

**EFFECTS OF *UNCARIA TOMENTOSA* ON THE GROWTH AND  
SURVIVAL OF B16-BL6 MOUSE MELANOMA CELLS**

**by**

**Ali Zari**

**A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science (MSc) in Biology**

**The Faculty of Graduate Studies  
Laurentian University  
Sudbury, Ontario, Canada**

**© Ali Zari, 2014**

**THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE**  
**Laurentian University/Université Laurentienne**  
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	EFFECTS OF UNCARIA TOMENTOSA ON THE GROWTH AND SURVIVAL OF B16-BL6 MOUSE MELANOMA CELLS		
Name of Candidate Nom du candidat	Zari, Ali		
Degree Diplôme	Master of Science		
Department/Program Département/Programme	Biology	Date of Defence Date de la soutenance	October 6, 2014

**APPROVED/APPROUVÉ**

Thesis Examiners/Examineurs de thèse:

Dr. Robert Lafrenie  
(Supervisor/Directeur(trice) de thèse)

Dr. Michael Persinger  
(Committee member/Membre du comité)

Dr. Kabwe Nkongolo  
(Committee member/Membre du comité)

Dr. Paul Spagnuolo  
(External Examiner/Examineur externe)

Approved for the Faculty of Graduate Studies  
Approuvé pour la Faculté des études supérieures  
Dr. David Lesbarrères  
M. David Lesbarrères  
Acting Dean, Faculty of Graduate Studies  
Doyen intérimaire, Faculté des études supérieures

**ACCESSIBILITY CLAUSE AND PERMISSION TO USE**

I, **Ali Zari**, hereby grant to Laurentian University and/or its agents the non-exclusive license to archive and make accessible my thesis, dissertation, or project report in whole or in part in all forms of media, now or for the duration of my copyright ownership. I retain all other ownership rights to the copyright of the thesis, dissertation or project report. I also reserve the right to use in future works (such as articles or books) all or part of this thesis, dissertation, or project report. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that this copy is being made available in this form by the authority of the copyright owner solely for the purpose of private study and research and may not be copied or reproduced except as permitted by the copyright laws without written authority from the copyright owner.

## Abstract

*Uncaria tomentosa* is a medicinal plant native to Peru that has been traditionally used in the treatment of various disorders, including cancer. *U. tomentosa* is one of the best-selling herbs in the world and is used as an immunomodulatory, anti-inflammatory and anti-cancer remedy. This study assessed the effectiveness of *U. tomentosa* on the growth and survival of B16-BL6 mouse melanoma cells. Both ethanol and PBS extracts of *U. tomentosa* extracts were tested *in vitro* and *in vivo* in order to evaluate their potential anticancer activity. In addition, different methods to measure the effect of treatment were used, including MTT assay, immunofluorescence (Ki67 protein and TUNEL assay) and the isogenic tumour transplantation model. The present results showed that *U. tomentosa* significantly inhibited cell proliferation and induced morphological changes *in vitro*. Furthermore, *U. tomentosa* was able to increase the percentage of apoptotic cells in a concentration-dependent manner. There was also decrease in the expression of Ki-67 (cell proliferation marker). Two experiments were performed to assess the ability of *U. tomentosa* to inhibit B16-BL6 cell growth *in vivo*. Mice were injected subcutaneously (on the top of the muscle) with B16-BL6 cells and tumours were allowed to progress for two weeks. In some animals, *U. tomentosa* extracts were injected intraperitoneal and intratumour. The animals were sacrificed and tumour diameters and weights were measured. The results showed that *U. tomentosa* caused a significant reduction in tumour weight but the tumor size was not significantly affected when compared to the controls. Interestingly, there were no significant differences in mouse weight for all treated groups. Overall, *U. tomentosa* showed promising results and caused a marked reduction in B16-BL6 melanoma cell growth.

**Keywords:** *Uncaria tomentosa*, B16-BL6 mouse melanoma cells, anticancer.

## **Acknowledgments**

I would like to express my special appreciation and thanks to my supervisor Dr. Robert Lafrenie whose expertise, understanding, and patience, added considerably to my graduate experience. I appreciate his knowledge and skills in many areas. I could not have completed my thesis without his advice, criticism, and corrections from the beginning up to the end of the writing. He has been supportive since I began working on his lab. He patiently provided the vision, encouragement and advice necessary for me to proceed through the master's program and complete my dissertation. I am very grateful for his motivation, enthusiasm, immense knowledge and for the useful comments and the remarks during the learning process of this master thesis.

Beside my supervisor, I would like to thank the members of my committee, Dr. Michael Persinger and Dr. Kabwe Nkongolo for their assistance, their brilliant comments and suggestions. Their guidance has served me well and I owe them my heartfelt appreciation. I am blessed to work with dynamic and intelligent committee members.

A special thanks to my family, words cannot express how grateful I am to my parents and my wife for the support they provided me through all my studies at University. For my father Dr. Talal Zari and my mother Ahlam Al-khateeb who raised me with a love of science, patience and supported me in all my pursuits. They were always supporting me and encouraging me with their best wishes. I would also like to thank my loving, supportive, and encouraging wife Nujood Kheshafaty for her faithful support during the final stages of this MSc. She was always there cheering me up and stood by me through the good times and bad.

# Table of Contents

<b>Abstract</b> .....	<b>iii</b>
<b>Acknowledgments</b> .....	<b>iv</b>
<b>Table of Contents</b> .....	<b>v</b>
<b>List of Tables</b> .....	<b>vii</b>
<b>List of Figures</b> .....	<b>viii</b>
<b>Abbreviations</b> .....	<b>ix</b>
<b>1. Introduction</b> .....	<b>1</b>
1.1 Cancer .....	1
1.2 Skin Cancer .....	2
1.3 Medical Plants.....	7
1.4 <i>Uncaria tomentosa</i> .....	10
<b>2. Objectives and Experimental Summary</b> .....	<b>17</b>
<b>3. Materials and Methods</b> .....	<b>18</b>
3.1 Tissue Culture .....	18
3.2 Plant Extraction .....	18
3.3 Cell Treatments.....	19
3.4 MTT Assay .....	19
3.5 Cell Morphology.....	20
3.6 Immunohistochemistry (Fluorescence) for ki-67 protein expression .....	20
3.7 TUNEL assay.....	21
3.8 Animal Experiments .....	21
<b>4. Results</b> .....	<b>24</b>
4.1 The effects of <i>U. tomentosa</i> on cell growth.....	24
4.2 Cell morphology.....	29
4.3 Inhibition of B16-BL6 cell proliferation using Ki-67 protein expression.....	40
4.4 Detection of apoptotic cells using TUNEL assay.....	45

4.5 Effect of <i>U. tomentosa</i> extracts on body weight, tumour weight and tumour size .....	54
<b>5. Discussion .....</b>	<b>70</b>
5.1 Effect of <i>U. tomentosa</i> extracts on cancer cell lines.....	71
5.2 Anti- proliferation activity of <i>U. tomentosa</i> extracts.....	73
5.3 Anti- proliferation activity of <i>U. tomentosa</i> extract (Ki-67 protein expression) .....	75
5.4 DNA fragmentation by <i>U. tomentosa</i> .....	76
5.5 <i>U. tomentosa</i> extract had significant effects on tumour weight and size .....	77
<b>6: Conclusion .....</b>	<b>79</b>
<b>References .....</b>	<b>80</b>

## List of Tables

Table 1: Shows the values of mean mouse weight (g), standard deviation and standard error of different groups (N=4-5).....	56
Table 2: Shows the values of mean tumour weight (g), standard deviation and standard error of different groups (N=4-5).....	56
Table 3: Shows the values of mean tumour size (cm), standard deviation and standard error of different groups (N=4-5).....	57
Table 4: Shows the values of mean mouse weight (g), standard deviation and standard error of different groups (N=3-5).....	57
Table 5: Shows the values of mean tumour weight (g), standard deviation and standard error of different groups (N=3-5).....	58
Table 6: Shows the values of mean tumour size (cm), standard deviation and standard error of different groups (N=3-5).....	58
Table 7: Shows the values of mean mouse weight (g), minimum, maximum, standard deviation and standard error of different groups (N=8-10). .....	59
Table 8: Shows the values of mean tumour weight (g), minimum, maximum, standard deviation and standard error of different groups (N=8-10). .....	59
Table 9: Shows the values of mean tumour size (cm), minimum, maximum, standard deviation and standard error of different groups (N=8-10). .....	59
Table 10: Regression statistics for tumour size (cm) as a function of tumour weight (g) (N=45).	60

## List of Figures

Figure 1: Effect of <i>U.tomentosaon</i> extracted with ethanol on the growth of the malignant B16-BL6 cell line.....	25
Figure 2: Effect of <i>U. tomentosaon</i> extracted with PBS on the growth of the malignant B16-BL6 cell line.....	27
Figure 3: Effect of <i>U. tomentosaon</i> extracted with ethanol on the morphology of the malignant B16-BL6 cell line.....	30
Figure 4: Effect of <i>U. tomentosaon</i> extracted with PBS on the morphology of the malignant B16-BL6 cell line.....	35
Figure 5: Effect of <i>U. tomentosaon</i> extracted with ethanol on cell proliferation .....	41
Figure 6: Effect of <i>U. tomentosaon</i> extracted with PBS on cell proliferation .....	43
Figure 7: Detection of DNA fragmentation in B16-BL6 cells treated with <i>U. tomentosa</i> using the TUNAL assay for 24 hours .....	46
Figure 8: Detection of DNA fragmentation in B16-BL6 cells treated with <i>U. tomentosa</i> using the TUNAL assay for 24 hours .....	48
Figure 9: Detection of DNA fragmentation in B16-BL6 cells treated with <i>U. tomentosa</i> using the TUNAL assay for 72 hours .....	50
Figure 10: Detection of DNA fragmentation in B16-BL6 cells treated with <i>U. tomentosa</i> using the TUNAL assay for 72 hours .....	52
Figure 11: Effect of <i>U. tomentosa</i> extracts on body weight .....	62
Figure 12: Effect of <i>U. tomentosa</i> extracts on tumour weight.....	64
Figure 13: Effect of <i>U. tomentosa</i> extracts on tumour size .....	66
Figure 14: The relationship between tumour size and tumour weight.....	68

## Abbreviations

BSA	bovine serum albumin
Ca <sup>2+</sup>	calcium ion
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
g	gram
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells (signaling molecule)
nm	nanometer
PBS	phosphate-buffered saline
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
μg	microgram
IFN	interferon
μl	microliter
U/ml	units per ml
TNF-α	interleukin-a
NO	nitric oxide
UV	ultraviolet
UVA	Ultraviolet A rays
UVB	Ultraviolet B rays
IL-2	interleukin-2
MTT	Methyl thiazol tetrazolium assay
HIV/AIDS	Human immunodeficiency virus infection / acquired immunodeficiency syndrome
BHT	butylated hydroxytoluene
BHA	butylated hydroxyanisole
TdT	terminal deoxynucleotidyl transferase

# 1. Introduction

## 1.1 Cancer

Cancer is a very serious public health problem and one of the common major diseases worldwide. It covers a large group of diseases and oncology research is considered a great challenge (Parkin *et al.*, 2005). Cancer involves over 200 diseases that affect all ages of humans and many of them are lethal (Alberts *et al.*, 1994). Cancer cells grow in an abnormal manner because of changes in the genetic information of cells leading to formation of a tumour, which can affect any part of the body. All cases of cancer are thought begin as a single cell that has lost its ability to grow normally and to replicate with inappropriate control (Cooper, 2000). Tumors are divided into two types: malignant and benign. Malignant tumours are characterized by their rapid growth rate and their ability to invade neighboring tissues. Metastasis refers to the spread of the tumour cells to other parts of the body through the lymphatic system or the bloodstream. Benign tumours, are not cancerous. Benign tumors do not spread and invade neighboring tissues of the body. As well, they usually have a slower growth rate (Liotta, 1984; Folkman and Klagsbrun, 1987; Hanahan and Weinberg, 2011). Four basic features have been identified to aid in diagnosis of benign and malignant tumours: differentiation and anaplasia, growth rate, metastasis, and local invasion (Moscow and Cowan, 2011).

Cancer is a major cause of death all around the world after cardiovascular diseases, and infectious disease and causes approximately 7.6 million deaths, which was 13% of all deaths in 2008 (Xiaomei and Herbert, 2006; Ferlay *et al.*, 2010). Patterns of cancer vary greatly from one region to another due to differences in lifestyle and

exposure to environmental agents (Parkin *et al.*, 2002). Several factors could contribute to increasing the risk of cancer development, including age, genetics, and environmental factors (Ames, 1998; Tzonou *et al.*, 1996; Wild, 2008). Eighty percent of all cancer cases appear in individual's aged 55 and older and result from deterioration in genome maintenance and low repair capacity (DePihno, 2000). Familial cancers are caused by inherited genetic mutation, and comprise approximately 5% of all cancer cases, but pose very high risk. Furthermore, environmental factors such as tobacco smoking, medications, radiation and some pro-carcinogenic foods have a significant involvement in cancer development with the high rate of deaths from lung cancer due to smoking as the biggest example (Hanahan and Weinberg, 2000). Understanding the causes of cancer is complex, since it can result from the interaction of many factors, particularly in old age (Smilenov, 2006).

## **1.2 Skin Cancer**

Skin cancer is a prevalent type among men and women, especially in populations of Caucasian origin (Buettner and Raasch, 1998; Diepgen and Mahler, 2002). Skin is the largest organ in the body and has two main layers, the dermis, and the epidermis. Skin plays an important role in preventing various environmental problems, such as dehydration, sunburn and damage due to pollution (Rycroft *et al.*, 2010). Statistics indicate that Australia has the highest rate of skin cancer, and constitutes about 80% of all malignant skin tumors while the lowest rates of skin malignancies have been reported for Black and Asian people (Jones, 1987; Rivers and Gallagher, 1995; Nggada *et al.*, 2003). According to Canadian Cancer Society (2007), in Canada one third of all new cancer

cases are skin cancers, and unfortunately the rate is on the rise. The increasing prevalence of skin cancer and its mortality rate could be due to stratospheric ozone depletion and atmospheric pollution (Williams and Kupper, 1996). Skin cancer has three main types, which are basal cell carcinoma, squamous cell carcinoma, and malignant melanoma (Leiter and Garbe, 2008). The majority of skin cancer cases are basal or squamous cell carcinomas while malignant melanoma cases are fewer (Weinstock, 1994).

Basal and squamous cell carcinomas are extremely common and often do not cause death because they usually do not spread to other parts of the body. However, malignant melanoma is the most serious form and can be fatal, causing 90 percent of skin cancer deaths (Garbea *et al.*, 2010). The Canadian Cancer Society reported 79,600 patients diagnosed with skin cancer in Canada in 2011 (5,500 with melanoma and 74,100 with non-melanoma skin cancer). It is suggested that melanoma cases will increase to 9,070 cases in 2031 and non-melanoma skin cancer will be about 201,302 cases. In the United States, malignant melanoma was the cause of about 9,000 deaths in 2009 (Kushiro and Núñez, 2011).

The main factor that contributes to the cause of skin cancer is exposure to ultraviolet (UV) rays from the sun or artificial sources, especially UVB with a wavelength of 290-320 nm (Williams and Kupper, 1996; Johnson, 2001). UVA and UVB play a critical role in shaping of melanoma. UVA can indirectly damage DNA through the generation of reactive oxygen species. UVB can directly damage DNA that leads to the apoptosis of keratinocytes or mutation in the sunburned cells (Situm *et al.*, 2007).

Melanoma has increased significantly among white populations since the 1950s (Mansson-Brahme *et al.*, 2002; Ulmer *et al.*, 2003; de Vries *et al.*, 2004; Lasithiotakis *et*

*al.*, 2006; Stang *et al.*, 2006). It is considered the sixth most common cancer in men and the seventh in women all over the world. In 1997, 40,300 new melanoma cases were estimated, and approximately 7,300 patients died of the disease (Parker *et al.*, 1997). In the United States classifies malignant melanoma as the sixth most common cancer, and approximately 44,200 new cases were reported in 1999 (Glocker-Reis *et al.*, 1994; Landis *et al.*, 1999). Linos *et al.* (2009) have shown the mortality of melanoma increasing annually at a rate of 0.4% from 1990 to 2004. In addition, increased annual mortality was 1.7% for men and women over age 65, which were approximately 14.3 deaths per 100,000 in 2004. It was noted that the mortality rate of men over 65 years was the highest.

Malignant melanoma occurs in melanocytes that are mutated to have uncontrolled growth. Melanocytes are the cells that produce pigment in the epidermis. Melanoma can occur anywhere that melanocytes are located, such as the skin, mouth, gastrointestinal tract and eyes. Melanoma develops when unrepaired DNA damage in skin cells causes mutations that cause the cells to multiply rapidly and promotes the formation of malignant tumours. Melanoma tumour progression generally invade tumour limited to the epidermis followed by the radial growth phase, and the vertical growth phase (Kanavy and Gerstenblith, 2011).

Melanoma has three general categories. The first and most common type of melanoma is called cutaneous melanoma (Mackie and Freudenberger, 1989). It occurs on the soles of feet and underneath the fingers and toenails. There are four types of cutaneous melanoma. Superficial spreading melanoma is considered the most common and can affect any site and all ages (Forman *et al.*, 2008). Nodular melanoma does not

show radial growth and can be pedunculated, nodular, or polypoid in form (Weinstock *et al.*, 1988). Lentigo maligna melanoma can occur on sun-exposed skin, face and upper limbs of older patients (Forman *et al.*, 2008). Acral lentiginous melanoma is common on plantar, palmar, and ungual skin (Lambert *et al.*, 1985). The second category is mucosal melanoma, which is a rare form. It can infect the mucous membranes of the body, such as the nasal passages, the vagina, oral cavity, the anus, head and neck (Epstein *et al.*, 1998). The third category is ocular melanoma also known as choroidal melanoma; which occurs in the eye (Gallagher *et al.*, 1984).

People with a large number of unusual moles, a family history of melanoma, light eye color, blond or red hair, fair skin, and freckles may have an increased risk of malignant melanoma (Bliss *et al.* 1995). There are many symptoms of melanomas including a spot or painful sore, a spot or lump that looks shiny, and a firm red lump especially these change in that shape, color and size. Melanoma can also appear as a new mole, and several signs have been determined to diagnose a mole as a cancer, such as asymmetry, a border that is irregular, uneven color, or a diameter that is increasing (Bishop *et al.*, 2007).

Melanoma treatments vary depending on the grade, size, location, and style of the melanoma and the patient's health (Neville *et al.*, 2007). For advanced metastatic (Stage IV) melanoma, there is currently no effective treatment, but early diagnosis can improve patient survival, especially in the young (Parker *et al.*, 1997). One treatment for melanoma can be a surgical wide excision, removing the melanoma and the normal skin around it. How much skin is taken will depend on the melanoma's thickness (Veronesi and Cascinelli, 1991; Balch and Urist, 1993). Several other options for treatment have

also been recommended including freezing, scraping, radiotherapy and chemotherapy (Neville, 2007). Some targeted therapy and immunotherapy forms can be effective, such as interferon- $\alpha$  (IFN) or interleukin-2 treatment (Eggermont *et al.*, 2008). Patients can receive doses of interferon- $\alpha$  (IFN) and interleukin-2 (IL-2) administered as adjunctive therapy in the second and third stages of melanoma (Creagan *et al.*, 1986; Moschos *et al.*, 2006). Using chemotherapy alone is not effective in melanoma as compared to biochemotherapy, which is a combination of cytotoxic chemotherapy and immunotherapy. Ives *et al.* (2007) found that biochemotherapy can increase response rates, such as reducing some symptoms, but has negative side effect of increased toxicity, as well as no improvement in survival.

Various treatments for melanoma may lead to side effects including redness, dryness, rashes, itching, peeling and acne. Side effects of interferon (IFN) can cause chills, fever, fatigue, nausea and vomiting which can begin within 30-120 minutes after interferon treatment and can continue for many hours (Quesada *et al.*, 1986). After a period of IFN treatment, patients often complain of memory loss, depression, cognitive slowing and sometimes-suicidal ideation (Trask *et al.*, 2000). The majority of anticancer drugs are given intravenously, which can cause a dark area in the skin, especially for people who have dark skin. Side effects of chemotherapy depend on the type of drug dose and the length of treatment. These side effects can include hair loss and mouth sores, tiredness and vomiting (Griffin *et al.*, 1996). According to the American Cancer Society, some chemo drugs can cause damaged nerve endings leading to symptoms, especially in the hands and feet, such as tingling sensations, sensitivity to cold or heat, or pain.

### 1.3 Medical Plants

Treatment with plant-derived medicines could provide lower cost options and may have some benefits over treatment with chemicals and other procedures as well as fewer side effects (Corner *et al.*, 2006; Vickers *et al.*, 2006). Various studies *in vitro* and *in vivo* have proved the anti-inflammatory and anticancer effectiveness of treatment with extracts from many medicinal herbs (Aravindaram and Yang, 2010). For thousands of years several communities have relied on nature for all of their basic needs, including food, protection, transport, clothing and therapeutic uses. In the field of treatment, herbs have been chewed to relieve pain, or wrapped around a wound to relieve individuals from pain or discomfort. As well, plants have been the basis of advanced traditional medicine in places such as Egypt, India, Greece, and China dating to the beginning of history. These medical systems have been the basis of some modern drugs (Hong-Fang *et al.*, 2009). Sumerians and Akkaidians used medicinal plants in 2600 BC (Samuelson, 1999). In Egypt more than 700 drugs were recorded dating from 1500 BC. In China, over 600 medicinal plants were reported in use around 1100 BC (Cragg *et al.*, 1997).

Compared to the beginning of time, when plants were the only form of medications for mankind, their use has now been relegated to a secondary role because advanced molecular biology and chemistry have permitted in the development of specific chemical compounds designed to target specific molecules. However, in recent years there has been a renewed interest in the use of natural products, which have played a major role in drug development (Hong-Fang *et al.*, 2009). People in many countries care deeply about the use of alternative therapies and products, especially natural health products. For example, Canadians spent approximately C\$ 2 billion on natural health

products in 1997 (Ghayur, 2010). Medicinal plants play a significant role in the fight against many diseases such as HIV/AIDS, Alzheimer's disease, malaria, improving lung function, and treating many infections (Balunas and Kinghorn, 2005; Singh *et al.*, 2010). It has been suggested that herbal remedies can help to prevent or treat cancer by enhancing cellular defenses such as detoxifying and antioxidant enzyme systems as well as the induction of anti-inflammatory and antitumor activity (Aravindaram and Yang, 2010).

There are approximately 250,000 species of plants and at least a thousand of these species have been shown to have significant anticancer properties (Mukherjee *et al.*, 2001). According to the United States Food and Drug Administration, the analysis of new drugs confirmed for anticancer therapy during the period of 1981-2002 showed that 62% of these cancer drugs were of natural origin. These therapeutically used drugs were obtained through structural modification of natural compounds, or by creating new compounds, and are developed by modeling from a natural compound (Gordaliza, 2007).

Krishnaiah *et al.* (2011) showed that several medicinal plants have significant antioxidant activity. Antioxidants decrease the oxidative stress in cells and are thus helpful in the treatment of many health problems such as cancer, cardiovascular diseases and inflammatory diseases. Studies show that there are significant antioxidant activities in extracts from the stems, roots, bark, leaves, fruits and seeds of several medicinal plants. Synthetic antioxidants, including butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are presently utilized as food additives and a wide array of plant species have similar antioxidant potentials compared to these synthetics. These species include *Diospyros abyssinica*, *Pistacia lentiscus*, *Geranium sanguineum* L.,

*Sargentodoxa cuneata* Rehd. Et Wils, *Polyalthia cerasoides* (Roxb.) Bedd, *Crataeva nurvala* Buch-Ham. *Acacia auriculiformis* A. Cunn, *Teucrium polium* L., *Dracocephalum moldavica* L., *Urtica dioica* L., *Ficus microcarpa* L. fil., *Bidens pilosa* Linn. *Radiata*, *Leea indica*, the *Lamiaceae* species, *Uncaria tomentosa* (Willd.) DC, *Salvia officinalis* L., *Momordica Charantia* L., *Rheum ribes* L., and *Pelargonium endlicherianum*. These natural antioxidants might be free of side effects and can be used as a substitute to synthetic antioxidants in the food industry and for preventive medicine uses (Krishnaiah *et al.*, 2011).

Ingredients extracted from plants played an essential role in the development of anticancer agents and have showed a relative lack of toxicity (Unnati *et al.*, 2013). Anticancer agents may work through a variety of mechanisms, including direct impact on cancer cell growth, by restriction of blood supply to tumors, or through stimulating an immune response (Hynes and Lane, 2005; Sharma *et al.*, 2011). Vinblastine, vincristine, irinotecan, topotecan, etoposide, podophyllotoxin, and paclitaxel are powerful anticancer agents that have been derived from plants (Cragg *et al.*, 1997; Newman and Cragg, 2007). Many medicinal plants have anticancer properties but most of these plants still have to be tested.

Herbs contain many components, which work together to produce useful outcomes (D'Epiro, 1999). There are several factors that determine the effectiveness of an herb, such as geographical location, growth temperature, seasonal changes and the type of environment (climate, bugs and soil quality). Furthermore, when and how it was harvested and processed as well as whether the environment includes pollutants or not are also important (Avato *et al.*, 2000; Sagdic, 2003). It has been found that natural

compounds are much more diverse and much more complex with regard to molecular structures compared to small molecular synthetic drugs and often have the ability to provide highly specific biological activities (Gonzales and Valerio, 2006).

#### **1.4 *Uncaria tomentosa***

*Uncaria* is a genus of flowering plants. It has about 40 currently known species and most of the native species are distributed in tropical areas such as Asia, Africa, the Mediterranean and the neotropics (Mabberley, 2008). *U. tomentosa* is a large woody vine from the Rubiaceae family and Cinchonoideae subfamily, known as Cat's Claw. It is widely used in the Amazon rainforest and throughout South and Central America (Rizzi *et al.*, 1993; Reinhard, 1999). Its name comes from the hook-like thorns that resemble claws, which are commonly found in the jungle. The leaves have a smooth edge and grow in two opposite whorls (Keplinger *et al.*, 1999). *U. tomentosa* has been traditionally used for medical therapies for decades. In Peru, many tribes such as the Aguaruna, Ashaninka, Cashibo, and Shipibo considered that *U. tomentosa* had a great number of uses and properties. In addition, they successfully used this species to treat various disorders (Keplinger *et al.*, 1999).

Cat's Claw may be useful in the treatment of several common diseases such as gastrointestinal disorders, diabetes, respiratory infections, several types of cancer, gastric ulcers, rheumatoid arthritis, hemorrhoids, herpes, multiple sclerosis and degenerative diseases (Heitzman *et al.*, 2005). Currently, Cat's Claw is more popular and is considered one of the best-selling herbs in the United States. According to the American Cancer Society, *U. tomentosa* is often recommended for its positive impact on health and has

become one of the most popular herbs in the United States and Europe. However, allergic reactions can potentially affect people who are allergic to plants in the Rubiaceae family or different species of *Uncaria*.

Since the 1970s, various studies have been conducted on *U. tomentosa* in Peru, Germany, Austria, England, Japan, Canada, the United States and other areas to learn more about the healing properties found in Cat's Claw extracts. Its components, including alkaloids, quinovic acid glycosides, polyhydroxylated triterpenes, flavonoids, catechins and sterols, can have great positive effects (De Matta *et al.* 1976; Aquino *et al.* 1989, 1991; Muhammad *et al.* 2001; Kitajima *et al.* 2004; Heitzman *et al.* 2005). It has also been shown that Cat's Claw can kill viruses, bacteria and other microorganisms that cause disease. These components can protect healthy cells from becoming cancerous and inhibit the development and proliferation of tumours (Lamm *et al.*, 2001; Åkesson *et al.*, 2003).

For two decades, researchers have examined many types and methods of extraction and monitored a large number of pharmacological properties of *U. tomentosa* including reduction of epithelial cell death in response to oxidant stress (Miller *et al.*, 2001), improvement in oedema through suppression of cyclooxygenase-1 and -2 (Aguilar *et al.*, 2002), cytoprotection by means of free radical scavenging, reduction of oxidative stress, and immediate suppression of TNF- $\alpha$  production (Sandoval *et al.*, 2000; Allen-Hall *et al.*, 2010). A large number of researchers have investigated the biological effects of *U. tomentosa* and the pentacyclic oxindole alkaloids that are present in this plant extract (Reinhard, 1999). Recently, laboratory experiments with many of these isolated alkaloids have demonstrated their antioxidant, immunomodulatory (Paniagua-Pérez *et al.* 2009) and anti-neoplastic properties (Bacheret *et al.*, 2005; García Prado *et*

*al.*, 2007). However, other studies have proved that compounds different from the oxindole alkaloids such as triterpenes and quinovic acid glycosides can be partially responsible for the observed effects (Aquino *et al.*, 1990, 1991). A third hypothesis indicates that the anti-inflammatory properties of *U. tomentosa* can be related to a synergy between a group of ingredients (Reinhard, 1999; Rizzi *et al.*, 1993). Various extracts and compounds found in *U. tomentosa* can be useful as inhibitors of the growth and proliferation of tumours (Dreifuss *et al.*, 2013).

Additionally, Sheng *et al.* (2005) described the active components of a water-soluble Cat's Claw extract, called C-Med-100, as preventing the proliferation of tumour cells and inflammatory responses by inhibiting cell growth and enhancing DNA repair. Quinic acid is a biologically active ingredient isolated from the bark of *U. tomentosa* that can be bioactive *in vivo*. Moreover, treatment with C-Med-100 can lengthen lymphocyte half-life and enlarge spleen cell numbers in mice given specific doses of this extract in their drinking water (Akesson *et al.* 2005). However, in other studies Cat's Claw extracts and its components have caused antiproliferative activity in many cancer cells, including acute lymphoblastic leukaemia, MCF7 breast cancer, glioma, neuroblastoma and premyelocytic leukemia (Riva *et al.*, 2001; Caballero *et al.*, 2005; Bacher *et al.*, 2006; Prado *et al.*, 2007; Fazio *et al.*, 2008; Rinner *et al.*, 2009; García Giménez *et al.*, 2010; Pilarski *et al.*, 2010).

*U. tomentosa* can be used as an antioxidant, has anti-apoptotic properties, and repairs cellular DNA damage (Sheng *et al.*, 2001). Farias *et al.* (2011) have shown that *U. tomentosa* can be used as an adjunct treatment for patients also being treated with chemotherapy by promoting DNA repair and reducing the harmful effects of the

chemotherapy such as weight loss, nausea, secondary infections, and hair loss, as well as by increasing the number of myeloid progenitors. *U. tomentosa* was extremely effective as an adjuvant treatment for breast cancer and reduced the neutropenia resulting from chemotherapy. It was also able to promote DNA repair (Farias *et al.*, 2012). This means the effects of *U. tomentosa* can be different on cell proliferation depending on the cell type involved. Dreifuss *et al.* (2013) compared the anti-neoplastic effects of a *U. tomentosa* brute hydroethanolic (BHE) extract with those of two different types of extracts. Walker-256 tumor cells injected into the pelvic limbs of male Wistar rats were used as a model. Results proved that the BHE extract and its BuOH fraction decreased tumour volume and weight, and modulated anti-oxidant systems. In addition, treatment increased the survival time for the animals that had tumours.

Pilarski *et al.* (2010) studied the activity of *U. tomentosa* preparations on cancer cells by calculating the IC50 values for preparations with different quantitative and qualitative oxindole alkaloid composition. They found a strong relationship between the total oxindole alkaloid content (from 0.43% to 50.40% d.m.) and the antiproliferative activity of the preparations (IC50 from >1000 µg/ml to 23.57 µg/ml). As well, animal experiments on mice with Lewis lung carcinoma showed the presence of significant inhibition of tumour growth by B/W37--bark extracted in water at 37 °C, for 21 days at daily doses of 5 and 0.5 mg. Gurrola-Díaz *et al.* (2011) studied the inhibitory mechanisms of *U. tomentosa* extracts on the Wnt-signaling pathway, a main organizer of development and tissue homoeostasis. Results showed that pharmacological action of *U. tomentosa* contributes to inhibition of the Wnt-signaling pathway, downstream of beta-Catenin activity.

Dreifuss *et al.* (2010) studied the antitumor and antioxidant effects of *U. tomentosa* hydroalcoholic extract on Walker-256 cancer cells. They found that *U. tomentosa* hydroalcoholic extract decreased tumour growth significantly and decreased the activity of AST. Treatment also increased CAT activity in the liver, while lowering it in the tumour tissue. In addition, SOD activity was reduced in the liver and in the tumour. Martino *et al.* (2006) examined the potential proapoptotic mechanism in three different tumoral cell lines using the activation of caspase3 in cells treated with root bark extracts of *U. tomentosa*. The results showed that treatment with a given extracts of *U. tomentosa* inhibited the cell growth of *U. tomentosa* aqueous extracts on SAOS (human osteosarcoma cell line), MCF7 (human breast carcinoma cell line) and HeLa cells (human cervical carcinoma cell line).

Cheng *et al.* (2007) used four solvents with different polarities to isolate extracts from Cat's Claw and then compared their relative effect on the proliferation of human premyelocytic leukemia, HL-60, cell lines. *U. tomentosa* n-hexane extracts (CC-H), ethyl acetate extracts (CC-EA) and n-butanol extracts (CC-B) had significantly higher effects on HL-60 cells compared to those extracted using methanol (CC-M). The study found that the ability of treatment with CC-EA to induce apoptosis in HL-60 cells could be very important for medicine evolution. Rojas-Duran *et al.* (2012) suggested that mitraphylline was the main pentacyclic oxindolic alkaloids existing in the bark of the chloroformic extract of *U. tomentosa*. An *In vivo* murine model was examined and the effect of the *U. tomentosa* extract on the expression of inflammatory cytokines was examined. Mitraphylline inhibited approximately 50% of the release of interleukins 1 $\alpha$ , 1 $\beta$ , 17, and

TNF- $\alpha$ , which are mediators of the immune response. Treatment also decreased 40% of the production of interleukin 4 (IL-4).

Nuclear factor kappa B (NF- $\kappa$ B) is a transcription factor, which is made of subunits that include the p50, p65 p52, Rel B, and cRel subunits. It is an important link between cancer and chronic inflammation (Li *et al.*, 2005). It has been shown that extracts of *U. tomentosa* inhibit NF- $\kappa$ B (Sandoval-Chacón *et al.*, 1998; Akesson *et al.*, 2003). Caballero *et al.* (2005) have shown that intraperitoneal (i.p.) injection of an aqueous extract of *U. tomentosa* suppressed TNF- $\alpha$  and IL-6 secretion in response to lipopolysaccharide (LPS) challenge, as well as inhibit the growth of tumours and metastasis in mice. The *in vitro* and *in vivo* effects of a hydroethanolic extract of *U. tomentosa* on the viability of B16-BL6 tumour cells and on the inflammatory response, including secretion of TNF- $\alpha$ , IL-6 and nitric oxide [NO], was examined in C57BL/6 mice. The results showed that *U. tomentosa* inhibited TNF- $\alpha$ , IL-6 and NF- $\kappa$ B activity *in vitro*. However, *U. tomentosa* did not demonstrate a significant cytotoxic effect *in vitro* at doses up to 300  $\mu$ g/ml, but did prevent tumor growth and metastasis *in vivo* (Fazio *et al.*, 2008).

Allen-Hall *et al.* (2007) showed that treatment of THP-1 monocyte-like cells with *U. tomentosa* extracts inhibited the MAP kinase signaling pathway and changed cytokine expression. ELISA assays showed that treatment with *U. tomentosa* extracts increased LPS-dependent expression of IL-1beta by 2.4-fold, while inhibiting the LPS-dependent expression of TNF-alpha by 5.5-fold. They observed that treatment of LPS-stimulated THP-1 cells with *U. tomentosa* extracts prevented ERK1/2 and MEK1/2 phosphorylation in a dose-dependent manner. Allen-Hall *et al.* (2010) have shown that treatment of

monocyte-like THP-1 cells with *U. tomentosa* inhibits the production of the pro-inflammatory cytokine TNF-alpha while increasing IL-1beta production. *U. tomentosa* inhibited the LPS-dependent activation of certain NF-kappaB and AP-1 subunits and improved cell death when NF-kappaB was inhibited.

Continuing research has explored the immunoregulatory and antiviral activities of *U. tomentosa* in samples that were examined in an *in vitro* DENV contagion model. DENV-2 infected human monocytes were incubated with *U. tomentosa* hydro-alcoholic extracts or pentacyclic oxindole alkaloid-enriched or non-alkaloid fractions. The results showed the presence of inhibitory activity in both extracts and the alkaloidal fraction, decreasing DENV-Ag+ cell rates in treated monocytes. A microbead immunoassay was used for cytokine determination (TNF-alpha, IFN-alpha, IL-6 and IL-10) in infected monocyte culture supernatants. Decreased level of TNF-alpha and IFN-alpha were also observed and there was a tendency towards IL-10 modulation. The most effective treatment for decreasing monocyte infection rates and affective cytokine levels was with the alkaloidal fraction of *U. tomentosa* (Reis *et al.*, 2008).

Many researchers have studied the effects of medicinal plants on B16-BL6 cells, an aggressive murine melanoma (Hasegawa *et al.*, 2002; Miyake *et al.*, 2010). This cell line was derived from a melanoma from the skin of a C57BL/6 strain mouse that displayed fibroblast-like characteristics and that produced melanin. Cells could lose their ability to produce pigmentation in the long term.

## 2. Objectives and Experimental Summary

The present study examines the effects of *U. tomentosa* on B16-BL6 cells *in vitro* and *in vivo* in order to evaluate its potential anticancer activity. Three different methods were used including, which are the MTT Cell Proliferation Assay, immunohistochemistry and the male isogenic tumour transplantation model. The MTT Cell Proliferation Assay determines the cell proliferation rate in cells treated with *U. tomentosa*. Immunohistochemistry detects specific target antigens for example the Ki-67 protein and TUNEL assay. The Ki-67 protein is a cell proliferation nuclear that evaluates the growth fraction of a treated cell population. TUNEL is a method used to detect DNA fragmentation by labeling the terminal end of nucleic acids through terminal deoxynucleotidyl transferase (TdT). The isogenic tumour transplantation model is performed and tumour tissues are collected in order to measure the tumour diameters and weights of animals injected with B16-BL6 cells treated *Uncaira* extracts. Furthermore, the research determines whether *U. tomentosa* is an effective treatment for melanoma cancer that may eventually be appropriate for human patients in the future.

### **3. Materials and Methods**

#### **3.1 Tissue Culture**

The B16-B16 cell line is an aggressive murine melanoma [American Type Culture Collection, Manassas, VA] that was maintained in tissue culture plates (Sarstedt, Laval, QC) in Dulbecco's modified Eagle's 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% CO<sub>2</sub> and subcultured 1:5 approximately every 3-4 days. For experiments, the cells were treated with media including *Uncaria tomentosa* extracts at various concentrations as specified. MTT assays and Immunofluorescence microscopy were performed in triplicate and the mouse experiments were performed and triplicate.

#### **3.2 Plant Extraction**

The *U. tomentosa* powder was obtained from Rosario Rojas (Peru) or as a natural product supplement (from the lowland Peruvian Amazon Region, Iquitos. Cat's Claw by now 500 mg, CODE 84618, Now Foods 60108) was extracted with PBS or 70% ethanol and the soluble fraction used in experiments. These extracts were shown to be very similar in cell growth studies and HPLC (not shown). For PBS extracts, 20 g of *U. tomentosa* powder was suspended in 180 ml water and 20 ml PBS and boiled for 1h. Bark debris was removed by centrifugation at 2000 x g for 10 min and the supernatant filtered through 0.22 µm filters. For the ethanolic extracts, 20 g of *U. tomentosa* powder was suspended in 200 ml 70% ethanol and boiled for 1h. The bark debris was removed by

centrifugation and the supernatant filtered through 0.22 µm filters.

### **3.3 Cell Treatments**

The B16-BL6 cell line cultured in Dulbecco's modified Eagle's medium (DMEM), was treated with various concentrations of *U. tomentosa* extracts and incubated at 37°C in 5% CO<sub>2</sub>. The PBS extraction (1000 mg/ml) and the ethanolic extract (1000 mg/ml) were used as two different preparations of *U. tomentosa*. Before treatment, the two extract were suspended in DMEM media. For treatments, various doses of these stock solutions were used to treat the cell cultures. The two extract were utilized at the same dose as the solvent-only controls for each experiment.

### **3.4 MTT Assay**

The B16-BL6 cells were plated on 96-well plates at a concentration of  $2 \times 10^3$  cells/ well and incubated overnight with 5% CO<sub>2</sub> at 37°C. These cells were treated in media with 0, 4 µg/ml, 20 µg/ml, 40 µg/ml or 200 µg/ml of *U. tomentosa* extracted with 70% ethanol or PBS on day 0. Cells were treated for four days in the continued presence of *U. tomentosa* without a media change. Furthermore, media was used as a negative control. On each day, cell viability was evaluated using the MTT assay. Each day, 10 µl/ml of MTT solution was added to each well of a plate at a final concentration of 0.25 mg/ml and incubated for 4 hrs. After the incubation, the media was removed and 100 µl/well of dimethyl sulfoxide (DMSO) was added to dissolve the converted crystals. The

absorbance was read at 540 nm using a (Spectramax 340PC 389) plate reader. Analysis of the effect of treatment with different concentrations of *U. tomentosa* was performed by ANOVA using Graph Pad Prism Software. Each experiment was performed at least 3 separate times.

### **3.5 Cell Morphology**

The B16-BL6 cells were suspended in culture media in 24-well plates and incubated at 37°C for 4 hrs. Cells were treated with control, 4 µg/ml (low dose), 40 µg/ml (middle dose) and 200 µg/ml (high dose) of *U. tomentosa* extracted with ethanol or PBS. Cells were imaged daily for 4 days to determine the cell morphology between various treatments.

### **3.6 Immunohistochemistry (Fluorescence) for ki-67 protein expression**

The B16-BL6 cells were plated on 6-well plates contains sterile glass coverslips in culture media and incubated at 37°C overnight. The B16-BL6 cells were treated with 0, 4 µg/ml, 20 µg/ml, 40 µg/ml or 200 µg/ml of *U. tomentosa* extract and incubated at 37°C for 72 hours. The treated cells were fixed with 4% formaldehyde (freshly prepared) for 5 minutes at room temperature, permeabilized by incubation in 0.1% Triton X-100 in PBS, and then blocked by incubation in 10% FBS, 0.5% BSA in PBS overnight at 4°C. The cells were stained with 100 µl primary anti-ki-67 antibody (titre 1:20) in block buffer for 60 minutes at room temperature. The primary antibody (solute) was removed and the

coverslips washed 3 times with PBS. The cells were then incubated with 100 µl goat anti-mouse IgG, secondary antibody- FITC (green) (titre 1:100) in 100 µl block buffer for 60 minutes at room temperature. Secondary antibody- FITC was removed and the coverslips were washed 3 times with PBS. Coverslips were then mounted on glass slides in 80% glycerol and analyzed under an LSM510 fluorescence microscope (Zeiss Canada).

### **3.7 TUNEL assay**

B16-BL6 cells were grown on glass coverslips overnight at 37°C. Cells were treated with *U. tomentosa* extract at concentrations of 4 µg/ml (low dose) or 200 µg/ml (high dose) for 72h. The B16-BL6 cells were also treated with 6 µg/ml of camptothecin (high dose) as a positive control for apoptosis. Treated cells were fixed by incubation with 4% formaldehyde (freshly prepared) for 5 minutes at room temperature. Cells were permeabilized with 0.1% TritonX-100 in 0.1% sodium citrate (freshly prepared) for 2 min on ice. Cells were washed twice with PBS and resuspended in 50 µl/well TUNEL reaction mixture (Roche). Cells were incubated for 60 min at 37°C. Sample was washed twice in PBS and the cover slips were mounted on glass slides in 80% glycerol. An LSM510 fluorescence microscope was used for analysis.

### **3.8 Animal Experiments**

A total of 50 syngeneic male C57BL/6 mice (8 weeks old) were used for these experiments (Charles River, Pointe-Claire, QB, Canada). Mice were received at 24-26

days of age and acclimatized in their cages for 3 weeks. All mice were placed in disposable plastic shoebox cages with 5 mice per cage using a high density, actively vented cage system (Innorack, Innovive Inc. San Diego, CA). The light: dark cycle was 12:12 with the onset at 08:00 h local time. Temperature was maintained between 20°C and 21°C. The mice were fed on a standard pellet diet and water provided ad libitum. No other experiments were conducted on these animals except this current research. Two experiments were performed with twenty-five mice used for each experiment. Tumours were induced by injection of B16-BL6 cells, an aggressive murine melanoma in the right flank of C57BL/6 mice subcutaneously (on the top of the muscle). The mice were injected with  $2 \times 10^5$  cells, suspended in 100  $\mu$ l of PBS.

All individual mice were injected with tumour cells and divided into 5 groups of 5 animals each and randomly assigned as follows: Group 1; control had only B16-BL6 tumours. Group 2; the mice were injected with 100  $\mu$ l of 200  $\mu$ g/ml ethanol extract of *U. tomentosa* intraperitoneal twice/week; Group 3; the mice were injected with 100  $\mu$ l of 200  $\mu$ g/ml ethanol extract of *U. tomentosa* intratumour twice/week; Group 4; the mice were injected with 100  $\mu$ l of 200  $\mu$ g/ml PBS extract intraperitoneal twice/week; and, Group 5; the mice were with 100  $\mu$ l of 200  $\mu$ g/ml PBS extract intratumour twice/week. Tumour growth progressed for 10 to 14 days. The mice were weighed and their health monitored every two days during the experiment.

In the first experiment, the intraperitoneal injection groups received the treatments for a period of 14 days (4 injections). The intratumour injection groups received the treatments for only week 2 (2 injections) after the appearance of palpable tumours. In the second experiment, all treatment groups (IP and IT) received the treatments for a period

of 14 days (4 injections). Following the treatment, the mice were euthanized for necropsy using CO<sub>2</sub> exposure, in compliance with the CCAC recommendations. Tumours were collected and their diameter and weight were measured. The tumours were fixed in EFA (720 ml ethanol (100%), 180 ml distilled H<sub>2</sub>O, 50 ml glacial acetic acid and 50 ml formaldehyde) for future analysis.

All processes were approved by the Laurentian University Animal Care Committee, protocol 2013-12-01, in accordance with guidelines established by the Canadian Council for Animal Care. Comparisons of mean values among different groups were performed using two-way analysis of variance (ANOVA) and post-hoc Tukey HSD tests (SPSS for windows). Differences were considered significant when  $p \leq 0.05$ .

## 4. Results

### 4.1 The effects of *U. tomentosa* on cell growth

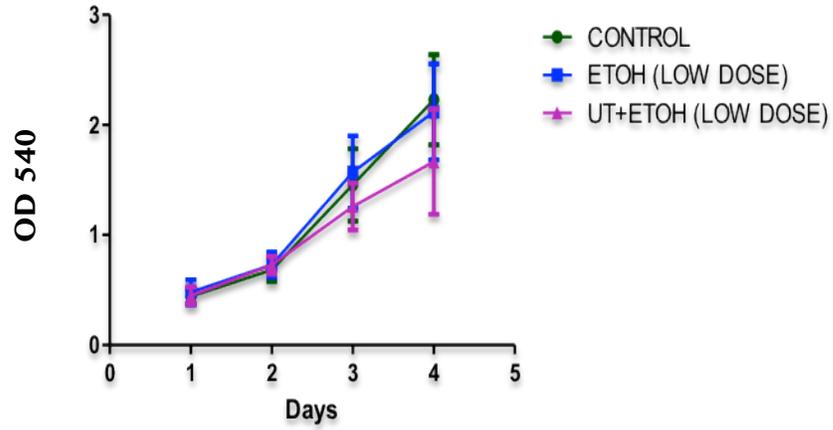
The effect of *U. tomentosa* on malignant cell growth was evaluated using the murine melanoma cell line, B16-BL6. There was significant inhibition in the B16-BL6 cell growth in response to *U. tomentosa* treatment over a period of four days. During the experiment, it was found that the decrease in cell growth was a function of the *U. tomentosa* dose. An MTT Cell Proliferation Assay was used to determine the changes in cell viability in response to treatment with *U. tomentosa* extracted using two different solvents. These preparations are the ethanolic extract and PBS extract. During the *in vitro* experiment, the results showed that *U. tomentosa* extracted with 70% ethanol can significantly kill B16-BL6 cells more effectively than *U. tomentosa* extracted with PBS. In brief, the treatment of *U. tomentosa* with ethanolic extracts showed a significant effect at the lowest concentration added, and completely inhibited cell growth when the highest concentration was added (Figure 1). Treatment with *U. tomentosa* PBS extracts did not have a significant effect at the lowest concentrations but caused significant inhibition of cell growth at the higher concentrations (Figure 2). Overall, *U. tomentosa* extracted with ethanol or PBS were able to effectively kill B16-BL6 cells, especially at  $\geq 200$   $\mu\text{g/ml}$ , which is the highest dose used. Finally, all experiments and data were run and analyzed with respect to triplicate independent experiments.

**Figure 1: Effect of *U. tomentosa* extracted with ethanol on the growth of the malignant B16-BL6 cell line**

*U. tomentosa* was added to B16-BL6 cells and growth measured using an MTT assay over a time of one-to-four days. The effect of *U. tomentosa* extracted with ethanol on the growth of the malignant B16-BL6 cell line at varying days compared to control (media) and ethanol suspended in DMEM media is shown. Cells were treated with low dose (4 µg/ml) Panel A, medium dose (40 µg/ml) Panel B, or high dose (200 µg/ml) Panel C *U. tomentosa* on day 0 and assessed for cell culture each day for 4 days. The data shows the results of a representative experiment from at least three independent experiments.

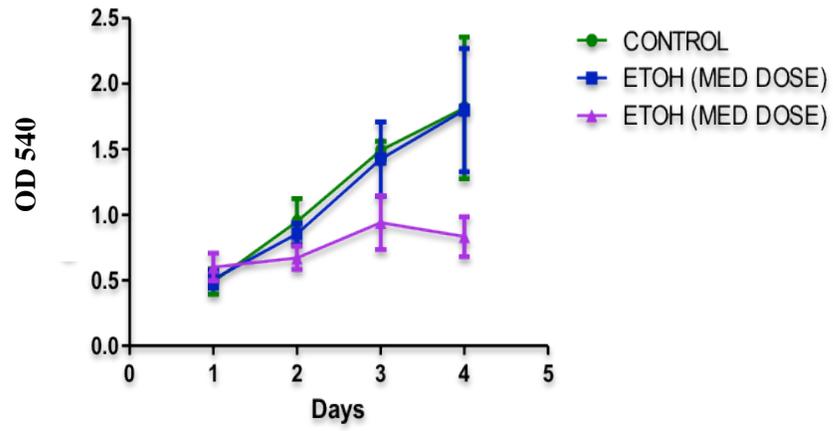
**A**

**ETOH**



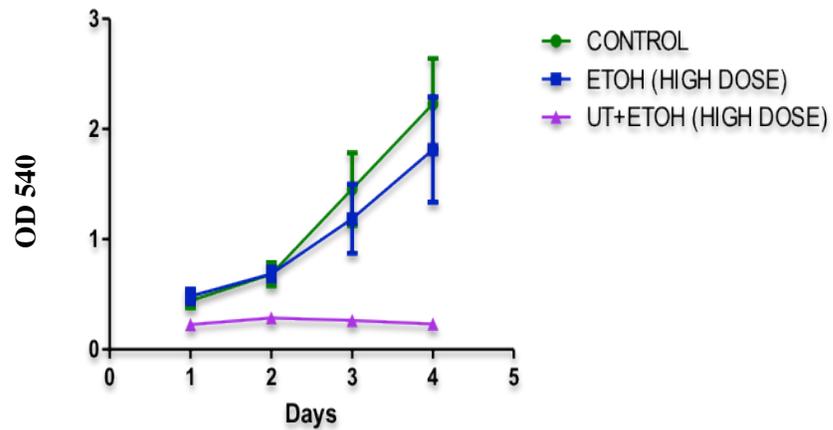
**B**

**ETOH**



**C**

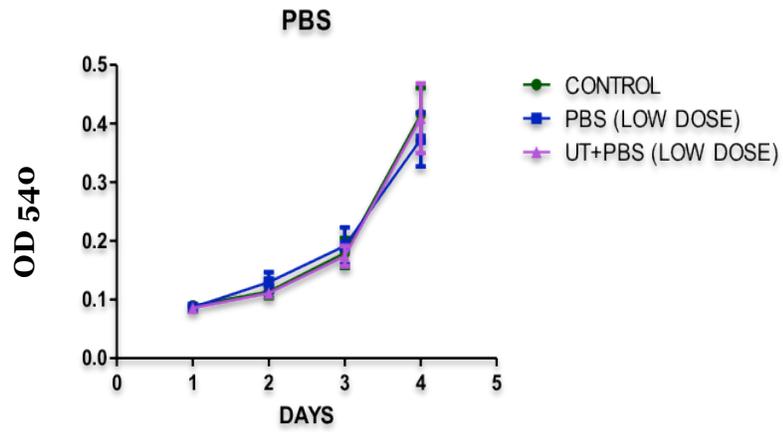
**ETOH**



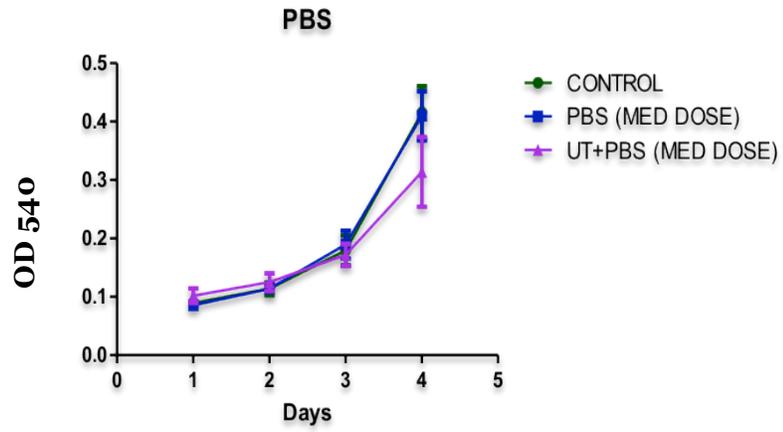
**Figure 2: Effect of *U. tomentosa* extracted with PBS on the growth of the malignant B16-BL6 cell line**

*U. tomentosa* was added to B16-BL6 cells and growth measured using an MTT assay over a time of one-to-four days. The effect of *U. tomentosa* extracted with PBS on the growth of the malignant B16-BL6 cell line at varying days compared to control (media) and PBS suspended in DMEM media is shown. Cells were treated with low dose (4 µg/ml) Panel A, medium dose (40 µg/ml) Panel B, or high dose (200 µg/ml) Panel C *U. tomentosa* on day 0 and assessed for cell culture each day for 4 days. The data shows the results of a representative experiment from at least three independent experiments.

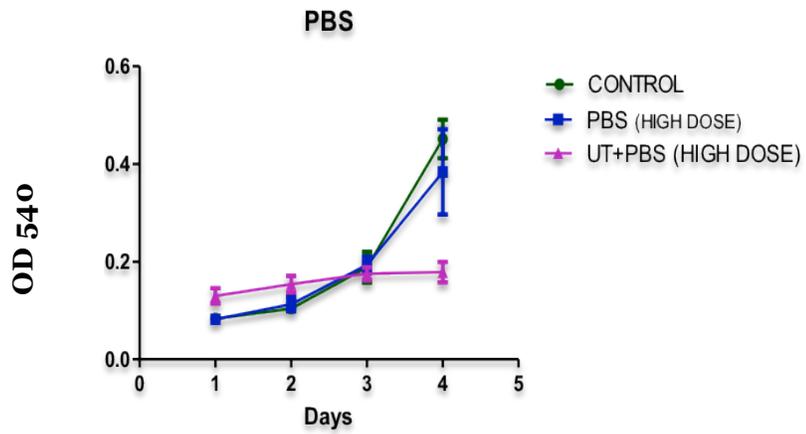
A



B



C



## 4.2 Cell morphology

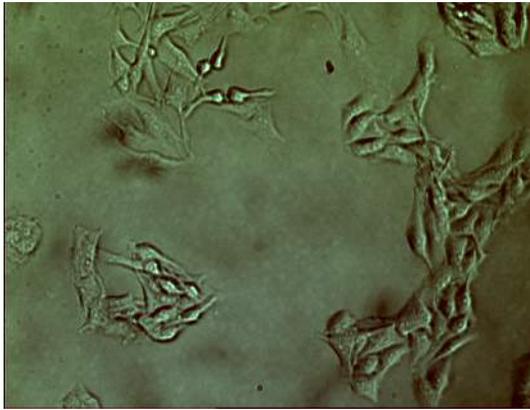
This experiment examined the morphology of the murine melanoma B16-BL6 cell line in terms of shape, structure and appearance over a period of one-to-four days. Furthermore, the pictures of the B16-BL6 cells treated with *U. tomentosa* consistent with the cell growth data that was collected using the MTT Assay. In brief, treatment with *U. tomentosa* ethanolic extracts showed a significant atrophy of the cells at all concentrations added, and it completely atrophied the B16-BL6 cells when highest concentrations added (Figure 3). Furthermore, the treatment with *U. tomentosa* PBS extracts showed no observed atrophy at the lowest concentrations but significantly atrophied the cells when the doses were increased (Figure 4). Overall, increasing the dose of *U. tomentosa* extracts both (ethanol and PBS) caused evident atrophy in B16-BL6 cell and decreased the cells number, especially at day 3 and 4.

**Figure 3: Effect of *U. tomentosa* extracted with ethanol on the morphology of the malignant B16-BL6 cell line**

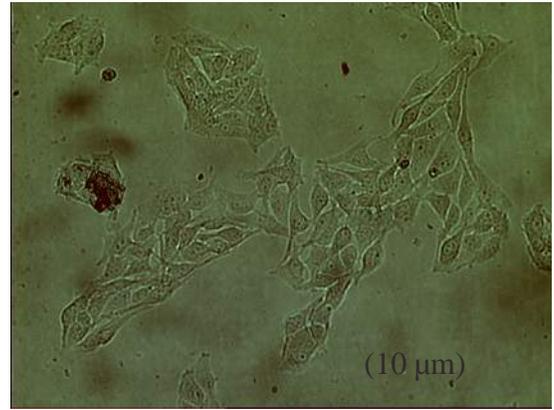
B16-BL6 cells were plated on 6-well plates tissue culture plated and grown in the presence of culture media (Panel A) 400x magnification, media supplemented with a low dose (4 µg/ml) (Panel B) 100x magnification, medium dose (40 µg/ml) (Panel C) 100x magnification or high dose (200 µg/ml) (Panel D) 400x magnification of *U. tomentosa* extracted in 70% ethanol. Images were taken using an Axiovert 100 microscope and Northern Eclipse Software.

Panel A

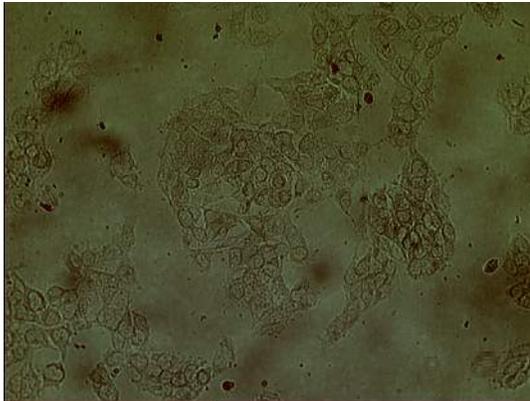
Day 1



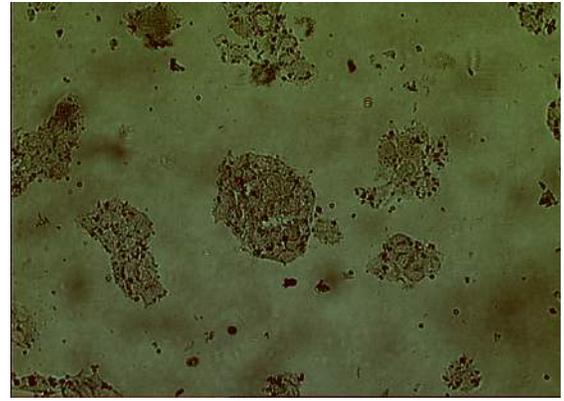
**(B16-BL6 cells) Control**



**(UT+ETOH) Low dose**



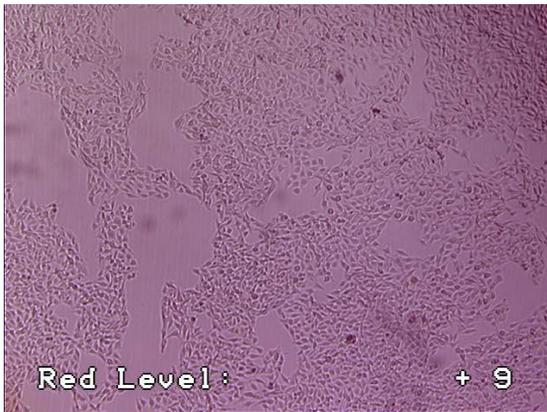
**(UT+ETOH) Med dose**



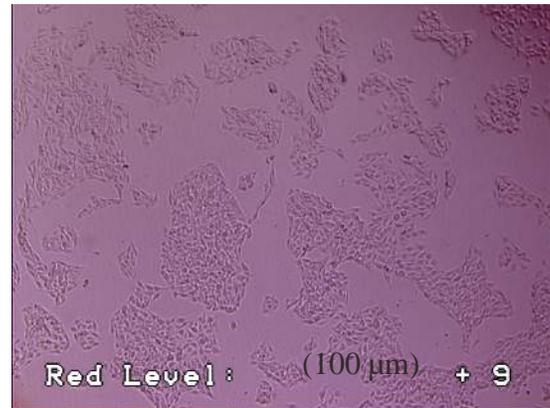
**(UT+ETOH) High dose**

Panel B

Day 2



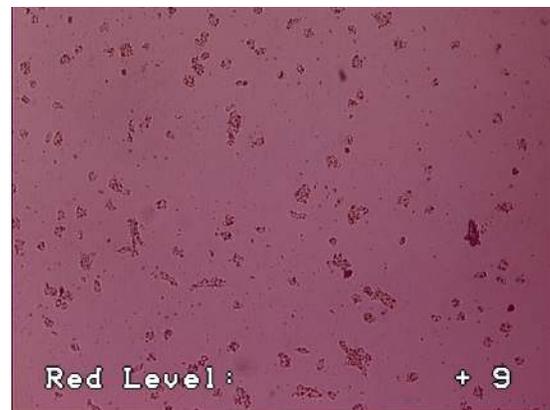
(B16-BL6 cells) Control



(UT+ETOH) Low dose



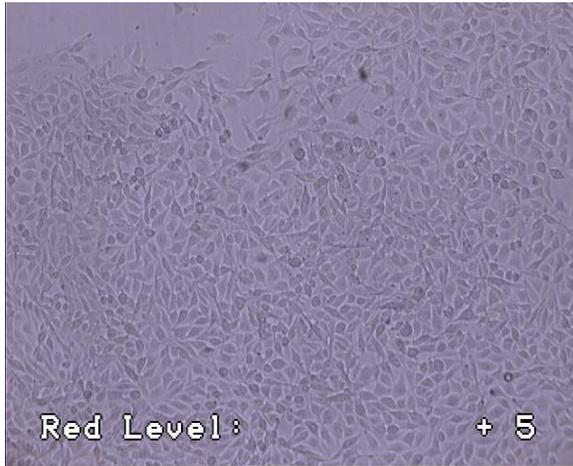
(UT+ETOH) Med dose



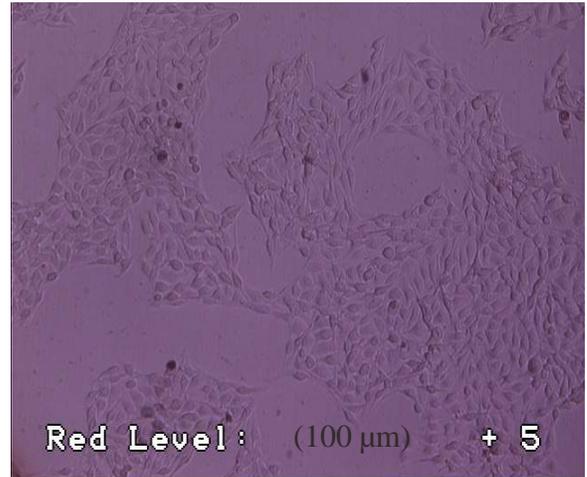
(UT+ETOH) High dose

Panel C

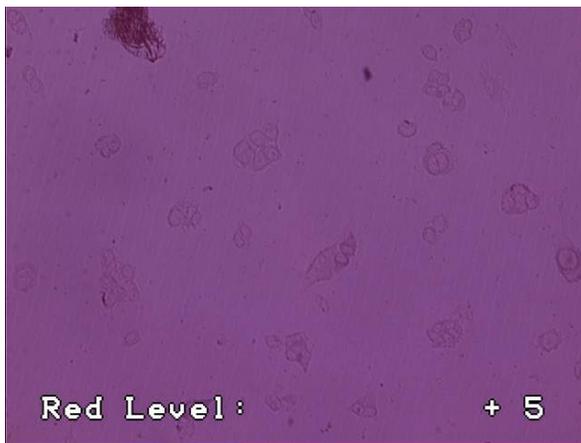
Day 3



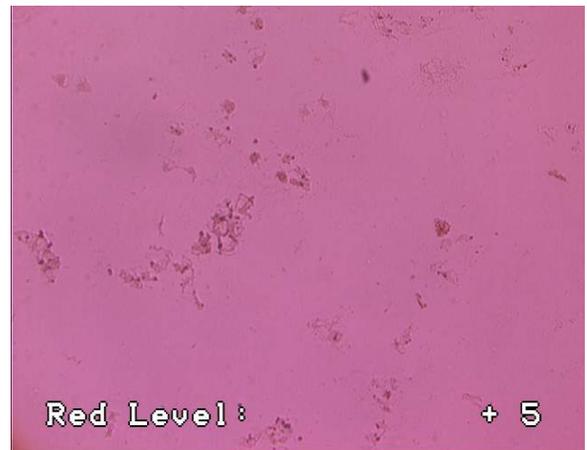
**(B16-BL6 cells) Control**



**(UT+ETOH) Low dose**



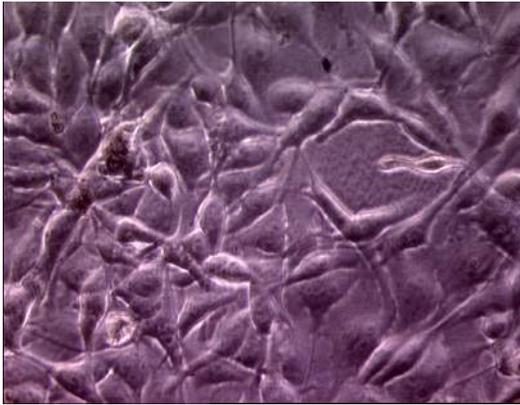
**(UT+ETOH) Med dose**



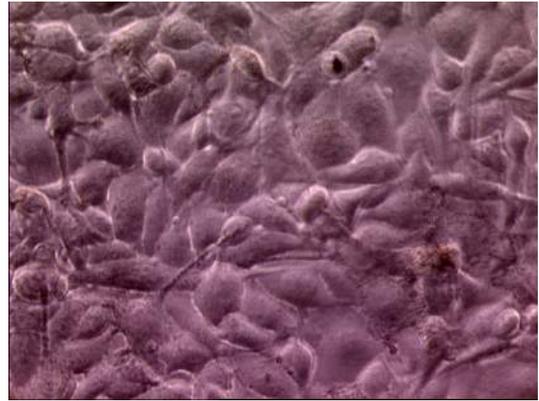
**(UT+ETOH) High dose**

Panel D

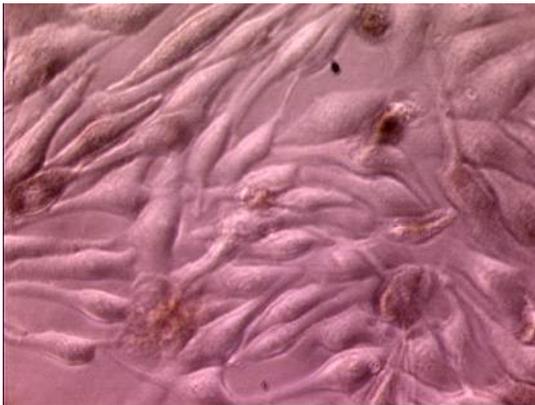
Day 4



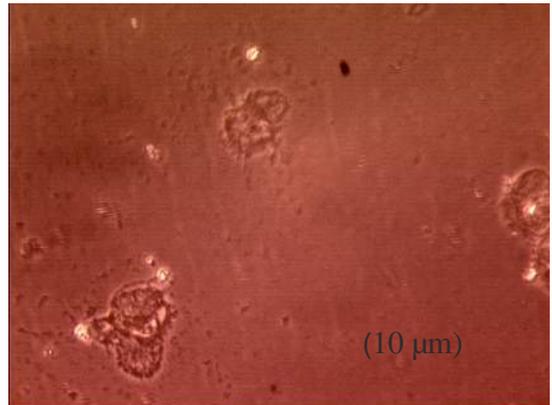
(B16-BL6 cells) Control



(UT+ETOH) Low dose



(UT+ETOH) Med dose



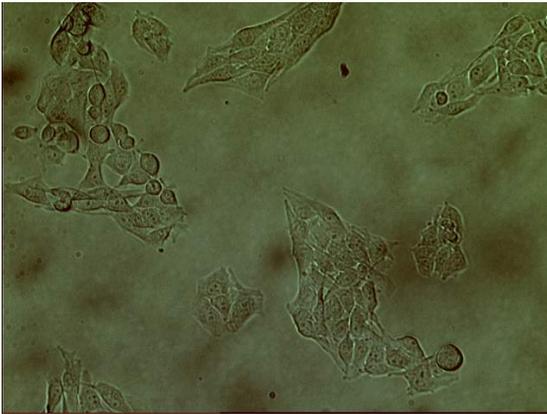
(UT+ETOH) High dose

**Figure 4: Effect of *U. tomentosa* extracted with PBS on the morphology of the malignant B16-BL6 cell line**

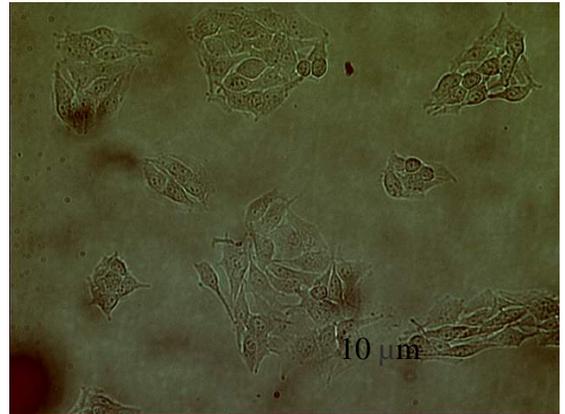
B16-BL6 cells were plated on 6-well plates tissue culture plated and grown in the presence of culture media (Panel A) 400x magnification, medium supplemented with a low dose (4 µg/ml) (Panel B) 100x magnification, medium dose (40 µg/ml) (Panel C) 100x magnification or high dose (200 µg/ml) (Panel D) 400x magnification of *U. tomentosa* extracted in PBS. Images were taken using an Axiovert 100 microscope and Northern Eclipse Software.

Panel A

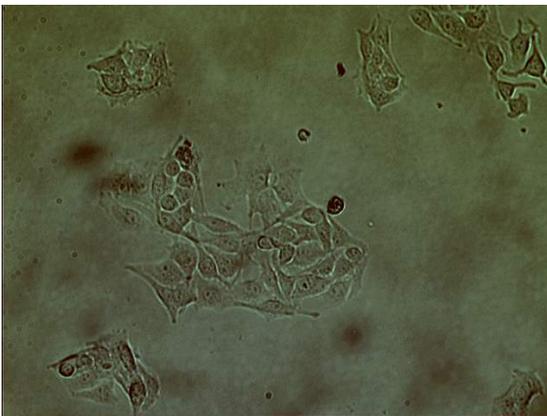
Day 1



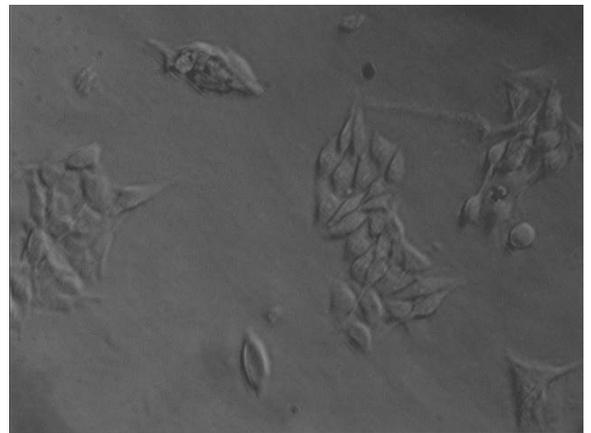
**(B16-BL6) Control**



**(UT+PBS) Low dose**



**(UT+PBS) Med dose**



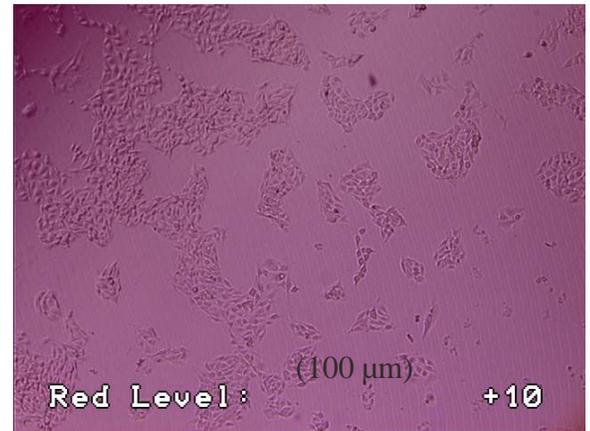
**(UT+PBS) High dose**

Panel B

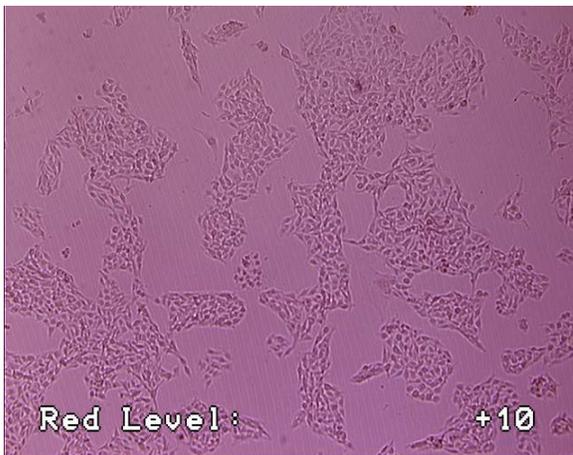
Day 2



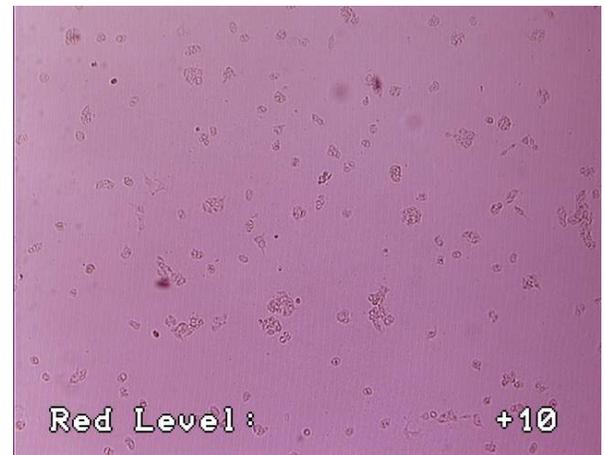
(B16-BL6) Control



(UT+PBS) Low dose



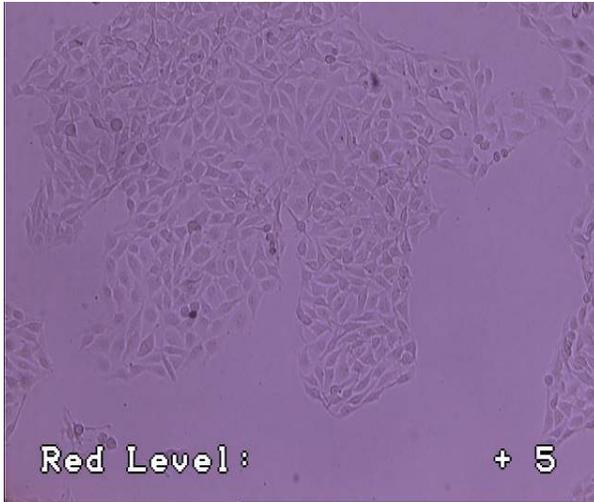
(UT+PBS) Med dose



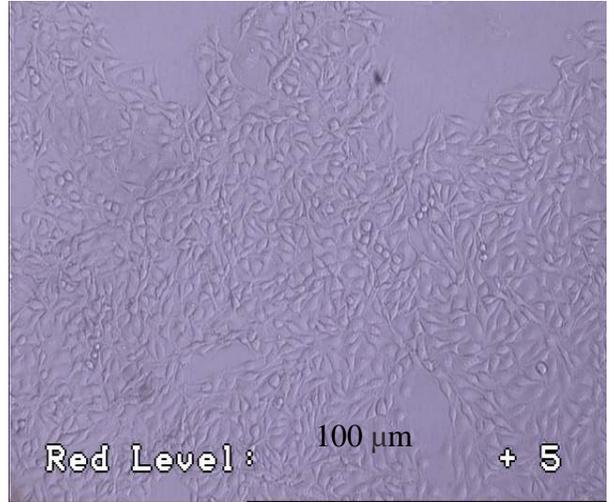
(UT+PBS) High dose

Panel C

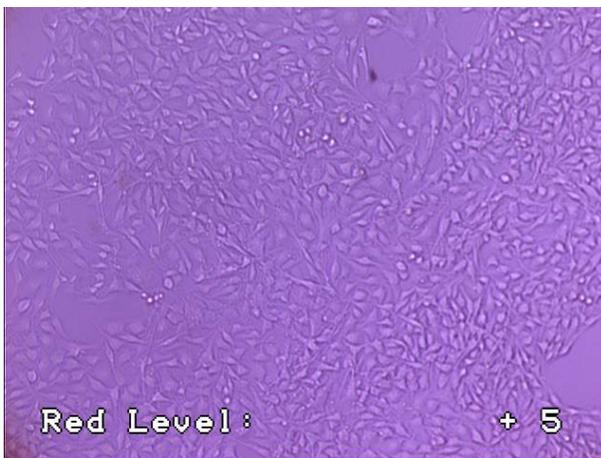
Day 3



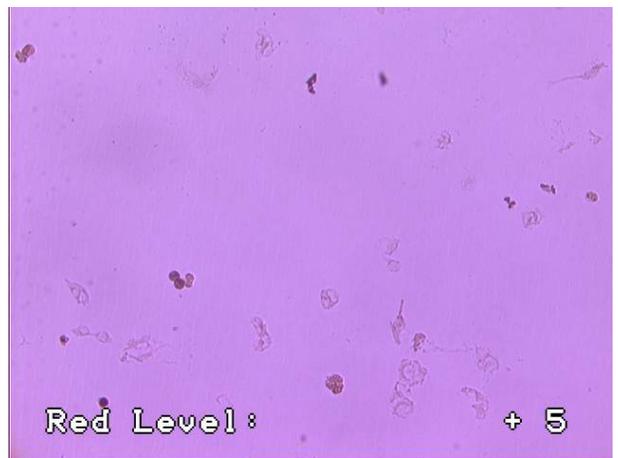
**(B16-BL6) Control**



**(UT+PBS) Low dose**



**(UT+PBS) Med dose**



**(UT+PBS) High dose**

Panel D

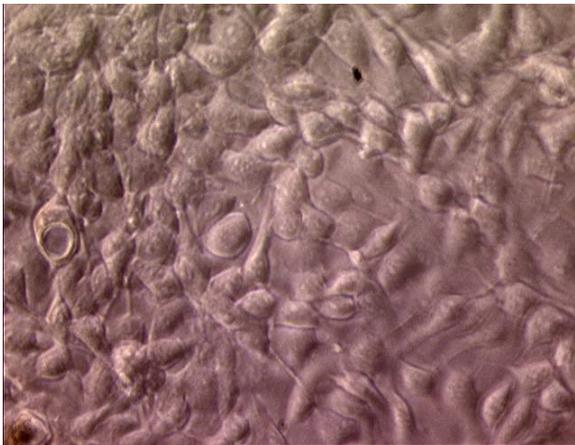
Day 4



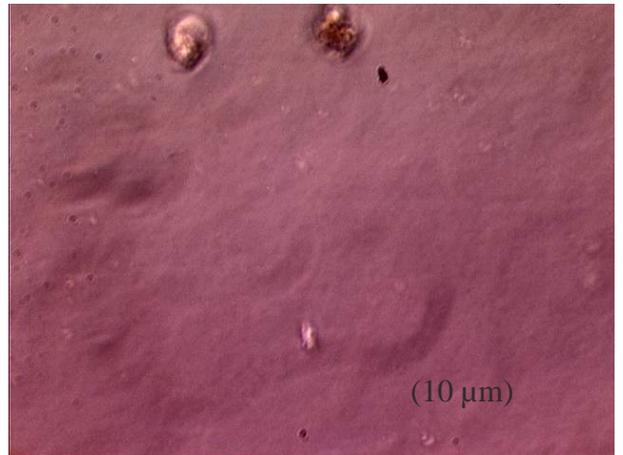
**(B16-BL6) Control**



**(UT+PBS) Low dose**



**(UT+PBS) Med dose**



**(UT+PBS) High dose**

### **4.3 Inhibition of B16-BL6 cell proliferation using Ki-67 protein expression**

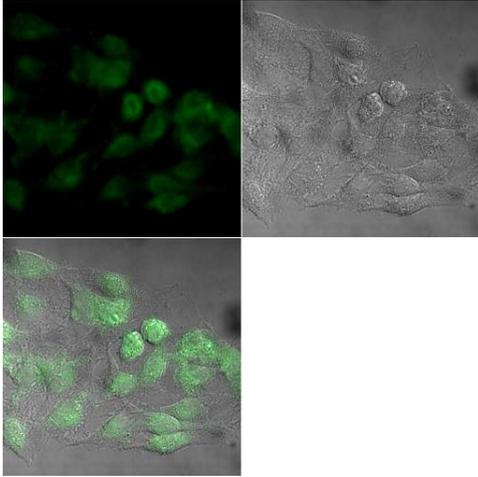
The effects of *U. tomentosa* extracted with ethanol or PBS on the expression of the ki-67 proliferation nuclear in treated B16-BL6 cells were examined. The primary antibody was applied to identify the growth fraction of a treated cell population and detected with a FITC labeled secondary antibody. The fluorescence was visualized using an LSM510 fluorescence microscope (excitation at 488 nm and emission at 520 nm) with constant settings. In brief, there was a significant inhibition in the number of ki-67-positive cells in cultures treated with the *U. tomentosa* extracts. Specifically, the cells stopped expression ki-67 especially at high doses of *U. tomentosa* extracted with ethanol (Figure 5). However, the treatment with *U. tomentosa* extracted with PBS showed a slight inhibition of ki-67 staining when compared to ethanol extracts. These results support our previous findings from the cell growth studies.

**Figure 5: Effect of *U. tomentosa* extracted with ethanol on cell proliferation**

Various concentrations of *U. tomentosa* were added to treat the B16-BL6 cells over one-to-three days. The cells were fixed and stained with an antibody against the cell proliferation nuclear ki-67 and a FITC labeled secondary antibody and images taken. The first panel shows the number of Ki-67-positive cells in cultures treated with the *U. tomentosa* extracted with ethanol represented by green fluorescence; The second panel shows the phase-contrast micrograph of the same field and the third panel shows the combination. Panel A: shows untreated cells. Panel B: shows a treated sample (low dose), Panel C: shows a treated sample (medium dose) and Panel D: shows a treated sample (high dose).

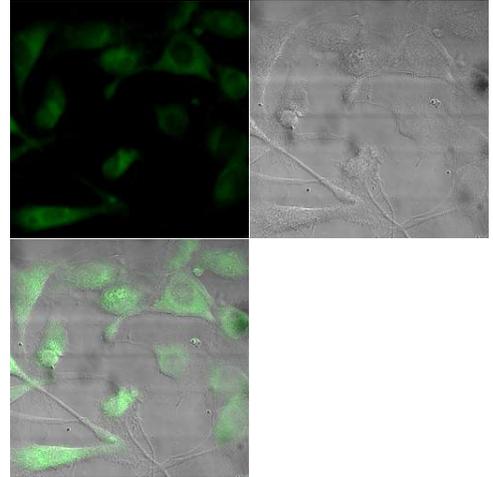
# Day 3

A



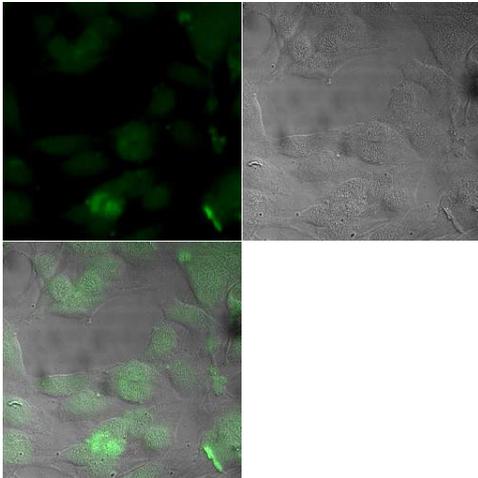
Control

B



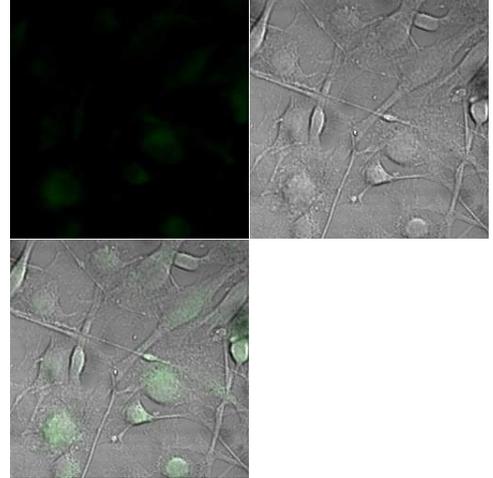
Low dose (UT+ETOH)

C



Med dose (UT+ETOH)

D



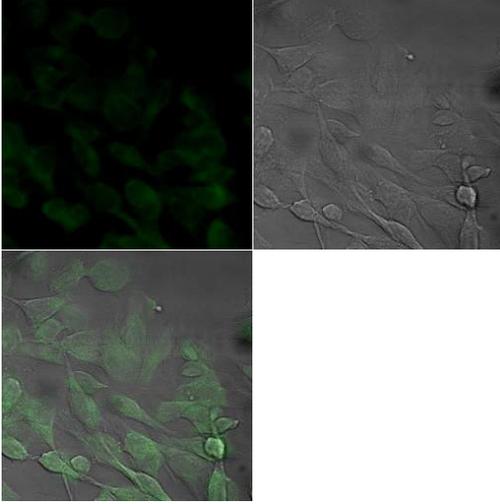
High dose (UT+ETOH)

**Figure 6: Effect of *U. tomentosa* extracted with PBS on cell proliferation**

Various concentrations of *U. tomentosa* were added to treat the B16-BL6 cells over one-to-three days. The cells were fixed and stained with an antibody against the cell proliferation nuclear ki-67 and a FITC labeled secondary antibody and images taken. The first panel shows the number of Ki-67-positive cells in cultures treated with the *U. tomentosa* extracted with PBS represented by green fluorescence; The second panel shows the phase-contrast micrograph of the same field and the third panel shows the combination. Panel A: shows untreated cells. Panel B: shows a treated sample (low dose), Panel C: shows a treated sample (medium dose) and Panel D: shows a treated sample (high dose).

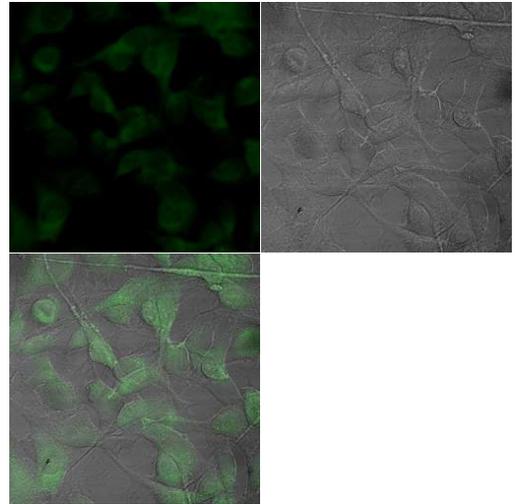
# Day 3

A



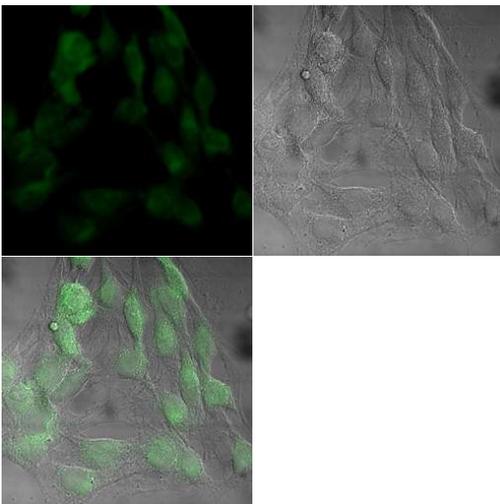
Control

B



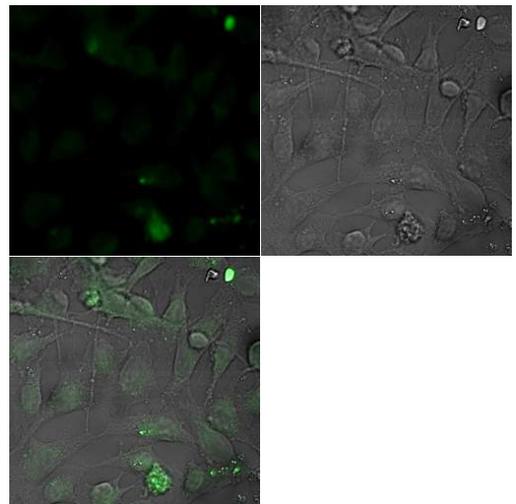
Low dose (UT+PBS)

C



Med dose (UT+PBS)

D



High dose (UT+PBS)

#### **4.4 Detection of apoptotic cells using TUNEL assay**

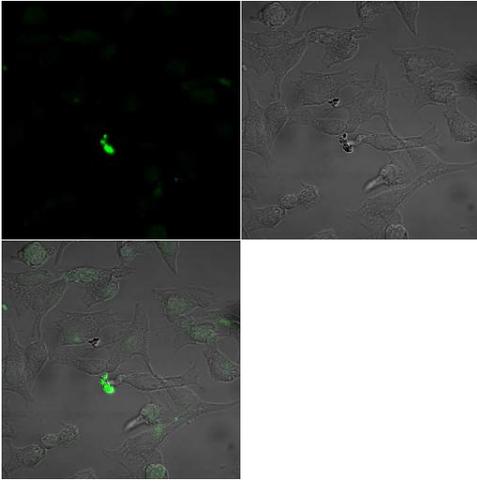
The TUNEL assay is used to detect DNA fragmentation, which is a hallmark of apoptosis, by labeling the terminal 3'-end of nucleic acids through terminal deoxynucleotidyl transferase (TdT). This method was used to confirm the previous results, which showed treatment with *U. tomentosa* extracts induced cell death. As the previous results indicated that *U. tomentosa* killed the B16-BL6 cells, the detection of apoptotic cells was confirmed using TUNEL assay and showed the presence of DNA fragmentation in the B16-BL6 cells following *U. tomentosa* treatment over a period of 72 hours. As well, this method was applied for 24 hours using two different preparations ethanol (Figure 7) and PBS (Figure 8) to evaluate and compare the duration of treatment. In brief, the results showed that the apoptotic cells significantly increased as a result of treatment with the high doses of *U. tomentosa* extracted with ethanol (Figure 9). However, treatment with *U. tomentosa* extracted with PBS had a lower effect (Figure 10). Finally, the treatment of 72 hours showed more positive cells especially when treated at the highest dose.

**Figure 7: Detection of DNA fragmentation in B16-BL6 cells treated with *U. tomentosa* using the TUNEL assay for 24 hours**

The effects of *U. tomentosa* extracted with ethanol on the B16-BL6 cells were evaluated using TUNEL assay to identify individual cells that were undergoing apoptosis. Various doses of *U. tomentosa* were added to treat the B16-BL6 cells for 24 hours. Cells were visualized and photographed using a fluorescence microscope: the green panel shows TUNEL stain, the second panel shows a phase-contrast micrograph of the cells, and the third panel shows the combination. At least five randomly chosen areas in every slide were used. Panel A: shows untreated cells. Panel B: shows a treated sample (low dose), and Panel C: shows a treated sample (high dose).

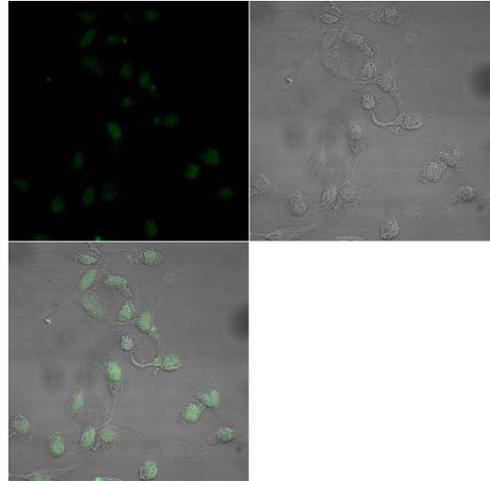
# Day 1

A



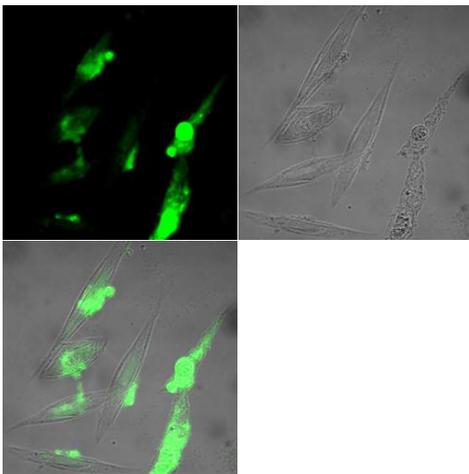
Control

B



Low dose (UT+ETOH)

C



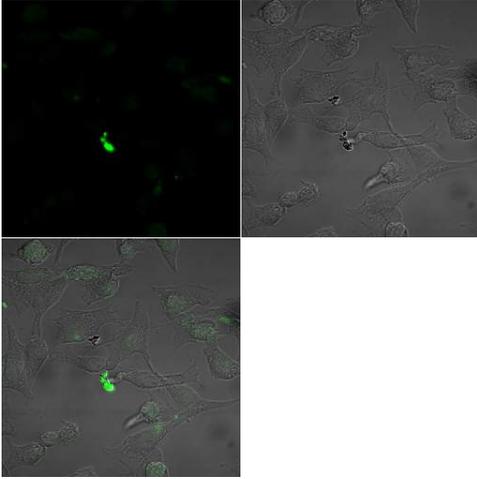
High dose (UT+ETOH)

**Figure 8: Detection of DNA fragmentation in B16-BL6 cells treated with *U. tomentosa* using the TUNEL assay for 24 hours**

The effects of *U. tomentosa* extracted with PBS on the B16-BL6 cells were evaluated using TUNEL assay to identify individual cells that were undergoing apoptosis. Various doses of *U. tomentosa* were added to treat the B16-BL6 cells for 24 hours. Cells were visualized and photographed using a fluorescence microscope: the green panel shows TUNEL stain, the second panel shows a phase-contrast micrograph of the cells, and the third panel shows the combination. At least five randomly chosen areas in every slide were used. Panel A: shows untreated cells. Panel B: shows a treated sample (low dose), and Panel C: shows a treated sample (high dose).

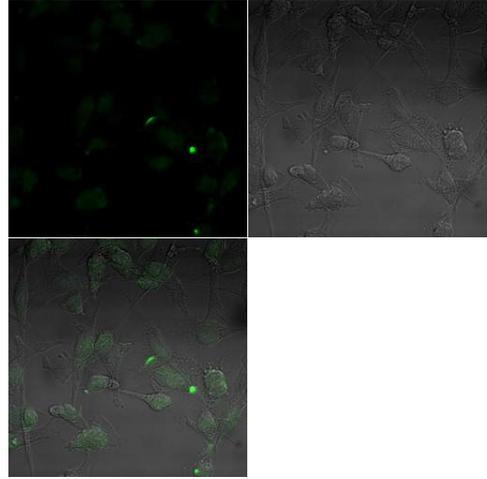
# Day 1

A



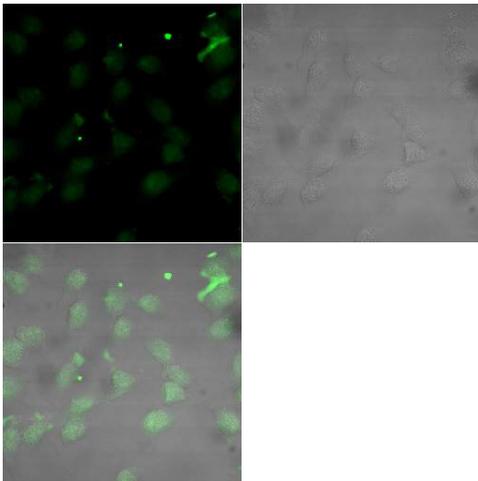
Control

B



Low dose (UT+PBS)

C



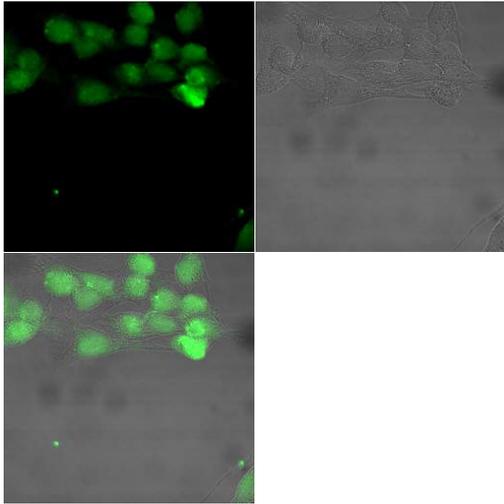
High dose (UT+PBS)

**Figure 9: Detection of DNA fragmentation in B16-BL6 cells treated with *U. tomentosa* using the TUNEL assay for 72 hours**

The effects of *U. tomentosa* extracted with ethanol on the B16-BL6 cells were evaluated using TUNEL assay to identify individual cells that were undergoing apoptosis. Various doses of *U. tomentosa* were added to treat the B16-BL6 cells for 72 hours. Cells were visualized and photographed using a fluorescence microscope: the green panel shows TUNEL stain, the second panel shows a phase-contrast micrograph of the cells, and the third panel shows the combination. At least five randomly chosen areas in every slide were used. Panel A: B16-BL6 cells treated with 6  $\mu\text{g/ml}$  camptothecin (high dose) were used as a positive control. Panel B: shows untreated cells. Panel C: shows a treated sample (low dose), and Panel D: shows a treated sample (high dose).

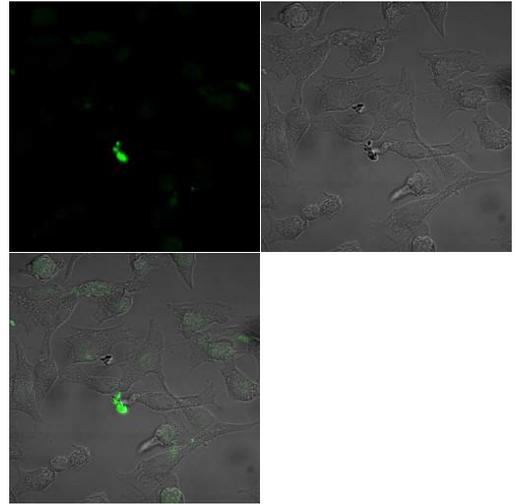
# Day 3

A



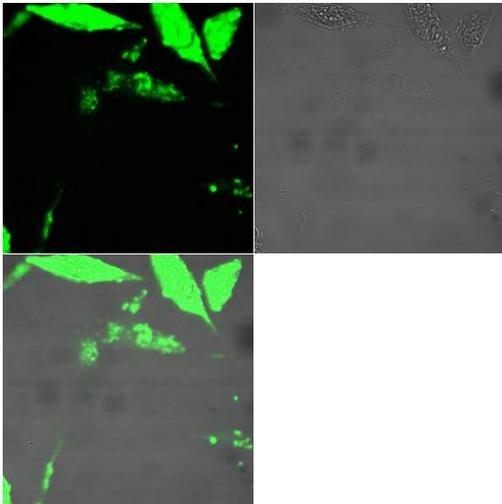
Positive control

B



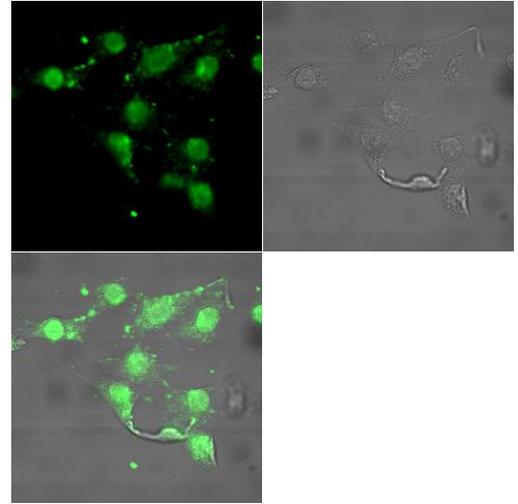
Negative control

C



High dose (UT+ETOH)

D



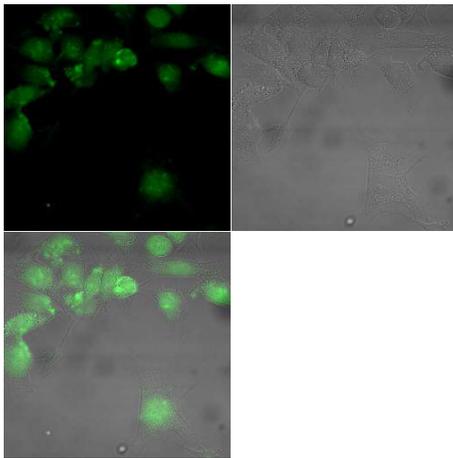
Low dose (UT+ETOH)

**Figure 10: Detection of DNA fragmentation in B16-BL6 cells treated with *U. tomentosa* using the TUNEL assay for 72 hours**

The effects of *U. tomentosa* extracted with PBS on the B16-BL6 cells were evaluated using TUNEL assay to identify individual cells that were undergoing apoptosis. Various doses of *U. tomentosa* were added to treat the B16-BL6 cells for 72 hours. Cells were visualized and photographed using a fluorescence microscope: the green panel shows TUNEL stain, the second panel shows a phase-contrast micrograph of the cells, and the third panel shows the combination. At least five randomly chosen areas in every slide were used. Panel A: B16-BL6 cells treated with 6 µg/ml camptothecin (high dose) were used as a positive control. Panel B: shows untreated cells. Panel C: shows a treated sample (low dose), and Panel D: shows a treated sample (high dose).

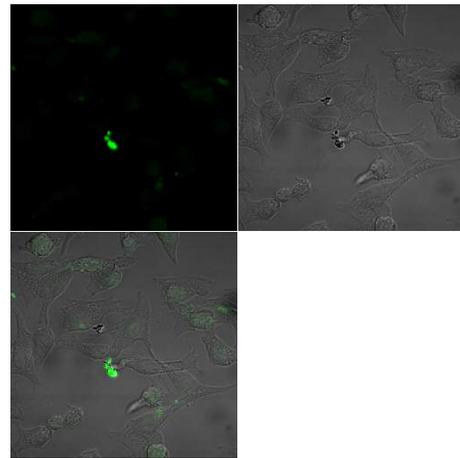
# Day 3

A



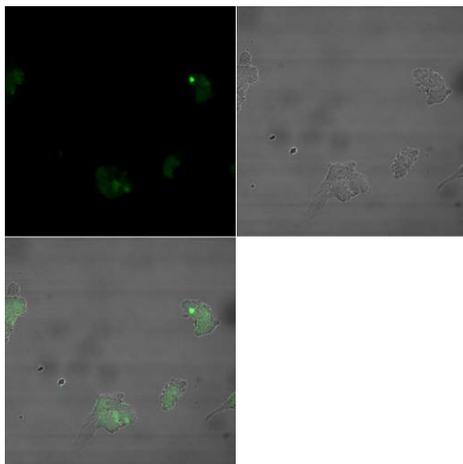
Positive control

B



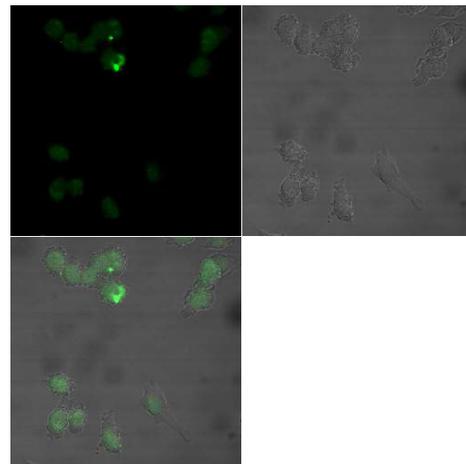
Negative control

C



Low dose (UT+PBS)

D



High dose (UT+PBS)

#### **4.5 Effect of *U. tomentosa* extracts on body weight, tumour weight and tumour size**

Two experiments were performed *in vivo* to determine the effect of *U. tomentosa* on the growth and survival of B16-BL6 mouse melanoma cells. Twenty-five male C57BL/6 mice were used for each experiment and divided into five groups. All individual mice were injected subcutaneously with  $2 \times 10^5$  cells, an aggressive murine melanoma, into the hind limb the right flank. In the first experiment, the intraperitoneal injection groups received the biweekly treatments during 14 days (4 injections). The intratumour injection groups received the treatments only for week 2 (2 injections) (Tables 1-3). However, in the second experiment all treatment groups (IP and IT) received the biweekly treatments during 14 days (4 injections) (Tables 4-6).

For statistical analysis, both experiments were combined using the two-way analyses of variance. The two-way analyses of variance indicated no significant differences between the five groups in terms of body weight (Table 7, Figure 11). In addition, two-way ANOVA showed no significant effects of experiment or group x experiment interaction on body weight.

However, animals treated with *U. tomentosa* had smaller tumours. Two-way ANOVA demonstrated significant effects of group ( $P < 0.001$ ) on tumour weight (Table 8, Figure 12). There was also a significant group x experiment interaction ( $P < 0.05$ ). However, two-way ANOVA showed no significant effects of experiment on tumour weight. Further analysis with Tukey HSD tests showed significant differences between each of the four treated groups and the control ( $P < 0.05$ ) but there were no significant differences between the four treated groups.

Furthermore, two-way ANOVA demonstrated significant effects of group ( $P < 0.01$ ) on tumour size (Table 9, Figure 13). However, two-way ANOVA showed no significant effects of experiment or group x experiment interaction on tumour size. Further analysis with Tukey HSD tests showed no significant differences between the four treated groups but there were significant differences between each of them and the control ( $P < 0.05$ ).

There was a significant relationship between tumour size and tumour weight ( $P < 0.001$ ). As the tumour weight was increased, the tumour size was increasing as well which indicated a direct proportional relationship (Table 10, Figure 14). The tumour size is significantly correlated to the tumour weight ( $r = 0.85$ ,  $P < 0.001$ ).

Furthermore, two-way analyses of variance showed there were no significant main effects or interactions for the two levels of treatment (injection type: IP vs IT and extract type: ETOH vs PBS), which means that all four treatments reduced tumour weight in the same manner.

**Table 1: Shows the values of mean mouse weight (g), standard deviation and standard error of different groups (N=4-5).**

Groups Weight	N	Mean	Standard deviation	Standard error of Mean
Control	5	27.04	.79	.35
ETOH IP	5	26.04	2.09	.93
PBS IP	5	25.96	1.63	.73
PBS IT	5	25.66	2.46	1.10
ETOH IT	4	25.83	2.21	1.11

**Table 2: Shows the values of mean tumour weight (g), standard deviation and standard error of different groups (N=4-5).**

Groups Tumour weight	N	Mean	Standard deviation	Standard error of Mean
Control	5	2.62	.98	.44
ETOH IP	5	1.66	.29	.13
PBS IP	5	.98	.51	.23
PBS IT	5	1.50	1.02	.46
ETOH IT	4	1.68	.62	.31

**Table 3: Shows the values of mean tumour size (cm), standard deviation and standard error of different groups (N=4-5).**

Groups Tumour size	N	Mean	Standard deviation	Standard error of Mean
Control	5	2.98	.77	.34
ETOH IP	5	1.96	.57	.25
PBS IP	5	1.74	.21	.09
PBS IT	5	1.66	.64	.29
ETOH IT	4	2.23	.29	.14

**Table 4: Shows the values of mean mouse weight (g), standard deviation and standard error of different groups (N=3-5).**

Groups Weight	N	Mean	Standard deviation	Standard error of Mean
Control	5	26.94	2.51	1.12
ETOH IP	5	24.48	1.94	.87
PBS IP	4	26.68	2.45	1.22
PBS IT	3	24.43	1.53	.88
ETOH IT	4	22.73	1.11	.55

**Table 5: Shows the values of mean tumour weight (g), standard deviation and standard error of different groups (N=3-5).**

Groups Tumour Weight	N	Mean	Standard deviation	Standard error of Mean
Control	5	3.10	.22	.10
ETOH IP	5	.72	.26	.12
PBS IP	4	1.53	.96	.48
PBS IT	3	2.13	.55	.32
ETOH IT	4	.88	.32	.16

**Table 6: Shows the values of mean tumour size (cm), standard deviation and standard error of different groups (N=3-5).**

Groups Tumour size	N	Mean	Standard deviation	Standard error of Mean
Control	5	2.74	.61	.27
ETOH IP	5	1.54	.57	.26
PBS IP	4	1.68	.71	.35
PBS IT	3	2.17	.32	.19
ETOH IT	4	1.50	.48	.24

**Table 7: Shows the values of mean mouse weight (g), minimum, maximum, standard deviation and standard error of different groups (N=8-10).**

Group	Count	Mean	Minimum	Maximum	Std. Deviation	Standard Error of Mean
Control	10	27.0	22.6	28.8	1.8	.6
ETOH IP	10	25.3	22.9	28.6	2.1	.7
PBS IP	9	26.3	24.0	29.1	1.9	.6
PBS IT	8	25.2	22.7	29.5	2.1	.8
ETOH IT	8	24.3	21.1	28.2	2.3	.8

**Table 8: Shows the values of mean tumour weight (g), minimum, maximum, standard deviation and standard error of different groups (N=8-10).**

Group (tweight)	Count	Mean	Minimum	Maximum	Std. Deviation	Standard Error of Mean
Control	10	2.9	1.5	4.2	.7	.2
ETOH IP	10	1.2***	.3	2.0	.6	.2
PBS IP	9	1.2***	.3	2.5	.7	.2
PBS IT	8	1.7**	.3	2.6	.9	.3
ETOH IT	8	1.3***	.4	2.6	.6	.2

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

**Table 9: Shows the values of mean tumour size (cm), minimum, maximum, standard deviation and standard error of different groups (N=8-10).**

Group (tsize)	Count	Mean	Minimum	Maximum	Std. Deviation	Standard Error of Mean
Control	10	2.9	1.8	4.3	.7	.2
ETOH IP	10	1.8**	.8	2.5	.6	.2
PBS IP	9	1.7**	.8	2.3	.5	.2
PBS IT	8	1.9**	.6	2.4	.6	.2
ETOH IT	8	1.9**	.8	2.6	.5	.2

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

**Table 10: Regression statistics for tumour size (cm) as a function of tumour weight (g) (N=45).**

**Model Summary**

Model	R	R Square	Adjusted R square	Std. Error of the Estimate
1	.852 <sup>a</sup>	.726	.720	.375

a. Predictors: (Constant), tweight

**ANOVA<sup>b</sup>**

Model	Sum of Squares	df	Mean Square	F	Sig.
1 Regression	16.060	1	16.060	114.177	P <.001 <sup>a</sup>
Residual	6.048	43	.141		
Total	22.108	44			

- a. Predictors: (constant). tweight
- b. Dependent Variable: tsize

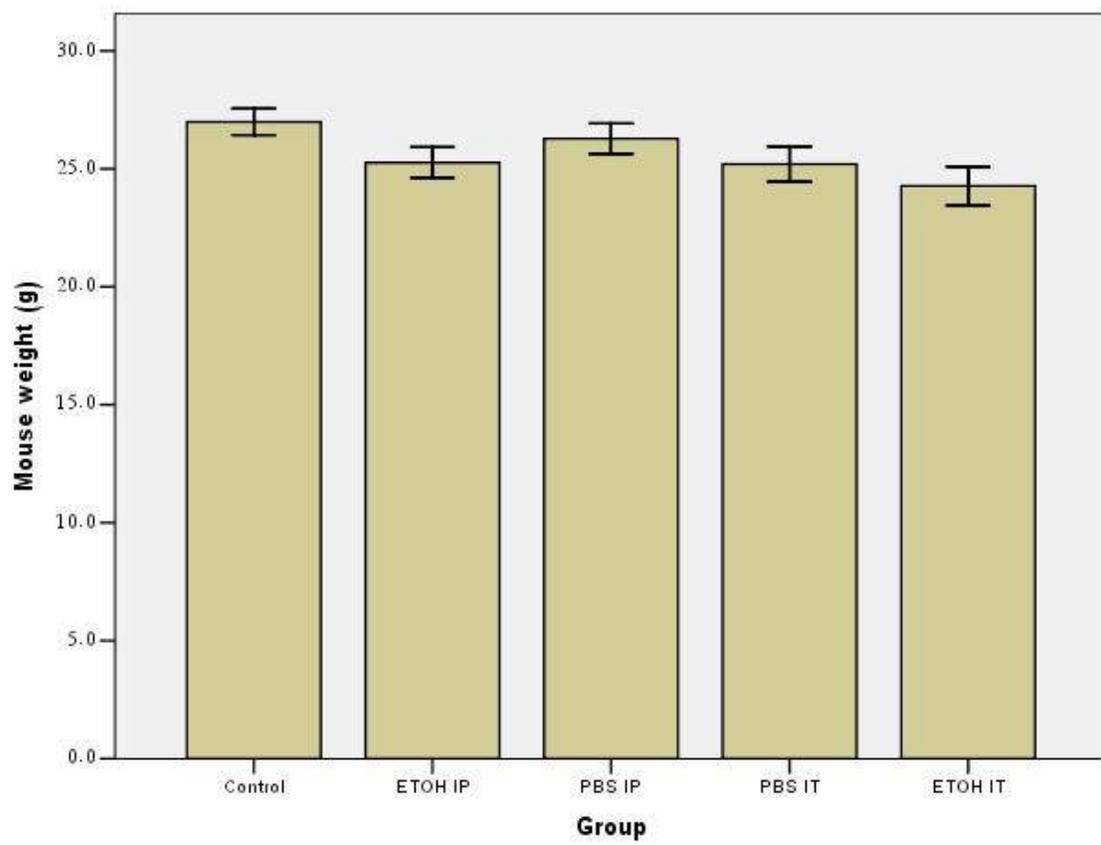
**Coefficients<sup>a</sup>**

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.958	.115		8.366	P <.001
	Tweight	.636	.060	.852	10.685	P <.001

- a. Dependent Variable: tsize

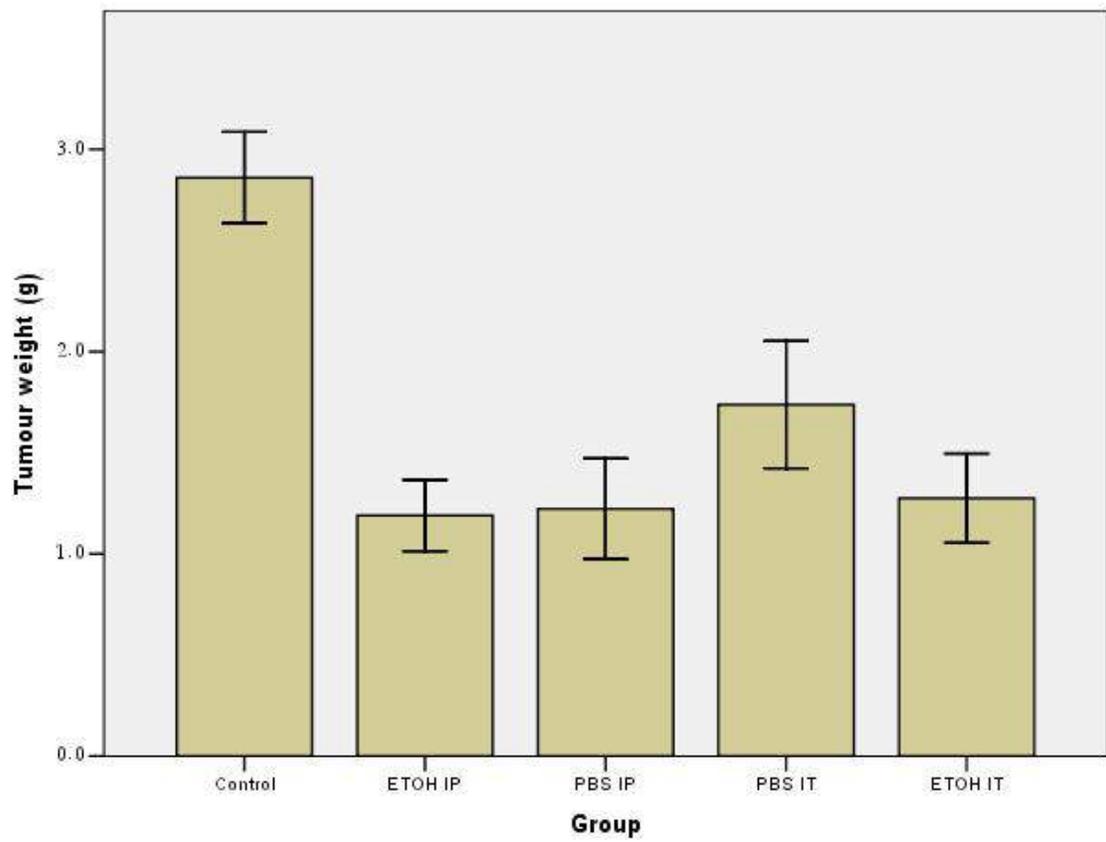
**Figure 11: Effect of *U. tomentosa* extracts on body weight**

At necropsy, the tumours were fully removed from the mice and the tumour diameters; weights and body weights were measured. Animals were sacrificed 13 to 14 days post injection of cancer cells and this was dependent upon the level of distress exhibited by the animal. The figure shows the total body weight in grams for the five groups ( $\pm$ standard error, N= 8-10). No significant differences were found between these groups.



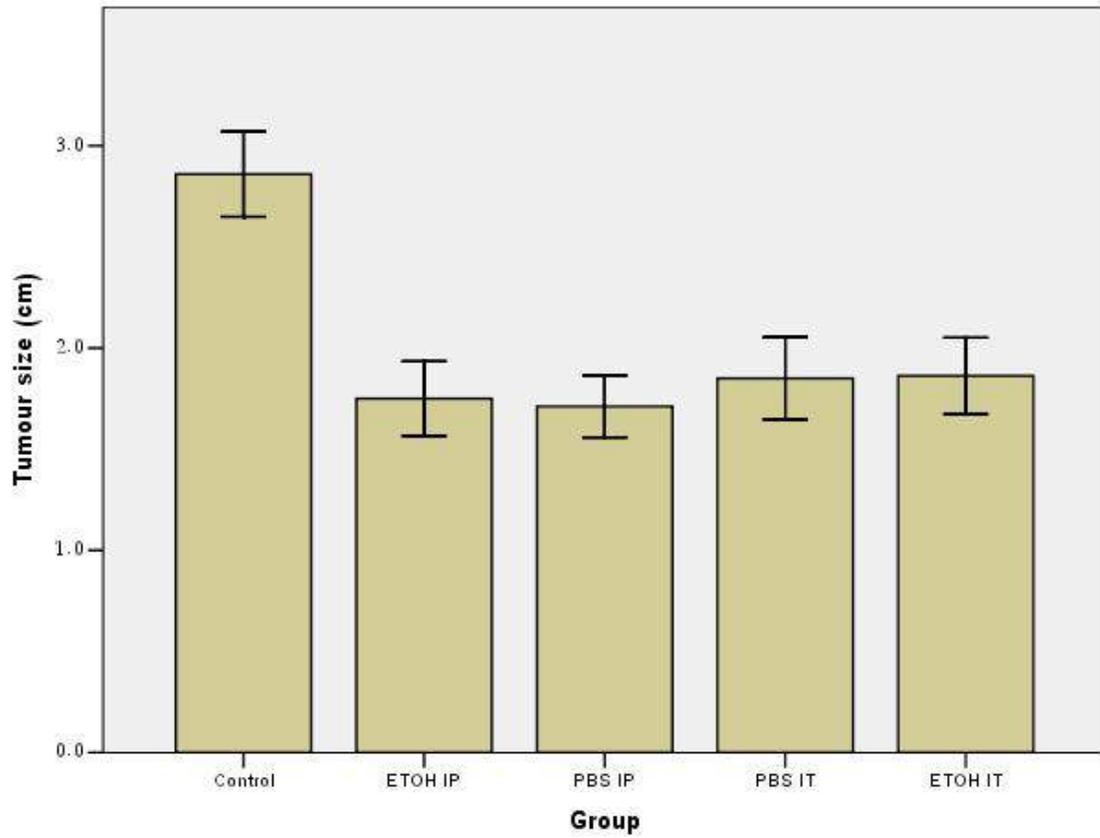
**Figure 12: Effect of *U. tomentosa* extracts on tumour weight**

At necropsy, the tumours were fully removed from the mice and the tumour diameters; weights and body weights were measured. Animals were sacrificed 13 to 14 days post injection of cancer cells and this was dependent upon the level of distress exhibited by the animal. The figure shows tumour weight in grams for the five groups ( $\pm$ standard error, N= 8-10). Significant differences were found in the four treated groups compared to the control.



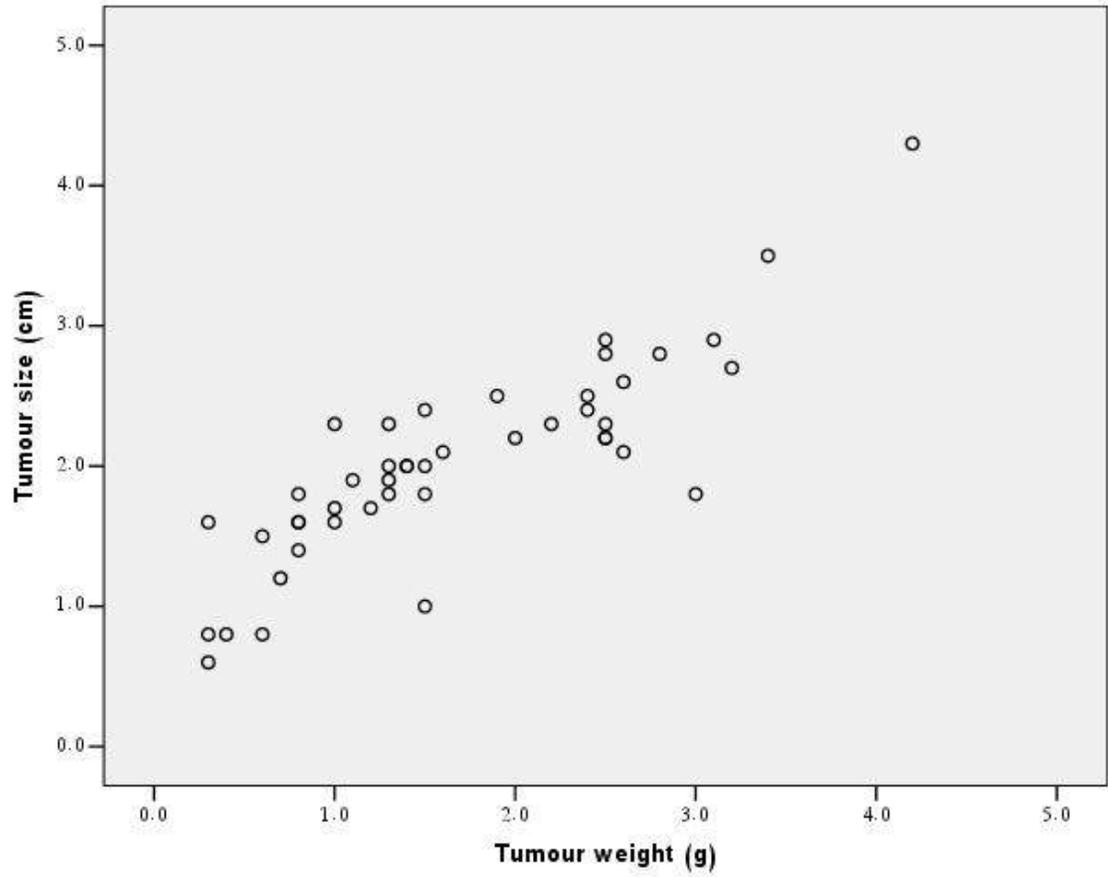
**Figure 13: Effect of *U. tomentosa* extracts on tumour size**

At necropsy, the tumours were fully removed from the mice and the tumour diameters; weights and body weights were measured. Animals were sacrificed 13 to 14 days post injection of cancer cells and this was dependent upon the level of distress exhibited by the animal. The figure shows the tumour size, the diameter in cm for the five groups ( $\pm$ standard error, N= 8-10). Significant differences were found in the four treated groups compared to the control.



**Figure 14: The relationship between tumour size and tumour weight**

As the figure shows there is a direct positive proportional relationship between tumour size and tumour weight, which means increasing the tumour weight led to an increase in tumour diameter. Therefore, this support the idea that both tumour weight and tumour size indicate differences in *U. tomentosa* treated mice.



## 5. Discussion

*Uncaria tomentosa* is a medicinal plant native to the Peruvian Amazon Basin. It has been used for decades in the treatment of various disorders and has been shown to inhibit the growth and proliferation of tumours. Researchers have tried to discover anti-cancer compounds that can kill cancer cells but that also lack toxicity to normal tissues. With this aim in mind, *U. tomentosa* is important because the bark and root of this species have been applied in traditional medicine and in adjuvant therapy to restore health (Dietrich *et al.*, 2014). In addition, its ingredients such as alkaloids, terpenes, quinovic acid glycosides, flavonoids and coumarins have powerful properties. The various effects of *U. tomentosa* preparations can be due to the several of bioactive chemical structures, which can be complementary and/or synergic in their actions (Wirth and Wagner, 1997). The pentacyclic oxindole alkaloids are considered biomarkers in alcoholic extracts of *U. tomentosa*. As well, they are immunostimulants and can inhibit many cancer cells. However, the tetracyclic oxindole alkaloids can cause many effects on the cardiovascular system and are involved in DNA protection. *Uncaria* polyphenols (flavonoids) have powerful antioxidant, anti-inflammatory and anti-tumour activities (Wirth and Wagner, 1997; Pilarski *et al.*, 2006).

The inhibitory effects of some aqueous *U. tomentosa* extracts on tumour cell growth can be mediated by the induction of apoptosis (Sheng *et al.*, 2000; De Martino *et al.*, 2006). Furthermore, *U. tomentosa* extracts are capable of decreasing the productions of proinflammatory cytokines TNF- $\alpha$  and IL-6 in response to immune system stimulate. In addition, they can inhibit nitric oxide production and NF- $\kappa$ B activity (Caballero *et al.*, 2005; Fazio *et al.*, 2008; Dreifuss *et al.*, 2010). Using an *U. tomentosa* hydroalcoholic

extract *in vivo*, Dreifuss *et al.* (2010) revealed that the anti-tumour activity is partially a consequence of the ability of *U. tomentosa* to restore redox and metabolism homeostasis via downregulation of the NF- $\kappa$ B transcription factor and superoxide dismutase (SOD) activities. Several substances appear to be effective in synergy with the oxindole alkaloids or even independently of them, in order to exert some degree of selectivity on its locale of action. Further studies are needed in order to assess the degree of participation of these substances, or others, in the mechanisms by which *U. tomentosa* exerts its anti-neoplastic effects (Dreifuss *et al.*, 2013).

### **5.1 Effect of *U. tomentosa* extracts on cancer cell lines**

The previous studies showed that *U. tomentosa* had positive effects when used as an adjuvant treatment for cancer patients. Sheng *et al.* (2001) determined the effect of C-Med-100 supplement on a human volunteer study. They observed *in vivo* that *U. tomentosa* water extracts (C-Med-100) reduced the adverse effects of chemotherapy by promoting DNA repair, mitogenic response and leukocyte recovery. In their study, twelve healthy adults were randomly selected and distributed to 3 groups with age and gender matched. One group was treated daily with a 250 mg tablet containing an aqueous extract of *U. tomentosa*, C-Med-100, and another group with a 350 mg tablet, for 8 weeks. There was a great decrease in DNA damage and an increase in DNA repair in the supplement groups (250 and 350 mg/day) when compared with controls. The results also showed an increased tendency of lymphocyte proliferation in response to treatment with the mitogen phytohemagglutinin in the treatment groups (Sheng *et al.*, 2001).

*U. tomentosa* water extracts are free of oxindole alkaloids, and have a wide range of biological activity such as DNA repair and anti-inflammatory properties. In another study, skin cultures were treated with or without 5 mg/mL C-Med-100, irradiated with 0-100 mJ/cm<sup>2</sup> UVB, and microscopically analyzed for necrosis, and for the level of pyrimidine dimers using immunofluorescent TT-dimer antibody staining. The results showed that co-incubation with C-Med-100 decreased skin cell death from UV exposure and increased DNA repair (Mammone *et al.*, 2006).

Araújo *et al.* (2012) studied the effect of *U. tomentosa* on reducing the harmful effects of chemotherapy via a randomized clinical trial. Patients that have Invasive Ductal Carcinoma-Stage II, who underwent a treatment regimen known as FAC (Fluorouracil, Doxorubicin, Cyclophosphamide), were distributed into two groups; the *U. tomentosa* treated with chemotherapy plus 300 mg dry *U. tomentosa* extract per day. The group treated only with chemotherapy and used as the control. Blood samples were collected and immunological parameters, antioxidant enzymes, and oxidative stress and blood counts were tested. The results showed that *U. tomentosa* decreased the neutropenia resulting from chemotherapy. As well, *U. tomentosa* was able to repair cellular DNA damage and increase survival rates amongst cancer patients.

Farias *et al.* (2012) examined the effect of *U. tomentosa* in decreasing the side effects of chemotherapy and improving the antioxidant status of colorectal cancer (CRC) patients. This study was performed on 43 patients who were undergoing adjuvant/palliative chemotherapy with 5-Fluorouracil/leucovorin + oxaliplatin (FOLFOX4). They were divided into two groups. The first group was treated with chemotherapy plus 300 mg *U. tomentosa* daily for 12 weeks. The other group received

only the FOLFOX4 chemotherapy and was used as a control. During the treatment, blood samples were collected before each of the 6 cycles of chemotherapy. Furthermore, hemograms, oxidative stress enzymes, antioxidants, immunologic parameters, and adverse processes were tested. They observed that using 300 mg *U. tomentosa* daily during 6 cycles of FOLFOX4 did not cause any changes in the most prevalent adverse events, which no toxic effects were found. Moreover, the comet assay was performed as a method for the detection of DNA damage, demonstrated no differences between groups. This suggests that the effects of *U. tomentosa* supplementation vary depend on cancer type or chemotherapy type.

However, the present results showed that *U. tomentosa* extracts inhibited the proliferation of B16-BL6 cells. In addition, *U. tomentosa* caused DNA fragmentation and increased the percentage of programmed cell death (apoptosis), which means its effects can be different on cell proliferation depending on the cell type involved. Therefore, different *U. tomentosa* extracts were examined *in vitro* and *in vivo* in order to evaluate their activity, with promising results.

## **5.2 Anti- proliferation activity of *U. tomentosa* extracts**

Here the study showed that ethanolic extracts had significantly higher inhibitory effect compared to PBS extracts in a concentration-dependent manner. Similarly, Bors *et al.* (2011) studied the effects of the ethanolic and aqueous extracts of *U. tomentosa* on human red blood cells as well as evaluating of the prophylactic effects of those extracts on hemolysis induction, hemoglobin oxidation, and changes in the level of reactive oxygen species (ROS), lipid peroxidation and catechol. The extracts of *U. tomentosa*

examined showed that the extent of hemoglobin oxidation and lipid peroxidation were reduced compared to ROS and hemolysis, provoked by treatment with 2,4-DCP. It has been suggested that ethanol extracts have more ability to inhibit oxidative processes in human red blood cells.

In the current study, MTT assay was used to measure the cell viability and proliferation rate *in vitro*. The MTT assay depends on the conversion of MTT into formazan crystals in living cells, which evaluates mitochondrial activity. For most of the cell populations the total mitochondrial activity is linked to the number of viable cells (van Meerloo *et al.*, 2011). MTT assays were conducted on multiple days to measure proliferation. According to the present experiment, the B16-BL6 cells malignant showed a significant inhibition in the cell growth in response to *U. tomentosa* treatment over a period of four days. Furthermore, *U. tomentosa* extracted with ethanol completely inhibited B16-BL6 cells especially at 200 µg/ml, which is the highest dose. As the doses were increased, the reduction of the cells was increased. For PBS extracts, the treatment with 200 µg/ml, which is the highest dose, killed most of the B16-BL6 cells. However, the treatment with lower doses did not cause any significant reduction in cell growth unlike the ethanol extraction. Thus, the ethanol extract is more effective as an anti-cancer agent compared to the PBS extract.

Chen *et al.* (2011) studied the anti-tumor effect of Cryptotanshinone, which is the major active component of *Salvia miltiorrhiza*, on two melanoma cell lines with low/high-metastatic capacity (B16/B16-BL6). Cells were treated with various concentrations of Cryptotanshinone (0, 1, 10 and 25 µM) and for different times (4, 8, 12, 16, 20 and 24 h) *in vitro* and MTT was used to determine the cell growth. Similar results

were obtained in B16-BL6 cells, suggesting that the treatment with Cryptotanshinone had greatly inhibited cell proliferation (Chen *et al.*, 2011). These findings support our hypothesis that natural products such as *U. tomentosa* inhibited the B16-BL6 cells in a period of four days.

### **5.3 Anti- proliferation activity of *U. tomentosa* extract (ki-67 protein expression)**

The expression of the Ki-67 protein is correlated to the regulation of the cell cycle and cell proliferation. It is expressed in proliferating cells during all active stages of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)). The expression of the ki-67 protein is considered one of the most promising biomarker for cell proliferation. This experiment was performed *in vitro* using the anti ki-67 primary antibody to assess the ability of *U. tomentosa* to inhibit B16-BL6 cell proliferation. As the results showed, *U. tomentosa* extracted with ethanol was greatly effective in inhibiting proliferation of B16-BL6 cells especially at high doses. At the low doses, the experiment showed that there was a slight decrease in the level of proliferation. For PBS extracts, the treatment with 200 µg/ml, which is the highest dose, reduced the cell proliferation. However, the treatment with low doses did not show any significant reduction *in vitro*.

Similar results using different natural products were found. Khan *et al.* (2014) showed that the oral administration of naturally occurring chitosan-based nanoformulated green tea polyphenol EGCG significantly inhibits prostate cancer cell growth in a xenograft model. Tumor from athymic nude mice was stained using polyclonal primary

antibody. The study showed that there was 4.1- to 5.9-fold less expression of Ki-67 and 5.5- to 6.8-fold less expression of PCNA in mice tissues that treated with Chit-nanoEGCG than control group. The apparent increase in ki-67 and PCNA immunostaining-positive cells was taken as clear evidence of carcinogenicity. Cell proliferation activity in tissues of non-lesion areas was increased. This study showed that the tumors in mice given Chit-nanoEGCG had lower ki-67 and PCNA-positive index when compared with EGCG-treated and control groups (Khan *et al.*, 2014).

#### **5.4 DNA fragmentation by *U. tomentosa***

The TUNEL assay is considered one of the major methods for detecting apoptotic programmed cell death. Specifically, it is used to detect apoptotic cells that undergo extensive DNA degradation in the late phases of apoptosis by labeling the terminal end of nucleic acids. The experiment showed that the apoptotic cells greatly increased as a result of the treatment with high doses of *U. tomentosa* extracted with ethanol *in vitro*. In addition, apoptotic cells were increased in B16-BL6 cells treated with *U. tomentosa* extracted with PBS at the highest dose but still showed a lower effect. At the low doses, a low level of apoptotic cells was found in B16-BL6 cells treated with *U. tomentosa* extracted with ethanol. However, no significant apoptotic cells were found at the low doses for PBS extracts.

Different results were obtained for B16 and B16-BL6 cells treated with Cryptotanshinone at different concentrations (0, 1, 10 and 25  $\mu\text{M}$ ) over a period of 24 hours. Then, cells were tested for apoptosis via TUNEL assay in order to evaluate the effect of Cryptotanshinone on B16 and B16-BL6 melanoma cell apoptosis. Similar

results were found for these two cell lines. The percentage of apoptotic cells was increased under the influence of Cryptotanshinone, but the effect was small. At 25  $\mu$ M, Cryptotanshinone caused apoptosis in about 4% of cells (Chen *et al.*, 2011).

### **5.5 *U. tomentosa* extract had significant effects on tumour weight and size**

Tumours were induced by the subcutaneous (s.c.) injection of  $2 \times 10^5$  B16/BL6 cells in 100  $\mu$ l PBS into the hind limb the right flank of C57BL/6 mice *in vivo*. Two experiments were performed with twenty-five mice used for each experiment and divided into five groups. The effect of *U. tomentosa* on tumour diameters and weights were measured. In addition, the same method was applied for both experiments. However, the number of injections was increased for IT groups in the second experiment. In terms of mouse weight, no significant differences were found between individual mice. There was a significant inhibition in tumour weight but the tumor size is not significantly affected. Furthermore, there are no significant main effects or interactions between the levels of treatment, including injection type and both extracts. This means that all treatment groups reduced tumor weight in the same manner.

Similarly, Fazio *et al.* (2008) studied the effect of a hydroethanolic extract of *U. tomentosa* on many cancer cells *in vitro* including B16/BL6 (murine melanoma), K1735 (amelanotic murine melanoma), HT29 (human colon carcinoma), A549 (human lung carcinoma), WEHI 164 (mouse fibrosarcoma), and LEC (mouse liver endothelial cell line) and on B16-BL6 cells *in vivo*. In the laboratory, the results showed that *U. tomentosa* inhibited the cell growth at high doses of 100 and 300  $\mu$ g/ml. These cell lines

(HT-29, K1735 and WEHI) as well as the three primary cell preparations (huPBMC, muSplen and muPM) were more response to the extract than the B16/BL6, A549 and LEC cells. In addition, the ethanolic extract of *U. tomentosa* reduced inflammation and B16-BL6 melanoma growth in female C57BL/6 mice injected intraperitoneal with *U. tomentosa*. Furthermore, *in vitro* *U. tomentosa* inhibited tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-6 (IL-6) and nitric oxide (NO) production. As well, NF- $\kappa$ B activity was inhibited.

Caballero *et al.* (2005) proved that an aqueous extract of *U. tomentosa* was not cytotoxic for many cells at concentrations up to 3 mg/ml. However, Fazio *et al.* (2008) showed that the hydroethanolic extract of *U. tomentosa* had more cytotoxic for some of the tumor cell lines and the primary cells, but not for the B16-BL6 melanoma cells. Allen-Hall *et al.* (2010) demonstrated that *U. tomentosa* is able to elicit a response through an NF- $\kappa$ B-dependent mechanism. More studies are required to describe the mechanism by which *U. tomentosa* can affect this pathway could offer a means to improve anti-TNF- $\alpha$  therapies.

## 6. Conclusion

The present results showed that *U. tomentosa* significantly inhibited the growth of B16-BL6 cells and induce morphological changes particularly with ethanol extracts *in vitro*. Extracts from plant was also able to increase the percentage of apoptotic cells in a concentration-dependent manner. In addition, there was a significant inhibition of proliferation as detected by a decrease in the percentage of tumor cells that were positive for Ki67 expression. The *in vivo* results showed that *U. tomentosa* caused a significant reduction in tumour weight but the tumor size is not greatly affected when compared to the controls. Interestingly, there were no significant differences in mouse weight for all treated groups. Overall, *U. tomentosa* extracts can provide an effective treatment for melanoma cancer that could be suitable for human patients. Therefore, it appears appropriate to perform new experiments, testing various *U. tomentosa* extracts against different neoplastic scenarios, both *in vitro* and *in vivo*, and thus achieve a better understanding of the overall therapeutic potential of this plant.

## References

Ames, B. N. (1998) Micronutrients prevent cancer and delay aging, *Toxicology Letters*, 102 103:5-18.

Allen-Hall, L., Cano, P., Arnason, J. T., Rojas, R., Lock, O., and Lafrenie, R. M. (2007) Treatment of THP1 cells with *Uncaria tomentosa* extracts differentially regulates the expression of IL1 $\beta$  and TNF- $\alpha$ , *Journal of Ethnopharmacology*, 109(2): 312-7.

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

Araújo, M., Farias, I., Gutierrez, J., Dalmora, S., Flores, N., Farias, J., Cruz, I., Chiesa, J., Morsch, V., and Schetinger, M. (2012) *Uncaria tomentosa*—Adjuvant Treatment for Breast Cancer: Clinical Trial, *Evidence-Based Complementary and Alternative Medicine*, 676984.

Avato, P., Tursil, E., Vitali, C., Miccolis, V., and Caddido, V. (2000) Allyl sulfide constituents of garlic Volatile oil as antimicrobial agents, *Phytomedicines*, 7: 239 – 243.

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

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994) *Molecular Biology of the Cell*, Garland Publishing, 22(3): 164.

Aguilar, J. L., Rojas, P., Marcelo, A., Plaza, A., Bauer, R., Reininger, E., Klaas, C. A., and Merfort I. (2002) Anti-inflammatory activity of two different extracts of *Uncaria tomentosa* (Rubiaceae), *Journal of Ethnopharmacology*, 81(2): 271-6.

Aquino, R., Simone, F. D., Pizza, C., Conti, C., and Stein, M. L. (1989) Plant metabolites. Structure and in vitro antiviral activity of quinovic acid glycosides from *Uncaria tomentosa* and *Guettarda platypoda*, *Journal of Natural Products*, 52(4): 679–685.

Aquino, R., Simone, F. D., Vincieri, F. F., Pizza, C., and Gaćs-Baitz, E. (1990) New polyhydroxylated triterpenes from *Uncaria tomentosa*, *Journal of Natural Products*, 53(3): 559–564.

Aquino, R., Feo, V. D., Simone, F. D., Pizza, C., and Cirino, G. (1991) Plant metabolites. New compounds and anti-inflammatory activity of *Uncaria tomentosa*, *Journal of Natural Products*, 54(2): 453–9.

Akesson, C., Lindgren, H., Pero, R. W., Leanderson, T., Ivars, F., (2003) An extract of *Uncaria tomentosa* inhibiting cell division and NF-kappa B activity without inducing cell death, *International Immunopharmacology*, 3(13-14): 1889-1900.

Åkesson, C., Lindgren, H., Pero, R. W., Leanderson, T., and Ivars, F. (2005). Quinic acid is a biologically active component of the *Uncaria tomentosa* extract C-Med 100®, *International Immunopharmacology*, 5(1): 219-29.

American Cancer Society Melanoma Skin Cancer, American Cancer Society; 2013.

Bors, M., Bukowska, B., Pilarski, R., Gulewicz, K., Oszmiański, J., Michałowicz, J., and Koter-Michalak, M. (2011) Protective activity of the *Uncaria tomentosa* extracts on human erythrocytes in oxidative stress induced by 2,4-dichlorophenol (2,4DCP) and catechol, *Food and Chemical Toxicology*, 49(9): 2202-11.

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

Balch, C. M., Urist, M. M., Karakousis, C. P., Smith, T. J., Temple, W. J., Drzewiecki, K., Jewell, W. R., Bartolucci, A. A., et al (1993) Efficacy of 2-cm surgical margins for intermediate-thickness melanomas (1 to 4 mm). Results of a multi-Institutional randomized surgical trial, *Annals of Surgery*, 218(3): 262–269.

Bishop, J. N., Bataille, V., Gavin, A., Lens, M., Marsden, J., Mathews, T., and Wheelhouse, C. (2007) The prevention, diagnosis, referral and management of melanoma of the skin: concise guidelines, *Clinical Medicine*, 7(3): 283-90.

Buettner, P. G., & Raasch, B. A. (1998) Incidence rates of skin cancer in Townsville, Australia, *International Journal of Cancer*, 78(5): 587–593.

Bliss, J. M., Ford, D., Swerdlow, A. J., Armstrong, B. K., Cristofolini, M., Elwood, J. M., Green, A., Holly, E. A., Mack, T., MacKie, R. M., et al. (1995) Risk of cutaneous melanoma associated with pigmentation characteristics and freckling: systematic overview of 10 case-control studies. The International Melanoma Analysis Group (IMAGE), *International Journal of Cancer*, 62(4): 367-76.

Balunas, M. J., and Kinghorn, A. D. (2005) Drug discovery from medicinal plants. *Life Sciences*, 78(5): 431-41.

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

Cragg, G. M., Newman, D. J., and Snader, K. M. (1997) Natural products in drug discovery and development, *Journal of Natural Products*, 60(1): 52-60.

Cheng, A., Jian, C., Huang, Y., Lai, C., Hsu, P., and Pan, M. (2007) Induction of apoptosis by *Uncaria tomentosa* through reactive oxygen species production, cytochrome c release, and caspases activation in human leukemia cells, *Food and Chemical Toxicology*, 45(11): 2206-18.

Chen, L., Zheng, S., Sun, Z., Wang, A., Huang, C., PUNCHARD, N. A., Huang, S. L., Gao, X., Lu, Y. (2011) Cryptotanshinone has diverse effects on cell cycle events in melanoma cell lines with different metastatic capacity, *Cancer Chemotherapy and Pharmacology*, 68(1): 17-27.

Creagan, E. T., Ahmann, D. L., Frytak S., Long, H. J., Chang, M. N., Itri, L. M. (1986) Phase II trials of recombinant leukocyte A interferon in disseminated malignant melanoma: Results in 96 patients, *Cancer Treatment Reviews*, 70(5): 619-24.

Caballero, M., Arsenak, M., Abad, M. J., Cesari, I. M., Taylor, P. G. (2005) Effect of 3 plant extracts on B16-BL6 melanoma cell growth and metastasis in C57BL/6 mice, *Acta Científica Venezolana*, 55: 21-27.

Cooper, G. M. (2000) *The Cell: A Molecular Approach*. 2nd edition, Boston University, Sinauer Associate, Sunderland, MA,

Corner, J., Harewood, J., Maslin-Prothero, S., and Lewith, G. (2006) A Study of the Use of Complementary and Alternative Therapies among People Undergoing Cancer Treatment: A Quantitative and Qualitative Study. Department of Health NHS Rand D Programme. Department of Health: London

Canadian Cancer Society's Advisory Committee on Cancer Statistics. (2014) Canadian Cancer Statistics 2014. Toronto, ON: Canadian Cancer Society.

Canadian Cancer Society's Steering Committee on Cancer Statistics. Canadian cancer statistics 2011. Toronto, ON: Canadian Cancer Society, 2011.

Dreifuss, A. A., Bastos-Pereira, A. L., Ávila, T. V., Soley, B. D., Rivero, A. J., Aguilar, J. L., and Acco, A. (2010) Antitumoral and antioxidant effects of a hydroalcoholic extract of cat's claw (*Uncaria tomentosa*) (Willd. Ex Roem. & Schult) in an in vivo carcinosarcoma model, *Journal of Ethnopharmacology*, 130(1): 127-33.

Dreifuss, A. A., Bastos-Pereira, A. L., Fabossi, I. A., Lívero, F. A., Stolf, A. M., et al. (2013) *Uncaria tomentosa* exerts extensive anti-neoplastic effects against the Walker-256 tumour by modulating oxidative stress and not by alkaloid activity, *PLoS One*, 8(2): e54618.

D'Epiro, N. W. (1999) An historical, regulatory, and medical use perspective on nine common herbs. In: Micozzi MS, Bacchus AN, eds. *The Physician's Guide to Alternative Medicine*. Atlanta, Ga: American Health Consultants, 21-30.

De Martino, L., Silva-Martinot, J. L., Franceschelli, S., Leone, A., Pizza, C., De Feo, V. (2006) Proapoptotic effect of *Uncaria tomentosa* extracts, *Journal of Ethnopharmacology*, 107(1): 91-4.

DePihno, R. A. (2000) The age of cancer, *Nature*, 408(6809): 248-54.

Dietrich, F., Kaiser, S., Rockenbach, L., Figueiró, F., Bergamin, L. S., da Cunha, F. M., Morrone, F. B., Ortega, G. G., Battastini, A. M. (2012) Quinovic acid glycosides purified fraction from *Uncaria tomentosa* induces cell death by apoptosis in the T24 human bladder cancer cell line, *Food and Chemical Toxicology*, 67:222-9.

Diepgen, T. L., and Mahler, V. (2002) The epidemiology of skin cancer, *British Journal of Dermatology*, 146 Suppl 61:1-6.

- de Vries, E., Schouten, L. J., Visser, O., Eggermont, A. M., and Coebergh, J.W. (2003) Rising trends in the incidence of and mortality from cutaneous melanoma in the Netherlands: a Northwest to Southeast gradient? *European Journal of Cancer* 39(10): 1439-46.
- de Matta, S.M., Monache, F. D., Ferrari, F., and Marini-Bettolo, G. B. (1976) Alkaloids and procyanidins of an *Uncaria* sp. from Peru. *Farmaco Sci*, 31(7), 527–535.
- Epstein, J. B., Gorsky, M., Wong, F. L., and Millner, A. (1998) Topical bleomycin for the treatment of dysplastic oral leukoplakia, *Cancer*, 83(4): 629-34.
- Eggermont, A. M., Suci, S., Santinami, M., Testori, A., Kruit, W. H., Marsden, J., Punt, C. J., Salès, F., Gore, M., Mackie, R., Kusic, Z., Dummer, R., Hauschild, A., Musat, E., Spatz, A., and Keilholz, U. (2008) Adjuvant therapy with pegylated interferon alfa-2b versus observation alone in resected stage III melanoma: final results of EORTC 18991, a randomised phase III trial, *Lancet*, 372(9633): 117-26.
- Farias, I., Araújo, M. D., Zimmermann, E. S., Dalmora, S. L., Benedetti, A. L., Alvarez Silva, M., Schetinger, M. R. (2011) *Uncaria tomentosa* stimulates the proliferation of myeloid progenitor cells, *Lancet*, 137(1): 856-63.
- Folkman, J., and Klagsbrun, M. (1987) Angiogenic Factors, *Science*, 235(4787): 442-7.
- Farias, I., Araújo, M., Farias, J., Rossato, L., Elsenbach, L., Dalmora, S., Flores, N., Durigon, M., Cruz, I., Morsch, V., and Schetinger, M. (2012) *Uncaria tomentosa* for Reducing Side Effects Caused by Chemotherapy in CRC Patients: Clinical Trial, *Evidence-Based Complementary and Alternative Medicine*, 892182.
- Fazio, A., Ballen, D., Cesari, IM., Abad, M., Arsenak, M., and Taylor, P. (2008) An ethanolic extract of *Uncaria tomentosa* reduces inflammation and B16-BL6 melanoma growth in C57BL/6 mice, *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, 7 (5): 217 – 224.
- Forman, S. B., Ferringer, T. C., Peckham, S. J., Dalton, S. R., Sasaki, G. T., Libow, L. F., and Elston, D. M. (2008) Is superficial spreading melanoma still the most common form of malignant melanoma? *Journal of The American Academy of Dermatology*, 58(6): 1013-20.

- Gurrola-Díaz, C. M., García-López, P. M., Gulewicz, K., Pilarski, R., and Dihlmann, S. (2011) Inhibitory mechanisms of two *Uncaria tomentosa* extracts affecting the Wnt-signaling pathway, *Phytomedicine*, 18(8-9): 683-90.
- Gonzales, G. F., and Valerio, L. G. (2006) Medicinal Plants from Peru: A Review of Plants as Potential Agents Against Cancer, *Anti-cancer Agents in Medicinal Chemistry*, 6(5): 429-44.
- Ghayur, M. N. (2010) Science Across Borders: 5th Annual Natural Health Product Research Conference—March 26–29, 2008, Toronto, Canada, *Evidence-based Complementary and Alternative Medicine*, 7(3): 391–395.
- Gordaliza, M. (2007) Natural products as leads to anticancer drugs, *Clinical and Translational Oncology*, 9(12): 767-76.
- García Giménez, D., García Prado, E., Sáenz Rodríguez, T., Fernández Arche, A., et al. (2010) Cytotoxic effect of the pentacyclic oxindole alkaloid mitraphylline isolated from *Uncaria tomentosa* bark on human Ewing's sarcoma and breast cancer cell lines, *Planta Medica*, 76(2): 133-6.
- Gallagher, R. P., Elwood, J. M., Rootman, J., Spinelli, J. J., Hill, G. B., Threlfall, W. J., and Birdsell, J. M. (1985). Risk factors for ocular melanoma: Western Canada Melanoma Study, *Journal of the National Cancer Institute*, 74(4): 775-8.
- Glocker-Reis, L. A., Miller, B. A., Hankey, B. F., et al. (1994) SEER Cancer Statistics Review, 1973–1991. Bethesda, Md.: National Cancer Institute, 1994, National Institutes of Health publication no, 94: 2789.
- Griffin, A. M., Butow, P. N., Coates, A. S., Childs, A. M., Ellis, P. M., Dunn, S. M., Tattersall, M. H. (1996) On the receiving end V: Patient perceptions of the side effects of cancer chemotherapy in 1993, *Annals of Oncology*, 7(2): 1 89-95.
- Garbe, C., Peris, K., Hauschild, A., Saiag, P., Middleton, M., Spatz, A., Grob, J. J., Malvehy, J., Newton-Bishop, J., Stratigos, A., Pehamberger, H., and Eggermont, A. Diagnosis and treatment of melanoma: European consensus-based interdisciplinary guideline, *European Journal of Cancer*, 46(2): 270-83.
- Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of Cancer: The Next Generation, *Cell*, 144(5): 646-74.

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

Hynes, N. E., and Lane, H. A. (2005) ERBB receptors and cancer: the complexity of targeted inhibitors, Nat Rev Cancer, 5(5): 341-54.

Hasegawa, H., Suzuki, R., Nagaoka, T., Tezuka, Y., Kadota, S., and Saiki, I. (2002) Prevention of growth and metastasis of murine melanoma through enhanced natural killer cytotoxicity by fatty acid conjugate of protopanaxatriol, Biological & Pharmaceutical Bulletin, 25: 861–866.

Hanahan, D., and Weinberg, R.A. (2000) The hallmarks of cancer, Cell, 100, 57–70.

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

Ives, N. J., Stowe, R. L., Lorigan, P., & Wheatley, K. (2007) Chemotherapy Compared With Biochemotherapy for the Treatment of Metastatic Melanoma: A Meta-Analysis of 18 Trials Involving 2,621 Patients, Journal of Clinical Oncology, 25(34): 5426-34.

Johnson, K. O. (2001) The roles and functions of cutaneous mechanoreceptors, Current Opinion in Neurobiology, 11(4): 455-461.

Jones, R. R. (1987) Ozone depletion and cancer risk, Lancet, 2(8556): 443-446.

Khan, N., Bharali, D. J., Adhami, V. M., Siddiqui, I. A., Cui, H., Shabana, S. M., Mousa, S. A., and Mukhtar, H. (2014) Oral administration of naturally occurring chitosan-based nanoformulated green tea polyphenol EGCG effectively inhibits prostate cancer cell growth in a xenograft model, Carcinogenesis, 35(2): 415-23.

Krishnaiah, D., Sarbatly, R., and Nithyanandam, R. (2011) A review of the antioxidant potential of medicinal plant species, Food and Bioproducts Processing, 89: 217–233.

Keplinger, K., Laus, G., Wurm, M., Dierich, M. P., and Teppner, H. (1999) *Uncaria tomentosa* (Willd.) DC. – Ethnomedicinal use and new pharmacological, toxicological and botanical results, Journal of Ethnopharmacology, 64(1): 23-34.

- Kitajima, M., Hashimoto, K., Sandoval, M., Aimi, N., and Takayama, H. (2004) New oleanan-type triterpene and cincholic acid glycosides from Peruvian “Una de Gato” (*Uncaria tomentosa*), *Chemical and Pharmaceutical Bulletin (Tokyo)*, 52(10), 1258–1261.
- Kanavy, H. E., and Gerstenblith, M. R. (2011) Ultraviolet radiation and melanoma, *Seminars in Cutaneous Medicine and Surgery*, 30(4): 222-8.
- Kushiro, K., Núñez, N. P. (2011) Ob/ob serum promotes a mesenchymal cell phenotype in B16BL6 melanoma cells, *Clinical and Experimental Metastasis*, 28(8): 877-86.
- Lambert, M. W., Lambert, W. C., Schwartz, R. A., Mesa, M. L., Brodtkin, R. H., Abbey, A. H., Potter, G. K., Little, W. (1985) Colonization of nonmelanocytic cutaneous lesions by dendritic melanocytic cells: a simulant of acral-lentiginous (palmar-plantar-subungual-mucosal) melanoma, *Journal of Surgical Oncology*, 28(1): 12-8.
- Lamm, S., Sheng, Y., Pero, R. W. (2001) Persistent response to pneumococcal vaccine in individuals supplemented with a novel water soluble extract of *Uncaria tomentosa*, *C-Med-100, Phytomedicine*, 8(4): 267-74.
- Li, Q., Withoff, S., Verma, I. M. (2005) Inflammation-associated cancer: NF- $\kappa$ B is the lynchpin, *Trends in Immunology*, 26(6): 318-325.
- Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. (1999) Cancer statistics, 1999, *A Cancer Journal for Clinicians*, 49(1): 8-31.
- Liotta, L. A. (1984) Tumor invasion and metastasis - role of the basement membrane, *American Journal of Pathology*, 117: 339-348.
- Leiter, U., and Garbe, C. (2008). Epidemiology of Melanoma and Nonmelanoma Skin Cancer—The Role of Sunlight, *Advances in Experimental Medicine and Biology*, 624:89-103.
- Linos, E., Swetter, S. M., Cockburn, M. G., Colditz, G. A., and Clarke, C. A. (2009) Increasing Burden of Melanoma in the United States, *Journal of Investigative Dermatology*, 129(7): 1666-74.
- Lasithiotakis, K. G., Leiter, U., Gorkiewicz, R., Eigentler, T., Breuninger, H., Metzler, G., Strobel, W., and Garbe, C. (2006) The incidence and mortality of cutaneous melanoma in Southern Germany: trends by anatomic site and pathologic characteristics, 1976 to 2003,

Cancer, 107(6): 1331-9.

Mammone, T., Åkesson, C., Gan, D., Giampapa, V., and Pero, R. W. (2006) A water soluble extract from *Uncaria tomentosa* (Cat's Claw) is a potent enhancer of DNA repair in primary organ cultures of human skin, *Phytotherapy Research*, 20(3): 178-83.

Månsson-Brahme, E., Johansson, H., Larsson, O., Rutqvist, L. E., and Ringborg, U. (2002) Trends in incidence of cutaneous malignant melanoma in a Swedish population 1976-1994, *Acta Oncol*, 41(2): 138-46.

Mabberley, D. J. (2008). *Mabberley's Plant-Book* third edition, Cambridge University Press, UK.

Muhammad, I., Dunbar, D. C., Khan, R. A., Ganzera, M., and Khan, I. A. (2001) Investigation of Una De Gato I. 7-Deoxyloganic acid and <sup>15</sup>N NMR spectroscopic studies on pentacyclic oxindole alkaloids from *Uncaria tomentosa*, *Phytochemistry*, 57(5): 781-5.

Martino, L. D., Martinot, J. L., Franceschelli, S., Leone, A., Pizza, C., and Feo, V. D. (2006) Proapoptotic effect of *Uncaria tomentosa* extracts, *Journal of Ethnopharmacology*, 107(1): 91-4.

Mackie, R., Freudenberger, T., Aitchison, T. (1989) Personal risk-factor chart for cutaneous melanoma, *Lancet*, 2(8661): 487-90.

Mukherjee, A. K., Basu, S., Sarkar, N., Ghosh, A. C. (2001) Advances in cancer therapy with plant based natural products, *Current Medicinal Chemistry*, 8(12): 1467-86.

Moschos, S. J., Edington, H. D., Land, S. R., Rao, U. N., Jukic, D., Shipe-Spotloe, J., and Kirkwood, J. M. (2006) Neoadjuvant treatment of regional stage IIIB melanoma with high-dose interferon alpha-2b induces objective tumor regression in association with modulation of tumor infiltrating host cellular immune responses, *Journal of Clinical Oncology*, 24:3164-3171.

Moscow, J. A., and Cowan, K. H. (2011) *Biology of cancer*. In Goldman L, Schafer AI, eds. *Cecil Medicine*. 24th ed. Philadelphia, Pa: Saunders Elsevier; chap 185.

Nggada, H.A., Na'aya, H.U. and Ali, N. (2003) A histological analysis of malignant tumors of the skin in University of Maiduguri Teaching Hospital, Nigeria, *Highland Medical Research Journal*, 1: 38-40.

- Miyake, K., Li, F., Tezuka, Y., Awale, S., and Kadota, S. (2010) Cytotoxic activity of quassinoids from *Eurycoma longifolia*, *Natural Product Communications*, 5(7): 1009-12.
- Newman, D. J., and Cragg, G. M. (2007) Natural products as sources of new drugs over the last 25 years, *Journal of Natural Products*, 70:461–477.
- Neville, J. A., Welch, E., and Leffell, D. J. (2007) Management of nonmelanoma skin cancer in 2007, *Nature Clinical Practice Oncology*, 4, 462-469.
- Pilarski, R., Filip, B., Wietrzyk, J., Kuras, M., and Gulewicz, K. (2010) Anticancer activity of the *Uncaria tomentosa* DC. preparations with different oxindole alkaloid composition, *Phytomedicine*, 17(14): 1133-9.
- Prado, E. G., Gimenez, M. D., Vázquez, R. D., Sánchez, J. L., and Rodríguez, M. T. (2007) Antiproliferative effects of mitraphylline, a pentacyclic oxindole alkaloid of *Uncaria tomentosa* on human glioma and neuroblastoma cell lines, *Phytomedicine*, 14(4): 280-4.
- Paniagua-Pérez, R., Madrigal-Bujaidar, E., Molina-Jasso, D., Reyes-Cadena, S., Álvarez González, I., Sánchez-Chapul, L., and Pérez-Gallaga, J. (2009) Antigenotoxic, Antioxidant and Lymphocyte Induction Effects Produced by Pteropodine, *Basic and Clinical Pharmacology and Toxicology*, 104(3): 222-227.
- Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. (1997) Cancer statistics, 1997, *A Cancer Journal for Clinicians*, 47(1): 5-27.
- Pilarski, R., Zieliński, H., Ciesiołka, D., & Gulewicz, K. (2006) Antioxidant activity of ethanolic and aqueous extracts of *Uncaria tomentosa* (Willd.) DC, *Journal of Ethnopharmacology*, 124: 18-23.
- Parkin, D. M., Bray, F., Ferlay, J., and Pisani, P. (2005) Global Cancer Statistics, 2002, *Ca-a Cancer Journal for Clinicians*, 55(2): 74-108.
- Quesada, J., Talpaz, M., Rios, A., Kurzrock, R. and Gutterman, J. (1986) Clinical Toxicity of Interferons in Cancer Patients, *Journal of Clinical Oncology*, 4(2), 234-243.
- Reis, S. R., Valente, L. M., Sampaio, A. L., Siani, A. C., Gandini, M., Azeredo, E. L., Kubelka, C. F. (2008) Immunomodulating and antiviral activities of *Uncaria tomentosa* on human monocytes infected with Dengue Virus2. *International Immunopharmacology*, 8(3): 468-76.

- Rycroft, R. J., Robertson, S. J., and Wakelin, S. H. (2010) Colour handbook of dermatology. London: Manson.
- Rizzi, R., Re, F., Bianchi, A., De Feo, V., De Simone, F., Bianchi, L., and Stivala, L.A. (1993) Mutagenic and antimutagenic activities of *Uncaria tomentosa* and its extracts, *Journal of Ethnopharmacology*, 38(1): 63-77.
- Reinhard, K.H. (1999) *Uncaria tomentosa* (Willd.) D.C.: cat's claw, uña de gato, or seventaro, *Journal of Alternative and Complementary Medicine*, 5(2): 143-151.
- Rojas-Duran, R., González-Aspajo, G., Ruiz-Martel, C., Bourdy, G., Doroteo-Ortega, V.H., Alban-Castillo, J., Robert, G., Auberger, P., and Deharo, E. (2012) Anti-inflammatory activity of Mitraphylline isolated from *Uncaria tomentosa* bark, *Journal of Ethnopharmacology*, 143(3): 801-4.
- Rinner, B., Li, Z.X., Haas, H., Siegl, V., Sturm, S., Stuppner, H., and Pfragner, R. (2009) Antiproliferative and pro-apoptotic effects of *Uncaria tomentosa* in human medullary thyroid carcinoma cells, *Anticancer Research*, 29(11): 4519-28.
- Rivers, J. K., Gallagher, R. P. (1995) Experience of the Canadian Dermatology Association Public education projects in skin cancer, *Cancer*, 75 (2 Suppl): 661-666.
- Reinhard, K. H. (1999) *Uncaria tomentosa* (Willd.) DC.: cat's claw, una de gato, or saventaro, *Journal of Alternative and Complementary Medicine*, 5(2): 143-51.
- Riva, L., Coradini, D., Di Fronzo, G., De Feo, V., De Tommasi, N., De Simone, F., and Pizza, C. (2001) The antiproliferative effects of *Uncaria tomentosa* extracts and fractions on the growth of breast cancer cell line, *Anticancer Research*, 21(4A): 2457-61.
- Smilenov, L. B. (2006) Tumor development: Haploinsufficiency and local network assembly, *Cancer Letters*, 240(1): 17-28.
- Sheng, Y., Bryngelsson, C., Pero, W. R. (2000) Enhanced DNA repair, immune function and reduced toxicity of C-MED-100 tm, a novel aqueous extract from *Uncaria tomentosa*, *Journal of Ethnopharmacology*, 69:115-126.
- Sheng, Y., Li, L., Holmgren, K., and Pero, R. W. (2001) DNA repair enhancement of aqueous extracts of *Uncaria tomentosa* in a human volunteer study, *Phytomedicine*, 8(4): 275-82.

Sheng, Y., Åkesson, C., Holmgren, K., Bryngelsson, C., Giamapa, V., and Pero, R. W. (2005) An active ingredient of Cat's Claw water extracts, *Journal of Ethnopharmacology*, 96(3): 577-84.

Sharma, P., Wagner, K., Wolchok, J. D., and Allison, J.P. (2011) Novel cancer immunotherapy agents with survival benefit: recent successes and next steps, *Nature Reviews Cancer*, 11(11): 805-12.

Sandoval, M., CHARBONNET, R. M., OKUHAMA, N. N., Roberts, J., Krenova, Z., Trentacosti, A. M., and MILLER, M. J. (2000) Cat's claw inhibits TNF $\alpha$  production and scavenges free radicals: role in cytoprotection, *Free Radical Biology & Medicine*, 29(1): 71-78.

Sandoval-Chacón, M., Thompson, J. H., Zhang, X. J., Liu, X., Mannick, E. E., Sadowska-Krowicka, H., Charbonnet, R. M., Clark, D. A., Miller, M. J. (1998) Antiinflammatory actions of cat's claw: the role of NF-KB, *Alimentary Pharmacology & Therapeutics*, 12(12): 1279-1289.

Sagdic, O. (2003) Sensitivity of four pathogenic bacteria to Turkish thyme and oregano hydrosols. *Food Science Technology*, 36: 467 – 473

Samuelson, G. (1999) *Drugs of Natural Origin: A Textbook of Pharmacognosy*. 4th edition, Swedish Pharmaceutical Press, Stockholm, Sweden.

Singh, A. K., Gupta, A., Mishra, A. K., Gupta, V., Bansal, P., Kumar, S. (2010) Medicinal Plant for Curing Alzheimer's Disease, *International Journal of Pharmaceutical and Biological*, 1(2): 108 – 114.

Situm, M., Buljan, M., Bulić, S. O., and Simić, D. (2007) The mechanisms of UV radiation in the development of malignant melanoma, *Coll Antropol*, 31 Suppl 1:13-6.

Stang, A., Pukkala, E., Sankila, R., Soderman, B., and Hakulinen, T. (2006) Time trend analysis of the skin melanoma incidence of Finland from 1953 through 2003 including 16,414 cases, *International Journal of Cancer*, 119:380–384.

Tzonou, A., Lipworth, L., Kalandidi, A., Trichopoulou, A., Gamatsi, I., Hsieh, C., Notaral, V., and Trichopoulos, D. (1996) Dietary factors and the risk of endometrial cancer: a case - control study in Greece, *British Journal of Cancer*, 73, 1284-1290.

- Trask, P. C., Esper, P., Riba, M., Redman, B. (2000) Psychiatric side effects of interferon therapy: prevalence, proposed mechanisms, and future directions, *Journal of Clinical Oncology*, 18(11): 2316-26.
- Unnati, S., Ripal, S., Sanjeev, A., and Niyati, A. (2013) Novel anticancer agents from plant sources, *Chinese Journal of Natural Medicines*, 11(1): 0016–0023.
- Ulmer, M. J., Tonita, J. M., and Hull, P. R. (2003) Trends in invasive cutaneous melanoma in Saskatchewan 1970–1999, *Journal of Cutaneous Medicine and Surgery*, 7:433–442.
- Veronesi, U., Cascinelli, N. (1991) Narrow excision (1-cm margin). A safe procedure for thin cutaneous melanoma, *Archives of Surgery*, 126 (4): 438-41, 1991.
- van Meerloo, J., Kaspers, GJ., and Cloos, J., (2011) Cell sensitivity assays: the MTT assay, *Methods in Molecular Biology*, 731:237-45.
- Vickers, K.A., Jolly, K.B., and Greenfield, S.M. (2006) Herbal medicine: women’s views, knowledge and interaction with doctors: a qualitative study, *BMC Complementary and Alternative Medicine*, 6: 40.
- Williams, I. R., and Kupper T. S. (1996) Immunity at the surface: homeostatic mechanisms of the skin immune system, *Life Sciences*, 58(18): 1485-1507.
- Wirth, C., and Wagner, H. (1997) Pharmacologically active phenolic compounds from the bark of *Mahonia aquifolium*, *Phytomedicine*, 4(4): 357-8.
- Wild, C. P. (2008) Environmental exposure measurement in cancer epidemiology, *Mutagenesis*, 408(6809): 248-54.
- Weinstock, M. A. (1994) Epidemiology of nonmelanoma skin cancer: clinical issues, definitions, and classification, *Journal of Investigative Dermatology*, 102(6): 4S-5S.
- Weinstock, M. A., Morris, B. T., Lederman, J. S., Bleicher, P., Fitzpatrick, T. B., and Sober, A. J. (1988) Effect of BANS location on the prognosis of clinical stage 1 melanoma: New data and meta-analysis, *British Journal of Dermatology*, 119(5): 559-65.