immunology (Second Edition), Pages 1409-1412.

McCredie, M.R., Dite, G.S., Porter, L., Maskiell, J., Giles, G.G., Phillips, K.A., Redman, S., and Hopper, J.L. (2001) Prevalence of self-reported arm morbidity following treatment for breast cancer in the Australian Breast Cancer Family Study, Breast, 10(6): 515-22.

McDonald, E., Clark, A., Tchou, J., Zhang, P., and Freedman, G. (2016) Clinical Diagnosis and Management of Breast Cancer, Journal of Nuclear Medicine, 57 Suppl 1:9S-16S.

Mettlin, C. (1999) Global breast cancer mortality statistics, A Cancer Journal for Clinicians, 49: 138–44.

Mellman, I., Coukos, G., and Dranoff, G. (2011) Cancer immunotherapy comes of age, Nature, 480(7378): 480–489.

Mondal, D., and Sharma, D. (2016) External beam radiation techniques for breast cancer in the new millennium: New challenging perspectives, Journal of the Egyptian National Cancer Institute, Pages 211-218.

Mukherjee P.K. (2002) Quality Control of Herbal Drugs: An Approach to Evaluation of Botanicals, New Delhi, India: Business Horizons.

Murphy, K., Travers, P., and Walport, M. (2008) Janeway's Immunobiology 7th. New York, NY, USA: Garland Science Taylor and Francis Group. Principles of innate and adaptive immunity, pp. 1–38.

Nagata S. (2000) Apoptotic DNA fragmentation, Experimental Cell Research, 256(1): 12-8.

Narod, S., Iqbal, J., Miller, A. (2015) Why have breast cancer mortality rates declined?, Journal of Cancer Policy, Pages 8-17

National Cancer Institute (NCI). “Types of Cancer Treatment”. April 6, 2017. Avaibale from: https://www.cancer.gov/about-cancer/treatment/types

National Cancer Institute (NCI). "Defining Cancer". 2007-09-17. 28 March 2018. Avaibale from: https://www.cancer.gov/about-cancer/understanding/what-is-cancer

Navarro Hoyos, M., Sánchez-Patán, F., Murillo Masis, R., Martín-Álvarez, P.J., Zamora Ramirez, W., Monagas, M. J., and Bartolomé, B. (2015) Phenolic assesment of *Uncaria tomentosa* L.(Cat’s Claw): Leaves, stem, bark and wood extracts, Molecules, 20(12), 22703-22717.

Navarro-Hoyos, M., Alvarado-Corella, D., Moreira-Gonzalez, I., Arnaez-Serrano, E., and Monagas-Juan, M. (2018) Polyphenolic Composition and Antioxidant Activity of

Aqueous and Ethanolic Extracts from *Uncaria tomentosa* Bark and Leaves, Antioxidants, 7(5), 65.

Nelson, JC., Beitsch, P.D., Vicini, F.A., Quiet, C.A., et al. (2009) Four-year clinical update from the American Society of Breast Surgeons MammoSite brachytherapy trial, The American Journal of Surgery, 198(1): 83-91.

O'Rourke, A., and Mescher, M. (1993) The roles of CD8 in cytotoxic T lymphocyte function, Immunology Today, 14(4): 183-8.

Pal, S.K., and Shukla, Y. (2003) Herbal medicine: current status and the future, Asian Pacific Journal of Cancer Prevention, 4(4): 281–8.

Pagan, J.A., and Pauly, M.V. (2005) Access to Conventional Medical Care and the Use of Complementary and Alternative Medicine, Health Affairs, 24(1): 255–62.

Paniagua-Pérez, R., Madrigal-Bujaidar, E., Molina-Jasso, D., Reyes-Cadena, S., Álvarez-González, I., et al. (2009) Antigenotoxic, antioxidant and lymphocyte induction effects produced by pteropodine, Basic and Clinical Pharmacology and Toxicology, 104: 222–227.

Palucka, K., and Banchereau, J. (2012) Cancer immunotherapy via dendritic cells, Nature Reviews Cancer, 12(4):265–277.

Peto, R., Parish, S.E, Gray, R.G. (1985) There is no such thing as ageing, and cancer is not related to it, IARC Publications Website, 43-53.

Peter, B., and Bernard, L. (2008) World Cancer Report, International Agency for Research on Cancer; WHO Press; Lyon, France.

Perez, B., Benitez, R., Fernandez, M.A., Oliva, M.R., Soto, J.L., Serrano, S., et al. (1999) A new beta 2 microglobulin mutation found in a melanoma tumor cell line, Tissue Antigens, 53(6): 569-72.

Pegram, M., Konecny, G., et al. (2004) Rational Combinations of Trastuzumab With Chemotherapeutic Drugs Used in the Treatment of Breast Cancer, Journal of the National Cancer Institute, Pages 739–749.

Pilarski, R., Poczekaj-Kostrzewska, M., Ciesiołka, D., Szyfter, K., Gulewicz, K. (2007) Antiproliferative activity of various *Uncaria tomentosa* preparations on HL-60 promyelocytic leukemia cells, Pharmacological Reports, 59: 565–572.

Pilarski, R., Filip, B., Wietrzyk, J., Kuraś, M., and Gulewicz, K. (2010) Anticancer activity of the *Uncaria tomentosa* (Willd.) DC. preparations with different oxindole alkaloid composition, Phytomedicine, 17(14), 1133-1139.

Pinho, S.S., and Reis C.A. (2015) Glycosylation in cancer: mechanisms and clinical implications, Nature Reviews Cancer, 15(9): 540–555.

Poortmans, P. (2007) Evidence based radiation oncology: breast cancer, Radiotherapy and Oncology, 84(1): 84–101.

Polgár, C., and Major, T. (2009) Current status and perspectives of brachytherapy for breast cancer, International Journal of Clinical Oncology, 14 (1): 7–24.

Podojil, J.R., and Miller, S.D. (2009) Molecular mechanisms of T-cell receptor and costimulatory molecule ligation/blockade in autoimmune disease therapy, Immunological Reviews, 229(1): 337–355.

Preston, D.L., Mattson, A.M., Holmberg, E., Shore, R., et al. (2002) Radiation effects on breast cancer risk: a pooled analysis of eight cohorts, Radiation Research,158: 220–235.

Principe, D.R., Doll, J.A., Bauer, J., Jung, B., Munshi, H.G., Bartholin, L., et al. (2014) TGF-beta: duality of function between tumor prevention and carcinogenesis, Journal of the National Cancer Institute, 106: djt369.

Qi, F., Li, A., Inagaki, Y., Gao, J., Li, J., Kokudo, N., et al. (2010) Chinese herbal medicines as adjuvant treatment during chemo- or radiotherapy for cancer, BioScience Trends, 4(6): 297–307.

Rajabi, M., and Mousa, S. (2017) The Role of Angiogenesis in Cancer Treatment, Biomedicines, 5(2): 34.

Rajesh, E., Sankari, L., Malathi, L., and Krupaa, J. (2015) Naturally occurring products in cancer therapy, Journal of Pharmacy and Bioallied Sciences, 7(Suppl 1): S181–S183.

Ramachandran, C., Rodriguez, S., Ramachandran, R., et al. (2005) Expression profiles of apoptotic genes induced by curcumin in human breast cancer and mammary epithelial cell lines, Anticancer Research, 25(5): 3293–302.

Reed, J.C. (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in haematologic malignancies, Semin Haematol, 34:9–19.

Reeder, J.G, and Vogel, V.G. (2008) Breast cancer prevention, Cancer treatment and research, 141: 149–64.

Reinhard, K.H (1999) *Uncaria tomentosa* (Willd.) D.C.: cat's claw, una de gato or saventaro, The Journal of Alternative and Complementary Medicine 5: 143–51.

Rinner, B., Li, Z., Haas, H., Siegl, V., Sturm, S., Stuppner, H., and Pfragner, R. (2009) Antiproliferative and Pro-apoptotic Effects of *Uncaria tomentosa* in Human Medullary Thyroid Carcinoma Cells, Anticancer Research, 29(11): 4519-28.

Rizzi, R., Re, F., Bianchi, A., De Feo, V., and De Simone F., et al. (1993) Mutagenic and antimutagenic activities of *Uncaria tomentosa* and its extracts, Journal of Ethnopharmacology, 38: 63–77.

Riva, L., Coradini, D., Di Fronzo, G., De Feo, V., De Tommasi, N., et al. (2001) The antiproliferative effects of *Uncaria tomentosa* extracts and fractions on the growth of breast cancer cell line, Anticancer Research, 21: 2457–2461.

Rosenberg, S.M., and Partridge, A.H. (2013) Premature menopause in young breast cancer: effects on quality of life and treatment interventions, Journal of Thoracic Disease, 5(Suppl 1): S55–S61.

Rojas-Duran, R., González-Aspajo, G., Ruiz-Martel, C., Bourdy, G., Doroteo-Ortega, V.H., Alban-Castillo, J., et al. (2012) Anti-inflammatory activity of Mitraphylline isolated from *Uncaria tomentosa* bark, Journal of ethnopharmacology, 143(3), 801-804.

Russo, J., Hu, Y., Yang, X., and Russo, I. (2000) Chapter 1: Developmental, Cellular, and Molecular Basis of Human Breast Cancer, Journal of the National Cancer Institute (JNCI) Monographs, Pages 17–37.

Saklani, A., and Kutty, S.K. (2008) Plant-derived compounds in clinical trials, Drug Discovery Today, 13(3-4): 161–71.

Scheuermann, R.H., and Racila, E. (1995) CD19 antigen in leukemia and lymphoma diagnosis and immunotherapy, Leukemia and Lymphoma, 18 (5–6): 385–97.

Schreiber, R.D., Old L.J., and Smyth, M.J. (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion, Science, 331(6024): 1565–1570.

Sheng, Y., Pero, R.W, Amiri, A., and Bryngelsson, C. (1998) Induction of apoptosis and inhibition of proliferation in human tumor cells treated with extracts of *Uncaria tomentosa*, Anticancer Research, 18: 3363–3368.

Sheng, Y., Pero, R.W., and Wagner, H. (2000) Treatment of chemotherapy-induced leukopenia in a rat model with aqueous extract from *Uncaria tomentosa*, Phytomedicine, 7(2): 137–143.

Sharma, G., Dave, R., Sanadya, J., Sharma, P., and Sharma, K. (2010) various types and management of breast cancer: an overview, Journal of Advanced Pharmaceutical Technology & Research, 1(2): 109–126.

Sharpe, A.H. (2009) Mechanisms of costimulation, Immunological Reviews, 229(1): 5–11.

Singletary, K.W., and Gapstur, S.M. (2001) "Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms", The Journal of the American Medical Association, 286 (17): 2143–51.

Smith-Garvin, J.E. Koretzky, G.A., and Jordan, M.S. (2009) T cell activation, Annual Review of Immunology, 27:591-619.

Sun, J. C., and Lanier, L. L. (2009) Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity? European Journal of Immunology, 39(8): 2059–2064.

Taraphdar, A.K., Roy, M., and Bhattacharya, R.K. (2001) Natural products as inducers of apoptosis: Implication for cancer therapy and prevention, Current Science, 80(11): 1387–96.

Terabe, M., and Berzofsky J.A. (2008) The role of NKT cells in tumor immunity, Advances in Cancer Research,101: 277–348.

Tsujimoto,Y., Finger, L.R., Yunis, J., Nowell,P.C., and Croce, C.M. (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation, Science, 226,1097–1099.

Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C.M. (1985) Involvement of the bcl-2 gene in human follicular lymphoma, Science, 228, 1440–1443.

Vaux, D.L., Cory, S., and Adams, J.M. (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells, Nature, 335, 440–442.

van Leeuwen, F.E., Klokman, W.J., Stovall, M., et al. (2003) Roles of radiation dose, chemotherapy, and hormonal factors in breast cancer following Hodgkin's disease, Journal of the National Cancer Institute, (13):971-80.

van Meerloo, J., Kaspers, G.J., and Cloos, J. (2011) Cell sensitivity assays: the MTT assay, Methods in Molecular Biology, 731:237-45.

Vinh-Hung, V., and Verschraegen, C. (2004) Breast-conserving surgery with or without radiotherapy: pooled-analysis for risks of ipsilateral breast tumor recurrence and mortality, Journal of the National Cancer Institute, 96:115–121.

Vogelstein, B., and Kinzler, K.W. (2004) Cancer genes and the pathways they control, Nature medicine, 10(8), 789-799.

Wagner, H., Kreutzkamp, B., and Jurcic, K. (1985) Alkaloids from *Uncaria tomentosa* and their phagocytosis enhancement effect, Planta Medica 5: 419–423.

Wallace-Brodeur, R.R., and Lowe, S.W. (1999) Clinical implications of p53 mutations. Cell Mol, Life Sciences, 55, 64–75.

Waldhauer, I., and Steinle, A. (2008) NK cells and cancer immunosurveillance, Oncogene, 27(45): 5932–5943.

Wang, R.F., Wang, X., Atwood, A.C., Topalian, S.L., and Rosenberg, S.A. (1999) Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen, Science, 284(5418): 1351-4.

Wheat, J., Currie, G. (2008) Herbal medicine for cancer patients: An evidence based review, International Journal of Complementary and Alternative Medicine, 5(2).

Winer E,P., Morrow, M., Osborne, C.K., and Harris, J.R. (2001) Cancer of the breast. In: Cancer: Principles and Practice of Oncology 6th edition. De Vita, V.T., Hellman, S., and Rosenberg, S.A. Philadelphia: Lippincott Williams and Wilkins, 1264-1333.

Wirth, C., and Wagner, H. (1997) Pharmacologically active phenolic compounds from the bark of Mahonia aquifolium, Phytomedicine, 4(4): 357-8.

Wong, R. (2011) Apoptosis in cancer: from pathogenesis to treatment, Journal of Experimental and Clinical Cancer Research, 30(1): 87.

Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., et al. (1995) A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma, Science, 269: 1281–410.

Workman, C., Szymczak-Workman, A., Collison, L., Pillai, M., and Vignali, D. (2009) The Development and Function of Regulatory T Cells, Cellular and Molecular Life Sciences, 66(16): 2603–2622.

World Health Organization (WHO). (2018a) Cancer Fact sheet N°297, Available from: https://www.who.int/en/news-room/fact-sheets/detail/cancer

World Health Organization (WHO). (2018b) Breast Cancer, Available from: http://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/

Wurm, M., Kacani, L., Laus, G., Keplinger, K., and Dierich, M.P. (1998) Pentacyclic oxindole alkaloids from *Uncaria tomentosa* induce human endothelial cells to release a lymphocyte-proliferation-regulating factor, Planta Medica, 64(8): 701–704.

Yang, H., Chen, P., and Wang, J. (2014) Chinese Herbs Containing Aristolochic Acid Associated with Renal Failure and Urothelial Carcinoma: A Review from Epidemiologic Observations to Causal Inference, BioMed Research International, (6): 569325.

Yankeelov, T., Pickens, D., and Price, R. (2011) Quantitative MRI in Cancer 1st Edition, CRC Press.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6, Nature, 352, 345–347.

Zhang, Q., Zhao, J.J., Xu, J., Feng, F., Qu ,W. (2015) Medicinal uses, phytochemistry and pharmacology of the genus Uncaria. Review. Journal of Ethnopharmacology; 173:48–80.

Zhu, J., Yamane, H., and Paul, W.E. (2010) Differentiation of effector CD4 T cell populations, Annual Review of Immunology, 28:445-89.

Zhu, S., Liu, W., Ke, X., Li, J., et al. (2014) Artemisinin reduces cell proliferation and induces apoptosis in neuroblastoma, Oncology Reports, Pages: 1094-1100.

Ziegler, U., and Groscurth, P. (2004) Morphological features of cell death, News in physiological sciences, 19: 124–128.