The effect of *Andrographis paniculata* on the growth of malignant cancer cells

By

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Abstract:

There are a variety of plants that have been recognized and used in traditional medicine for their health benefits. Among these plants is *Andrographis paniculata,* commonly known as the king of bitters because of its bitter taste. In the past, *Andrographis paniculata* has been used to treat various ailments, including respiratory infections, the common cold, snake bites, inflammation and diarrhea. We have tested the effect of *Andrographis paniculata* extracts on the growth of malignant cancer cells and its potential benefits towards cancer treatment. Cancer cells, including B16- BL6, MCF-7, MDA-MB-231, 4T1, and ASPC-1 cells, were treated with 70% ethanol extracts or aqueous extracts of *Andrographis paniculata* with a range in concentration from 0.1 to 1%. Treatment with the ethanol extract of *Andrographis paniculata* inhibited cell growth when added at 0.25% to 1%. The aqueous extract was less potent and inhibited cell growth at only 1%. Treatment of B16- BL6 cells with 1% of the aqueous extract of *Andrographis paniculata* for 48 h induced apoptosis as detected using the acridine orange/ ethidium bromide cell staining assay. On the other hand, treatment with the ethanol extract had a much stronger effect and cell staining showed late stage apoptosis and loss of membrane integrity. We have shown that treatment of B16- BL6 cells with *Andrographis paniculata* extracts altered cell survival pathways and promoted apoptosis by using western blot analysis for ERK ½, phosphorylated-ERK and Caspase 3. Similarly, the treatment of B16-BL6 with the ethanol extract for 48 h increased the number of cells in the Sub-G1 phase, in comparison to treatment with the aqueous extract, by using flow cytometry of propidium iodide-stained cells. Overall, this study demonstrated that *Andrographis paniculata* had successfully suppressed the growth of malignant cancer cells.

Keywords: *Andrographis paniculata*, Malignant cancer cells, Apoptosis, Caspase 3, Sub-G1.

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Abbreviations

***A. paniculata*:** *Andrographis paniculata.*

ANOVA: Analysis of variance.

AO/ EB: Acridine orange/Ethidium bromide staining**.**.

AP1: andrographolide.

AP3:14-deoxy-11,12-didehydroandrographolide.

AP6: 14-deoxyandrographolide.

APS: Ammonium persulfate.

ASPC-1: Pancreatic cancer.

ATCC: American Type Culture Collection

B16-BL6: Murine Melanoma cells.

BRCA1 gene: breast cancer-associated Gene 1.

BSA: Bovine Serum Albumin.

°C: degree Celsius.

C6: glioma cells.

CDDP: cisplatin.

COX-2: cyclooxygenase 2.

CT scanning: computed tomography.

DMEM: Dulbecco's Modified Eagle Medium (culture medium).

DMSO: Dimethyl sulfoxide.

ECL: Enhanced chemiluminescence.

EGFR: epidermal growth factor receptor.

ER & PR: Estrogen and Progesterone hormones.

ERK1/2: Extracellular Signal-Regulated Kinases 1 and 2.

EtOH: Ethanol.

FBS: fetal bovine serum.

FTLEE: first true leaf ethanol extract.

HCL: hydrogen chloride.

HER2: human epidermal growth factor receptor 2.

HHV8: human herpes virus 8.

IKK: Inhibitor of Kappa Kinase.

IRF-3: Interferon Regulatory Factor 3.

JNK: Jun N-terminal protein kinase.

Lovo cells: human colon cancer.

MCF-7: Human Breast Cancer (Positive Triple).

MDA-MB-231: Human Breast Cancer (Negative

Triple).

mg: milligram.

ml: milliliter.

MLWE: mature leaf water extract.

MRI scanning: magnetic resonance

imaging.

mRNA: messenger ribonucleic acid.

MTT: Methyl thiazol tetrazolium assay.

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

NO: nitric oxide.

P-ERK: Phosphorylated Extracellular Signal-Regulated Kinase.

PARP: poly adenosine diphosphate ribose polymerase.

PBS: Phosphate buffered saline.

PI: Propidium iodide.

RIPA: Radio immunoprecipitation Assay Buffer.

RPMI: Roswell Park Memorial Institute (culture medium).

SDS: Sodium dodecyl sulfate.

TBS: Tris-Buffered Saline.

TBST: Tris-Buffered Saline and Tween 20.

TD-47: human breast cancer.

TNF-a: tumor necrosis factor-a.

4T1: Mouse Breast Cancer (Negative Triple).

u/ml: micro per milliliter.

ul: microliter.

UV: Ultraviolet.

µg/ml: microgram per milliliter.

µm: micrometer.

Chapter 1: Introduction

1.1 Cancer as a Problem

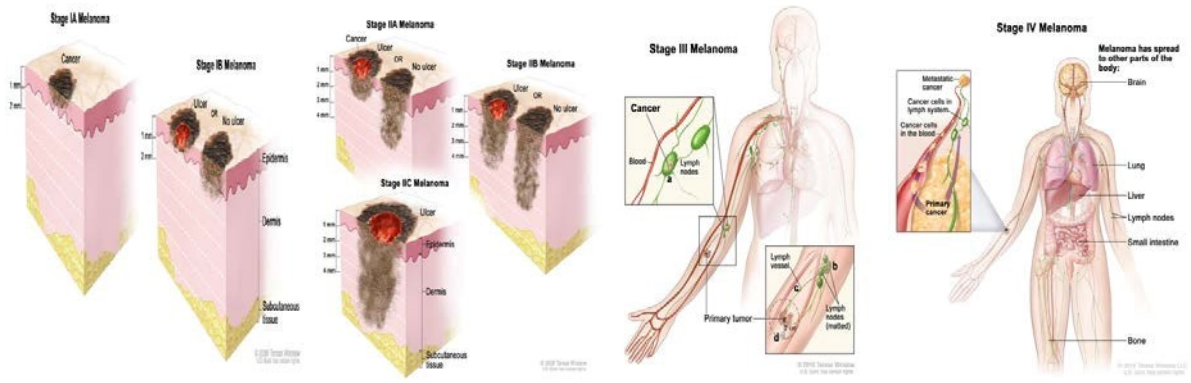
Cancer comprises of over two hundred diseases that have similar characteristics. One common characteristic of these diseases is that they are marked by uncontrolled multiplication and growth of cells (Edmunds, 2007; Gabriel, 2007). Cancer is a top cause of death in the United States and in parts of Europe, ranking second behind cardiovascular disease (Ruddon, 2007). In 2016, approximately 1.7 million new cancer diagnosis and 600,000 deaths are expected in the United States, according to the American Cancer Society estimates. Exposures to environmental carcinogenic agents and lifestyle factors are thought to account for differences in geographical rates of cancer incidence. Some of these lifestyle factors include diets with high fat, calories and red meat (Pardee et al., 2009).

Any cell has a chance of becoming malignant (cancerous) when there is disruption or failure of the standard cell control mechanisms (Gabriel, 2007). Cancer development is a consequence of a destabilization in the balance between cell division, differentiation and death. When there is uncontrolled division, a tumor resulting from the growth of abnormal cells can result and these growths become cancer if they have the ability to invade neighboring tissues (Lambert et al., 2009). In addition to abnormal division, malignant cells can result from an accumulation of gene mutations and tumors can appear 20 years or more after the first mutations that initiated the cancer. Mutation hampers the ability for cell differentiation and facilitates the spread to other locations (Pardee et al., 2009).

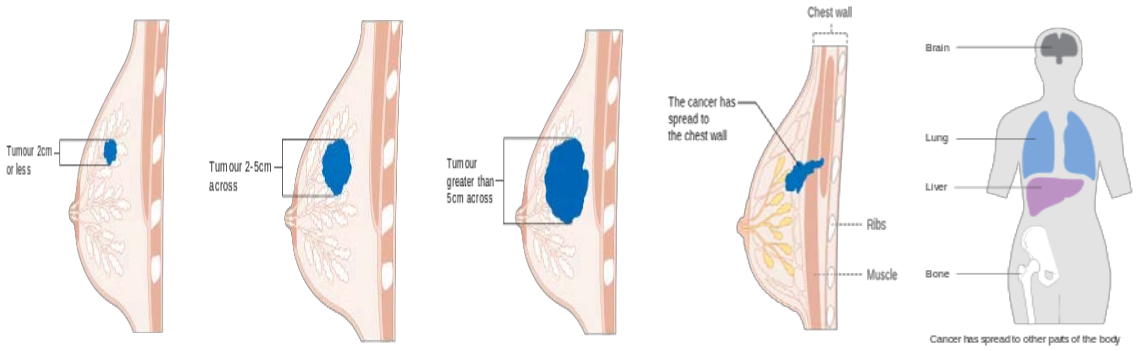
Treatment of patients with cancer is usually through the surgical removal of the tumor. However, this is not always successful because of the microscopic spread of cancer cells to surrounding tissues which may be undetected or appear noncancerous to the surgeon. If left untreated, the cancers can then re-emerge and spread. Replication of malignant cells leads to

infiltration of the surrounding tissues including blood vessels and lymphatics thus enabling them to be carried to other parts of the body where they can continue the process of growth and replication (Gabriel, 2007). Cancer cells can mutate in ways that enable them to evade the surveillance of the immune system. For example, some cancer cells can secrete transforming growth factor-beta, a cytokine that interferes with anti-cancer immune system cell function (Pienta, 2009). Consequently, the cancer cells can relentlessly, colonize other locations and present an enormous challenge in treatment (Eleni & Christopher, 2009).

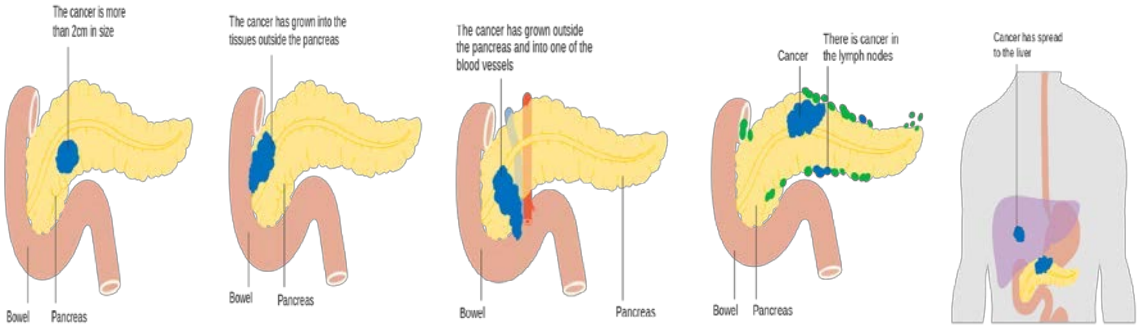
Cancer as a Problem



Malignant melanoma (skin cancer) stages (National cancer institute., 2016).
https://www.cancer.gov/types/skin/patient/melanoma-treatment-pdq#section/_96



Breast cancer stages (https://en.wikipedia.org/wiki/Breast_cancer).



Pancreatic cancer stages (https://en.wikipedia.org/wiki/Pancreatic_cancer

1.2. Malignant Melanoma

Melanoma is one of the cancers that shows an increasing rate of incidence and mortality. Early detection of the disease contributes to the survival of the patient. The tumor's thickness has been regarded as the most significant factor to affect the patient's survival. The five-year rate of survival for patients is 94% for melanoma thinner than 1mm compared to less than 50% for those with a tumor thicker than 3mm. Melanoma is characterized as a tumor with an aggressive potential for replication and a high possibility to spread. While excessive exposure to the sun remains the primary risk factor, others risks include blistering sunburns, atypical mole syndrome, UV light exposure, immune suppression and a history of the condition in a first-degree kin (Rigel et al., 2000). The incidence of the disease varies with latitude, increasing in frequency with proximity to the equator. Lifestyle changes, characterized by increasing outdoor recreation and less skin coverage, also increase incidence. Other risk factors include childhood sunburns, and prolonged exposure to fluorescent lighting or to polychlorinated biphenyl compounds (Bailin et al., 2002).

There are specific patient phenotypes that increase the risk of the disease including blue eyes, sun sensitivity, light skin complexion, red hair, and diagnosis of a non-melanoma skin cancer (Rigel et al., 2000; Bailin et al., 2002). A multivariate study singled out six factors that independently affected the risk of developing the disease. The factors include; a family history of the disease, red hair, marked freckling present on the upper back, a history of more than three blistering sunburns before 20 years of age, outdoor jobs for three or more years for teenagers, and the existence of actinic keratosis (Rigel et al., 2000). Skin prototype is also a risk factor, and the condition is predominant among whites, accounting for about 70 % of melanoma cases which are largely credited to sun-exposure (Barnhill, 2003).

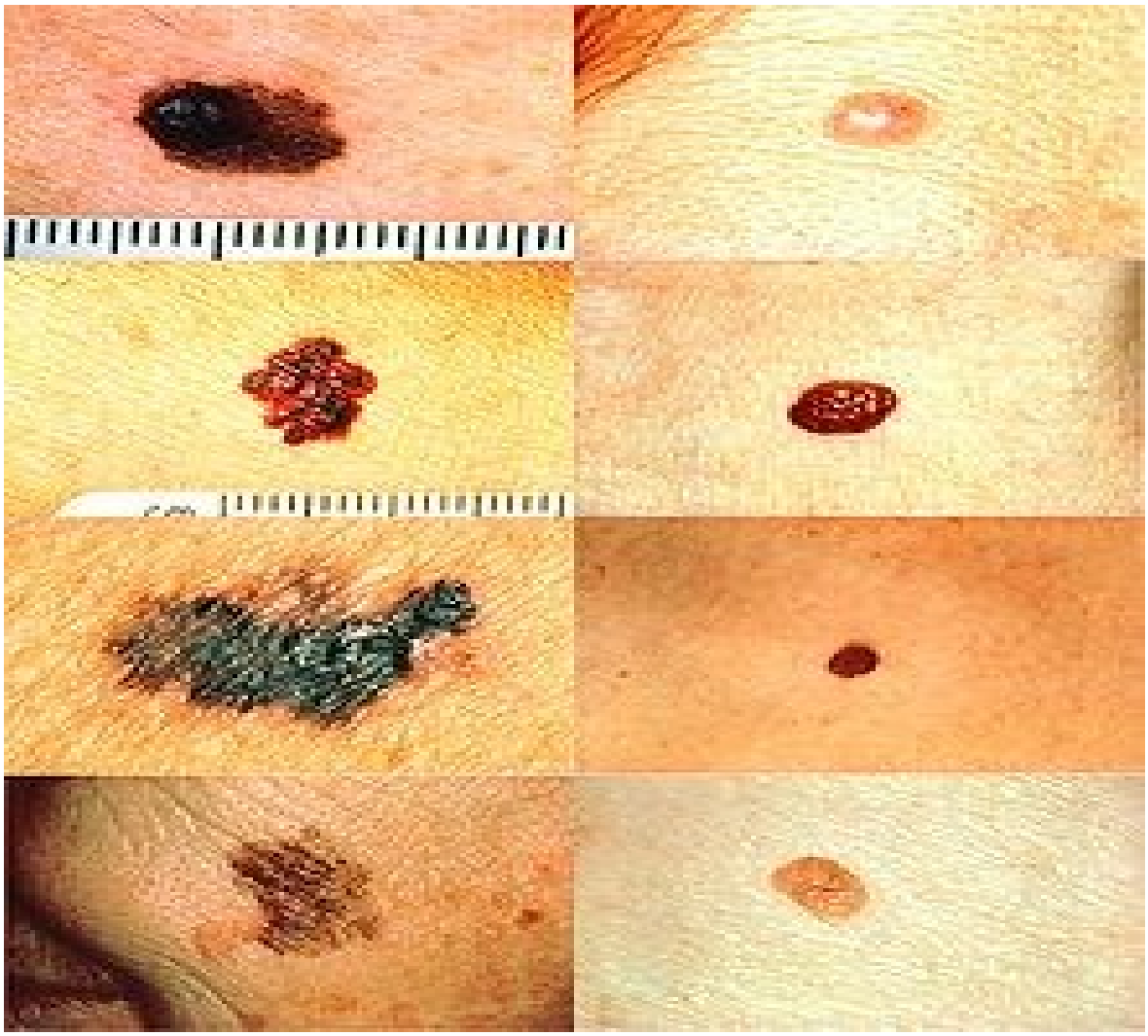


Figure 1.1. The ABCD melanoma guideline for diagnosis (Asymmetry pattern, Border irregularity, Color variegation and Diameter) https://en.wikipedia.org/wiki/Melanoma

According to Barnhill (2003), the common symptoms for diagnosis of melanoma include a change in the color and size of skin lesions, bleeding and ulceration of the lesions, and as the cancer advances it becomes itchy. Early recognition of the condition can be done through an ABCD guideline (Figure 1) which includes; Asymmetry patterns that result from the uneven growth of lesions, which in turn leads to Border irregularity. Such inconsistency causes Color variegation forming shades of black and light and dark brown. Lesions that have the ABC features coupled with a Diameter that exceeds 6 mm are to be considered suspicious of melanoma (Rigel et al., 2000).

Because a skin biopsy is a relatively simple procedure, lesions that are suggestive of cancerous melanoma should be assessed for early detection. Moreover, self-examination by patients also contributes to early detection and seeking a physician's advice if they notice new pigmented lesions increases early diagnosis. Surgical excision is the primary and most efficient method of treating early tumors. More advanced tumors are also treated with chemotherapy, adjuvant therapy, and vaccines (Rigel et al., 2000). The limitations to early treatment include delayed medical assessment by patients who have suspicious lesions due to denial, fear or because they lack knowledge. The existence of nodal metastases reduces the 5-yr rate of survival by at least 40 %. In addition, it is prudent to follow the patients' condition after diagnosis in order to detect recurrences and to continue treatment to enhance long-term survival. Unfortunately, recurrent disease is not always treatable and treatment of melanoma is not necessarily curative. Although it has not yet been proved experimentally, it is thought that patient follow-up and the rate of survival are directly linked. There is a greater risk of recurrence of melanoma in the first year following the original diagnosis and treatment, which declines steadily over time. Most recurrences are symptomatic, and therefore, self-examination can play a crucial role in seeking medical attention (Bailin et al., 2002).

1.3. Breast Cancer

According to Afonso (2009), the approaches to identifying women at high risk of developing breast cancer include evaluating family history, clinical history records, and assessing mammographic density. Further, the study notes that inherited factors account for only 1 to 2% of all breast cancer cases. The incidence of breast cancer increases as the individual ages with a majority of diagnosis in women after 40 years old. Delayed childbirth has been suggested to be a contributing factor for women past 35 years while childbearing is associated with risk for women below 35 years.

For diagnosis, ultrasound is considered to be more efficient than a mammogram in the detection of breast cancer among young women and it can distinguish between solid masses and cysts. A breast MRI screening is recommended for women with a 20-25% lifetime risk including those with a familial syndrome and those having received chest radiation. Treatment of cancer often includes breast conserving surgery coupled with radiation. Surgery as a treatment method is determined, by among other things, the preference of the patient, the size of the tumor, prior radiation and the likelihood of attaining an acceptable cosmetic outcome. Breast conservation, especially among young women, is associated with an increased risk of recurrence compared to older women (Gabriel & Domchek, 2010).

Chemotherapy as a method of treatment is insufficient on its own, and endocrine therapy should be incorporated in the treatment plan. Alternatively, a combination of tamoxifen with ovarian suppression could be used in place of other treatments. Chemotherapy can lead to permanent menopause and is a concern in the treatment of young women. Additionally, women would have to delay pregnancy until after the tamoxifen treatment is completed due to teratogenicity. This means a women might have to wait 5 years, which is the optimal duration of treatment, before getting pregnant although her fertility decreases as she advances in age.

Pregnancy following early diagnosis has no negative influence on the rate of patient survival. For women who prefer to bear children after treatment, there is a need for fertility procedures to be commenced before therapy since treatment is likely to cause genetic damage to oocytes. Therefore, such patients should be referred to a fertility specialist before systematic therapy is initiated. Such fertility measures include ovarian preservation, embryo cryopreservation or oocyte cryopreservation (Gabriel & Domchek, 2010).

To prevent recurrence, women with a history of the disease should have annual mammogram screening. Maintaining a healthy weight and physical activity reduces the risk of recurrence. Deficiency in vitamin D has been identified as a contributor to recurrence and death in patients diagnosed with an early stage of the disease. Diagnosis can be distressing for women. Those who experience treatment-induced menopause have lower health perspectives compared to their peers. The situation has been interpreted to imply that diagnosis at a young age comes with an emotional shock and physical demands which make it more difficult to endure treatment. Furthermore, concerns about fertility loss, sexual dysfunction and deteriorating body image for women who undergo mastectomy increases the emotional strain on survivors. Thus, early counseling and support needs to be incorporated into the management of these patients (Gabriel & Domchek, 2010).

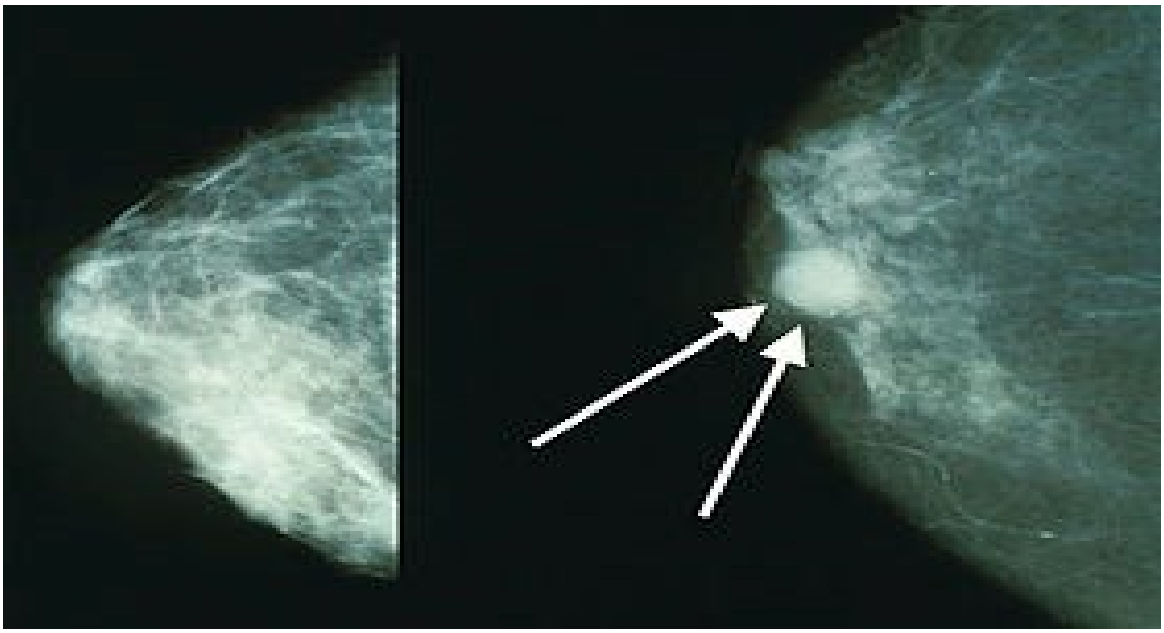


Figure 1.2. Breast cancer was detected by mammography in the right breast compared to the normal breast in the left side. https://en.wikipedia.org/wiki/Breast_cancer

Estrogen receptor (ER) is a key focus in breast cancer treatment and prevention. The risk of estrogen-dependent breast cancer can be minimized through exercise and reduction in body weight, cessation of alcohol intake among postmenopausal women and increased dietary folate (Afonso, 2009). Menopausal hormones are thought to be a cause of breast cancer. Moreover, the association between estrogen and breast cancer implies that assessment needs to be done before counseling women on methods of relieving menopausal symptoms to ensure that the choices do not increase their susceptibility to chronic diseases (Colditz, 1998).

1.3.1. ER Positive Breast Cancer & HER2 positive breast cancer:

Patients with ER-positive tumors are more common than patients with ER-negative ones across all ages and the incidence of positivity increases with age. Consequently, breast cancers in women of postmenopausal age are mostly ER positive. ER positive tumors are more likely to be smaller, low-grade and lymph node negative. The type is more prevalent among Caucasian

women and recurs at a low, steady rate. The estimated long-term recurrence is about 2% per annum. Moreover, a diagnosis of ER-positive breast cancer is associated with better prognosis except for black women diagnosed with ER positive and HER2 negative breast cancer. Patients with ER positive tumors who have partial irradiation have a reduced chance of experiencing recurrence.

In HER2 positive breast cancer; the oncogene HER2 is overexpressed. The type accounts for about 20 percent of breast cancers. HER2 positive breast cancer has a high likelihood of manifesting itself symptomatically. These tumors are more prevalent among the young and have more lymph node involvement. The median survival for patients with advanced HER2 positive breast cancer is over two years. If treatment involves conserving surgery, recurrences are more common for this type than any other type, such as ER+ and HER2- due to residual disease at re-excision (Cadoo, Fornier & Morris, 2013).

1.3.2. Triple negative breast cancer (TNBCs):

This type of tumor does not express the ER, PR or HER-2 genes. The main treatment for patients with triple negative breast cancer includes chemotherapy, and recent developments includes the application of EGFR inhibitors as a therapeutic option although with mixed results (Hudis & Gianni, 2011). Although the triple-negative subtype accounts for a minority of breast cancer cases, it has a disproportionately higher number of deaths. Since the triple-negative type consists of tumors that do not have targeted therapeutics, clinicians exclusively rely on nonspecific cytotoxic agents (Schneider et al., 2008). Cytotoxic chemotherapy is the standard mode of care. The subgroup accounts for about 15% of all breast cancer incidences with prevalence being highest among African-Americans, young women, and patients with mutations in the BRCA1/BRCA2 genes. The type manifests with an aggressive phenotype and poor prognosis. Triple negative breast cancers are unlikely to have any involvement of lymph nodes.

Patients diagnosed with triple negative breast cancer in the early stages, with no incidences of tumor relapse in the first five years after treatment have a high likelihood of being cured. However, patients with triple negative breast cancer have a higher risk to develop metastases during the first five years following diagnosis. Further, there is a significant risk of both CNS and pulmonary involvement as sites of recurrent disease while bone involvement is less likely (Cadoo, Fornier & Morris, 2013).

1.4. Pancreatic Cancer

The American Cancer Society predicts that in 2016, pancreatic cancer will kill approximately 41,780 people (20,330 women and 21,450 men). The reason for the high rate of mortality is because less than 20% of patients have localized, more easily treated tumors, when they are diagnosed (Hidalgo, 2010). A major problem is that patients with pancreatic tumors do not show symptoms until the tumors are very advanced and therefore diagnosis is usually very late in cancer progression. The contributing factors for risk of pancreatic cancer are smoking, male gender, African-American ethnicity, diabetes mellitus, history of chronic pancreatitis in the family, obesity, advanced age, and non-O blood group (Vincent et al. 2011). Twenty percent of tumors result from cigarette smoking and tumors in smokers have higher mutation rates than those from non-smokers. Also, family history accounts for between 7 and 10 % of the disease incidence with the first-degree relatives of a person's familial type having a nine times more risk compared to the general populace. For kindreds with at least three first-degree relatives of the disease, the risk is increased 32-fold (Vincent et al. 2011).

In patients with a strong family history of pancreatic cancer, identification of the predisposing gene could allow for testing screening, and chemoprevention in identified relatives. Genetic testing for pancreatic cancer is underutilized because there is the lack of recognition of

the family history and secondly because there is usually inadequate recording of family history (Vincent et al. 2011). For most patients diagnosed with pancreatic cancer, life expectancy is a matter of months. High morbidity results from the fact that the cancer disseminates to distant sites early and is resistant to most currently available treatments (Li et al., 2004). Diagnosis of patients for pancreatic cancer should be performed using a CT scan and endoscopic ultrasonography. The disease's manifestations are vague and nonspecific and lead to a delay in diagnosis. When it presents, it may be in the form of weight loss, jaundice and abdominal pain (Zhang et al., 2016). Various options for diagnosis exist where pain without jaundice is experienced, but a CT scan remains critical for observing pathology within the pancreas. Symptoms of tumors in the pancreas result from compression of surrounding organs including the bile duct, duodenum and nerves and these effects imply that diagnosis can be made rather quickly. The main symptoms include back and abdominal pain, diabetes mellitus, and obstructive jaundice (Li et al., 2004).

For cases of locally advanced disease with the tumor encasing a vascular structure but with no proof of distant metastatic disease in the liver, peritoneum, and chest, the conventional treatment is fluorouracil-based chemotherapy and radiation (Li et al., 2004). In patients with metastatic disease, survival depends on the situation and the burden exerted by the tumor. Chemotherapy at this stage is palliative rather than curative, and its impacts on quality of life need to be considered in contrast to the toxic effects (Li et al., 2004). Over 80 % of patients with pancreatic cancer have unrespectable tumors because the disease has metastasized. The use of chemotherapeutic agents on patients with advanced disease results in a 5-year survival rate of 1015% (Nunes & Lobo, 2007).

1.5. **Cancer Treatment**

The options for cancer treatment recommended by a doctor depend on the type as well as the stage of the disease, probable side effects, and the patient's overall health. Once a tumor has been identified, various options can be pursued to treat the cancer. These options include surgery, radiotherapy, hormonal therapy, and stem cell transplants, among others. The options can be used to cure cancer as well as to provide palliative care to cancer patients by alleviating the symptoms of the disease (Canadian Cancer Society, 2016).

1.5.1. Chemotherapy:

Chemotherapy is the utilization of cytotoxic medicine to kill cancer cells which most commonly targets fast growing cells by disrupting DNA replication or cell division and causing cell death. However, since the chemotherapy drugs are powerful, they can lead to damage of any growing cells, including healthy ones such as blood cells, immune cells, and fast reproducing cells in the gastrointestinal tract. As such, the damage causes many side effects (Healthline Media, 2016). Briefly, some of the short-term side effects include fatigue, pain (stomach ache, headache, and muscle pain), throat and mouth sores, diarrhea, vomiting, and nausea, constipation, blood disorders, nervous system effects, changes in memory and thinking, reproductive and sexual issues, loss of appetite, and hair loss. Nonetheless, most effects disappear after treatment although some can last for months after chemotherapy ends (Healthline Media, 2016). In addition, some kinds of chemotherapy may lead to permanent damage of the reproductive system, kidneys, liver, lungs, and heart. Moreover, some individuals develop problems in concentrating, memory, and thinking that can persist for months or years after treatment (University of Rochester Medical Center Rochester, 2016).

1.5.2. Radiotherapy:

Radiotherapy is employed as a primary cancer treatment, and it is often given after surgery to eliminate any residual cancer cells (Schulz, 2005). The process can be conducted either externally or internally. External radiation with high energy, such as from x-rays, electron beams and gamma rays have adequate energy to significantly damage cells. When it happens, it can result in enough damage to ultimately destroy the cells. Thus, its critical goal is to obliterate the cancer cells, which slow down tumor growth, but to avoid damaging nearby healthy tissues (American Cancer Society, 2016). One of the main research aims of radiation therapy is to improve targeting of the tumors to create the least amount of damage to neighboring tissues. However, similar to other treatment options, this method also causes a variety of side effects depending on the type of cancer, general health, location, and dose. Some of the common short-term side effects include skin problems and fatigue. However, even though some of the effects may disappear after treatment, some may progress for months after treatment ends (National Cancer Institute, 2016; American Cancer Society, 2016). For example, exposure to radiotherapy is also a risk factor for new cancers.

1.5.3. Surgery:

The major goal of surgery is the physical removal of the tumor as well as a small amount of surrounding tissue (the tumor margin) during an operation. The goals of surgery vary as it can also be utilized at the diagnostic stage, where a sample of tissue is removed from the patient's body and examined for any traces of cancer (biopsy). Accordingly, the surgical removal of the tumor may be the only treatment, or it may be combined with chemotherapy or radiotherapy, or other alternatives that may be available before or after surgery. Similar to other treatment options, this method also has its side effects that may include pain, fatigue, limb swelling, bleeding, infection and organ dysfunction (Canadian Cancer Society, 2016).

1.6. Natural products as anti-cancer agents

The rapid expansion of synthetic protein kinase inhibitors and monoclonal antibodies against cancer targets for the treatment of cancer in the late 1990s led to anticancer natural products being disregarded by the pharmaceutical industries (Bailly, 2009). However, in 2007 three new drugs developed from natural products were approved for clinical use signaling the re-emergence of promising antitumor compounds derived from microorganisms, and the increasing significance of novel formulations of recognized natural product-derived medicines, resulting in a new wave of natural products in oncology (Bailly, 2009). Further, there is a clear indication that the escalating use of microbial sources has increased the discovery of medically helpful natural products (Bailly, 2009).

Natural products have been an irresistible success in society. They have long been used to minimize pain and suffering in addition to revolutionizing medicine by facilitating organ transplantation. Natural products have been the source of most of the currently used essential anti-inflammatory and anticancer agents. Accordingly, over 60% of approved drugs and new drug application candidates are either natural products or are derived from them (excluding biologicals, such as monoclonal antibodies and vaccines) (Cargg et al., 1997; Demain & Zhang, 2005). Many natural products are used as chemoprotective agents against numerous common cancers worldwide. Correspondingly, a primary collection of such products include powerful antioxidants, others are phenolic in nature, and some have specific reactive groups, which can confer protective properties. The natural products have been most frequently found in studies looking at herbs, plant extracts, fruits, and vegetables. Even though the mechanisms of action for many of these agents are not clear, the idea that the use of vegetables and fruits reduces the incidence of carcinogenesis is widely supported (Reddy et al., 2003).

Natural products for anticancer agents are derived from divergent natural sources. For example, one of the most notable examples of an anticancer agent isolated from plants is the vinca alkaloid family (e.g. Vinblastine) isolated from the *Periwinkle Catharanthus roseus* found in the Amazon rain forest (Noble.,1990). Another excellent example is etoposide, derived from a microbe, that has led to high rates of cure in testicular cancer when utilized together with bleomycin and cisplatin that are derived from natural products as well (Da Rocha et al., 2001; Williams et al., 1987). Moreover, marine sources have also been significant sources of anticancer agents. Other sources include marine organisms, soft corals, sponges, seaweeds, and marine invertebrates (Mann, 2002; Faulkner, 2000; Faulkner,2001). Many ecteinascidins have been isolated from the marine tunicate *Ecteinascidia turbinate*. According to pre-clinical research, one of the ecteinascidins (ET-743) is toxic to many tumor cell lines in nanomolar to subnanomolar concentrations (Da Rocha et al., 2001; Rinehart, 2000). *Andrographis paniculata* has also been found to be a useful plant having medicinal properties and anti-cancer compounds. Such plants are believed to possess defensive mechanisms, including toxins that act against parasites and insects, which can be used to prevent and treat human cancer (Da Rocha et al., 2001).

The introduction of the natural active agents into the cancer armamentarium has changed the natural history of most kinds of human cancer. Experimental agents from natural products are offering a significant chance to discover not only entirely new anticancer agents' chemical classes but also new and potentially relevant mechanisms of action (Da Rocha et al., 2001).
In addition, the incidence of cancer can be substantially minimized by changing diets. A diet rich in legumes, vegetables, and fruits which has an immense amounts of antioxidants, can protect against the harmful activity of free radicals that may promote the development of cancer. It has also been illustrated that while synthetic cancer medicines often lead to the non-specific destruction of cells, natural products may contain more selective agents and may have reduced

cytotoxicity for noncancerous cells and may be able to promote nutrient repletion in compromised individuals (Reddy et al., 2003).

1.7. Andrographis Paniculata

Andrographis paniculata, commonly referred to as the “king of the bitters”, is a herbaceous plant belonging to the Acanthacease family (Jayakumar et al., 2013). It is mainly found throughout subtropical and tropical India, Southeast Asia, and Asia. Moreover, its extracts, alongside purified Andrographolide, show pharmacological activities that are immunostimulatory (Kumar et al., 2004), antibacterial (Singha et al., 2003), and antiviral (Calabrese et al., 2000; Jayakumar et al., 2013). In traditional medicine, Andrographolide is broadly utilized to reduce body heat, dispel toxins from the body, avert the common cold and infections of the upper respiratory tract (Gabrielian et al., 2002), and as a snake and insect poison antidote (Samy et al., 2008; Joselin & Jeeva, 2014). Additionally, it has been suggested to have potential therapeutic action in treating colds, frequent coughs, and liver disorders in humans (Geethangili et al., 2008). The distinctive secondary metabolites made by the plant have significant value in the area of medicinal plants (Joselin & Jeeva, 2014).

Figure 1.3. Andrographis paniculata morphology (Jayakumar et al., 2013)

https://www.hindawi.com/journals/ecam/2013/846740/

Andrographis paniculata is an annual, branched, herbaceous plant rising to a height of 30 to 100 cm in humid dark areas (Joselin & Jeeva, 2014). Moreover, the stem is quadrangular; much branched, and is easy to break due to its brittle texture. Its leaves are simple, glabrous, lanceolate, 2 to 12 cm long and 1 to 3cm broad with an entire sharp margin (Joselin & Jeeva, 2014). In addition, inflorescence is axillary and terminal in panicle, 10 to 30 mm long, with a short pedicel and small bract (Joselin & Jeeva, 2014). The flowers have calyx with five sepals that are linear and small. The corolla tubes are narrow, approximately 6 mm long, the upper lip oblong, bilabiate, and white with a yellowish top while the lower tips are widely cuneate, 3 lobed, and white with violet markings (Joselin & Jeeva, 2014).

The aerial part of the plant has been commonly utilized for its medicinal value. Nonetheless, the whole plant, including the roots, is mentioned for particular limited usages in some manuscripts (Akbar, 2011). Traditionally, it was used as a decoction, powder, or an infusion, either in combination with other medicinal drugs or alone. However, in contemporary times, the

commercial preparations used in controlled clinical tests have tended to be standardized extracts of the entire plant (Akbar, 2011).

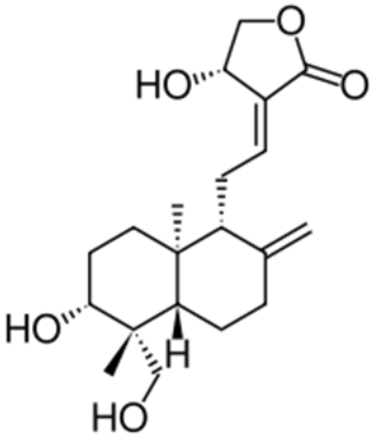


Figure 1.4. The chemical structure of andrographolide (Jayakumar et al., 2013)

https://www.hindawi.com/journals/ecam/2013/846740/

https://en.wikipedia.org/wiki/Andrographolide

Andrographolide is a key bioactive phytoconstituent of *Andrographis paniculata* and is found in various parts of the plant but mostly in the leaves (Chao & Lin, 2010). Andrographolide, has been found to treat variety of diseases including melanoma, lung cancer, leukemia and breast cancer (Jayakumar et al., 2013; Nanduri et al., 2004; Rajagopal et al., 2003). *Andrographis paniculata* has numerous pharmacological properties. Recent research has suggested that it has some immunological potential in that the extract may be capable of interfering with the viability of HIV. It is also a candidate therapeutic anticancer pharmacophore which has dual properties, acting both indirectly and directly on cancer cells (Jarukamjorn & Nemoto, 2008). The methanolic extract of the plant has shown toxicity against lymphocytic and human epidermoid leukemia cell lines (Siripong et al., 1992). Different studies have indicated that andrographolide efficiently induces cell-cycle arrest at the G0/G1 checkpoint in cancer cells

(Geethangili et al, 2008).

Furthermore, extracts containing andrographolide are recognized to have an anti-inflammatory potential (Chiou et al., 1998; Chiou et al., 2000). Plant extracts were also found to inhibit the proliferation of *Plasmodium berghei*, one of the insects known to transmit malaria (Misra et al., 1992). It was also established that *Andrographis paniculata* extracts can cause pregnancy termination, and has anti-fertility effects (Kamal et al., 2003; Deshpande et al., 2014).

1.7.1. Safety and Dosage of Andrographis Paniculata

Andrographis paniculata has been shown to be safe in conventional Chinese medicine. Even though human trial and error may not be regarded scientific, it is a method of establishing whether a substance is harmful or useful. Apparently, when the safety of the plant was under scrutiny from scientists, formal toxicological research in animal models alongside human clinical tests substantiated that andrographolide together with the other constitutes had exceedingly low toxicity (Joselin &Jeeva, 2014).

According to an antifertility test conducted on rats administered with *Andrographis paniculata*, no toxicity was found even at a high dose. The LD50 of andrographolide in male mice administered through the intraperitoneal route was recorded to be 11.46 g/kg (Handa & Sharma., 1990; Jayakumar et al, 2013). Further, in research carried out on HIV-positive individuals, a dose of 1,500 to 2,000 mg andrographolide was administered on a daily basis for six weeks (Jayakumar et al., 2013). The study was concluded early in spite of some enhancements in the count of CD4 and the observation that the side effects were ordinary (Calabrese et al., 2000). Additionally, 10 mg/kg of andrographolide was administered to rabbits, and did not result in changes to the liver, heart, spleen, and kidney (Guo et al., 1988). Moreover, administering andrographolide does not cause cytotoxic impacts to platelets at concentrations between 35 and 150 mM (Lu et al., 2011; Jayakumar et al., 2013). Additionally, research also

indicates that pre-treatment with *Andrographis paniculata* and andrographolide at 500 mg/kg body weight and 125 mg/kg body weight, respectively, could reduce toxicity (Singha et al., 2007; Jayakumar, et al., 2013).

However, similar to all herbs, some people can have an allergic reaction to *Andrographis paniculata*. Nevertheless, the present evidence has shown andrographolide is a naturally occurring compound that has minimal toxicity. Accordingly, it is suggested that it should be clinically tried as a pharmaceutical agent and that using it for health promotion or as an alternative medical therapy should be done cautiously (Jarukamjorn & Nemoto, 2008; Jayakumar, et al., 2013).

1.7.2. Anticancer activities of *Andrographis paniculata*

Andrographis paniculata has a wide variety of uses in traditional medicine in several countries of Southeastern Asia (Lin et al., 2013). The main components of this herb are diterpene lactones, with about 70% of the extract being made up of andrographolide, which has several pharmacological properties and is widely used for the clinical treatment of inflammation, fever, diabetes, cold, diarrhea, among other infectious ailments (Lin et al., 2013). Findings from studies carried out recently suggest that extracts of *Andrographis paniculata* have anticancer as well as immunoregulatory activities (Lin et al., 2013). It can potentially be applied during chemotherapeutic management of various medical conditions (Lin et al., 2013). Despite the wide applications of andrographolide in the traditional management of various conditions, the precise mechanisms involved in its actions are still vague (Shen et al., 2013). This chapter presents a review of several publications reporting findings on the mechanisms of action involved in the anticancer activities displayed by extracts from *Andrographis paniculata*.

1.7.3. Human Colorectal Carcinoma:

In their publication, Lin et al, (2013) present the issue of colorectal cancer, which is among the leading causes of death due to cancer around the globe. When discovered in its early stages, this cancer may be cured by surgical procedures. However, in advanced stages, it often causes death due to recurrence, even among patients treated with combination chemotherapy. Oxaliplatin, CDDP (cisplatin), and other platinum drugs are key chemotherapy agents utilized against various cancers including colorectal, cervical, lung, and testicular cancers, and are particularly important in combination chemotherapy. However, the clinical application of CDDP is greatly limited because of the associated side effects including hepatotoxicity, resistance, and nephrotoxicity. In their article, Lin et al acknowledge the potential of *Andrographis paniculata* as the anticancer agent of choice for use in treatment of colon carcinoma and prevention of side effects. The authors begin with a background review of the findings in recent studies assessing the anticancer effects of the herb widely used in the traditional treatment of a variety of medical conditions in countries in Southeastern Asia (Lin et al., 2013). The findings revisited by these researchers in their publication indicated that extracts from the plant induce arrest of the cell cycle and inhibit growth, cause death of various cancerous cells by apoptosis, increases the cytotoxic potency of cancer drugs such as TRAIL and fluorouracil against cancerous cells, and suppresses the invasion as well as migration of cancerous cells and may thus be used as an anti-metastasis treatment (Lin et al., 2013). Moreover, a study recently demonstrated that andrographolide increases chemosensitivity of CRC cells to doxorubicin by inhibiting the STAT3 pathway (Zhou et al., 2010).

Taken together, the findings from these studies indicate that andrographolide has several targets and is strongly effective in increasing the cytotoxicity of drugs against cancer cells. The authors conducted experimental studies to assess the effect of andrographolide on the

effectiveness of CDDP using the MTT assay and described the specific signaling pathway involved in the apoptotic effects of a combination of CDDP and extracts from *Andrographis paniculata* in colorectal carcinoma cells. The results presented show a reduced viability of cells exposed to andrographolide together with low concentrations of CDDP in comparison with cells treated with either of the compound alone. The findings confirmed activation of apoptosis by Lovo cells exposed to CDDP and andrographolide. These cells show typical morphological changes corresponding to apoptosis such as shrinking of cells and blebbing of membranes, a rise in hypodiploid cells, and movement of phosphatidylinositol towards the outer cell membrane layer in the early stages of apoptosis, as well as caspase-3, caspase-8 and caspase-9 cleavage (Lin et al., 2013). From these observations, the authors concluded that the increase in the cytotoxicity of CDDP, caused by andrographolide, contributes to apoptosis induction in Lovo cells (Lin et al., 2013).

1.7.4. Glioma Cells:

One of the most commonly occurring tumors affecting the central nervous system are gliomas, which may involve astrocytes, ependymal cells, oligodendrocytes, and other glial cells. These tumors are extensively infiltrative and frequently affect essential areas of the brain, and therefore surgical removal is not possible. Management of glioma patients relies heavily on adjuvant treatments including chemotherapy and radiotherapy. Other agents that target specific molecules and factors such as anti-vascular endothelial growth factor and epidermal growth factor receptor are also applied in the control of gliomas. However, patient management in these disease states is associated with an eventual treatment failure. Glioblastoma, a kind of glioma, is associated with very poor prognosis, and is the most highly malignant and common form of glioma. It has a 7.5 % two-year survival rate that drops to a five percent five-year survival rate, with most patients affected by glioblastoma dying within two years. The development of better

agents for the chemotherapeutic management of malignant gliomas are mandatory (Yang et al., 2014).

In their report, Yang and co-authors studied the antitumor activities of andrographolide on C6 glioma cells, a glioblastoma experimental model, and the mechanisms underlying this activity (Yang, et al., 2014). The methodology of this study involved cell culture of C6 glioma cells, cell survival assays, apoptosis detection assays, transfection of small interfering RNA for p53, western blotting, and statistical analysis. The researchers found that andrographolide induced apoptotic death among C6 glioma cells, triggered caspase and PARP cleavage and activation and increased expression of p53. Treatment with andrographolide resulted in the inhibition of growth of the tumors and their regression, which is mediated by the apoptotic cell death induced by andrographolide. This compound's cytotoxicity is selective to cells that are cancerous, and andrographolide has no effect on normal astrocytes, and as such its potential for clinical utilization as an antitumor agent is great (Yang et al., 2014).

1.7.5. Intrahepatic Cholangiocarcinoma:

In their publication, Suriyo et al (2014) describe the inhibitory effects of different diterpenoids isolated from *Andrographis paniculata* on the growth of cancerous cells in intrahepatic cholangiocarcinoma. The main medical issue presented by the authors is cholangiocarcinoma, which includes intrahepatic, perihelia, as well as distal extrahepatic tumors affecting the epithelium of the bile ducts. This form of primary liver cancer is relatively rare, but its incidence rates in southeastern Asia, particularly in Thailand, are significantly high. Epidemiologic studies conducted recently have indicated an increase in the cholangiocarcinoma incidence rates and mortality rates around the globe. Cholangiocarcinoma is a highly aggressive malignancy that is typically characterized by poor prognosis as well as a persistent unresponsiveness to many radiotherapeutic and chemotherapeutic techniques. The treatment of

this condition is quite challenging. Therefore, there is an urgent need for an alternative effective therapy for cholangiocarcinoma (Suriyo et al., 2014).

The potential for use of *Andrographis paniculata* in the management of these cancer has renewed interest among researchers in search of medicinal herbs for use as anticancer agents. Together with its primary constituent diterpenoids, particularly AP1, *Andrographis paniculata* has been found to possess anticancer activity in models of different types of cancers. Suriyo, et al report the inhibitory effects of this herb and its main diterpenoids components at different stages in the growth of cancerous hepatic and bile duct cells (Suriyo et al., 2014).

The researchers prepared extracts of the herb at varying stages of plant growth and determined their diterpenoid content by use of HPLC. Analysis of MLWE (Mature Leaf Water Extract) and FTLEE (First True Leaf Ethanol Extract) from *Andrographis paniculata* revealed a higher content of AP1 in the Mature Leaf Water Extract than in the First True Leaf Ethanol Extract, while both AP3 and AP6 were present at relatively higher concentrations in the First True Leaf Ethanol Extract. By using an MTT assay, the cytotoxic activity of the two extracts on hepatic cell carcinomas and on intrahepatic cholangiocarcinoma were evaluated. The results showed a fourfold higher cytotoxicity of FTLEE on all cell lines in comparison to MLWE. A consequent analysis of the cytotoxic effects of the different diterpenoids on the cancerous hepatocytes and cholangiocytes demonstrated that AP1 had a significantly higher cytotoxic effect than the rest of the diterpenoid components of *Andrographis paniculata*. Due to these observations, Suriyo, et al speculated that the cytotoxic effect that *Andrographis paniculata* has on cancerous hepatocytes and cholangiocytes is a feature of AP1. An interesting finding made by these researchers is the difference in cytotoxic potency of the extracts obtained at different stages of growth of the plant (Suriyo et al., 2014). FTLEE, which was found to have a high AP6 content but low AP1 content in comparison to MLWE, inhibited growth more potently among the

cancerous hepatic and bile duct cells than its counterpart. While the presence of AP1 in MLWE explained its cytotoxic activity against cancer cells, this does not account for the difference that was observed for the cytotoxicity of the extracts. The authors hypothesized there were interactions occurring between the main active diterpenoids in the extracts, particularly AP1 and AP6, which have a role in the compounds' growth inhibitory effect. The effect of other diterpenoids apart from the four on which this study focused could account for the difference in cytotoxic potency between FTLEE and MLWE. This hypothesis is not conclusive, and calls for further studies in this direction (Suriyo et al., 2014).

This study showed that the cytotoxic effect of FTLEE relied on cell cycle arrest and increased apoptosis in the cancerous cells. FTLEE induced arrest of the cell cycle at GO/G1 as well as in G2/M phases, and was followed by a decrease in the expression of cyclin D1. Experiments that were designed to assess the cytotoxic activity of AP1 in HuCCA-1 and RMCCA cells, revealed a significantly higher cytotoxic potency of this compound. This difference was hypothetically attributed to the varying etiology of the cancerous cells used, particularly their inflammatory background. The role of cyclooxygenase 2 in the inhibition of apoptosis mediated by Fas and the difference in the cyclooxygenase content between f HuCCA-1 and RMCCA cells were also implicated in the observed differences in the cytotoxic potency of AP1 against these cancerous cell types (Suriyo et al., 2014). These studies demonstrate the necessity for supplementary studies to complement this publication and the dependence on the findings from other studies.

1.7.6. Inflammation and cancer:

Studies have shown an association between inflammation and the development and progression of cancer (Rakoff -Nahoum, 2006). Inflammation is the host response to infection by microbes and mediates the repair and regeneration resulting from tissue damage. The

inflammatory response involves the release of several growth and activating factors by the immune system that can result in cell activation. Inflammation can also promote tissue damage in addition to that caused by infection. Epidemiological evidence suggests a correlation between the inflammatory responses and predisposition to cancer development. Prolonged inflammation causes cell dysplasia. Moreover, an estimated 15% of cancer incidence in the global population is linked to infection by microbes or viruses. Chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and human papilloma virus (HPV) can cause hepatocellular carcinoma and cervical cancers. Opportunist infection by human herpes virus-8 (HHV 8) may result in the development of Kaposi's sarcoma. Inappropriate responses by the immune system in some individuals result in the development of gastric cancer following colonization by Helicobacter pylori or colon-cancer development secondary to prolonged inflammatory bowel disease that are caused by microflora in the intestines (Rakoff-Nahoum, 2006).

In other cases, chronic irritation together with the inflammation that follows, predispose to cancer, as is the case with chronic exposure to asbestos, silica, and cigarette smoke. Signs of inflammation like leukocyte infiltration are a key feature of almost all types of tumors. The utilization of non-steroidal anti-inflammatory drugs in preventing the spontaneous formation of tumors in individuals having familial adenomatous polyposis presents another piece of evidence on the role of inflammation in cancer (Rakoff-Nahoum, 2006).

Inflammation and cancer development are linked by epidemiological evidence, histopathological changes, inflammatory profiles, as well as the effectiveness of anti-inflammatory agents in cancer prophylaxis (Rakoff-Nahoum, 2006). Agents that are effective in the control of inflammatory responses are of considerable importance in the overall management of various cancers, and andrographolide, the most active component isolated from *Andrographis paniculata,* is one of these agents (Chen et al., 2014). Analysis shows that andrographolide has

inhibitory effects on vascular smooth muscle cells after exposure to a stimulus that causes inflammation (Chen et al., 2014). The main issue presented here is vascular inflammation, which at some critical level causes cardiovascular illnesses such as atherosclerosis, hypertension, and vascular dysfunction. After stimulating smooth muscle cells with tumor necrosis factor-α, andrographolide was administered, resulting in the suppression of inducible NO synthase expression in a manner that was concentration-dependent and involved induction of JNK-Akt and p65 phosphorylation (Chen et al., 2014).

These findings, were complemented by observations made following administration of LY294002 (an inhibitor of Akt activation), suggested that andrographolide can be effectively used in the therapy of inflammatory diseases affecting blood vessels by inhibiting NF-kB activity through the JNK-Akt and p65 signaling cascade (Chen et al., 2014). A similar study was conducted by Shen and co-authors in 2013 to determine the molecular mechanisms underlying andrographolide's anti-inflammatory activity. The researchers used kinase assays, measurements of transcription factor levels in the nucleus, and luciferase reporter-promoter gene expression assays to determine the molecular targets of andrographolide. Andrographolide was found to suppress the release of NO, prostaglandin E2, and decreased tumor necrosis factor-α, interferon β, cyclooxygenase, and inducible NO synthase mRNA in peritoneal macrophages as well as in RAW264.7 cells activated by lipopolysaccharide, in a concentration-dependent manner (Shen et al., 2013). Andrographolide was also found to ameliorate symptoms of hepatitis induced by lipopolysaccharide and gastritis induced by HCl in mice. The study revealed that andrographolide's anti-inflammatory effects were mediated by the IKKε/ IRF- 3 pathway. The authors concluded that there are novel pathways used by andrographolide in suppression of inflammatory responses (Shen et al., 2013). Therefore, as indicated by findings from these

studies, andrographolide is a potential anti-inflammatory agent having two pharmacological targets (Shen et al., 2013).

1.8. Apoptosis

Apoptosis is derived from “dropping off” in Greek and refers to the falling of tree leaves in autumn (Wong, 2011). Moreover, the term, apoptosis, is used to explain the condition whereby a cell vigorously pursues a course toward death on receiving certain stimuli (Kerr, 1991; Wong, 2011). Clearly, the idea that apoptosis is a gene-directed program to cause the death of a cell has had significant implications for the understanding of tissue homeostasis and in developmental biology (Lowe & Lin, 2000). The ability to induce apoptosis implies that the number of cells can be controlled by specific processes in addition to those that regulate differentiation and proliferation. Further, the genetic basis for apoptosis implies that the death of a cell, similar to any other developmental or metabolic program, can be upset by mutation (Lowe & Lin, 2000). Apoptosis pathway defects are now thought to be key contributors to numerous human illnesses, ranging from malignancy to neurodegenerative disorders (Lowe & Lin, 2000; Thompson,1995). A tumor is the result of aberrant growth of cells and can result from increases in cell proliferation or by disruption of normal processes of cell death and apoptosis.

1.8.1. Morphological changes:

Apoptosis is characterized by a very specific set of morphological characteristics that includes changes in nuclear shape, condensation of chromatin, alongside the fragmentation of the nucleus that are accompanied by cellular volume reduction, cell rounding and retraction of pseudopods (Kroemer et al., 2005; Wong, 2011). The condensation of the chromatin begins at the nuclear membrane periphery, forming a ring or crescent-like structure. It further condenses

until it breaks up inside the cell even though the plasma membrane remains intact, a characteristic known as karyorrhexis (Manjo & Joris., 1995). Notably, although the plasma membrane remains intact during most of the process, there are numerous morphological features in the late phases of apoptosis which include loss of membrane integrity, organelles, cytoplasmic ultrastructure modification, and membrane blebbing (Kroemer et al., 2005). Typically, phagocytic cells engulf apoptotic cell fragments prior to the release from the apoptotic bodies (Wong, 2011).

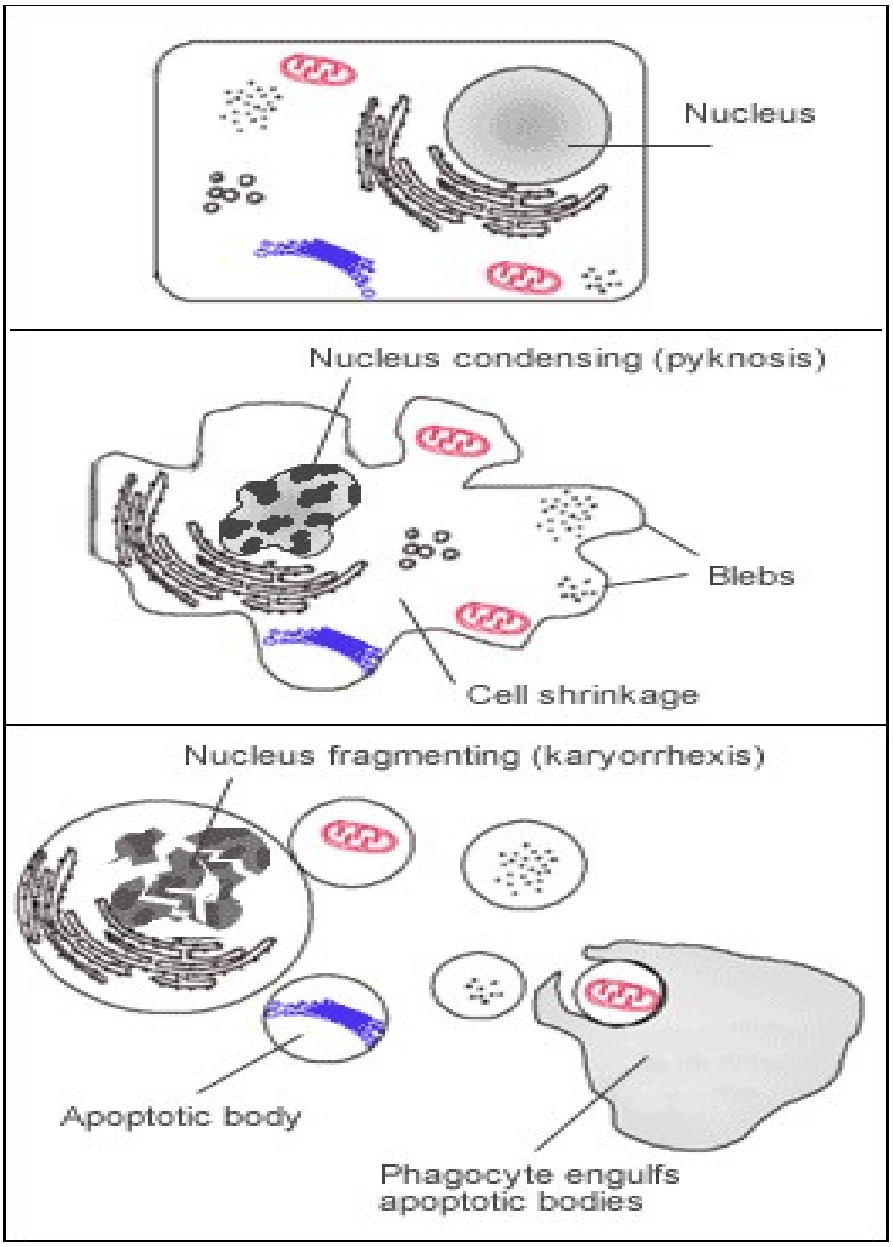


Figure 1.5. Morphological changes in apoptotic cells.

https://en.wikipedia.org/wiki/Apoptosis

1.8.2. Biochemical changes:

Three key biochemical changes can be observed during apoptosis which includes; the activation of caspases, protein and DNA breakdown, and membrane changes to promote recognition by phagocytic cells (Wong, 2011; Kumar et al., 2014). In the early stages of apoptosis, phosphatidylserine (PS) becomes expressed on the exterior side of the plasma membrane because it has been flipped from the cytoplasmic side (Gerl & Vaux, 2005; Wong, 2011). The presence of PS on the surface of the cell (and apoptotic bodies) permits macrophages to recognize and phagocytose the apoptotic cells before they can release DNA or proteins which would activate the immune system and promote the release of pro-inflammatory cytokines (Hengartner, 2001). PS expression on the cell surface is followed by DNA breakdown into large kilobase pieces (Vaux-Silke, 2003) and later, by internucleosomal cleavage of the DNA into oligonucleosomes of approximately 150 bp (Wong, 2011). Additionally, another feature of apoptosis is the activation of a group of enzymes that belong to the family of cysteine protease called caspases. Activated caspases cleave numerous essential cellular proteins in addition to breaking up the nuclear scaffold and cellular cytoskeleton. Furthermore, caspases can activate DNAase that further degrade nuclear DNA (Lavrik et al., 2005; Wong, 2011).

1.8.3. Apoptotic Mechanisms:

The apoptotic process can be induced either by external or internal cell signals. In the case of the external death (extrinsic) pathway, apoptosis is initiated when extracellular proteins called death ligands attach to the death receptor on the target cell. Even though there are numerous known death receptors, the most commonly recognized ones are the type 1 TNF receptor (TNFR1) and a related protein known as Fas (CD95). Additionally, their ligands are TNF-like molecules and Fas ligands (FaSL) (Hengartner, 2003; Wong, 2011). Death receptors bind the death ligands and result in the formation of a death-inducing signaling complex in the target cell

cytoplasm which initiates the apoptotic process by activating caspase 8. When caspase 8 is activated, it is able to activate other downstream caspases to ultimately destroy the cells (Wong, 2011).

On the other hand, the interior activated (or intrinsic) pathway begins inside the cell. Accordingly, internal stimuli including irreparable genetic destruction, escalated concentrations of cytosolic calcium, hypoxia, and severe oxidative stress are some triggers that initiate the pathway (Wong, 2011; Karp et al., 2014). Although there are many different activators, they all result in an increase in mitochondrial permeability and the release of pro-apoptotic molecules including cytochrome-c into the cytoplasm (Danial-Korsmeyer, 2004). Cytoplasmic cytochrome c, in turn, attaches to cytoplasmic proteins including Apaf-1 and caspase 9, a process that results in the formation of the apoptosome. Finally, the formation of the apoptosome activates the caspases responsible for initiating the apoptosis process (Kroemer et al., 2007; Wong, 2011).

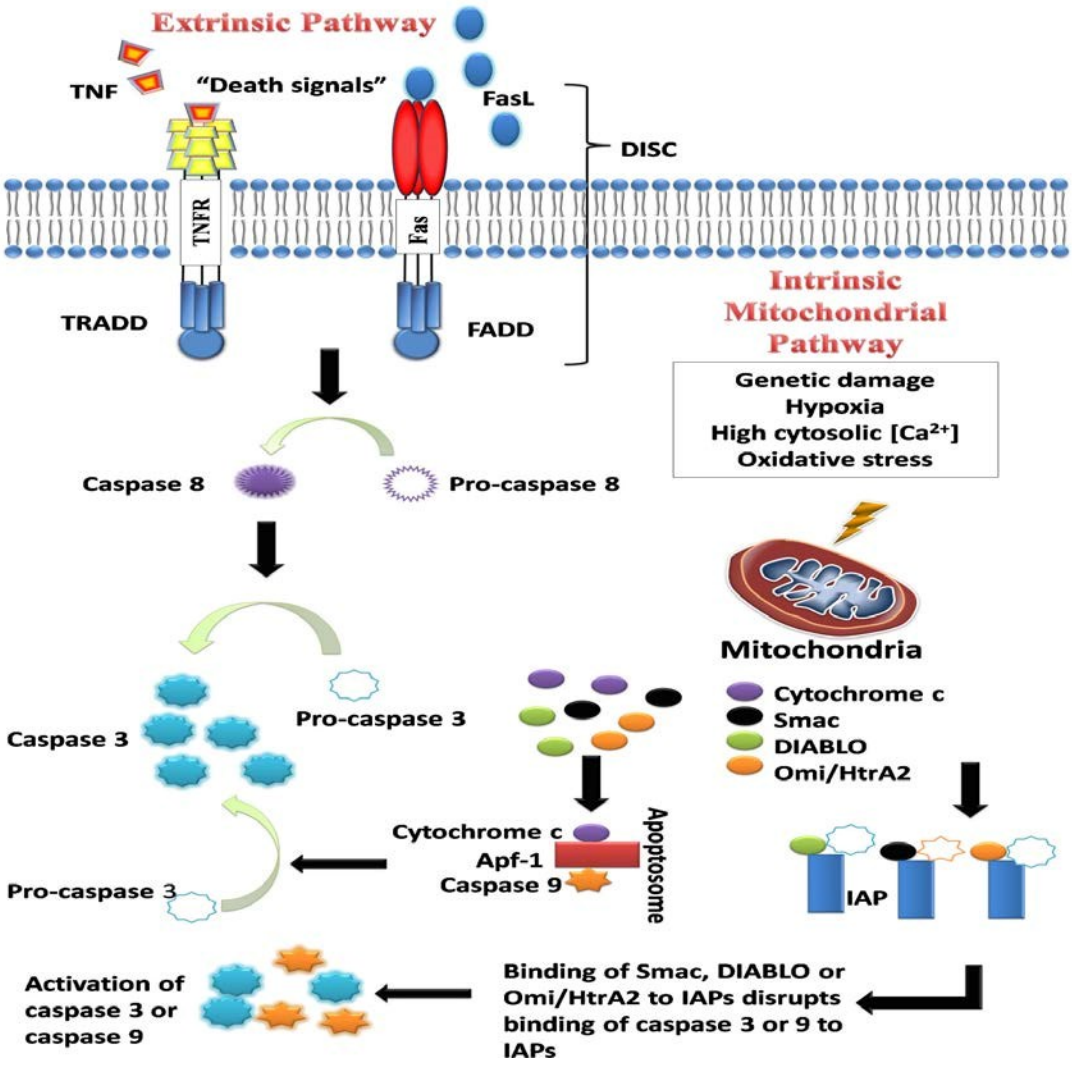


Figure 1.6. The apoptosis pathways [extrinsic, intrinsic] (Wong., 2011).

http://jeccr.biomedcentral.com/articles/10.1186/1756-9966-30-87

1.8.4. Caspases:

Before its activation, a caspase exists within a cell in an inactive form known as procaspase. The procaspase contains an N-terminal leader peptide that acts to inhibit its enzymatic activity. The activation of the procaspase involves enzymatic cleavage of this peptide to allow activity: active caspases are always smaller than procaspases. There are different types of caspases, and the formation of the apoptosome activates some of them, including caspase-8, caspase-9, 10 and 2. These initiator caspases, in turn, cleave and activate other caspases, including caspase-3, caspase-6 and caspase-7. Once activated, the effector caspases degrade the cell's components, resulting in the destruction of its DNA, chromosomes, and membrane. The cell dies and is removed by phagocytosis (Fink& Cookson, 2005; Wong, 2011).

Summary:

Andrographolide, which is the most abundant and active component isolated from extracts of *Andrographis paniculata*, has a wide variety of pharmacological activities and has been utilized in the traditional management of several medical conditions in countries in Southeastern Asia. This component of *Andrographis paniculata* has been shown to have anticancer effects against several cancer types, including intrahepatic cholangiocarcinoma, gliomas, and human colorectal carcinoma, and the mechanism mainly involves enhancement of apoptosis and arresting of the cell cycle. By use of mechanisms that inhibit NO production and expression of factors such as TNF and cyclooxygenase, Andrographolide also has anti-inflammatory activity.

1.9. Research objectives:

This research centers around the effect of *Andrographis paniculata* on the growth of malignant cancer cells and examines the mechanisms underlying these effects. The research questions are fashioned based on the actions of 70% ethanol extracts and aqueous extracts of *Andrographis paniculata* on malignant cancer cells, especially B16-BL6 cells. A key hypothesis of this study is that *Andrographis paniculata* extracts suppress the growth or induce the death of the malignant cancer cells.

- **The purposes of this research are to:**

1- Study the effect of 70% ethanol extract and aqueous extract of *Andrographis paniculata* on the growth of malignant cancer cells including B16-BL6, ASPC-1, 4T1, MCF-7, and MDA-MB-231 cell lines.

2- Determine the effect of *Andrographis paniculata* extracts on B16-BL6 murine melanoma cell morphology.

3- Examine caspase 3 activation and inhibition of ERK phosphorylation to investigate whether *Andrographis paniculata* extracts block survival signals and induce apoptosis as a mechanism of action in B16-BL6 murine melanoma cells.

4- Determine the effect of 70% ethanol extract and aqueous extract of *Andrographis paniculata* on the B16-BL6 cell cycle profile.

Chapter 2: Materials and Methods

2.1. Cancer cell lines:

The malignant cancer cell lines presented in this chapter are the following:

1. **B16-BL6 cell line:** These cells originated from malignant mouse epidermal melanocytes and have the property of invading secondary organs like liver, lungs, and brain (Kushiro& Nunez., 2012).
2. **ASPC-1 cell line:** Isolated from the ascites cells from a 62-year-old Caucasian women with pancreatic cancer. The cells were shown to be pancreatic cancer cells that were able to express carcinoembryonic antigen (CEA) (Public Health England., 2016).
3. **MCF-7 cell line:** These cells were derived in 1970 from the breast tissue, of a 69-yearold Caucasian woman with triple positive cancer who presented the malignant adenocarcinoma in the pleural effusion (Altogen Biosystems., 2016).
4. **4T1 cell line:** These are triple negative murine breast cancer cells that are transplantable, extremely tumorigenic and can metastasize from mammary gland tumors to secondary organs like lymph nodes, brain, bone, liver and lungs (Pulaski-Rosenberg., 2001).
5. **MDA-MB-231 cell line:** These cells were derived from the pleural effusion of a 51-yearold woman with triple negative breast cancer malignant breast cancer (Public Health England., 2016).

2.2. Tissue culture:

B16-BL6, MCF-7, MDA-MB-231, ASPC-1 and 4T1 cell lines were purchased from the American type culture collection (Manassas, VA 20110, United States) an American company that specializes in the sale of cells, microorganisms, culture reagents, and other related products.

B16-BL6, MCF-7 and MDA-MB-231 cell lines were maintained 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). ASPC-1 and 4T1 were maintained in RPMI medium (Hyclone, Logan, UT), supplemented with 10% fetal bovine serum (Hyclone) 100 μg/ml streptomycin, and 100 U/ml penicillin. Thereafter, all cell lines were incubated in a humid atmosphere at 37°C and 5% CO_2. The cells were subcultured by removing the media from the cell culture plate, washing the cells with PBS to remove all media traces, adding 7 ml of trypsin to the cell culture plate and placed in the incubator for five minutes, collecting the trypsin and a wash of 7 ml of culture media into a centrifuge tube, and then pelleting the cells by centrifugation at 400xg for 10 min. New media was added to the cells and an aliquot added to a new culture plate containing media and then incubated in a humid incubator at 37˚C, 5% CO_2. During the incubation period, the cells were subcultured every three-to-five days, depending on the growth rate of the cells.

2.3. Plant Materials:

Different sources of *Andrographis paniculata* were used in these experiments:

1- *Andrographis paniculata* containing 33% andrographolide and derived from the whole plant. This plant preparation was purchased from Durham Natural Foods (1191 Montrose Avenue, Sudbury, Canada, Ontario: 80040811).

2- *Andrographis paniculata* extract containing 10% andrographolide and derived from the aerial parts of plant. This plant preparation was purchased from Nutrition House (Dundas St W, Toronto, Canada, ON: 80029281).

2.4. Plant extract preparation:

Two categories of *Andrographis paniculata* extraction prepared for these experiments:

1- Preparation of aqueous (PBS) extract of *Andrographis paniculata*:

600 mg of *Andrographis paniculata* powder mixed with 6 ml of PBS.

2- Preparation of 70% Ethanol extract of *Andrographis paniculata*:

600 mg of *Andrographis paniculata* mixed with 6 ml of 70% EtOH.

Both mixtures were boiled for one hour, made up to the appropriate level with solvent, and then filtered through a 0.22 uM syringe filter. The extracts were aliquoted and stored at -80˚C. To evaluate the stability of the plant, some of the extracts stored at 4˚C and tested at multiple time points.

2.5. MTT cell viability assay (Methyl Tetrazolium Blue):

The overall aim of the experiment was to measure the effects of the aqueous or ethanol extracts of *Andrographis paniculata* on cell proliferation. To achieve this aim, the proliferation of the B16-BL6, ASPC-1, MDA-MB-231, MCF-7 and 4T1 cell lines was used to examine the effect of the extracts of *Andrographis paniculata* (33% andrographolide) stored at -80°C. Additionally, *Andrographis paniculata* extracts stored at 4°C for 3 months were tested on the B16-BL6 cell line to assess the stability of the plant extract. However, another source of *Andrographis paniculata* containing 10% andrographolide was also tested on B16-BL6 cell line and compared to the other source. Moreover, media controls (untreated cells) were used as a negative control. Some cells were tested with equivalent amounts of PBS in media or ethanol in media to control for the solvent used to extract the *Andrographis paniculata*.

Method:

Over 10^3 cells/well were plated in each well of the 5 different 96 well plates and allowed to grow in the media at 37°C and 5% CO_2 for 24 hours. Thereafter, some cells were treated with the PBS extract of *Andrographis paniculata* while other cells were treated with the 70% Ethanol extract of *Andrographis paniculata* at various concentrations (0.1, 0.25, 0.50, 0.75, and 1%). During a five-day incubation period, one of the plates was subjected to the MTT assay each day. For the assay, 10 ul of MTT solution (0.125 mg of MTT powder mixed with 50 ml of PBS and stored at 4°C in the dark) added to each plate and incubated for 4 hours. The MTT solution was removed from the wells and 100 ul DMSO (dimethyl sulfoxide) added to each well to solubilize the formazan crystals. Subsequently, the absorbance corresponding to the MTT product, corresponding to cells treated with different concentrations of *Andrographis paniculata* extracts was analyzed by performing ANOVA using Graph Pad Prism Software.

2.6. Cell Morphology:

The overall aim of this experiment was to assess the effect of *Andrographis paniculata* extracts (aqueous-ethanol) on the morphological changes in the cells (shape and appearance) under the microscope. To realize this objective, *Andrographis paniculata* extracts were used to treat B16-BL6 and MDA-MB-231 cell lines for two days.

Method:

The B16-BL6 and MDA-MB-231 cell lines were cultured and allowed to grow in DMEM media at 37°C and 5% CO_2 for 24 hours. Thereafter, some of the cells were treated with the PBS extract of *Andrographis paniculata* while other cells were treated with the ethanol extract of *Andrographis paniculata* at low and high concentrations for 48 hours. Media controls (untreated cells) were used as a negative control. After 48 hours, an Olympus DP80 microscope was used to

image the treated and untreated cells. Thereafter, the observations were recorded with the aid of Cell Sens Dimension computer software.

2.7. Acridine Orange/Ethidium Bromide staining assay:

The acridine orange and ethidium bromide staining assay was used to visualize nuclear changes (morphological changes) and apoptotic body formation that are characteristic of apoptosis in B16-BL6 cells.

- Acridine orange: is a vital stain that stains both live and dead cell's nucleic acids green.
- Ethidium bromide: is a vital stain that stains DNA red only in cells that have lost membrane integrity.

Method:

B16-BL6 cells were plated on sterile glass cover slips in 60x15 mm tissue culture plates in DMEM culture media and allowed to grow in the incubator at 37°C and 5% CO_2 for 24 hours. The cells were then divided into three groups. The first group of cells were treated with the ethanol extract of *Andrographis paniculata* while the second group of cells was treated with the aqueous extract at low and high concentrations for 48 hours. Media controls (untreated cells) were incubated for 48 h and used as a negative control. After the 48-hour treatment, the cells were stained by incubation in culture media containing 10 ug/ml of Acridine orange (Sigma Aldrich) and 10 ug/ml of Ethidium bromide (Sigma-Aldrich) for 15 minutes. The coverslips were washed with PBS and mounted in a drop of (80% glycerol in PBS) on the microscopic slides. Then, clear nail polish was added on the slides to seal the coverslips. The fluorescence was visualized using an LSM510 microscope.

2.8. Western Blot Analysis:

The overall aim of this experiment was to analyze changes in specific proteins from the cell lysate. To achieve this aim, *Andrographis paniculata* extracts were tested on B16-BL6 cells and cell lysates prepared and analyzed.

Method:

B16-BL6 cells were cultured and maintained in DMEM media and allowed to grow at 37°C and 5% CO_2 for 24 hours. The cell cultures were divided into four groups. The first group of the cells was treated with the ethanol extract of *Andrographis paniculata* at various concentrations and the cells in the second group were treated with the PBS extract of *Andrographis paniculata* at various concentrations for 24 hours and 48 hours. In addition, a UV exposed plate of cells was utilized as a positive control for induction of cell apoptosis and a media control (untreated cells) was used as a negative control. After treatment, the B16-BL6 cells were harvested, collected by centrifugation, and homogenized in 300 ul of lysis buffer (RIPA buffer: 150 mM sodium chloride, 50 mM Tris-HCl, pH 7, 1% Triton x-100, 0.5% sodium dodecyl sulphate, 0.5% sodium deoxycholate) with ¼ tablet of protease inhibitors (Roche). Subsequently, a 10% of polyacrylamide gel containing sodium dodecyl sulphate (SDS) (10% AcryI Bis, 0.125 M Tris- HCl, pH 8.8, 2% SDS, 0.005% TEMED, 0.01% APS) was utilized to separate the proteins in the cell lysate. A sample of each cell lysate, was normalized for cell number, boiled in sample buffer (0.125 M Tris-HCl, pH6.8, 4% SDS, 20% glycerol, and 0.1 mg/ml bromophenol blue), and loaded into the wells of the gel. The gels were electrophoresed in running buffer (Tris-Glycine/SDS) at 90 volts for approximately 1 ½ hours. Thereafter, the proteins were transferred from the gel to a nitrocellulose membrane using a Bio Rad semi-dry transfer machine for 45 minutes at 12v. The membrane was then stained with 0.1% red ponceau S in 1% acetic acid stain for 10 min and then rinsed with sterile water. A gel documentation

system was utilized to take a photo of the membrane to prove that the lanes of the gel were equally loaded. The membrane was blocked with 5% BSA in Tris-buffered saline containing Tween-20 (TBST, 50 mM Tris-HCl, pH7.5, 150 mM sodium chloride, 0.1% Tween-20) solution and incubated at room temperature for 1 hour. Next, the blots were incubated with one of the following primary antibodies (1:1000 dilution of caspase 3 monoclonal mouse antibody, 1:1000 dilution of phosphorylated-ERK monoclonal mouse antibody or 1:1000 dilution of ERK1/2 polyclonal goat antibody) overnight at 4°C on a rocker platform. The membrane washed with TBST three times for 10 min each. A suitable secondary antibody (1:10.000 dilution of goat anti-mouse IgG-HRP or rabbit anti-goat IgG-HRP conjugate) in 5% BSA in TBST was incubated with the membrane at 4°C for 1 hour. The membrane was washed with TBST for three times for 10 min each. A TBS solution was incubated with the membrane for 5 mins. The membrane was placed face down in the ECL reagent for 5 mins and face up for 2 mins and then inserted in a plastic sheet into the X-ray cassette with a sheet of X-ray film placed on top of the membrane, and then exposed. The film was developed using an XOMAT film developing machine.

2.9. Flow Cytometry:

The objective of this experiment was to determine the effect of *Andrographis paniculata* (PBS and ethanol extracts) on the cell cycle distribution of B16-BL6 cells by measuring the DNA content.

The propidium iodide (staining solution) is a fluorescent dye that intercalates into the DNA so that the level of fluorescence corresponds to the amount of DNA. It can also stain the apoptotic cells, which have decreased DNA content due to nuclear fragmentation (creating a sub G1 peak).

Method:

B16-BL6 cells were cultured in 60x15 mm tissue culture plates by incubating them at 37°C and 5% CO_2 for 24 hours. Then, the cell cultures were divided into four groups. One group of cells was treated with the PBS extract of *Andrographis paniculata* at low and high doses while the cells in the second group were treated with the ethanol extract of *Andrographis paniculata* at low and high concentrations. Further, untreated cells were used as a negative control whereas a UV exposed sample was used as a positive control for apoptosis. After treatment, the cells were washed with PBS and trypsin was used to harvest them. The cells were pelleted by centrifugation at 400xg for 5 minutes and washed with PBS. The B16-BL6 cells were then fixed by incubation in cold 70% ethanol and stored at -20°C until used. Subsequently, the cells were washed with PBS and then 0.5 ml of PBS and 0.5 ml of PI solution (distilled water, 1 ug/ml RNAase, 10 ug/ml propidium iodide, 0.05% Triton 100, and 150 mM NaCl) were added to each centrifuge tube and incubated for 30 min in the dark. Finally, the samples were filtered through a 0.5 um filter sheet with the aid of a filter syringe and then transferred to the flow tube. The stained B16-BL6 cells were analyzed using a Beckman coulter LS600 flow cytometer.

Chapter 3: Results

3.1. Cancer cell viability (70% ethanol extract versus PBS extract):

The MTT assay was used to determine the effect of *Andrographis paniculata* extracts on the cancer cell's viability. B16-BL6 murine melanoma cells were treated with two different extracts (ethanol & PBS) of *Andrographis paniculata* containing 33% andrographolide at low concentration, middle and high concentration. Treatment of cells with the ethanol extract of *Andrographis paniculata* revealed a significant inhibition of cell growth at high concentration and middle concentration while the low dose of the ethanol extract promoted an much smaller decrease in cell number (Figure 3.1.). However, treatment of the cells with the aqueous extract of *Andrographis paniculata* showed a significant decrease in the cell number only at the highest concentration while treatment with the PBS extract at the middle concentration showed a slight decrease in cell number while the cell number at the low dose of PBS extract was the same as the untreated cells (Figure 3.2.).

To evaluate the stability of the drug, B16-BL6 cells treated with *Andrographis paniculata* extracts (ethanol, aqueous) that had been stored at 4˚C for approximately 3 months. The findings suggested that the viability assay of cells treated with the ethanol extract and aqueous extract was similar to the drug extracts that were stored at -80˚C (Figure 3.3. and 3.4.). Consequently, the effects of *Andrographis paniculata* extracts were stable in the extracts when stored at 4˚C and still had the ability to inhibit cell growth.

In addition, B16-BL6 cells that were treated with different source of *Andrographis paniculata* containing 10% andrographolide extracts (aqueous, ethanol), presented results that were similar to extract made for *Andrographis paniculata* obtained from the second source (Figure 3.5.and 3.6.). However, this plant extract displayed a little difference, which suggested that the ethanol extracts have a similar ability to inhibit cell growth at all concentration levels

while the aqueous extract only inhibited cell growth at the highest concentration and middle concentrations.

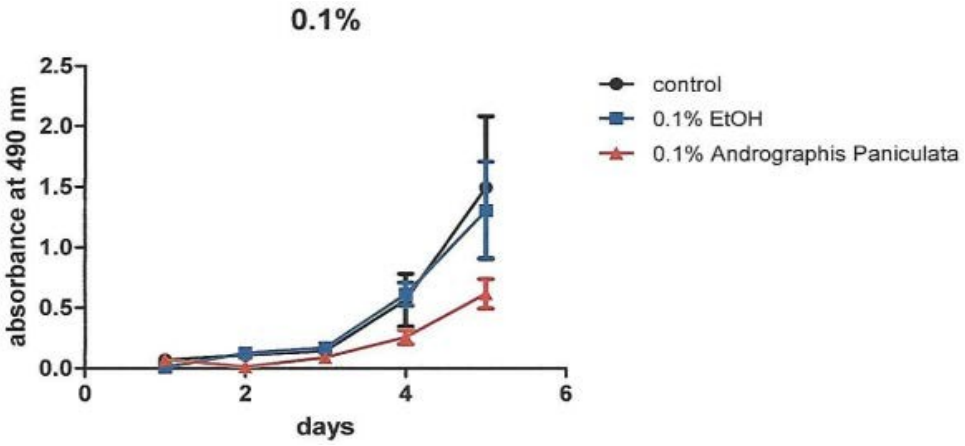
ASPC-1 cells that were treated with the ethanol extract of *Andrographis paniculata* showed an inhibition of cell growth at low, middle, and high concentration in comparison to untreated cells that showed a typical increase in cell number (Figure 3.7.). In addition, an analysis of the effect of the aqueous extract of *Andrographis paniculata* on ASPC-1 cells at high concentration suggested that there was a decline in the cell number (Figure 3.8.). However, treatment with the aqueous extract at low and middle concentration demonstrated that there was a much weaker effect on cell growth. MCF-7 (human breast cancer-triple positive) cells that were treated with *Andrographis paniculata* extracts showed strong inhibition of growth when treated with high, medium and low concentrations of the ethanol extract (Figure 3.9.) but only strong inhibition when treated with high concentration of the aqueous extract (Figure 3.10.), similar to the observations with the ASPC-1 cells.

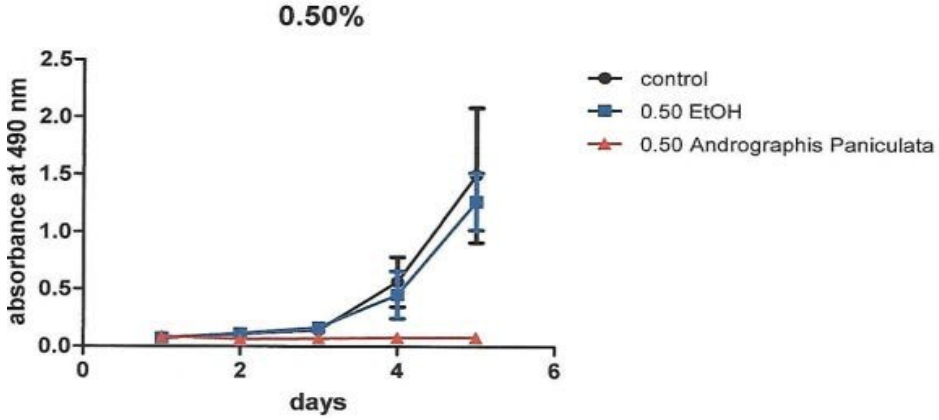
However, 4T1 (mouse breast cancer-triple negative) and MDA-MB-231 (human breast cancer-triple negative) cells treated with the ethanol extract of *Andrographis paniculata* showed a significant inhibition in cell growth at high and middle concentrations but a weaker effect at the low concentration (Figure 3.11. and 3.13.). In contrast, treatment of 4T1 and MDA-MB-231 cells with the aqueous extract of *Andrographis paniculata* did not significantly inhibit the cell growth, which implies that the cells continue proliferation (Figure 3.12. and 3.14.).

In summary, these findings demonstrated that the effect of ethanol extract of *Andrographis paniculata* on the cancer cells was greater than the effect of the aqueous extract. Further, the results suggested that there were significant variation in sensitivity between the cell lines.

Figure 3.1. The effect of ethanol extract of *Andrographis paniculata* containing 33% andrographolide on B16-BL6 cells growth.

B16-BL6 murine melanoma cells were treated with the ethanol extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant, at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The B16-BL6 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 490 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
days
control
0.1% EtOH
0.1% Andrographis Paniculata


0.50%
absorbance at 490 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
days
control
0.50 EtOH
0.50 Andrographis Paniculata

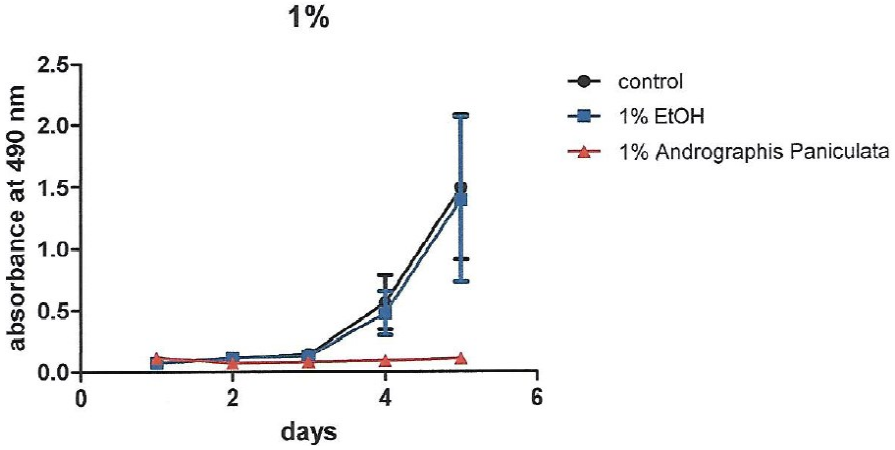
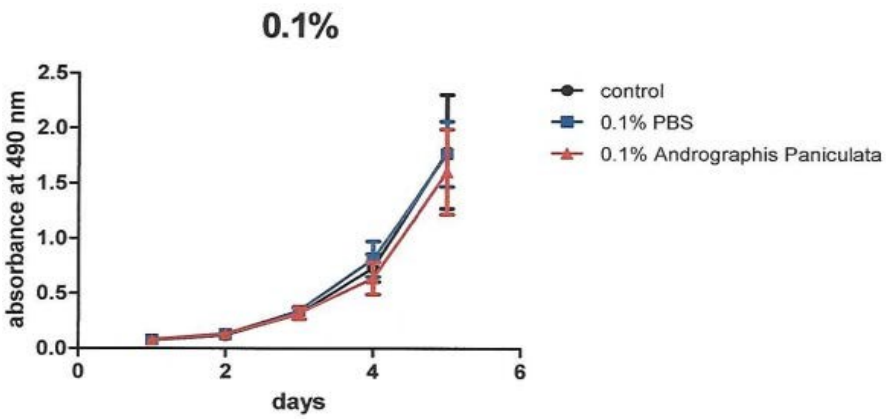
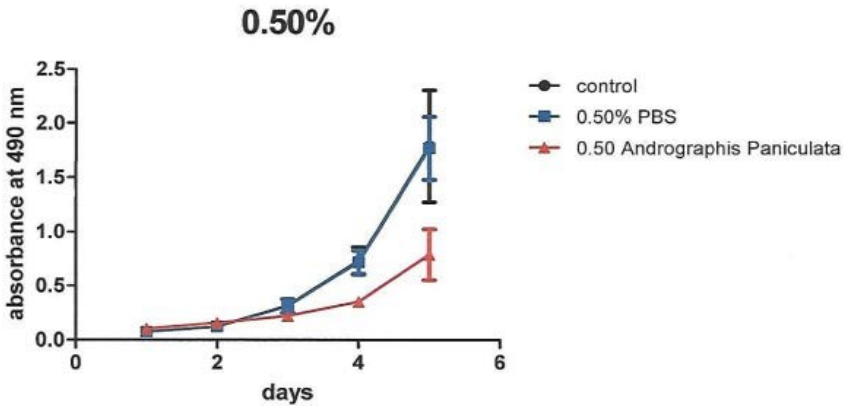

1%
absorbance at 490 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
days
control
1% EtOH
1% Andrographis Paniculata

Figure 3.2. The effect of aqueous extract of *Andrographis paniculata* containing 33% andrographolide on B16-BL6 cells growth.

B16-BL6 murine melanoma cells were treated with the PBS extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant, at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The B16-BL6 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 490 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
days
control
0.1% PBS
0.1% Andrographis Paniculata


0.50%
absorbance at 490 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
days
control
0.50% PBS
0.50 Andrographis Paniculata

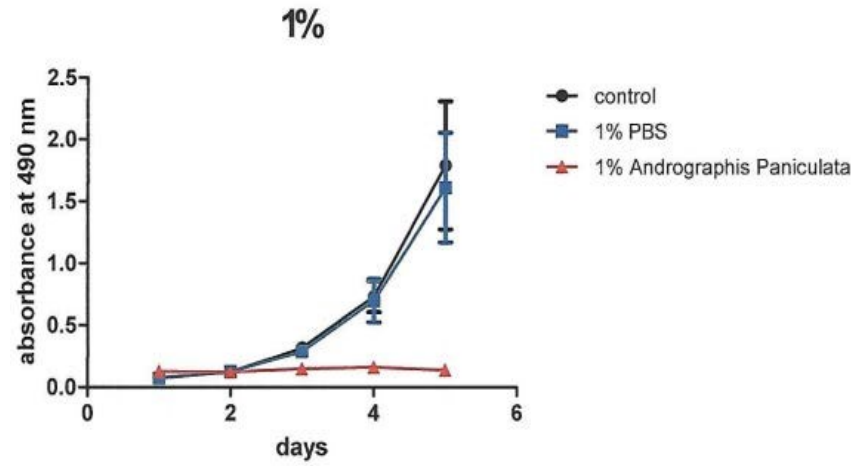
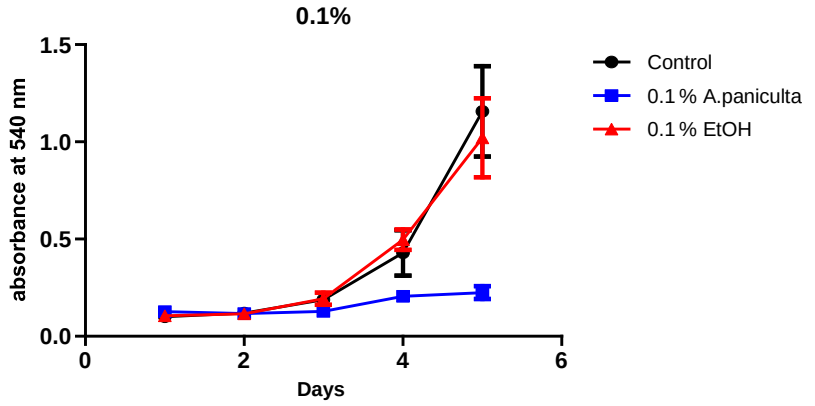
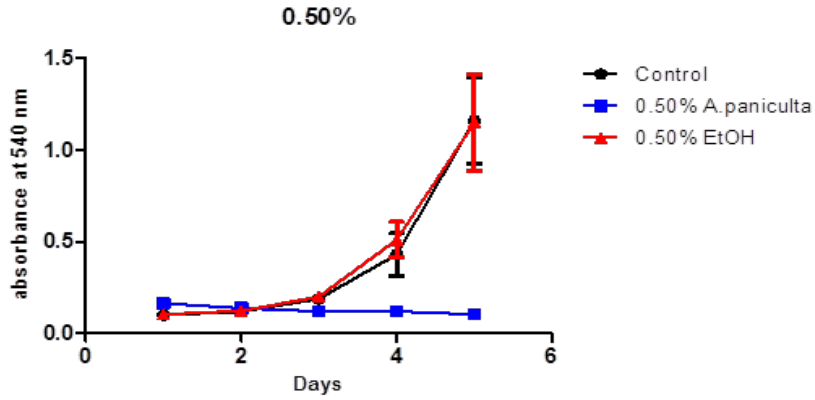

1%
absorbance at 490 nm
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
days
control
1% PBS
1% Andrographis Paniculata

Figure 3.3. The stability of ethanol extract of *Andrographis paniculata* that stored at room temperature for 3 months.

B16-BL6 murine melanoma cells were treated with the ethanol extract of *Andrographis paniculata* (33% andrographolide) that had been incubated at 4˚C for approximately 3 months. The cells were incubated with low (0.1%), middle (0.50%) and high (1%) concentrations of the extract for five days. The B16-BL6 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.1% A.paniculta
0.1% EtOH


0.50%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.50% A.paniculta
0.50% EtOH

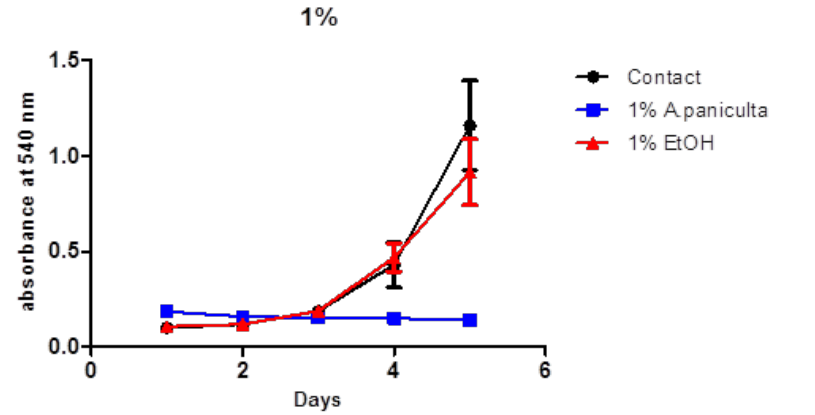
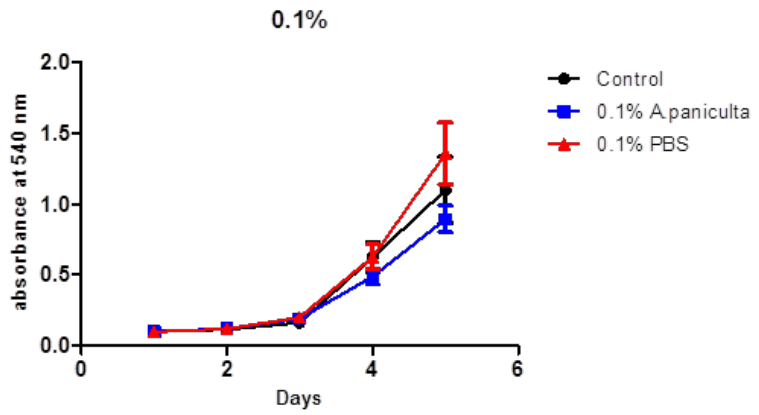
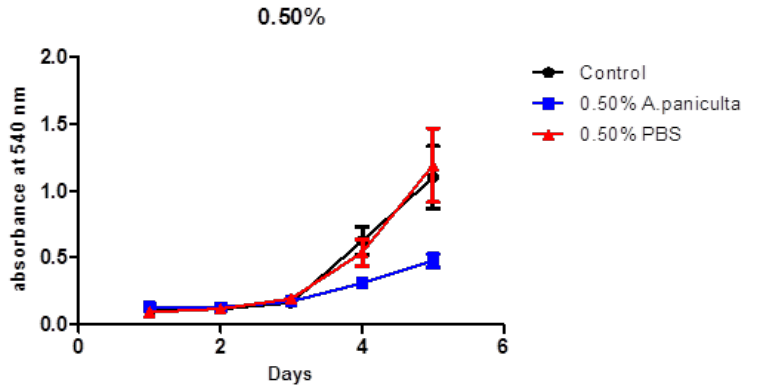

1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Contact
1% A.paniculta
1% EtOH

Figure 3.4. The stability of aqueous extract of *Andrographis paniculata* that stored at room temperature for 3 months.

B16-BL6 murine melanoma cells were treated with the PBS extract of *Andrographis paniculata* (33% andrographolide) that had been incubated at 4˚C for approximately 3 months. The cells were treated with low (0.1%), middle (0.50%) and high (1%) concentrations of the extract for five days. The B16-BL6 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.1% A.paniculta
0.1% PBS


0.50%
absorbance at 540 nm
2.0
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.50% A.paniculta
0.50% PBS

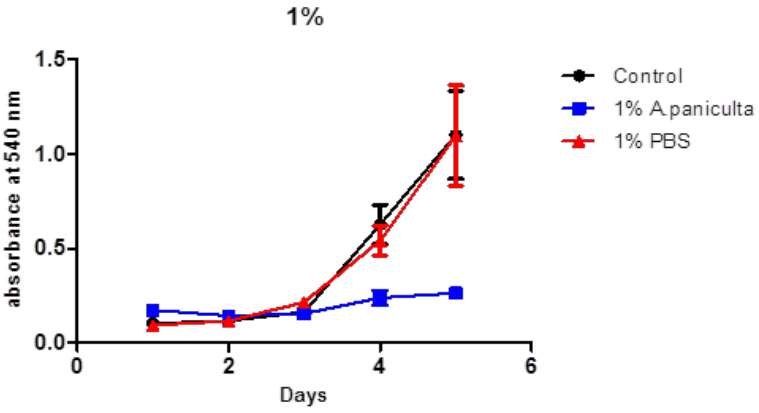
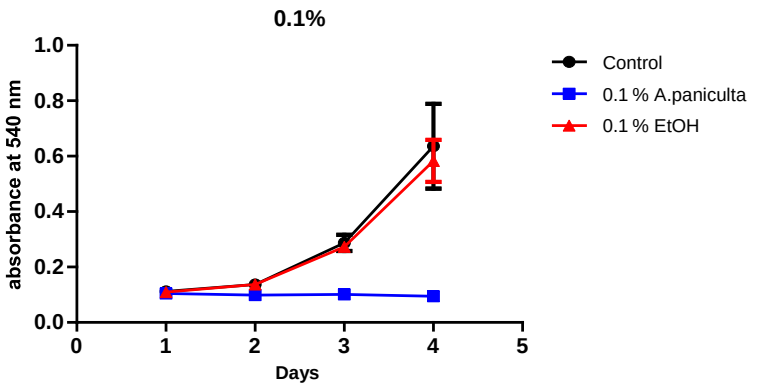
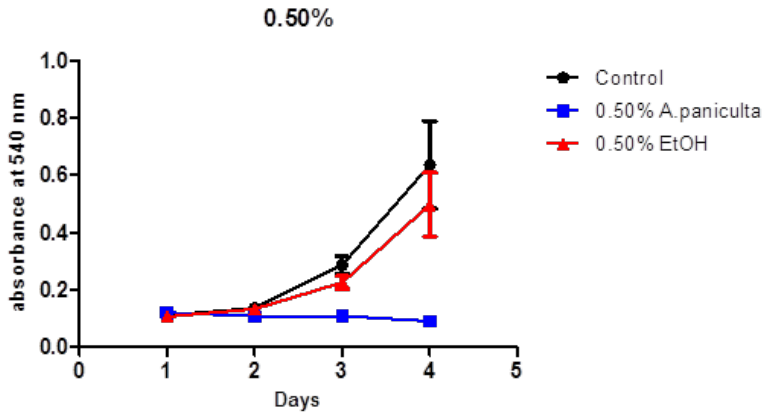

1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
1% A.paniculta
1% PBS

Figure 3.5. The effect of ethanol extract of *Andrographis paniculata* containing 10% andrographolide on B16-BL6 cells growth.

B16-BL6 murine melanoma cells were treated with the ethanol extract of *Andrographis paniculata* (10% andrographolide) that was derived from the aerial part of the plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The B16-BL6 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.1 % A.paniculta
0.1 % EtOH


0.50%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.50% A.paniculta
0.50% EtOH

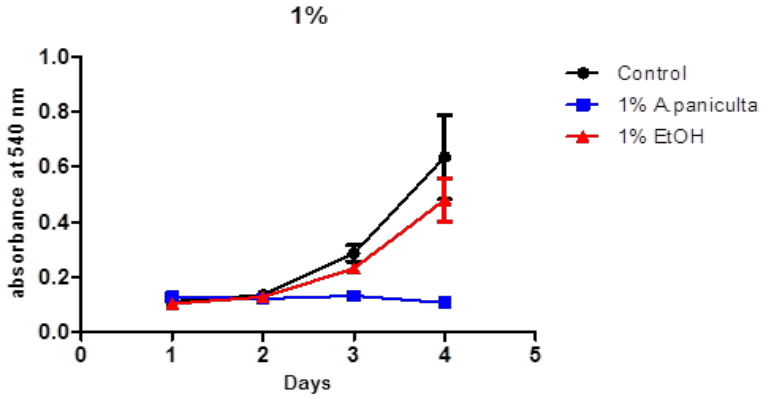
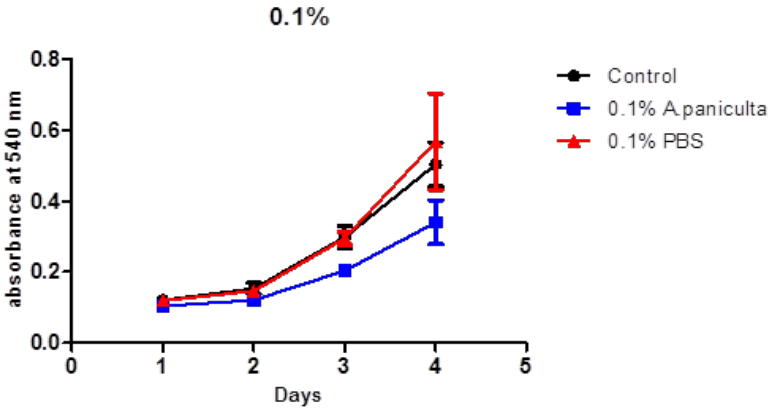
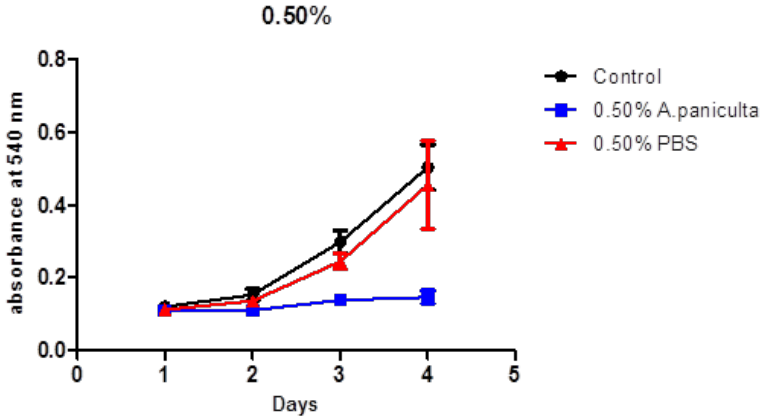

1%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
1% A.paniculta
1% EtOH

Figure 3.6. The effect of aqueous extract of *Andrographis paniculata* containing 10% andrographolide on B16-BL6 cells growth.

B16-BL6 murine melanoma cells were treated with the PBS extract of *Andrographis paniculata* (10% andrographolide) that was derived from the aerial part of the plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The B16-BL6 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.1% A.paniculta
0.1% PBS


0.50%
absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.50% A.paniculta
0.50% PBS

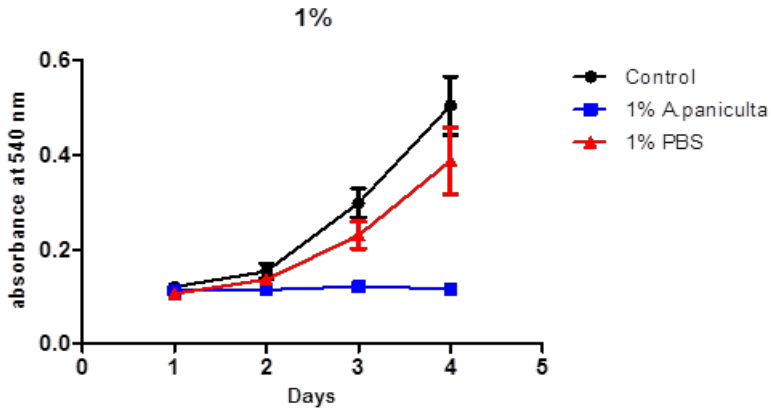
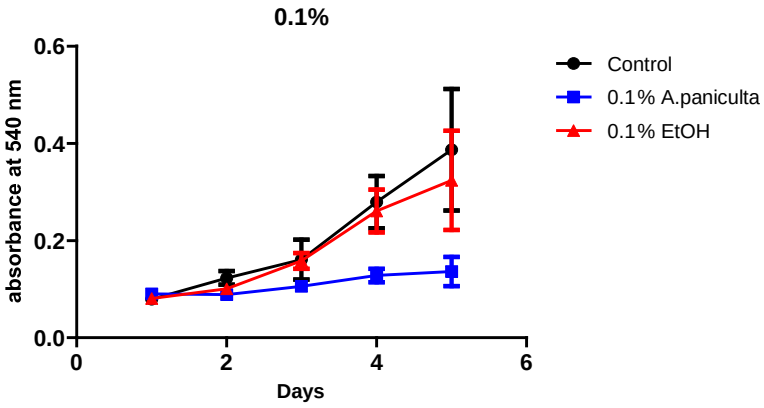
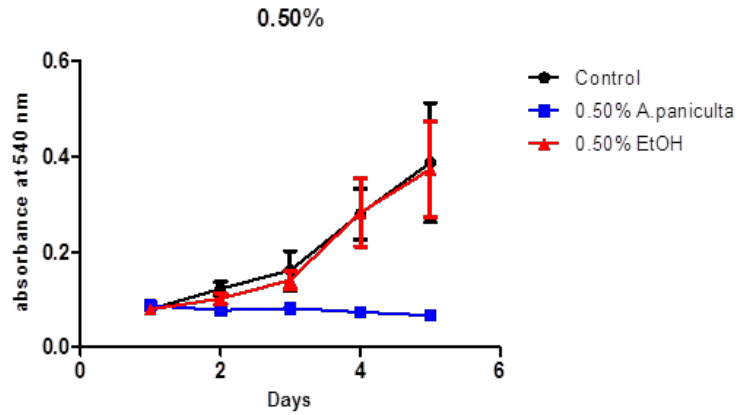

1%
absorbance at 540 nm
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
1% A.paniculta
1% PBS

Figure 3.7. The effect of ethanol extract of *Andrographis paniculata* on ASPC-1 cells growth.

ASPC-1 (pancreatic cancer cells) were treated with the ethanol extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The ASPC-1 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
0.1% A.paniculta
0.1% EtOH


0.50%
absorbance at 540 nm
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
0.50% A.paniculta
0.50% EtOH

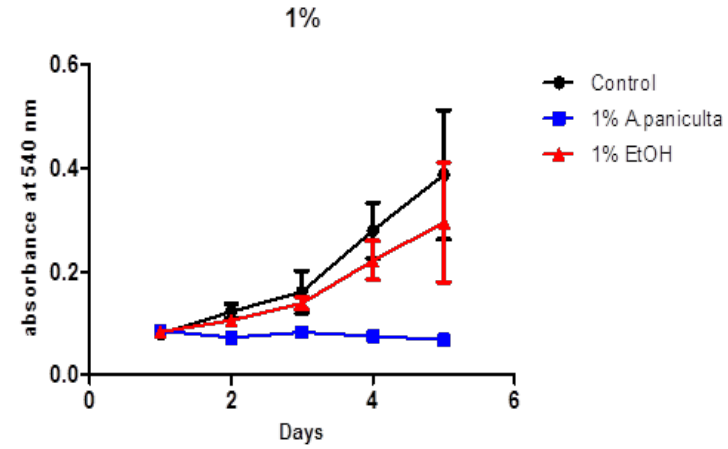
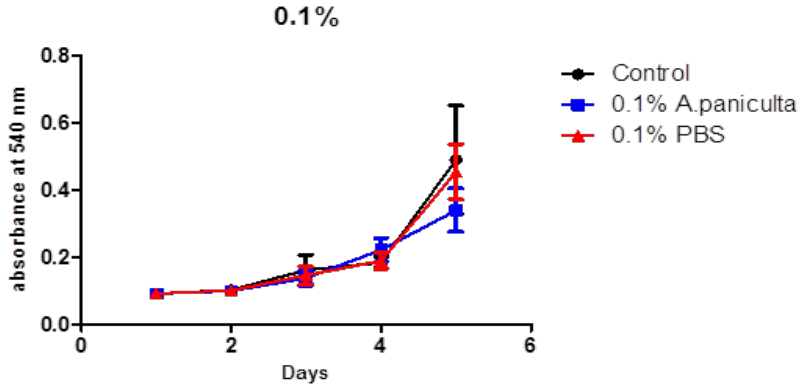
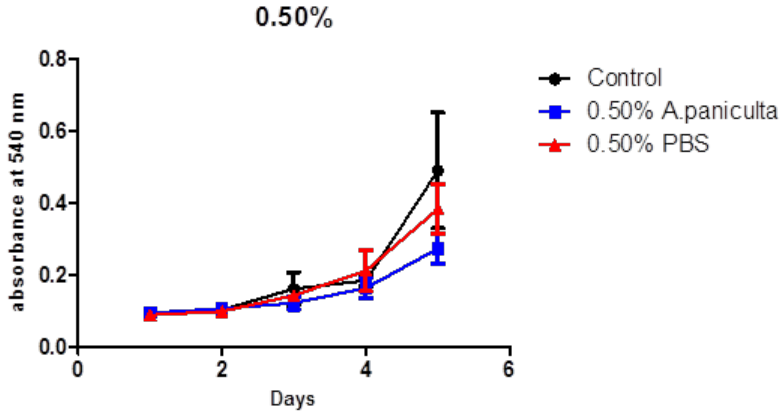
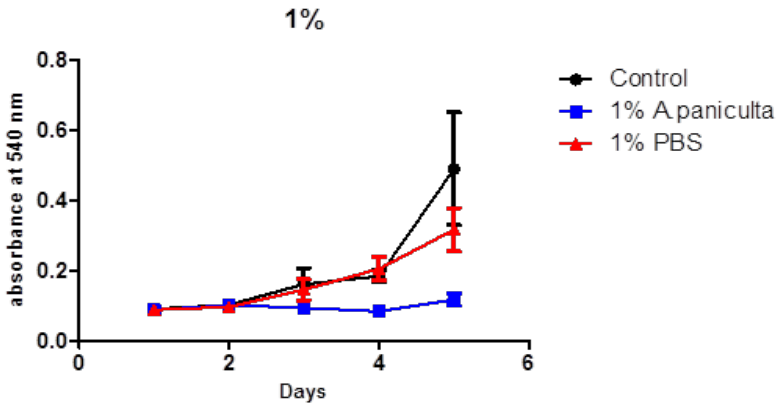

1%
absorbance at 540 nm
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
1% A.paniculta
1% EtOH

Figure 3.8. The effect of aqueous extract of *Andrographis paniculata* on ASPC-1 cells growth.

ASPC-1 (pancreatic cancer cells) were treated with the PBS extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The ASPC-1 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
0.1% A.paniculta
0.1% PBS


0.50%
absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
0.50% A.paniculta
0.50% PBS


1%
absorbance at 540 nm
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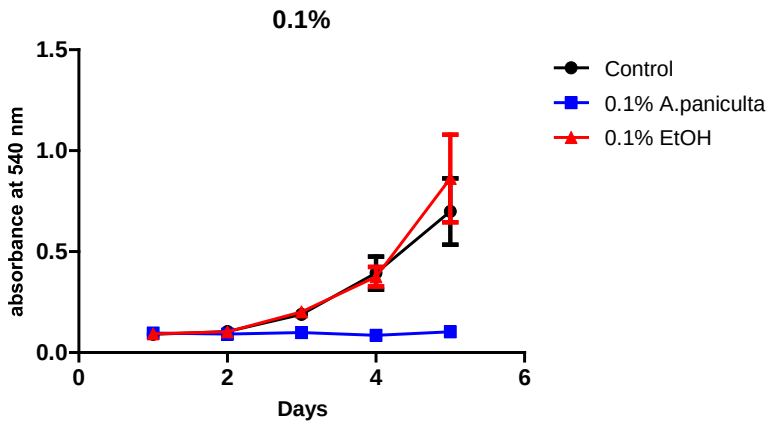
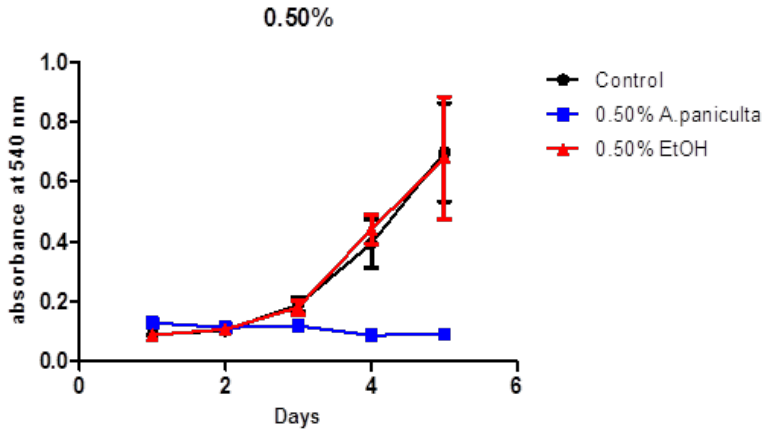
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
1% A.paniculta
1% PBS

Figure 3.9. The effect of ethanol extract of *Andrographis paniculata* on MCF-7 cells growth.

MCF-7 (human breast cancer cells- triple positive) were treated with the ethanol extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The MCF-7 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.1% A.paniculta
0.1% EtOH


0.50%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
Days
Control
0.50% A.paniculta
0.50% EtOH

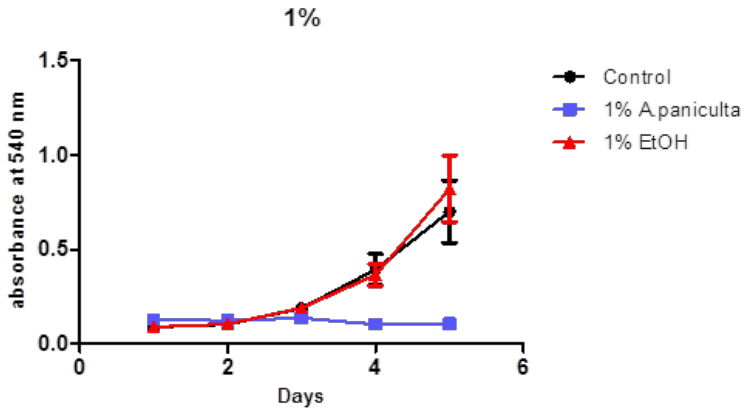
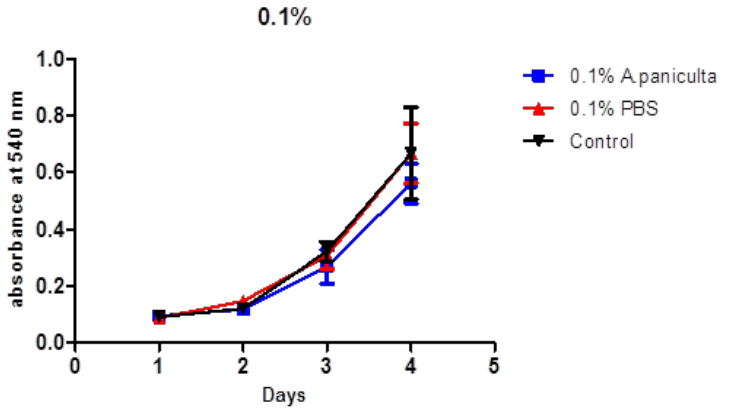
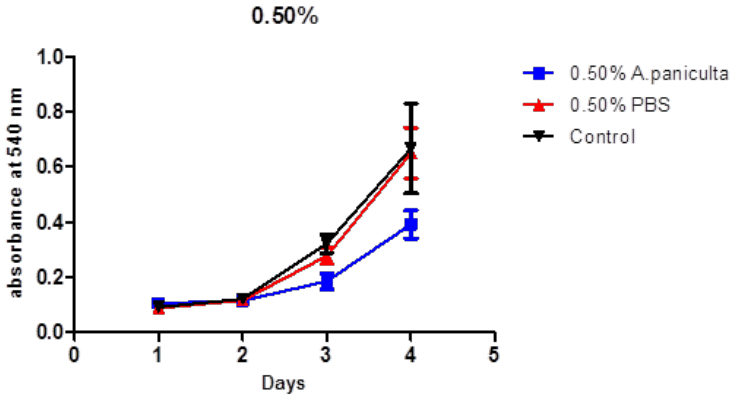
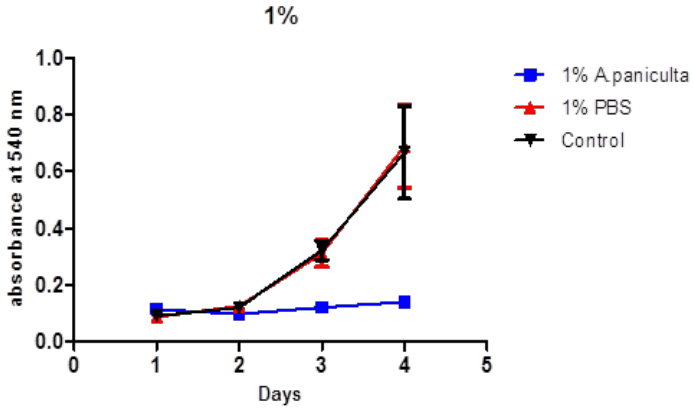

1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
1% A.paniculta
1% EtOH

Figure 3.10. The effect of aqueous extract of *Andrographis paniculata* on MCF-7 cells growth.

MCF-7 (human breast cancer cells- triple positive) were treated with the PBS extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The MCF-7 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
0.1% A.paniculta
0.1% PBS
Control


0.50%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
0.50% A.paniculta
0.50% PBS
Control


1%
absorbance at 540 nm
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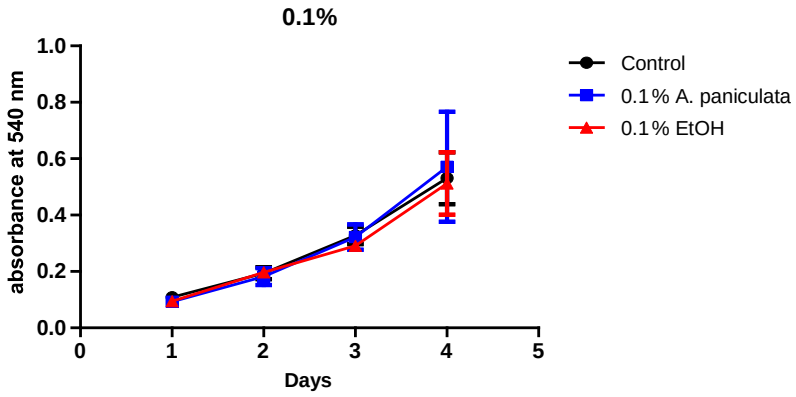
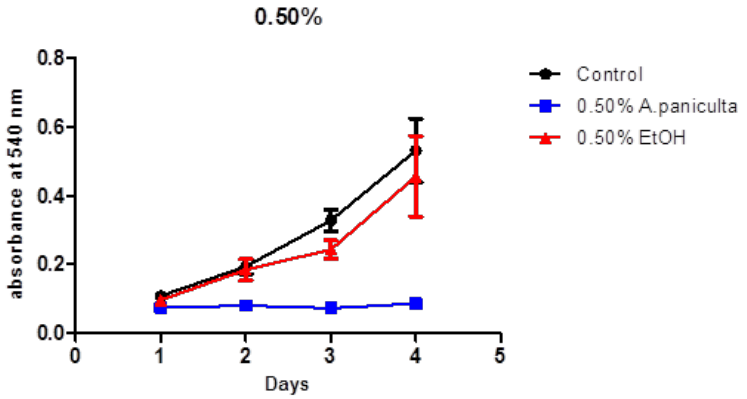
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
1% A.paniculta
1% PBS
Control

Figure 3.11. The effect of ethanol extract of *Andrographis paniculata* on 4T1 cells growth.

4T1 (mouse breast cancer cells- triple negative) were treated with the ethanol extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The 4T1 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.1% A. paniculata
0.1% EtOH


0.50%
absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.50% A.paniculta
0.50% EtOH

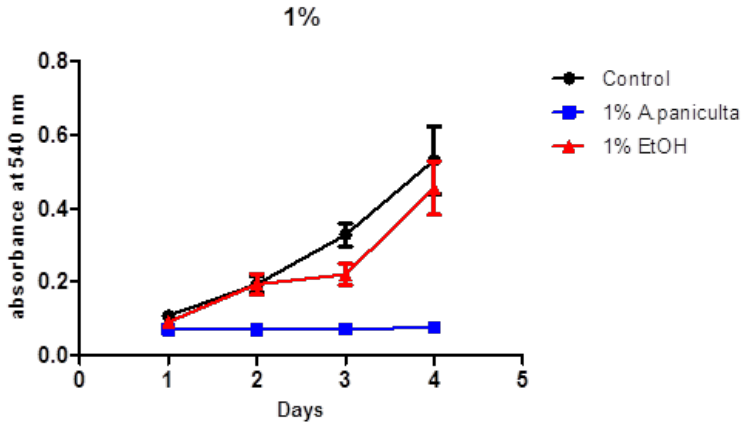
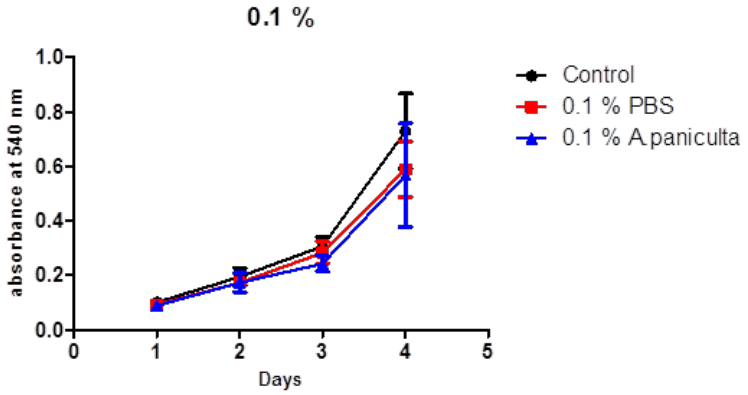
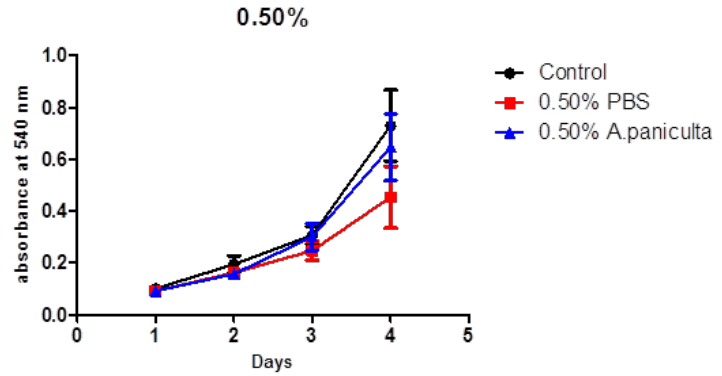

1%
absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
1% A.paniculta
1% EtOH

Figure 3.12. The effect of aqueous extract of *Andrographis paniculata* on 4T1 cells growth.

4T1 (mouse breast cancer cells- triple negative) were treated with the PBS extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The 4T1 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1 %
absorbance at 540 nm
Days
Control
0.1 % PBS
0.1 % A.paniculta


0.50%
absorbance at 540 nm
Days
Control
0.50% PBS
0.50% A.paniculta

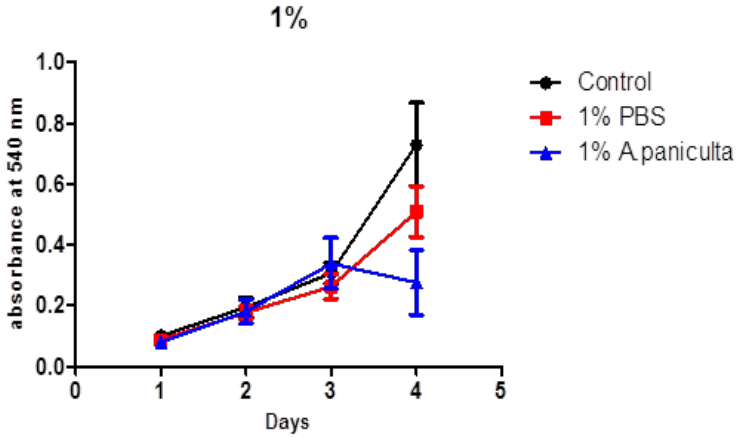
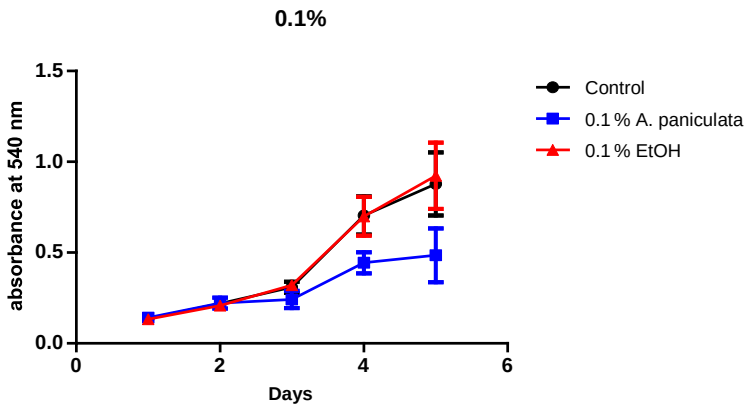
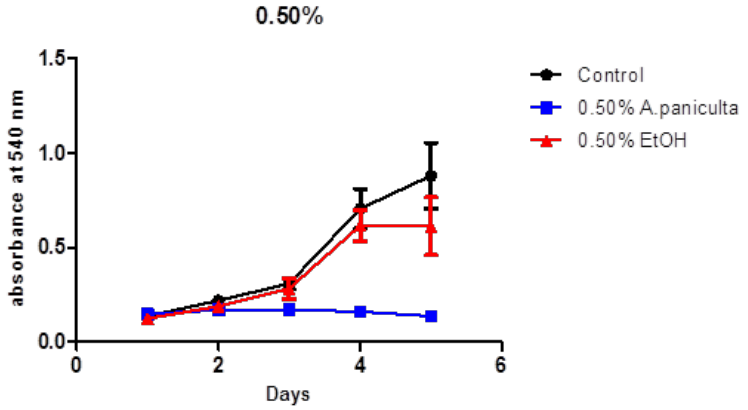

1%
absorbance at 540 nm
Days
Control
1% PBS
1% A.paniculta

Figure 3.13. The effect of ethanol extract of *Andrographis paniculata* on MDA-MB-231 cells growth.

MDA-MB-231 (human breast cancer cells- triple negative) were treated with the ethanol extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The MDA-MB231 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.1% A. paniculata
0.1% EtOH


0.50%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
0.50% A.paniculta
0.50% EtOH

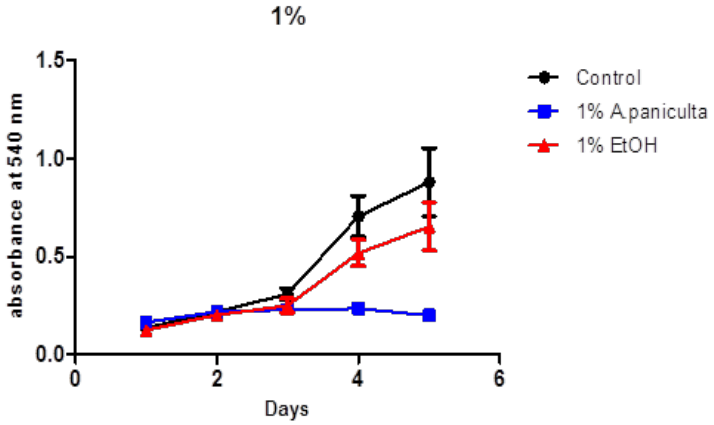
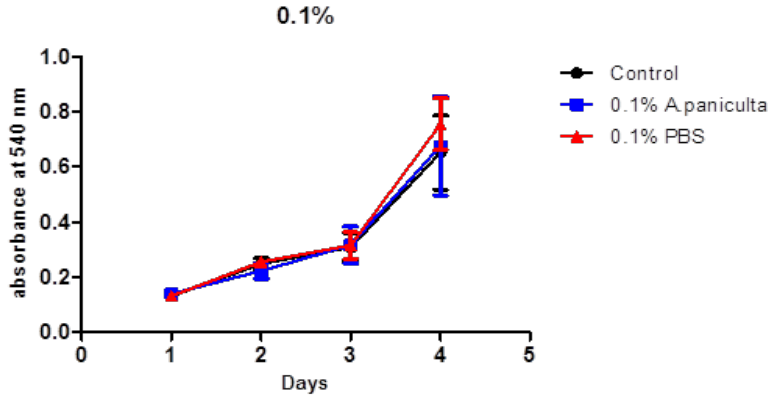
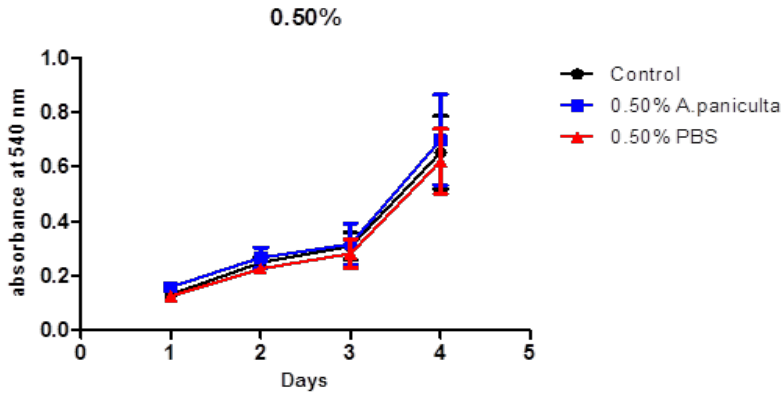

1%
absorbance at 540 nm
1.5
1.0
0.5
0.0
0
2
4
6
Days
Control
1% A.paniculta
1% EtOH

Figure 3.14. The effect of aqueous extract of *Andrographis paniculata* on MDA-MB-231 cells growth.

MDA-MB-231 (human breast cancer cells- triple negative) were treated with the PBS extract of *Andrographis paniculata* (33% andrographolide) that was derived from the whole plant at low (0.1%), middle (0.50%) and high (1%) concentrations for five days. The MDA-MB-231 cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.


0.1%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.1% A.paniculta
0.1% PBS


0.50%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
0.50% A.paniculta
0.50% PBS

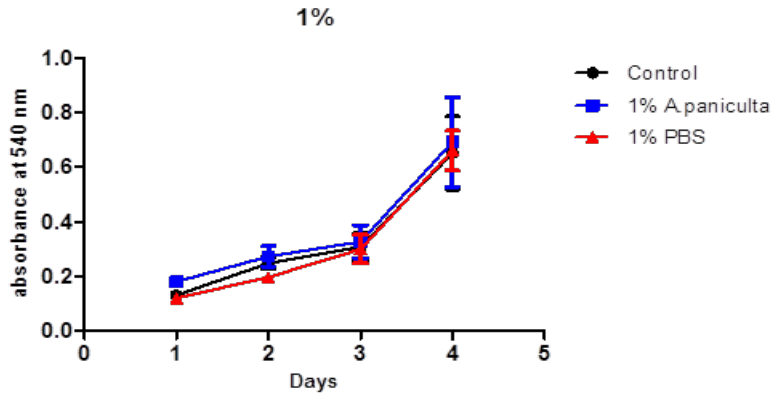
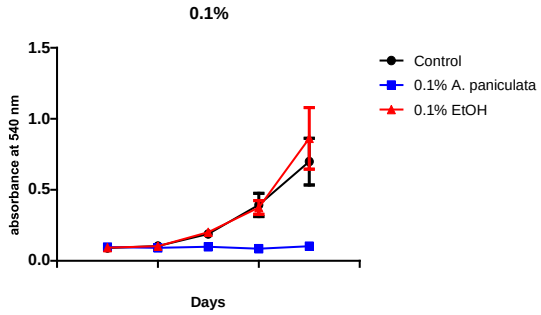
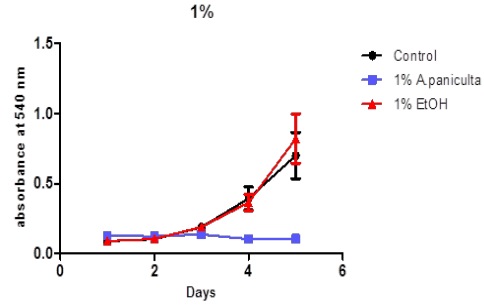

1%
absorbance at 540 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days
Control
1% A.paniculta
1% PBS

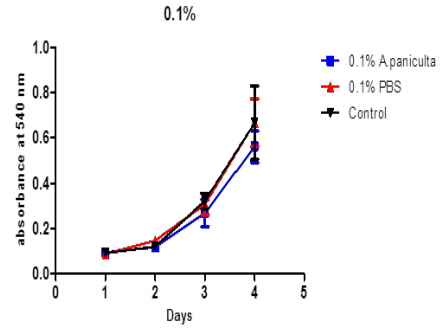
Figure 3.15. Comparison of the effect of *Andrographis paniculata* extracts on MCF-7 (triple positive breast cancer) and (MDA-MB-231) triple negative breast cancer.

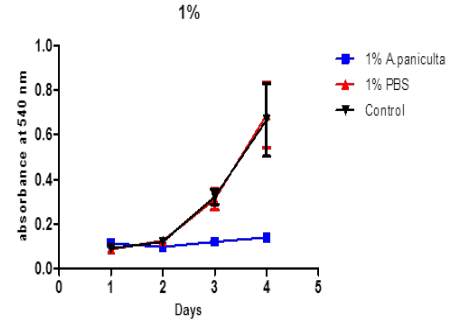
MDA-MB-231 (human breast cancer cells- triple negative) and MCF-7 (human breast cancer cells- triple positive) were treated with *Andrographis paniculata* extracts (ethanol and PBS) at middle (0.50%) and high (1%) concentrations for five days. The cells viability was determined by MTT assays each day for five days. The data were analyzed using Graph Pad Prism for 3 independent experiments.

MCF-7 cell line

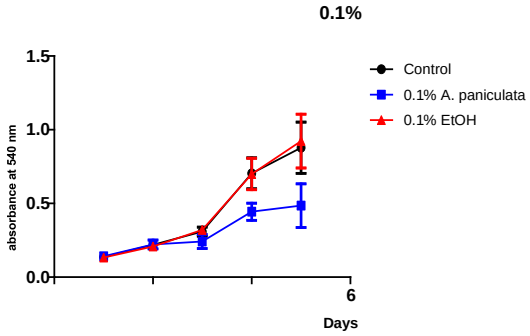


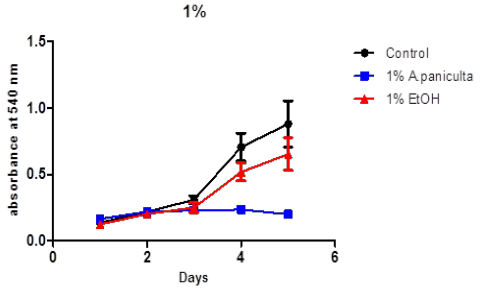


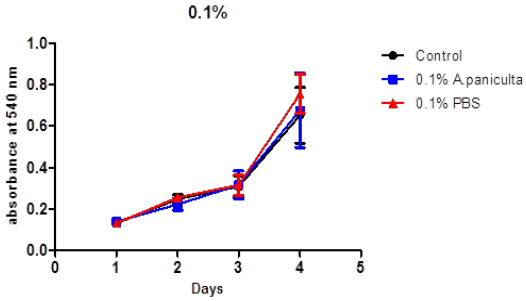


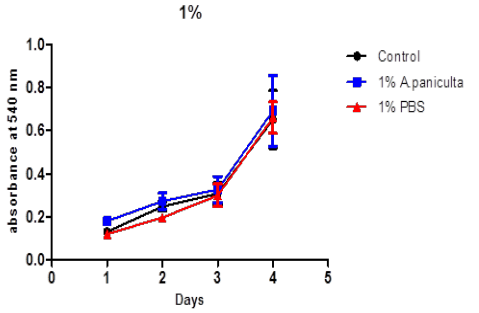


MDA-MB-231 cell line









3.2. Morphological changes of B16-BL6 & MDA-MB-231 cells upon exposure to *Andrographis paniculata* extracts:

B16-BL6 and MDA-MB-231 cells were treated with various doses of *Andrographis paniculata* extracts (both ethanol & aqueous) for 48 hours to assess the effect of treatment on B16-BL6 and MDA-MB-231 cell morphology using an Olympus DP80 microscope. The results demonstrated that the B16-BL6 cells treated with a high dose of the ethanol extract exhibited a significant decrease in cell number, the appearance of tangled apoptotic cells, and a decline in the cellular content of the cells (Figure 3.16.). This outcome was similar to that seen following the treatment with a high dose of the aqueous extract of *Andrographis paniculata* (Figure 3.16.). However, the decline in cells in the sample treated with the aqueous extract was not as great as the cell number decline that was observed with ethanol extracts. B16-BL6 that were treated with the ethanol extract and aqueous extract of *Andrographis paniculata* at low doses showed similar results. The results demonstrated that the B16-BL6 cells treated with the ethanol extracts and aqueous extracts of *Andrographis paniculata,* at low doses, exhibited cell shape changes, which manifested in the form of an elongated shape. Based on the appearance of the elongated cells, we concluded that they were under physiological stress.

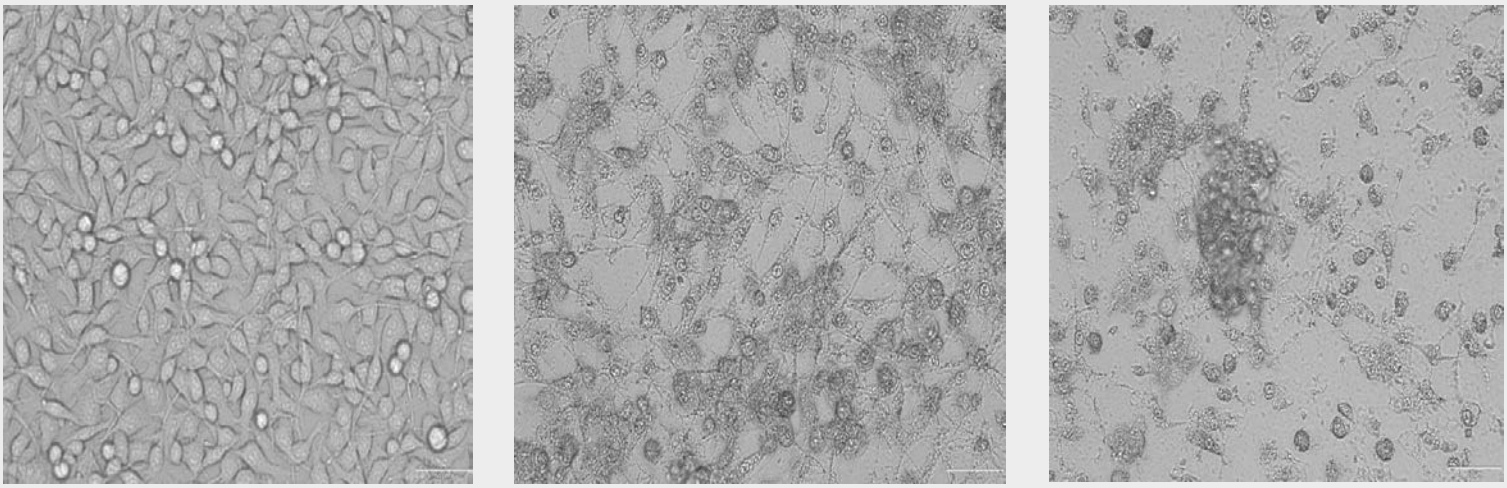
In contrast, the MDA-MB-231 cells treated with high and low doses of the aqueous extract of *Andrographis paniculata* did not exhibit any significant changes in cell shape (Figure 3.17). The results demonstrated that, at low and high concentrations, the cells retained their shape and number throughout the experiment. In fact, their appearance was similar to untreated cells. However, exposure of MDA-MB-231 cells to high doses of the ethanol extract of *Andrographis paniculata* showed a significant decline in cell number in comparison to treatment with the low dose of the ethanol extract.

Figure 3.16. The effect of *Andrographis paniculata* extracts on the morphology of B16-BL6 murine melanoma cells.

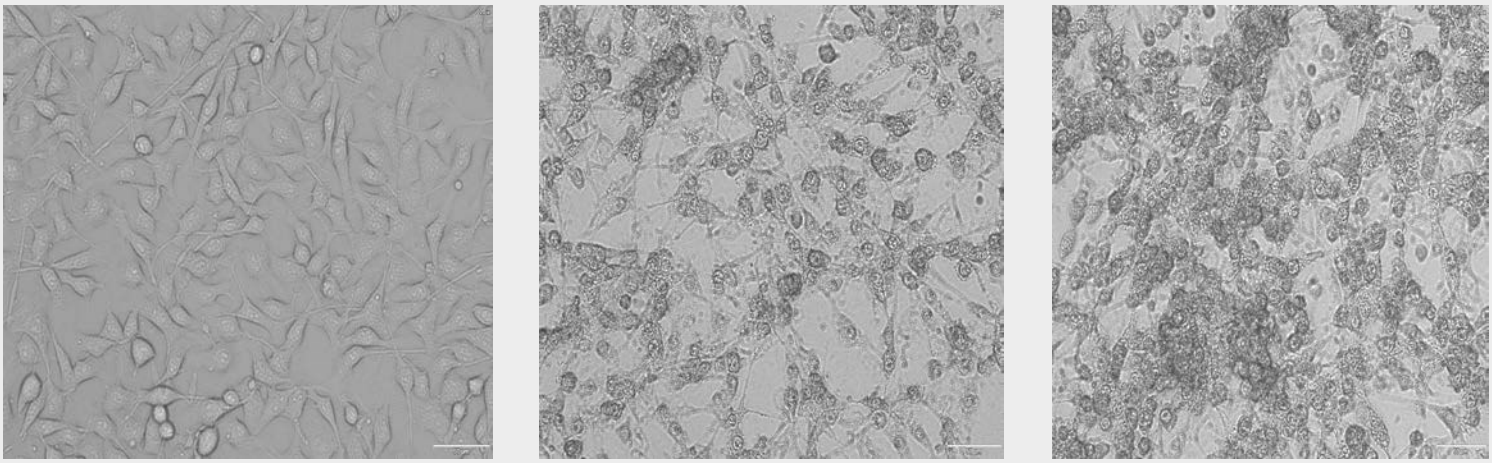
B16-BL6 cells were treated with *Andrographis paniculata* extracts (ethanol and PBS extracts) at low and high doses for 48 h. Untreated cells were used as a negative control. The experiment was run 3 time and the results were observed under Olympus DP80 Microscope. The bar in the bottom corner marks a field size of 20 μm.

A

Negative Control | Low Dose of ethanol extract | High Dose of ethanol extract

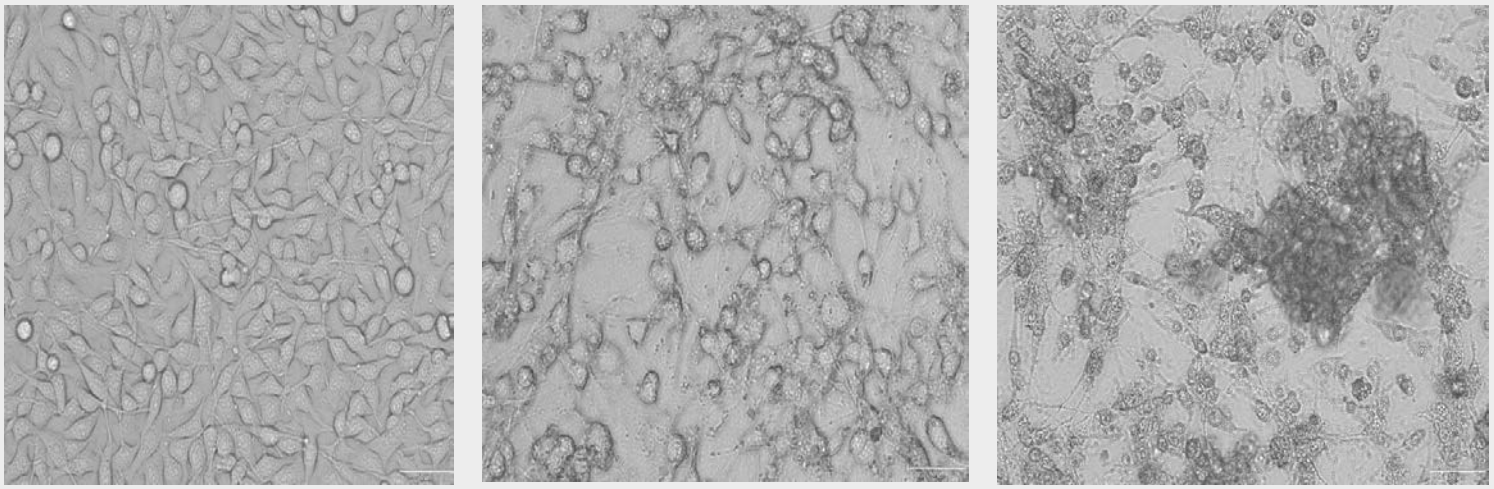


Negative Control | Low Dose of ethanol extract | High Dose of ethanol extract



B

Negative Control | Low Dose of PBS extract | High Dose of PBS extract



Negative Control | Low Dose of PBS extract | High Dose of PBS extract

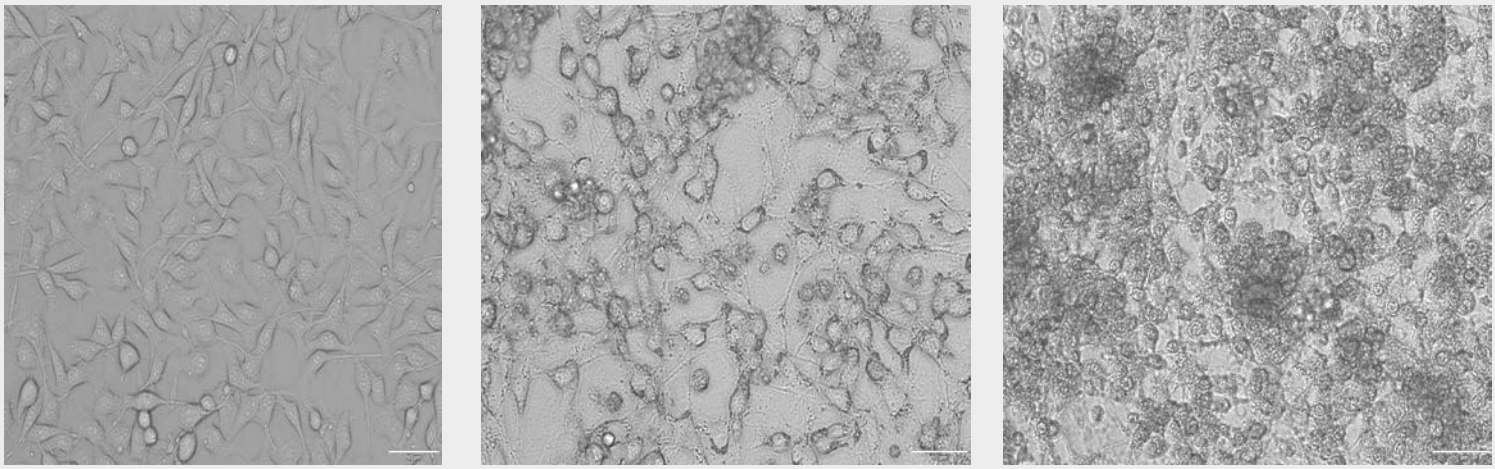
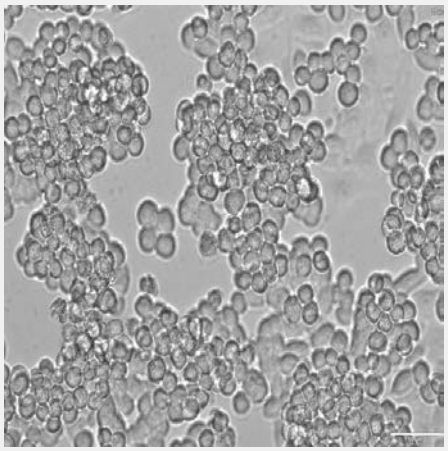


Figure 3.17. The effect of *Andrographis paniculata* extracts on the morphology of MDAMB-231(human breast cancer-negative triple).

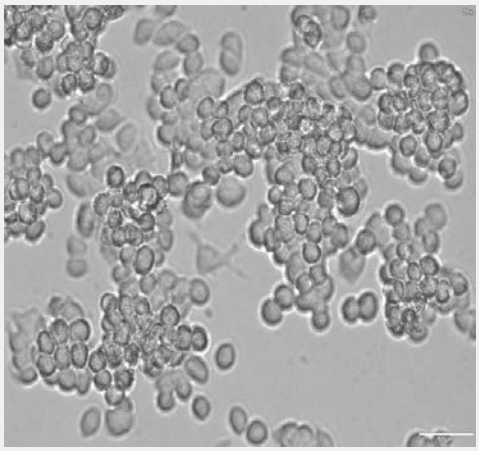
MDA-MB-231 cells were treated with *Andrographis paniculata* extracts (ethanol and PBS extracts) at low and high doses for 48 h. Untreated cells were used as a negative control. The experiment was run 3 times and the results were observed under Olympus DP80 Microscope. The bar in the bottom corner marks a field size of 20 μm.

A

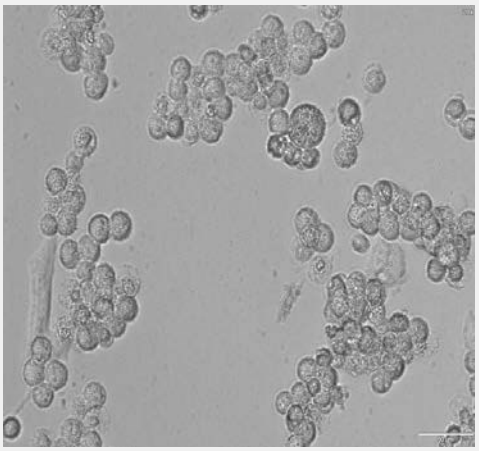
Negative Control



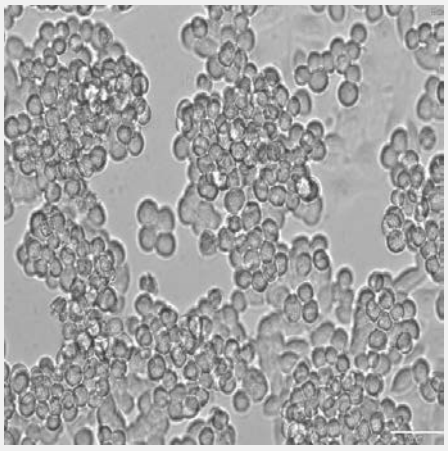
Low Dose of ethanol extract



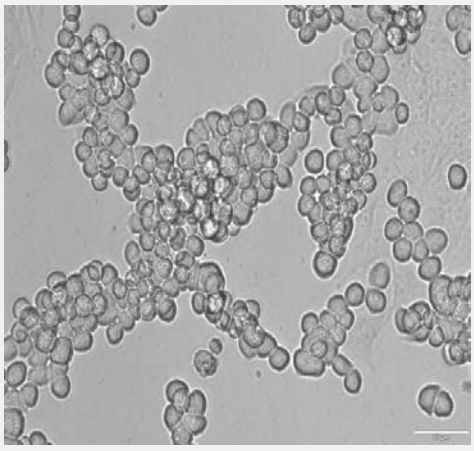
High Dose of EtOH extract



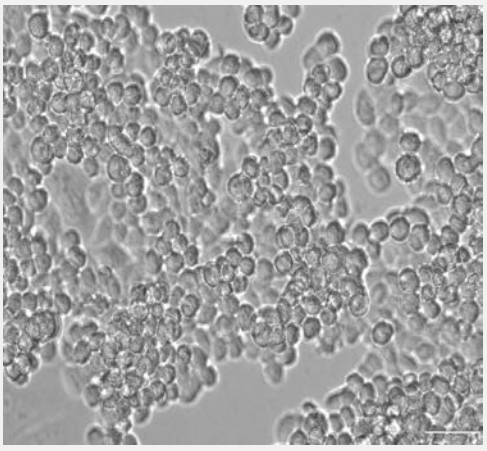
Negative Control



Low Dose of PBS extract



High Dose of PBS extract



3.3. Treatment of B16-BL6 cells with *Andrographis paniculata* extracts for > 48h resulted in apoptotic morphology:

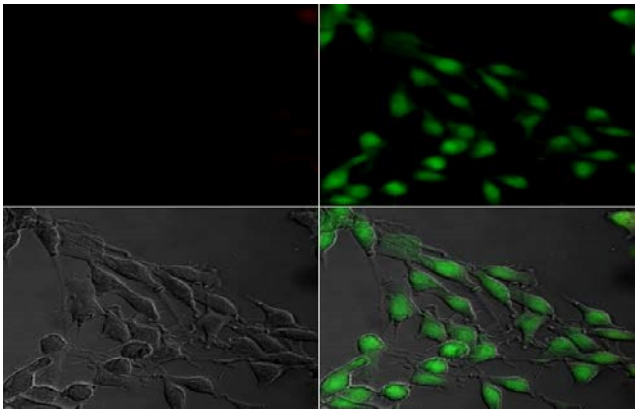
The acridine orange and ethidium bromide staining assay was used to test the effect of *Andrographis paniculata* extracts on B16-BL6 cell morphology. Untreated B16-BL6 cells showed a relatively even green stain in the nucleus, corresponding to acridine orange binding. The absence of any red staining corresponding to ethidium bromide staining was evidence that the plasma membrane was intact (Figure 3.18). The B16-BL6 cells that were treated with a high dose of the ethanol extract of *Andrographis paniculata* presented significant changes in cell morphology. The observed changes included the complete destruction of cell structure, and the appearance of orange staining in cells because of the loss of membrane integrity. However, we did not observe any morphological changes in the B16-BL6 at low dose of ethanol extract. (The cells appeared uniformly green; a clear sign for an intact nucleus in the cells). The exposure of B16-BL6 cells to a high concentration of the aqueous extract of *Andrographis paniculata* suggested that treatment led to the appearance of green cells with bright green dots in the nuclei because of chromatin condensation and nuclear fragmentation (Figure 3.19.). This result was evidence an early apoptosis compare to untreated cells, which showed normal nuclear staining.

Figure 3.18. The treatment of B16-BL6 murine melanoma cells with the ethanol extract of *Andrographis paniculata* > 48 h resulted in apoptotic morphology.

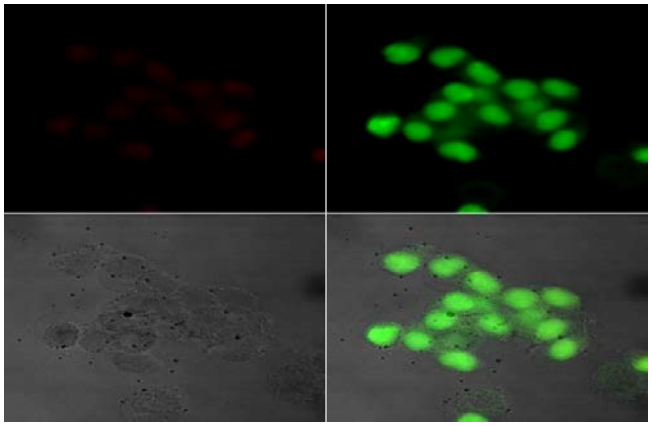
B16-BL6 cells were treated with the ethanol extract of *Andrographis paniculata* at low and high concentrations for 48 h. Untreated cells were used as a negative control. Acridine orange and ethidium bromide were used to stain B16-BL6 cells to look at nuclear morphology and plasma membrane integrity under the fluorescence microscope. This represents one of more than three independent experiments.

A

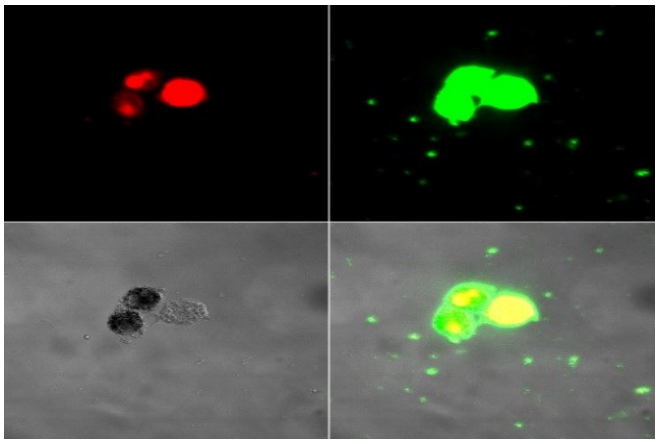
Negative Control



Low Dose of ethanol extract



High Dose of ethanol extract



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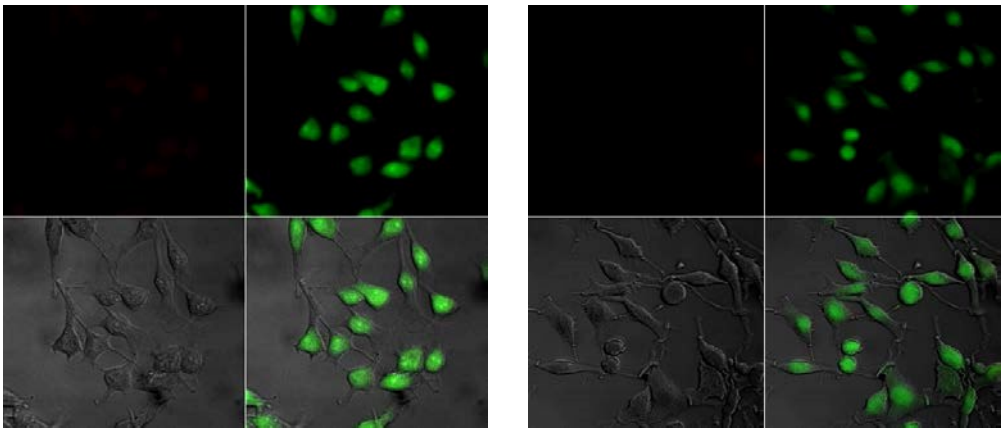
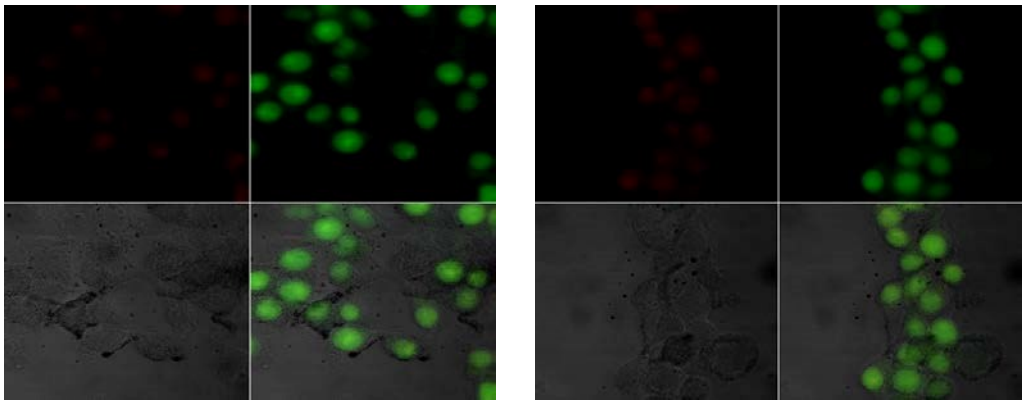
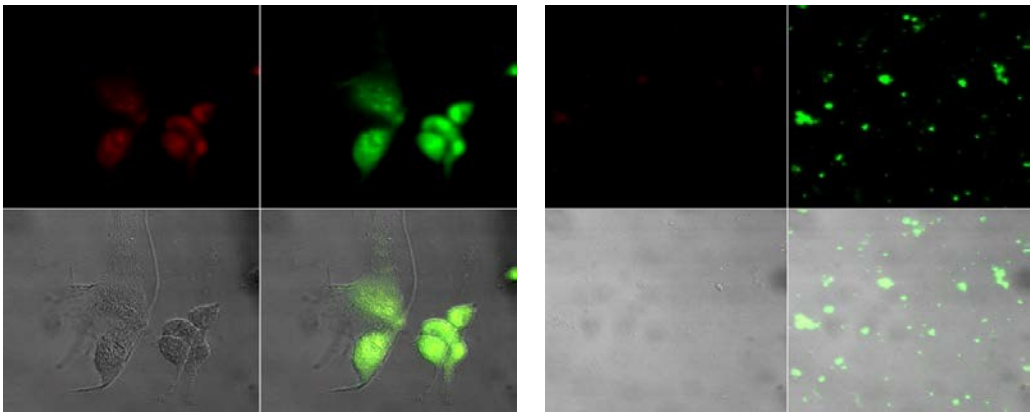


Low Dose of ethanol extract



High Dose of ethanol extract



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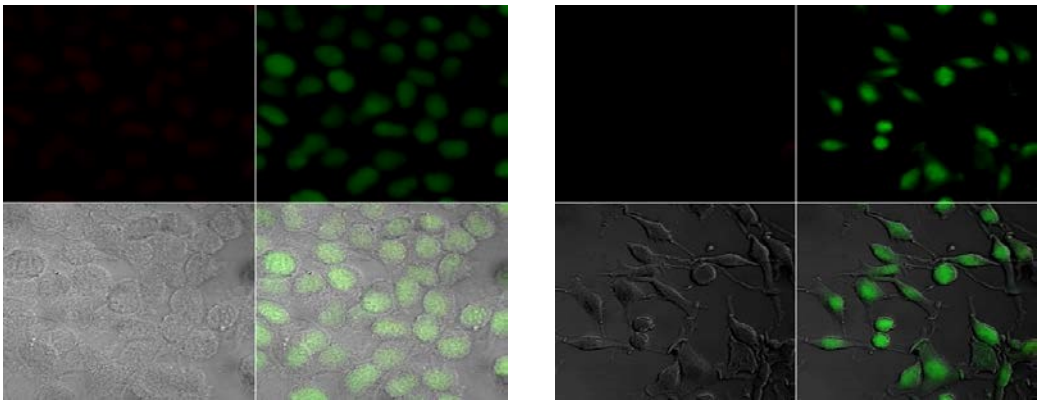
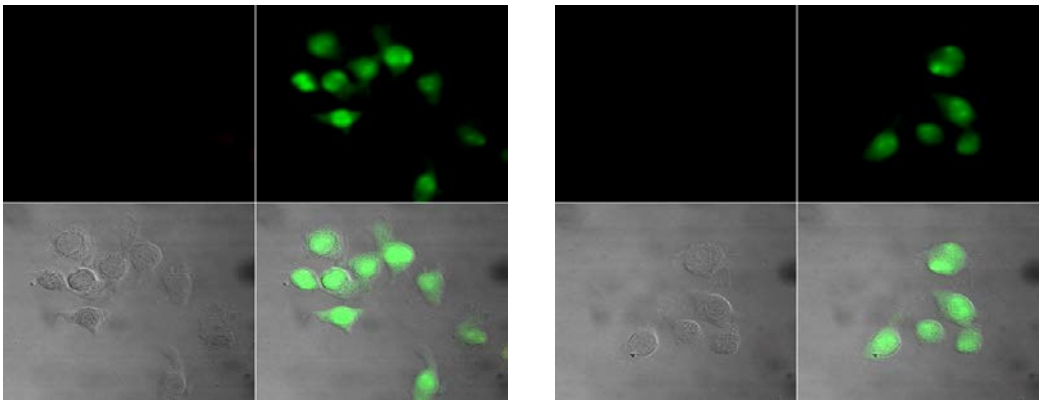


Low Dose of ethanol extract



High Dose of ethanol extract

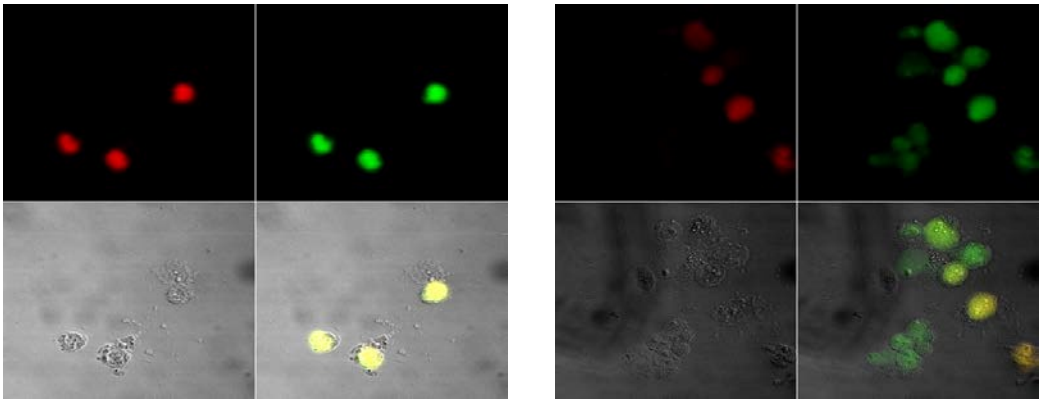
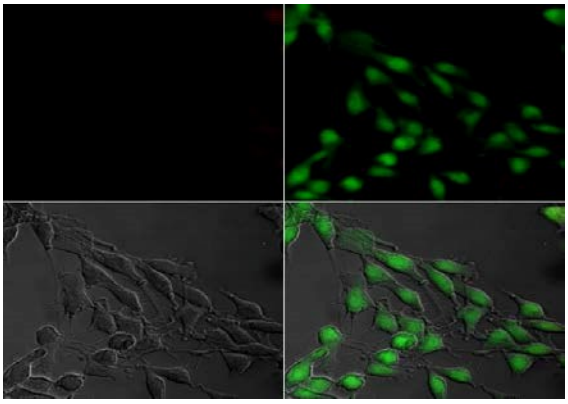


Figure 3.19. The treatment of B16-BL6 murine melanoma cells with the aqueous extract of *Andrographis paniculata* > 48 h resulted in apoptotic morphology.

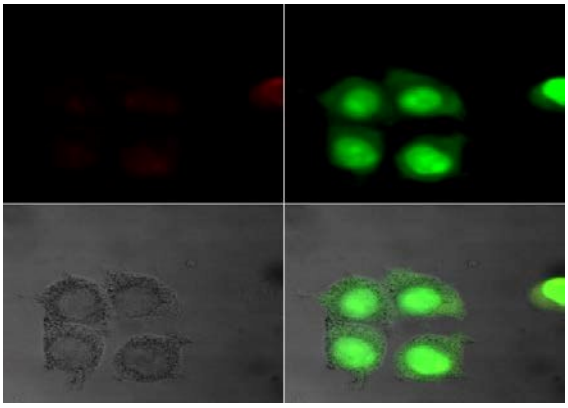
B16-BL6 cells were treated with the PBS extract of *Andrographis paniculata* at low and high concentrations for 48 h. Untreated cells were used as a negative control. Acridine orange and ethidium bromide staining were used to detect B16-BL6 cell death under fluorescence microscope. The experiment was run three separate times with similar results.

B

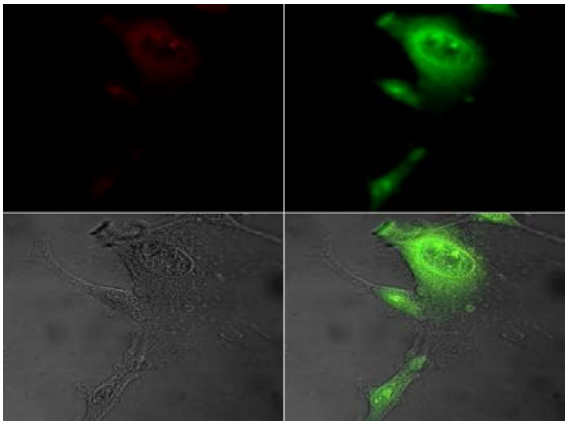
Negative Control



Low Dose of PBS extract



High Dose of PBS extract



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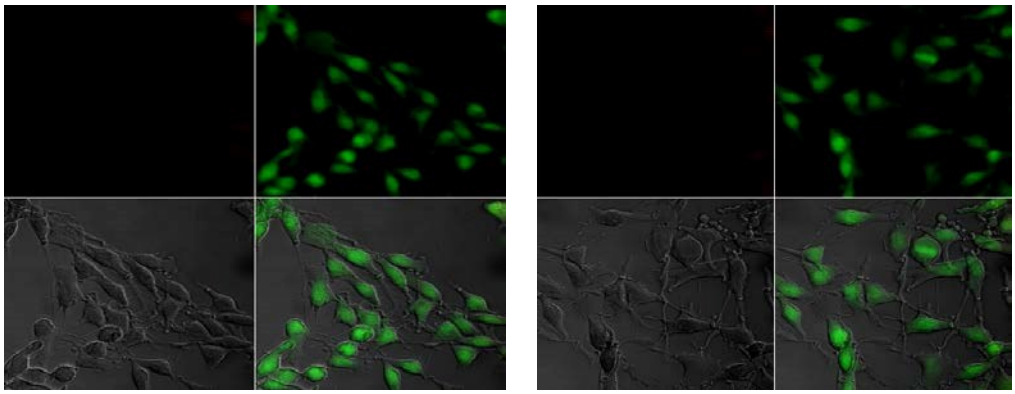
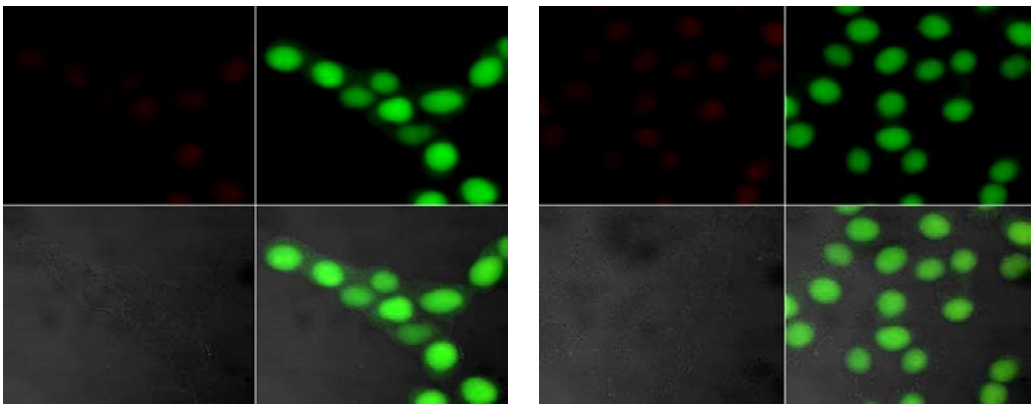
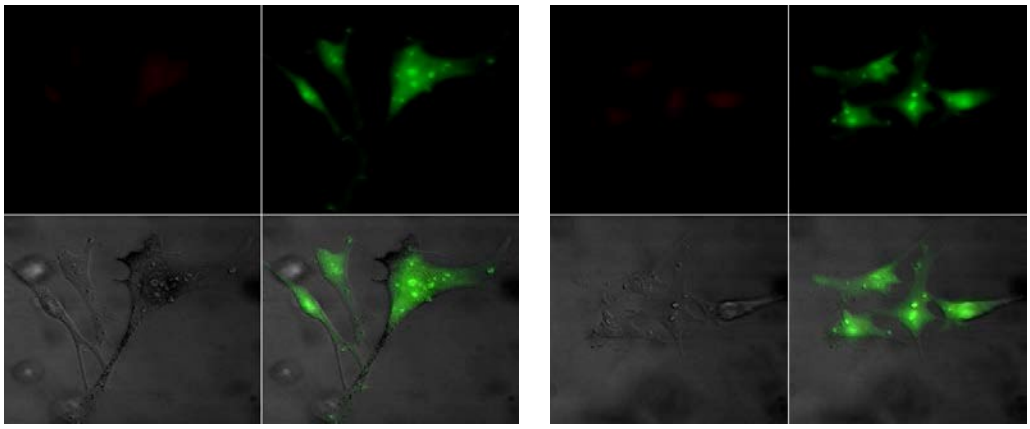


Low Dose of PBS extract



High Dose of PBS extract



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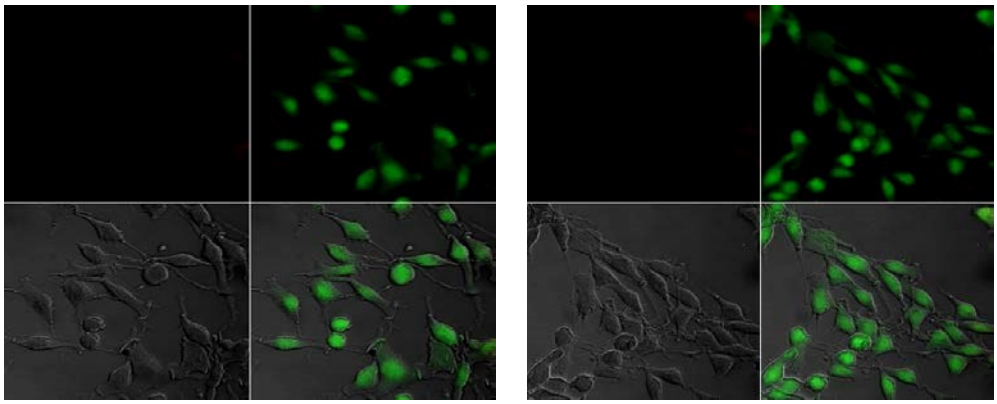

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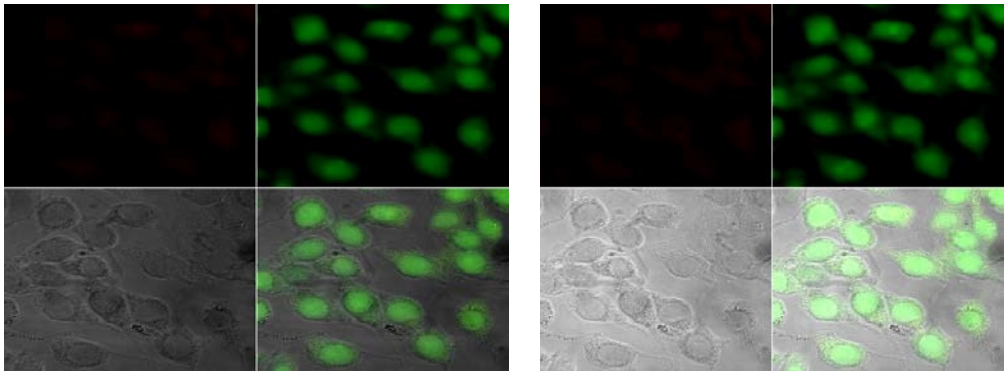
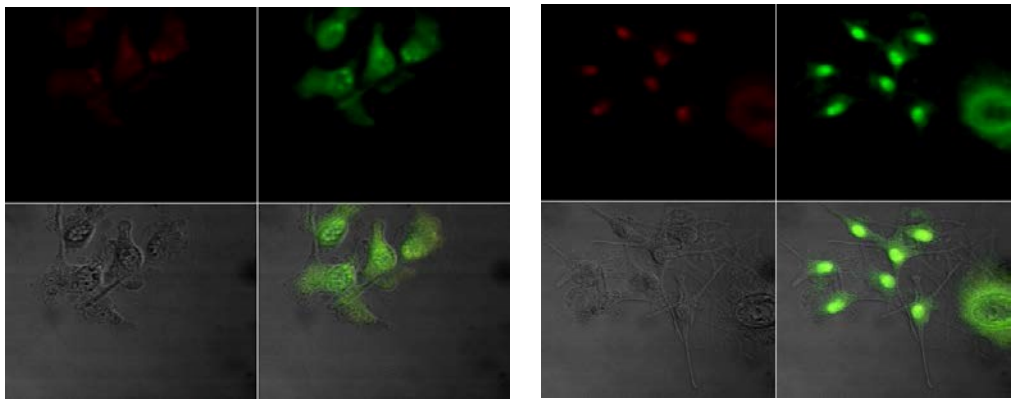


High Dose of PBS extract



3.4. Treatment of *Andrographis paniculata* extracts inhibited ERK phosphorylation and promoted caspase 3 cleavage:

Caspase 3 activation and inhibition of ERK phosphorylation were examined to determine whether *Andrographis paniculata* extracts inhibited survival signals and induced apoptosis as a mechanism of action. B16-BL6 cells were treated with *Andrographis paniculata* extracts (ethanol, aqueous) at varying concentrations. In addition, untreated cells were utilized as a negative control while UV-treated cells were used as a positive control for apoptosis. The treatment of B16-BL6 cells with the ethanol extract showed caspase-3 cleavage after 24 h of treatment (Figure 3.20). The expression of uncleaved caspase 3 bands were prominent at the low concentrations but disappeared at the highest concentrations because of cell death and the removal of unattached floating cells during the medium removal step. Further, B16-BL6 cells that were treated with the ethanol extract of *Andrographis paniculata* at various concentrations promoted a significant inhibition in the phosphorylation of ERK (P-ERK) relative to the ERK1/2 bands, which allows apoptosis. Clearly, the expression of P-ERK is decreased at the high concentrations of extract because of the cell's death. Furthermore, B16-BL6 cells that were treated with various concentrations of the aqueous extract of *Andrographis paniculata* exhibited a very weak band corresponding to caspase-3 cleavage after 24-h treatment (Figure 3.21.). After 48 h, the B16-Bl6 cells were shown to be undergoing apoptosis by the presence of a strong band of caspase-3 cleavage. In addition, the treatment with the aqueous extract showed inhibition of PERK compared to the constant expression of ERK1/2.

Figure 3.20. The treatment of B16-BL6 cells with the ethanol extract of *Andrographis paniculata* for 24 h promoted caspase-3 cleavage, and inhibited ERK phosphorylation. B16-BL6 murine melanoma cells were treated with the ethanol extract of *Andrographis paniculata* at various concentrations for 24h, altered cell survival pathway and induced apoptosis by using western blot analysis with three different antibodies: caspase-3, P-ERK and ERK1/2.

A

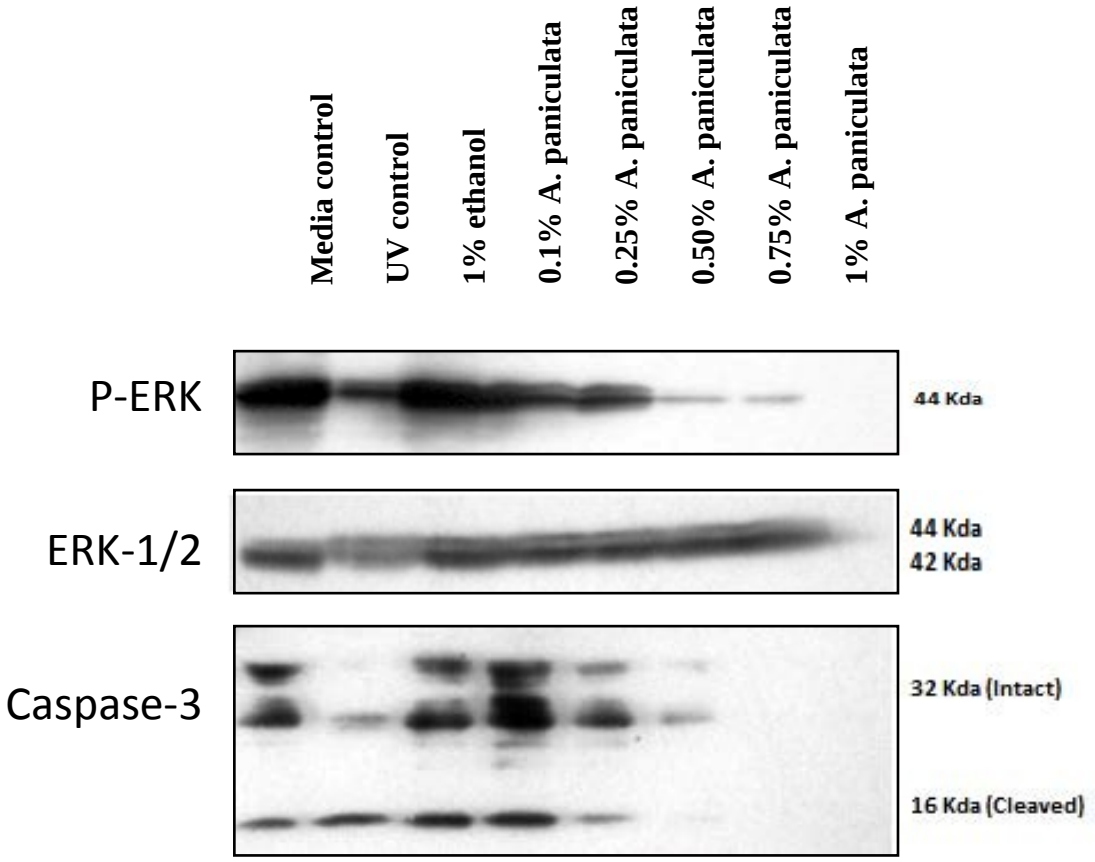
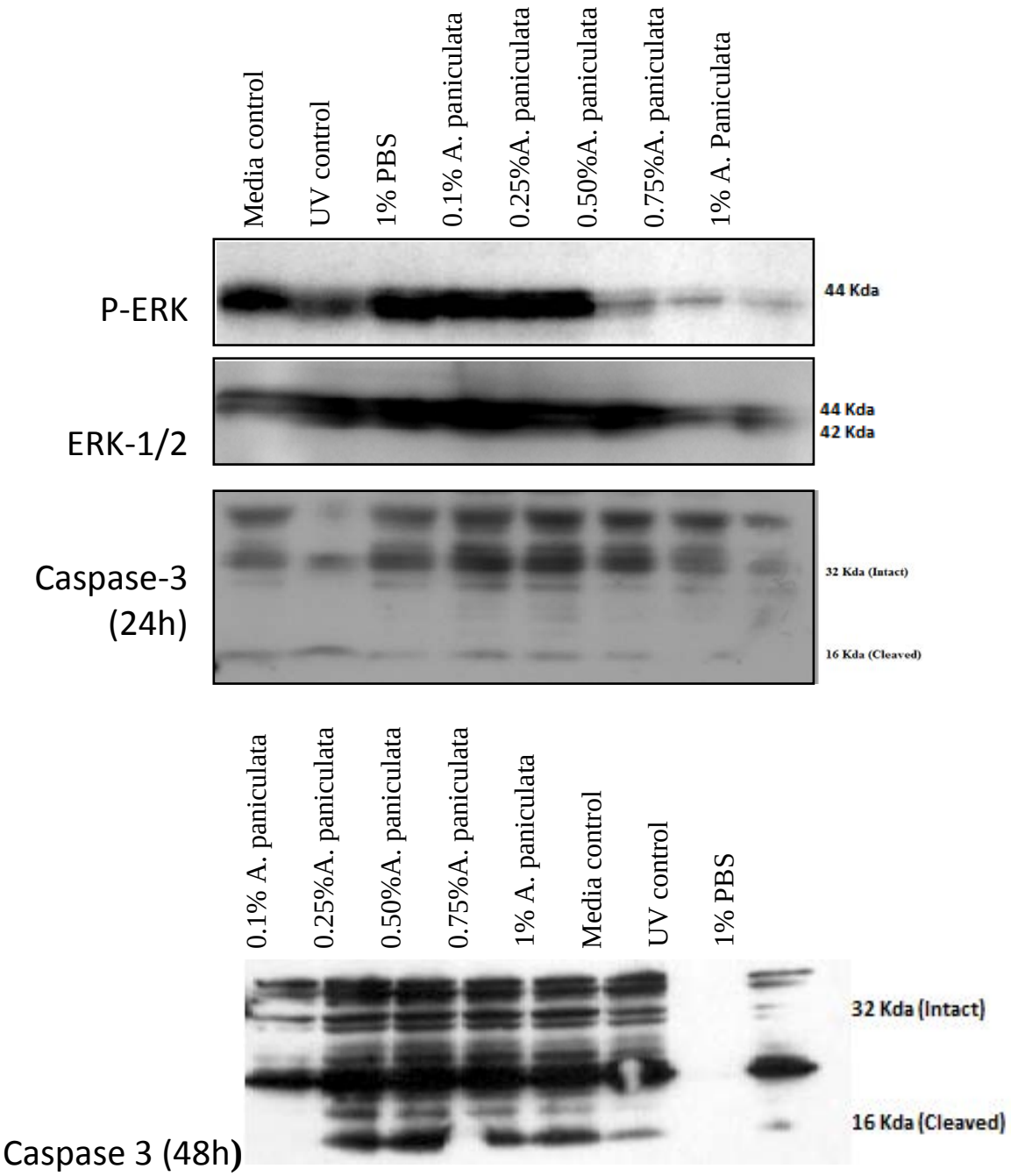

Media control
UV control
1% ethanol
0.1% A. paniculata
0.25% A. paniculata
0.50% A. paniculata
0.75% A. paniculata
1% A. paniculata
P-ERK
44 Kda
ERK-1/2
44 Kda
42 Kda
Caspase-3
32 Kda (Intact)
16 Kda (Cleaved)

Figure 3.21. The treatment of B16-BL6 cells with the aqueous extract of *Andrographis paniculata* promoted caspase-3 cleavage, and inhibited ERK phosphorylation. B16-BL6 murine melanoma cells were treated with the PBS extract of *Andrographis paniculata* at various concentrations for 24 h and 48 h, altered cell survival pathway and induced apoptosis by using western blot analysis with three different antibodies: caspase-3, P-ERK and ERK1/2.

B


Media control
UV control
1% PBS
0.1% A. paniculata
0.25%A. paniculata
0.50%A. paniculata
0.75%A. paniculata
1% A. Paniculata
P-ERK
44 Kda
ERK-1/2
44 Kda
42 Kda
Caspase-3
(24h)
32 Kda (Intact)
16 Kda (Cleaved)
0.1% A. paniculata
0.25%A. paniculata
0.50%A. paniculata
0.75%A. paniculata
1% A. paniculata
Media control
UV control
1% PBS
32 Kda (Intact)
16 Kda (Cleaved)
Caspase 3 (48h)

3.5. The effect of *Andrographis paniculata* extracts on the B16-BL6 cell cycle profile (Sub-G1):

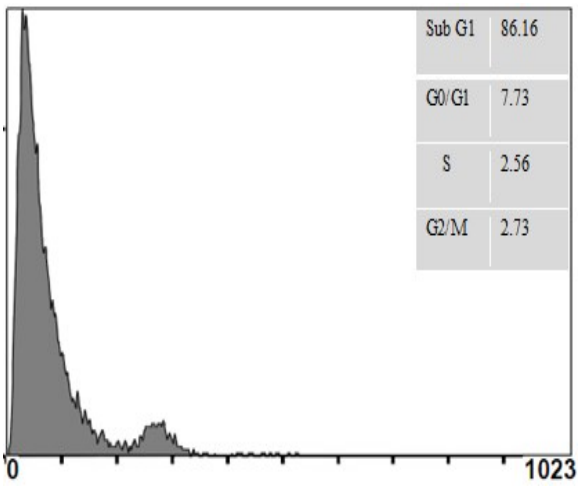
B16-BL6 cells were treated with a high concentration of the ethanol extract of *Andrographis paniculata* for 24 h. The results of PI staining showed that 16.63% of the cells were apoptotic, in the sub-G1 phase, which represents the percentage of cells with fragmented DNA. This percentage increased to 82.09% after 48 h of treatment with the ethanol extract which reveals a late stage of apoptosis. This outcome was similar to the UV control, which showed a high percentage of apoptotic cells around 86.16%. In contrast, the cells treated with the low dose of the ethanol extract showed a small percentage of apoptotic cells (<20%) and a high percentage of living cells (Figure 3.22.). It is also interesting to note that cells treated with the low dose of the ethanol extract for 24 h, and the high dose of the ethanol extract for 24 h and 48 h also showed cells in the S and G2/M phases of the cell cycle. This indicates that treatment caused apoptosis without first blocking the cell cycle. Further, B16-BL6 cells that were treated with the aqueous extract of *Andrographis paniculata* at high dose for 24 h exhibited a lower percentage of apoptotic cells of around 6% compare to the UV control (Figure 3.23.). The treatment with the aqueous extract for 24 h also showed a regular cell cycle distribution. These findings suggested that B16-BL6 cells were more sensitive in response to ethanol extract than aqueous extract. *Andrographis paniculata* induced apoptosis in B16-BL6 cells in a drug concentration-, time- and extract type-dependent manner.

Figure 3.22. The effect of the ethanol extract of *Andrographis paniculata* on B16-BL6 cell cycle profile (Sub-G1).

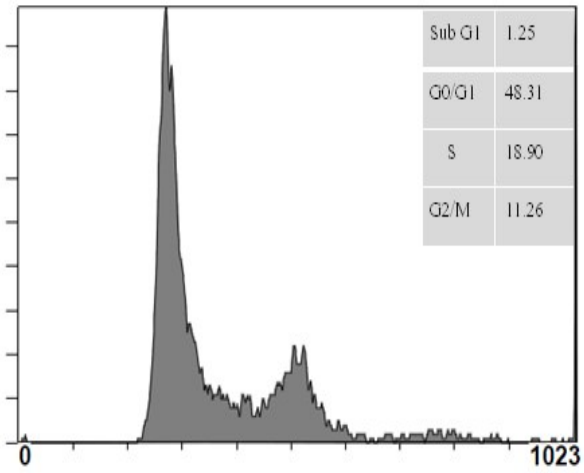
B16-BL6 murine melanoma cells were treated with the ethanol extract of *Andrographis paniculata* at a low dose for 24 h and at high dose for 24 h and 48 h to measure the DNA content of each cell following propidium Iodide staining. The results were analyzed by using a flow cytometer and the experiment was run 3 separate times. The graph shows the relative number of cells in each phase of the cell cycle (including a sub-G1 population).

A

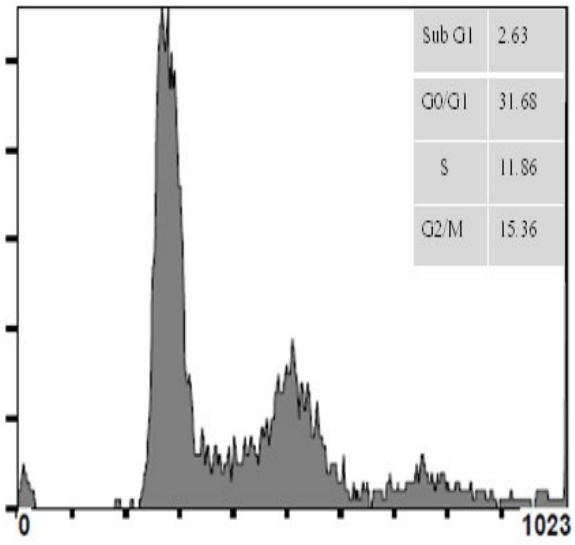
UV Control



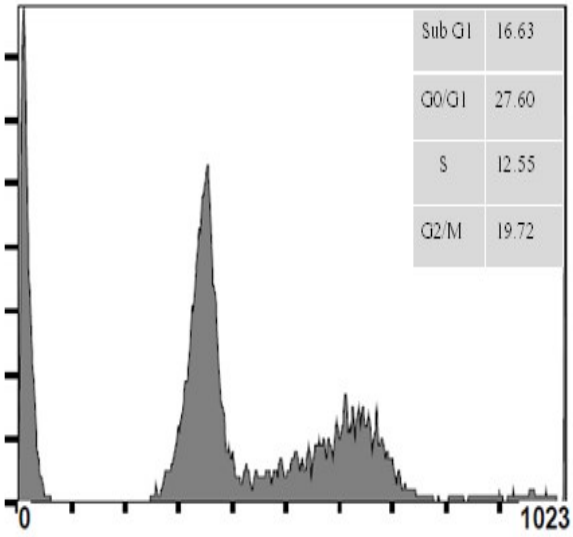
Negative Control



Low Dose of ethanol extract (24h)

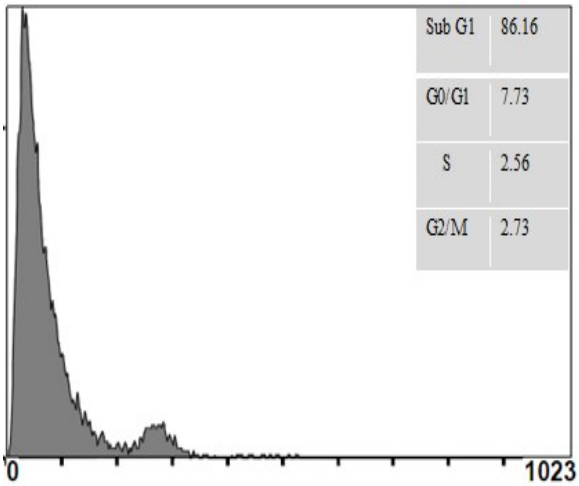


High Dose of ethanol extract (24h)



A1

UV Control



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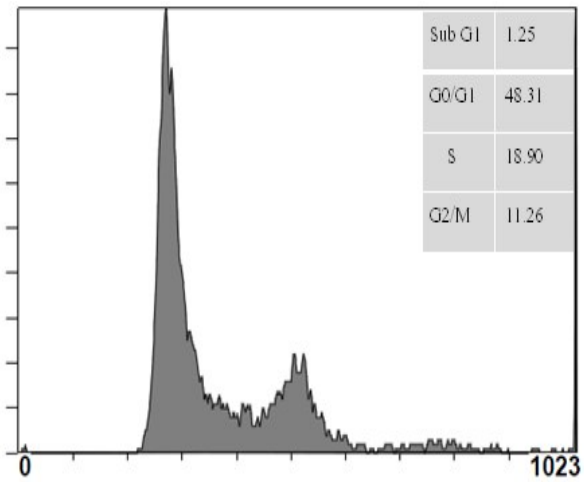
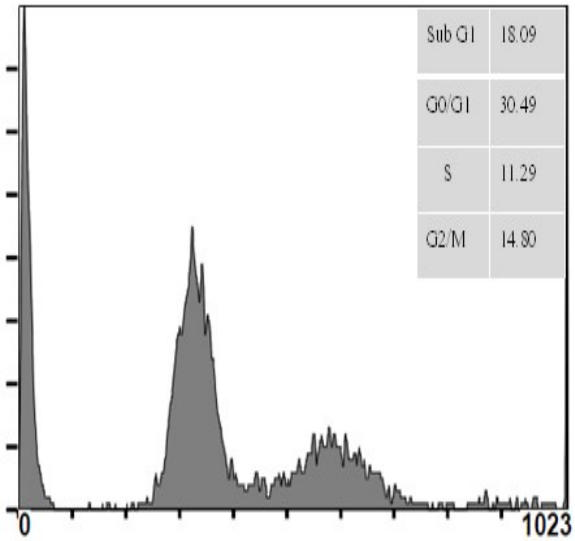


High Dose of ethanol extract (24h)



High Dose of ethanol extract (48h)

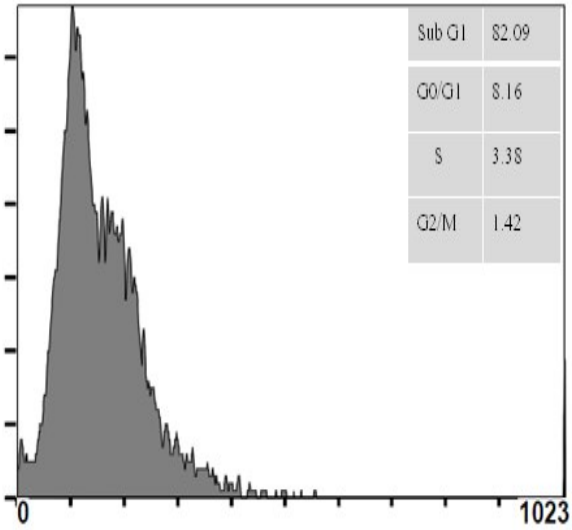
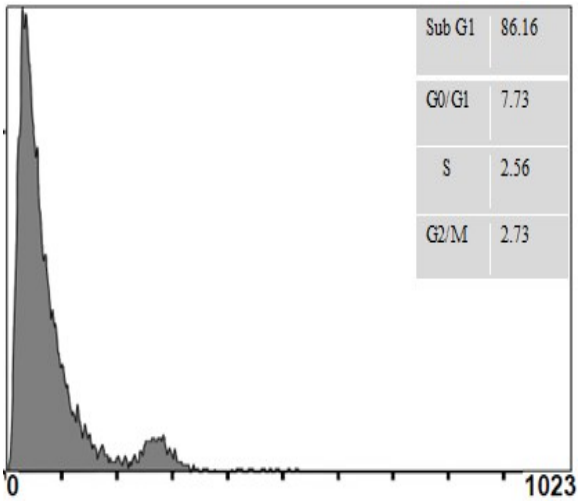


Figure 3.23. The effect of the aqueous extract of *Andrographis paniculata* on B16-BL6 cell cycle profile (Sub-G1).

B16-BL6 murine melanoma cells were treated with the PBS extract of *Andrographis paniculata* at low and high doses for 24 h and the DNA content of each cell was measured using propidium iodide staining. The results were analyzed by using flow cytometer and the experiment was run 3 separate times.

B

UV Control



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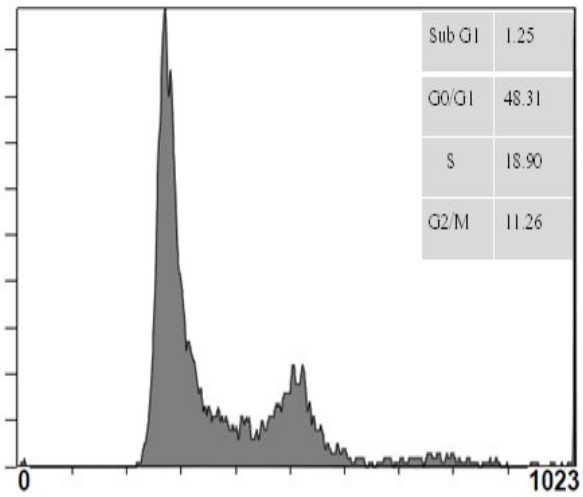
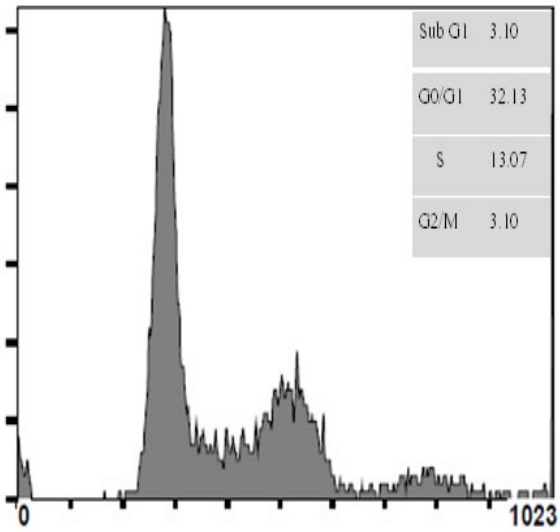
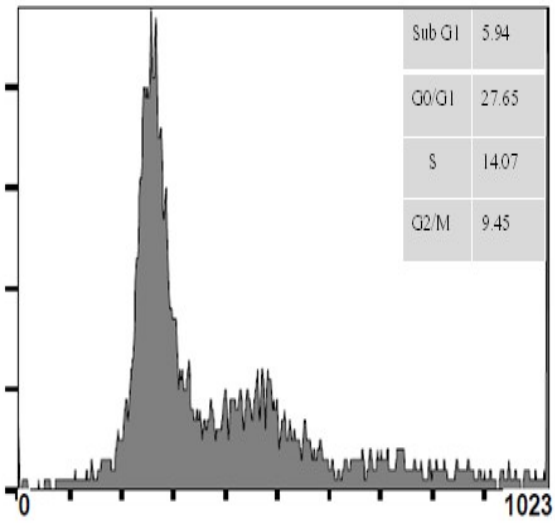


Low Dose of PBS extract (24h)



High Dose of PBS extract (24h)



Chapter 4: Discussion

Cancer is one of the many progressive disorders known today. Unregulated cellular proliferation is the cause of cancer and approximately 6 million new cancer cases are registered globally on a yearly basis. The majority of these cases (over 70%) are found among people from low- and middle-income countries. According to the world health organization, the potential risk factors for cancer include; prolonged tobacco and alcohol consumption, inadequate intake of fruits and vegetables, and prolonged infections from some viruses like human papillomavirus (HPV), hepatitis C virus (HCV) and hepatitis B virus (HBV). It is estimated that deaths caused by cancer will continue to increase globally in the coming years and reach 12 million/year by 2030 (Butler., 2008). With the rise in cancer incidence, there is an increasing search for natural products that may be used as remedies. Such products are already being recognized as effective against several disorders. More specifically, natural therapeutic agents have been useful for drug discovery in the case of cancer and several infectious diseases (Choudhury& Paddar., 1985; Joselin& Jeeva., 2014). For cancer chemotherapy, plant-derived agents including vinblastine, paclitaxel, vincristine, epipodophyllotoxin and camptothecin are part of the treatment choices that are available for physicians. Nevertheless, the search for new and more efficacious therapeutics is a constant endeavor, and a wide variety of flora from all over the world being studied to identify potential anticancer agents (Varma, Padh & Shrivastava., 2011).

In this regard, a plant called *Andrographis paniculata*, which has been used for many years in Indian and Chinese traditional medicine, has come under focus in recent cancer research. The primary bioactive agent derived from this plant is called andrographolide and several research studies have shown its anti-tumor activities (Varma, Padh & Shrivastava, 2011). Using *in* vitro studies, we have demonstrated that *Andrographis paniculata* is capable of inhibiting the growth of a variety of cancer cells. *Andrographis paniculata* is also able to induce apoptosis in B16-BL6

mouse melanoma cell line in a time and dose-dependent manner. The following sections will discuss our findings, in addition to other studies in detail.

4.1. Anti-proliferative Activity of *Andrographis paniculata* Extracts:

The first study with *Andrographis paniculata* involved the analysis of an ethanol extract and a PBS (aqueous) extract and it ability to inhibit the proliferation of the B16-BL6 murine melanoma, ASPC-1 pancreatic cancer, MCF-7 human breast cancer, 4T1 murine breast cancer, and MDA-MB-231 human breast cancer cell lines over a period of 5 days by using the MTT assay. We found that the ethanol extract showed a significant inhibition of all the cell lines across all concentrations. On the other hand, the PBS extract was only effective in stopping cell proliferation at the highest concentration, and then only in the B16-BL6, ASPC-1, and MCF-7 cell lines. There was no inhibition of cell proliferation observed for the 4T1 and MDA-MB-231 cell lines with the aqueous extract.

These findings were in agreement with Suriyo et al., who analyzed the anti-proliferative effects of *Andrographis paniculata* extracts on (HepG2 and SK-Hep1) hepatocellular carcinoma, and (HuCCA-1 and RMCCA-1) intrahepatic cholangiocarcinoma cell lines by using the MTT assay. An ethanol extract of the first true leaf (FTLEE) and aqueous extract of the mature leaf (MLWE) of *Andrographis paniculata* were applied to these cell lines. They showed that FTLEE, which was found to have a high concentration of 14-deoxyandrographolide but low andrographolide content in comparison to MLWE, inhibited growth more potently among the cancerous hepatic and bile duct cells than its counterpart. While the presence of AP1 in the MLWE extract explained its cytotoxic activity against cancer cells, this does not account for the difference that was observed in cytotoxicity between the extracts. The authors hypothesized that interactions occurring between main active diterpenoids in the extracts, particularly AP1 and

AP6, have a role in the compounds' growth inhibitory effect. The effect of other unknown diterpenoids apart from the two on which this study focused could account for the difference in cytotoxic potency between FTLEE and MLWE (Suriyo et al., 2014). In addition to the potent anti-proliferative effects of *Andrographis paniculata* extracts (ethanol and aqueous) at different growth stages, different cancer cell lines showed variable sensitivity to these extracts. For example, Suriyo et al. found that the HuCCA-1 intrahepatic cholangiocarcinoma cell line was twice as sensitive to the extracts in comparison to the other cell lines (Suriyo et al., 2014). Similarly, our laboratory findings showed that the MCF-7 human breast cancer (triple positive) cell line possessed greater sensitivity to the extracts in comparison to the MDA-MB-231 human breast cancer (triple negative) and 4T1 mouse breast cancer (triple negative) cell lines. We theorize that these differences in the ethanol and aqueous extracts of *Andrographis paniculata* could be due to the differences in the chemical constitutions of these extracts. More specifically, we believe that some unknown compounds present in the ethanol (and not the aqueous) extract could play an important role in the differences in cell toxicity effects. In addition, we believe that the differences in hormone (estrogen) responsiveness between the MCF-7, MDA-MB-231 and 4T1 cell lines may be responsible for their variable sensitivities to the *Andrographis paniculata* extracts.

There are several studies that have been published to study the anti-cancer effect of andrographolide, the major active component of *Andrographis paniculata,* on different cancer cell types. In a more recent study, Yang et al., (2010) exposed four different lymphoma cell lines (SUDHL4, HF-1, Granta, and Ramos) to 0-100 µmol/L of andrographolide for 2 days. As the dosage increased, the rate of cell death also increased and the IC50 (which is defined as the concentration of a toxic agent that results in 50% cell death) for two days was noted at 15, 20,

30, and 40 µmol/L for HF-1, Ramos, SUDHL4, and Granta cell lines, respectively (Yang et al., 2010).

Furthermore, the anti-proliferative effect of andrographolide has been observed under *in vivo* conditions. Yang et al. injected C6 glioma cells into both ear pinna of ICR mice and allowed the cells to grow into tumors for 3 days. At day 3, the tumors on each ear had grown to similar sizes. Phosphate buffered saline (negative control) was injected into the left ear and 20 µmol/L of andrographolide was injected in the right ear. At day 9, the weight of the tumor in the right ear was reduced by 67% suggesting that andrographolide is an effective anti-cancer agent against glioma cells (Yang et al., 2014).

In summary, these studies present evidence for the anti-tumor effect of *Andrographis paniculata*. This could be attributed to the presence of andrographolide which has been shown to possess anti-proliferative properties.

4.2. Apoptosis induction & cell morphological changes by *Andrographis paniculata* extracts:

Apoptosis is a highly regulated process that ensures cellular homeostasis. It is one of the hallmarks of cancer treatment and an effective therapeutic response would involve specific destruction of cancer cells, and not the neighboring normal cells. Apoptotic cells display a specific change in cell morphology and gene expression profiles (Taraphdar, Roy & Bhattacharya, 2001). In our study, B16-BL6 cells were treated with varied concentrations of *Andrographis paniculata* extracts for 48 h. The cells were then stained with acridine orange and ethidium bromide to determine the morphological changes of the cells using a microscope. These dyes are used as indicators of apoptosis. We found that the treatment of B16-BL6 cells with a high dose of aqueous extract followed by staining with the dyes caused the presence of bright green dots in the nuclei, which was indicative of the fragmentation of the nuclear membrane and

condensation of the chromatin. When these cells were treated with a high dose of ethanol extract of *Andrographis paniculata*, the complete destruction of the cells was seen, suggestive of end stage apoptosis. These findings were supported by Harjotaruno et al., (2007), who assessed cell apoptosis in TD-47 human breast cancer cells by treating them with different concentrations of andrographolide and then staining these cells with acridine orange and ethidium bromide. Under low doses of andrographolide, the TD-47 cells showed an early-stage of apoptosis with the nuclei staining with bright green spots within nuclei. However, under high doses of andrographolide, the cells become completely fragmented and incorporated ethidium bromide, appearing orange compared to the uniform non-fluorescent appearance of the negative control cells (Harjotaruno et al., 2007).

Further investigation into the mechanism of action of *Andrographis paniculata* was done. Western blot analysis was used to examine whether *Andrographis paniculata* extracts are able to initiate apoptotic activity by caspase activation and downregulation of the cell survival signal, phospho-ERK. Our findings showed that treatment of B16-BL6 with the ethanol extract of *Andrographis paniculata* induced apoptosis by promoting caspase-3 activation and by inhibiting phosphorylation of ERK. Additionally, the activity of *Andrographis paniculata* extracts has been shown to be concentration- and time-dependent. When the B16-BL6 cell line was treated with the aqueous extract of the plant, we observed that the number of cells that became apoptotic increased from 24 to 48 h. Similar findings have been noted by Harjotaruno et al., (2007) which described the treatment of TD-47 human breast cancer cells with andrographolide for 24, 48, and 72 h. The results showed an increase in caspase-3 levels that was associated with a dosage dependent increase.

Further support for the apoptosis activity of andrographolide was found in Yang et al., (2014). In this study, the C6 glioma cell line was exposed to 10, 15, and 20 μM concentrations of

andrographolide for 24 and 48 h and the levels of caspase-7 and its downstream target, poly (ADP-ribose) polymerase (PARP) were determined. The authors found that treatment with 20 μM andrographolide for 12 and 24 h caused the levels of caspase-7 to increase by 1.8 and 2.2 times, respectively. The initial values, were suggestive of inductive effects of andrographolide on caspase-7 expression and activation (Yang et al., 2014). Furthermore, they found that the levels of cleaved PARP protein were increased 1.5, 3.5, and 3.8 times by treatment for 12, 24, and 48 h, respectively. This suggested that andrographolide induced cell death through the caspase-7 – PARP signaling pathway, and that activated caspase-7, in turn cleaved and inactivated PARP in a dose and time dependent way (Yang et al., 2014).

In another study, Yang et al., (2010) analyzed the effect of treating lymphoma cell lines (Ramos, Granta, SUDHL4 and HF-1), primary lymphoma patient samples (MCL, DLBCL and FL), and normal human lymphocytes with andrographolide for different periods of time and at different concentrations. Induction of apoptosis was determined by the activation of caspase-7 and downstream cleavage of PARP. While andrographolide did not result in significant cell death in the normal human lymphocytes, all of the cancer cell lines and patient samples that were tested were affected at all doses of the agent and at all periods of time, but to different extents (Yang et al., 2010). In addition, the affected cells showed the presence of cleaved PARP and activation of other downstream caspases (caspase-8, caspase-9, and caspase-3) in a dosage and time dependent way (Yang et al., 2010). Remarkably, this study found that cancer cells from patients had a greater sensitivity to andrographolide in comparison to the cancer cell lines, suggesting that this agent may have greater anti-cancer potential in clinical trials in comparison to *in vitro* studies (Yang et al., 2010).

In summary, our results showed that *Andrographis paniculata* (33% andrographolide) was effective in stimulating apoptosis as measured by caspase 3 activation and P-ERK inhibition.

These results were in agreement with earlier studies which revealed and confirmed that andrographolide induced apoptosis measured by caspase activation in various cancer cell types.

4.3. *Andrographis paniculata* and Cell Cycle Analysis*:*

The cell cycle is comprised of four phases, a G1 phase which is variable in duration due to the presence of the G0 checkpoint, followed by a synthetic S phase, a G2 phase, and a short mitotic M phase, during which the cell grows, doubles its genetic content, and finally divides. Controlling the cell cycle in the G1 phase has recently been identified as a potential target for cancer treatment and research, since blocking cell division early in the cycle is important in developments in cancer treatment. It has been discovered that a large number of the regulatory genes associated with the G1 phase have significant effects on differentiation, proliferation, apoptosis and oncogenic transformation (Owa et al., 2001). In our study, flow cytometry with propidium iodide staining was employed to determine the presence and the changes in cell cycle and to assess the number of apoptotic (sub-G1) cells. Upon exposure to the high dose ethanol extract for 24 hours, the number of apoptotic cells accumulating in the sub-G1 phase measured 17%. Prolonged exposure for 48 hours dramatically increased the number of sub-G1 cells to 82%. This was in contrast to the exposure to high dose of aqueous extract for 24 hours, which caused apoptotic changes in only about 6% of the cells. This further reinforced the previous finding that the ethanol extract was more potent in inducing apoptosis than the aqueous extract and both caused apoptosis in a dose- and time-dependent manner.

Suriyo et al. treated the intrahepatic cholangiocarcinoma cell lines (HuCCA-1, RMCCA1) with an ethanol extract of the first true leaf of *Andrographis paniculata*. The cell cycle arrest and apoptotic cells were detected by using flow cytometry. It was found that a cell cycle arrest was induced in the G0/G1 and the G2/M phases (Suriyo et al., 2014). Furthermore, apoptosis was

dose-dependent in both cell lines. Approximately 7.9-37.8% of cells died upon exposure to FTLEE at doses from 0.2 mg/mL to 0.8 mg/mL in the HuCCA-1 line and 10.9-50.9% of cells died at doses from 0.4 mg/mL to 1.0 mg/mL in the RMCCA-1 cell line (Suriyo et al., 2014). This study suggested that *Andrographis paniculata* extracts stimulate apoptosis and cell cycle arrest in the HuCCA-1 and RMCCA-1 cells (Suriyo et al., 2014).

Wong et al. subjected human pancreatic cancer, PC-3 cells to 1, 3, 10, and 30 µM concentrations of andrographolide for 24 hours and performed flow cytometry to determine the cell cycle disruption. It was found that this treatment resulted in PC-3 cells accumulating at the G2/M phases and declining in the G1 and S phases (Wong et al., 2011). Interestingly, the authors also found that treatment with higher concentrations of andrographolide also caused the accumulation of cells in the sub-G1 phase, which indicated the presence of apoptotic cells (Wong et al., 2011).

In another study, Banerjee et al. determined the cell cycle arrest in the MDA-MB-231 triple negative breast cancer cell line following treatment with 30 µM andrographolide for 24, 36, and 48 h, using flow cytometry analysis. It was found that most cells accumulated in the S phase and only a few cells were present in the G1/G0 phase. The number of apoptotic cells increased as the duration of andrographolide exposure increased with 41% sub-G1 cells at 48 h (Banerjee et al., 2016). Other studies have also shown similar findings in hepatoma cells HepG2 (Li et al., 2007), colorectal carcinoma, Lovo cells (Shi et al., 2008) and acute myeloid leukemia, HL-60 cells (Manikam & Stanslas, 2009; Jayakumar et al., 2013). In summary, our findings were in line with the previous studies which indicated that *Andrographis paniculata* and its major bioactive component andrographolide effectively induced cell cycle arrest and apoptosis in variety cancer cell lines.

5. Conclusion:

Cancer is a multi-faceted disorder with several different etiologies and mechanisms for pathogenesis. Therefore, there is a constant need for novel therapeutics that could act alone or in addition to current remedies. Given the harsh nature of many current cancer therapeutic agents, there is a shift towards identifying naturally-derived agents for cancer treatment. In this regard, *Andrographis paniculata*, which is used extensively in South Asian traditional medicine, and has been cited in the Ayurveda as having anti-cancer potential, has become a promising agent for cancer therapeutic research (Varma et al., 2011). In this study, specific inhibition of cell growth and proliferation has been observed in several cancer cell lines, including B16BL6, ASPC-1, MCF-7, MDA-MB-231, 4T1 upon exposure to different concentrations and different periods of time to *Andrographis paniculata* extracts. Additionally, it has been determined that the ethanol extract is more potent than the aqueous extract in inducing anti- proliferative and apoptotic effects. Treatment with *Andrographis paniculata* extracts induced caspase activation and P-ERK inhibition. Further, it has been found that the properties of this agent is dependent on the type of extract, and the type of cancer cell lines.

However, there is a need for continued research on this agent before it can be proclaimed as a cancer therapeutic. In this study, the primary mode of action of *Andrographis paniculata* extracts was apoptosis, while the cell death pathway involved is still unknown. In addition, the dosage differences that produce effective cytotoxic responses in animal and human cancer cell lines need to be further investigated. Again, *Andrographis paniculata* has exciting prospects as a potent anti-cancer agent, but it needs to be thoroughly investigated before it can be put forward as a suitable candidate for cancer drug discovery.

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