

**THE EFFECT OF *UNCARIA TOMENOSA* ON THE
MURINE MELANOMA CELL LINE, B16-BL6**

By

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of the requirements for the degree of
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Abstract

Uncaria tomentosa, commonly known as Cat's claw, is a medicinal plant native to Peru. It has been used for decades in the treatment of various inflammatory disorders. Treatment with *Uncaria tomentosa* has been shown to have effective anti-inflammatory activities. Recent studies show that treatment of cells with extracts of *Uncaria tomentosa* can inhibit the MAP kinase, Akt, and Wnt signaling pathways, suggesting it has specific anticancer therapeutic properties. Previous work from our laboratory has shown that the effect of *Uncaria tomentosa* on the monocyte-like THP-1 cell line can block activation of these immune cells. We are now investigating the effect of the *Uncaria tomentosa* as an anti-cancer therapy. We have shown that *Uncaria tomentosa* can inhibit the growth of cell cultures and can induce apoptosis in the murine melanoma cell line B16- BL6. Extracts of *Uncaria tomentosa* with 70% ethanol were more efficient at inducing apoptosis than aqueous extracts. Apoptosis induction was evident as early as 24 h after treatment and almost all cells treated with the ethanolic extract of *Uncaria tomentosa* were apoptotic by 72h. Treatment with *Uncaria tomentosa* caused an increase in DNA fragmentation (TUNEL assay), caspase-3 cleavage, sub G1 peaks in flow cytometry, and apoptotic morphology. Our experimental results indicate that *Uncaria tomentosa* can effectively kill melanoma cancer cells *in vitro*, in a dose-dependent manner, by enhancing apoptosis.

Keywords

Uncaria tomentosa, B16- BL6 cell line, Apoptosis, TUNEL assay, caspase-3, sub G1

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Abbreviations

DNA	Deoxyribonucleic acid
EMT	Epithelial-mesenchymal transition
MET	Mesenchymal-epithelial transition
COX- 2	cyclooxygenase 2 inhibitors
SERMs	Selective estrogen receptor modulators
RXRs	retinoid X receptors
RARs	retinoic acid receptor
Bcl- 2	B-cell lymphoma 2
NPH	natural health product
P53	tumor suppressor p53
PARP	poly ADP ribose polymerase
B16- BL6	Murine Melanoma B16- BL6
HEK293T	Human Embryonic Kidney 293 cells
HSG	Human salivary gland cell
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle Medium
CO2	Carbon dioxide
HPLC	High-performance liquid chromatography
MTT	Methyl thiazol tetrazolium assay
AO/ EB	Acridine orange/ethidium bromide staining
LSM510	The Zeiss LSM 510 Meta Confocal Microscope
TUNEL	Terminal deoxynucleotidyl transferase

PBS	Phosphate buffered saline
PI	Propidium iodide
BCA	Bovine Serum Albumin
SDS- PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris-Buffered Saline and Tween 20
TBS	Tris-Buffered Saline
ECL	Enhanced chemiluminescence
EtoH	Ethanol
THP-1	A human monocytic cell line

Chapter 1: Introduction

1.1. Cancer

Cancer is a major public health problem and it is the main cause of death in many parts of the world. In Canada, cancer is the second leading cause of death. According to Canadian statistics, 2 of 5 Canadians will be diagnosed with cancer and 1 in 4 Canadians will die from cancer. In 2013, it was estimated that 187,600 Canadians will develop cancer in their lifetime and 75,500 will die from it. The concern of that is 52% of new cancer cases will be lung, breast, and prostate, which are considered to be the main cancer types that cause death. Moreover, in Canada, men are diagnosed with cancer more often than women and most of them are over the age of 50 years. Based on these statistics, the cancer cases among the Canadian population will increase from 34.9 million cases in 2012 to 47.7 million cases by 2036. These statistics are also important for planning health services for cancer patients in the future and provides incentives to increase cancer research to discover therapies that cure and prevent cancer in order to improve survival rates (1).

Cancer is one the diseases that develops by going through a multistep process (7). First, the transformation of normal cells to neoplastic cancer cells is widely studied in order to understand the causes of cancer progression (2). The initiation of carcinogenesis is usually associated with environmental stressors that require a long and repeated exposure to many exogenous agents such as chemicals, radiation, poor diet, risky life styles, and individual habits (2). This process can also be affected by intracellular factors

such as hormones, family history, and a genetic predisposition (7). In addition, viruses, chromosomal abnormalities, non-healing wounds, and failure of immunological surveillance can also promote cancer risk (4). Generally, carcinogenic promoters will cause a modification in the DNA sequence which can generate mutations that are involved in the development of cancer (4). These mutations, which are the first phenomena associated with cancer disease, usually involve neoplastic genome mutations that affect the genes that are involved in controlling cellular proliferation, cellular development, apoptosis, and differentiation (7). Complete transformation, the next step in developing a malignant cell is called the progression step in cancer development (7). According to this idea, cancer is defined as a genetic disease (5) caused by mutations in critical genes that result in uncontrolled cell proliferation or division (4) and decreased expression of programmed cell death (apoptosis) (6). Cancer cells can be characterized by several specific properties such as loss of specialization, de-differentiation, loss of the normal interaction with the basement membrane, and the ability to move and invade other organs to form a new malignant tumor, called metastasis (11). The metastatic ability of a cancer cell allows cancer to occur in every organ (12).

One of the most important cancer characteristics is metastasis, or invasion, which is considered to be the main cause of death because from cancer. This idea of metastasis is when the tumor cells lose their ability to adhere to each other and migrate from the primary tumor site to surrounding tissue and then grow to form a new tumor in the new tissue (9). One main mechanism in the metastatic process is Epithelial Mesenchymal Transition (EMT). EMT occurs when cancer cells lose adhesion to each other (via E-cadherin downregulation) and increase adhesion to the basement membrane promoting

cell migration. Ultimately, the basement membrane will be fragmented because of the increased secretion of proteases during cancer progression. After the cells migrate from their primary tissue, they can enter the blood or lymph vessel, and then spread to distant or neighboring tissues. Once in the new tissue the cells then return to their original epithelial morphology by Mesenchymal-Epithelial Transition (MET) and grow to form new tumors (10).

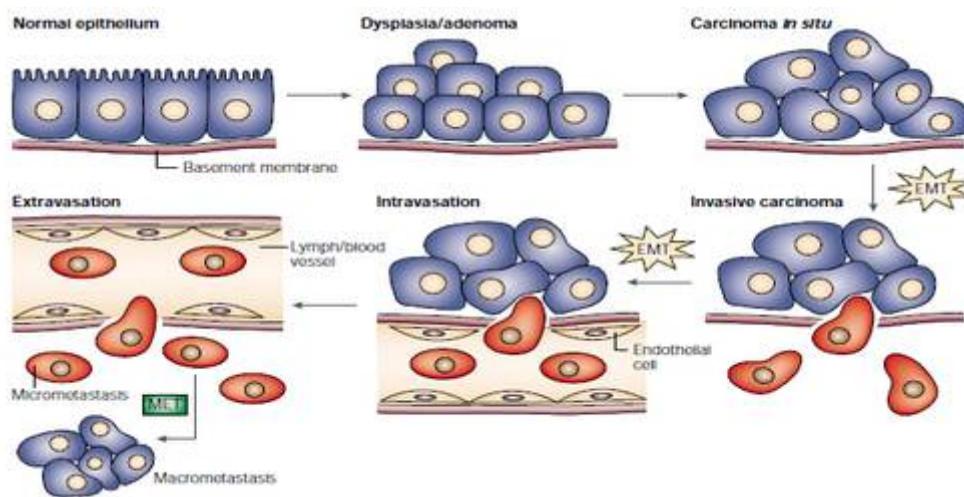


Figure 1.1: The main mechanism in the metastatic process (Epithelial Mesenchymal Transition, EMT)

1.2. Cancer therapy

The second step after cancer diagnosis is determining an appropriate approach to treatment. The ethical idea of figuring out which treatment method is suitable for each patient requires a plan to minimize the pain and discomfort experienced by the patient while expanding the patients' lifespan. Traditionally, the three common form of cancer treatment are surgery, radiation and chemotherapy although other novel methods, such as

immunotherapy are becoming more common. Choosing the right treatment is based on some important factors like the type of cancer, the cancer's stage, the evidence of metastasis, patient age, and the presence of any known genetic mutations.

Surgical treatment is basically where the damaged or diseased organ is removed from the body. This treatment is always for specific organs or locations. As long as the evidence of metastasis is not present after surgery this treatment gives the greatest hope of a successful cure. One of the most important and concerning side effects of surgery is formation of a secondary tumor. In some cases, the eradication of the primary tumor will initiate the growth of a metastatic tumor and can help to promote the growth of the secondary tumor.

A second type of treatment is to use X- rays (or other high energy radiation) in order to destroy the tumor, called radiation therapy. The theory is that the X- rays target the DNA molecules and cause extensive damage to the DNA of the cancer cells, which cannot be properly repaired, disrupting cell division and increasing cancer cell death. Radiation therapy is used for tumors with a specific location, similar to surgery. There are two types of radiation therapy, external beam radiation therapy and internal radiation therapy. External beam radiation therapy is done by exposing the tumor directly to X- rays beams. However, internal radiation therapy is an alternative method of delivering radiation and that done by implanting some radioactive pellets or seeds directly on the cancer tissue. The side effects of radiation therapy are general malaise, skin redness, hair loss, and mouth sores resulting from damage of adjacent tissues; and, for some women it can also cause menopause because of the decreased production of estrogen. Sometimes

radiation-dependent damage to the genetic information in adjacent healthy cells can cause oncogenic transformation that will lead to development a new cancer.

Chemotherapy is a commonly used anti-cancer therapy involving the use of cytotoxic drugs which have anti-cancer properties and are capable of inhibiting cancer cell proliferation or can directly kill these cells. The mechanism of actions of these drugs starts when the cytotoxic agent is delivered through the circulatory system and diffuses to the cancer cells and targets the activities of some important molecules in cancer progression such as the molecules that are required for cancer cell division (including DNA). The benefit of chemotherapy is that it is focused on systemically killing the cancer cells and decreasing their ability to grow independent of the location of the tumor in the body. Chemotherapy also decreases the chance of a metastatic tumor forming in the future. Chemotherapy, like other therapies, has side effects on the healthy adjacent tissue and these effects can be more extensive than radiation therapy. The most significant effect of chemotherapy is its ability to kill the fast growing cells of the hematopoietic system (blood plus immune system) and gastrointestinal tract.

Other treatments that are also used in treating cancer include biological therapy, which is also known as immunotherapy because it is focused on the body's immune system. This treatment involves using interferon, cytokines, colony- stimulating factors, and gene therapy approaches. Minimizing or eliminating the side effects of cancer treatment is an important component in helping to develop improved methods and increase the effectiveness of chemoprevention (13).

1.3. Chemoprevention

Using natural compounds, synthetic agents, or biological chemical agents in order to prevent tumor development and formation of metastatic (malignant) tumors is commonly known as chemoprevention (14). Chemopreventive agents mainly target the carcinogenic process that is driven by specific mutations. Those mutations can be identified in patients early in life, even more than 20 years in advance of metastasis. The mechanisms by which the chemopreventive agents work focuses on blocking mutagenic damage to DNA. Consequently, many of the chemoprevention agents have been developed based on their mechanism of action; four classes of new agents were developed clinically and experimentally according to their action in the cell. Chemoprevention agents are also classified based on their function such as antigenic, anti-proliferative, and anti-hormonal agents (16).

The first class of chemoprevention agents was developed based on the relationship between inflammation and carcinogenesis to inhibit the function of COX-2 enzyme activity. COX-2 is involved in synthesizing the inflammatory prostaglandins produced by arachidonic acid, and the overexpression of the COX-2 gene is linked to colon carcinogenesis. Celecoxib, which is a selective inhibitor of the activity of the COX-2 enzyme, is one of these agents.

Selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, were developed to prevent prostatic carcinogenesis and tested experimentally on rats and shown to work by controlling the binding between the estrogen receptor and estrogen analogs.

Rexinoids (RXRs) are the third important class of chemoprevention agents. These agents are able to heterodimerize with retinoic acid receptor (RARs), vitamin D receptors, the thyroid receptor, and orphan receptors that are called the nuclear receptor superfamily. The RXRs agents are used in the prevention of mammary carcinogenesis and have been evaluated experimentally in rat cancer models.

The last important class of chemoprevention agents is the orphan nuclear receptor, PPAR- γ , which was developed to prevent colon carcinogenesis because of its high ability to bind to fatty acids and prostaglandins (15).

These chemopreventive components are capable of stimulating programmed cell death (apoptosis) in some cancer cells. Studies have tested their ability to regulate the intrinsic or extrinsic apoptotic pathways. These agents always target the intrinsic pathway and control the Bcl-2 family proteins and inhibit Bcl-2 expression or induce Bax expression, to regulate mitochondrial permeability transition (18).

1.4. Natural products and drug discovery

Natural products have been used as medicines to cure a variety of diseases and illnesses for a long time. In developing countries, the herbal and vitamin medicines are still used in primary health care. In developed countries there is a belief that herbal remedies have fewer side effects (19). Approximately, 71% of the Canadian population consumes a natural health product (NPH) (20), such as traditional Chinese medicine, traditional Japanese medicine, and traditional Indian medicine (21) and believe these

medicines to be safe. The structures of some of the widely used NHPs, including curcumin and resveratrol are shown in figure 2.

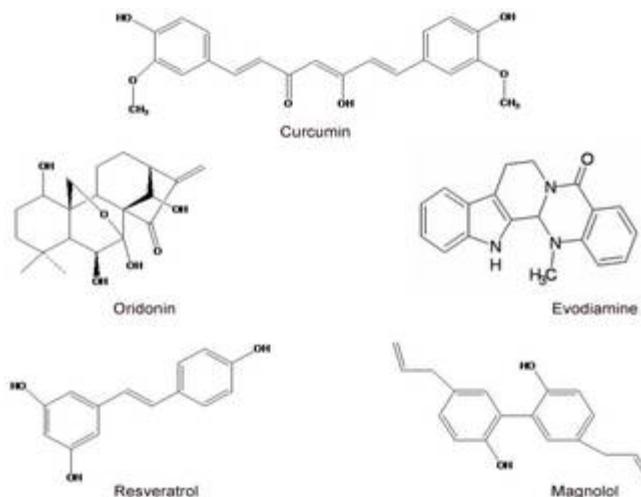


Figure 1.2: The structure of Natural compounds (32)

Apparently, after screening many natural products, antioxidant nutrients and phytochemicals were identified which had a low toxicity and were active as anti-oxidant chemoprevention drugs (17). The combination of most phytochemicals with vitamins and phenolic agent alkaloids has played a very important role in the discovery of many cancer prevention drugs. These compounds have a role in inhibiting the rapid proliferation of cancer cells, inducing apoptosis or promoting differentiation, consistent with their chemoprevention properties. Several may also have antioxidant activities which are used as anti-carcinogenesis drugs, specifically for skin carcinogenesis (17). Mainly, these natural products are extracted from plants, animals, and microorganisms and many of these are found to be effective in treating more than 60 human cancer cell

lines, including solid tumors like lung cancer, kidney cancer, prostate cancer, and breast cancer (22).

As a result, natural products became the best source for the development of anti-cancer drugs once they showed the biological activity in screening assay. These materials provided the source to identify active chemicals, which were then used as the basis for synthesizing of new drugs. This process is one of the most important processes in developing cancer preventive drugs. Most of the anti-cancer drugs that were originally extracted from natural products sources need an accurate analysis of their physiochemical features in order to better understand more their anti-cancer activities. The process of discovering an efficient anti-cancer drug started when the natural products extractions were purified to identify their active components such as alkaloids (e.g. morphine, quinine, and atropine), which are still extensively used (23).

The prior advantages of purifying the active component for the natural extractions are useful in standardizing the dose amount and for reducing the side effects of these ingredients. In drug discovery processes, these natural product extracts must be tested by further biological experiments and by fractionations of these extractions to identify further active ingredients, which can then be characterized. The active components identified by this processes can then go through further synthesis by chemists to create new, more effective small molecule drugs in order to target diseases based on identifying the molecular lesion (23). Approximately, 119 chemical components isolated from plants have been highly used as drugs in many parts of the world (24).

1.5. Natural products and cancer chemotherapy

Natural products have contributed to the discovery of new drugs used in cancer chemotherapy. This has required a proper understanding of the idiosyncratic mechanisms of action of the oncology drugs originally derived from natural products in order to improve their effectiveness (23). First, in order to identify any new chemotherapy drug, it is important to understand the molecular changes that are involved in the development of cancer cells in order to identify target molecules or pathways. This requires a careful study of the genes that are related to cancer and then to identify any specific mutation which might change activity. Intelligent drug design requires that the structure of the mutant proteins can be determined and modeled to the potential drug. Optimal structures that bind the mutant proteins can then be modeled; this selected molecule can be tested experimentally *in vitro* and *in vivo* by testing angiogenesis inhibition, signal transduction, or activation of protein kinase activity (24). Some of the anti-cancer effects mediated by drugs can be based on their mechanism of action. For example, some anti-cancer drugs target the DNA molecules, protein receptors, or enzymes and interfere with normal function (23).

Developing natural products into drugs based on their mechanism of action is a relatively new approach to target cancer cells. For instance, antibody-directed enzyme pro-drug therapy is a technique involving the combination of antibody modulated tumor targeting and natural products. The details of this technique involve using specific antibodies that bind to an enzyme or other important molecule that accumulates on the cancer cell surface. After a short treatment time, the cells are exposed to a non-cytotoxic pro-drug and the enzyme/antibody-binding complex is able to convert the pro-drug to

generate the cytotoxic anti-cancer drug at the site of antibody binding. Therefore, the anti-cancer drugs are released close to the targeted tumor cells and non-specific cytotoxic effects are reduced. Examples of some of natural product-derived drugs that have been used in this technique are the Vince alkaloids and Taxols, which were originally, isolated from Cephalosporin- alkaloid pro drugs (22).

1.6. Alkaloids and their effects on cancer

Alkaloids extracted from some natural products have been shown to have anti-proliferation and anti-metastasizing activities on many kinds of cancer cells. Consequently, alkaloids have provided a rich resource for drug discovery. For example, camptothecin and vinblastine, which have been experimentally developed as anti-cancer drugs, were originally isolated as alkaloids from natural sources. A ring structure and nitrogen located in the heterocyclic ring, structurally characterize alkaloids. The alkaloids are classified into different group according to their biosynthetic pathways, such as the alkaloids that exist in plants belonging to Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae, and Loganiaceae (25).

The alkaloids are also diverse based on their biological activities. For example, the action of ephedrine on asthma, the analgesic action of morphine, and the anti-cancer effect of vinblastine all have different mechanisms of action. The typical natural alkaloids that show anti-cancer activity are berberine, evodiamine, matrine, piperine, sangllinarine, and tetrandrine. These molecules have been widely studied in terms of their anti-cancer activities on many cancer cells and have contributed in the development

of anti-cancer drugs. Basically, the above alkaloids are isolated from different plant families and have different biosynthetic activities and different pharmacological activities. For example, piperine can block some inflammatory activities and mainly has activity in cancer prevention, whereas other alkaloids, such as berberine are also focused on promoting anti-proliferative activity in the cancer cells.

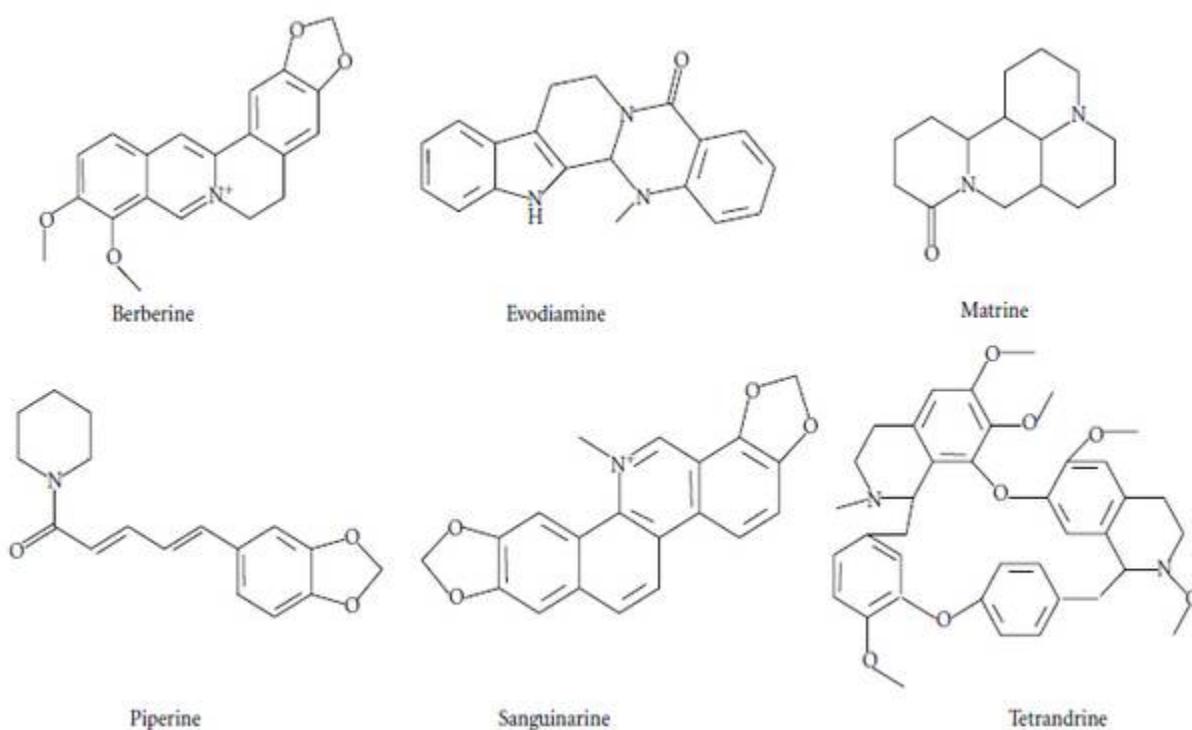


Figure 1.3: The typical natural alkaloids

The anti-cancer alkaloids have further characteristics that are important in understanding their anti-cancer activities (25). First, the alkaloid concentration that enhances anti-cancer effects is very important. For example, the previous alkaloids must

be presented to the cancer cells at high concentration compared to other chemotherapeutic drugs, such as vinblastine. On the other hand, marine alkaloids need only millimole concentrations to show their anti-cancer activity. Most of the natural alkaloids require structural modification before they are useful as chemotherapy drugs because they have low water solubility and poor bioavailability and are a difficulty to get to the cancer site. Finally, the toxicity of these alkaloids must be studied because, like other chemicals, they have side effects. For example, berberin causes side effects including anaphylaxis, constipation, and skin allergies and can also cause kernicterus. Consequently, the transformation or modification of these alkaloids' chemical structures is required to limit the toxicities of these alkaloids while maintaining their anti-cancer activities (25).

1.7. Natural products as inducers of apoptosis

The mechanism of cell death can be classified into different types which are differentiated based on the cellular morphological characteristics and by biochemical and cellular parameters. The main types of cell death that are associated with distinct morphological changes are apoptosis, necrosis, and autophagy. However, the most extensively studied are apoptosis and necrosis.

Necrosis is defined morphologically by an increase in the cell volume, swelling of the organelles, fragmenting of the plasma membrane and release of the intracellular contents including DNA. Programmed cell death, known as apoptosis, is a controlled physiological process to remove the damaged cells. Apoptosis is characterized by several

structural modifications such as rounding up of the cell, decrease on cellular volume, chromatin condensation, DNA fragmentation, and blebbing of the plasma membrane. The result is that the cell contents are packaged into membrane-bound vesicles, called apoptosomes, which are consumed and degraded by macrophages. Relatively early during the process of apoptosis a group of proteins called caspases, which degrade cellular proteins and DNA, are activated. However, caspase activation is not responsible for the execution of cell death (42).

Apoptosis is mainly controlled by two core pathways that are able to induce apoptosis, the extrinsic" death receptor" pathway and intrinsic" mitochondrial" pathway. The extrinsic pathway is triggered by signals that are activated by binding of ligands to death receptors in the plasma membrane. The intrinsic pathway is regulated at the mitochondria and is initiated by the release of cytochrome c to the cytoplasm (figure 4) (43).

The natural products show an important role in this field of research because some have been shown to induce apoptosis without damaging normal cells. There are plants extracts used as a traditional medicine that are focused on inducing apoptosis in abnormal cells. For example, the aqueous extract of *Selaginella tamariscina* has been shown to increase the expression of the p53 gene and elicit a G1phase cell cycle arrest and cause DNA fragmentation in human leukaemia cell lines. *Salanum muriatum* extracts also induced apoptosis by causing DNA fragmentation and PARP cleavage; which is one of the apoptosis hallmarks, in prostate cancer (26).

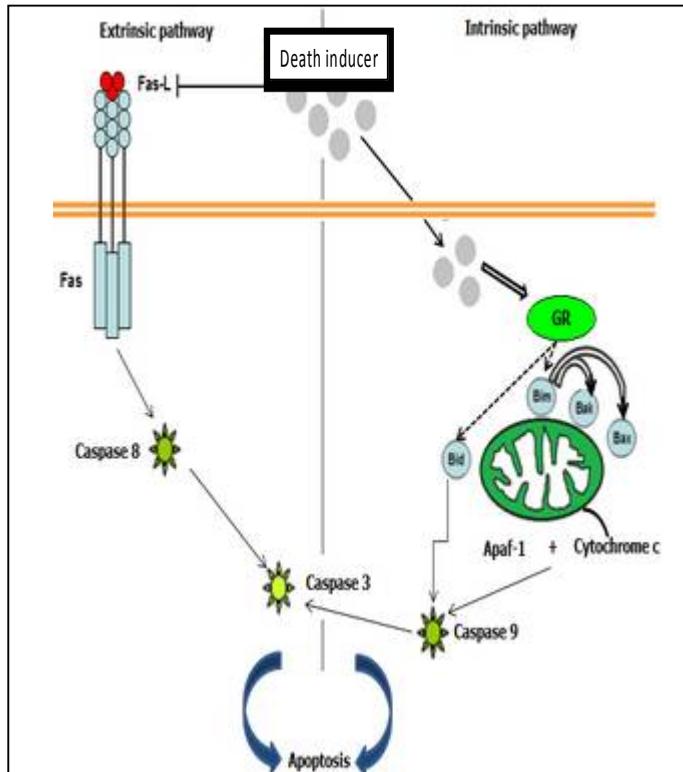


Figure 1.4: Apoptosis pathways (47).

The mechanism by which natural chemopreventive agents are found to work is by regulating processes related to xenobiotic biotransformation, or by stimulating apoptosis in premalignant and malignant cells. Because, most of these agents target signal transduction pathway that regulate apoptosis, understanding how apoptosis is regulated is important. First apoptosis, or a programmed cell death, is a process that causes destruction of damaged or abnormal cells (49).

To enhance apoptosis, the chemopreventive agents must target one of the effector mechanisms that activate apoptosis. These effector mechanisms consist of several components that correlate with caspase activation or activation of death receptor-mediated receptors generally known as the extrinsic pathway of apoptosis. The intrinsic

pathway of apoptosis is mainly focused on mitochondrial mediators such as the Bcl family of proteins that disrupt mitochondria function and activate of a caspase cascade (49).

The role of most chemopreventive agents is to block or slow cell transformation by inducing apoptosis processes. These agents could also be beneficial for healthy people who might be at increased cancer risk. One concern raised by many researchers is that long- term use of these chemopreventive agents might raise the incidence of drug resistance for many cancers. Alternatively, chemoprevention agents may be used only to target the tumor cells in patients with cancer by using short-term treatment to enhance cellular apoptosis (26).

The apoptosis process decreases the life span of both normal and cancer cells. The aim of most chemotherapy and chemopreventive agents is to preferentially kill cancer cells with a lesser effect on normal cells. Therefore, it is important to screen for anti-cancer agents that induce apoptosis in cancer cells to a greater extent than normal cells.

1.8. *Uncaria tomentosa* (Cat's claw)

Uncaria tomentosa is a woody vine, in the *Rubiaceae* family (30), commonly known as Cat's Claw (27). It was originally found in the Amazon rainforest and in various areas of South and Central America (30). The native Indians used *Uncaria tomentosa* as a tea made from its bark or roots (28) which are the most commonly used

parts of *Uncaria tomentosa* (29). It has been more recently found that the organic components of *Uncaria tomentosa* have cytotoxic and anti-inflammatory activities (28), and that the *Uncaria tomentosa* bark is rich in alkaloids, quinovic acid, glycosides, and phenolic compounds (29) which have been used medicinally to treat several diseases such as inflammation, rheumatism, arthritis, and cancer (31).

Several studies have shown the anti-inflammatory activity of *Uncaria tomentosa* *in vivo* and *in vitro* and have identified a role for *Uncaria tomentosa* in inhibiting tumor initiation and growth. *Uncaria tomentosa* is one of the anti-inflammatory drugs that has been shown to possess effective action in cancer therapy and prevention. *Uncaria tomentosa* was shown to inhibit NF- κ B, which is an important transcription factor in both chronic inflammation and cancer and it is an important target in cancer therapy (32).

Different preparations of *Uncaria tomentosa* have been found to have different anti-inflammatory activities such as reducing the production of the inflammatory cytokine TNF α . Also, it has a role in eliciting the production of factors that control lymphocyte-proliferation by human endothelial cells. *Uncaria tomentosa* extracts have also been shown to have the ability to enhance DNA repair and protect lymphocytes from apoptosis (34).

Recently, many studies have determined the biological activities of *Uncaria tomentosa*. These studies have found that these activities are mediated by the presence of different effective components such as tetracyclic and pentacyclic oxindole alkaloids. In addition, phenolic components such as flavonoids and phenolic acids were shown to be responsible for the high antioxidant activity (33).

Chapter 2: Thesis objectives

The objectives of this thesis are to:

- 1- Study the effect of *Uncaria Tomentosa* on the melanoma cell line, B16-BL6.
- 2- Determine the mechanism of action of *Uncaria Tomentosa* in promoting cancer cell death.

Chapter 3: Materials and Methods

3.1. Tissue culture

The B16-BL6 (murine melanoma), HEK293T(human embryonic kidney cells) and HSG (human salivary gland) cell lines were obtained from ATCC [American Type Culture Collection, Manassas, VA] and maintained in Dulbecco's Modified Essential Medium (DMEM, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Burlington, ON). Cells were incubated at 37 °C in 5% CO₂. For experiments, the cells were treated with media containing *Uncaria tomentosa* or the individual component at various concentrations as indicated. All experiments were run in triplicate and data were analyzed from three independent experiments.

3.2. Extracts of *Uncaria tomentosa*

Uncaria tomentosa was provided as a dried powder of the roots by Rosa Rosales (Lima, Peru) or purchased as a NHP (Cat's Claw extract, Now Foods, Bloomington, IL. code 84618). The powder was extracted with either water or 70% ethanol and the soluble fraction used in experiments. Equivalent results were obtained for the *Uncaria tomentosa* acquired from both sources. For the aqueous extraction, 1 g of *Uncaria tomentosa* powder was suspended in 10 ml water and boiled for 1h. For the ethanolic extraction, 1g of *Uncaria tomentosa* powder was suspended in 10 ml 70% ethanol and

boiled for 1h. The insoluble material was removed by centrifugation at 10,000 x g for 10 min and then the supernatant filtered through a 0.22 µm syringe filter.

For some experiments the *Uncaria tomentosa* was fractionated over an ethanol-water gradient. Fifty grams of *Uncaria tomentosa* powder was suspended and boiled in 500 ml of 70% ethanol for 1 h. The mixture was filtered through 1mm Whatman filter paper and the filtrate freeze-dried (a sample of dried powder was removed). The *Uncaria tomentosa* extract was then resuspended in 200 ml water and mixed with 20 g of poly(vinylpyrrolidone) matrix (Sigma Chemical, St Louis, MO). The mixture was poured into a column and 50 ml fractions taken. The column was sequentially eluted with 200 ml each of water, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 100% ethanol. The fractions were either freeze-dried or evaporated to a powder for use in cell growth and HPLC experiments.

3.3. Cell treatments

The B16-BL6, HEK 293T, and HSG cells, cultured and maintained in DMEM containing 10% fetal calf serum, were treated with *Uncaria tomentosa* extracts at various concentrations and allowed to grow in 5% CO₂ at 37°C. For treatments, the cells were collected by centrifugation at 1000xg and plated onto 96 well plates at approximately 20% confluence (2000 cells/well). Two different preparations of *Uncaria tomentosa* were used; the aqueous extract (1000 mg/ml) and the ethanolic extract (1000 mg/ml). The two extracts were compared by HPLC in order to ensure equivalent quantities of the detected components. The two extracts were resuspended in DMEM media before

treatment. For treatments, different doses of these stock solutions were applied to the cell cultures. For each experiment, water and ethanol were used at the same dose to generate negative controls.

3.4. MTT assay (Methyl Tetrazolium Blue)

B16-BL6, HEK293T, and HSG cells were plated on 96-well plates at a concentration at approximately 2×10^3 cells per well & incubated for 24 hours (5% CO₂ 37°C). Cells were treated with 0.01% (low dose) or 1% (high dose) *Uncaria tomentosa* extract in media on day 0; media alone served as a negative control. Cells were treated for varying times (1-5 days) without a media change. Cell viability was measured daily using the MTT assay. To each well, 10 µl/ml of 0.25 mg/ml MTT was added followed by a 4 hour incubation. The media was removed and 100 µl DMSO was added to solubilize the crystals. Absorbance at 450 nm was measured on a plate reader (Spectramax 340PC 389).

3.5. Wound healing migration assay

Cells were plated on 6 well plates at an approximate confluence of 1×10^6 cells/well and cultured overnight in media. Wounds were made in the confluent monolayers using a 200 µl plastic pipet tip. Cells were treated with culture media in the presence of 0.01 or 1% *Uncaria tomentosa* extract. Digital photographs of the wounds

were taken (using an Axiovert 100 microscope) at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h post treatment and changes in the distant spanned by the wounds were measured.

3.6. Acridine orange and ethidium bromide staining

B16-BL6 cells were plated on glass cover slips in culture media overnight at 37°C to allow the cells to adhere. Cells were treated with *Uncaria tomentosa* extract (0.01% low dose or 1% high dose) on day 0. B16-BL6 cells treated with 6 µg/ml of camptothecin as a positive control for apoptosis. Cells were treated for 6 h, 12 h, 24 h, 48 h, or 72 h. Cells were stained with 100 µg/ml acridine orange (Sigma-Aldrich) for 5 min and then stained with 100 µg/ml of ethidium bromide (Sigma-Aldrich) for 5 min. Coverslips were washed in PBS, gently mounted onto a glass slide, and sealed with clear nail polish. Fluorescence was visualized using an LSM510 microscope.

3.7. TUNEL assay of DNA fragmentation

B16-BL6 cells were plated on glass cover slips overnight at 37°C to allow the cells to adhere. Cells were treated with *Uncaria tomentosa* extract (0.01% low dose or 1% high dose) on day 0 and incubated for 72h. B16-BL6 cells treated with 6 µg/ml of camptothecin were used as a positive control for apoptosis. Cells were fixed with 4% paraformaldehyde (freshly prepared) for 5 min. Cells were then permeabilized by incubation with 0.1% TritonX-100 in 0.1% sodium citrate (freshly prepared) for 2 min on ice. The cell cultures were washed with PBS and treated with in 50 µl/well TUNEL

reaction mixture (Roche, Laval, QB). The reaction was incubated for 60 min at 37°C in a humidified atmosphere in the dark. The cells were washed twice in PBS, pH 7.4, and then the cover slips were transferred to glass slides. Samples were directly analyzed using a fluorescence microscope.

3.8. Flow cytometry

B16-BL6 cells grown in 60 x 15 mm tissue culture plates. The cells were treated with *Uncaria tomentosa* at 0.01% for the low concentration, 0.1 % for medium and 1 µl/ml for high concentration. B16-BL6 cells treated with 6 µg/ml camptothecin as a positive control for the detection of apoptosis. The cells were then washed with PBS, harvested by incubation in 0.25% (w/v) trypsin-EDTA and then collected by centrifugation at 400 x g for 10 min. The cells were fixed by adding cold 70% ethanol and incubated at -20°C. The cells were then subjected to centrifugation at 400 x g for 10 min and washed twice with PBS. The cells were re-suspended in 500 µl of propidium iodide (PI) staining solution (165 mM NaCl, 10% Triton 100, 50 µg/µl RNase A, 200 µg/ml of propidium iodide). Samples were analyzed on a Beckman Coulter LS600 flow cytometer.

3.9. Western blot analysis

Cell monolayers were cultured on 100 mm² plates and treated with *Uncaria tomentosa* extracts for 1-3 days. The cells were collected in RIPA (0.5% SDS, 0.5%

sodium deoxycholate, 1% Triton X-100, 150 mM sodium chloride, and 250 mM Tris-HCl, pH 7.4) and lysed. Protein concentrations were determined using a BCA assay and 25 µg of cell lysate was separated by on a 10% polyacrylamide gel containing SDS (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane (Whatman, Mandel Sci., Burlington, ON) using a BioRad semi-dry transfer machine. Blots were stained with 0.5% Ponceau S in 1% acetic acid to confirm uniform loading. The membranes were blocked by incubation in blocking buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 0.05% Tween-20 (TBST) and 5% bovine serum albumin (BSA, Sigma). After washing with TBST, the membranes were incubated in anti-caspase-3 mouse antibody (Cell Signaling Inc, Lake Placid, NY, titre 1:1000). The blots were washed and incubated with an appropriate secondary antibody (goat anti-mouse IgG- HRP) (Santa Cruz Biotechnology, Santa Cruz, CA; titre 1: 10,000) for 1 hour and then washed twice with TBST and once with TBS. The blots were then incubated for 5 min in enhanced chemiluminescence reagent (ECL, Pierce Chemical Co., Toronto, ON) and then exposed to X-ray film.

3.10. HPLC (High-performance liquid chromatography)

Uncaria tomentosa extracts were fractionated on poly (vinylpyrrolidone) columns into different fractions starting by the aqueous fraction, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 100% ethanol. The alkaloid composition of these fractions was determined by using High-performance liquid chromatography (HPLC) on a Breeze 2 chromatography system (Waters Inc, Toronto, ON). Each fraction

was separated by using a Sunfire C18 column 3.5 μm resin 4.6x100 mm. The solvents used were; (A) 60 volumes 10 mM phosphate buffer, pH 6.6, 20 volumes acetonitrile, and 20 volumes methanol, and (B) 30 volumes 10 mM phosphate buffer, pH 6.6, 35 volumes acetonitrile, and 25 volumes methanol. Solvent was pumped through the column at 1ml/min. First, the solvents were mixed using a gradient starting with 100% buffer A and finishing with 100% B over a time period of 40 min. Then, 100% buffer B was pumped through the column for 10 min and finally a gradient starting with 100% B and finishing with 100% was pumped through the column for 5 min. The components in the *Uncaria tomentosa* extract were detected using a wavelength of 245 nm and the detected peaks compared to a group of standards (Planta Analytical).

Chapter 4: Results

4.1. Inhibition of B16- BL6 cell proliferation treated with *Uncaria tomentosa*

The effect of *Uncaria tomentosa* treatment on the growth of B16- BL6 cell cultures was determined. B16-BL6 treated with two different extracts (water and 70% ethanol) of *Uncaria tomentosa* showed significant inhibition of proliferation. The number of cells in cultures treated with *Uncaria tomentosa* was significantly reduced at day 3 in a dose-dependent manner. The number of cells in high dose (1%)-treated cultures was approximately 50% of the suspending media control (**Figures 4.1 and 4.2**). B16-BL6 cell proliferation was consistently reduced for at least 5 days after the treatment. At the highest doses, the number of cells did not increase compared to day 0.

Treatment of the non-malignant cell lines, HEK293 and HSG cells, with extracts of *Uncaria tomentosa* also significantly inhibited cell proliferation in a dose-dependent manner (**Figures 4.3, 4.4, 4.5, and 4.6**). In all cases, treatment with the ethanolic extract was more effective in inhibiting cell proliferation than was the aqueous extract. In addition, there were some differences in the sensitivity of the cell lines to *Uncaria tomentosa*. The HSG cells were much less sensitive to the aqueous extract compared to HEK293T and B16-BL6 cells although all three cell lines showed similar sensitivity to the ethanolic extract (**Figure 4.7**).

Figure 4.1: Effect of the ethanolic extract of *Uncaria tomentosa* on B16- BL6 cell growth.

B16- BL6 cells were treated with different concentrations of *Uncaria tomentosa* extracted with 70% ethanol (0.01, 0.05, 0.1, 0.5, and 1% *Uncaria tomentosa* in media). Cell proliferation was measured using the MTT assay. The cells were incubated with the *Uncaria tomentosa* extract for five days and absorbance was determined each day. Data were analyzed for three independent experiments.

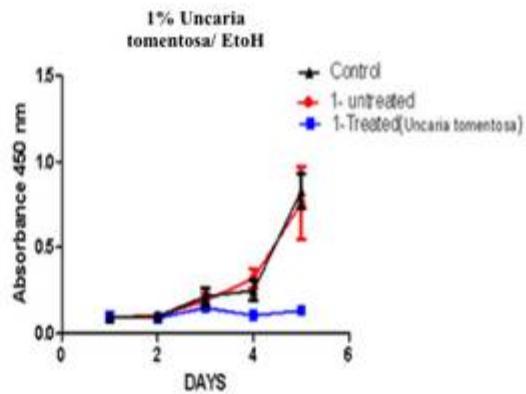
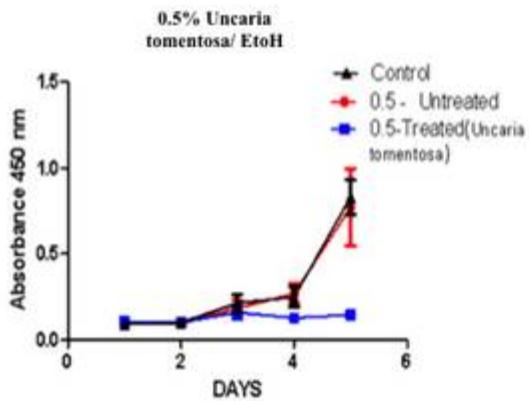
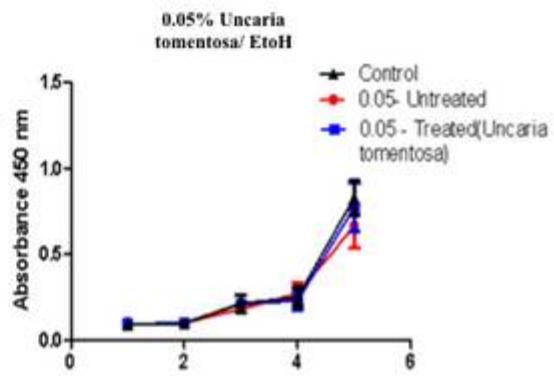
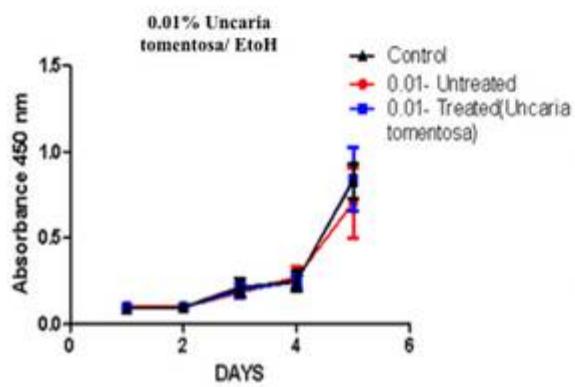


Figure 4.2: Effect of the aqueous extract of *Uncaria tomentosa* on B16-BL6 cell growth.

B16- BL6 cells were treated with different concentrations of *Uncaria tomentosa* extracted with water (0.01, 0.05, 0.1, 0.5, and 1% *Uncaria tomentosa* in media). Cell proliferation was measured using the MTT assay. The cells were incubated with the *Uncaria tomentosa* extract for five days and absorbance was determined each day. Data were analyzed for three independent experiments.

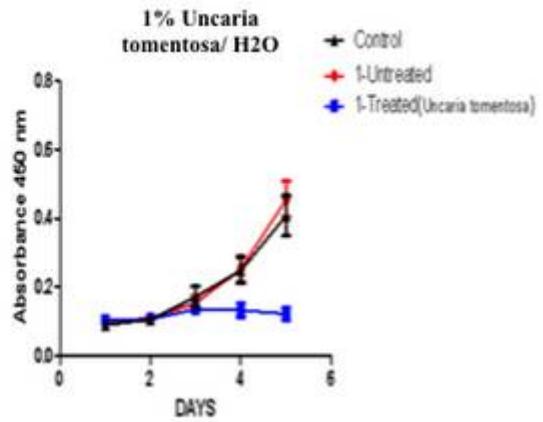
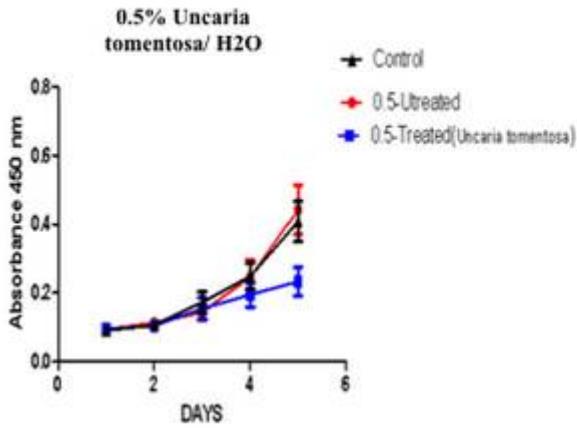
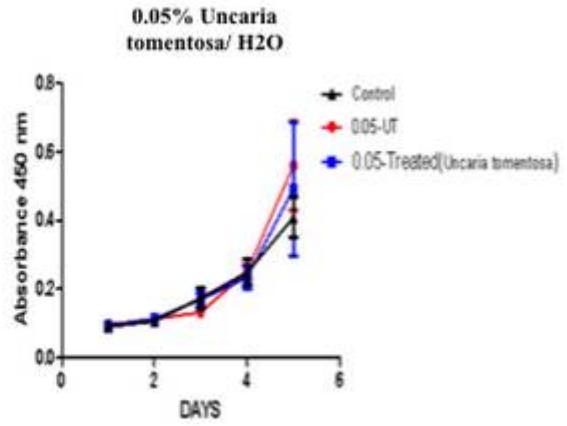
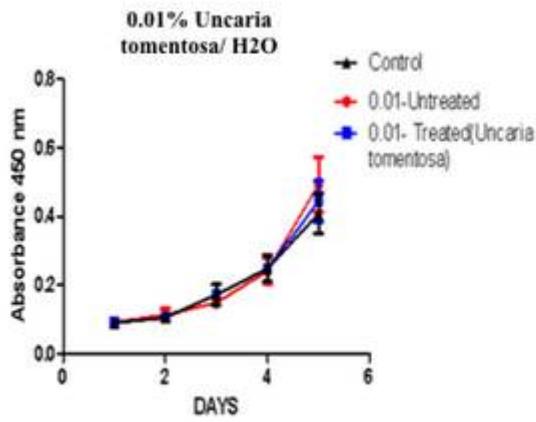


Figure 4.3: Effect of ethanol extracts of *Uncaria tomentosa* on non-malignant HEK293T cell growth.

HEK293T cells were treated with different concentrations of *Uncaria tomentosa* extracted with 70% ethanol (0.01, 0.05, 0.1, 0.5, and 1% *Uncaria tomentosa* in media). Cell proliferation was measured using the MTT assay. The cells were incubated with the *Uncaria tomentosa* extract for five days and absorbance was determined each day. The graphs represent one of at least three independent experiments.

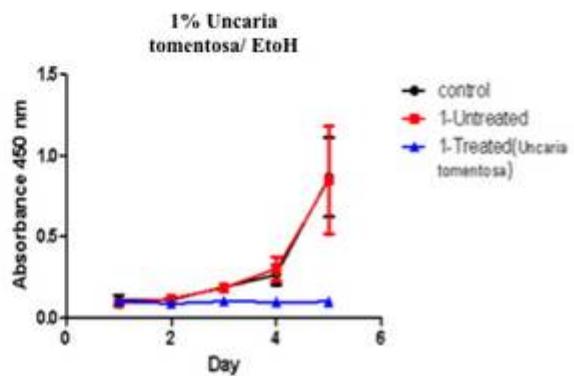
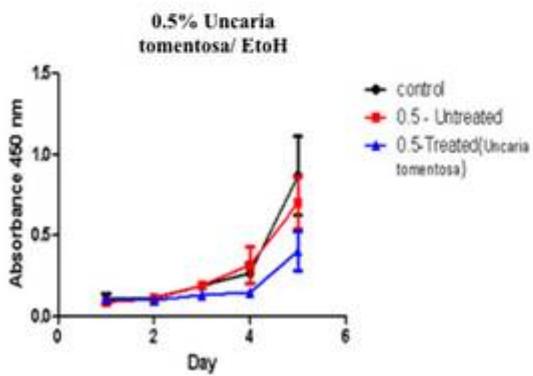
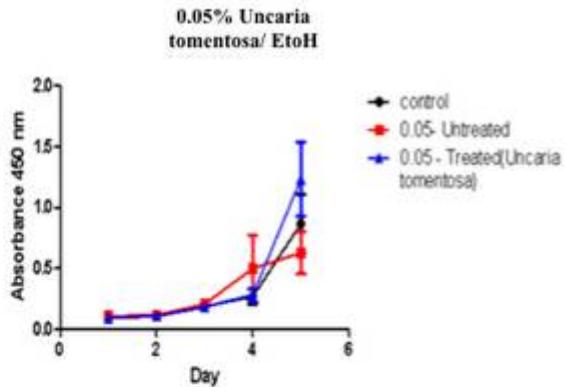
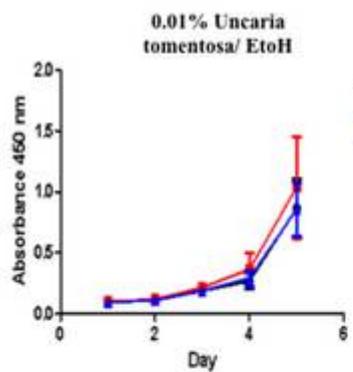


Figure 4.4: Effect of aqueous extracts of *Uncaria tomentosa* on non-malignant HEK293T cell growth.

HEK293T cells were treated with different concentrations of *Uncaria tomentosa* extracted with water (0.01, 0.05, 0.1, 0.5, and 1% *Uncaria tomentosa* in media). Cell proliferation was measured using the MTT assay. The cells were incubated with the *Uncaria tomentosa* extract for five days and absorbance was determined each day. The graphs represent one of at least three independent experiments.

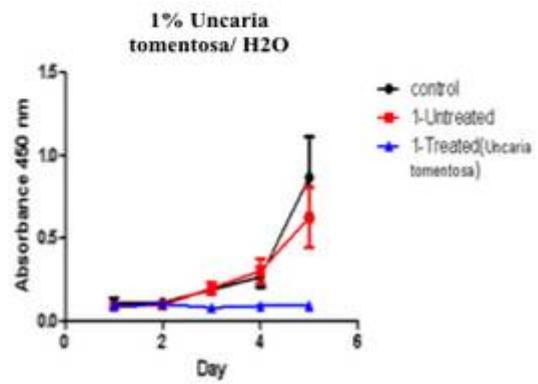
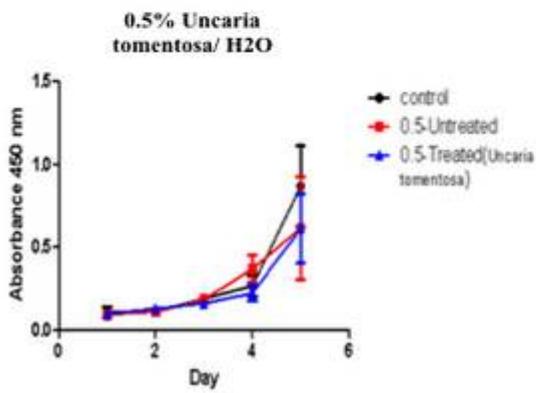
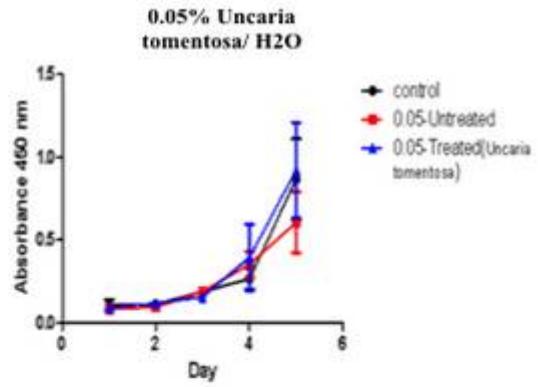
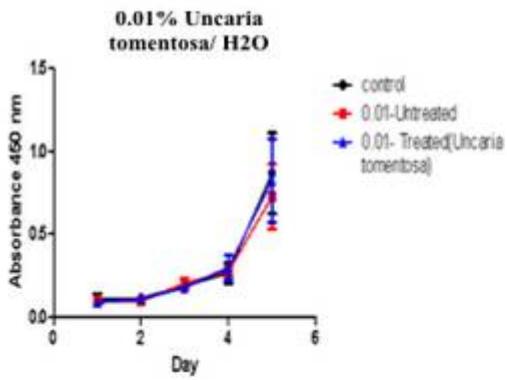


Figure 4.5: Effect of ethanol extracts of *Uncaria tomentosa* on non-malignant HSG cell growth.

HSG cells were treated with different concentrations of *Uncaria tomentosa* extracted with 70% ethanol (0.01, 0.05, 0.1, 0.5, and 1% *Uncaria tomentosa* in media). Cell proliferation was measured using the MTT assay. The cells were incubated with the *Uncaria tomentosa* extract for five days and absorbance was determined each day. The graphs represent one of at least three independent experiments.

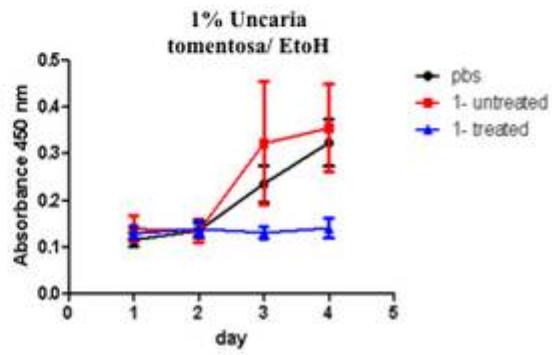
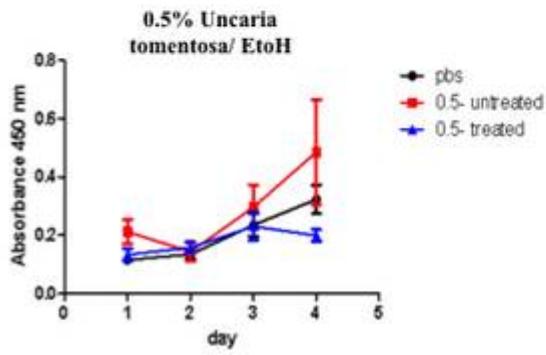
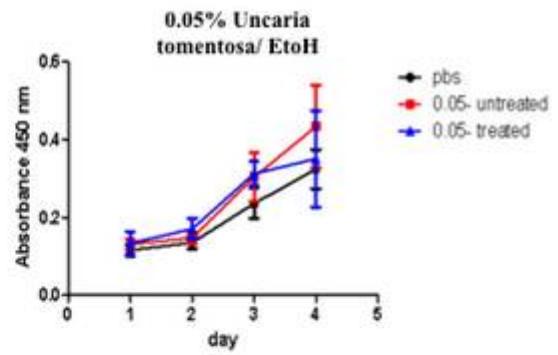
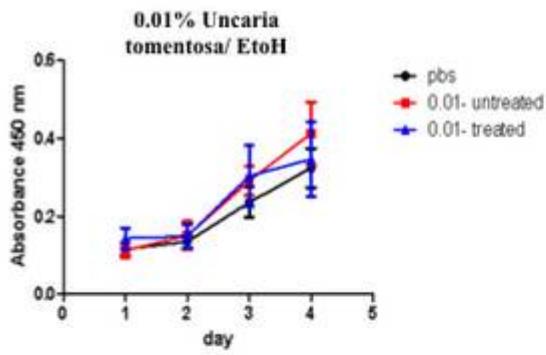


Figure 4.6: Effect of aqueous extracts of *Uncaria tomentosa* on non-malignant HSG cell growth.

HSG cells were treated with different concentrations of *Uncaria tomentosa* extracted with water (0.01, 0.05, 0.1, 0.5, and 1% *Uncaria tomentosa* in media). Cell proliferation was measured using the MTT assay. The cells were incubated with the *Uncaria tomentosa* extract for five days and absorbance was determined each day. The graphs represent one of at least three independent experiments.

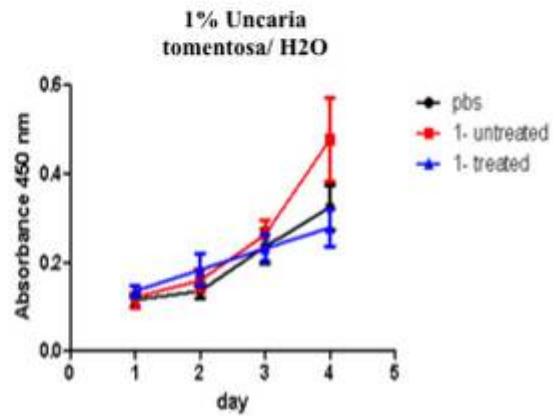
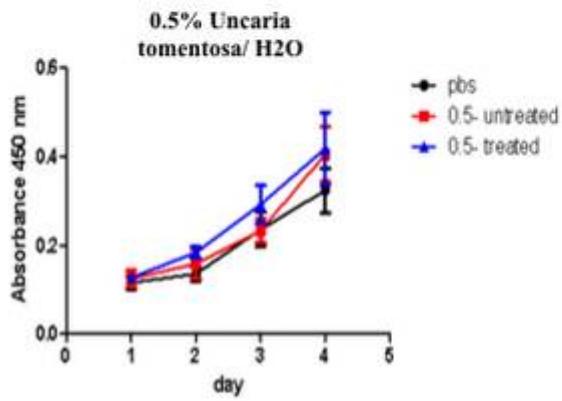
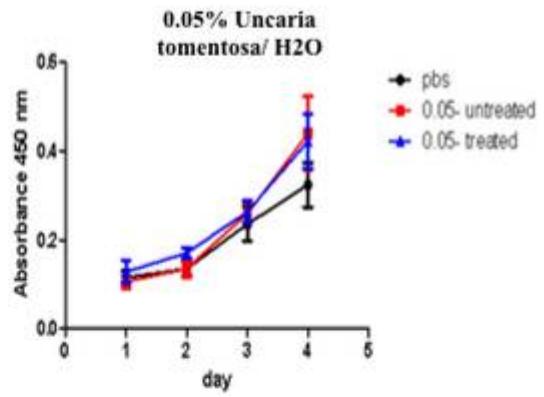
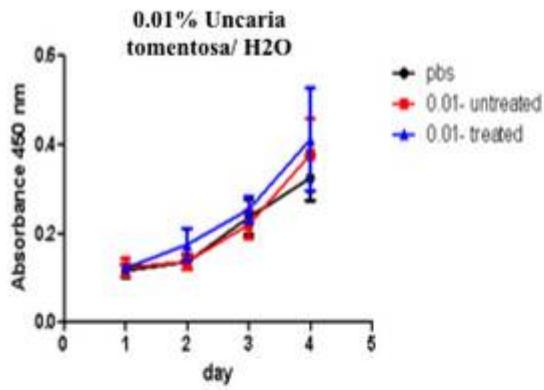
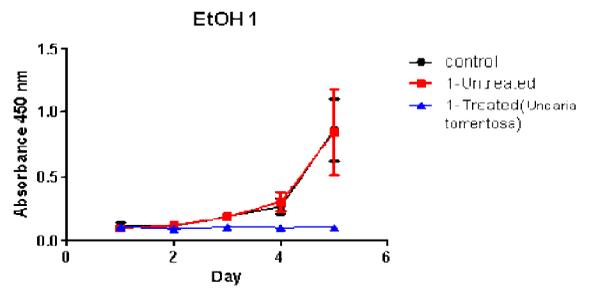
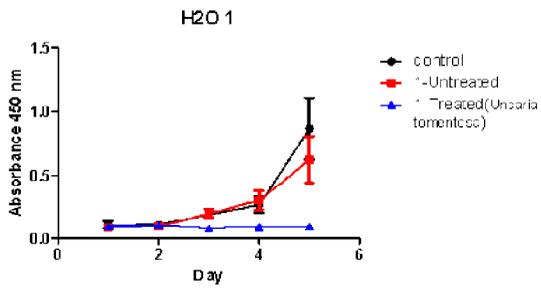
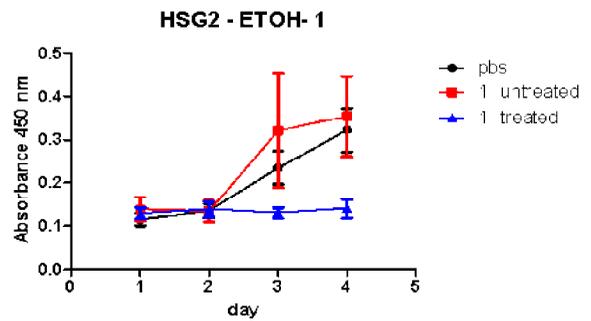
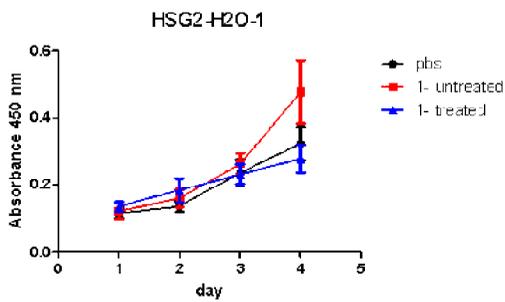


Figure 4.7: Comparison of the effect of *Uncaria tomentosa* extract on multiple cell lines.

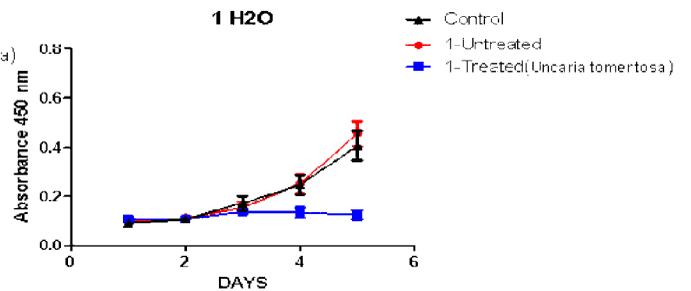
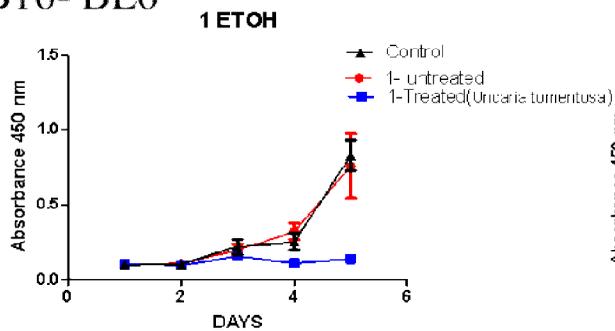
HEK- 239T



HSG



B16- BL6



To show the stability of the active agents in the extracts, we stored the different extracts of *Uncaria tomentosa* at 4° C for four weeks. Each week, the effect of the extracts on the proliferation of B16- BL6 cells was tested. The results of these studies showed that the *Uncaria tomentosa* extracts were stable and promoted a similar level of cell growth inhibition for at least four weeks (**Figures 4.8. and 4.9.**).

4.2. Treatment with *Uncaria tomentosa* inhibits cell migration

The effect of the *Uncaria tomentosa* on B16- BL6 migration was determined by using a wound healing migration assay. Cells treated with both extracts of *Uncaria tomentosa* showed a significant inhibition in the cell migration especially in the high concentration of the ethanolic extract of *Uncaria tomentosa* during different time points starting from 0 h and extending until 72 h after treatment (**Figure 4.10 and 4.11.**). Treatment with the aqueous extract was less effective and the diameter of the wound was reduced to approximately 50% within 72 h. Treatment with the positive control, camptothecin, almost completely inhibited cell migration while cells treated only with suspending media completely closed the “wound” within 24 h.

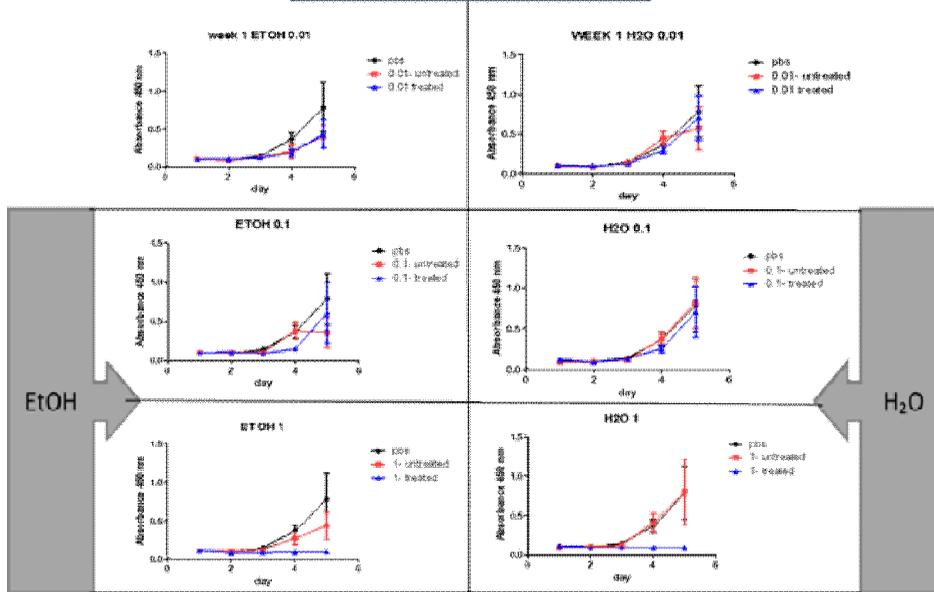
4.3. *Uncaria tomentosa* effects B16-BL6 morphology

B16-BL6 cells treated with both extracts of *Uncaria tomentosa* for 72 h showed a significant change in the cell morphology. The treated cells showed an elongated and stellate morphology especially in the cells treated with the high concentration of the ethanol extract (**Figure 4.12.**)

Figure 4.8: Stability of the *Uncaria tomentosa* extracts (2 weeks).

Uncaria tomentosa was extracted in 70% ethanol and these stored at 4 C. Each week, the effect of the extract on the proliferation of B16-BL6 cells was treated as described in Figure 4.1.

WEEK 1



WEEK 2

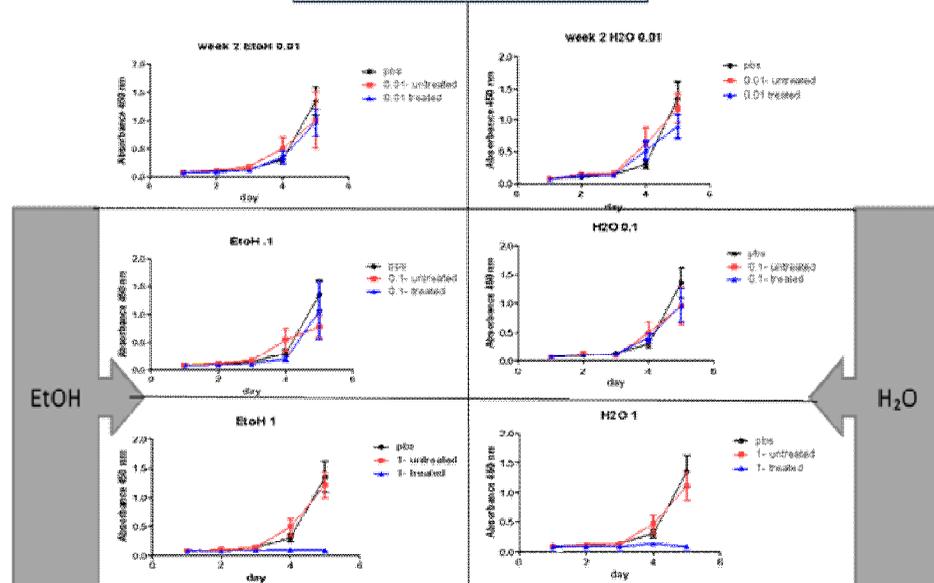
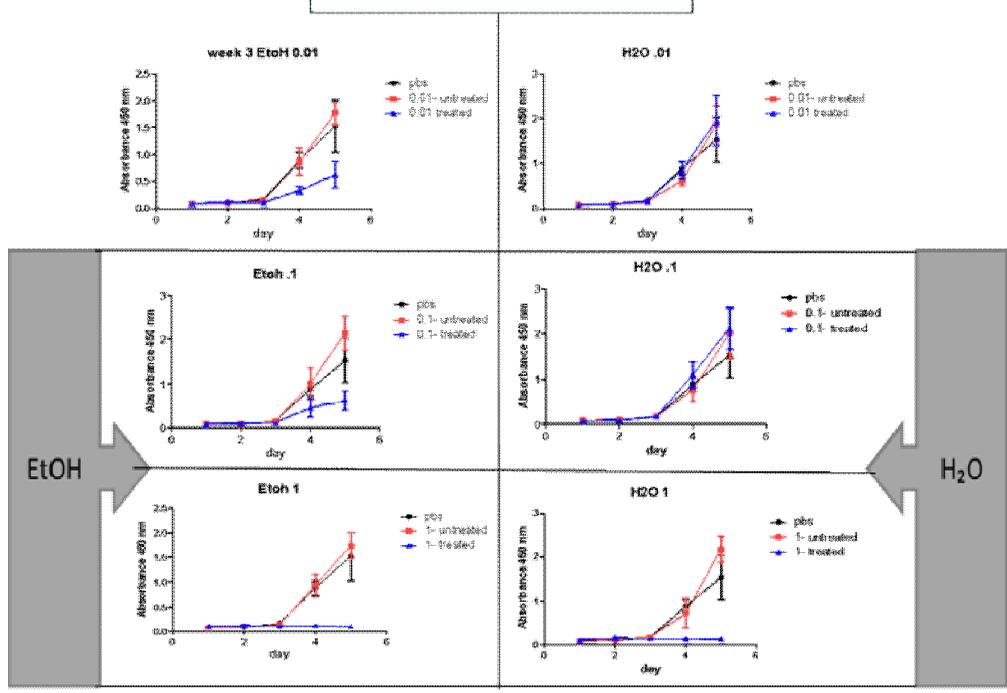


Figure 4.9: Stability of the *Uncaria tomentosa* extracts (4 weeks).

Uncaria tomentosa was extracted in 70% ethanol and these stored at 4 C. Each week, the effect of the extract on the proliferation of B16-BL6 cells was treated as described in Figure 4.1.

WEEK 3



WEEK 4

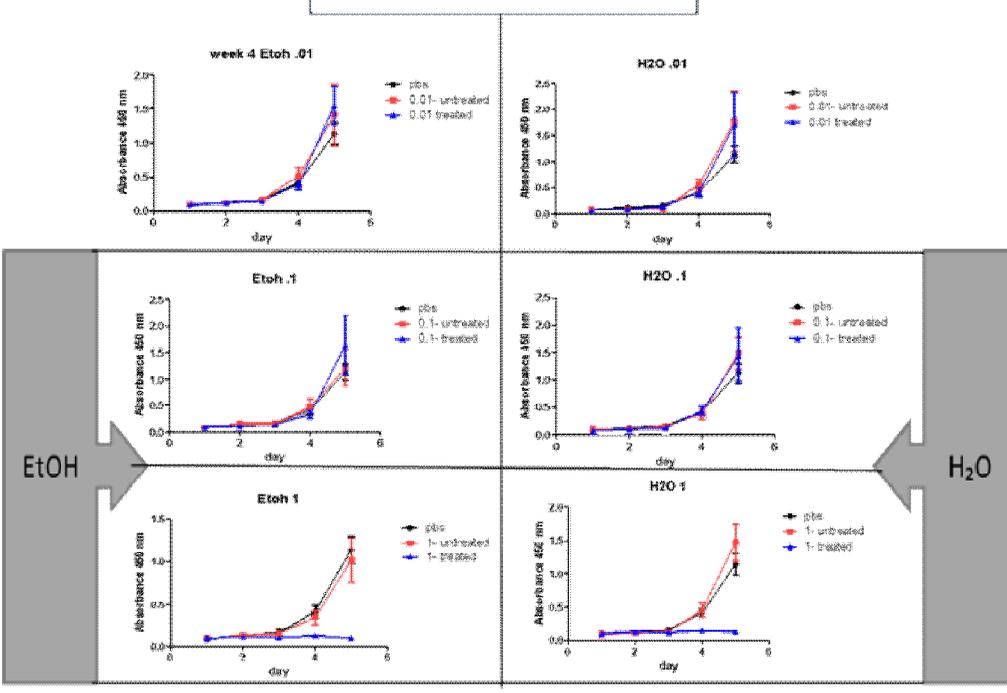


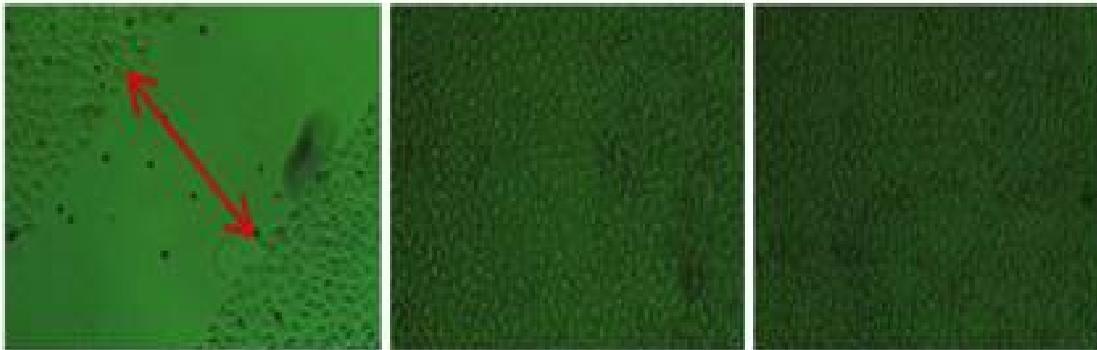
Figure 4.10: Effect of *Uncaria tomentosa* on the migration of B16-BL6 cells.

The effect of *Uncaria tomentosa* on B16-BL6 migration was determined by using wound healing migration assay. A 200 µl yellow pipet tips was used to score a wound in the monolayer (width = 45 mm). The cells were then incubated with various doses of *Uncaria tomentosa* and the width of the wound measured at various time of incubation. Cells were treated with low dose (0.01%) and high dose (1 %) of the two extracts of *Uncaria tomentosa*. Cells were treated with a positive control of Camptothecin and a media negative control. Digital photographs of the wounds were taken at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h post-treatment. The treatment with a high concentration of the ethanolic extract showed an inhibition on cell migration. The experiment was done three times.

Camptothecin

media

0.01% Uncaria/H2O



1% Uncaria/ H2O

0.01%Uncaria/ EtoH

1%Uncaria/ EtoH

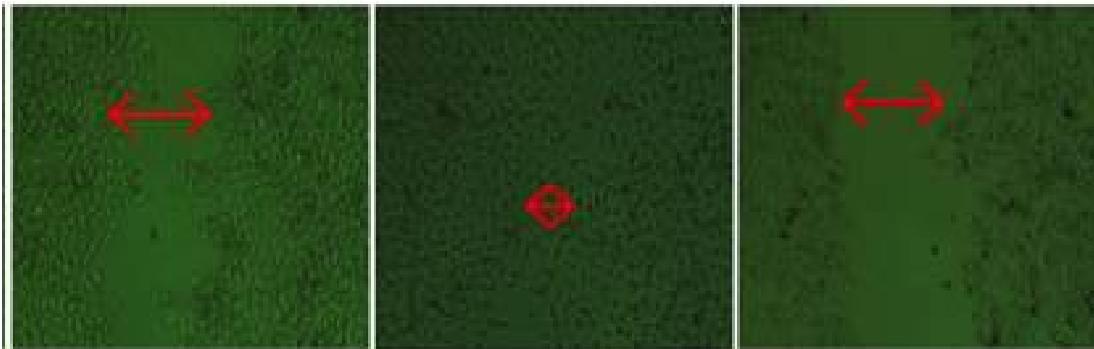


Figure 4.11: Effect of *Uncaria tomentosa* on the migration of B16-BL6 cells.

A graphical presentation of the effect of the *Uncaria tomentosa* extracts on B16-BL6 migration as determined by using wound healing migration assay described in Figure 4.10. The experiment was done three times.

Wound healing migration

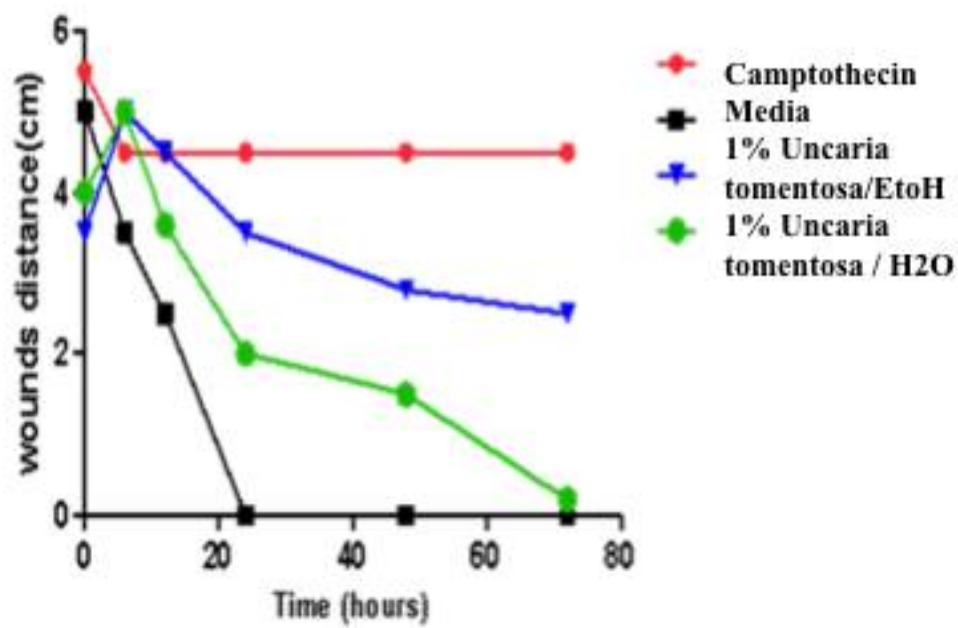


Figure 4.12: Changes in B16-BL6 morphology caused by *Uncaria tomentosa* treatment.

B16-BL6 cells were treated with low dose (0.01%) and high dose (1 %) of *Uncaria tomentosa* extract and incubated for three days before pictures were taken. *Uncaria tomentosa* treated B16- BL6 cells showed a decrease in number and a slight change in morphology. The experiment was done three times.

Media

0.01%Uncaria/ EtoH

1% Uncaria/ EtoH



0.01%Uncaria/ H2O

1% Uncaria/H2O



4.4. Treatment with *Uncaria tomentosa* effects cell DNA content

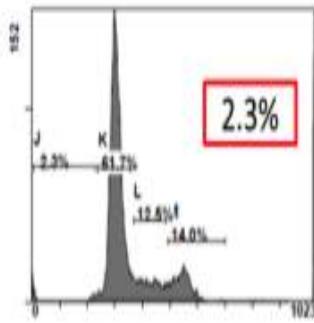
The effect of the *Uncaria tomentosa* extract on B16-BL6 cell cycle distribution was determined by using flow cytometry. A propidium iodine staining solution was used to measure the amount of DNA in each cell. The number of cells with DNA content less than normal G1 cells (the sub-G1 population) was increased in cells treated with extracts of *Uncaria tomentosa* in a dose-dependent manner (**Figure 4.13**). The presence of cells in the sub-G1 peak is a marker of an apoptotic cell population. Cells treated with the ethanolic extract of *Uncaria tomentosa* showed a significant increase in sub-G1 cells which increased to over 70% of the cells following treatment for 72h. The aqueous extract was less active and showed an increase to 10% of the cells in the sub-G1 peak after 72 h. Treatment with camptothecine for 24 h increased the sub-G1 population to approximately 25%. It is of some interest to note that cells treated with lower concentrations of *Uncaria tomentosa* extracts for 72 h still showed a small G2 DNA peak (approximately 50% the proportion of cells in media controls) which suggests that individual cells were still able to go through the cell cycle.

4.5. Treatment with *Uncaria tomentosa* induces apoptosis

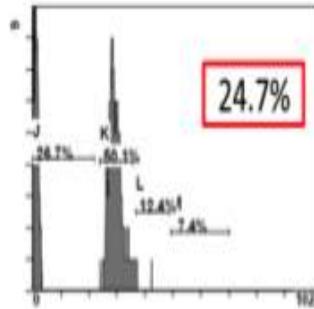
To examine the ability of the ethanolic extract of *Uncaria tomentosa* treatment to induce apoptosis in B16- BL6 cells, a cell staining assay (Acridine orange/ Ethidium bromide) was used. Treatment of B16-BL6 cells with the ethanolic extract of *Uncaria tomentosa* showed morphological changes, such as membrane blebbing and chromatin condensation as visualized by fluorescence microscopy (**Figures 4.14 and 4.15**).

Figure 4.13: The effect of *Uncaria tomentosa* on cell cycle profiles (sub-G1 peaks).

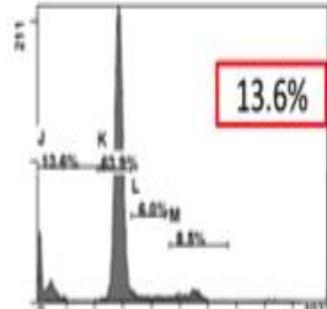
The effect of *Uncaria tomentosa* on DNA content was determined by flow cytometry. B16-BL6 were treated with both extracts of *Uncaria tomentosa* at various concentrations (0.01%, 0.1%, and 1%) or treated with 6 µg/ml camptothecin as a positive control. Cells were collected after three days of treatment and incubated with propidium iodide staining solution for 60 min. The samples were analyzed on a Beckman-Coulter LS600 flow cytometer. The percentage of cells in the Sub-G1 (apoptotic cells) peak is shown on the histogram.



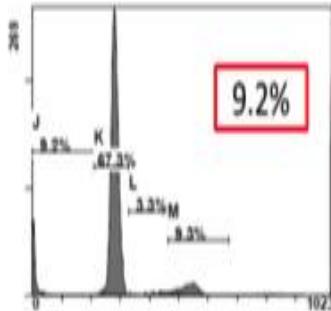
Media



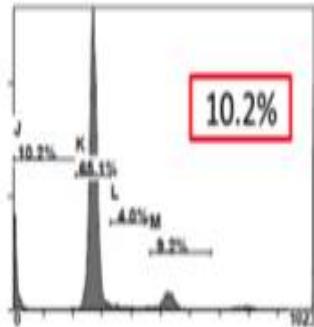
Camptothecin



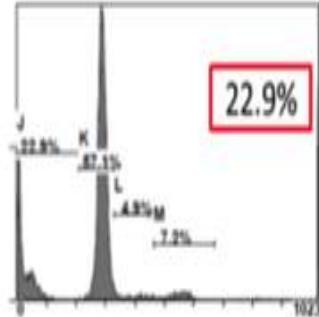
0.01%Uncari/ H2O



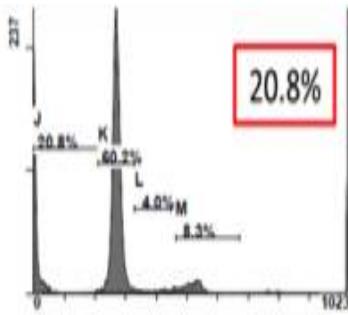
0.1% Uncaria/H2O



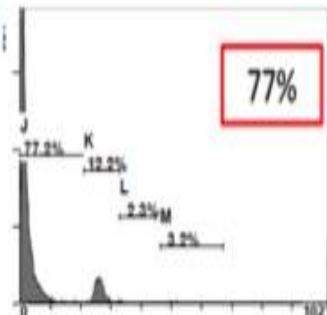
1%Uncaria/ H2O



0.01% Uncaria/ EtOH



0.1%Uncaria/ EtOH

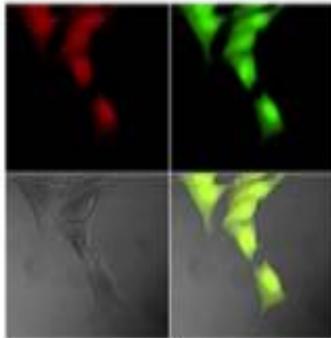


1%Uncaria/ EtOH

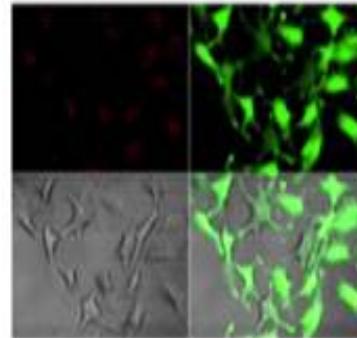
Figure 4.14: Detection of apoptosis in B16-BL6 cells using acridine orange and ethidium bromide staining.

Cells were cultured on glass coverslips and treated with different concentrations (0.01% and 1%) of both extracts of *Uncaria tomentosa*. Cells were treated with camptothecin as a positive control to detect apoptosis and with media as the negative control. Cells were stained by incubation in a mixture of 100 µg/ml acridine orange and 100 µl/ml ethidium bromide for 10 min. The morphological changes in the apoptotic cells were visualized by using an LSM510 microscope. *Uncaria tomentosa* appears to cause apoptosis at high concentration. The experiment was done three times.

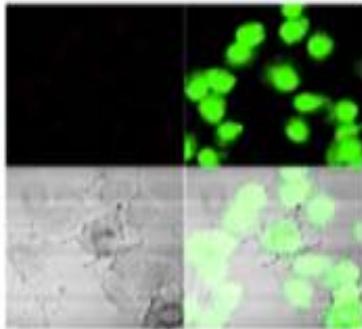
Media



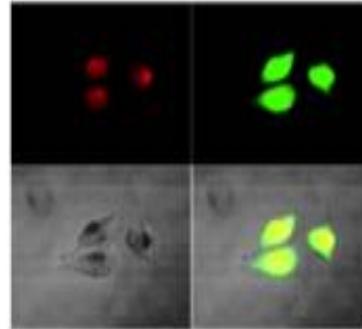
Camptothecin



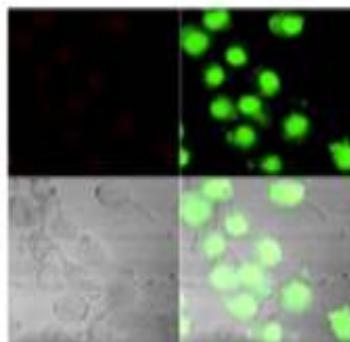
0.01% Uncaria/H2O



1%Uncaria/ H2O



0.01%Uncaria/ EtOH



1%Uncaria/ EtOH

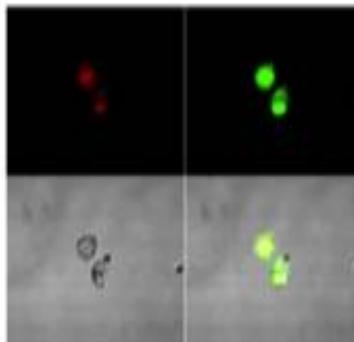
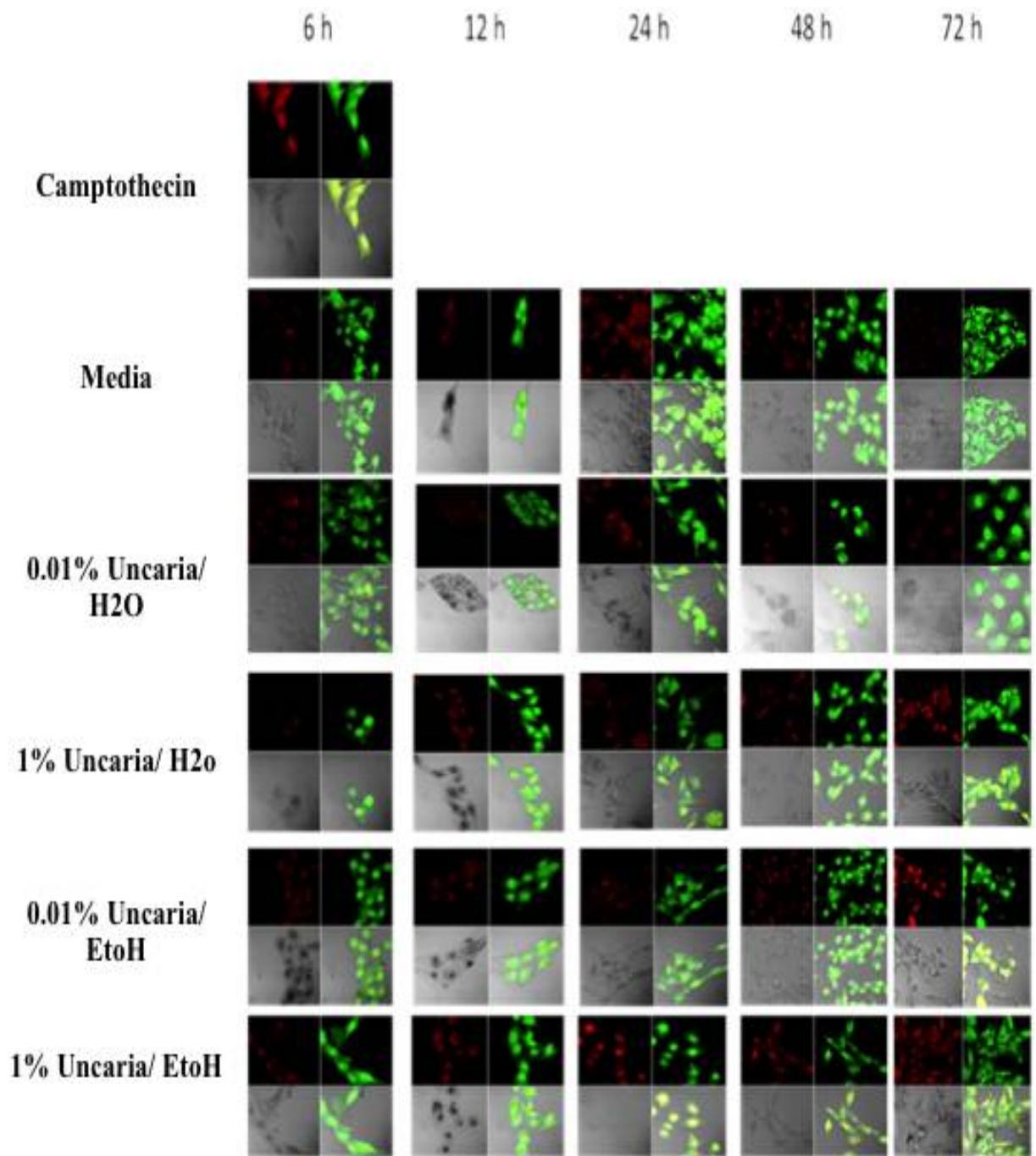


Figure 4.15: Detection of apoptosis in B16-BL6 cells using acridine orange and ethidium bromide staining.

Cells were cultured on glass coverslips and treated with different concentrations (0.01% and 1%) of both extracts of *Uncaria tomentosa*. Cells were treated with camptothecin as a positive control to detect apoptosis and with media as the negative control. Cells were stained by incubation in a mixture of 100 µg/ml acridine orange and 100 µg /ml ethidium bromide for 10 min. The morphological changes of the apoptotic cells were visualized by using an LSM510 microscope. *Uncaria tomentosa* appears to cause apoptosis at high concentration. The experiment was done three times.



The TUNEL assay was also used to identify DNA fragmentation induced by *Uncaria tomentosa* treatment, which is another hallmark of apoptosis. B16-BL6 were treated with the ethanolic extract of *Uncaria tomentosa* for 72 h and then stained with terminal transferase by the TUNEL reaction. The nuclear staining was visualized by fluorescence microscopy. The results showed that cells treated with the ethanolic extract of *Uncaria tomentosa* induced DNA fragmentation in treated cells after 3 days (**Figures 4.16. and 4.17**). Treatment with the aqueous extract of *Uncaria tomentosa* was not as effective at inducing DNA fragmentation. This is consistent with the results from the flow cytometry and acridine orange/ ethidium bromide staining results. This supports the idea that treatment of cells with *Uncaria tomentosa* extracts induces apoptosis which decreases the growth of treated cell cultures.

4.6. Treatment with *Uncaria tomentosa* causes caspase-3 cleavage

Since these results have supported the idea that the treatment with the ethanolic extract of *Uncaria tomentosa* induced apoptosis, we further examined the effect of treatment with the ethanolic extract of *Uncaria tomentosa* on caspase-3 cleavage. Caspase-3 cleavage is a classic indicator of apoptosis. Cells treated with the ethanolic extraction of *Uncaria tomentosa* showed caspase-3 cleavages after 72 h treatment compared to the positive control (camptothecin), providing further evidence that the *Uncaria tomentosa* mechanism of action to kill cells is by apoptosis (**Figure 4.18.**). Treatment of cells with the aqueous extract of *Uncaria tomentosa* showed a very weak band corresponding to the cleaved form of caspase-3, consistent with the previous results.

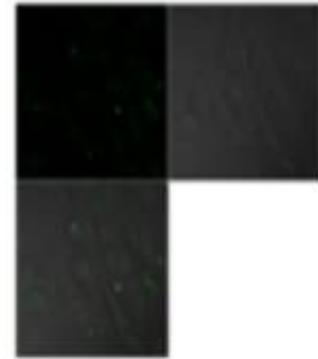
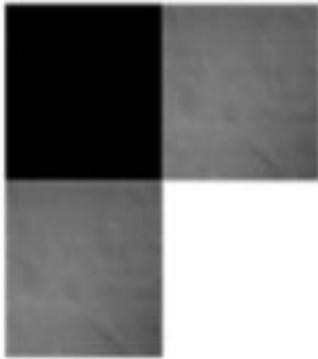
Figure 4.16: Detection of DNA fragmentation in B16-BL6 cells treated with *Uncaria tomentosa* using TUNEL assay (0 h).

Detection of DNA fragmentation after treatment with *Uncaria tomentosa* was done by using the Terminal deoxynucleotidyl transferase (TUNEL) assay. Cells were plated on glass coverslips and treated with different concentrations (0.01% and 1%) of *Uncaria tomentosa* extracts. Cells were treated with camptothecin as a positive control and with culture media as a negative control. At 0 h of treatment, the cells were incubated with 50 μ l of TUNEL reaction mixture for 60 min at 37 C° in the dark. The samples were analyzed using a fluorescence microscope to detect labelled cell nuclei. *Uncaria tomentosa* does not cause DNA fragmentation at time 0 h.

Media

0.01% Uncaria/ EtoH

1% Uncaria/ EtoH



0.01% Uncaria/ H2O

1% Uncaria/H2o

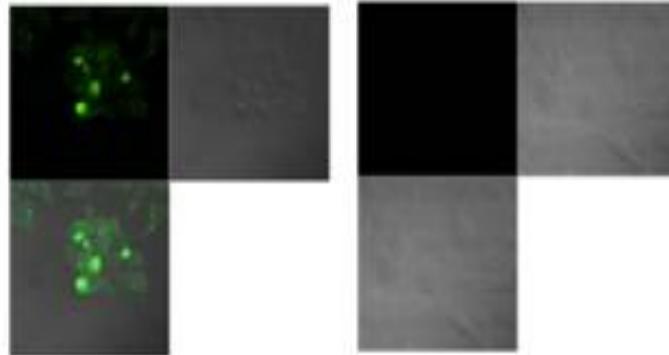


Figure 4.17: Detection of DNA fragmentation in B16-BL6 cells treated with *Uncaria tomentosa* using the TUNEL assay (72 h).

Detection of DNA fragmentation after treatment with *Uncaria tomentosa* was done by using the Terminal deoxynucleotidyl transferase (TUNEL) assay. Cells were plated on glass coverslips and treated with different concentrations (0.01% and 1%) of *Uncaria tomentosa* extracts. Cells were treated with camptothecin as a positive control and with culture media as a negative control. At 72 h of treatment, the cells were incubated with 50 µl of TUNEL reaction mixture for 60 min at 37 C° in the dark. The samples were analyzed under a fluorescence microscope to detect labelled cell nuclei. *Uncaria tomentosa* appears to cause DNA fragmentation in the high concentration of ethanolic extract after three days of treatment.

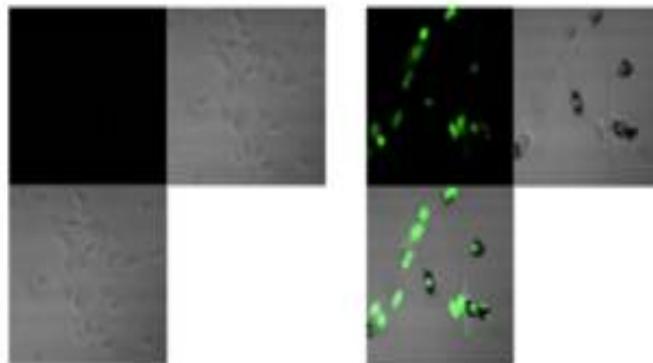
Camptothecin

media



0.01% Uncaria/ EtoH

1% Uncaria/ EtoH



0.01% Uncaria/ H2O

1% Uncaria/H2O

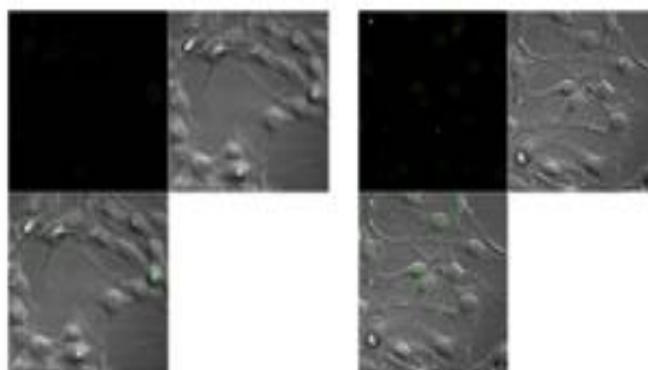
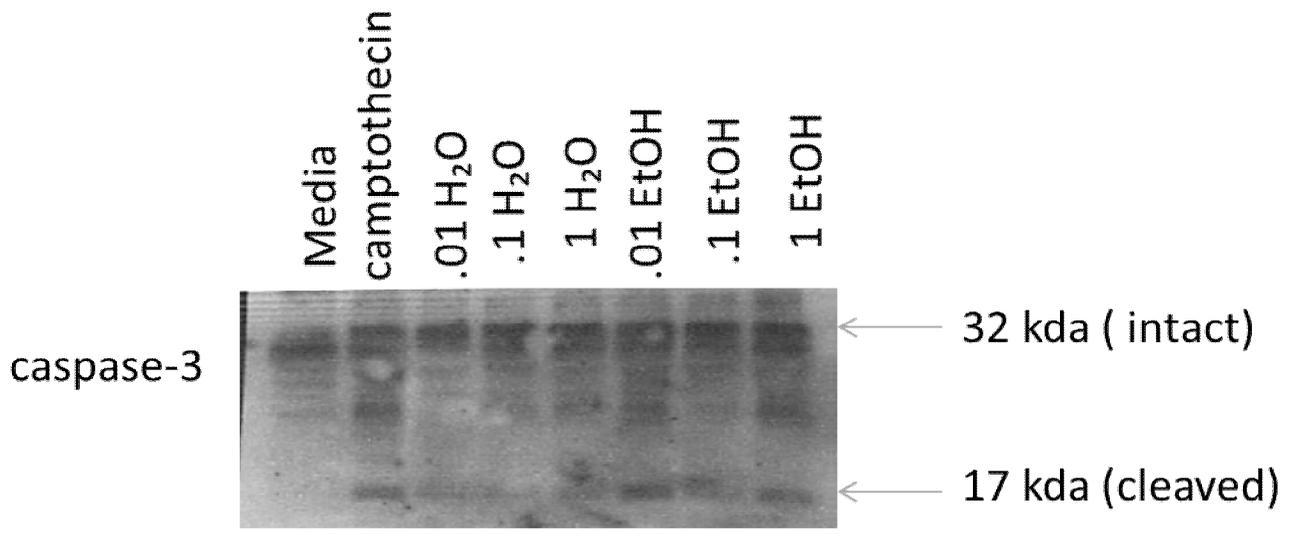


Figure 4.18: Effect of *Uncaria tomentosa* treatment of B16-BL6 cells on caspase-3 cleavage.

The effect of *Uncaria tomentosa* on apoptosis was determined by using western blot analysis and by analyzing Caspase cleavage. Cells were treated with two extracts of *Uncaria tomentosa* at different concentrations (0.01%, 0.1%, and 1%) and treated with 6 µg/ml camptotecin as a positive control and media as negative control. The cells lysates were electrophoresed on a 15% polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibody (anti-Caspase-3) followed by incubation with a secondary antibody. The treatment with *Uncaria tomentosa* especially the ethanolic extraction showed Caspase-3 cleavage.



4.7. Identifying the major components of the *Uncaria tomentosa* extracts

Uncaria tomentosa extractions fractions were analyzed by using HPLC in order to identify the major components. *Uncaria tomentosa* fractions were subjected to HPLC and the chromatographs (**Figure 4.19.**) showed several peaks which appear similar in both the ethanol and aqueous extracts. Several of these peaks correspond to the known standard alkaloid components of *Uncaria tomentosa*; uncarine C, uncarine D, uncarine E, mitraphylline, isomitraphylline, and rhynophylline although a few unidentified peaks are also seen. Separation of the ethanolic extract of *Uncaria tomentosa* on an ethanol gradient also showed the ability to separate the various alkaloids. The pure aqueous fraction (H₂O), and 20% ethanol fractions showed similar patterns of alkaloids compared to the parent ethanol extract (**Figure 4.20.**). The 40 % ethanol and 60% ethanol fractions showed a significant decrease in the amount of alkaloid detected and showed a change in the proportions of the various standard compounds that were present. The 80% and 100% ethanol fractions showed very low levels of alkaloids. The ability of the ethanol gradients to separate out some of the components of the *Uncaria tomentosa* extracts suggests that this method might be useful in identifying the active components that affect cell apoptosis in future experiments.

Figure 4.19: HPLC analysis of *Uncaria tomentosa* extracts.

Uncaria tomentosa extracts were analyzed by using HPLC to characterize the major components. *Uncaria tomentosa* powder was extracted in water or 70% ethanol solutions and freeze-dried. The resulting powder was resuspended in 50% ethanol at 50 mg/ml and subjected to the HPLC. The spectra show the relative position of several standard alkaloids fractionated under the same conditions. The identification of some additional peaks will be investigated further.

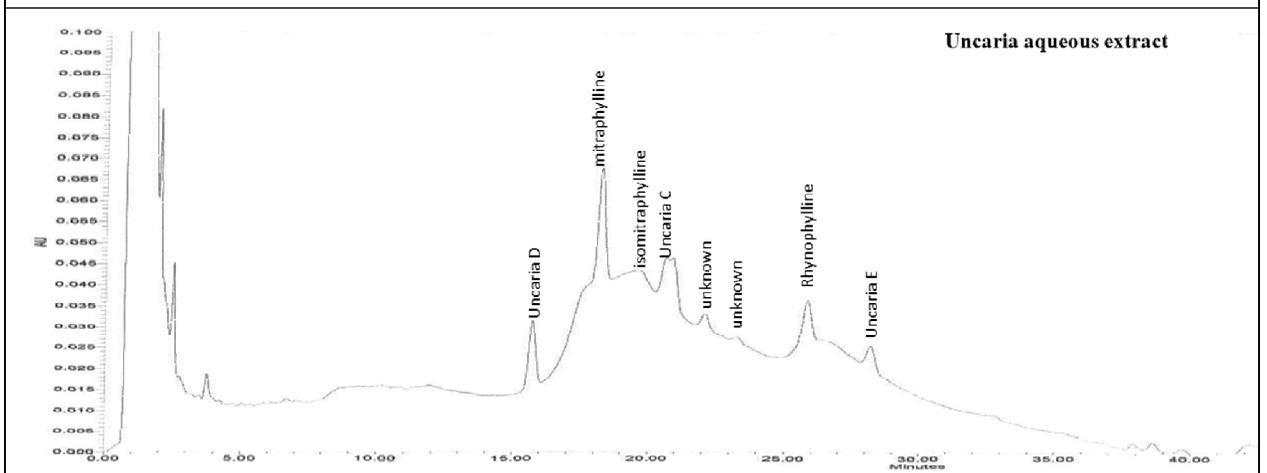
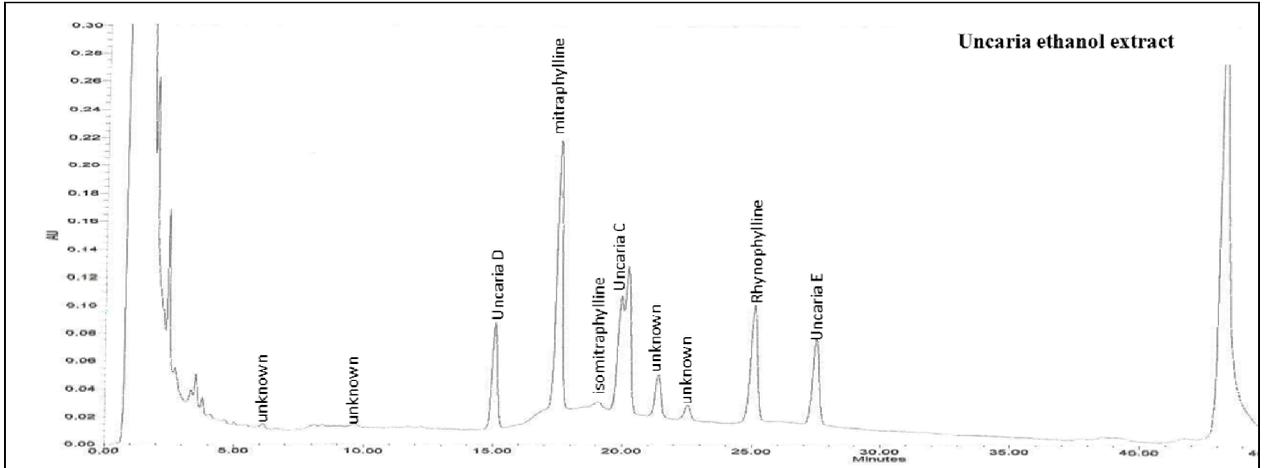


Figure 4.20: HPLC analysis of fractionated *Uncaria tomentosa* extracts

Uncaria tomentosa powder was extracted in 70% ethanol and then fractionated into different fractions using an ethanol gradient. The various fractions were analyzed using the HPLC in order to identify the major ingredients. The HPLC analysis of the *Uncaria tomentosa* fractions showed several alkaloid peaks in water, and 20% ethanol fractions which decreased in amount in the 40 % ethanol and 60% ethanol fractions. The identification of those peaks will be under investigation in the future.

Chapter 5: Discussion

The results of these studies showed that treatment with *Uncaria tomentosa* inhibited the proliferation of the murine melanoma cell line B16-BL6. However, the *Uncaria tomentosa* extract showed a similar effect on non-malignant cell lines in reducing cell growth. Thus, indicating that *Uncaria tomentosa* is an unselective drug because of it can inhibit the growth of cells that reproduce rapidly whether they are malignant or non-malignant. Moreover, treatment with *Uncaria tomentosa* was shown to stimulate programmed cell death (apoptosis) in cancer cells.

Most of the natural products that have been shown to have medicinal value have an inhibitory effect on cell proliferation and hormone metabolism; or can induce cell differentiation (35). Consequently, natural products such as green tea, garlic, and curcumin can be developed into chemoprevention agents because it is believed that these products are mostly non-toxic and show an inhibitory effect on carcinogenesis in rodents (36).

In order to identify the effectiveness and the mode of action of these medicinal plants, some biological experiments *in vivo* and *in vitro* must be done. *Uncaria tomentosa*, or “cat’s claw”, has been shown to have anti-cancer and anti-inflammatory activities, which are the most important activities required for treating different diseases such as gastric illnesses, arthritis, rheumatism, and cancer (37).

Several studies confirm that *Uncaria tomentosa* has some effects on the treatment of these disorders. Akesson's study reported that *Uncaria tomentosa* bark is capable of inhibiting the growth of several cancer cell lines (38). Further, results from this study showed that *Uncaria tomentosa* reduced cancer cell proliferation in a dose-dependent manner compared to the control cultures. Moreover, this result was observed in several malignant and non-malignant cell lines. They concluded their study by suggesting that *Uncaria tomentosa* can control cell growth and cell cycle progression (38).

5.1. Anti-proliferation activity of *Uncaria tomentosa* extracts

The anti-proliferative properties of *Uncaria tomentosa* have been confirmed by many studies. Fazio's study reported the inhibition of cancer cell proliferation follows treatment for 24h and that inhibition was less than 50% among the different tumor cell lines that were used in his study (36). Otherwise, *Uncaria tomentosa's* anti-proliferative activity has been demonstrated in immune cells such as THP-1 monocytes. However, THP-1 monocytes treated with *Uncaria tomentosa* extracts did not show any increase in the proportion of dead cells (37).

In our study, we tested two different preparations of *Uncaria tomentosa*; the ethanolic and water extracts, for their anti-proliferation activities. The ethanolic extract was prepared by using 70% ethanol as the extraction solvent. These extractions were mainly tested in the murine melanoma cell line (B16-BL6) and in some other non-malignant cell lines (HSG and HEK923T). In order to demonstrate the bioactivity of

these extracts, they were tested at different concentrations started from the lower (0.01%) to the higher concentrations (1%) (38).

The proliferation rates of the cells were measured using the Methyl-tetrazolium assay (MTT) (39). The main principle of the MTT assay is counting the total number of the viable cells that have active mitochondria. The MTT solvent is reduced by these active mitochondria to produce formazan products. The ability of the cytotoxicity drugs to inhibit cell proliferation is varied between the cell lines according to the results from these experiments (40). The various results showed different activity between the two types of extracts and between different cell lines (41). The *Uncaria tomentosa* extracts added to the different cancer cell lines also showed a significant decrease in cell proliferation. This is an expected activity of the *Uncaria* extracts as an anti-proliferative drug in cancer cells as described previously (39).

Both of the extracts were made by suspending 0.1 g/ml of *Uncaria tomentosa* bark powder in either water or 70% ethanol. When the extract was dried, approximately 50% of the original weight was recovered. When added to the cells at a concentration of 1% (the higher concentration) both extracts showed a significant decrease in the growth for malignant and non-malignant cell lines. However, at concentrations of 0.01- 0.1% of *Uncaria tomentosa*, extracts showed lower levels of inhibition in cell proliferation in all tested cell lines. According to this result, ethanol extracts of *Uncaria tomentosa* had the greatest anti-proliferative activity. The concentration of 1% *Uncaria tomentosa* extracts significantly inhibited the proliferation of B16- BL6, HEK-293T and slightly inhibited HSG cell proliferation.

Additional assays were done to prove the effects of the *Uncaria tomentosa* extracts on the growth and movement of the cells from one area to another. This assay is the wound healing migration assay and it confirmed that the ethanol extract of *Uncaria tomentosa* showed a significant effect in reducing the migration of the cells from one area to another.

5.2. Dose and time dependency of *Uncaria tomentosa*

The activity of *Uncaria tomentosa* in inhibiting cancer metastasis to the lung by the murine melanoma cell line B16-BL6 had been shown in mice. The mice were inoculated with B16-BL6 cells via the tail vein and then injected with 50 µg of *Uncaria tomentosa* daily for 20 days. Consequently, the size of the lung tumors found in the mice treated with 50 µg of *Uncaria tomentosa* was significantly inhibited; notably, treated tumors measured approximately 75% less than the control up to day 16 (36).

In our study, the results showed a dose-dependent inhibition of growth for the murine melanoma cell line B16-BL6 *in vitro*. The low doses of *Uncaria tomentosa* showed a range of growth inhibition of between 10% to 30% fewer cells. However, more than a 50% reduction in the number of cells was seen when the cultures were treated with the high dose of *Uncaria tomentosa* for 48 h treatment. These data support the idea that there is an effective anti-cancer component in *Uncaria tomentosa*.

5.3. Apoptosis induction by *Uncaria tomentosa*

Natural products have been found that are able to regulate cell death by eliciting or inhibiting apoptosis. The aim of studying the effect of natural components on apoptosis is to identify and improve the critical apoptotic component that is capable of inducing programmed cell death in cancer cells. Consequently, examining these compounds may help to discover a new anti-cancer drug. Many studies support the ability of natural products to control both the intrinsic and extrinsic apoptosis pathways and our experimental results showed that *Uncaria tomentosa* could induce and control apoptosis.

To identify the mechanism of action of *Uncaria tomentosa* and whether it is an apoptosis inducer or not, several experiments were done to identify any morphological and biochemical modifications. To determine the morphological changes, the cells were stained with a combination of acridine orange and ethidium bromide. Acridine orange is capable of staining both live and dead cells and they appear green under the microscope. The nuclei morphology is visible in the stained cells and nuclear condensation or fragmentation can be detected. However, ethidium bromide stains only the dead cells that have lost membrane integrity and are a good indicator of necrosis or late apoptosis in cells treated for many days (44). Therefore, the cell staining assay is one of the apoptosis assays that shows the nuclear changes due to chromatin condensation and DNA fragmentation (44).

In both extracts, the cells showed chromatin condensation after three days treatment. However, treatment with the ethanolic extract of *Uncaria tomentosa* caused

the cells to show membrane blebbing early on and chromatin condensation was present by 48 h treatment. In contrast, treatment with the aqueous extract did not show early membrane blebbing and chromatin condensation was not evident until 72 h of treatment. This shows that the ethanolic extract of *Uncaria tomentosa* is more effective as an apoptosis inducer than the aqueous extract of *Uncaria tomentosa*.

During apoptosis, DNA fragmentation is one of the most important structural changes. To show whether DNA fragmentation is present or not, a TUNEL assay was used. Basically, free 3'-OH terminal ends of DNA will appear due to DNA strand breaks during DNA fragmentation and these ends will be labeled using terminal transferase and the TdT label and appear as stained nuclei. Consequently, this will be visualized under the fluorescence microscope (45). The cells were treated with *Uncaria tomentosa* at different concentrations and different time points then incubated with TUNEL mixture and then the fluorescence microscope was used to visualize them. After 72 h, the cells treated with the ethanolic extract showed DNA fragmentation compared to control cells and the *Uncaria tomentosa* aqueous extracts. As a result, the ethanolic extract of *Uncaria tomentosa* was shown to be more effective in causing DNA fragmentation than the water extracts.

To confirm that the cells were dying by apoptosis in response to treatment with *Uncaria tomentosa* extract, more biological experiments were done such as flow cytometer by propidium iodide (PI) staining. This experiment shows apoptotic cells as an increase in the sub-G1 DNA peak because the apoptotic cell fragments have lower DNA content (46). The quantification of the cells in the sub-G1 population was determined after treatment with 1% of both extractions of *Uncaria tomentosa*. After 72 h treatment,

especially with the ethanolic extract, the percentage of apoptotic cells was around 70% compared to the negative control. This confirms the ability of the ethanolic extract of *Uncaria tomentosa* to induce a large sub-G1 peak due to the increase in the apoptotic cell population.

Further studies such as immunoblot assays were used to show that *Uncaria tomentosa* treatment of B16-BL6 cells able to initiate the activity of the pro-apoptotic caspase-3 and that will confirm the apoptosis induction by *Uncaria tomentosa*. Our results showed that cells treated with 1% of ethanolic extract of *Uncaria tomentosa* showed cleavage of caspase-3.

5.4. Characterization of *Uncaria tomentosa*

Identifying the components of *Uncaria tomentosa* extracts by HPLC helps to profile the phytochemical characteristics of *Uncaria tomentosa*. In previous studies, the HPLC results showed different active components, which played an important role in the biological activities. For example, the alkaloids were shown to express an immune stimulating activity and many of the quinovic acid glycosides have an anti-inflammatory activity. Also, many studies reported the presence of polyhydroxylated triterpenes in *Uncaria tomentosa* that has activity in this plant and its extracts. Both pharmacological and clinical demonstration of this plant showed that the alkaloids and quinovic acid glycosides have the most important biological activities. Most of the commercial drugs from this plant must contain those classes of compounds. (51)

In our study, the main objective of the *Uncaria tomentosa* separation was to identify unknown components that are responsible for the anti-cancer activity in *Uncaria tomentosa*. Profiles of each fraction of the *Uncaria tomentosa* were created to characterize those fractions for future characterization and insure their bioactivity by themselves or in combination with other active components.

Chapter 6: Conclusion

The treatment of murine melanoma B16- BL6 cells with *Uncaria tomentosa* extracts significantly inhibited cell proliferation and both ethanolic and aqueous extracts had similar anti-proliferative effects on malignant and non-malignant cell lines. From our experimental results, we could identify the mechanism of action of *Uncaria tomentosa* and these results showed that the ethanolic extract appeared to kill cells by apoptosis. From the cell staining we saw the morphological modifications that emphasized the apoptosis characteristics, such as membrane blebbs and chromatin condensation. In addition, the increase in the sub-G1 peak, because of the increase in the number of apoptotic cells, would support the idea that the cells undergo apoptosis by studying the cell cycle profile. Moreover, the TUNEL assay confirmed one of the most important characters of apoptosis, which is DNA fragmentation. This experiment showed DNA fragmentation in cells treated with the ethanolic extract of *Uncaria tomentosa*. All of these experiments confirmed that treatment of cells with *Uncaria tomentosa* promotes cell death via enhancing cellular apoptosis.

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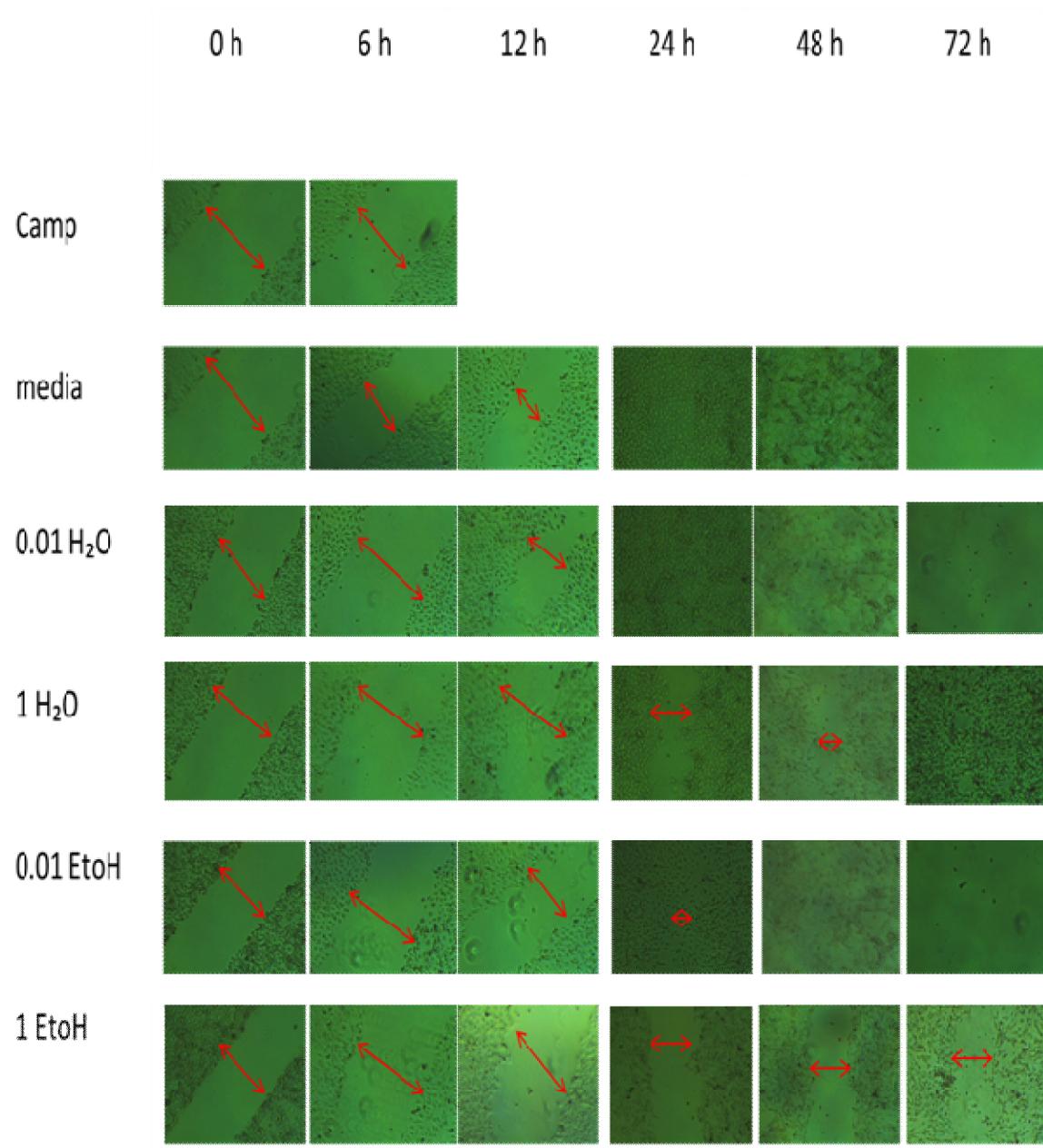
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Appendix



***Uncaria tomentosa* inhibits cell migration in high concentration of ethanol extract in different time points**

