Screening of Medicinal Plants on Skin Cancer Melanoma Cell Lines A375 and B16 using Cell Viability Assay

Oneza Saleem

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Abstract

According to the cancer research UK (2017), 14.1 million new cases of cancer were registered leading to the death of 8.2 million patients in 2012. It is estimated that by 2030, the incidence rate of cancer will rise to 23.6 million cases per year. (2017). Skin cancer melanoma is the 5th most common cancer in the UK as per Cancer Research UK (2015). This study focuses on skin cancer melanoma due to its increasing mortality rate. This study aims to screen medicinal plants from across the world against skin cancer melanoma. 26 medicinal plants were extracted with chloroform and methanol. 52 extracts of 26 plants were screened for anti-proliferation against human skin cancer melanoma cell line A375 and mice skin cancer melanoma cell line B16, using a colorimetric assay MTT. Plants like Horsetail and *Melissa officinalis* have shown significant results in decrease of cell viability. *Melissa officinalis* Lemon Balm has majorly shown a significant decrease in % cell viability in methanol extracts (IC$_{50}$= 0.39µg/ml) in comparison to positive control (IC$_{50}$= 0.65µg/ml) on B16 cell line. *Equisetum arvense* Horsetail shows less than 25% cell viability across all the extracts and cell lines. This study unveils interesting anticancer activity of some medicinal plants from across the world.

Declaration

This thesis is a presentation of my own work. References are included, to acknowledge the contribution of others and literature. Soxhlet extracts of the plants samples were provided by fellow PhD student Holly Siddique.

The work has been carried out under the supervision of Dr Mukhlesur Rahman and Prof. Olivia Corcoran at the University of East London.

Name: Oneza Saleem

Date:

Signed:

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Last but not the least, I would like to extend my deepest gratitude to my mother; Late Mrs Saima Saleem and would like to dedicate this research to her in search for treatment for cancer.

Chapter 1: Introduction

Cancer has emerged as a fatal disease worldwide, increasing demand for cure and treatment for cancer. According to World Health Organisation (2018), one in three cancer diagnosed is skin cancer. Around 232,000 people were diagnosed with skin cancer melanoma in 2012 as per Cancer Research UK (2018).

1.1 Cancer

Cancer is defined as the disease caused by uncontrolled division of abnormal cells leading to the formation of a tumour (National Cancer Institute, 2018). It is the malfunctioning of the cell cycle where cells keep growing and dividing without dying leading to the formation of a tumour mass in parts of the body. Failure to treat or prevent the spread of cancerous tumour which eventually leads to death. Several factors are responsible to cause the deadly disease including infectious organisms, unhealthy diet, environmental toxins, genetic mutations, immune conditions and hormones (Asadujjaman and Mishuk, 2013).

1.1.1 Types of cancer

There are many types of cancer that are sub divided into 6 categories (Table 1 adapted from; Asadujjaman and Mishuk, 2013)

Table 1.1: Different types of cancer

Category	Types	Examples
Carcinoma	Adenocarcinoma Squamous cell carcinoma Transitional cell carcinoma Clear cell carcinoma (CancerCenter.com, 2018)	Lung, colon, breast Head neck, anal, skin Bladder Renal (CancerCenter.com, 2018)
Leukaemia	Myelogenous or granulocytic Lymphatic, lymphoblastic (CancerCenter.com, 2018)	Myeloid and granulocytes Lymphoid and lymphocytic (CancerCenter.com, 2018)
Lymphoma	Hodgkin lymphoma Non-Hodgkin lymphoma (Asadujjaman and Mishuk, 2013)	Stomach, brain, breast (Asadujjaman and Mishuk, 2013)
Myeloma		Plasma cells in bone marrow (Asadujjaman and Mishuk, 2013)

Mixed types	Adenosquamous carcinoma Mixed mesodermal tumour Carcinosarcoma (Asadujjaman and Mishuk, 2013)	Breast, Lung, Colon, Prostate (Asadujjaman and Mishuk, 2013)
Sarcoma	Osteosarcoma (bono sarcoma) Soft tissue sarcoma (CancerCenter.com, 2018)	Bone Smooth muscles, connective tissues, blood vessels (Asadujjaman and Mishuk, 2013)

(Note: the top of the first visible row reads "(Keyword-suggest-tool.com, 2018)" in the rightmost column, continuing from the previous page.)

1.2 Carcinoma: Skin Cancer

Skin Cancer is the most common type of cancer in the world (Craythorne and Al-Niami, 2017). As of 2015, 15400 new melanoma skin cancer cases were registered accounting for 8100 in men and 7800 in women. (Cancer Research UK, 2018). There are 2400 melanoma skin cancer deaths in UK, as of 2016 as per Cancer Research UK.

1.2.1 Types of Skin Cancer

Skin cancer consist of two categories; non melanoma skin cancer (derived from epidermal cells) and melanoma skin cancer (derived from melanocytes) accounting for 95% of skin cancer types. (Craythorne and Al-Niami, 2017). Figure 1 below shows the different categories and sub categories of skin cancer.
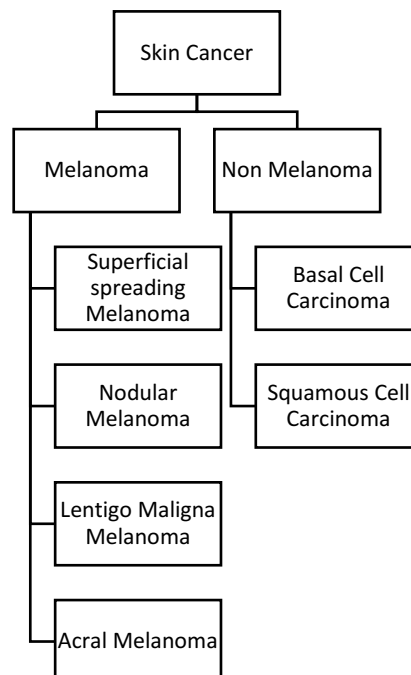

Figure 1.1: Different types of Skin Cancer (Simões, Sousa and Pais, 2015)

1.2.1.1 Non Melanoma Skin Cancer

Non melanoma skin cancer (NMSC) is the most common form of cancer. There are two types of NSMC; basal cell carcinoma accounting for 75% cases of NSMC and squamous cell carcinoma accounting for 25% (Samarasinghe and Madan, 2012)

1.2.1.2 Melanoma Skin Cancer

Melanoma Skin cancer is the deadliest type of skin cancer. Melanoma skin cancer is further divided into four types; superficial spreading melanoma (a slow growing melanoma), nodular melanoma (fast growing melanoma), lentigo malignant melanoma (affects areas of skin that has been extensively exposed to sun, generally of older people) and acral melanoma (occurs on soles of feet and palms of hand). (Skin cancer: Types, diagnosis and prevention, 2013)

Malignant melanoma occurs only in 4% of the population yet it causes 65% of skin cancer related deaths (Porter et al 2011) Malignant melanoma originates from epidermal melanocytes and is induced through various mechanism such as suppression of immune system of the skin, damage of melanocyte and induction of melanocyte cell division (Cummins DL, 2006). Melanocyte cells produces melanin; which is responsible for the pigment in skin. (Porter et al 2011)

1.2.2 Anatomy of normal skin

In order to understand skin cancer in depth, the structure of skin; largest organ in the body is studied in detail. The skin comprises of two main layers, the dermis and the epidermis. The different parts of the body have a varied thickness of dermis and epidermis, ranging from 2mm to 4mm.
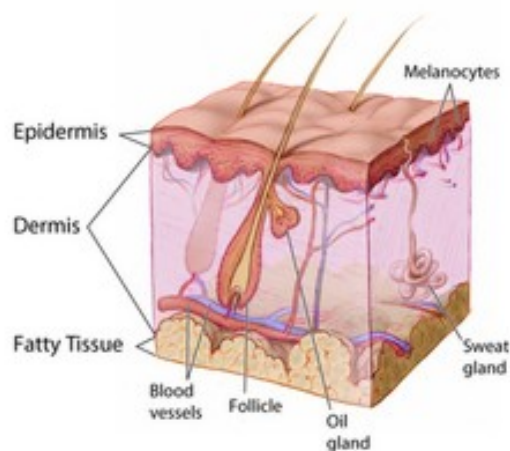

Figure 1.2: Anatomy of skin, highlighting the epidermis and dermis layer (Bliss, 2010)

1.2.2.1 Epidermis

The epidermis layer of the skin is made up of three types of cells, squamous cells, basal cells and melanocytes. Majority of the epidermis is made up of the squamous cells, the basal cells are round and found at the bottom of the squamous cells, melanocytes produces a pigment called melanin and is found between basal cells and other cells (Samarasinghe and Madan, 2012).

1.2.2.2 Dermis

The dermis of the skin layer consists of skin vasculature, nerves, sebaceous; which produces sebum to keep the skin moist and waterproof and sweat glands. The collagen and elastin in the skin gives it strength and elasticity.

1.2.3 Causes of Skin Cancer

Skin is the largest organ of the body and acts as a barrier and protects the body not only physically but also chemically against the harmful environmental agents such as pathogens ultraviolet radiation, chemicals and temperature fluctuations (Penta, Somashekar and Meeran, 2017). Most skin cancers are caused by exposure to sun, accounting for 65% of skin cancer across the world (Armstrong and Kricker, 1995). Factors that can contribute to cause skin cancer include family history, personal characteristics such as blue eyes, fair and/or red hair, sun exposure, atypical mole syndrome or socioeconomic status. (Heistein and Acharya, 2018)

1.2.4 Symptoms

Skin cancer melanoma can develop in any part of the body; especially those areas which have been exposed to sun like face, legs and arms. One of the first symptoms include change in a normal looking skin, whether colour, texture, development of new mole or changes to previous mole such as discharge, discolouration or rapid increase in size.

1.2.5 Diagnosis

An initial assessment is carried out using the ABCDE rule by the clinician's unaided eye, see table 1.2 (Skin cancer: Types, diagnosis and prevention, 2013). To limit human error, false negative cases and to improve efficacy, new detection and diagnostic techniques such as skin surface microscopes are used which allows improved visualisation of lesion. (Kittler H, 2002) Other advanced development diagnostic techniques include; MEDS, an automated melanoma diagnosis system used to analyse different measurement and characteristics of patient lesions to produce effective diagnosis. (Sboner et al., 2003) Other diagnostic techniques used for detection of skin cancer include, X ray, computed tomography scan or positron emission tomography scan, ultrasonography of regional lymph nodes or lymph node biopsy (Board, 2018).

Table 1.2: The ABCDE rule used by clinicians to carry out initial assessment

Asymmetry	The mole has an irregular shape
Border	The border of the mole is irregular or has jagged edges
Colour	There is a mix of colours in the mole
Diameter	The diameter of the mole is greater than 7mm
Evolution	The mole has changed size shape or colour

1.2.6 Stages

The stages of skin cancer melanoma is dependent on the thickness of the cancer and its spread. The thicker the cancer and/or the more it has spread the serious the cancer and therefore the higher the stage (Clinic, 2018). Letters T N M are used to determine the severity; (Kaufman, 2018).

- T is used for the extent of the tumour. Ulceration is the breakdown of the skin over the tumour which is not visible to the naked eye but is visible under a microscope during a biopsy is a sign of danger. In general, the thicker the tumour the harder it is to cure the cancer. (Kaufman, 2018).

- N is used for lymph node. Lymph nodes contain white blood cells, if melanoma is spread to nearby lymph nodes than its an advanced stage cancer (Kaufman, 2018).

- M is metastasis, cancer that has spread to other organs or distant lymph nodes is very dangerous (Kaufman, 2018).

Skin cancer melanoma is divided into four stages, see table below. (nhs.uk, 2017)

Table 1.3: Different stages of skin cancer melanoma

Stage	Description
Stage 0	Melanoma is on surface of skin (nhs.uk, 2017)
Stage 1	1A: The melanoma is less than 1mm thick (nhs.uk, 2017)
	1B: The melanoma is 1-2mm thick or less than 1mm (nhs.uk, 2017)
Stage 2	2A: The melanoma is 2-4mm thick or its 1-2mm thick and ulcerated (nhs.uk, 2017)
	2B: the melanoma is thicker than 4mm or its 2-4mm thick and ulcerated (nhs.uk, 2017)

	2C: The melanoma is thicker than 4mm and ulcerated (nhs.uk, 2017)
Stage 3	3A: The melanoma has spread to 1 to 3 nearby lymph nodes, but they are not enlarged, the melanoma is not ulcerated and has not spread (nhs.uk, 2017)
	3B: The melanoma has spread to 1 to 3 nearby lymph nodes and is ulcerated but they are not enlarged, or the melanoma is enlarged but not ulcerated and has spread to 1 to 3 nearby lymph nodes, or the melanoma has not spread to nearby (nhs.uk, 2017)
	3C: The melanoma is ulcerated and enlarged and has spread to 1 to 3 nearby lymph nodes, or it has spread into 4 or more lymph nodes nearby (nhs.uk, 2017)
Stage 4	The melanoma cells have spread to other parts of the body such as brain or lungs or to other parts of the body (nhs.uk, 2017)

1.2.7 Treatment

The treatment varies for every patient, depending on the type of cancer, the stage of cancer; as to how far it has spread or the size and general health. Surgery is the main treatment for skin cancer melanoma. (nhs.uk, 2017). Currently there are five different types of standard treatment options that includes surgery, chemotherapy, radiation therapy, immunotherapy and targeted therapy; available to treat skin cancer patients (Board, 2019).

1.2.7.1 Surgery

The primary treatment option for all stage of melanoma is to remove tumour via surgery. In order to remove the melanoma tumour, a wide local excision is carried out. In some cases, skin grafting; which is taking part of skin from another part of the body and replacing it on top of the skin that is being removed; is done to cover the wound caused by surgery (Board, 2019).

The surgery is carried out to improve patient's quality of life (Board, 2019). In some cases, patients are given chemotherapy post the surgery to kill any cancer cells that are left, whereas in other cases chemotherapy is given to lower the risk of the cancer returning, this is also known as adjuvant therapy (Board, 2019).

1.2.7.2 Chemotherapy

Chemotherapy is a treatment for cancer, that uses drugs to stop the growth of cancer cells either by killing them or stopping their growth (Board, 2019). Chemotherapy is administered in various ways such as via injection to veins or muscles, or placed directly to the organ or cerebrospinal fluid or body cavity, or directly to the arm or leg where the cancer is in. The administration of chemotherapy is dependent on type and stage of cancer (Board, 2019). Dacarbazine also known as DTIC, is one of the alkylating agents used to treat skin cancer, by sticking to cancer cell and damaging the DNA. Other drugs include; Temozolomide, Nab-paclitaxel, cisplatin, vinblastine etc. Vinblastine is a vinca alkaloid that binds to tublin and inhibit assembly of microtubules causing M phase cell cycle arrest.

1.2.7.3 Radiation Therapy

A high energy x-rays or other types of radiation is used in a radiation therapy to treat cancer by either killing the cancer cells or stopping their growth (Board, 2019). There are two types of radiation therapy:

- Internal Radiation therapy, which uses needles or catheters sealed with radioactive substance to be placed inside or near the cancer (Board, 2019).
- External Radiation therapy; sends radiation towards the cancer while the body is inside the machine (Board, 2019).

The type of radiation given is dependent on type and stage of cancer.

1.2.7.4 Immunotherapy

Immunotherapy is another type of standard treatment for cancer which uses patient's immune system to fight cancer. The body's natural defence is boosted by substances made by the body or in the laboratory to fight against cancer (Board, 2019). Currently there are four types of immunotherapy being used to treat melanoma; immune checkpoint inhibitor therapy, Interferon, Interleukin-2 (il-2) and tumour necrosis factor (TNF) (Board, 2019). The common immunotherapy currently in use is Nivolumab and Ipilimumab, this is used when cancer cannot be removed by surgery. (30)

1.2.7.5 Targeted Therapy

Targeted therapy is used to treat cancer with drugs that only target the cancer cells and cause less harm to normal cells in comparison to chemotherapy and radiotherapy (Board, 2019). Some of the targeted therapy being used to treat melanoma include; signal transduction inhibitor, oncolytic virus therapy and angiogenesis inhibitors.

### 1.2.7.6 New Treatment in Clinical Trials

New treatments are currently being studied to find a treatment for skin cancer, to improve quality of life of patients. One of the main treatments under study is the vaccine therapy

for stage 3 melanoma; which is to use of substances for immune system to respond to tumour and kill it (Board, 2019).

1.3 Medicinal Plants as source of anticancer drugs

The use of medicinal plants for treating diseases goes back to one of the traditional methods of healing. Since 1980 to 2002, 69% of the approved anticancer drugs have been developed from naturals sources (Cragg and Newman, 2007). The use of allopathy, using drugs to cause an opposite effect to the symptom (definition of allopathy in English by oxford dictionaries, 2017), in treating cancer brings along complications and adverse effects due to toxic levels. The demand for naturally derived drugs from medicinal plants is increasing day by day due to low levels of toxicity in comparison to current therapies (Greenwell and Rahman, 2015)

Vinca alkaloids are a group of compounds derived from a medicinal plant and the second most common therapy drug used to treat cancer. Four vinca alkaloids namely, vinblastine, vincristine, vindesine and vinorelbine, act as anti- tumour and antiluekemic agents. They are extracted from the pink periwinkle plant, *Catharanthus roseus G*. Though they are classified as alkaloid based on the formation and bonding of oxygen, carbon, hydrogen and nitrogen, some of the alkaloids do not pose alkaline properties. Vinblastine, a vinca alkaloid binds to tublin, inhibit assembly of microtubules causing M phase specific cell cycle arrest, hence stopping the development and growth of cancerous cells. It was discovered in 1950 for the first time and are known as cancer fighter's due to its cytotoxic effects. Recently in 2008 another alkaloid has been developed and is used in Europe for treatment called vinflunine. These alkaloids are not only used for treating cancer but are also used in treating high blood pressure and diabetes due to hypoglycaemic properties. The mechanism of action of vinca alkaloids involve interaction with tubulin and disruption of microtubules. . (Moudi et al., 2013). The vinca alkaloid bind to the site of tubulin separate from taxannes disrupting microtubule congregation leading to metaphase arrest hence stopping the growth of cells. They interact with the mitotic spindles stopping the replication and growth of cancer cells leading to the death in process of division. Though the stop the growth of cell, but the side effect is that they vinblastine causes toxicity in white blood cells. (Moudi et al., 2013). Although these are found to be the most effective cancer cell treatment as the replication of cells is stopped instantly after interaction, but they also affect normal healthy cells. These alkaloids are generally used in combination for the purpose of treating cancer. (DeGregorio and Wiebe, 1999).
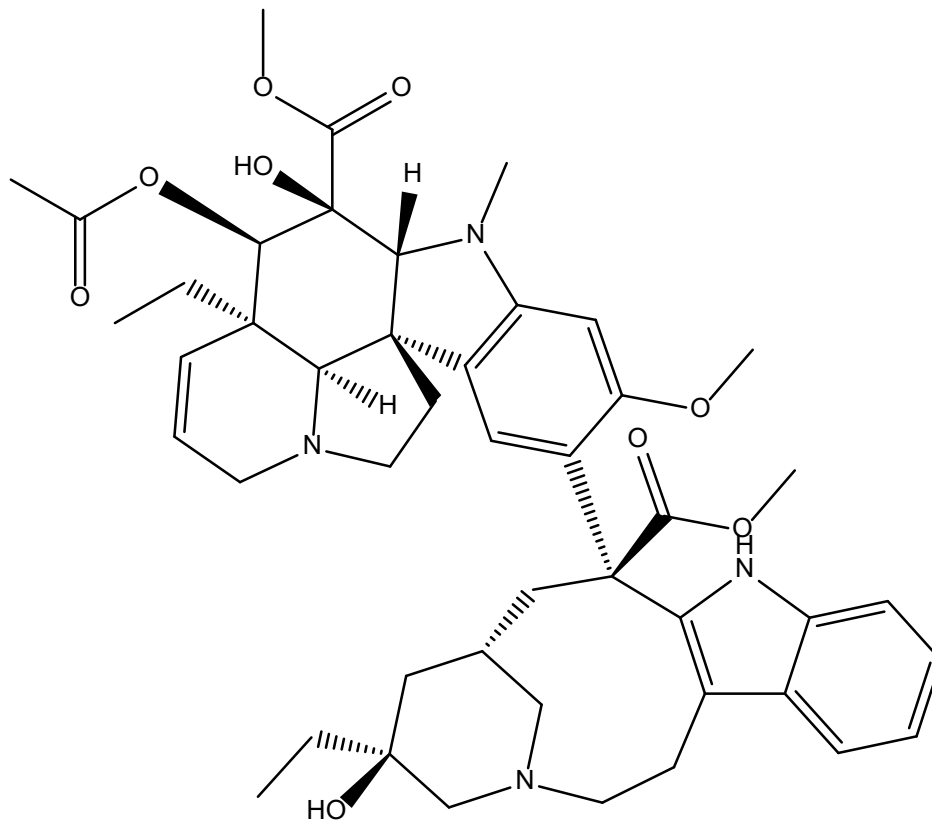
Structure of Vinblastine
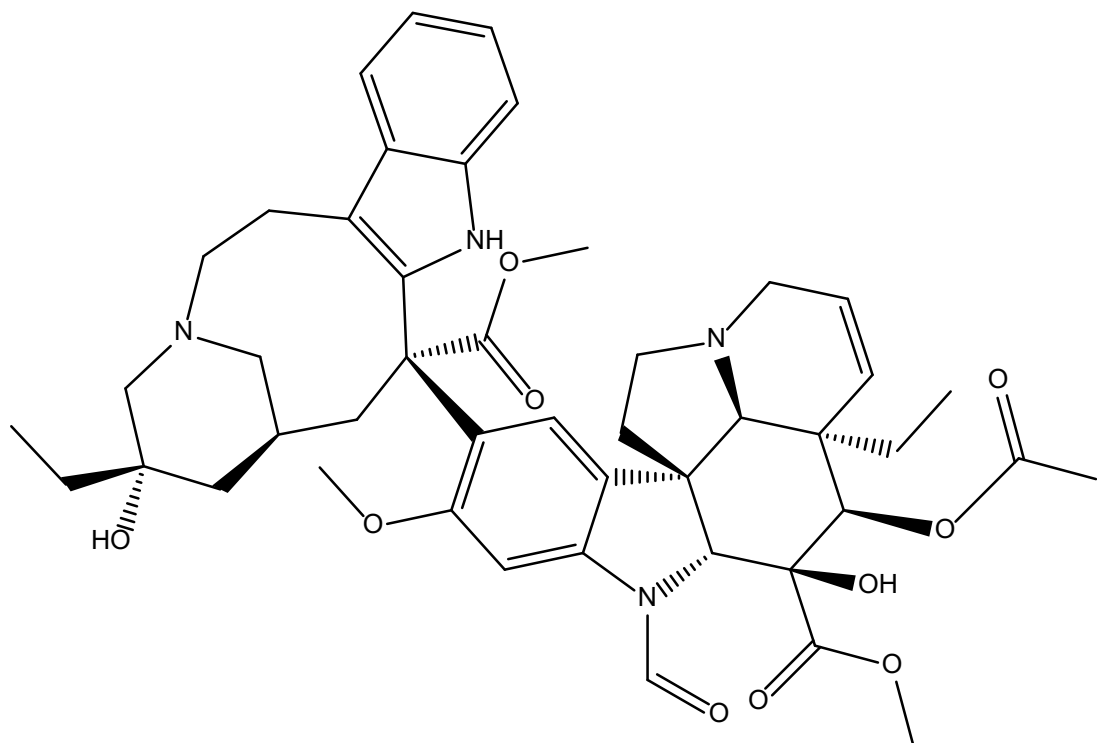
Structure of Vincristine

Taxol also known as Paclitaxel is derived from a natural product called Pacific yew tree, is used to treat several types of cancer by blocking the growth of the cells. (Weaver 2014). Chlormethine also known as Mustargen is an alkylating agent used to treat cancer. This was discovered accidently in 1940's when mustard gas was released and inhaled by people, it was noticed that the white blood cells count has decreased, hence was further investigated and it is currently being used to treat lymphatic cancer called Hodgkin's lymphoma (Singh et al., 2018).
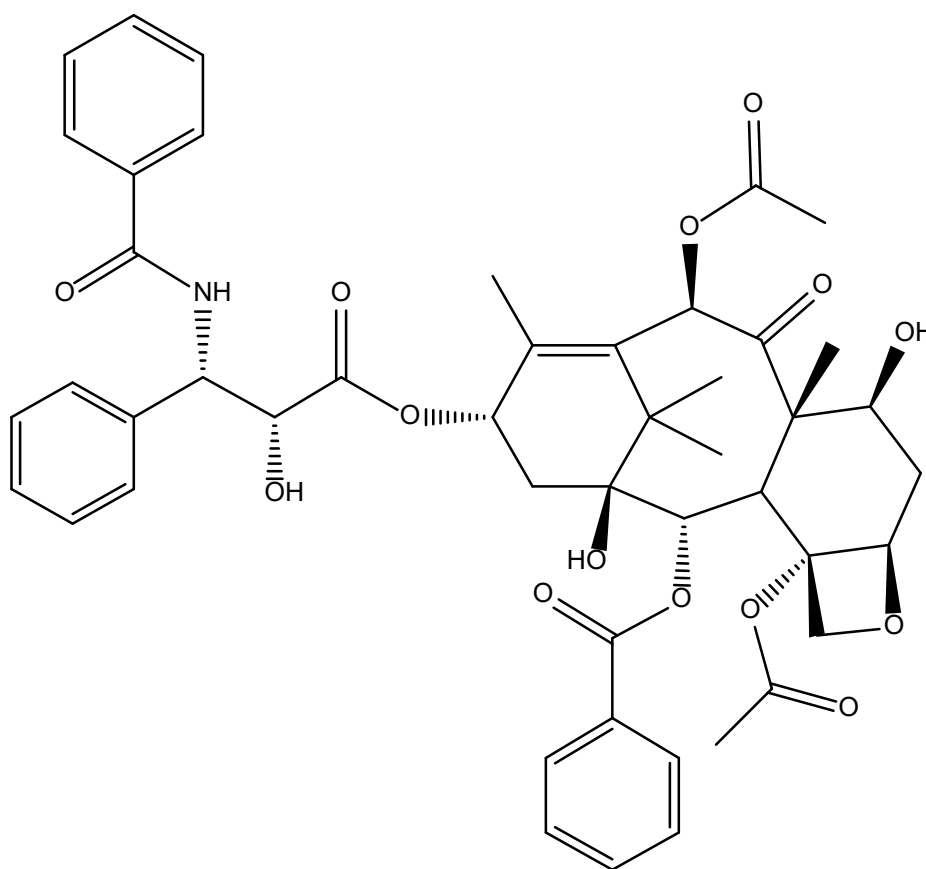
Structure of Taxol

1.3.1 Selection of plants:

In house plants were selected on the basis of literature review, traditional uses and previous research carried out. The selection of plants is based on exploring research papers about their properties and traditional use to treat cancer by searching on various sites; PubMed, Google Scholar, Science Direct, and Medline. The review of plants looked at papers from various years by searching various keywords; such as name of the plant, cancer, treatment, traditional use, medicinal plants and properties. The inclusion criteria

was looking at traditional use of the plants within a timespan of around 30-40 years focusing on treatment and medicinal use. The excluding criteria was to ignore diagnostic or causative use of the plants. The study looked at specific properties of each plants mainly focusing on anti cancer activity. Some properties of the plants with respect to cancer can be seen in the table 1.4. These plants were identified by a qualified taxonomist and the voucher specimens of these plants were submitted to the Bangladesh National Herbarium.

Table 1.4: Name of plants, the family and properties

Name of Plant	Family	Properties	Source
Agropyron cristatum Wheatgrass	Poaceae	Anticancer (Gore, 2017)	Proline Botanicals
Andrographis paniculata Kalmegh	Acanthaceae	Antitumor and antimetastic (Yue et al., 2019)	Bangladesh
Angelica archangelica Angelica	Apiaceae	Antitumor (Oliveira et al., 2019)	Proline Botanicals
Apium graveolens Celery	Apiaceae	Antitumor (Hazafa et al., 2019)	Proline Botanicals
Borage root Starflower	Boraginaceae	Anti-inflammatory (Akinbo et al., 2018)	Proline Botanicals
Agathosma, Buchu Leaf	Rutaceae	Anti-inflammatory (Drugs.com, 2019)	Proline Botanicals
Equisetum arvense Horsetail	Equisetum	Antioxidant and antiproliferative (Četojević-Simin et al., 2010)	Proline Botanicals
Feronia limonia Wood Apple	Rutaceae	Antioxidant (Thirugnanasampandan and David 2014)	Bangladesh
Glycyrrhiza glabra Liquorice	Fabacea	Antiproliferative (Gioti et al., 2019)	Proline Botanicals

Hemidesmus indicus Anantmool	Apocynaceae	Anticancer (Turrini et al., 2018)	Bangladesh
Hypericum perforatum St John's Wort	Hypericaceae	Anxiolytic (Howe et al., 2016)	Proline Botanicals
Hyssopus officinalis Hyssop	Lamiaceae	Antioxidant and antiproliferative (Nile, Nile and Keum, 2017)	Proline Botanicals
Lavandula Lavender	Lamiaceae	Antioxidant antibacterial and antiproliferative (Nunes et al., 2016)	Proline Botanicals
Matricia recutita Chamomile	Asteraceae	Antioxidant and anti cancer (Al-Dabbagh et al., 2019)	Proline Botanicals
Melissa officinalis Lemon Balm	Lamiaceae	Anxiolytic (Howe et al., 2016)	Proline Botanicals
Rosa gallica Rose	Rosaceae	Anti-inflammatory and antioxidant (Lee et al., 2018)	Proline Botanicals
Rosmarinus Rosemary	Lamiaceae	Antitumor (Pérez-Sánchez et al., 2019)	Proline Botanicals
Salix alba White Willow	Salicaceae	Antitoxic and antitumor (Aksinenko et al., 2015)	Proline Botanicals
Salvia Sage	Lamiaceae	Antiproliferative (Jiang, Zhang and Rupasinghe, 2017) antioxidant (GARCIA et al., 2016)	Proline Botanicals
Swertia cirayita Felworts	Gentianaceaea	Antiproliferative (Wang et al., 2018)	Bangladesh
Tilia x europea Lime	Malvaceae	Anxiolytic and anti fungal (Howe et al., 2016)	Proline Botanicals
Turnera diffusa Damiana	Passifloraceae	Antibacterial antioxidant and anticancer (Avelino-Flores et al., 2015)	Proline Botanicals

Tussilago farfara, Coltsfoot	Asteraceae	Anticancer and antimicrobial (Lee et al., 2019)	Proline Botanicals
Valeriana, Valerian	Caprifoliaceae	Antitumor (Honma et al., 2019)	Proline Botanicals
Viola tricolor Heartsease	Violaceae	Anticancer (Sadeghnia et al., 2014)	Proline Botanicals
Zingiber officinale wild Ginger	Zingiberaceae	Anti leukemic, anticancer, Kalantari et al., 2019) antioxidant and antimicrobial (Rahimi Babasheikhali, Rahgozar and Mohammadi, 2019)	Bangladesh

A recent study shows the use of two traditional medicinal plants in Srilanka that pose anticancer properties. *Zingiber officinale* commonly known as wild ginger is used as an anticancer agent to prepare traditional medicine for treatment of gastrointestinal, liver and oesophageal cancer (Singh, 2007). *Hemidesmus indicus*, commonly known as Indian Sarsaprilla is also used as an anticancer agent as it poses poly herbal properties Eleonora Turrini and Manuele, 2018).

1.4 Aim

The overall aim of this study is to investigate selected traditional medicinal plant extracts against human skin cancer melanoma cell line A375 and mice skin cancer melanoma cell line B16.

1.4.1 Objectives

To prepare chloroform and methanol extracts using Soxhlet extraction.

To maintain human skin cancer melanoma cell line A375 and mice skin cancer melanoma cell line B16.

To treat A375 and B16 cell line with different concentrations of different plant extracts.

To test cell viability of A375 and B16 cell line using colorimetric assay, MTT.

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Chapter 2: Materials and Methods

2.1 Plant Materials

In house plants, available in the lab, were screened for anti-cancer activity during this study. Majority of the plants were purchased from commercial suppliers including Proline Botanicals (currently Herb in a Bottle). Some plants were also collected by a PhD student Holly Siddique from Bangladesh and voucher specimens of these plants were kept in the Bangladesh national Herbarium.

2.2 Experimental Design

Plants were extracted sequentially with chloroform and methanol using Soxhlet apparatus. These were screened against human and mouse cell lines from different origins to analyse cell viability using colorimetric assay.

Table 2.1: Cell lines used in this study and related disease

	Cell Lines	Cell Types (Organ)	Disease	Source
Mice	A375	Epithelial (skin)	Malignant Melanoma	(Atcc.org, 2018)
Human	B16	Mixture of spindle shaped epithelial cells (skin)	Melanoma	(Lgcstandards-atcc.org, 2018)
	DU145	Epithelial (prostate, derived from metastatic site: brain)	Prostate Carcinoma	(Lgcstandards-atcc.org, 2018)
	MCF-7	Epithelial (mammary gland, breast, derived from metastatic site: pleural effusion)	Breast Adenocarcinoma	(Lgcstandards-atcc.org, 2018)

2.3 Plant Extraction

Soxhlet extraction, the most common method of extracting compounds from natural products, involves a solvent to extract the desired compounds from the solid material, therefore is known as a form of continuous solid liquid extraction.

Dried plant materials were crushed using a mortar and pestle to give larger surface area before undergoing soxhlet extraction. The solid materials (plant samples; 1-2 gm) were

placed in thimble before being placed in the main chamber of the extractor and the solvent (chloroform or methanol; 50-100 ml) was placed in the distilled flask at a temperature of 4°C. The plant sample was extracted by heat reflux for 120min. The extracts were dried off using a rotary vapour evaporation and stored at 4°C before use.

These extracts were used for cell viability study against different cancer cell lines.

2.4 Plant Sample Preparation

Using a weighing scale 10mg of crude extracts were transferred to labelled vials and stored at 4°C. Crude extracts were dissolved in DMSO to give a stock solution of 40mg/ml and further diluted to the required starting concentration using complete growth medium when required.

2.5 Cell Lines and Growth Conditions

The complete medium also known as growth medium was prepared in a sterile flow hood and stored at 4C for all the cell lines. Human skin melanoma; A375, Rat skin melanoma; B16 and Prostate cancer; DU145 cells were cultured in RPMI 1640 cell culture media, supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. All the cell lines were grown in humidified incubator at 37°C with 5% CO_2. These cells were provided by School of Pharmacy, University College London.

2.6 Cell culture

The cell lines were grown in complete growth medium until 80-90% confluent to be used. In this assay the nature of the cells was adherent. The medium was discarded, and the cells were washed with PBS solution. 1ml trypsin was added to the cell culture flask and incubated for 5min for cells to be suspended. The cells were viewed under microscope to check for dissociation, once fully dissociated, 10ml fresh medium was added. The cells were then centrifuged at 300*g for 5min. Then the medium was removed, and cells were resuspended in 5ml fresh medium. 0.025ml cell sample was mixed with 0.475ml trypan blue in a 1.5ml Eppendorf tube, 0.1ml of this solution was loaded onto the Neubauer cell counting chamber to count the cells. The cells were seeded into a clear 96 well cell culture plate at the optimum seeding density for all cell lines (1x10 cells/ml). The cells were incubated for 24h at 37°C and 5% CO_2 before the treatment was added. The cell lines used were maintained in Complete Medium and every 48h the media was refreshed. The cells were passaged when 80-90% confluence.

2.7 Comparison between different Assays

Table 2.2: Comparison between different types of assays to determine cell viability.

	SRB	XTT	MTT
Principle	SRB (Sulforhodamine B) assay is a colorimetric assay, used to determine cytotoxicity. The SRB assay consists of four stages; the preparation of treatment followed by incubation of cells with treatment, cell fixation, SRB staining and absorbance measurement. (Orellana and Kasinski, 2018)	XTT assay is a colorimetric assay used to determine cell proliferation. (Sigma-Aldrich, 2018)	MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is a colorimetric assay, used to determine cell viability. (Ogbole et al., 2018)
Mechanism	SRB (Sulforhodamine B) assay is a colorimetric assay, which in acidic conditions binds to the protein but under basic conditions can be extracted (Orellana and Kasinski, 2018)	The colourless or slightly yellow XTT dye is reduced to brightly orange soluble formazan derivative. (Wang, Yu and Wickliffe, 2011) (Berridge, Herst and Tan, 2005)	The yellow MTT dye (tetrazolium) is reduced by the succinate dehydrogenase to form a purple coloured insoluble compound known as formazan. The amount of formazan produced is proportional to the number of viable cells. (George et al., 2010) DMSO (dimethyl sulfoxide), a solubilising solution is added to dissolve the purple formazan containing cells which is measured by spectrophotometry.
Uses		It is used to measure cell proliferation,	It is used to measure cell viability and proliferation. (Ogbole et al., 2018)

		cytotoxicity and apoptosis. (Wang, Yu and Wickliffe, 2011)	
Limitation	It is a long process Expensive	Expensive	

2.8 MTT Assay

MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is a colorimetric assay, used to determine cell viability of plant extracts (Ogbole et al., 2018). The yellow MTT dye (tetrazolium) is reduced by the succinate dehydrogenase to form a purple coloured insoluble compound known as formazan. The amount of formazan produced is proportional to the number of viable cells (George et al., 2010). DMSO (dimethyl sulfoxide), a solubilising solution is added to dissolve the purple formazan containing cells which is measured by spectrophotometry. A comparison of a few cell viability assay was carried out as can be seen in table 2.3 above. MTT assay was selected for cell viability assay due to its cost effectiveness and quicker results.

The cells were seeded in a 96 well plate with a density of 1×10^5 cells/ml in a 100ul complete growth medium and incubated for 24h at 37C and 5% CO_2. The cells were treated with 100ul of 1:20 diluted extracts to give concentrations of 500, 250, 125, 62.5 and 31.25µg/ml along with vehicle control and positive control (doxorubicin) and incubated for 4h, 24h and 48h at 37C and 5% CO_2.After 24h incubation 10ul of yellow MTT reagent from 5mg/ml stock solution was added to each well and the 96 well plates were wrapped around in foil to maintain MTT sensitivity to light and incubated for 2h to 4h at 37C and 5%CO_2. The precipitated crystals were dissolved in 100ul of DMSO. The absorbance was read using a microplate reader at a wavelength of 570nm. Every experiment included a set of vehicle control (cells+ media+ DMSO) and negative control (cells + media). The experiment was performed using technical replicates of 4 on the plate (Figure 3).

A comparison of different colorimetric assays; SRB, XTT and MTT was carried out for determination of cell viability as can be seen in Table 6. MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide)) assay was carried out to determine the cell viability of plant extracts on rat and human skin cancer melanoma cell lines A375 and B16, respectively. MTT assay involves fewer steps; hence is not complicated, uses fewer materials; therefore, is cheaper and doesn't have any radioactive waste disposal. On the

contrary SRB assay is limited to manual or semiautomatic screening and the process is very complicated and XTT assay is very expensive. (Riss et al., 2018)

2.8.1 Vehicle

Traditionally water is used to dissolve plant extract, but complete aqueous dissolution was not possible. Therefore DMSO (Dimethyl sulfoxide) was used as a vehicle control to dissolve all the controls and plant extracts. DMSO was used as it is a routinely used solvent in laboratories. (Rodríguez-Burford C, 2003)

2.8.2 Control

Positive controls were used to show that the assay is working as well as to account the effects of vehicle control. Positive control was tested against both the cell lines to determine cell viability using MTT assay. The positive control selected is already in use to treat cancer, therefore showed that the system worked well.

Doxorubicin is generally used as a positive control to determine cell viability or cell proliferation via MTT assay. It is a chemotherapeutic agent that has been in use to treat cancer since 1960s. (Johnson-Arbor and Dubey, 2018). All the 52 extracts from 26 plants screened against A375 and B16 at a concentration of 500µg/ml (as this showed the least cell viability), were compared to Doxorubicin; the positive control as can be seen in figure 30 to figure 33.
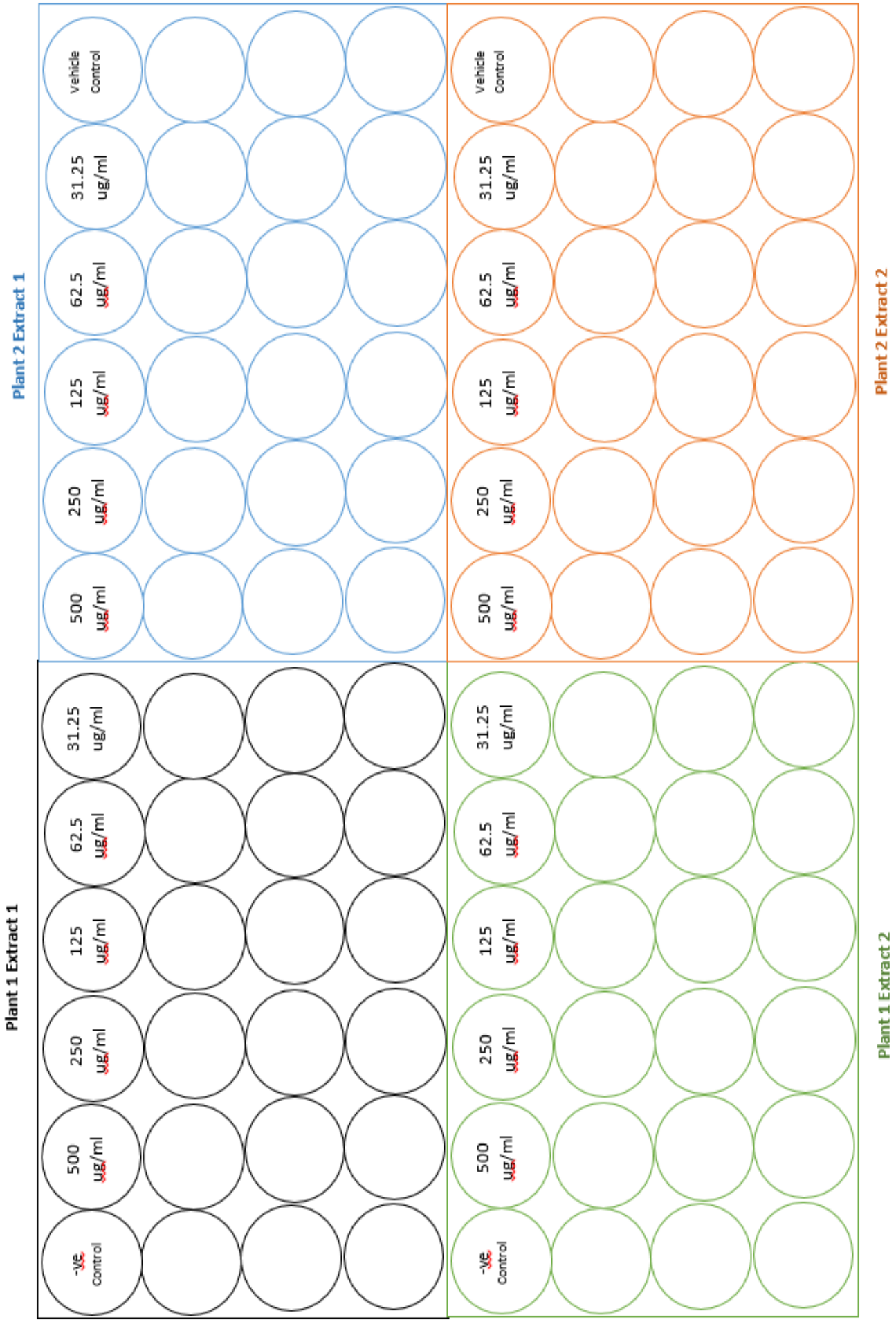
2.8a 96 well plate template

Figure 2.1: The 96 well template used to determine cell viability of different plant extracts on different cell lines at different concentrations along with negative and vehicle control.

2.9 Statistical Analysis

The results were expressed as means and ±standard deviation of the technical replicates (n=1; 4 technical replicates on the plate) followed by calculating %cell viability using the formula below. One way analysis of variance (ANOVA) was performed for comparing the data. All statistical analysis were performed using GraphPad Prism 4.02. The differences were accepted as statistically significant at ***$p<0.001$, **$p<0.01$ and $p<0.05$.

% Cell Viability = (absorbance for treated cells / absorbance of control cells) * 100

Chapter 3: Results

To investigate the cytotoxic effects of medicinal plants on human malignant melanoma cell line A375 and rat melanoma cell line B16, a total of 26 plants were extracted with chloroform and methanol using Soxhlet extraction. 52 extracts from 26 plants were screened against A375 and B16 using a colorimetric assay, MTT to determine cell viability. The absorbance was read using a microplate reader at a wavelength of 570nm.

3.1 Determination of Cell Viability

52 extracts from 26 plants at five different concentrations (31.25, 62.5, l25, 250, 500µg/ml) were screened against A375 and B16 using a colorimetric assay, MTT to determine cell viability. The vehicle control DMSO was also tested to ensure the cell viability in extract treated cells is not compromised. It can be seen that each plant treatment showed a different degree of cell death as the concentration increased. Almost all treatment achieved an IC50 at different concentration. Methanol extracts have overall shown a decrease in cell viability as per its high polar and strong attractive forces nature.

3.1.1 Overview of Results obtained

The results obtained from this study shows that activity of all plant extracts is concentration dependant. Except, *Hemidesmus indicus*, all other plants have shown cell viability less than 50% when treated with 500µg/ml across both cell lines as can be seen in figures 3.1-3.8; which shows the presence of an active phytochemical agent. However, almost all plant extracts have shown cell viability more than 75% when treated with 31.25µg/ml across both cell lines. Most extracts have shown stronger cytotoxic activity against B16 than A375. The percentage cell viability decreased as the concentration of plant extracts increase, hence resulting in negative correlation. Methanol extracts tend to show lower % cell viability in comparison to chloroform such as *Andrographis paniculata* methanol extracts show a % cell viability of 0.2% in both cell lines; b16 and A375, whereas chloroform extracts showed around 10% and 0.3% cell viability in A375 and B16 respectively as can be seen in figure 3.6. Overall methanol extracts have shown the least % cell viability in comparison to chloroform extract.
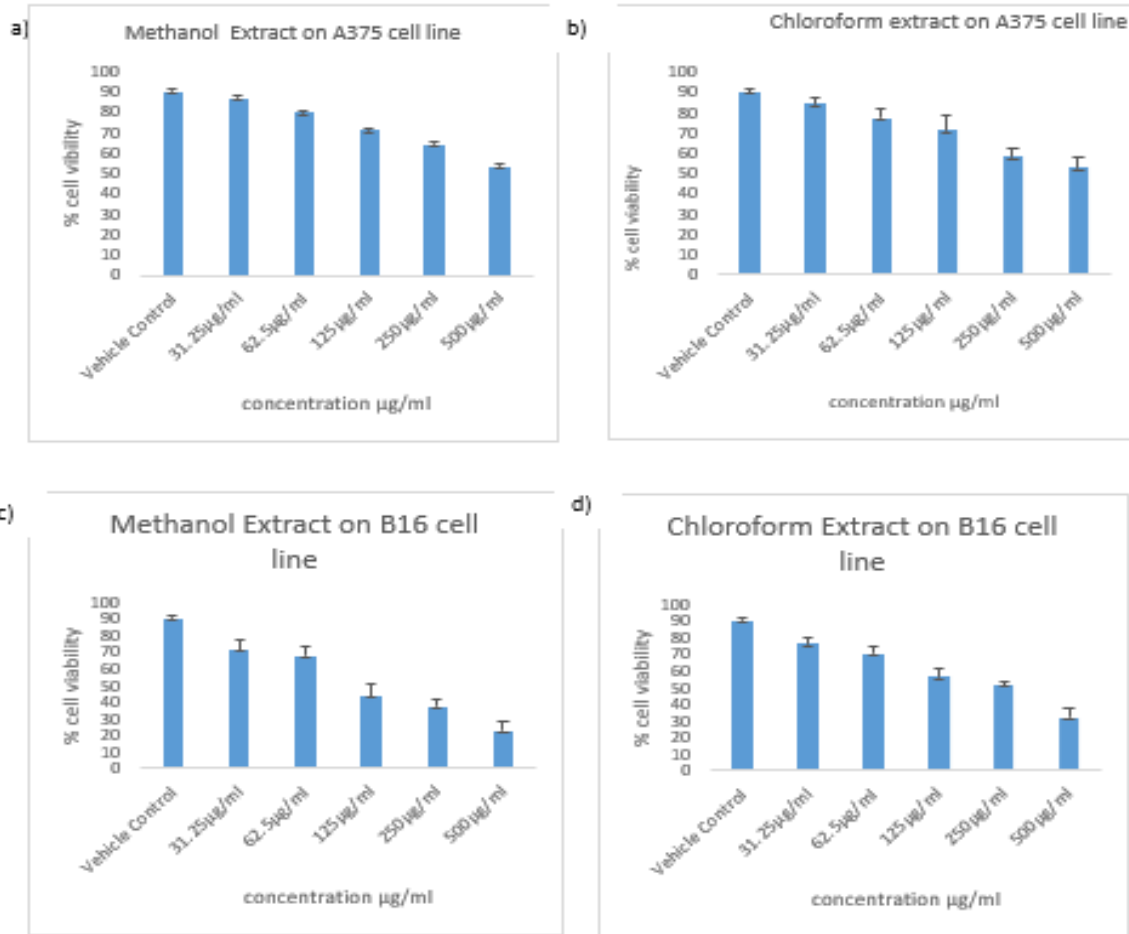
% Cell Viability of Plant Zingiber officinale Wild Ginger

Figure3.1:Determination of Cell Viability. % Cell Viability of *Zingiber officinale* Ginger (a) Methanol extract on A375 human melanoma, (b) Chloroform extract on A375 human melanoma, (c) Methanol extract on B16 rat melanoma and (d) Chloroform extract on B16 rat melanoma, via MTT assay 24hr after treatment at different concentrations. Samples were compared using one way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Zingiber officnale commonly known as ginger pose many medical properties such as antibacterial, antifungal anti-inflammatory and anticancer (Mbaveng and Kute, 2017). *Zingiber officinale* chloroform and methanol extract have shown a decrease in percentage cell viability as the concentration increases as can be seen above. Both the extracts have shown relatively low % cell viability on both cell lines; however, methanol extract has shown the least cell viability of 25% on rat melanoma B16 cell line at 500µg/ml concentration as can be seen in figure c.

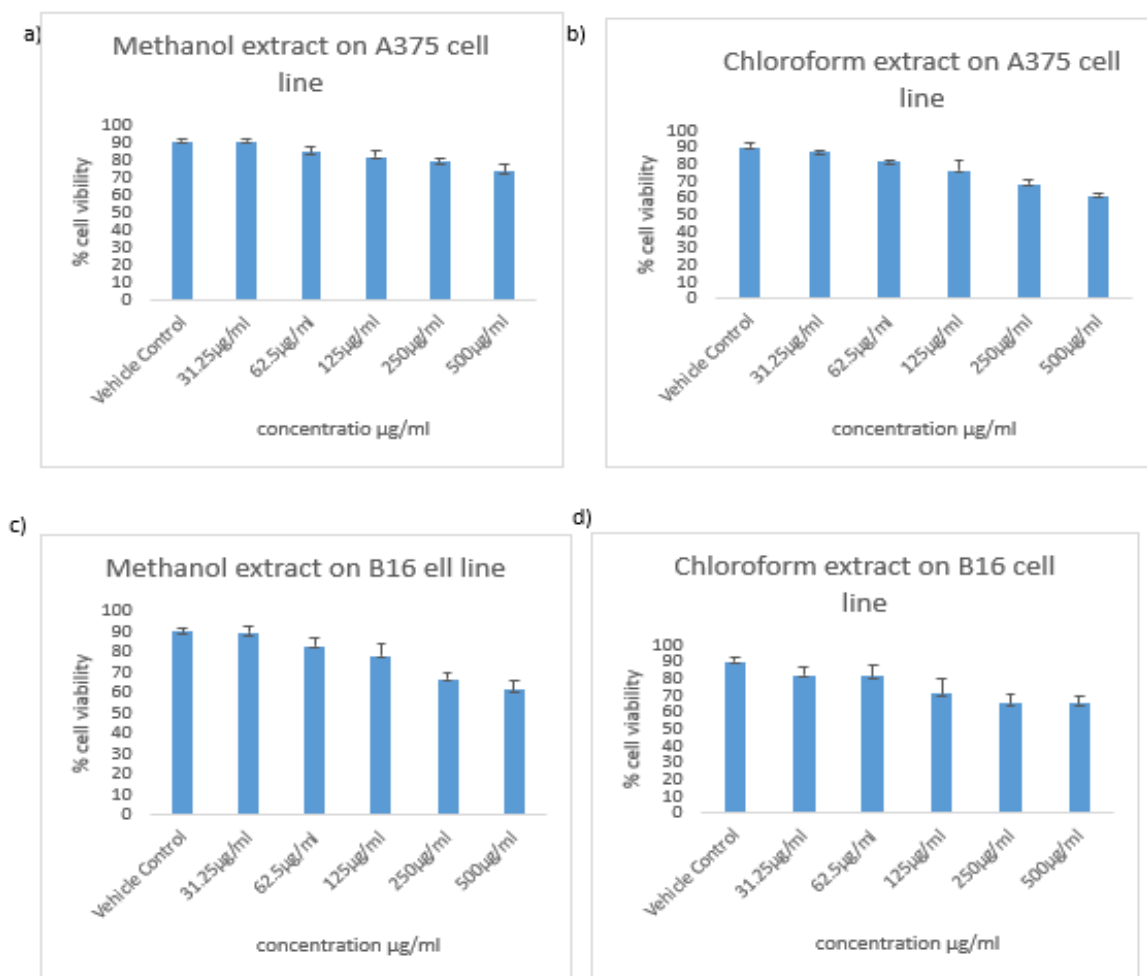% Cell Viability of Plant *Hemidesmus indicus* Anantmool

Figure 3.2: Determination of Cell Viability. % Cell Viability of *Hemidesmus indicus* Anantmool (a) Methanol extract on A375 human melanoma, (b) Chloroform extract on A375 human melanoma, (c) Methanol extract on B16 rat melanoma and (d) Chloroform extract on B16 rat melanoma, via MTT assay 24hr after treatment at different concentrations. Samples were compared using one way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Hemidesmus indicus chloroform and methanol extract have shown a decrease in percentage cell viability as the concentration increases. The methanol extract has shown the least % cell viability of 75% on B16 at 500μg/ml as can be depicted in figure c. However, these extracts have not had much impact on the cell viability of both the cell lines showing not much significance. Only 25% cell death is induced at max, that could be caused due to other factors, hence it is observed that Hemidesmus doesn't contain active phytochemicals.

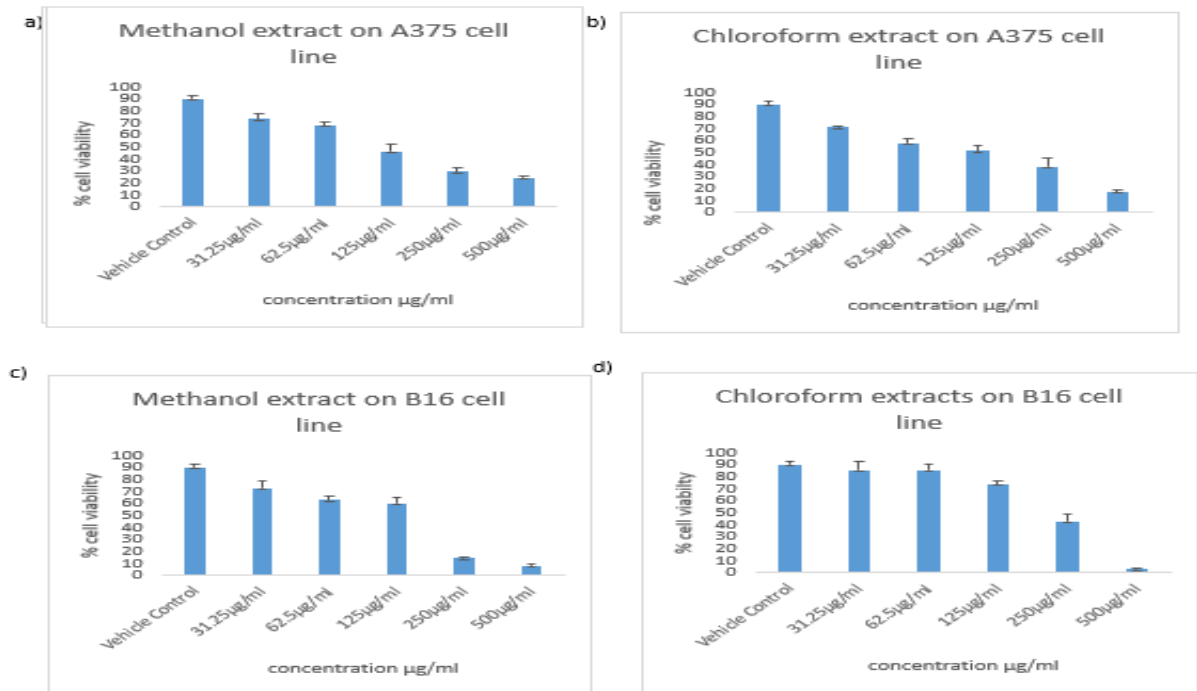% Cell Viability of Plant *Tussilago farfara* Coltsfoot

Figure 3.3: Determination of Cell Viability. % Cell Viability of *Tussilago farfara* Coltsfoot (a) Methanol extract on A375 human melanoma, (b) Chloroform extract on A375 human melanoma, (c) Methanol extract on B16 rat melanoma and (d) Chloroform extract on B16 rat melanoma, via MTT assay 24hr after treatment at different concentrations. Samples were compared using one way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Overall *Tussilago farfara* chloroform and methanol extract have shown a decrease in percentage cell viability as the concentration increases. Both the extracts have shown relatively low % cell viability on both cell lines; however, Chloroform extract has shown the least cell viability on rat melanoma B16 cell line at 500µg/ml concentration as can be seen in figure d.

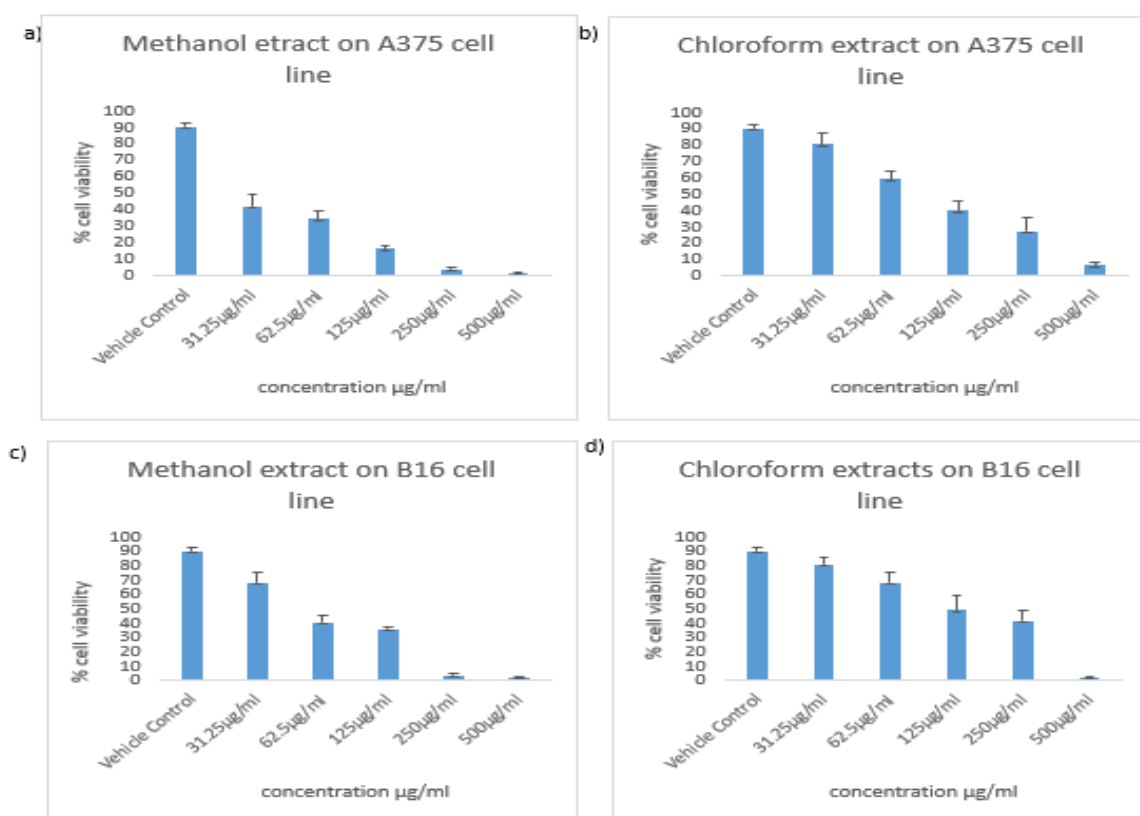% Cell Viability of Plant *Andrographis paniculata* Kalmegh

Figure 3.4: Determination of Cell Viability. % Cell Viability of *Andrographis paniculata* Kalmegh (a) Methanol extract on A375 human melanoma, (b) Chloroform extract on A375 human melanoma, (c) Methanol extract on B16 rat melanoma and (d) Chloroform extract on B16 rat melanoma, via MTT assay 24hr after treatment at different concentrations. Samples were compared using one way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***$p<0.001$, **$P<0.01$, *$P<0.05$, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Overall *Andrographis paniculata* chloroform and methanol extract have shown a decrease in percentage cell viability as the concentration increases. Both the extracts have shown relatively low % cell viability on both cell lines; however, methanol extract has shown the least cell viability of 1% on rat melanoma B16 cell line at 500µg/ml concentration as can be seen in figure c. The methanol extracts show a less than 50% cell viability at 62.5µg/ml and onwards in comparison to chloroform extracts which shows almost 75%.

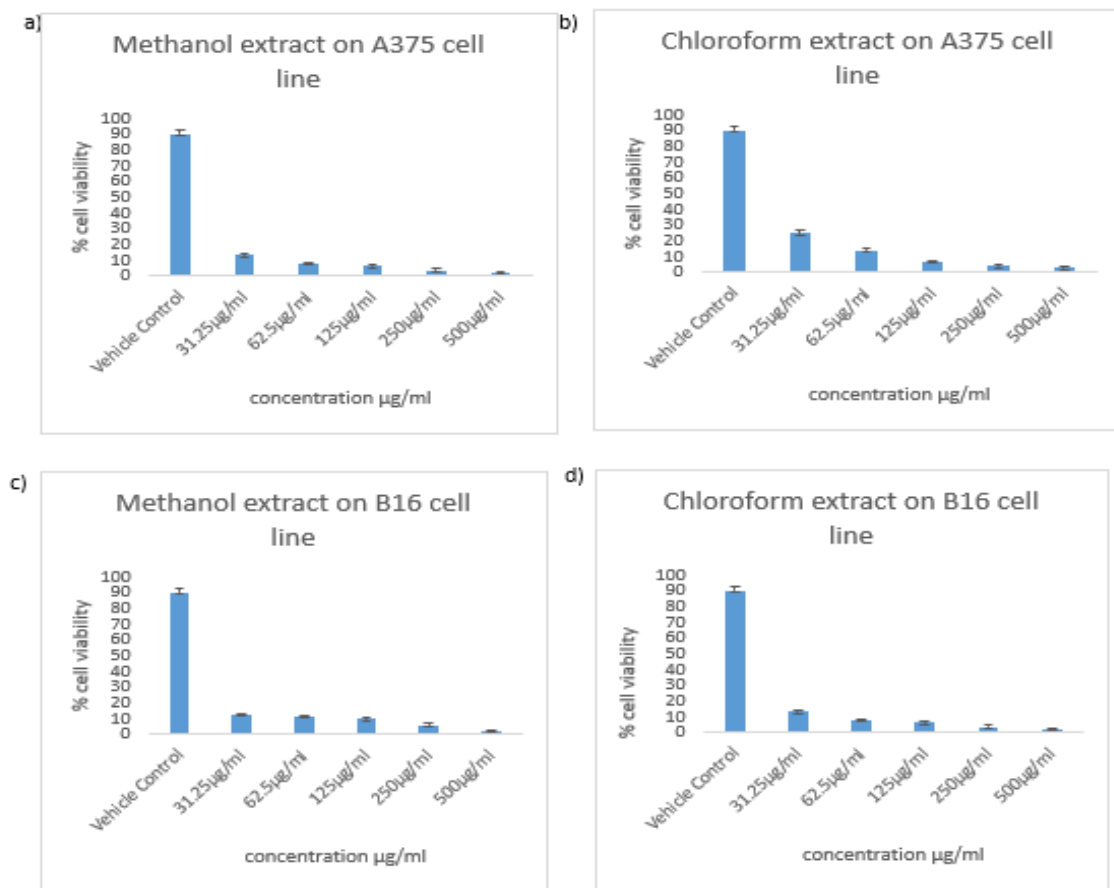% Cell Viability of Plant *Equisetum arvense* Horsetail

Figure 3.5: Determination of Cell Viability. % Cell Viability of *Equisetum arvense* Horsetail (a) Methanol extract on A375 human melanoma, (b) Chloroform extract on A375 human melanoma, (c) Methanol extract on B16 rat melanoma and (d) Chloroform extract on B16 rat melanoma, via MTT assay 24hr after treatment at different concentrations. Samples were compared using one way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Equisetum arvense chloroform and methanol extract have shown a decrease in percentage cell viability as the concentration increases. Both the extracts have shown relatively low % cell viability on both cell lines; however, methanol extract has shown the least cell viability of 1% on rat melanoma B16 cell line at 500µg/ml concentration as can be seen in figure c. These extracts resulted in significant results, as both extracts on both cell lines shows %cell viability of less than 25%. It can be seen above that there is an active phytochemical compound present in these extracts that can be a future potential as its shows the least cell viability at the least concentration.

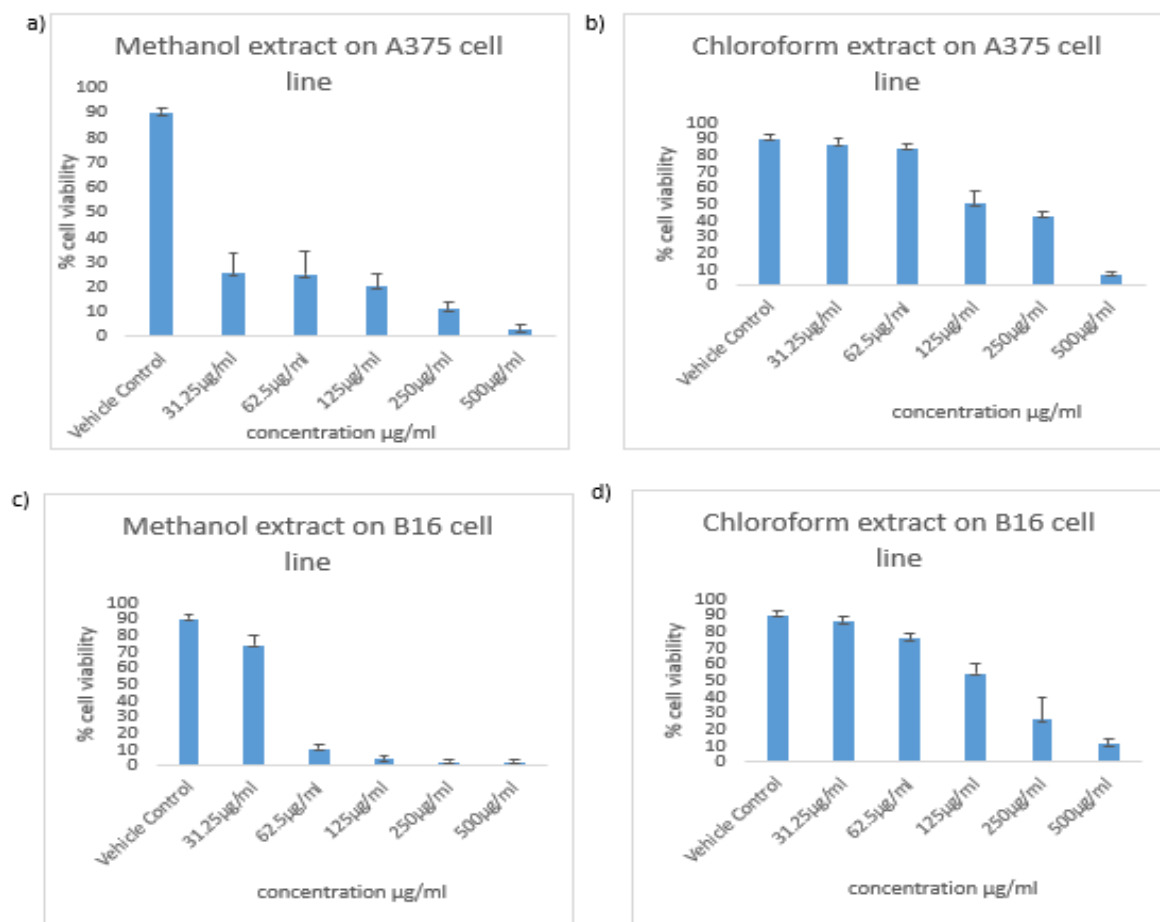% Cell Viability of Plant *Melissa officinalis* Lemon Balm

Figure 3.6: Determination of Cell Viability. % Cell Viability of *Melissa officinalis* Lemon Balm (a) Methanol extract on A375 human melanoma, (b) Chloroform extract on A375 human melanoma, (c) Methanol extract on B16 rat melanoma and (d) Chloroform extract on B16 rat melanoma, via MTT assay 24hr after treatment at different concentrations. Samples were compared using one way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Overall *Melissa officinalis* chloroform and methanol extract have shown a decrease in percentage cell viability as the concentration increases. The methanol extract has shown the least % cell viability about 2% on A375and B16 at 500µg/ml as can be depicted in figure a and c respectively.

3.2 Comparison with Positive Control

The 52 extracts from 26 different plants screened against A375 and B16 were compared with the positive control Doxorubicin (1uM) at the highest concentration of 500µg/ml showing the least % cell viability.

Figure 3.7: Determination of cell viability of positive control doxorubicin vs methanol extracts at concentration of 500µg/ml on A375 human melanoma via MTT assay 24hr after treatment at different concentrations. Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.

Figure 3.8: Determination of cell viability of positive control doxorubicin vs chloroform extracts at concentration of 500μg/ml on A375 human melanoma via MTT assay 24hr after treatment at different concentrations. Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.
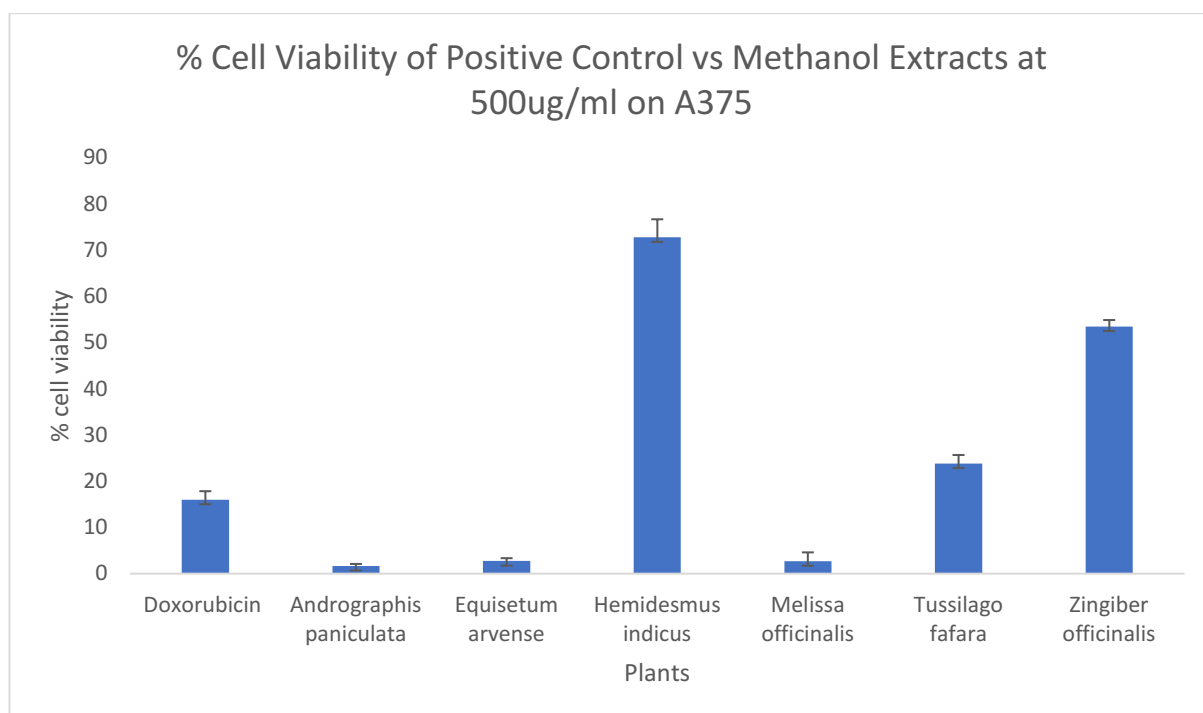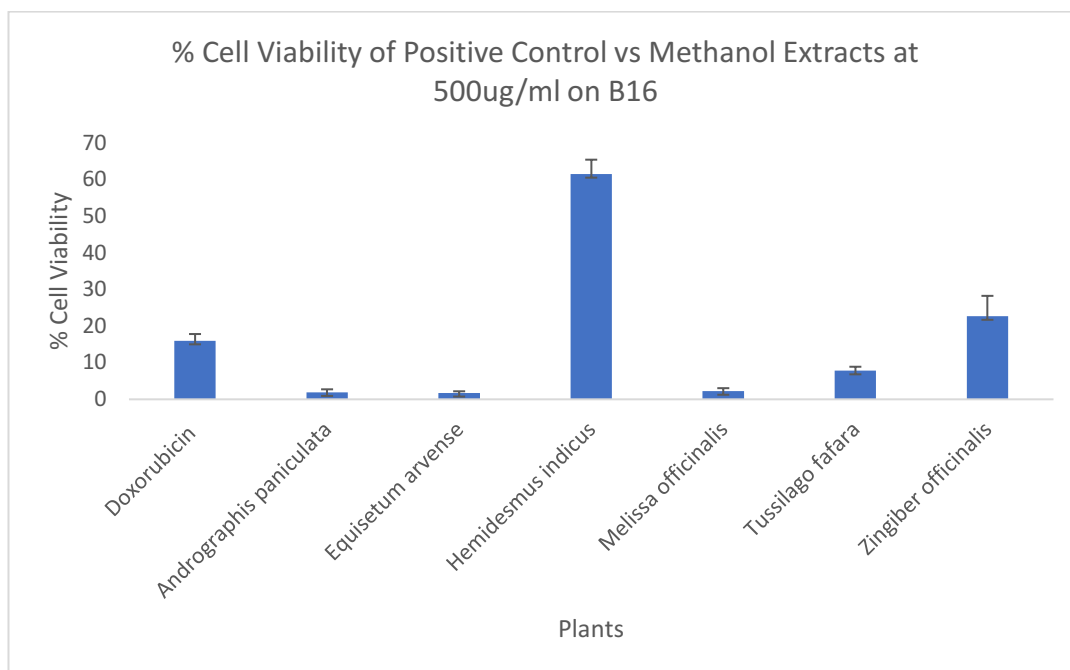
Figure 3.9: Determination of cell viability of positive control doxorubicin vs methanol extracts at concentration of 500µg/ml on B16 rat melanoma via MTT assay 24hr after treatment at different concentrations. Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.
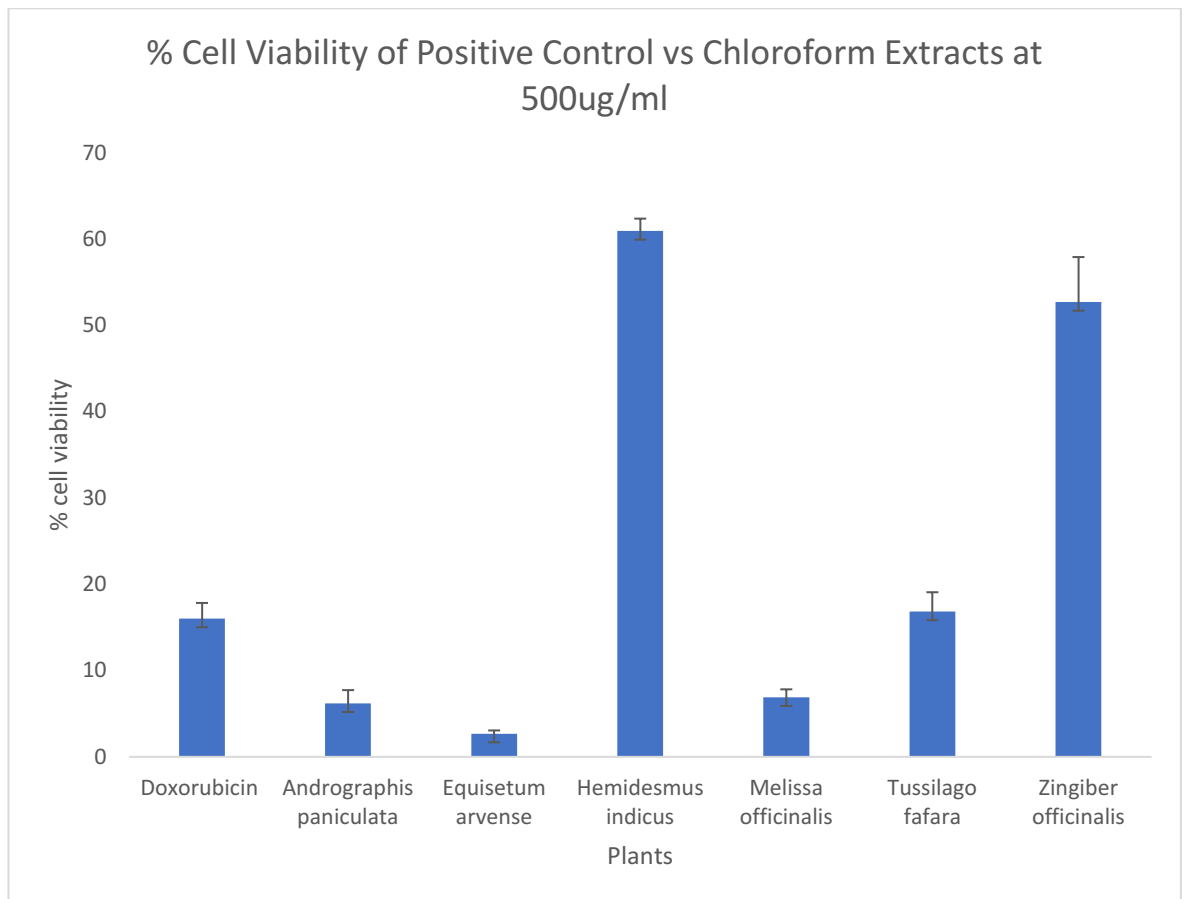
% Cell Viability of Positive Control vs Chloroform Extracts at 500ug/ml on B16

Figure 3.10: Determination of cell viability of positive control doxorubicin vs chloroform extracts at concentration of 500μg/ml on B16 rat melanoma via MTT assay 24hr after treatment at different concentrations. Samples were compared using one-way ANOVA. Error bars indicated mean ± SD, (n=1; 4 technical replicates on the plate), ***p<0.001, **P<0.01, *P<0.05, in comparison to vehicle control DMSO at a final concentration of 0.1%.
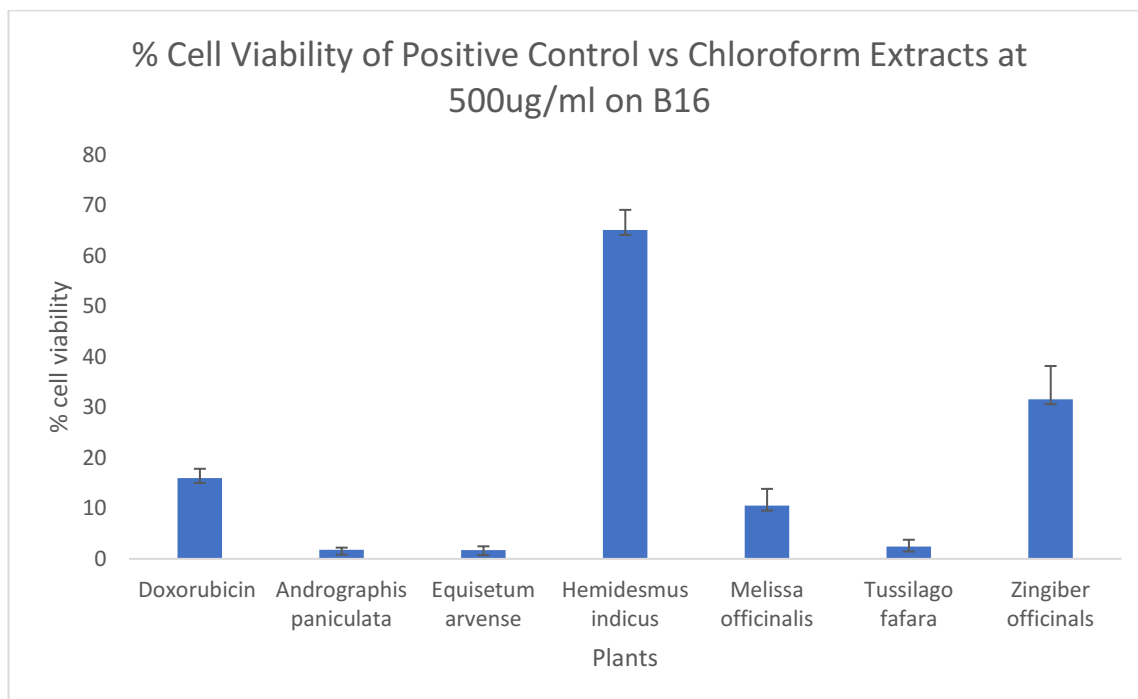
Chapter 4: Discussion

Cancer is the second leading cause of death across the globe and is responsible for every sixth death in the world (Who.int, 2019). It has resulted in approximately 9.6 million deaths in 2018, which is an increase of nearly 10% since 2015 (8.8 million). (Who.int, 2019). This is not only affecting the quality of life of patients but also creates a significant burden on global economy.

An intervention to find a treatment for cancer has become the utmost focus of research due to the impact on global economy and increase in number of cancer deaths. Natural products have been in use to treat cancer for over 40 years (Demain and Vaishnav, 2010). Therefore, the purpose of this study was aimed at understanding the role of medicinal plants in treatment of cancer and to lay foundations for future work as well as to assess if the constituent of the plant could be used as a possible treatment option for cancer patients.

Cancer is the second leading cause of mortality worldwide (2018). The survival rate of cancer patient is increasing due to use of chemotherapy and radiotherapy but this lease to several toxic effects (Qamar, Rehman and Chauhan, 2019). This has led to search for new anticancer agents that are more effective and cause less side effects. recent study's findings show anticancer drugs derived from medicinal plant to pose minimum cytotoxic values in a dose dependent MTT assay as they inhibit tumour growth. (Qamar, Rehman and Chauhan, 2019). Hence there has been an incline towards finding active phytochemicals in medicinal plants in search for anticancer drugs with minimal side effects.

This study evaluated 52 extracts from medicinal plants (Table 1 in methods) on A375 and B16 cell lines using MTT. Overall most of the plant extracts have shown a decrease in cell viability against both the cancer cell line A375 and B16. It Is also very evident by looking at the figures in result section that as the concentration increases the % cell viability decrease, hence, %cell viability is inversely proportional to the concentration and shows negative correlation. *Hemidesmus indicus* has shown anticancer properties in previous studies (Turrini et al., 2018). For example, a 2011 study has shown to induce cell death at all concentrations against Jukrat cells. (Fimognari et al., 2011). However, this study presented doesn't show significant decrease in cell viability in both the cell lines against *Hemidesmus indicus*. Despite increasing the concentration of dose, the % cell viability is more than or around 75% just about inducing cell death in 25% of cell, that could be possibly caused due to other possible factors such as natural death, contamination or technical errors.

Plants such as *Equisetum arvense* Horsetail and *Andographis paniculata* Kalmegh extracts have shown a massive decrease in cell viability against both the cell lines as can be seen in figures 7 and 6 respectively. *Equisetum arvense* component EA1 has shown decrease in cell viability of A549, lung carcinoma using MTT and has manifested cytotoxicity (Mohammed, Paray and Rather, 2017). A recent study shows, that *Equisetum arvense* extracts can be potentially used as chemo preventive agent or coadjuvant chemotherapy due its nature of preventing chromosomal damage in induced mice. (Koue et al., 2017). A 2014 study resulted in 1.09µg/ml IC50 of Equisetum arvense however this study shows an IC50 0f 0.56µg/ml, which means that Equisetum arvense is more potent to skin cancer cell line A375. *Andographis paniculata* has shown anti tumor, anti metastic and immunomodulatory properties in esophagael cancer animal models, by supressing growth of cells and increasing cell death without causing severe side effect like other chemotherapeutics (Yue et al., 2019). A recent study also shows that *Andographis paniculata* plays a major role in treatment for prostate cancer by causing DNA damage post inhibiting PCa (Forestier-Roman et al., 2019). Hence the results obtained from this and previous study shows pharmacological significance and need to be further investigated in search for potential anticancer agent.

It is observed that *Melissa officinalis* Lemon Balm extracts out of the 52 extracts were highly lethal to both the cell lines A375 and B16 (figure6). This shows that this compound contains bioactive substance and if studied in detail could provide active compounds that pose antitumor or antiproliferative properties. *Melissa officinalis* is currently in use in the market to treat a disease called herpes. Herpes is a viral condition, in which blisters are developed around different parts of the body majorly around lip area, the anti-viral cream made of *Melissa officinalis* is used to treat that by peeling of the dead skin. Looking at the theory of use of *Melissa officinalis* to treat herpes, it can potentially be used to treat skin cancer formed above the skin, following the same process of applying it over the affected area and letting it peel off as a dead skin. However, further investigation needs to be carried out before it comes in the market.

In comparison to the positive control Doxorubicin, *Swertia cirayita* Felworts both extracts chloroform and methanol have shown the most significance against both the cell lines A375 and B16 with the p value ranging between 0.01 and 0.5. Though the % cell viability of *Swertia cirayita* has decreased for both the extracts against both the cell lines but is above 50%, hence it is seen that although the cell viability drops with administration of *Swertia cirayita* viability, but it might be due to other contributing factors.

Figures 3.7-3.10 shows a comparison between the positive control Doxorubicin and both plant extracts on A375and B16. Overall it is observed that Hemidesmus indicus and Zingiber officinalis shows significantly higher %cell viability on both cell lines in comparison to positive control. Tussilago farfara shows a varied %cell viability across both cell lines; with low % cell viability of chloroform extract on B16 of 4% whereas a high % cell viability of methanol extract of 35% on A375. On the other hand, the other 3 plants Andrographis paniculata, Equisetum arvense and Melissa officinalis both extracts shows a significant decrease in % cell viability in comparison to positive control Doxorubicin on both A375 and B16 cell lines.

Future Work

Continuing with this research, it is wise to screen these extracts at a larger scale by carrying out other assays then MTT such as SRB assay to determine cell viability in order to ensure quality of data as well as gathered data. Other than cell viability, other factors such as morphological changes in the shape and size of cell, protein content should be observed using techniques like electron microscopy, western blotting and Elisa.

One of the other things would be to repeat the experiments carried out number of times to ensure the quality of results and data obtained. This will not only qualify the data, but the data produced will be more reliable and accurate as well as discard any human, standard or technical errors. Due to time constraints, the assays were carried out at time lapse of 24hrs only, the assays can be carried out over a time lapse of 4hrs, 8hrs, 12hrs and 48hrs to ensure the exact time of effect of medicinal plant extract cause the viability of cells to decrease. This will help to determine the effect of medicinal plant after administration hence will be beneficial in deciding the course of action of medications in future.

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