

**TREATMENT WITH EXTRACTS OF *UNCARIA TOMENTOSA*
PROMOTES APOPTOSIS IN THE HUMAN BREAST CANCER
CELL LINE, MCF7**

By

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**A thesis submitted in partial fulfillment
of the requirements for the degree of
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Abstract

Uncaria tomentosa is a medicinal plant native to Peru which has been used traditionally for the treatment of various inflammatory disorders and cancer. Some studies have shown that treatment with *Uncaria tomentosa* promotes repair of cellular DNA in patients treated with chemotherapy drugs, preventing mutations and cell damage. Treatment with *Uncaria tomentosa* also inhibits inflammatory responses by inhibiting the proliferation of T and B-lymphocytes and decreasing the production of pro-inflammatory cytokines (IL-1, IL-6, and TNF- α). We have examined the effects of *Uncaria tomentosa* extracts on the growth of malignant cells such as MCF-7 and MDA-MB-231 cells, human breast cancer cell lines and non-malignant cells such as HBL-100, HEK 293T and HSG cells. Our results have shown that treatment of malignant cells and non-malignant cells with *Uncaria tomentosa* extracts inhibits their proliferation and promotes cell death in a dose-dependent manner. Further, extracts produced by boiling the ground bark in 70% ethanol are much more effective than extracts produced by boiling in water. *Uncaria tomentosa*-ethanol extracts potently induce cellular apoptosis as measured by changes in cell morphology, chromatin condensation (Acridine Orange/Ethidium Bromide staining assay) and DNA fragmentation (TUNEL assays) within 24 h of treatment. Overall, *Uncaria tomentosa* appeared to kill breast cancer cells effectively *in vitro* by increasing cellular apoptosis.

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Abbreviations

AO/ EB	Acridine orange/ethidium bromide staining
ATCC	American Type Culture Collection
Bcl- 2	B-cell lymphoma 2
BRCA1	Breast cancer type1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BCT	Breast conservation therapy
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Estrogen receptor
EtOH	Ethanol
HBL100	Non-malignant human breast cells
HEK293T	Human Embryonic Kidney 293 cell line
HELA	Human cervical carcinoma cell line
HER2	Human epidermal growth factor receptor 2
HRT	Hormone replacement therapy
HSG	Human salivary gland cell line
MCF7	Human ER-positive Breast cancer cell line
MDA-MB-231	Human triple negative breast cancer cell line
MTT	Methyl thiazol tetrazolium assay
P53	Tumor suppressor p53

PBS	Phosphate buffered saline
PR	Progesterone receptor
ROS	Reactive oxygen species
SAOS	Human osteosarcoma cell line
SDS- PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-Buffered Saline
TUNEL	Terminal deoxynucleotidyl transferase

Chapter 1: Introduction

1.1. Cancer

Cancer is a worldwide killer; is a primary cause of death among many populations and is a major health burden in Canada. Cancer accounts for about a quarter of all deaths in Canada and is the second common reason for death after heart disease (Canadian Cancer Society, 2014). Almost half of Canadians will be diagnosed with cancer at some time during their lives. According to estimates in 2014, 191,300 cases of cancer were diagnosed in all ages, mostly over the age 50, and most of these cases were lung, breast, prostate and colorectal cancers. By 2020, the population worldwide is expected to have risen to 7.5 billion; approximately 15 million of this number will be diagnosed with cancer and 12 million cancer patients will die from different types of cancer (Anand et al., 2008).

Cancer can be defined simply as uncontrolled cell division with the ability to spread either by invasion or metastases (Gaaib et al., 2014). Invasion refers to the movement of cancer cells from the primary site to surrounding tissues (Carey et al., 2012). Metastasis is defined as the transported of malignant cells to distant organ of the body which is the main reason for cancer related-mortality (Banfalvi, 2012)

Cancer is a complex disease initiated in normal cells because of a series of genetic changes such as activation of oncogenes or inactivation of *tumour* suppressor genes and epigenetic changes that promote the stimulation of cell proliferation (Weinberg, 1989; Vogelstein and Kinzler, 2004). It involves abnormal cell growth and proliferation through

a variety of pathways leading to *tumour* formation with the potential to spread throughout the body, which is called metastases (Orr et al., 2000).

The formation of cancer includes a multistep process cellular and molecular which involving mutation and uncontrolled cell division (Weinberg, 1989; 2007). These processes take many years to develop. Fundamentally, cancer is a genetic disease that arises from the accumulation of multiple mutations and altered expression of genes in the normal cells leading misbehaves to these cells and additional DNA damaged (Vogelstein and Kinzler, 2004).

Changes in the activity of various genes and proteins that regulate cell growth and differentiation are involved in the progressive transformation of normal cells into highly malignant cells (Weinberg, 1989; 2007; Vogelstein and Kinzler, 2004). The malignant cell is characterized by specific properties: resistance to apoptosis; invasive cell division, increased cell mobility, and metastases (Baba and Cătoi, 2007). However, not all *tumours* are malignant. There are two types of *tumours*; benign or malignant with benign tumours defined as lacking the ability or invade or spread. Most malignant *tumours* are carcinomas, which represent 90% of human cancer and arise from epithelial tissues such as those in skin, lungs, and mammary glands. Unlike carcinomas, sarcomas are malignancies that occur at lower frequency and arise from non-epithelial cells such as osteoblasts and fibroblasts (mesophyll cells) (Weinberg, 2007).

Different internal factors and acquired factors contribute to the development of human cancers. In most cases, cancers are largely the result of exposure to environmental factors and unhealthy lifestyle (90–95%). The direct contribution of

genetic predisposition such as inherited mutation and hormones is estimated at 5–10% (Frank, 2004).

Environmental carcinogens, defined broadly to include tobacco use, diet, sun exposure, radiation, stress and infectious diseases all enhance cancer risks. Unhealthy lifestyle which known as lack of activity, obesity, poor diet and alcohol consumption also contribute to the formation of tumours. In other words, the process of *tumour* formation is a multifactorial and results from a complex interaction between several factors that influence the development of cancer particularly in an ageing population (McPherson et al., 2000).

Improving survival rates and decreasing mortality by increasing prevention efforts leads to reduced incidence rates or result in earlier diagnosis and treatment is the current goal of most anticancer programs (Canadian Cancer Statistics, 2013).

1.2. Breast cancer

Breast cancer is the most commonly cancer diagnosed among Canadian females and is the second most frequent type of cancer that increases the mortality rate in women (Canadian Cancer Statistics, 2013). Breast cancer has been the subject of many epidemiological and genetic research studies, which has shown it is a heterogeneous group of diseases. Male breast cancer is less common and accounts for 1% of breast cancer cases and deaths. However, the mortality of this disease hasn't changed for the past five decades among the female populations (Russo, 2000). According to the Canadian Cancer Society, breast cancer is the most frequently cancer in the Canadian

women, with an estimated 24,400 new cases diagnosed in 2014 and 5000 deaths. Despite importance advances in early diagnosis and treatment, it continues to develop and is the most frequent cause of cancer deaths globally in women (Jemal et al., 2011). Therefore, extensive research has been done to explore treatment options for this disease. Moreover, it is estimated that 1 in 9 women will develop breast cancer at some point in her lifetime and that 1 in 30 will die of it (The Breast Cancer Foundation, 2014). Importantly, breast cell division is stimulated by estrogen that can increase chances of developing breast cancer because of the high level of estrogen production in women's bodies (Hilakivi-Clarke et al., 2001).

1.3. Types of breast cancer

Each breast is made up of 15 to 20 glands called lobes, which have small structures called lobules. These lobes are connected to the nipple by tiny canals called ducts. The rest of the breast is fat. Breast cancer starts in the tissues of the breast which can extend up to the collarbone and from the armpit to the breastbone. Breast cancers are classified depending on which breast tissues develop the *tumours*. Ductal carcinoma arises from epithelial cells in the milk ducts tubes that carry breast milk to the nipple and can metastasize to surrounding tissues. It is the most common histological form of breast cancer (Albrektsen et al., 2010) and represents roughly 85% of all invasive breast cancers. Lobular carcinoma arises from epithelial cells in milk glands where the milk is produced and can also spread to other parts of the body. It accounts for around 15% of all invasive breast cancer cases (Reed et al., 2015).

Invasive ductal and lobular carcinomas refer to breast cancer where the breast cells can metastasize over time to the lymph nodes and other areas of the body through the bloodstream or lymphatic system. On the other hand, *in situ* ductal and lobular breast cancers don't have the ability to invade beyond their original location (Harold et al., 1998). Due to this reason, *in situ* ductal carcinoma is considered an early stage of breast cancer so treatment will be effectively for most women. However, it requires treatment because even though it is not life-threatening it increases the risk of developing into an invasive breast cancer. Recently, the rate of women who are diagnosed with DCIS has increased dramatically because of widespread screening with mammograms (Lenavo et al., 2014). However, *in situ* lobular cancer is not a true malignant cancer but is an indicator that a female is at a high risk to develop invasive cancer later in life.

There are also other rare types of invasive breast cancer beside these common types of invasive cancer such medullary, tabular, mucinous, etc. (Winer et al., 2001). Medullary carcinoma accounts for 5% of all cases of invasive breast cancer and are more common in women in their late 40s and 50s. Mucinous carcinoma represents less than 2% of all breast cancers (Zhang et al., 2014) and it tends to be less aggressive than other type of invasive carcinoma (Schneider et al., 2011). Tabular carcinoma is an uncommon type of breast carcinoma (Romano et al., 2015). It makes up about 1% to 4% of all breast cancer and usually grows slowly (Rakha et al., 2009).

1.4. Risk factors for breast cancer

There are several risk factors that promote the chances to develop breast cancer. Every woman is at risk of developing a breast cancer in her lifetime. Breast cancer is a

complex disease that results from multiple environmental and hereditary risk factors (Perera, 1997). Genetic factors are thought to be a relatively weak contributor to breast cancer (Venitt, 1994) and accounts for about 5% to 10% of all breast cancer risk (Perera, 1997). However, environment factors are highly associated with an increased risk of breast cancer incidences (Strumylaitė et al., 2009). There are several environmental conditions that play more important roles in the growing incidence of breast cancer, which can be classified as modifiable risk factors and non-modifiable factors (Petracci et al., 2011). The modifiable risk factors include body weight, alcohol consumption, radiation exposure, smoking, physical activity, and hormone replacement therapy. The non-modifiable risk factors are female gender, advanced age, an early menarche, late menopause, breast density, and family history.

1.4.1. Non-modifiable risk factors

Although men can be diagnosed with breast cancer, the risk of developing the disease is 100 times more likely for a female (Meister et al., 2002). Women have significantly higher breast cancer incidences because they have more breast tissue than men.

The rates of breast cancer incidence clearly increase with age (McPherson et al., 2000). For every 10 years of age, the incidence of breast cancer doubles for women before menopause. Women with early menarche (12 years or younger) and late menopause (55 years or older) are at significantly higher risk of developing breast cancer. Early menarche is associated with early onset of regular menstrual cycles that increases the hormone exposure that associates with ovulatory cycles. Moreover, women with

menarche prior to 12 years of age produce relatively high estrogen concentrations compared to those starting menarche later (Kelsey et al., 1993). Women who first menstruated at age 15 had a 23% lower risk of breast cancer than those who start menarche at age 12 or younger (Brinton et al., 1988).

Mammograms measure the proportion of fat (which has low density) and the presence of areas of high cell concentration in the breast. Breast density is related to age with an estimated 50% of women of age between 40-49 and 30% of women aged 70 or more shown to have dense breast tissue (50 %) (Harvey and Bovberg, 2004). A dense breast has the potential to be use as a predictor of breast cancer risk especially in older women (McCormack and dos Santos Silva, 2006). The increasing ratio of developing breast cancer is a four to six fold (White, 2000) for women with 75% dense breast (Bolan, 2013). In addition, the risk percentage of breast cancer attributable to any dens breast is 40% (Harvey and Bovberg, 2004).

1.4.2. Family history of cancer

Risk associated with family history of breast cancer varies depends on the number of affected family members, the age of the family member at diagnosis, and the degree of relatedness. A single case of breast cancer among first-degree relative, increases the risk of developing cancer up to two–to threefold (Hulka et al., 2001). This indicates that the individual risk among women with a family history of breast cancer is higher than their age-associated risk. However, the vast majority of women who get breast cancer have no such family history of the disease.

However, family history is a key of risk factor in women that develop invasive breast cancer and this risk is highly increased when first-degree relative have developed breast cancer before the age of 50 years, bilateral breast cancer, ovarian cancer or male relatives with breast cancer (Warner et al., 2011). It provides clues as to hereditary breast cancer, which accounts for approximately 7% of overall breast cancer risk (Hulka et al., 2001). Importantly, about 20% to 25% of the excess familial risk is contributed by the genetic factors that are known to be associated with mutations in the BRCA1 and BRCA2 genes (Laloo and Evans, 2012).

BRCA1 and BRCA2 are high-penetrance breast cancer susceptibility genes and it is well known as their mutations confer a high risk of hereditary breast cancer (Afonso, 2009). These genes are highly associated with developing breast cancer at both early and later age. Therefore, a mutated BRCA1 dramatically increases the average risk of breast cancer risk by age of 70 to between 55% and 85%, while the average range is between 37% and 85% for BRCA2 mutations (Afonso, 2009). However, it is estimated that about 55–70% of individuals who do not have family risk but carry mutation in BRCA1 are at high risk of developing breast cancer (Laloo and Evans, 2012). In contrast, the likelihood for women with no family history and without the BRCA1 or 2-gene mutations is 12% (Antoniou et al., 2003).

BRCA1, which plays a critical role in genomic stability, was identified in 1990 and appears to be linked to breast cancer and ovarian cancer (National Center for Biotechnology Information, 1998). BRCA2 appears to be linked to male breast cancer (National Center for Biotechnology Information, 1998). These two human genes produce

tumour suppressor proteins that repair damaged DNA or destroy cells when DNA cannot be repaired. When one of these genes is altered, the protein will not function properly and DNA damage may not be repaired properly, which can generate cancer-causing mutations.

Furthermore, harmful mutations in both genes raise the chances of developing different types of cancers in addition to breast and ovarian cancer, such as prostate cancer for men (Levy-Lahad et al., 2007) and pancreatic cancer for both genders (Ferrone et al., 2009).

1.4.3. Modifiable risk factors for breast cancer

Many studies have suggested that a high level exposure to ionizing radiation or repeated chest fluoroscopies or examination by x-ray can induce breast cancer. The risk of developing cancer varies according to dose of exposure, age of exposure, and time after exposure (Land et al., 1980). Females between 10 and 20 years are at greater risk of developing breast cancer following radiation exposure as the breast tissue is rapidly developing and responds significantly to the maximum effects of radiation. Additionally, this risk is highly increased during adolescence and remains high for 10 to 15 years after the initial exposure (Singletary, 2003). Therefore, this risk decreases by age 30 (Kelsey et al., 1998). This association has been derived from studies of both atomic bomb survivors and women who received radiation therapy (Singletary, 2003).

Breast cancer incidence appears to rise during the use of hormonal replacement therapy (HRT). Although HRT, which involves treatment with estrogen with or without

progestin, has been used to relieve menopausal symptoms and prevent osteoporosis, the risk of breast cancer was found to be relatively increased by 2.3% for every year of use by women without breast cancer (Colditz, 1998). Importantly, the National Institutes of Health and Epidemiologic Studies have shown that recent use of HRT was associated with a greater risk of breast cancer than for those who stopped this treatment for a long period of time (Singletary, 2003).

Multiple studies indicate that moderate alcohol intake has no significant effect on breast cancer incidence rates (Singletary, 2003; Longnecker, et al., 1988). However, numerous epidemiologic studies support a positive but not strong association between alcohol consumption and breast cancer (Longnecker et al., 1988; Zhang, et al., 2007). A meta-analysis of 53 epidemiologic studies showed that a comparison of drinkers who drink more than 45 grams of alcohol daily versus non-drinkers revealed 1.5 times more risk of developing breast cancer (Hamajima et al., 2002).

1.5. Tumor receptors

There are three major function *tumour* receptors, which can be used to indicate cancer status and which play a critical role in *tumour* development and progression (Mankoff et al., 2008). These receptors are the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Cadoo et al., 2013). Additionally, these receptors have been used for diagnosis, classification of breast cancer subtypes, and *tumour* specific therapy.

Overexpression of hormone receptors is related with neoplastic proliferation for some *tumour* types including breast cancer (Huber et al., 2009). Most breast tumours express ER hormone receptor, which accounts for 75% of all invasive breast cancer followed by PR, which accounts for 65% of breast cancers. Both ER and PR receptors are the targets for endocrine therapy such as tamoxifen treatment. On the other hand, HER-2 expression accounts for 15-20% of all invasive breast cancers. HER2- positive expression in *tumours* tends to indicate more aggressive tumours that are more responsive to drug treatment (Marty et al., 2005). Triple negative breast cancer is characterized as estrogen receptor-negative; progesterin receptor-negative and human epidermal growth factor receptor-negative (Hudis, 2011). It represents 10-20% of all invasive breast *tumours* (Schwentner et al., 2013). This type of *tumour* does not respond to hormone therapy, or to chemical therapy. Thus, it is typically treated with combinational treatment and still has a poor prognosis.

1.6. Treatment of breast cancer

Breast cancer is a heterogeneous disease that becomes a target for several treatments divided into local and systemic therapies, according to the breast cancer types, stages, and sites of the *tumour* as well as other patient-related factors such as age and general health. Local treatment options include surgery and radiotherapy that are applied to specific areas of the body. Systemic treatments involve the entire body, where the *tumour* has spread and includes chemotherapy, hormonal therapy, and HER2-targeted therapies. Usually, the best results are obtained when two or more methods are used in combination (Verma et al., 2004).

1.6.1. Surgery

In the early stage of breast cancer, surgery is the first treatment (Early Breast Cancer Trialist's Collaborative Group, 2006). Usually, such early disease is detected in breast only or in regional lymph nodes and can be removed surgically. Additionally, it can be diagnosed by screening mammography and treated with just local treatments (Hortobagyi, 1998). The aim of surgery is basically to remove all *tumour* tissues and nearby margins to reduce the local recurrence. However, there are two options of surgery, mastectomy or breast-conserving surgery, followed by radiation with some factors to guide the decision including *tumour* size, location, cosmetic achievement post-surgery, and pre-exposure to radiation (Gabriel and Domchek, 2010).

Mastectomy includes removal of the entire breast tissue and chest wall tissue with axillary lymph nodes (Kaviani et al., 2013). Traditionally, it is used when local recurrence risk is increased and is associated with a size more than 5 cm³, existence of four or more positive lymph nodes and occurrence of vascular invasion (Kaviani et al., 2013). Even after mastectomy, the risk of local recurrence can continue and it can be reduced by post-mastectomy radiotherapy which causes a reduction in long term breast mortality although some studies have shown that post-mastectomy radiotherapy may only have a slight effect within the first few years on breast cancer death. Therefore, it is suggested to treat patients at high risk of local reoccurrence with radiotherapy after mastectomy (Siegel et al., 2012).

Breast conservation therapy (BCT) removes the cancerous lump with a portion of the breast tissue surrounding the cancerous lump without increasing the chance of local

recurrence (Gabriel and Domchek, 2010). Additionally, the lymph nodes under the arm might be removed if the cancer has spread (Kroman et al., 2004). However, the breast itself remains relatively undamaged. The National Institutes of Health recommended BCT with radiation in 1990 as an appropriate treatment for the most women with early-stage breast cancer (Lee et al., 2009). This recommendation was made after early-randomized trials showed that there was no statistically significant difference in survival rate and local recurrence between patients treated with BCT and those undergoing a mastectomy (Early Breast Cancer Trialists Group et al., 2011).

Currently, around 60% of patients with early-stage breast *tumour* are treated with BCT (Early Breast Cancer Trialists Group et al., 2011). According to some studies young women experience local recurrence more frequently than middle-old women following BCT (Kroman, et al., 2004; Harrold et al., 1998). Women aged 35 years or less at diagnosis that receive BCT represent more than 30% of 10-year local recurrence rates while middle-aged and older women represent less than 10% of 10-year local recurrence rates (Kroman et al., 2004). However, in two big randomized trials analysis, results found that young women treated with mastectomy did not show a rise in local recurrence compared to old patients (Gabriel, 1998). These studies display that the disease-free survival rate from breast cancer is the same for patients undergoing mastectomy or undergoing BCT along with radiotherapy particularly in women with early stage Breast cancer (Stages I and II) (Kaviani et al., 2013).

Numerous publications have analyzed the risk of local recurrence associated with early stage of breast cancer. In mastectomized patients, local recurrence to the chest wall accounts for 2-10%, whereas local recurrence is 3-10% after BCT (Kaviani et al., 2013).

The vast majority of women with local recurrence after BCT can be recovered with mastectomy and further recurrence after mastectomy is about 5%.

1.6.2. Radiotherapy

Radiotherapy is one of the local treatment methods that destroy the *tumours* by targeting the DNA of the cancer cells. Moreover, it is usually given to younger patients after BCT and following mastectomy for patients with a greater risk of local recurrence (Early Breast Cancer Trialists Group et al., 2011). Results from the Oxford overview analysis indicated that the 5-year local recurrence risk rate for patients treated with post-surgery with radiotherapy was 7%. This proportion is significantly increased to 26% in women undergoing breast-conserving surgery only. Moreover, the 15-year breast cancer mortality rate in patients who underwent radiotherapy following surgery was 30.5% whereas for women treated with surgery alone the mortality rate was raised to 35.9% (Ring et al., 2011). Additionally, the incidences of local and regional recurrences were reduced by 50-to75% after post mastectomy radiotherapy, but according to a meta-analysis of several trials this decrease was not accompanied by increased survival (Gabriel and Domchek, 2010). For that reason, radiotherapy after mastectomy is suggested only for women at great risk for local or regional recurrence (patients with lots of positive axillary lymph nodes and those with large *tumours* invading the chest wall or the skin of the breast). However, recent randomized trial indicated that overall long-term survival rate in patients treated with chemotherapy before radiotherapy was significantly effective (Gabriel and Domchek, 2010).

1.6.3. Chemotherapy

Chemotherapy usually includes cytotoxic drugs, which can either directly kill cancer cells or inhibit *tumour* cell proliferation. For adjuvant therapy, which is usually given after surgery, combination chemotherapy is more successful than single-drug therapy as it reduces the risk of mortality by about 20% annually (Gabriel et al., 1998).

One of the early combination protocols with proven efficacy was composed of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) (Bonadonna et al., 1995). The use of anthracyclines in chemotherapy protocols is now used extensively in the treatment of breast cancer (Crozier et al., 2014).

Anthracyclines appear to act by inducing covalent topoisomerase II – DNA complexes that result in single or double strand DNA breaks. Anthracyclines, such as doxorubicin and epirubicin, are used in adjuvant and neoadjuvant chemotherapy and are the frequently preferred primary line of chemotherapeutic treatment for locally advanced breast cancer (Bonadonna et al., 1995). According to the Early Breast Cancer Trialists' Collaborative Group (2005) poly-chemotherapy acts to decrease disease associated with increasing age, showing a decrease in mortality of 30, 15, 9 and 13% for the age groups 40–49, 50–59, 60–69 and 70 and older, respectively (Ring et al., 2011). Additionally, age was found to be associated with chemotherapy related deaths: 0.2% for age 50, 0.7% for ages (51-64), and 1.5% for those over age 65 (Ring et al., 2011).

1.6.4. Hormone therapy

Hormone therapy is another systemic therapy used beside chemotherapy to treat

patients with breast cancer. Hormone therapy uses drugs that block hormone binding and deprives the *tumour* cells of hormone. Since many breast cancer cells grow faster and spread more in the presence of female sex hormones blocking hormone binding results in inhibition of *tumour* cell regrowth (Liedtke and Kiesel, 2012). Hormonal therapy is confirmed to greatly reduce the risk of recurrence and mortality. Currently, hormone receptor positive breast cancers make up around 75% to 80% of the diagnosed breast cancers (Cadoo et al., 2013).

Tamoxifen is the most commonly used antagonist and is an effective treatment for estrogen receptor (ER) positive breast cancers. Tamoxifen has been shown to significantly reduce the risks of recurrence for breast cancer for all age groups (Gabriel et al., 1998) and decreases the breast cancer death rate in hormone receptor-positive breast cancer patients by up to one third (Shiavon and Smith, 2014). Patients who take tamoxifen for around 5 years have a decrease in annual recurrence of 47% and a decrease in mortality of 26% (Early Breast Cancer Trialists' Collaborative Group, 1998)

Importantly, only women with ER-positive *tumours* can benefit from tamoxifen while patients with ER-negative *tumours* appear to show no benefits from tamoxifen (Gabriel et al., 1998).

1.6.5. Side effects of chemotherapy treatments

Attempts to cure breast cancer by using a variety of treatments that destroy *tumour* cells can also affect healthy cells. Therefore, chemotherapy has potential benefits and also possible risks. The various side effects associated with chemotherapy are

divided into short and long term. Long-term side effects involve issues of treatment occurring after the conclusion of chemotherapy while short-term side effects include those toxic effects occurring during treatment. Short term side effects include depression, nausea, vomiting, decreased muscular strength, weakness, fatigue, decreased aerobic capacity, cardiac dysfunction, and weight gain (Partridge et al., 2001). However, the negative side effects of breast cancer treatment have increased attention on the need to develop more effective agents that can prevent and treat metastasis of breast cancer with minimum side risks and less painful therapy such treatments derived from natural medicine.

1.7. Natural products and cancer

Different breast cancer treatments have limited options of drug combinations. Consequently, there is a growing need to discover new methods that effectively management breast cancer with less toxic effects (Nabavi et al., 2015). Most chemotherapy drugs have nonselective toxic effects on normal tissues leading to neutropenia (a decrease in the number of neutrophils) the most commonly observed adverse reaction, which raises the chances of infections (Santos Arujo et al., 2012).

Natural products provide a diversity of potential anticancer compounds that can be used for inhibition of breast cancer cells or prevention of breast cancer. Additionally, the World Health Organization (WHO) has estimated that 80% of the population consume traditional medicine as an alternative treatment for their diseases and most of these medicines involve plant-derived medicine and their active components (Nabavi et al., 2015; Krishnaiah et al., 2011).

Natural products (NP) are described as chemical substances or groups of substances, produced by a living organism that has pharmacological activities that are used without chemical modification (Gollahon et al., 2011). Natural products also play a highly significant role in cancer treatment (Newman et al., 2003) since several natural products can work to decrease the side effects of therapy while improving survival (Kado et al., 2012). Treatment with natural products may have some benefits over treatment with other cancer treatment and may provide lower charge selections.

Under stress, human bodies produce more reactive oxygen species than antioxidants and this imbalance can cause many health problems. As result, consuming health products is high among patients with chronic health problem since natural products contain many antioxidants that can quench the reactive free radicals (Krishnaiah et al., 2011). Today, extensive numbers of anticancer agents are natural products or are derived from natural products and play a relevant role in cancer treatment (Nobili et al., 2009).

1.8. *Uncaria tomentosa*

Uncaria tomentosa is one of the herbal medicines that is widely used for treatment of several diseases such as gastric illnesses, cancer, viral infections, arthritis and other inflammatory disorders (Aguilar et al., 2002; Goncalves et al., 2005).

Uncaria tomentosa, which is also known as cat's claw, grows in the Amazon forests of Peru. It is a large woody vine from the Rubiaceae family and Cinchonoideae subfamily. It is traditionally used as a therapy for different disorders such as respiratory infections, diabetes, gastrointestinal disorders, gastritis, and rheumatoid arthritis

(Heitzman et al., 2005). The Peruvian population has used *Uncaria tomentosa* for centuries as treatment for several diseases (Ccahuana et al., 2007). Currently, several nutritional formulations contain *Uncaria tomentosa* to treat a variety of health issues (Sheng et al., 2005).

The root bark of cat's claw contains secondary metabolites such as polyphenols, oxindole alkaloids, and quinovic acid glycosides (Aguilar et al., 2002). Additionally, bark extracts have been traditionally used for their anticancer and anti-inflammatory prosperities.

Uncaria tomentosa extracts have antibacterial, immunostimulating, antiviral, antirheumatic, and anti-oxidative and anticancer effects (De Martino et al., 2006; Bors et al., 2012). *Uncaria tomentosa*' constituents have also showed cytotoxic effects on breast cancer cells (Bacher et al., 2006).

Treatment of cancer cells with *Uncaria tomentosa* causes inhibition of TNF α production and nuclear transcription factor NF- κ B (Akesson et al., 2003). Moreover, several studies have reported that *Uncaria tomentosa* has anti-proliferative effects on different cell lines (De Martino et al., 2006). Sheng et al (2005) reported that an aqueous extract of *Uncaria tomentosa* causes cell growth inhibition without cell death which increase the opportunities for DNA repair leading to anti-inflammatory activity, cancer prevention, and immune stimulation (De Martino et al., 2006). A water extract of *Uncaria tomentosa* decreased proliferation of normal mouse T and B-lymphocytes and this reduction was not because of apoptosis or toxicity (Akesson et al., 2003).

Sheng et al (1998; 2000) also stated that a water extract of *Uncaria tomentosa*

decreases *tumour* cell division *in vitro* and induces apoptosis, which was confirmed by DNA fragmentation and morphological changes (De Martino et al., 2006). A clinical trial demonstrated that breast cancer patients who take *Uncaria tomentosa* along with traditional cancer therapies have fewer side effects than those who were just treated with chemotherapy or radiation (Araújo et al., 2012). Moreover, extracts of *Uncaria tomentosa* have been shown to promote DNA repair and prevent mutation and cell damage in breast cancer patients (Araújo et al., 2012). *Uncaria tomentosa* treatment minimizes the neutropenia resulting from chemotherapy and was effective as an adjuvant treatment for breast cancer (Araújo et al., 2012).

1.9. Apoptosis

Apoptosis, necrosis, and autophagy are the most common classes of cell death that can be catalyzed according to the cell's morphological appearance (Kroemer et al., 2009). Necrosis, in contrast to apoptosis, is a passive form of cell death. It is initiated by environmental distresses and results in uncontrolled release of inflammatory cellular contents (Fink and Cookson, 2005; Edinger and Thompson, 2004). Necrosis is characterized morphologically by the breakdown of the plasma membrane, increased cell volume and the presence of inflammation around the dying cells (Edinger and Thompson, 2004).

The first appearance of apoptosis in the biomedical literature was in 1972 (Wyllie, 1997). It is one of the essential biochemical processes and is responsible for eliminating damaged cells and controlling cell division (Sheng et al., 2005). Apoptosis is a process of programmed cell death that is a main component of various processes and that plays an

important role in different physiological processes in adult tissues including the function and development of the immune system, chemically-induced cell death, normal cell turnover, and during embryonic development (Reed, 2000; Elmore, 2007). It also occurs during aging to maintain a stable cell population.

Apoptosis is a physiological process of cellular suicide and is characterized by morphological phenomenon including plasma membrane blebbing, chromatin condensation, cell shrinking, and cellular fragmentation (Elmore et al., 2007). Eventually, the morphological features of apoptosis includes: cell shrinking and separation from its neighbours; the nucleus starts to break apart, and the DNA cleaves into inter-nucleosomal fragments; then the cell fragments into several apoptotic bodies each with surrounding membrane and extensive plasma membrane blebbing; and, the apoptotic bodies are cleared by phagocytic cells which secret cytokines to inhibit inflammation around the dying cells (Edinger and Thompson, 2004; Reed, 2000; Wyllie, 1997

The extrinsic (death receptor) and intrinsic (mitochondrial) pathways represent the two main distinct subtypes of apoptosis. These two pathways can induce apoptosis through diverse biochemical routes although the results are morphologically similar (Edinger and Thompson, 2004; Elmore, 2007).

The extrinsic pathway is mediated by ligation of death receptors on the plasma membrane with ligands leading to activation of caspase 8, which directly activates caspase 3 and 7 causing apoptosis. The intrinsic pathway is activated by permeablization of the mitochondrial outer membrane by Bcl-2 family members that results in the release

of cytochrome C that activates caspase 9 and 3 to cause apoptosis (Elmore 2007; Tait et al., 2010).

1.10. *Uncaria tomentosa* induces apoptosis

Phytochemical studies of *Uncaria tomentosa* have identified three main fractions of chemicals secondary metabolism that have antitumor effects; the polyphenols, alkaloids and triterpene derivatives (Dietrich et al., 2014). In addition, some studies have shown that the inhibitory effects of *Uncaria tomentosa* on the proliferation of different cancer cell lines, such as breast cancer, cervical carcinoma and osteosarcoma, are mediated through caspase-dependent apoptosis (Dietrich et al., 2014).

C-Med-100 is a novel water extract of *Uncaria tomentosa* that can inhibit the *in vitro* growth of K562 and HL60 (human leukemia cell lines) cancer cells and human EBV-transformed B lymphoma cell lines through the induction of apoptosis confirmed by inter-nucleosomal DNA fragmentation and characteristic morphological changes (Sheng et al., 1997).

De Martino et al (2006) examined the apoptotic effect of *Uncaria tomentosa* bark extracts on three *tumour* cell lines, MCF7 (breast cancer), HeLa (human cervical carcinoma cell line) and SAOS (human osteosarcoma cell line). The results provided evidence that treatment with the *n*-BuOH- soluble fraction of the water extracts of *Uncaria tomentosa* were able to induce apoptosis through activation of caspase 3 in a dose –dependent manner (De Martino et al., 2006).

1.11. Apoptosis and chemotherapy

Several types of chemotherapy increase cellular stress resulting in the induction of apoptosis (Fulda et al., 2010). Many reports have suggested that chemotherapy induced apoptosis involves increased expression of death receptor ligands, particularly the Fas ligand. Other reports have shown that chemotherapy causes apoptosis by stimulating release of cytochrome C from mitochondria (Kaufmann and Earnshaw, 2000).

Fas ligation causes activation of cell death caspases. This initiates a proteolytic cascade that leads to the cellular fragmentation and death (Crowe and Yoon, 2002).

The cytotoxic effects of chemotherapy induce DNA damage, which in turn activates P53. Activation of P53 is result in induction of apoptosis by both intrinsic and extrinsic singling pathways (Seitz et al., 2010).

1.12. Thesis objectives

Previous studies have shown that treatment with extracts of *Uncaria tomentosa* was able to inhibit growth of some cancer cells. However, it was not clear if treatment with *Uncaria tomentosa* was active against human breast cancer cells or if was able to preferentially kill cancer cells compared to non-malignant cells. This study was performed to examine the effects of *Ucaria tomentosa* extracts (alcoholic versus Phosphate-buffered saline extracts) on the proliferation of malignant breast cell lines (MCF7 and MDA-MB-231) and non-malignant cell lines (HBL-100, HSG, and HEK293). In addition, studies were designed to identify the mechanisms by which *Uncaria tomentosa* causes inhibition of MCF7 cell growth or increases in cell death and

to determine if *Uncaria tomentosa* has potential as a treatment for patients with breast cancer.

Chapter 2. Materials and Methods

2.1. Tissue culture

The MCF7 (human ER-positive breast cancer), MDA-MB-231 (human triple negative breast cancer), HBL 100 (human breast), HEK293T (human embryonic kidney) and HSG (human salivary gland) cell lines were purchased from the American Type Culture Collection (Manassas, VA). All of these cells were cultured in Dulbecco's Modified Essential Medium (DMEM, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 µg/ml streptomycin, and 100µ/ml penicillin (Invitrogen, Burlington, ON). Cells were incubated in a humidified atmosphere in a 5% CO₂ incubator at 37°C.

2.2. Drug preparation

Uncaria tomentosa was purchased as Natural Health Product NHP (Cat's Claw extract, Now Foods, Bloomington, IL. Code 84618) or obtained as a dried powder prepared from the bark of the plant by Rosa Rosales (Lima, Peru). For the preparation of ethanol extracts, 1g of *Uncaria tomentosa* powder was dissolved in 10 ml of 70% ethanol and boiled for 60 minutes. For preparation of PBS extracts, 1g of *Uncaria tomentosa* was suspended in 9 ml of water and 1ml of 10X PBS stock and then boiled for 60 min. Both suspensions were centrifuged for at 800xg for 10 min to remove the insoluble material. The supernatant was then filtered through 0.22 µm syringe filters and stored in aliquots at -80 °C

2.3. Cell Treatments

The MCF7, MDA-MB-231, HBL 100, HEK293T, and HSG cells, maintained in Dulbecco's modified Eagle's medium (DMEM), were harvested using trypsin (0.25 ug/ml, Life Technologies) and resuspended in culture media at 2×10^4 cells/ml. The cells were plated on a 5x96 well plates; 100 μ l of cell suspension (2000 cells/well) were added to each well. The cells were then incubated in a 5% CO₂ incubator at 37°C. On day 1, the cells were treated with the *Uncaria tomentosa* extracts and maintained for the duration of the experiment without a media change. The cells were treated with different concentrations of both the ethanolic and PBS extracts (0.05, 0.1, 0.25, 0.5, and 1%), as indicated with a media-only negative control. On each day, for 5 days, the relative cell number was determined using the MTT assay.

2.4. MTT assay (Methyl Tetrazolium Blue)

The antiproliferative effects of *Uncaria tomentosa* were measured by determining changes in cell viability using the MTT assay. The MTT assay is based on the ability of living cells to convert a yellow MTT solution into an insoluble formazan purple salt in the cell cytoplasm. Thus, color formation serves as convenient marker only the viable cells. Malignant cells (MCF7, MDA-MB-231) and non-malignant cells (HBL-100, HEK 293T, and HSG) for cultured on 96 well plates were treated with various concentrations of *Uncaria tomentosa* extracts and cell viability measured each day for five days. Each day, 10 μ l/ml of 0.4 μ g/ml MTT in PBS, pH 7.4, was added to each well and incubated for 4 hr. Following the incubation, the media was removed and 100 μ l of dimethyl sulfoxide (DMSO) was pipetted into each well to dissolve the formazan crystals into

solution. The absorbance was measured at 540 nm using a plate reader (SpectraMax 340 PC 389) and the relative absorbance reported for each treatment condition.

2.5. Cell Morphology

The MCF7, MDA-MB-231, HBL 100 (human breast), HEK293T and HSG cells were cultured in 35 mm² culture plates and incubated overnight at 37°C. The cells were then treated with suspending media (negative control), a low dose (0.01%) or a high dose (1%) of either the alcoholic or PBS extracts of *Uncaria tomentosa*. The morphological change in the cells was imaged daily for 5 days using an Inverted Phase Contrast Axiovert 100 Microscope (Zeiss Microscopes) and recorded using Northern Eclipse Software.

2.6. Wound healing migration assay

The MCF7 cells were plated on 6 well plates and incubated for (24) hours to allow formation of a confluent cell monolayer. Then, the monolayer was gently scratched with a 200- μ l plastic pipet tip to create a “wound” of cleared cells. The cells were then treated with suspending media (control) or with the high dose of either the alcoholic or PBS extracts of *Uncaria tomentosa* extracts (1%). Digital images were documented each day after treatment using an Axiovert 100 microscope and Northern Eclipse software and the diameter of the gap measured. The changes in the gap were plotted for each day to determine the effect of treatment on “wound healing”.

2.7. Cell Staining assay (Acridine Orange/Ethidium Bromide)

This experiment was performed to visualize any nuclear changes associated with apoptosis in response to treatment with *Uncaria tomentosa* extracts. The MCF-7 cells were plated on sterile glass coverslips in 6-well plates in DMEM culture media and incubated overnight at 37°C. The cells were treated with suspending media or the high dose of *Uncaria tomentosa* ethanol or PBS extracts (1%) for different time points (24, 48, and 72 h). Cells were treated with 6 µg/ml of camptothecin for 24 h as a positive control for apoptosis. The cells were then stained with 10 µg/ml of acridine orange (Sigma-Aldrich) and 10 µg/ml of ethidium bromide (Sigma-Aldrich) for 15 minutes. The coverslips were washed with PBS and gently mounted onto glass slides in 80% glycerol in PBS. The fluorescently labelled cells were visualized on an LSM 510 fluorescence microscope (Zeiss) and images obtained showing acridine orange (green), ethidium bromide (red), and phase contrast fields.

2.8. TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling)

DNA fragmentation due to apoptosis was detected using a TUNEL assay according to the manufacturer's instructions (Roche, Laval, QB). MCF7 cells were grown on sterile glass cover slips in DMEM culture media and incubated overnight at 37°C. Cells were treated with suspending media or with media containing 1% *Uncaria tomentosa* ethanolic or PBS extracts and incubated for three different time points (24hrs, 48hrs and 72hrs). Cells were treated with 6 µg/ml of camptothecin for 24 h and used as a positive control for apoptosis. After treatment, the cells were fixed by incubation in 1ml

of 4% freshly prepared formaldehyde for 5 minutes at 4°C. The fixative was removed and the cells were rinsed with PBS and then permeabilized by incubation in 1% Triton X- 100 in PBS at 4°C for 3 minutes. The cells were rewashed twice with PBS. The cells were then treated with 50 µl of the TUNEL reaction mixture and incubated for 60 minutes at 37°C. The stained coverslips were rinsed in PBS, pH 7.4, twice and then mounted on glass slides. The slides were directly viewed using an LSM 510 fluorescence microscope and images of the fluorescent label (green) and phase contrast images obtained.

2.9. Caspase activity assay (caspase colorimetric protease sampler kit)

The Caspase activity assay (Life Technology) was used to detect the activity of caspases-3, -8, and -9 in apoptotic cells. MCF7 cells were plated on 150x20 cm plates. Duplicate cell plates were set up for each concentration. Cells were treated with low dose (0.01%) and high dose of ethanol extract of (1%) for 72 hours. 1% of only ethanol was used as negative control for apoptosis. The cells were harvested and 50 µl of chilled Cell Lysis Buffer (provided in the kit) were added to $3-5 \times 10^6$ cells/sample and then incubated for 10 minutes on ice. The cell lysates were centrifuged for 1 minute at 10,000-x g and the supernatants transferred to new tubes and placed on ice. Protein concentration was detection using a BCA assay and the sample diluted to a concentration of 50-200 µg protein per 50 µl Cell Lysis Buffer (1- 4 mg/mL). 50 µl of 2x Reaction Buffer/DTT mix (provided in the kit) was added to each sample and 5 µl of the 4 mM colorimetric substrate (200 µM final concentration) was added. Samples were incubated at 37 °C for 2 hours and the absorbance read on a (SpectraMax 340 PC 389) at 405 nm. The increase in caspases -3, -8, -9 activity were measured by comparison with an un-

induced control.

2.10. Statistical analysis

The cell viability data were expressed as mean \pm standard deviation of three separate experiments and were analyzed using Graph Pad Prism program and significant differences confirmed by ANOVA * ($P < 0.05$). Post-hoc analysis for differences between treatments was done using Tukey tests. The caspase assay tests were done in triplicate, each experiment normalized (controls set to 1), and the average and standard deviation for each assay reported. The difference between groups was assessed using a Students t-test.

Chapter 3. Results

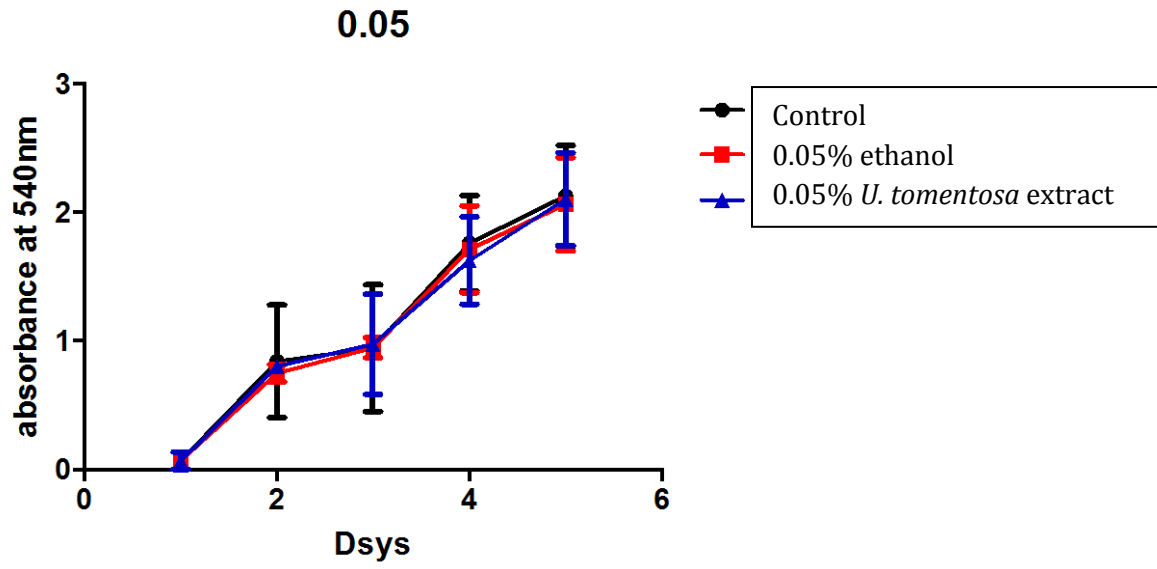
3.1. Cell proliferative assay

The MTT assay was used for *in vitro* experiments to determine the effects of treatment with *Uncaria tomentosa* extracts on the viability of malignant cell lines (MCF7 and MDA-MB-231 cells), and non-malignant cell lines (HBL100, HEK 239T, and HSG cells). Treatment with a high dose (1%) of 70% ethanol extract of *Uncaria tomentosa* significantly inhibited cell growth of MCF7 cells by ~65% (**Figure 3.1**) and of MDA-MB-231 by ~35% (**Figure 3.3**) ($p < 0.05$) particularly at day 3 compared to the low concentration (0.05%), which showed only a slight decrease in cell proliferation compared to the untreated control cells. There was a small level of inhibition in MCF7 cell growth (30%) after treatment with high dose (1%) of PBS extracts of *Uncaria tomentosa* ($p < 0.05$) (**Figure 3.2**) compared to the low concentration, which did not affect the proliferation of the cells compared to the untreated control cells. Treatment of MDA-MB-231 cells with the PBS extract of *Uncaria tomentosa* had no significant effect on proliferation compared to untreated control cells (**Figure 3.4**).

The growth of the non-malignant cells was completely reduced after treatment with the high dose of ethanolic *Uncaria tomentosa* extract compared to the low dose. Treatment of HBL100 cells with 1% of the ethanolic extract of *Uncaria tomentosa* for 5 days decreased the cell number to below the starting concentration (**Figures 3.5**) while treatment of HEK293T cells decreased cell number by approximately 80% compared to

Figure 3.1. The effect of the ethanolic extract of *Uncaria tomentosa* on the proliferation of the malignant MCF7 cell line. MCF7 cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with 70% ethanol, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures are shown for each experiment. This data shows one representative experiments of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



B

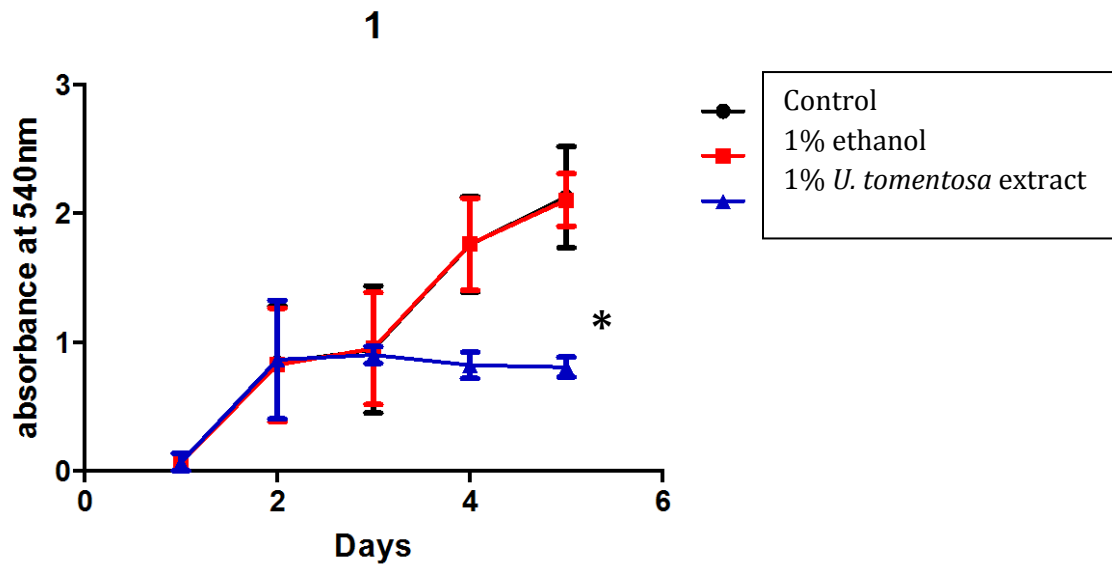
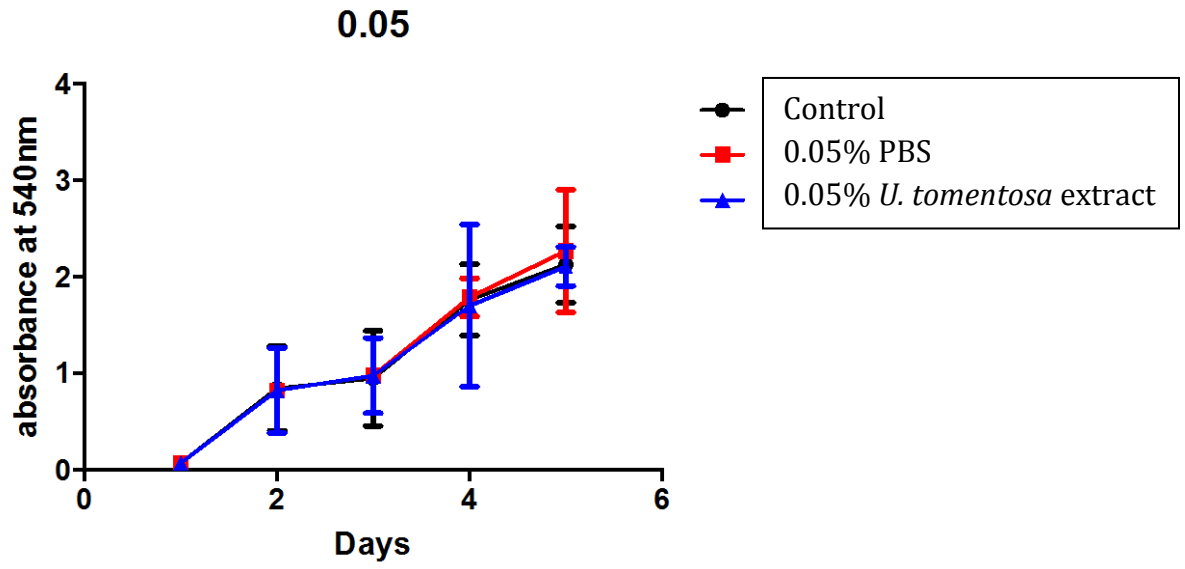


Figure 3.2. The effect of the PBS extract of *Uncaria tomentosa* on the proliferation of the malignant MCF7 cell line. MCF7 cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with PBS, pH 7.4, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures are shown for each experiment. This data shows one representative experiment of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

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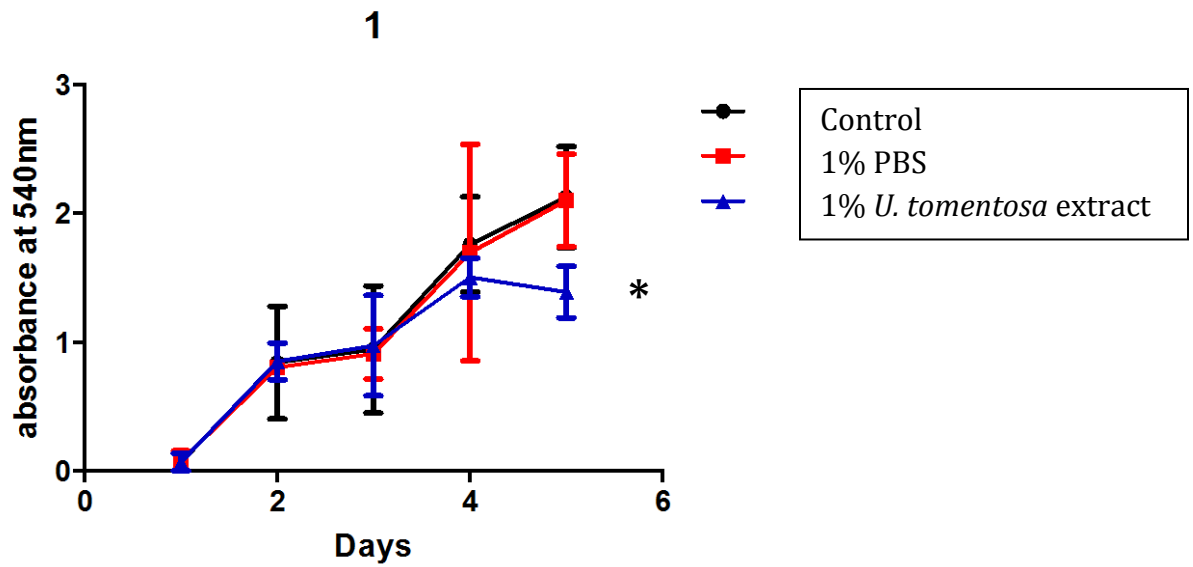
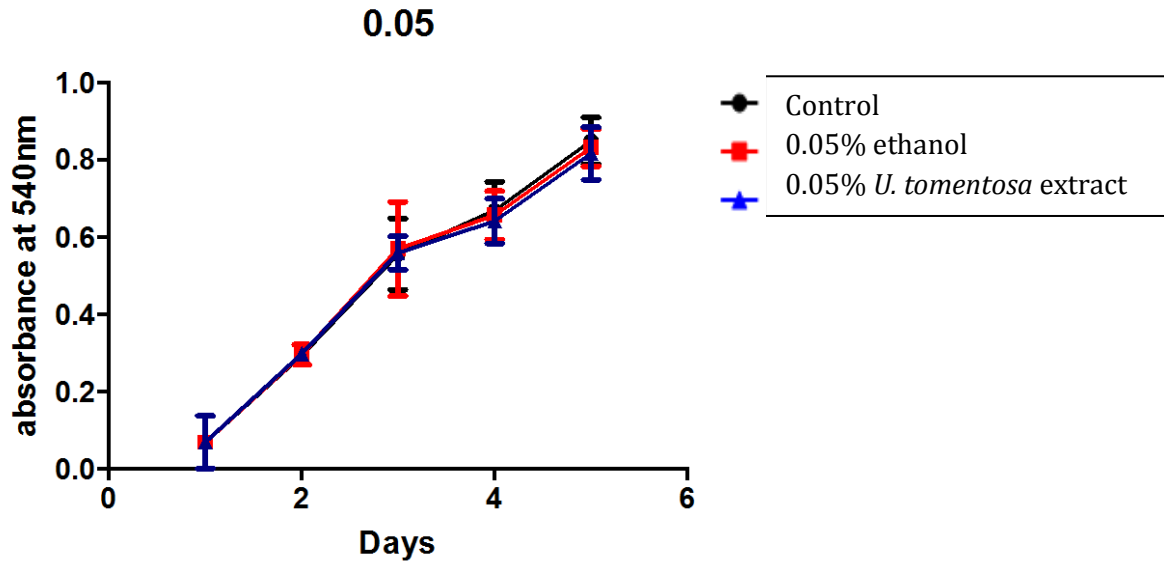


Figure 3.3. The effect of the ethanolic extract of *U. tomentosa* on the proliferation of the malignant MDA-MB-231 cell line. MDA-MB-231 cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with 70% ethanol, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures is shown for each experiment. This data shows one representative experiment of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

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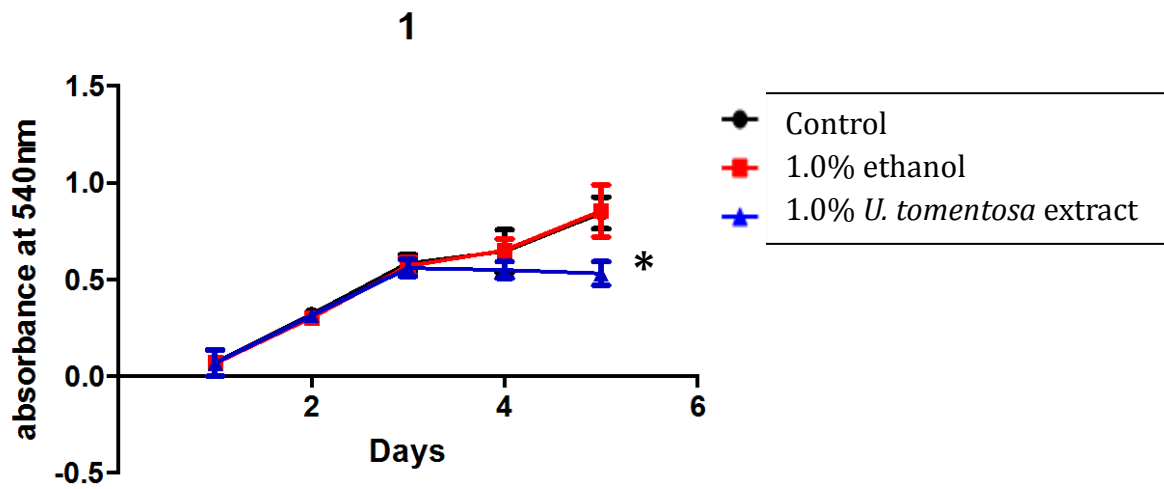
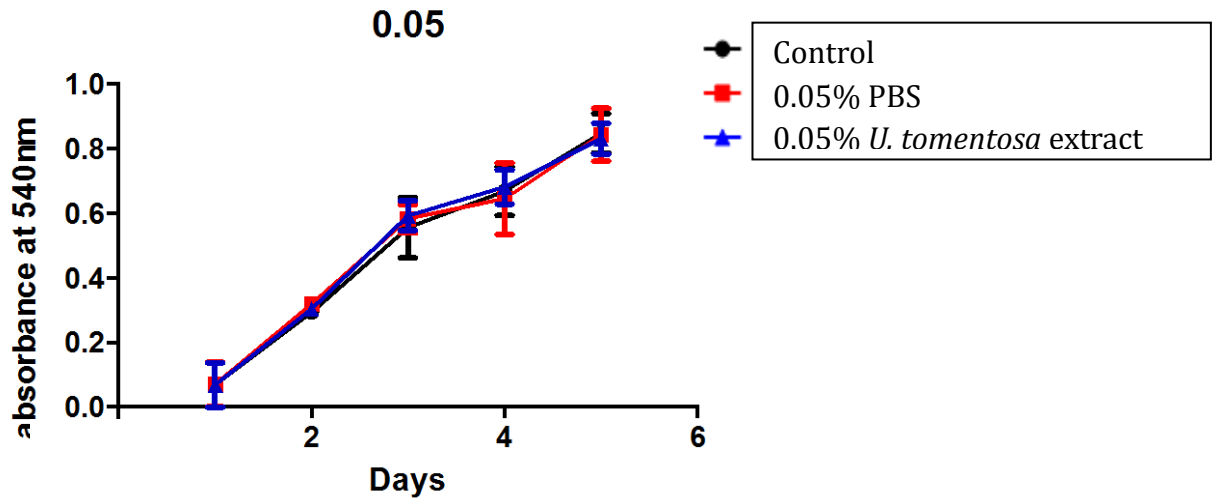


Figure 3.4. Effect of the PBS extract of *U. tomentosa* on the proliferation of the malignant MDA-MB-231 cell line. MDA-MB-231 cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with PBS, pH 7.4, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



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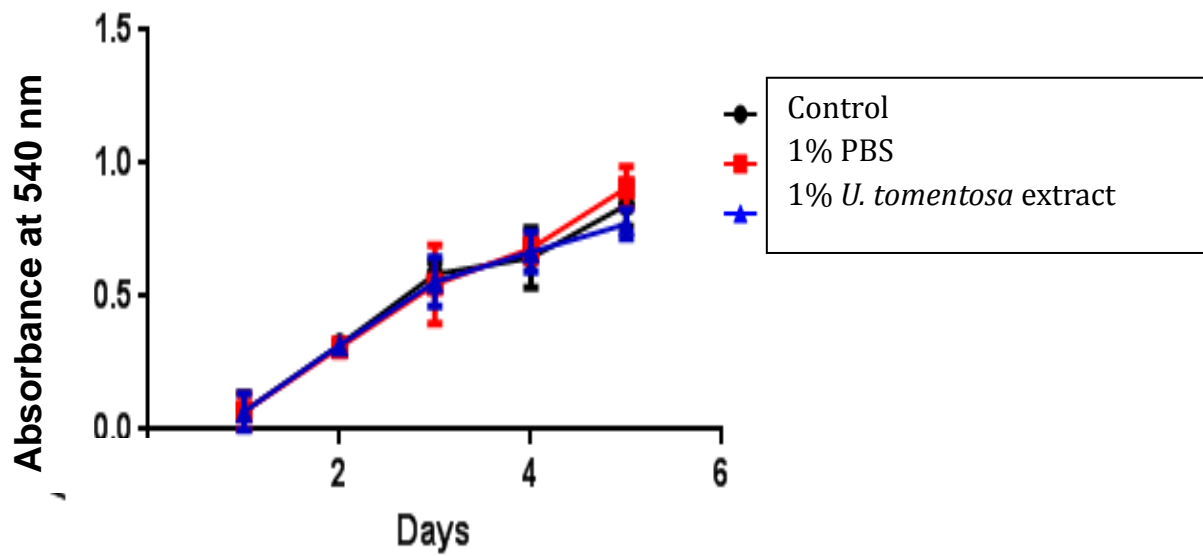
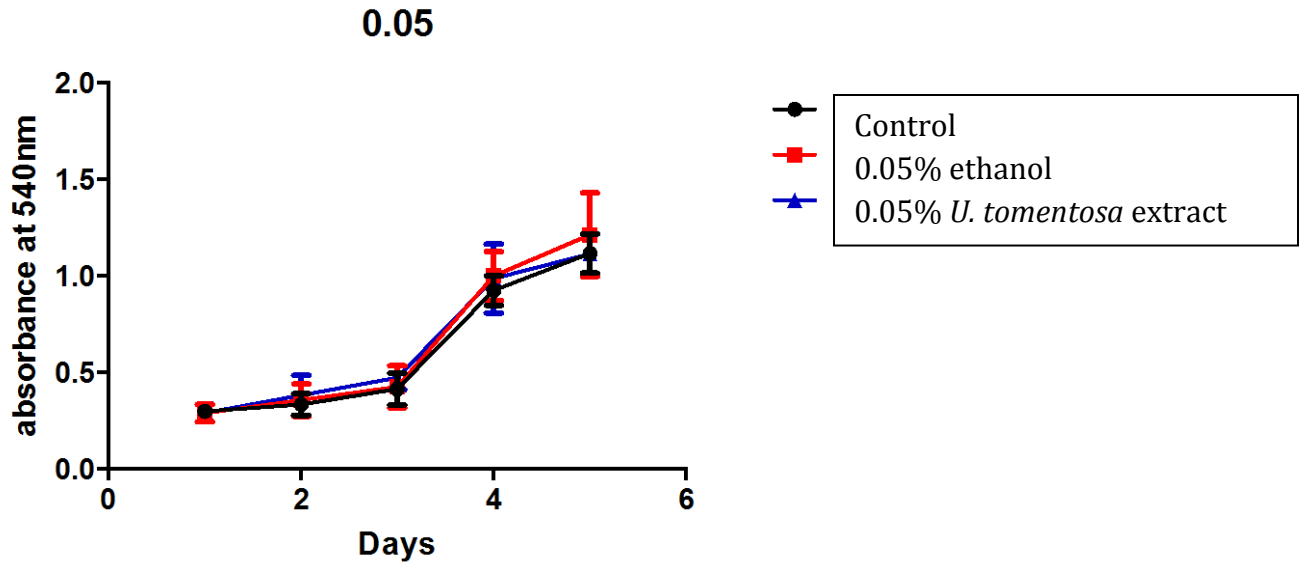
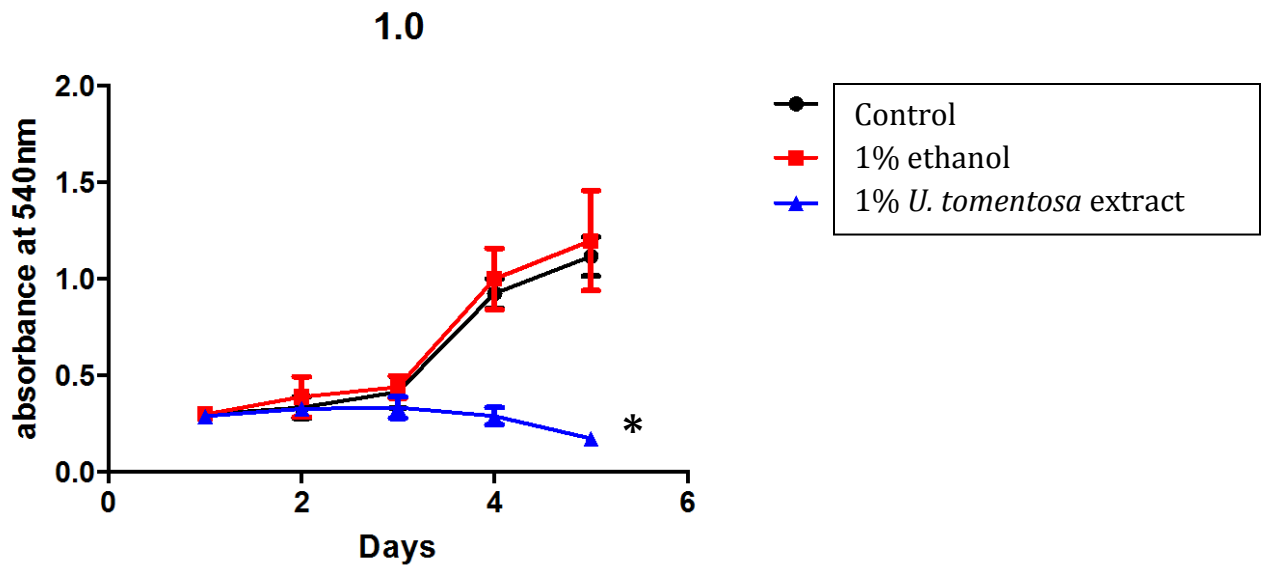


Figure 3.5. Effect of the ethanolic extract of *Uncaria tomentosa* on the proliferation of the non-malignant HBL100 cell line. HBL-100 non-cancer cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with 70% ethanol, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



B



control (**Figure 3.7**) and treatment of HSG cells decreased cell number by more than 70% (**Figure 3.9**). Treatment with both the high (1%) and low (0.05%) doses of the PBS extract did not cause a decrease in cell proliferation for the HBL100 (**Figure 3.6**), HEK293T (**Figure 3.8**), or HSG cells (**Figure 3.10**).

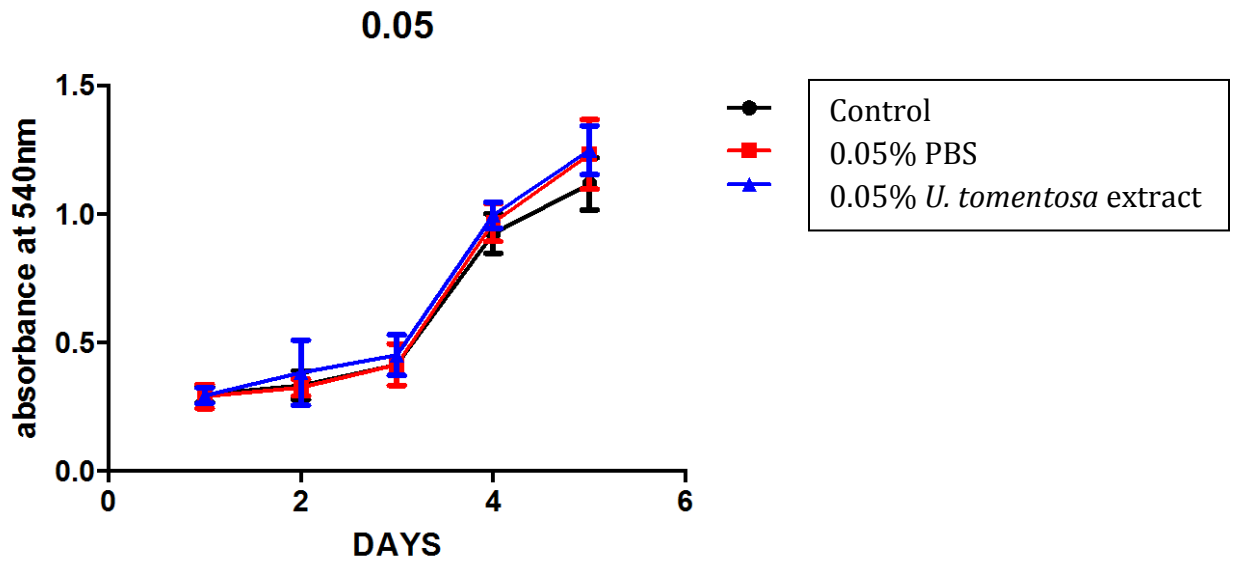
In brief, the ethanolic extract of *Uncaria tomentosa* was able to effectively kill different cell types to a greater extent than the PBS extract (**Figure 3.11**). All experiments were run at least three independent times. In addition, the sensitivity to *Uncaria tomentosa* varied between cell lines. The largest effect of the ethanolic extract of *Uncaria tomentosa* was seen with HBL100 cells, as it caused a significant inhibition of cell proliferation by 83% (**Figure 3.5**). In addition, the ethanolic extract of *Uncaria tomentosa* caused a significant decrease in the growth of HEK 293T cells by 75% (**Figure 3.7**).

3.2. Stability of both *Uncaria tomentosa* extracts.

To test the stability of functioning components of *Uncaria tomentosa* extracts, the ethanolic and PBS preparations were prepared and then stored at 4 °C. Every week for four weeks the same experiment was performed: the MCF7 cells were treated with different doses of *Uncaria tomentosa* extracts (0.05%, 1%) and cell number measured each day. The antiproliferative effect of each extract was tested for 5 days using the MTT assay (**Figure 3.12**). The results showed similar levels of cell growth inhibition for each of the four weeks, which indicated the stability of the active agents in the extracts.

Figure 3.6. Effect of the PBS extract of *Uncaria tomentosa* on the proliferation of the non-malignant HBL100 cell line. HBL-100 cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with PBS, pH 7.4, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

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B

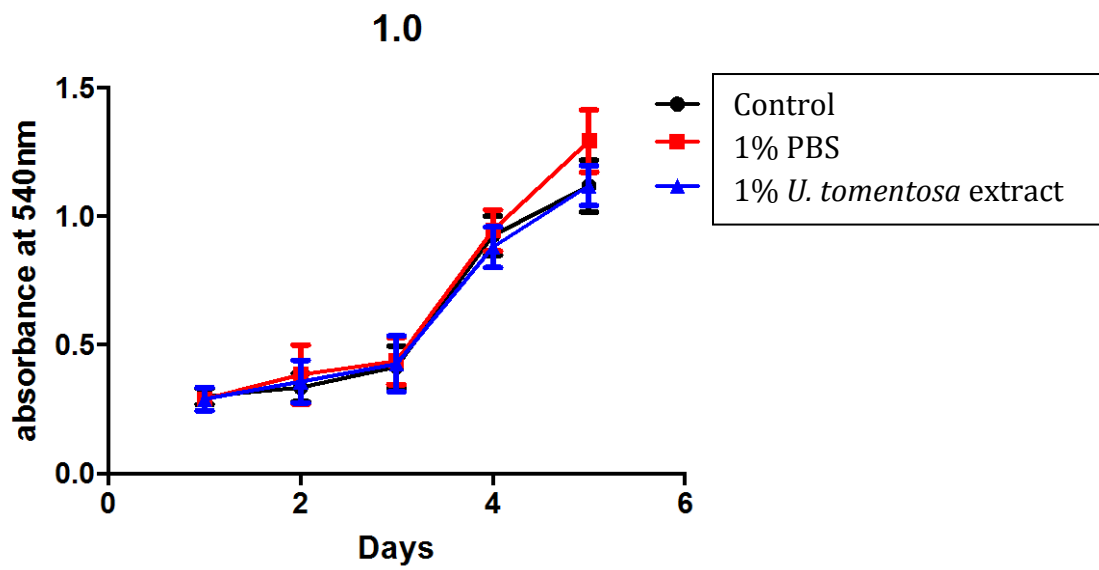
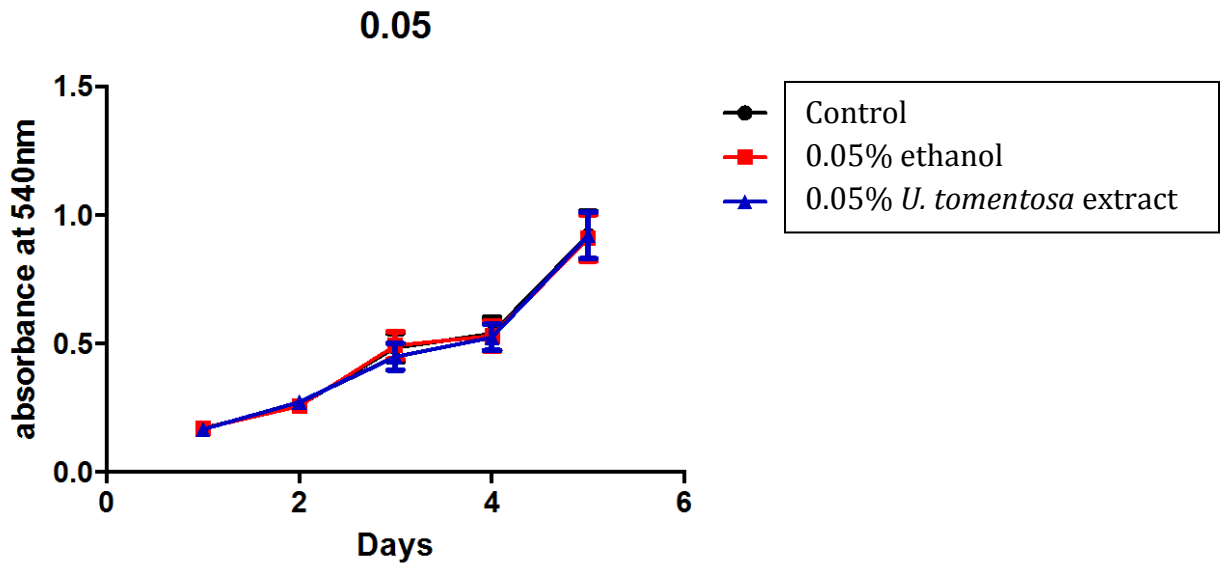


Figure 3.7. Effect of the ethanolic extract of *Uncaria tomentosa* on the proliferation of the non-malignant HEK 293T cell line. HEK293T cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with 70% ethanol, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



B

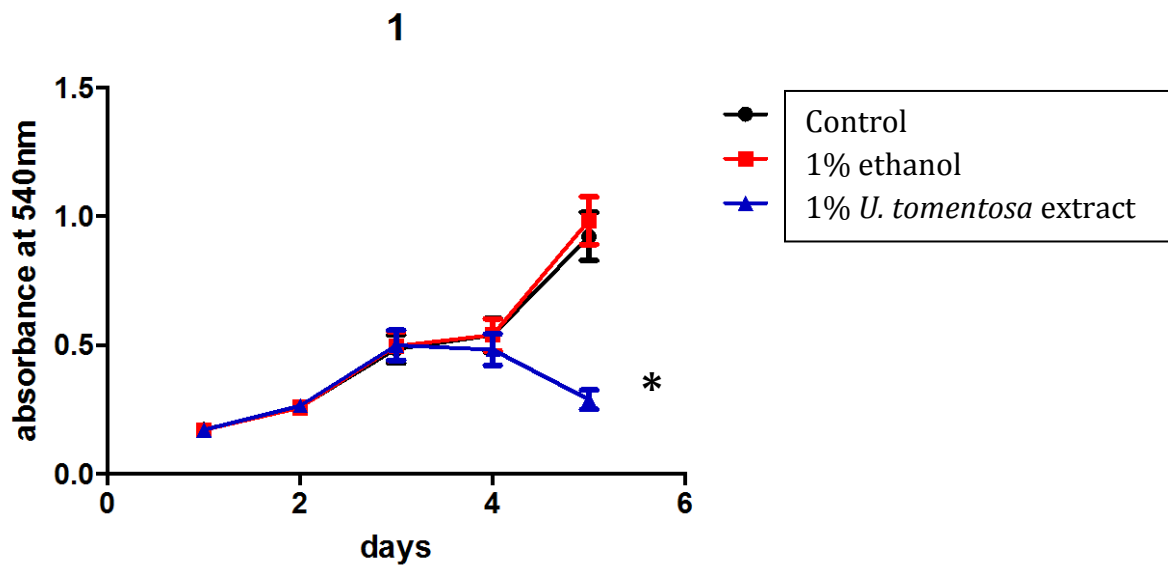
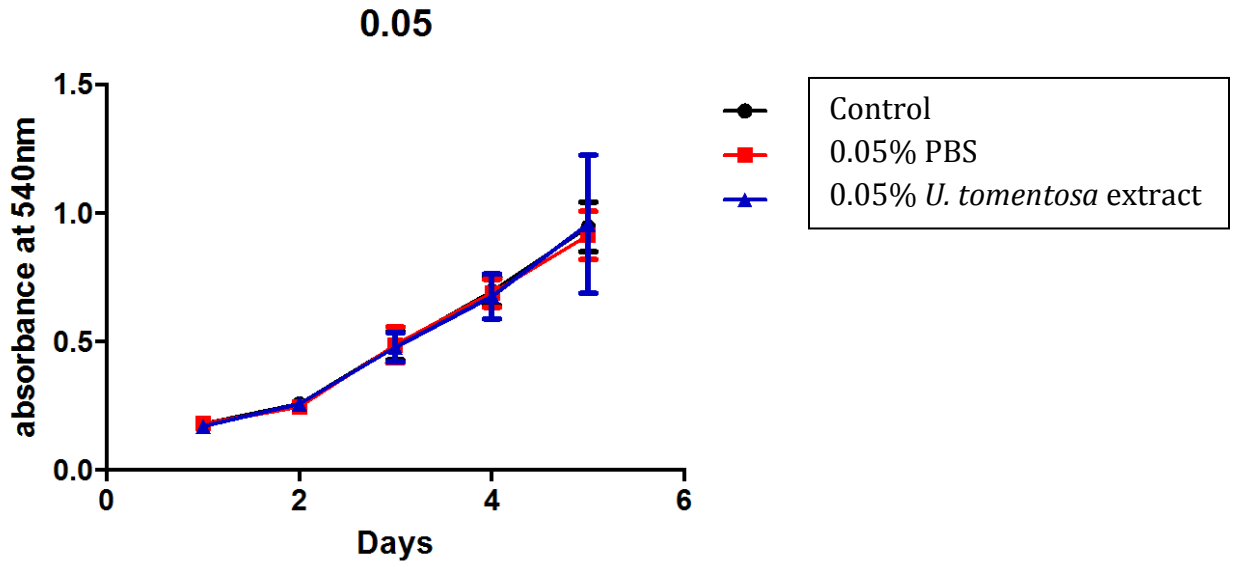


Figure 3.8. Effect of the PBS extract of *Uncaria tomentosa* on the proliferation of the non-malignant HEK 293T cell line. HEK293T cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with PBS, pH 7.4, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



B

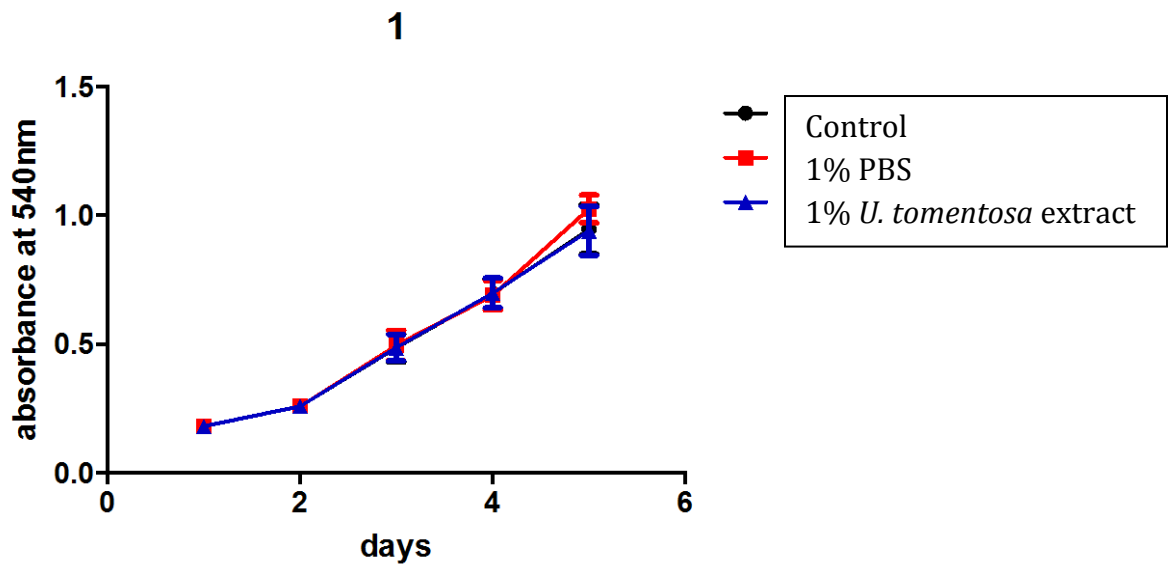
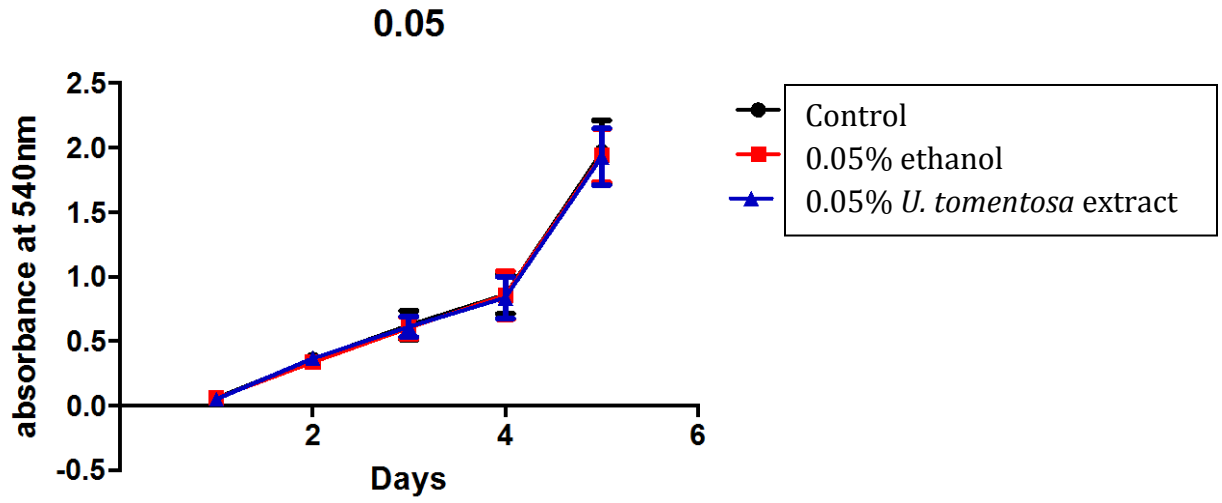


Figure 3.9. Effect of the ethanolic extract of *Uncaria tomentosa* on the proliferative of the non-malignant HSG cell line. MCF7 cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with 70% ethanol, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



B

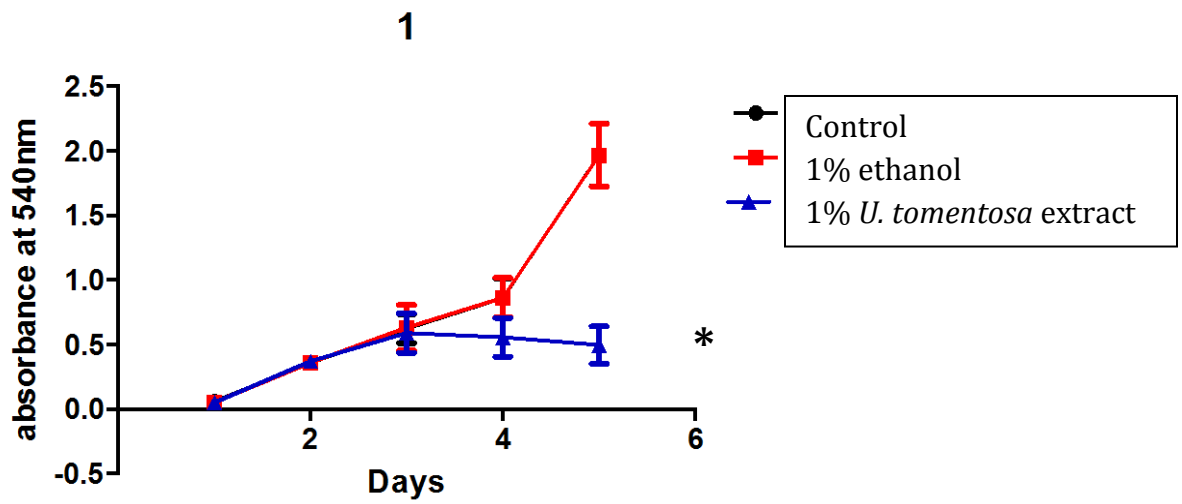
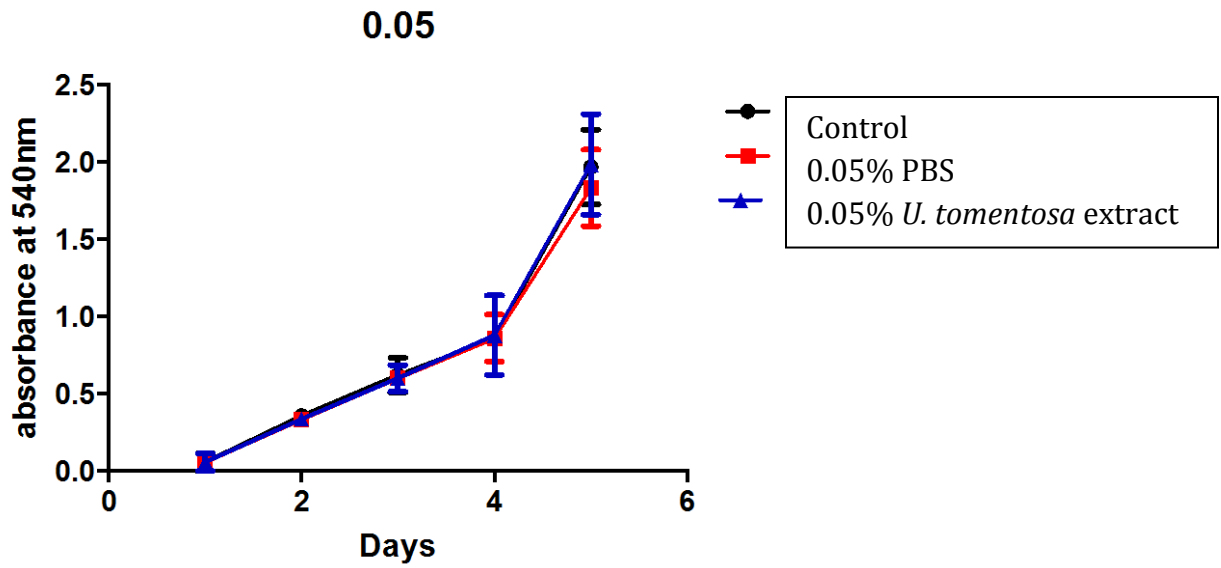


Figure 3.10. Effect of PBS extract of *Uncaria tomentosa* on the proliferation of the non-malignant HSG cell line. HSG cells were incubated with increasing concentrations of *Uncaria tomentosa* extracted with PBS pH 7.4, over 5 days and the cell growth was measured using the MTT assay. Absorbance at 540 nm was read each day. Mean values \pm standard deviation (SD) for 8 separate measures per experiment. This data shows one experiments but 8 experimentations of at least 3 independent experiments. The data were analyzed using graph pad prism and significant differences confirmed by ANOVA * ($P < 0.05$).

A



B

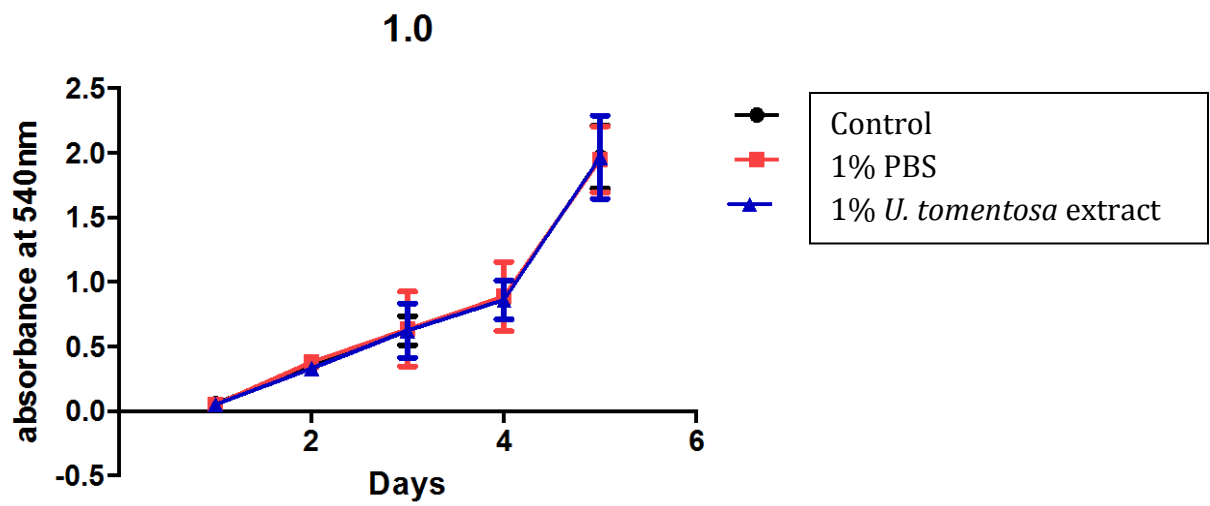


Figure 3.11. Comparison of the effect of both extracts of *Uncaria tomentosa* on various cell lines. Malignant and non-malignant cell lines were cultured for five days. Each day, the change in cell viability in response to treatments with the high concentration (1%) of both ethanolic and aqueous extracts of *Uncaria tomentosa* was assessed by MTT assay and compared.

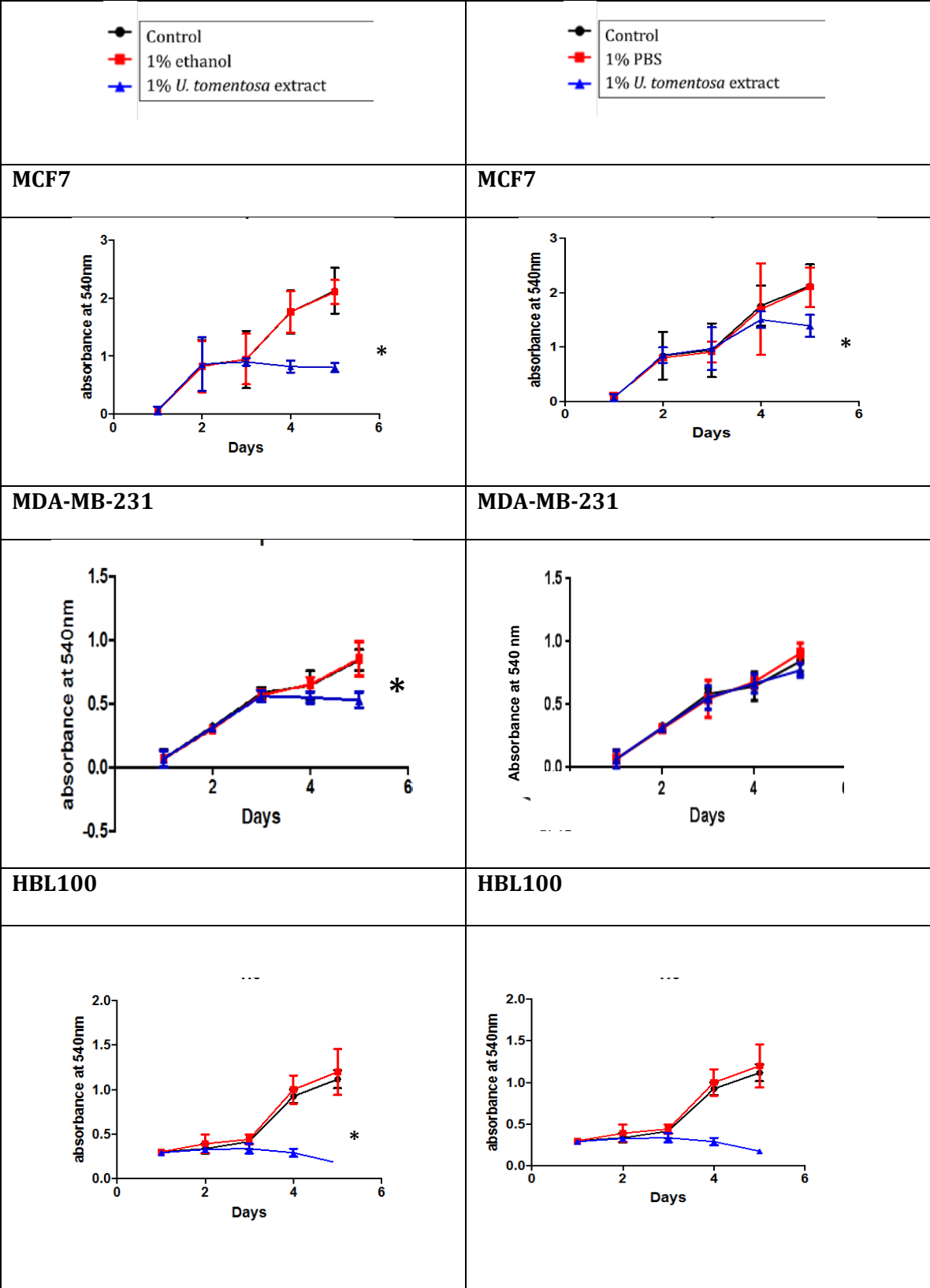


Figure 3.12. Stability of both *Uncaria tomentosa* extracts. Each week, the effect of the *Uncaria tomentosa* ethanolic extract on MCF7 growth was examined for four weeks. MCF7 cells were treated with the high and low dose of ethanolic extracts of *Uncaria tomentos*. The high dose of ethanolic extract of *Uncaria tomentosa* caused a significant decrease in the number of MCF7 cells by ~65%, 60%, 40%, 30% for week 1, week 2, week 3 and week 4, respectively

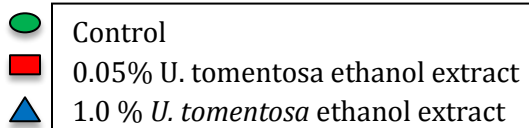
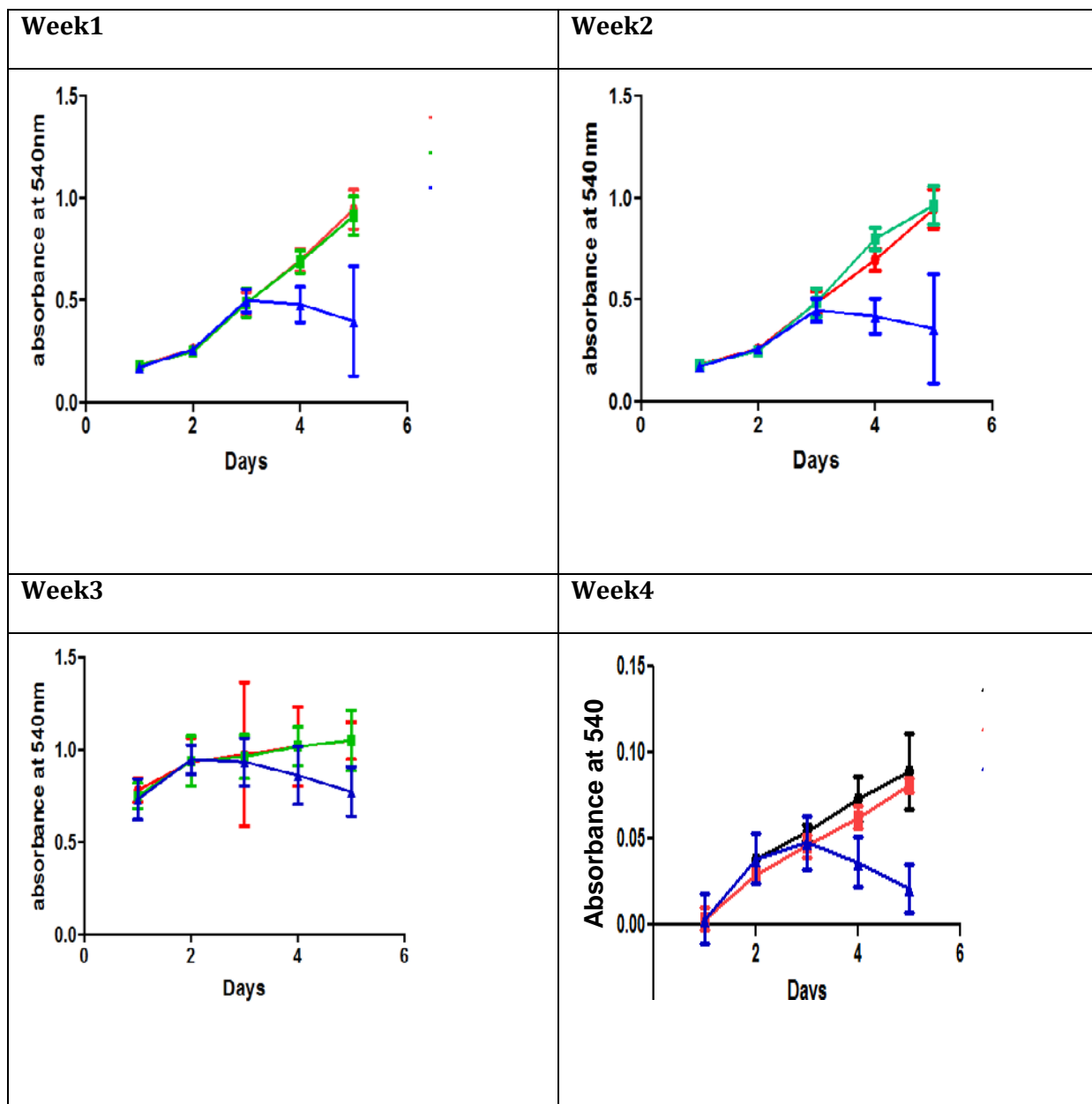
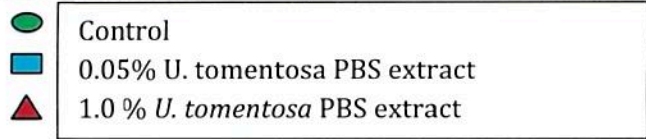
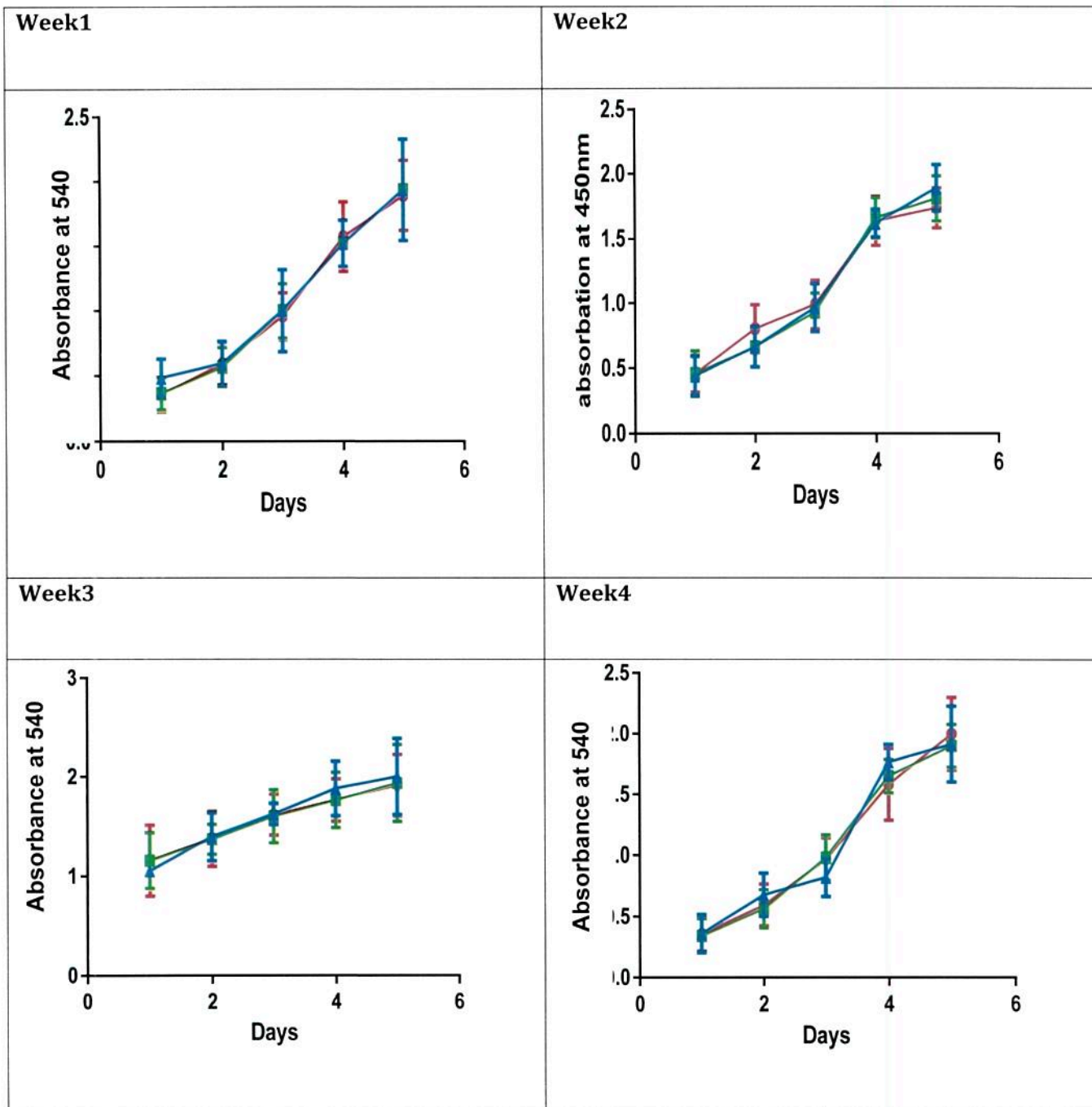


Figure 3.13. Stability of both *Uncaria tomentosa* extracts. Each week, the effect of the PBS extract of *Uncaria tomentosa* on MCF7 growth was examined for four weeks. MCF7 cells were treated with the high and low dose of the PBS extracts of *Uncaria tomentosa*.

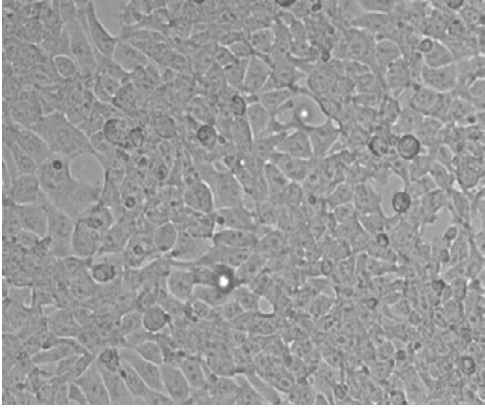
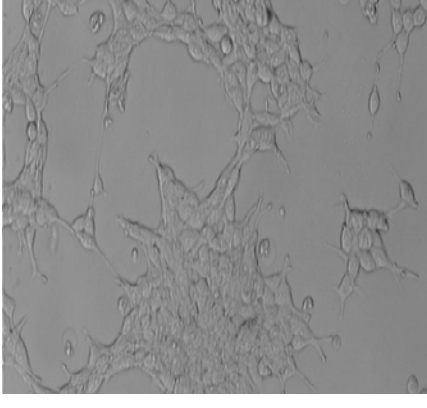
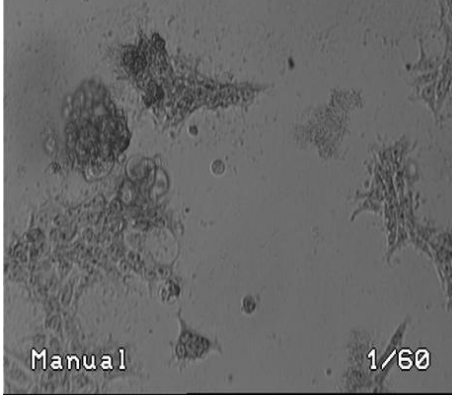


3.3. Treatment with *Uncaria tomentosa* changed the morphology of malignant MCF7 cells

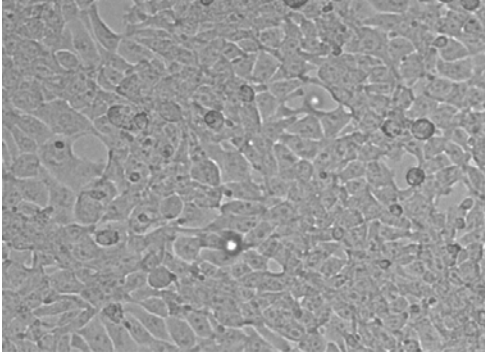
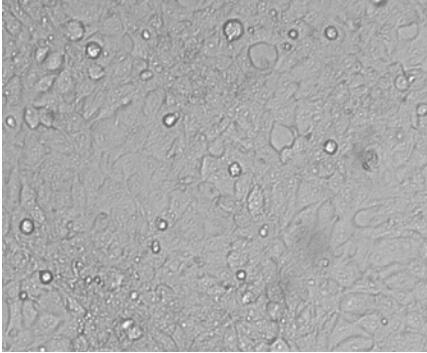
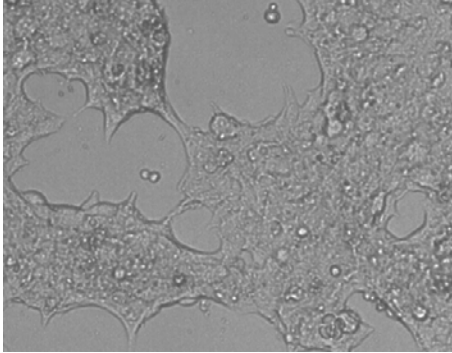
The morphology of MCF7 cells was examined after treatment with *Uncaria tomentosa* extracts to determine if treatment caused any changes in the appearance of the cell cultures or in cell morphology. Treatment of the MCF7 cell cultures with different doses of *Uncaria tomentosa* extracts for 72 h had significant effects on the appearance of the cultures and on the morphology of the cells compared to cells treated with suspending media (**Figure 3.14**). Furthermore, there was a significant decrease in cell number in the treated cultures. The control cultures (both ethanol and PBS suspending media) contained flattened cells at high confluence where there was little space between individual cells. Treatment of the cells with the ethanolic extracts showed a decrease in the confluence of the cells corresponding to a decrease in cell number as well as a shift in the appearance of the cells to a more rounded appearance (**Figure 3.14A**). These changes in number, shape, and structure of the cells were most obvious when the high dose (1%) of ethanolic extract was added. In contrast to the high dose, cells treated with the low dose (0.05%) of the ethanolic extracts showed only a smaller change in cell morphology and cell confluence of the MCF7 cells compared to the control. Treatment with the PBS extracts of *Uncaria tomentosa* also affected MCF7 cultures (**Figure 3.14B**). Treatment with the high dose of *Uncaria tomentosa* PBS extracts (1%) showed a significant change in MCF7 cells and reduced cell viability, whereas treatment with the low concentration of PBS extracts (0.05%) did not show any significant effect on the cells compared to the control cells. In brief, the morphology of treated cells significantly changed with

Figure 3.14. Treatment with *Uncaria tomentosa* changed the morphology of malignant MCF7 cells. MCF7 cells were plated on 6 well plates and treated with the high dose and low dose concentration of *Uncaria tomentosa* extracts. Pictures were taken daily using an Inverted Phase Contrast Axiovert 100 Microscope (Zeiss Microscopes) and recorded using Northern Eclipse Software. The experiments were run in triplicate. Figure **A** is the ethanolic extract of *Uncaria tomentosa*. Figure **B** is the PBS extract of *Uncaria tomentosa*.

A

Control (ethanol)	0.05%	1%
 <p data-bbox="461 835 565 865">(100 μm)</p>		 <p data-bbox="1122 764 1224 793">Manual</p> <p data-bbox="1463 764 1528 793">1/60</p>

B

Control (PBS)	0.05%	1%
 <p data-bbox="461 1654 565 1684">(100 μm)</p>		

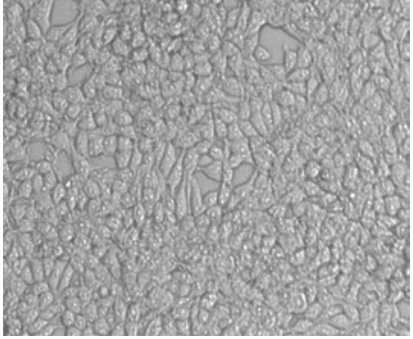
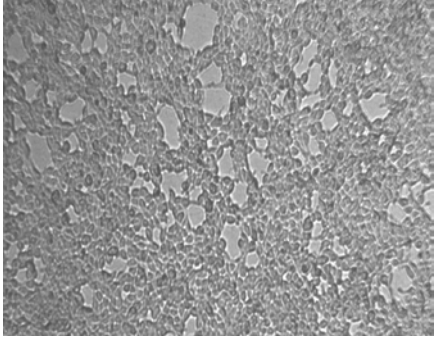
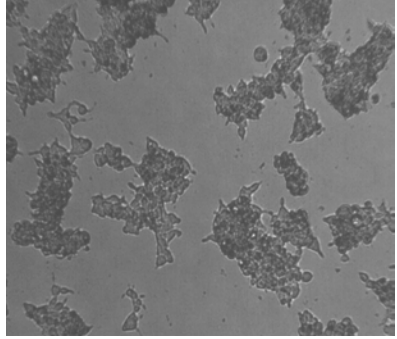
increasing concentration of both ethanolic and aqueous extracts of *Uncaria tomentosa*, after 72 hours.

3.4. Treatment with *Uncaria tomentosa* changed the morphology of non-malignant HEK293T cells

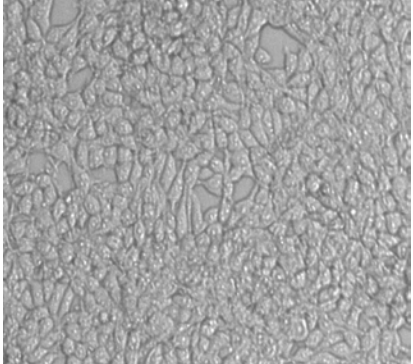
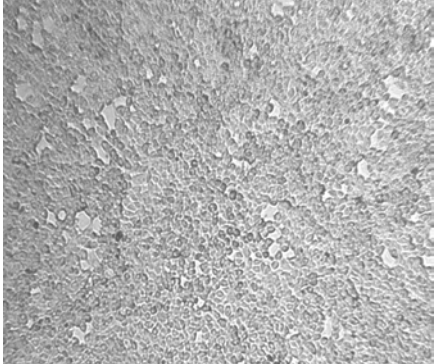
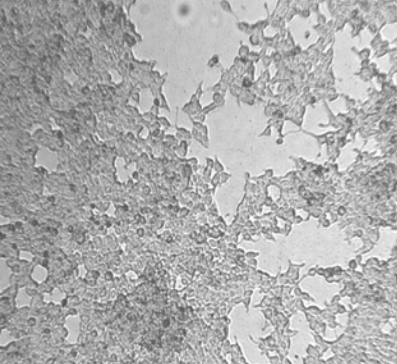
Treatment of the HEK293T cell cultures with different doses of *Uncaria tomentosa* extracts for 72 h had similar effects to the effects on MCF7 cells (**Figure 3.15**). The control cultures (both ethanol and PBS suspending media) contained flattened cells at high confluence where there was little space between individual cells. Treatment of the HEK293T cells with the ethanolic extracts showed a decrease in the confluence of the cells corresponding to a decrease in cell number as well as a shift in the appearance of the cells to a more rounded appearance (**Figure 3.15A**). Cells treated with the high dose (1%) of ethanolic extract were much fewer in number and appeared very condensed in appearance. In contrast, cells treated with the low dose (0.05%) of the ethanolic extracts showed a decrease in confluence of the monolayer which was “patchy” in appearance when compared to the control. Treatment with the PBS extracts of *Uncaria tomentosa* also affected MCF7 cultures (**Figure 3.15B**). Treatment with the high dose of *Uncaria tomentosa* PBS extracts (1%) showed a significant change in HEK293T cells and reduced cell viability, whereas treatment with the low concentration of PBS extracts (0.05%) showed some areas of cell loss with the cells appearing to be less spread on the substrate. In brief, the morphology of treated cells significantly changed with treatment with extracts of *Uncaria tomentosa*.

Figure 3.15. Treatment with *Uncaria tomentosa* changed the morphology of non-malignant HEK 293T. HEK 293T cells were plated on 6 well plates and treated with the high dose and low dose concentrations of *Uncaria tomentosa* extracts. Pictures were taken daily using an Inverted Phase Contrast Axiovert 100 Microscope (Zeiss Microscopes) and recorded using Northern Eclipse Software. The experiments were run in triplicate. Figure **A** is the ethanolic extract of *Uncaria tomentosa*. Figure **B** is the PBS extract of *Uncaria tomentosa*

A

Control	0.05%	1%
 <p data-bbox="441 886 568 924">(100 μm)</p>		

B

Control	0.05%	1%
 <p data-bbox="425 1711 552 1749">(100 μm)</p>		

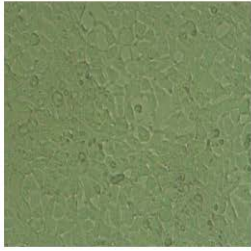
3.5. Effect of *Uncaria tomentosa* extracts on MCF7 cell migration.

The wound healing migration assay was used to determine the effect of *Uncaria tomentosa* on MCF7 cells behaviour. MCF7 cells were plated on 6 well plates for 24 hours then a wound of cleared cells was created in the monolayer with a 200 µl yellow pipet tip. The monolayers were treated with different concentrations of both the ethanol and PBS extracts of *Uncaria tomentosa* and digital photographs of the wounds taken at each day of treatment (day 4 is shown in **Figure 3.16**). Under normal culture conditions the cells migrate into the wound to recreate a confluent monolayer within 4 days. Camptothecin is a chemotherapy drug that promotes cell death and prevents cell migration and was used as positive control which showed no cell migration into the wound. The cells treated with the high concentrations of either ethanolic or PBS preparations of *Uncaria tomentos* (1%) showed a significant decrease in cell migration as shown by the presence of a large gap in the monolayer after 4 days of culture. The cells treated with the low concentration of the ethanolic extraction showed a small decrease in migration while the cells treated with the low concentration of the PBS extract looked very similar to the control cells. The experiments were performed in triplicate and the width of the gap was measured each day and plotted to determine the effect of treatment on “wound healing” (**Figure 3.17**). Treatment with the high dose of the ethanolic extract showed significant inhibition of cell migration. Treatment with the high concentration of the PBS extract of *Uncaria tomentosa* also showed inhibition of MCF7 cell migration but to a lesser extent.

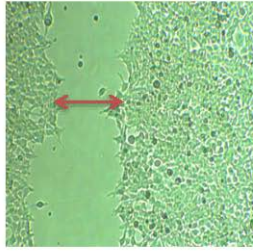
Figure 3.16. Effect of *Uncaria tomentosa* extracts on MCF7 cell migration.

MCF7 cells were cultured on 6 well plates for 24 hours then a wound of cleared cells was created in the monolayer with a 200 µl yellow pipet tip. The cells were cultured in suspending media (control) or with the high concentrations of either ethanolic or PBS preparation of *Uncaria tomentosa* (1%) and the monolayers visualized each day for 5 days. The width of the gap was measured each day. The images show the gap in the monolayer after 72 h of treatment. Camptothecin was used as positive control since it is expected to induce cell death and prevent cell migration. The experiments were performed in triplicate.

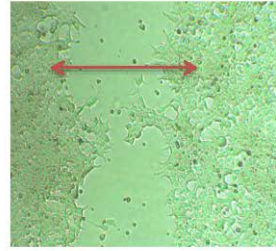
media



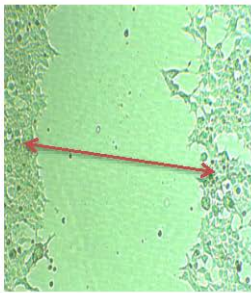
1%Uncaria/PBS



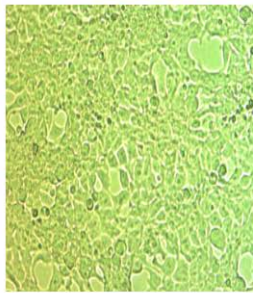
1%Uncaria/EtOH



Camptothecin



0.05%Uncaria/PBS



0.05% Uncaria/EtOH

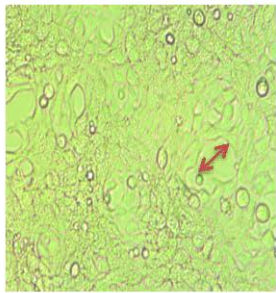
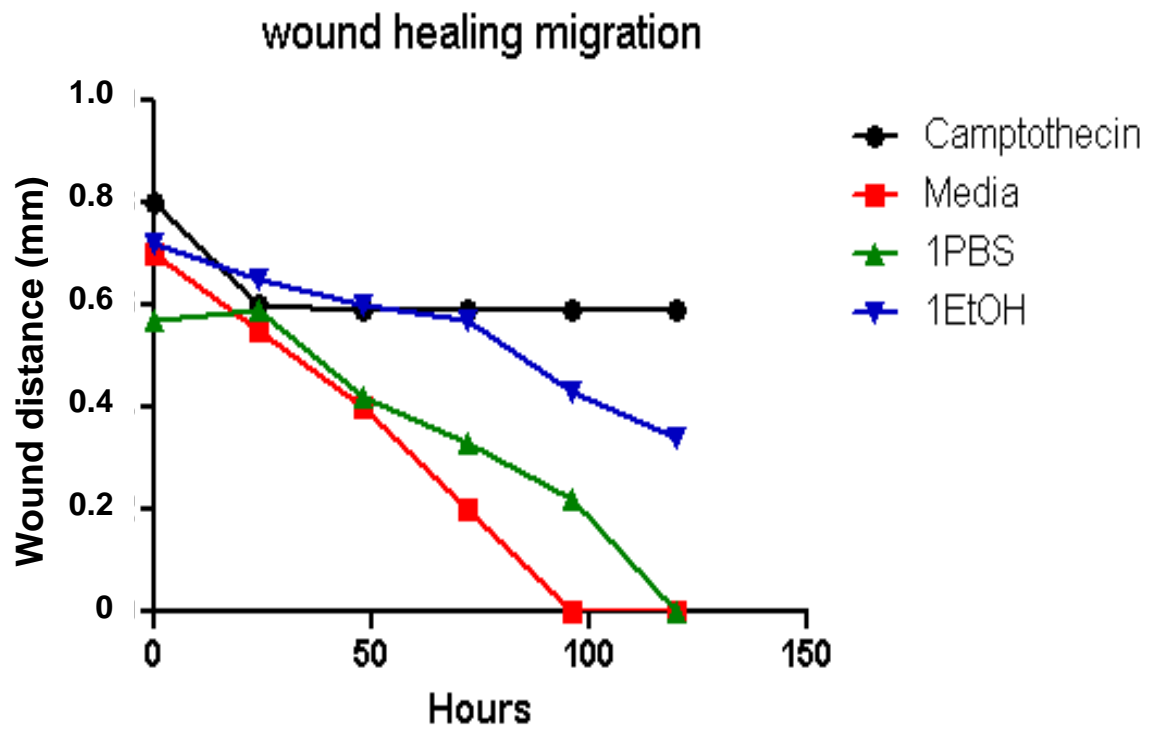


Figure 3.17. Effect of *Uncaria tomentosa* extracts on MCF7 cells migration.

A graphical representation of the effect of the *Uncaria tomentosa* extracts on wound healing of MCF7 cells as described in Figure 3.16.

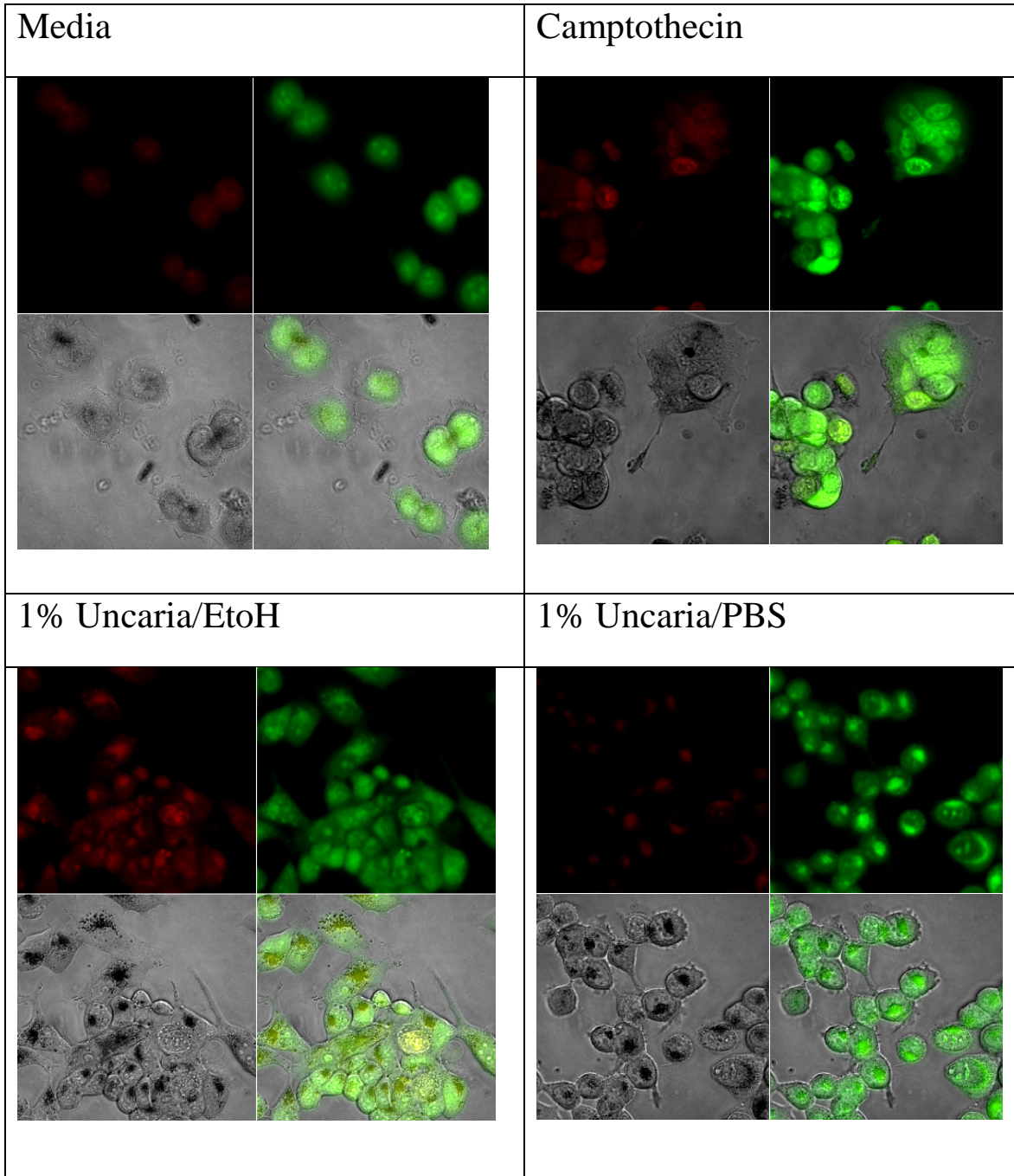


3.6. Induction of apoptosis and DNA fragmentation following treatment with *Uncaria tomentosa* extracts

A cell staining assay using acridine orange and ethidium bromide was used to examine the effect of *Uncaria tomentosa* extracted with ethanol or PBS on the morphological changes in treated MCF7 cells. MCF7 cells were treated with a high dose (1%) of *Uncaria tomentosa* extracts then incubated for different durations (24 and 72 hours) and digital photographs taken. Cells treated only with suspending media for 24 h show primarily nuclear staining with acridine orange and no ethidium bromide staining. Treatment with the high dose of ethanolic extracts for 24 h showed a change in cell morphology including changes in nuclear staining corresponding to condensation and the presence of some staining with ethidium bromide (**Figures 3.18A and 3.19A**). MCF-7 cells treated with the PBS extract for 24 h showed some smaller changes in nuclear staining but did not show ethidium bromide staining. Cells treated with suspending media for 72 h also showed nuclear staining with acridine orange and no ethidium bromide staining which was very similar to the cells treated for 24 h. MCF-7 cells treated with 1% ethanol extracts of *Uncaria tomentosa* showed significant nuclear fragmentation and apoptotic body formation as well as ethidium bromide staining of the nucleus (**Figures 3.18B, 3.19B**). However, the treatment with high dose of *Uncaria tomentosa* PBS extracts for 72 hours showed an earlier stage of apoptosis as indicated by nuclear condensation and some nuclear fragmentation although there was very little ethidium bromide staining. Cells treated with the chemotherapy drug, camptothecin showed significant nuclear condensation and fragmentation along with ethidium bromide staining and was used as a positive control for apoptosis.

Figure 3.18. Detection of apoptosis and DNA fragmentation in MCF 7 cells treated with *Uncaria tomentosa* using acridine orange and ethidium bromide staining. MCF7 cells were cultured on glass coverslips and treated with the high concentration (1%) of *Uncaria tomentosa* extracted with PBS or ethanol and incubated for 24 and 72. Camptothecin was used a positive control to detect apoptosis. Cells were stained with a mixture of acridine orange and ethidium bromide for 10 min. The fluorescently labelled cells were visualized on an LSM 510 fluorescence microscope (Zeiss) and images obtained showing acridine orange (green), ethidium bromide (red), and phase contrast fields. Experiments were run in triplicate. Figure **A** is the ethanolic extract and PBS extract of *Uncaria tomentosa* for 24 hours. Figure **B** is the ethanolic extract and the PBS extract of *Uncaria tomentosa* for 72 hours. Images of Media and Camptothecin were taken after 24hours.

A



B

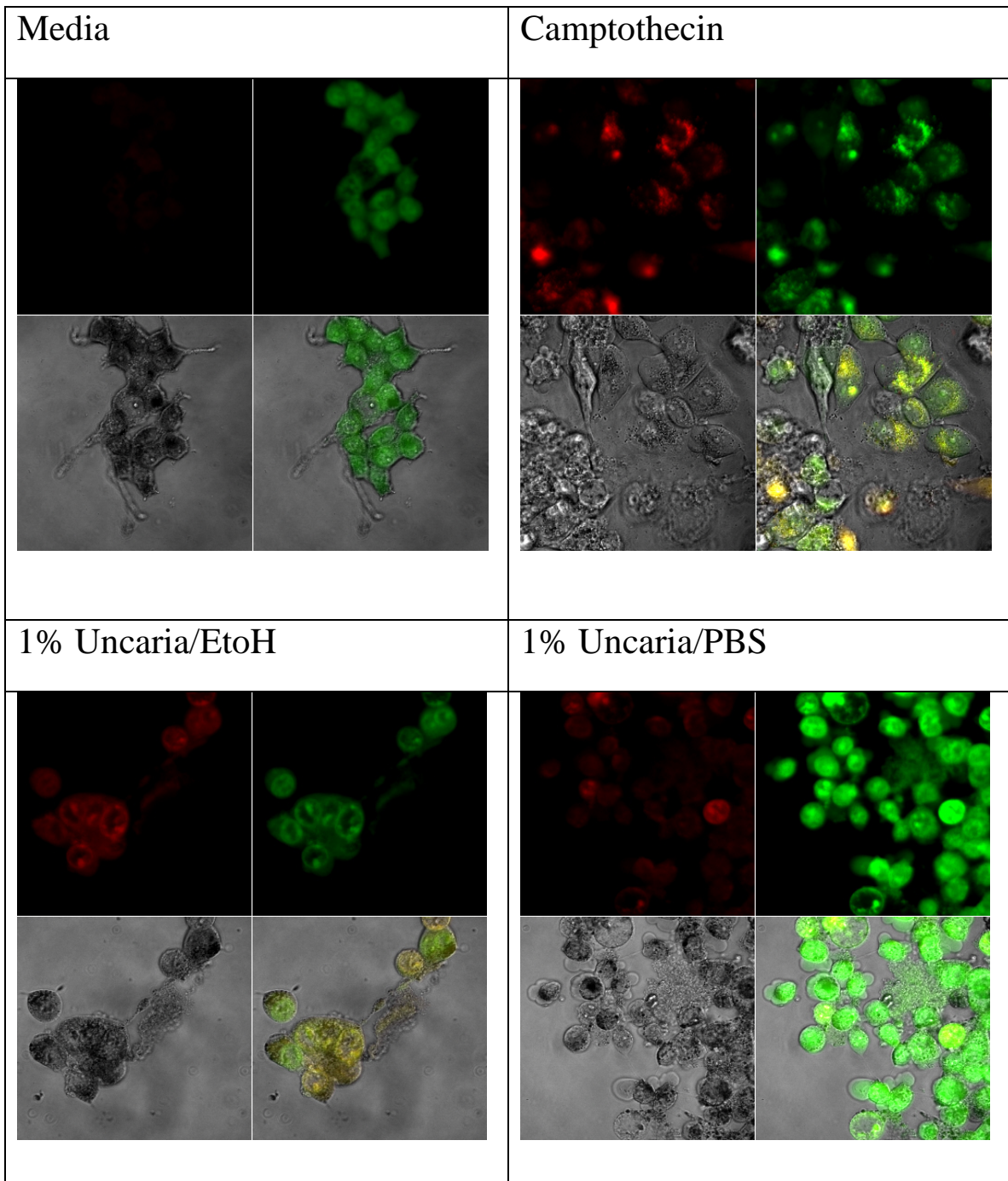
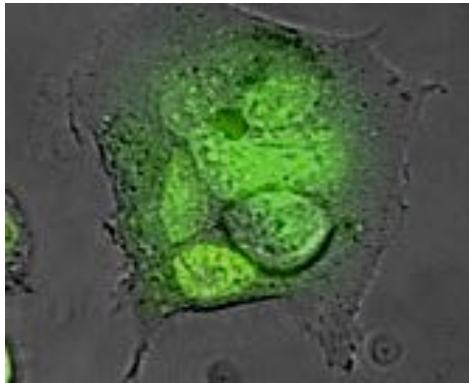


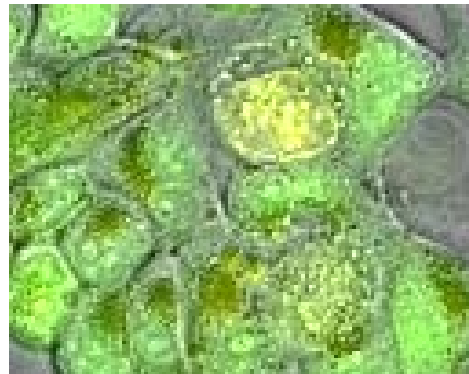
Figure 3.19. Detection of apoptosis and DNA fragmentation in MCF 7 cells treated with *Uncaria tomentosa* using acridine orange and ethidium bromide staining. MCF7 cells were cultured on glass coverslips and treated with the high concentration (1%) of *Uncaria tomentosa* extracted with PBS or ethanol and incubated for 24 and 72. Camptothecin was used a positive control to detect apoptosis. Cells were stained with a mixture of acridine orange and ethidium bromide for 10 min. The fluorescently labelled cells were visualized on an LSM 510 fluorescence microscope (Zeiss) and images obtained showing acridine orange (green), ethidium bromide (red), and phase contrast fields. Experiments were run in triplicate. Figure **A** is the apoptotic cells after treatment with the high concentration of both extracts of *Uncaria tomentosa* for 24 hours. Figure **B** is the apoptotic cells after treatment with the high concentration of both extracts of *Uncaria tomentosa* for 72 hours. Images of media and camptothecin were taken after 24hours.

A

camptothecin



1% Uncaria tomentosa ethanol extract

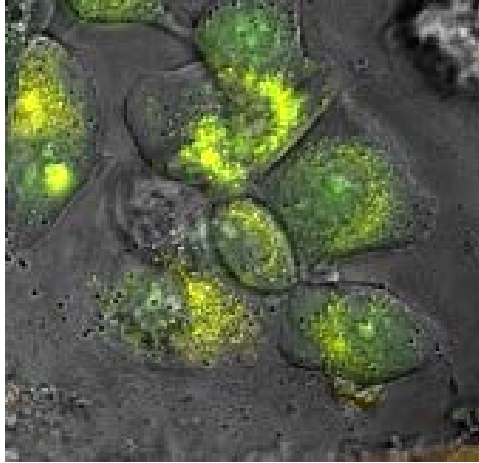


1% Uncaria tomentosa PBS extract

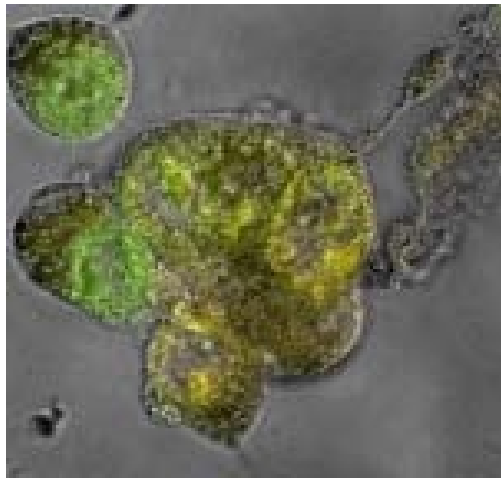


B

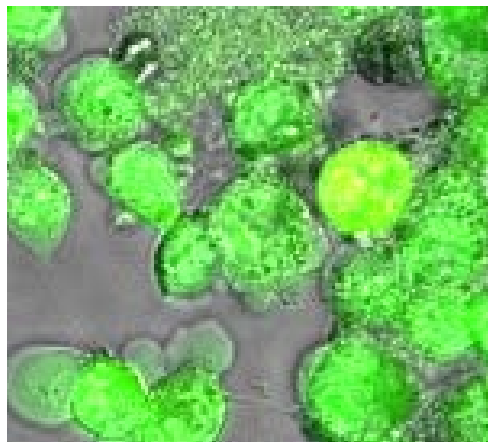
Camptothecin



1% Uncaria tomentosa ethanol extract



1% Uncaria tomentosa ethanol extract



The TUNEL assay was used to detect apoptosis, which is characterized by DNA fragmentation that occurs during late apoptosis. MCF7 cells were treated with the high dose of both ethanol and PBS extracts of *Uncaria tomentosa* (1%) for 24 and 72 hours and then stained with the TUNEL reaction mixture. The presence of DNA fragmentation is seen as a green-stained nucleus as is present in the camptothecin-treated positive control cells. Treatment of the cells with suspending media did not cause the cells to stain with the TUNEL reagent and there were very few stained cell present. Treatment with the high dose of *Uncaria tomentosa* ethanolic extract for 24 h (**Figure 3.20A**) only showed a low level of TUNEL staining but treatment for 72 h caused a significant increase in TUNEL staining and DNA fragmentation where almost every cell was stained indicating induction of apoptosis after 72 hours of treatment (**Figure 3.20B**). Treatment with the high dose of the PBS extract for 24 hours showed only very low levels of staining with TUNEL while treatment for 72 h showed significant levels of staining in the majority of the cells.

These results confirm our previous findings from the cell viability experiments that treatment of cells with *Uncaria tomentosa* decreased cell number by increasing cellular apoptosis, particularly for treatment with the ethanolic extracts of *Uncaria tomentosa*.

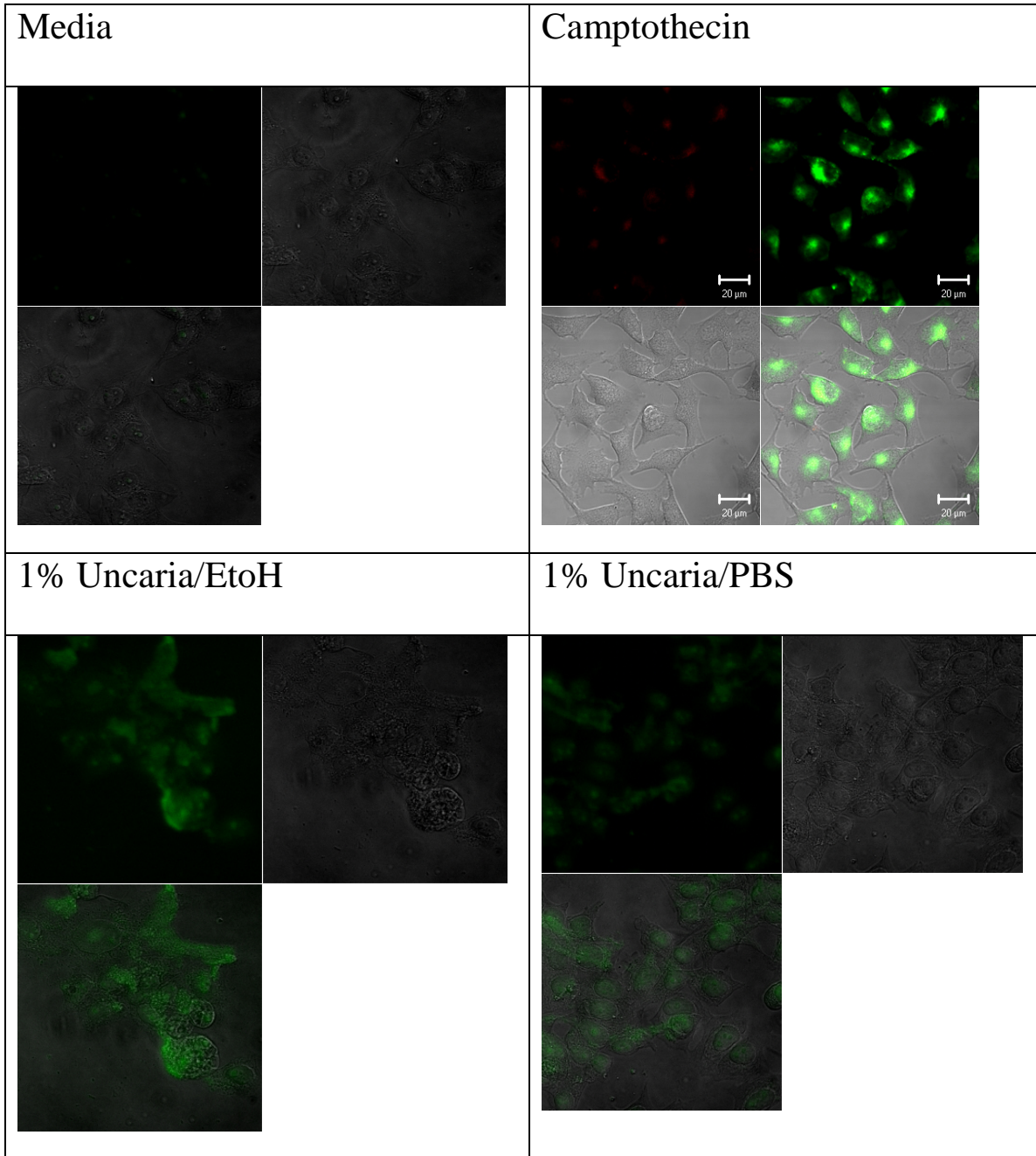
Figure 3.20. Detection of apoptosis and DNA fragmentation in MCF 7 cells treated with *Uncaria tomentosa* using TUNEL staining. MCF7 cells were cultured on glass coverslips and treated with different concentration (0.01% and 1%) of *Uncaria tomentosa* extracted with PBS or ethanol for 24 and 72 hours. MCF7 cells were treated with the high dose of *Uncaria tomentosa* extracts and incubated for 24 and 72 hours. Cells were directly analyzed using a LSM 510 fluorescence microscope to detect labelled cells (green). Experiments were run in triplicate. The first panel displays TUNEL stain, the second panel displays MCF7 as seen under phase-contrast micrograph, and the third panel displays MCF7 with combined fluorescence and phase contrast microscopy. .

Figure **A** is the ethanolic extract and PBS extract of *Uncaria tomentosa* for 24 hours.

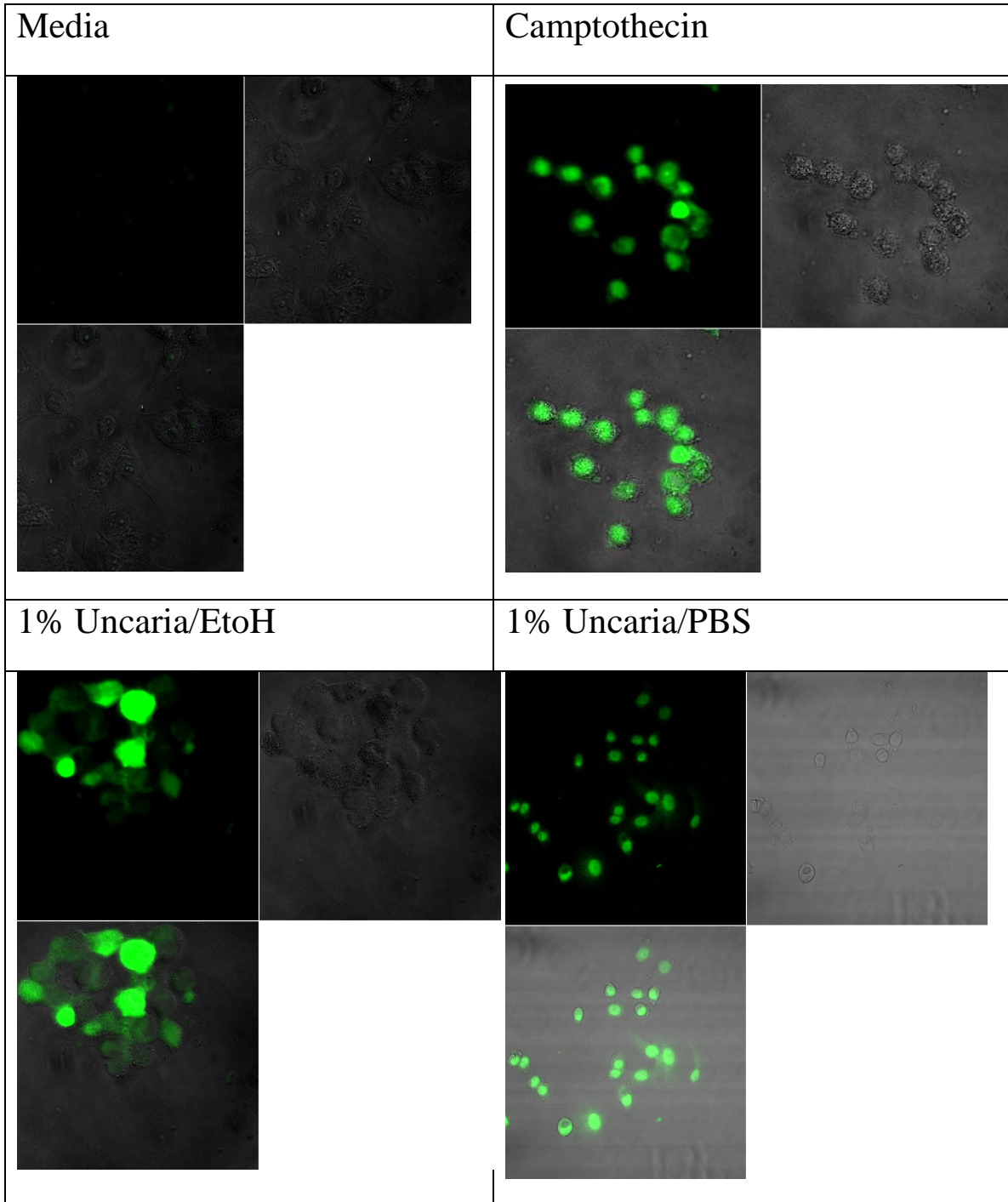
Figure **B** is the ethanolic extract and the PBS extract of *Uncaria tomentosa* for 72 hours.

Images of Media and Camptothecin were taken after 24hours.

A



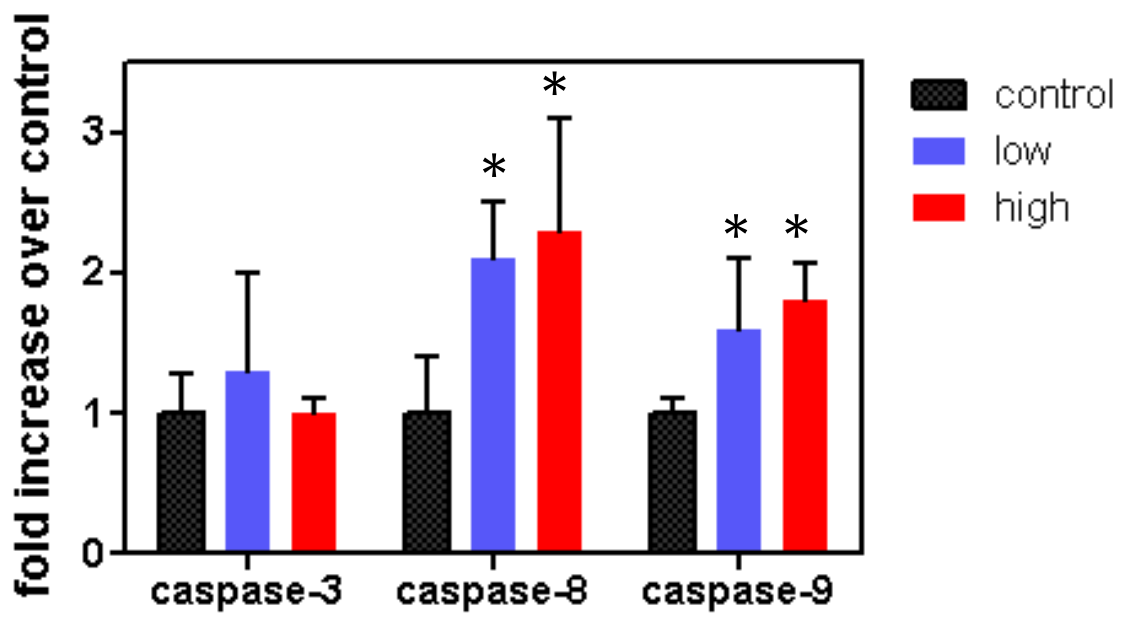
B



3.7. Detection of activated caspase-3, 8 and 9 in MCF7 cells treated with *Uncaria tomentosa*.

The presence of activated caspase proteases is a measure of the activation of apoptosis. To detect presence of active caspase -3, 8, and 9 in treated MCF7, a caspase activation assay was used. This assay measured the caspase enzyme activities that recognize and cleave the artificial substrates with the amino acid sequence, DEVD (for caspase-3), IETD (for caspase-8), and LEHD (for caspase-9). The activity of caspase-3, 8, and 8 was associated with the release of p-Nitroanilid (pNA) from the labeled peptides. The cells were treated with suspending media, low dose (0.05%) and high dose (1%) of *Uncaria tomentosa* extracted with ethanol and incubated for 72 hours. The treatment with the high dose of ethanol (1%) was previously shown to induce apoptosis. Duplicate cell plates were set up for each concentration. The increase in caspase-3, -8 and -9 activity was measured by comparison with un-induced control. MCF7 cells treated with the high dose of *Uncaria tomentosa* extracted with ethanol showed a significant increase in caspase 8 and 9 compare to control cells, with caspase 8 having the highest activity (**Figures 3.21**). However, cells treated with the high dose of ethanolic extracts of *Uncaria tomentosa* did not result in significant activation of caspase 3.

Figure 3.21. Detection of activated caspase -3,8, and 9 in treated MCF7 with *Uncaria tomentosa* extracted with ethanol. A colorimetric assay was used to detect active caspase 3,8 and 9 in apoptotic cells using activation kits (Life Technology). MCF7 cells were treated with a low dose (0.05%) and a high dose (1%) of ethanolic extracts of *Uncaria tomentosa* for 72 hours. Cells were treated with 1% ethanol only as negative control for apoptosis. The increases in caspase activities were determined in triplicate by comparison of changes in absorbance at 410 nm using a (SpectraMax 340 PC 389) plate reader. Data for three independent experiments was normalized by setting the control activity to 1.0 (+ SD) and the fold change in activity (\pm SD) determined for the treated cell samples. * mark significant increases ($p < 0.05$) in activity compared to control-treated cells. The increase in activity of caspase-8 was the highest compared to the control cells.



Chapter 4: Discussion

Breast cancer is the most common disease-affecting woman in the world. Different therapies are recommended for breast cancer that can involve treatment with a single drug or a combination of drugs (Santose et al., 2012). However, the toxic side effects of these therapies are not selective for cancer cells and can affect normal tissues as well (Santose et al., 2012; Dreifuss et al., 2010). Therefore, the search for novel therapeutic approaches with low systemic toxicity is needed. Herbal medicines have gained more interest because they are thought to be less toxic and because several plant-derived medicines have been identified, have been shown to have pharmacological properties, and have been shown to be promising agents to treat cancer and reduce the side effects of cancer therapies (Dreifuss et al., 2010).

Uncaria tomentosa is one of the herbal medicines that have been consumed for decades because of its reputed anti-cancer and anti-oxidant properties. *Uncaria tomentosa* has been reported to reduce the side effects of chemotherapy in cancer patients. It also helps to restore cellular DNA, prevent cell damage, and decrease mutations caused by chemotherapy (Santose et al., 2012). *Uncaria tomentosa* can also kill cancer cells or inhibit the growth of cancer cells with little toxicity to normal cells. My results showed the ability of *Uncaria tomentosa* extracts to inhibit cell growth of breast cancer cells *in vitro* as shown by the anti-proliferative effects.

Uncaria tomentosa extracts from the bark and roots have been successfully

applied for inhibiting nitrite and TNF production that is related to immunomodulatory effects (Dreifuss et al., 2010). Some researchers have reported that the anti-inflammatory activities possessed by *Uncaria tomentosa* could be related to a synergic combination of different compounds in the plant medicine (Dreifuss et al., 2010).

Several experiments have been published that study the effects of *Uncaria tomentosa* on different cell lines. Sheng et al (1998) has suggested that treatment with the aqueous extracts of *Uncaria tomentosa* significantly reduced the proliferation of HL60 human leukemia cells. Treatment also enhanced DNA repair activity. Furthermore, the (C-Med-100) aqueous extract from *Uncaria tomentosa* inhibited the proliferation of tumour cells by inducing apoptosis *in vitro* and by improving the immune response and increasing white blood cells *in vivo* as observed in both human volunteers and rats supplemented with the aqueous extract (Sheng et al., 2001; 2000).

Riva et al (2001) were the first that demonstrated the effects of treatment with *Uncaria tomentosa* bark extractions and fractions on the MCF7 breast cancer cell line (Dreifuss et al., 2010). More results from this study suggested that *Uncaria tomentosa* had anti-proliferative effects against neoplastic *tumours*. More recently, researchers have stated that mitraphylline, a component of *Uncaria tomentosa*, has anti-*tumour* activity on human cellular neuroblastoma and glioma cells *in vitro* (Dreifuss et al., 2010).

4.1 Effects of *Uncaria tomentosa* extracts on cell proliferation

The anti-proliferative effects of *Uncaria tomentosa* have been confirmed in many studies on different cell lines (De Martino et al., 2006). Prado's study reported the dose –

dependent anti-proliferative and cytotoxic activities of mitraphylline pentacyclic alkaloid isolated from *Uncaria tomentosa* on human neuroblastoma SKN-BE and glioma GAMG cells (Prado et al., 2007).

Rinner's study found anti-proliferative effects of *Uncaria tomentosa* extracts against medullary thyroid carcinoma (MTC) cells and anti-enzyme activities against mitochondrial dehydrogenase after three days of treatment. Further, Rinner's study found that the alkaloids, isopteropodine and pteropodine, significantly induced pro-apoptosis in MTC-SK cells that were derived from a solid tumour. Moreover, treatment with *Uncaria tomentosa* extracts of isopteropodine and pteropodine showed a dose and time-dependent increase in the expression of caspase-3 and caspase-7 while the level of bcl2 stayed constant (Rinner et al., 2009).

Pilarski's study reported the effects of different preparations of *Uncaria tomentosa* on the proliferation of HL-60 promyelocytic leukemia cells (Pilarski et al., 2007). In Pilarski's study, the MTT assay (thiazolyl blue tetrazolium bromide) was used to determine cell viability after treated with different preparations of *Uncaria tomentosa*. The MTT assay, which was also used in my experiments, is based on formation of dark purple formazan crystals from soluble MTT salts by the mitochondrial enzymes of living cells (Sylvester, 2011). The results from the MTT assay indicated that the ethanolic preparation had a higher cytotoxic effect than the aqueous extract of *Uncaria tomentosa* since it suppressed the proliferation of HL-60 cells more than aqueous preparation after exposure to preparations for 24, 48 and 72 hours (Sylvester, 2011).

In the present study, MTT assays were conducted over five days to measure the

proliferation rate of different cell lines after treatment with two preparation of *Uncaria tomentosa*: ethanolic and aqueous. The results showed that treatment with *Uncaaria tomentosa* extracts killed the tumor cell lines and non- malignant cell lines with the same efficacy which indicated the unselectively character of *Uncaria tomentosa*. Moreover, the results showed a complete inhibition of cell proliferation after exposure to the highest concentration (1%) of the *Uncaria tomentosa* ethanolic extract in both malignant cells (MCF7, MDA-MB-231) and non-malignant cells (HBL100, HEK239T and HSG). However, the highest concentration (1%) of the PBS preparation of *Uncaria tomentosa* caused a significant, but incomplete, reduction in cell proliferation of malignant cells, but not in non-malignant cells, which keep growing after treatment with various doses of *Uncaria tomentosa* extracted with PBS. This indicated that both extracts had anti-proliferative potency. However, *Uncaria tomentosa* extracted with 70% ethanol had strongest anti-proliferative effect due to the different compounds in both extractions. The ethanolic extract was much more effective as the cells showed the highest mortality after exposure to it. Indeed, the ethanolic extract may be enriched with petropedine and isopteropodine, which could be active inducers of apoptosis in MCF7 cells, a phenomenon which requires further investigation going forward.

In Rinner's study, the morphology of MTC-SK cells was changed after treatment with *Uncaria tomentosa* extracts isopteropodine and pteropodine as measured by chromatin condensation, cell shrinking and apoptotic bodies (Rinner et al., 2009). Future studies to determine if these components also moderate the changes in cell proliferation should be performed.

In our study, there were morphological changes in all of cell lines tested after

treatment with the high concentrations of *Uncaria tomentosa* extracts. There was significant damage to the MCF7 cells as indicated by differences in cell shape and the decrease in cell number after treatment with 1% of *Uncaria tomentosa* ethanol extracts by 72 hours. However, treatment with the high dose of PBS extract was less effective than the ethanolic extract in MCF7 cells in terms of changes in cell shape and number. This supports the potency of the ethanolic extracts of *Uncaria tomentosa* as an anti-cancer drug.

Additional confirmation was done using a wound healing migration assay to demonstrate the effects of *Uncaria tomentosa* extracts on MCF7 cell migration *in vitro*. The wound healing migration assay measures the ability of the cells to migrate into and fill a wound created by “scratching” a cell monolayer. There was an obvious reduction of MCF7 migration into the wounded area when the cells were treated with the high concentration of ethanol extracts of *Uncaria tomentosa*. This effect is dose-dependent, as the low dose of the ethanolic extract showed minimal MCF7 migration. The high dose of PBS extract caused less migration than the high dose of ethanol, while no migration was visible with the low dose of PBS extract. This emphasizes the potent effects of the alcoholic extracts of *Uncaria tomentosa* on tumor cells.

4.2 Induction of Apoptosis by *Uncaria tomentosa*

Plant-derived components have played a critical role for long time in treatments against cancer particularly by inducing death in malignant cells through the induction of apoptosis. It has become increasingly evident that plant-derived drugs may be valuable in the prevention and treatment of cancer (Taraphdar et al., 2001). Many studies suggest

that plant-derived natural products contain various anti-cancer components with different mechanisms of action, but almost all are based on their ability to induce apoptosis (Taraphdar et al., 2001). Our findings confirm that treatment with *Uncaria tomentosa* ethanol extracts can induce apoptosis in breast cancer cells.

Many experiments were done to investigate the mode of action of *Uncaria tomentosa* and measure various features of apoptosis including acridine orange/ethidium bromide staining and TUNEL assays. Acridine orange /ethidium bromide staining is a morphological staining to detect apoptotic morphology of cells *in vitro* (Ribble et al., 2005). Acridine orange stains nucleic acids (in particle nuclei) in both live and dead cells and early apoptotic cells show structural changes in the green-stained nucleus (Kasibhatla et al., 2006), while ethidium bromide stains only dead cells with compromised-plasma membranes allowing the nucleus to stain red.

An apoptotic cells shows condensed and fragmented staining of the nuclei (green) with no ethidium bromide staining. However, late in apoptosis, the plasma membrane breaks down and the cells also stain red. Our results showed an early apoptosis was introduced in a small number of cells after 24 h in MCF7 treated with the high dose of the ethanolic extract of *Uncaria tomentosa*. In contrast, the high dose of PBS did not show any early apoptotic features by 24 h. However, treatment with either extract of *Uncaria tomentos* caused chromatin condensation and late apoptosis by 72 hours although the number of affected cells was increased in cells treated with the ethanol extract. The ethanolic extract was much more effective than the PBS extract of *Uncaria tomentosa* at inducing apoptosis.

An additional assay was performed to detect apoptosis- related extensive DNA fragmentation. The TUNEL DNA fragmentation assay used terminal transferase to transfer a labelled nucleotide to any available DNA end. When DNA fragmentation occurs the number of free DNA ends increases dramatically and the presence of labelled DNA increases. Therefore, in the experiments, the MCF7 cells were treated with *Uncaria tomentosa* extracts for 1-3 days and labelled using TUNEL reactions. The results presented a significant increase in apoptotic cells due to DNA fragmentation after 72 h in MCF7 cells treated with the ethanolic extract of *Uncaria tomentosa* that was greater than that seen for the PBS extract. This confirms the effectiveness of ethanolic extract of *Uncaria tomentosa*.

Similar results were found in other studies in the laboratory that measured the effects of *Uncaria tomentosa* extracts on B16-BL6 cells. The results showed that treatment with *Uncaria tomentosa* extracts greatly induced DNA fragmentation and increased the number of apoptotic cells in B16-BL6 cells treated with 200 µg/ml of *Uncaria tomentosa* extracted with ethanol (Zari A, 2014).

Since the ethanolic extract of *Uncaria tomentosa* showed a significant induction of apoptosis, a caspase activity assay was done to confirm that the ethanolic extract mediated MCF7 cell killing via apoptosis. Apoptosis is characterized by activation of caspases (Zhang et al., 2011). In addition, the activation of specific caspases can indicate the pathway by which apoptosis is activated: the extrinsic pathway which involves activation of “ death receptors” on the cell surface results in activation of caspase-6 and-8 while activation of the intrinsic pathway following disruption of the mitochondria often as a result of Bcl-2 family protein interactions results in activation of caspase-9. Another

study suggested that *Uncaria tomentosa* greatly increased the activity of caspases 8, 1 and of the effector caspase 3 in HT29 cells (de Oliveira et al., 2014).

The results of these studies showed that MCF7 cells exposed to various concentrations of *Uncaria tomentosa* extracted with ethanol for 72 h caused a significant increase in the activity of caspase-8 and caspase-9 compared control. This indicates that *Uncaria tomentosa* extracts induced apoptosis by activation of both intrinsic and extrinsic pathways. The level of caspase-3 activation was relatively low and was not induced by treatment with *Uncaria tomentosa* extracts. Since MCF7 cells have been shown to be genetically deficient in the caspase-3 gene, it is not surprising there is low caspase-3 activity.

Overall, our result confirmed the anti-proliferative effects of both *Uncaria tomentosa* extracts against malignant and non-malignant cells. The ethanol extract was more potent than the PBS extract and showed significant results against MCF7 cells in terms of inhibition of cell growth and induced apoptosis. This could be due to different active component in both extracts.

Aguilar study's had similar results and demonstrated that both extracts of *Uncaria tomentosa* bark have an anti-inflammatory activity. The tested hydroalcoholic extract was more effective, which suggested that this effectiveness could be because of the presence of pentacyclic OAs acting alone or with other metabolites (Aguilar et al., 2002).

4.3. Conclusion

Plant derived component have played a critical role in improve health for a long

time. *Uncaria tomentosa* is one of the natural medicines has been used for decades for its pharmacological prosperities. Our present results showed that ethanolic extract of *Uncaria tomentosa* caused significant inhibition in terms of cell growth and number of both malignant and non-malignant cells in vitro by 72h. Also, it also induced morphology changes in MCF7 cells. However, the PBS extract was able to decrease the cell proliferation of malignant cells but to a lesser extent. Moreover, further confirmations were done using assay such as TUNEL assay and acridine orange/ethidium bromide staining. The results showed an increase in DNA fragmentation and apoptotic cells in MCF7 cells treated with both extracts particularly ethanolic extract. Since the ethanolic extract was more effective, a caspase activity assay was performed to emphasize the apoptosis. The data confirmed that MCF7 cells were undergoing apoptosis. Overall, The treatment with both extracts of *Uncaria tomentosa* caused major inhibition in cell proliferation of malignant and non-malignant cell lines. Furthermore, the ethanol extract derived from *Uncaria tomentosa* showed a significant inhibition of MCF7 cells in a dose-dependent manner and its effect was stronger than the PBS extract. Therefore, further testing required in different neoplastic cells in the future in order to develop a novel anticancer drug.

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