Study on the cytotoxic effect of *Amomum subulatum* in cancer cells

Dissertation Report

Submitted in the partial fulfillment of the requirement for

the award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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JULY 2014

CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled "**Study on the cytotoxic effect of *Amomum subulatum* in cancer cells**" in partial fulfillment of the requirements for the award of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala is an authentic record of my own work during a period from August 2013 to July 2014, under the supervision of Dr. Manoj Baranwal and Dr. Vikas Handa Assistant Professor, Department of Biotechnology, Thapar University, Patiala. The Report has not been submitted for the award of any other degree or certificate in this or any other university.

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This is to certify that the above statement given by the candidate is correct and true to the best of our knowledge.

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CERTIFICATE

This is to certify that the thesis entitled "**Study on the cytotoxic effect of *Amomum subulatum* in cancer cells**"being submitted by **Vivek Sharma**, Registration No. **601204030** in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is a bonafide work carried out under my supervision and guidance. The thesis has not been submitted for award of any other degree or certificate in this or any other university.

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CONTENTS

ABSTRACT

Cancer is a disease of uncontrolled cell growth and proliferation. Standard therapy which targets cancer cell cytotoxicity also causes non-specific cytotoxicity to normal cells so there is need of new bioactive agents which targets the cancer cells and has lesser side effects on the normal cells. The aim of this project work was initially to screen the different extract of *Amomum subulatum* for cytotoxic activity against MCF-7 and HeLa cells. The extracts were prepared with varying polarity of solvents (Hexane, Diethyl ether, Ethyl acetate, Ethanol, Acetone and Water) through soxhlet apparatus. Hexane and Ethyl acetate extracts were foundto exhibit maximum cytotoxic activity against MCF-7 and HeLa cells. Cytotoxic effect of hexane and ethyl acetate extracts becomes more pronounced with increase in both concentration and time in both MCF-7 and HeLa cells. Hexane and ethyl acetate extracts have also shown inhibition of stimulated (pokeweed mitogen treated) and unstimulated PBMC which shows that these extracts may exhibit immunosuppressive effect.

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CHAPTER 1: INTRODUCTION

Cancer is a group of diseases caused by various means leading to death worldwide around 13% of all death and accounting for 8.2 million deaths in 2012. (Globocan 2012 IARC). Extensive studies have been done which has revealed that cancer is caused by dysregulation of about 500 gene products (Sung *et al* 2012). Over 5% to 10% of cancers are caused by inheritance of mutated genes and somatic mutations, whereas the remaining 90–95% have been linked to lifestyle factors (like tobacco use, food carcinogens from grilled meat) and the environmental factors like ultra violet light, radon gas (Anand *et al.,* 2008*)* Breast cancer and cervical cancer are the most prevalent cancers among females all over world in developed and developing countries. The reason behind is the lack of early diagnosis and the poor and expensive treatments. Breast cancer caused by the mutated genes *BRCA1* or *BRCA2,* a type of tumor suppressor genes and acquired mutation in these genes while cervical cancer is caused by human papillomavirus (HPV). Chemotherapy remains the principal mode of treatment for various cancers. Although many anticancer drugs available around the globe which are of plant or microbial origin, but all the drugs have their own side effects too hence there is a need of some complementary and alternative medicine which has lesser side effects. Herbs and spices are used as medicines since ancient time by various means in traditional medicines system such as in Ayurveda and Unani in which polyherbal formulation were used to treat the disease. Nowadays, reverse pharmacology serve for isolation of new bioactive agents for their use to target human diseases.

As reported the incidence of the various types of cancer between the United States and India, the United States was found to have much higher rates of colorectal cancer. In 2000, the United States had 356 colon cancer cases reported and 139 deaths per 1 million people. In contrast, India only had 40 reported cases of colon cancer and 26 deaths per 1 million people. The reason behind the less cancer incidence in India than in most Western countries is not fully understood, but the high spice consumption may be one of the contributing factors (Kaefer *et al.,* 2008*) Amomum subulatum is* one of the commonly used spice is used in cuisines for taste and aroma. It is also known as bari elachi or greater cardamom or large cardamom which is member of Zingberaceae family mostly grows in

tropical areas and it is well known for flavouring spice in Ayurveda *Amomum* is commonly used to treat disease like nausea vomiting cough dyspepsia and for throat infect. Present study is oriented towards the study of effect of *Amomum subulatum* on growth of cancer and immune cells.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Cancer

Cancer originates from a single cell and is described as an abnormal growth of cells. The transformation of a normal cell to a tumor cell is a cascade of events which eventually leads to the cancer progression, as a result of change in the genetic makeup of a cell or external environmental factors. Cancer mortality rate can be reduced by early diagnosis and treatment. Majority of cancer cases (90–95%) have their roots in the environment and lifestyle such as diet and cigarette smoking, whereas the remaining 5–10% of all cancer cases are attributed to genetic defects (Anand *et al*., 2008). In developing countries, up to 20% of cancer deaths could be prevented by immunization against the infection of Hepatitis B virus and Human Papilloma Virus.

Among females, breast cancer is a leading cause of death in developed or developing countries. Genetics plays a limited but important role in the progression of breast cancer. Only 5% to 6% of breast cancers are considered hereditary. *BRCA-1* and *BRCA-2* accounts for an estimated 80% of hereditary breast cancer cases. Estrogen is the primary stimulant for breast cell, along with progesterone. Increased exposure to estrogen and/or progesterone such as early menarche, late menopause and related obesity, hormone replacement therapies are major causes of breast cancer (Frei.*et al.*, 2003). Cervical cancer is the second most common type of cancer among women. 70% of cervical cancers are due to sexually acquired infection with certain types of Human papillomavirus (HVP) (WHO/HPV/cervical cancer/ factsheet/2013). Cancer of the cervix is a cancer involving the squamous cells of the cervix. They grow at an abnormally fast rate function differently and start looking different from the normal squamous cells of the cervix. At a more advanced stage, the cancer cells spread to surrounding tissue and even spread to distant tissue such as bones and lungs through blood stream.

2.2 Cancer therapies

The main goal of a cancer diagnosis and treatment is to cure or considerably prolong the life of patients and to ensure the best possible quality of life to cancer survivors. Different type of

treatment modalities available against cancer includes surgery, chemotherapy, radiotherapy, targeted therapy, immunotherapy and systemic therapy.

Cancer chemotherapy is an important alternative to surgery and radiation to treat successfully some types of solid tumors, lymphomas and leukemias. Chemotherapeutic drugs are classified into various categorized such as alkylating agents, antimetabolites, antitumor antibiotics, topoisomerase inhibitors, mitotic inhibitors and Corticosteroids. Major side effects associated with chemotherapeutics treatment of cancer includes gastrointestinal distress, hair loss and fatigue (Drug Delivery in Oncology, 2013). Other than these, other side effects include.

a) Depression of the immune system, often by paralysing the bone marrow and leading to a decrease of white blood cells, red blood cells, and platelets, which may potentially lead to fatal infections.
b) Infertility (Brydoy *et al*., 2007)
c) Teratogenicity (Arnon *et al*., 2001)
d) Chemotherapy-induced Peripheral Neuropathy (del Pino, 2010)
e) Secondary neoplasm (Ruther, 2000)

Other limitations of cancer chemotherapy include affordability of the treatment, Cost effectiveness and resistance against chemotherapeutic drugs. Besides cost, safety is a major concern. For instance, tamoxifen is a chemotherapeutic drug, which has been approved by FDA for breast cancer treatment is not very well accepted, owing to its association with increased risk of endometrial cancer and thrombotic events (Meyskens *et al*., 2011).

The lack of safety and high cost of monotargeted therapies have encouraged alternative approaches based on plant or natural extract.

2.3 Prospects of Anticancer drugs based on spices

The Spices have been consumed in many cultures over centuries. They were primarily consumed because of their taste and aroma. However, the recent scientific studies have proved their biological activities beyond their taste and smell

Many plant based phytochemicals have been identified to have anticancer and therapeutic properties, such as curcumin from Curcuma *longa* (Figure 1).

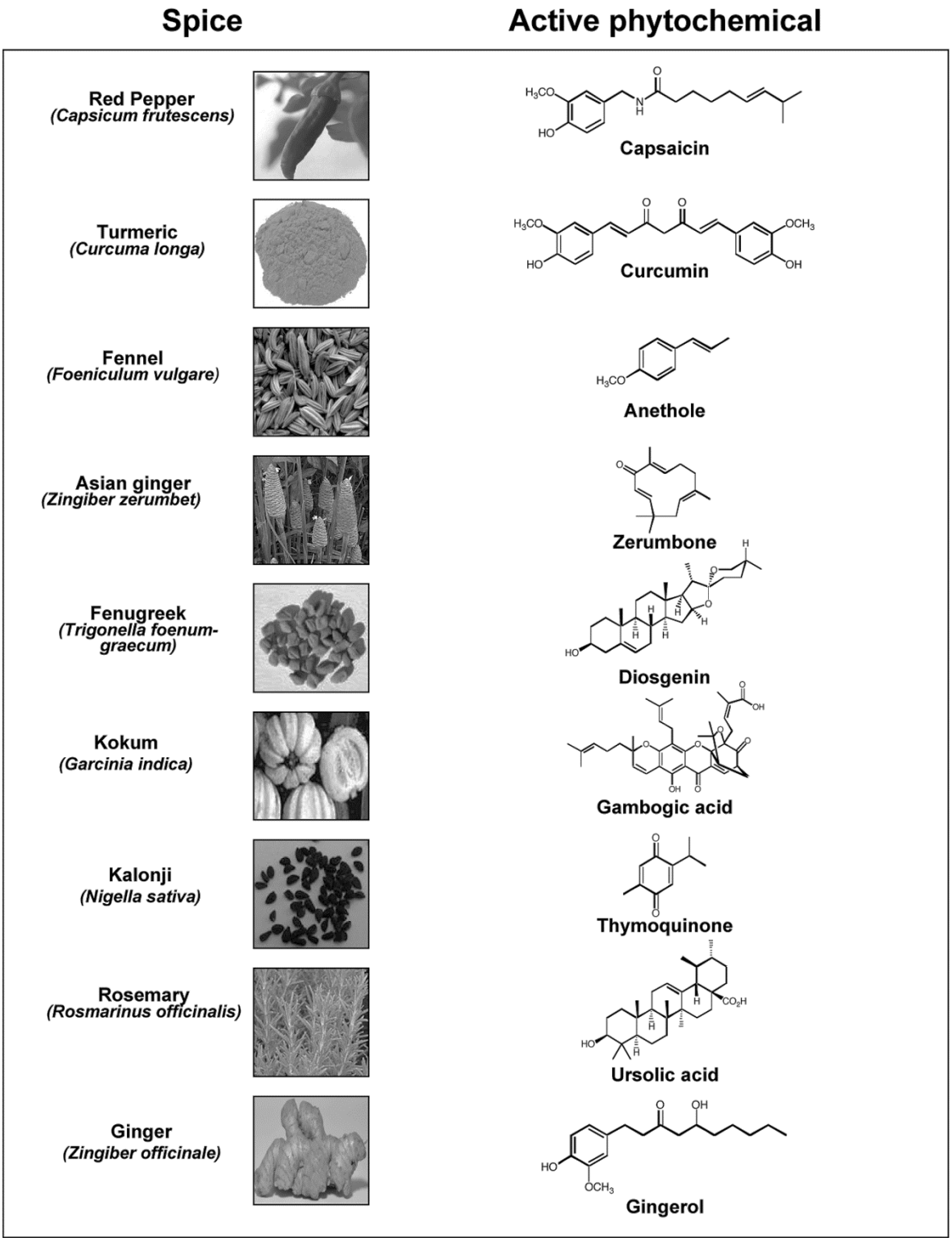


Figure 1: Major spices and their active phytochemicals. (Aggarwal *et al*., 2008)

Curcumin has been extensively studied for its anticancer activity in multiple human carcinomas including melanoma, prostate, breast, colon, pancreatic, ovarian and head and neck cancers (Mukhopadhyay *et al*.,2011, Mehta *et al*.,1997, Hanif *et al*., 1997, Elattar *et al*.,2000 , Lin *et al*., 2007, Wang *et al*., 2008). Curcumin has been found to have inhibitory role in initial stages of cancer development because of its potent anti-oxidant and free-radical quenching properties. Studies show that curcumin bears the ability to suppress UV irradiation-induced DNA mutagenesis and induction of cellular SOS functions (Oda, 1997).

In the area of cancer prevention, plants consumption such as spices and their constituents as potential chemopreventive agents remains an extensive research topic. Cardamom (*Elettaria cardamomum*) and black pepper (*Piper nigrum*) have been studied for their immunomodulatory activity by Majdalawieh and Carr (2010). Aqueous extracts were reported to significantly enhance splenocyte proliferation in a dose-dependent manner, especially when combined in a dose-dependent, synergistic fashion. While the effects of cardamom and black pepper were the opposite on Th1 and Th2 cytokine release by splenocytes, the presence of both spices significantly enhanced the cytotoxic activity of natural killer cells against YAC-1 lymphoma cells. These findings provide evidence that cardamom may have anticancer benefits by modifying immunocompetence.

Intake of spices is one of the most effective, convenient and economical ways of fortifying oneself against infectious diseases and related cancers (Steinmetz& Potter, 1996).

2.4 Introduction to *Amomum subulatum*

Amomum subulatum Roxb. (Zingiberaceae), also known as Black cardamom is a rhizomatous herb native to eastern Himalayas. In India it is mainly grown in Sikkim and Darjeeling (West Bengal). Nepal, Bhutan and Central China are also the producers of Black cardamom. India is the largest producer of *A. subulatum* with a 54% share in world production (Berrig *et al*. 1993). Its large, dried capsule is well known for its smoky and camphorous flavour. In India it is used extensively as a culinary spice. The seeds are reported to possess stimulant, stomachic, alexipharmic and astringent properties and are used in folklore medicine for the treatment of indigestion, vomiting, biliousness abdominal pains and rectal diseases. The seeds are found to promote elimination of bile and are used to treat congestive jaundice they are also used in gonorrhea, while the pericarp has been reported to be useful in treating headache and stomatitis.

The aromatic oil extracted from the seeds is applied to the eyes in cases of inflammation. *Amomum* seeds are considered as an antidote to scorpion or snake venom (Pruthi, 1979).

Its fruits are prescribed for the treatment of indigestion, vomiting, biliousness, abdominal pains, rectal diseases, throat troubles, congestion of the lungs, inflammation of the eyelids, pulmonary tuberculosis, loss of appetite and liver complaints (Nadkarni 1976; Jafri *et al*. 2001; Verma *et al*. 2010).

2.5 Biological activities of *Amomum subulatum*

The Spices have been consumed in many cultures over centuries. They were primarily consumed because of their taste and aroma. However, the recent scientific studies have proved that their biological activities beyond their taste and smell.

The antimicrobial activity of *A. subulatum has* been reported by Agnihotri and Wakode (2010). Methanolic extracts from fruit and rind and essential oils from the whole fruit were tested for their antimicrobial activity against *Bacillus pumilus, Bacillus subtilis, Stephylococcus aureus, Micrococcus epidermidis, Stephylococcus epidermidis, Escherichia coli, Pseudomonas aerugenosa, and fungal strains Candida albicans, Aspergillus niger, Saccharomyces cerevisiae*. Similar studies were carried out by Aneja and co-workers (2009) on microorganism causing dental carries. The acetone, ethanol and methanol extracts of *A. subulatum* exhibited antimicrobial activity against all tested microorganism except L. acidophilus. The most susceptible microorganism was found to be *S. aureus* followed by *S. mutans, S. cerevisiae* and *C. albicans*

In vivo studies conducted on mice have shown that methanolic extract of *Amomum subulatum* significantly prevents the damage to liver mitochondria through regulation of VDAC (Voltage dependent anion channel) expression reported by (Parmar *et al*., 2011).

A. subulatum has been studied for its gastroprotective action in mice A crude methanolic extract and its different fractions, viz. essential oil, petroleum ether (60-80 degrees), ethyl acetate and methanolic fractions, were studied in mice for their ability to inhibit the gastric lesions induced by aspirin, ethanol and pylorus ligature. The crude methanolic extract of *A. subulatum* inhibited gastric lesions induced by ethanol, whereas, ethyl acetate fraction increased the wall mucus in

pylorus ligated rats. The results suggest a direct protective effect of ethyl acetate fraction on gastric mucosal barrier.

Ethanolic and aqueous extracts of leaves of *A. subulatum* were evaluated for antioxidant activity by the 1,1Diphenyl -2-picrylhydrazyle (DPPH) free radical scavenging activity, β-carotene bleaching assay and total phenolic contents methods (Khare *et al*., 2010). Similar work was done by Kikuzaki and colleagues (2001) in which, ethyl acetate-soluble fraction showed a high radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Extensive studies on antimicrobial, antioxidant, immunomodulatory and other biological activities of *Amomum subulatum* and related plants underscores the need to explore it as a Compound isolated from hexanes and chloroform soluble extracts of *A. aculeatum* leaves were tested on Lu1, LNCaP and MCF-7 cancer cell lines. Aculeatin A was found to be active potential source of anticancer drug (Chin *et al*., 2008).

2.6 Extraction of bioactive compounds from plants

Bioactive compound is secondary plant metabolites eliciting pharmacological or toxicological effects in human and animals (Bernhoft, 2010). Croteau *et al*. (2000) categorisedbioactive compounds of plants are divided into three main categories: (a) terpenes and terpenoids (b) alkaloids and (c) phenolic compounds. There are four major pathways for synthesis of secondary metabolites or bioactive compounds: (1) Shikimic acid pathway, (2) malonic acid pathway, (3) Mevalonic acid pathway and (4) non-mevalonate (MEP) pathway (Tiaz and Zeiger, 2006).Bioactive compounds can be isolated and characterized from various plant parts such as leaves, stem, flower and fruits.

Choice of appropriate extraction technique is essential in order to conduct the qualitative and quantitative studies on bioactive compounds from plant materials (Sasidharan *et al*., 2011). Extraction is the first step of any medicinal plant study, plays a significant and crucial role on the final result and outcome. Selection of solvent, temperature, pressure, time and matrix properties of the plant part are the vital aspects of extraction processes (Hernández *et al*., 2009).

Huge technological and technical improvements have resulted in the advent of improved extraction procedures in terms of reduced requirement of solvents and operational time, and

better yield and quality of extract. But still, conventional methods like Soxhlet are still considered as one of the reference method to compare success of newly developed methodology.

Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet (1879). Soxhlet extraction is employed where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent.

Extraction efficiency of any conventional method like soxhlet mainly depends on the choice of solvents (Cowan, 1999). The polarity of the targeted compound is the most important factor for solvent choice. Arrays of solvents have been used for extraction of bioactive compounds from plants (Table 1). Solute dissolves best in a solvent that has a similar chemical structure to itself i.e. similar polarity. Molecular affinity between solvent and solute, mass transfer, environmental safety and human toxicity should also consider in selection of solvent for bioactive compound extraction (Table 2).

Table 1: Example of some extracted bioactive compounds by different solvents (adapted from Cowan (1999)).

Water	**Ethanol**	**Methanol**	**Chloroform**
Anthocyanins	Tannins	Anthocyanin	Terpenoids
Tannins	Polyphenols	Terpenoids	Flavonoids
Saponins	Flavonol	Saponins	
Terpenoids	Terpenoids	Tannins	
	Alkaloids	Flavones	
		Polyphenols	
	Dichloromethanol	**Ether**	**Acetone**
	Terpenoids	Alkaloids	Flavonoids
		Terpenoids	

Table 2: Solvent polarity

Relative Polarity	Formula	Group	Solvents
Non-polar	R-H	Alkanes	Petroleum ethers, hexanes, ligroin
	Ar-H	Aromatics	Toluene
	R-O-R	Ethers	Diethyl ether
	R-X	Alkyl halides	Tricholoromethane, chloroform
	R-COOR	Esters	Ethyl acetate
	R-CO-R	Aldehydes and ketones	Acetone, MEK
	R-NH2	Amines	Pyridine, triethylamine
	R-OH	Alcohols	MeOH, EtOH, IPA, Butanol
	R-COHN2	Amides	Dimethyformamide
	R-COOH	Carboxylic Acid	Ethanoic Acid
Polar	H-O-H	Water	

2.7 Cell Viability and Cytotoxicity assays

Screening the effects of compounds on the viability of cells grown in culture is widely used as a predictor of potential toxic effects in whole animals. Assays based on various cell functions such as enzyme activity, nucleotide uptake, cell permeability, ATP production are used to estimate the viable cell count (Figure 1).

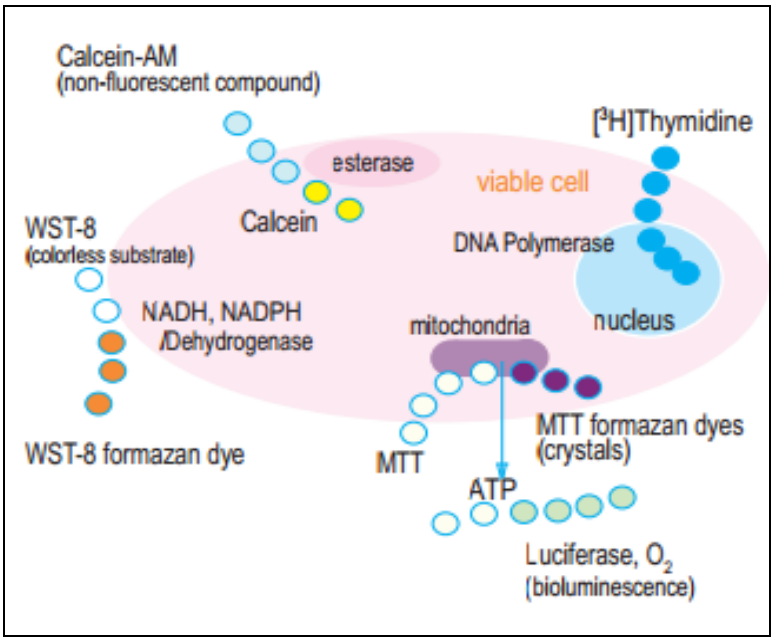


Figure2: Reagents for cell viability detection

Tritium-Labeled Thymidine Uptake method assay and 51Cr release assay are highly sensitive in determining low level of cytotoxicity but, the use of radioisotopes causes problems in handling, storage, and disposal. Enzyme based MTT and WTS are the colorimetric assay based on reductive coloring reagent and dehydrogenase in the viable cells. These methods are easy-to-use, safe and highly reproducible.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS) (Mosmann in 1983) . In this assay, the yellow tetrazolium MTT is reduced by metabolically active cells to purple colored formazan, The exact cellular mechanism of MTT reduction into formazan is not well understood, but it likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT. Purple colored formazan product thus formed is directly to proportional to the number of viable cells. The formazan product accumulates as an insoluble precipitate inside cells, near the cell surface and in the culture medium. Various solubilization methods include using: acidified isopropanol, DMSO, dimethylformamide, SDS, and combinations of detergent and organic solvent can be used to solubilize formazan crystals. Absorbance is recorded at 570nm (Riss *et al*., 2013).

2.8 Cell Culture for Cytotoxic studies

MCF-7 and HeLa cell line and PBMCs were used in the current cytotoxic studies.

Characteristics of MCF-7 cells

MCF-7 is a human breast cancer cell line isolated in 1970 from a 69-year-old woman (Figure 3a). MCF-7 is the acronym of Michigan Cancer Foundation-7 referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers. The MCF-7 breast cancer cell line was derived from a pleural effusion taken from a patient with metastatic breast cancer (Soule *et al.*, 1973). It was the first hormone response breast cancer cell line (Levenson and Jordan, 1997). MCF-7cell line has epithelial origin. It grows as adherent cell line having doubling time of around 38 hours.

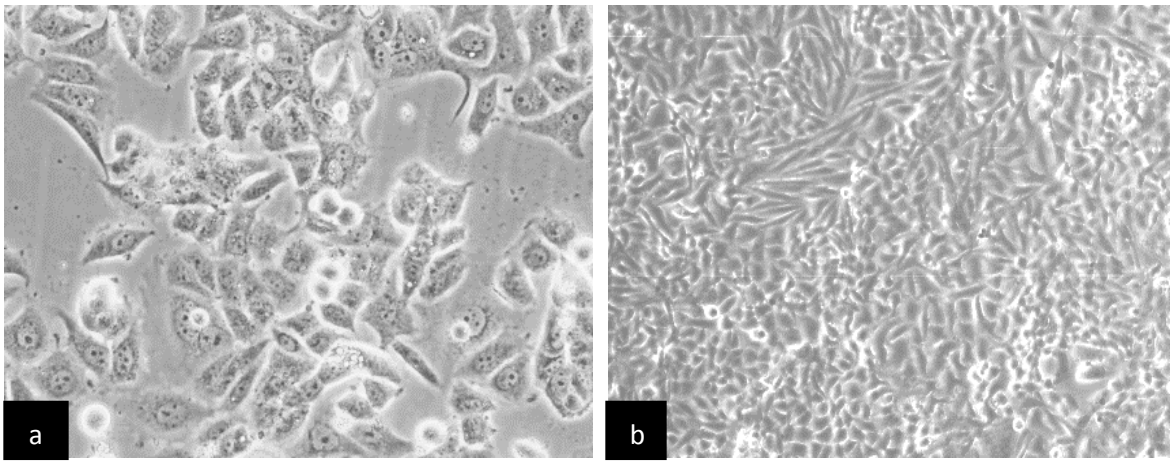


Figure3**. Cell lines a. MCF-7 b. HeLa**

Characteristics of HeLa cells

HeLa is the oldest, immortal and most widely used cell lines in research (Figure 3b). HeLawas derived from aggressive adenocarcinoma (epithelial) of the cervix of a 31 year old lady named Henrietta Lacks, from where cell line derives its name (Lucey *et al*., 2009). It was established in 1951 as first human cancerous cell line. The cells are easily propagated and grow rapidly having doubling time of 24 hours. A tissue biopsy obtained for diagnostic evaluation yielded additional tissue for Dr George O. Gey's tissue culture laboratory at Johns Hopkins (Baltimore, Maryland).

The cancer cells, now called HeLa cells grows as an adherent culture with a doubling time of almost 24 h.

Characteristics of PBMC's

Peripheral blood mononucleated cells (PBMC) isolated from the blood of a healthy donor. PBMCs include lymphocytes (consists of T cells CD4 and CD8, B cells and NK cells) monocytes, and dendritic cells. PBMC divide in vitro in the presence of stimulus, which can be any antigen or mitogens such as Phytohaemagglutinin (PHA), Pokeweed mitogen (PWM), Concanavalin A (Con-A). Isolated PBMCs are used in vitro to evaluate a variety of functions of lymphocytes. Proliferation Activation of monocytes or macrophages by small molecules cytokines and pathogen components can also be monitored. PBMCs can also be used for a variety of structural and functional studies for addressing issues in human immunology such as release of cytokines as in vitro. They grow under *in vitro* condition as a suspension culture.

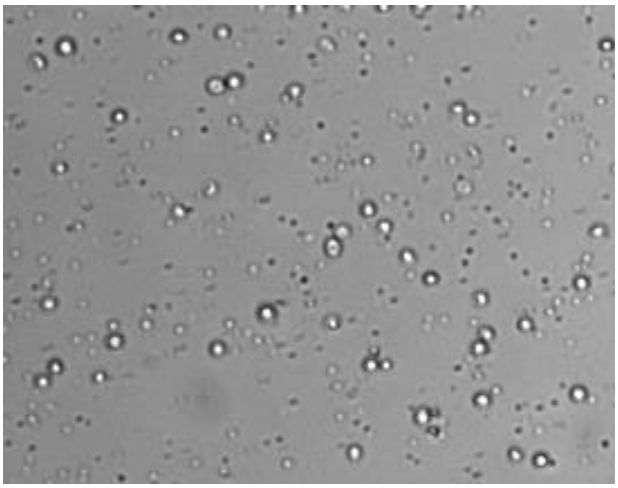


Figure 2. Freshly isolated PBMCs

CHAPTER 3: OBJECTIVES

1. Preparation of *Amomum subulatum* extracts in different solvents based on varying polarity
2. Study on cytotoxicity effect of extracts on breast (MCF-7) and cervical (HeLa) cancer cells
3. Study on the effect of extracts on proliferation of immune cells (peripheral blood mononuclear cells)

CHAPTER 4: MATERIAL AND METHODS

4.1 Material

All chemicals and reagents required during the current project work are given below

Chemicals/Reagents	Make
1) DMEM	Himedia
2) FBS	Gibco®Life technologies
3) Streptomycin	Himedia
4) Penicillin G	Himedia
5) Amphotericin	Himedia
6) Tylosin	Sigma-Aldrich
7) Trypsin/Accutase	Himedia
8) Pokeweed mitogen	Sigma Aldrich
9) Histopaque 1077	Sigma Aldrich
10) Sodium bicarbonate	Himedia
11) L-glutamine	Himedia
12) DMSO	SRL
13) MTT	Sigma Aldrich
14) Trypan Blue	Himedia
15) Hexane	EMPARTA® Merck
16) Diethyl ether	EMPARTA® Merck
17) Ethyl acetate	EMPARTA®Merck
18) Ethanol	EMPARTA® Merck
19) Acetone	EMPARTA® Merck
20) Liquid Nitrogen	

4.2 Preparation of *Amomum subulatum* crude extracts

The fruits of *Amomum subulatum* (25g) were made free from external impurities and then dried at temperature 37^{o}C for 5 days. The dried fruits were transferred to a clean, dry porcelain pestle and mortar and were grinded in the presence of liquid nitrogen to fine powder. Powdered fruit material was subjected to Soxhlet extraction in six different solvents based on increasing polarity (Figure1) and further removal of solvents under reduced pressure.

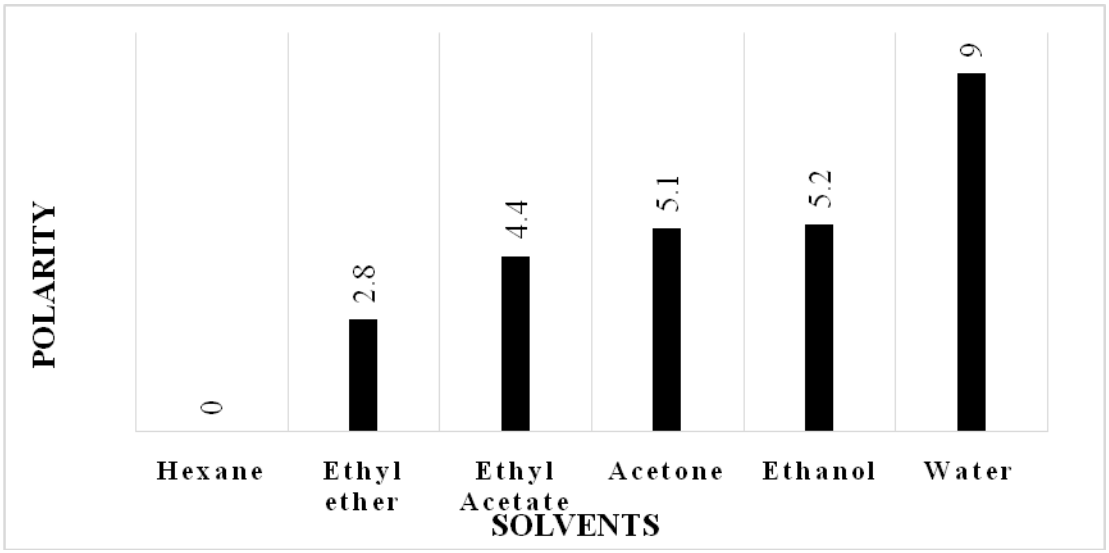


Figure 5: Solvents used and their Polarity Index

Dried and powdered sample of *A. subulatum* (whole fruit/ seeds and rind) was weighed and transferred into the extraction thimble and placed the thimble in soxhlet extractor. Extractor was connected to cooling condenser on top and distillation flask containing 150ml of the desired solvent along with glass beads on the bottom (Figure 2). The whole assembly was placed on heating mantle. Condenser was connected to inlet and outlet pipes for the continuous supply of water. Temperature of the mantle was adjusted according the boiling point of solvent (-10^{o}C below the boiling point). Twenty cycles of extraction were carried out at a fixed temperature for a particular solvent.

Table 3: Boiling point of solvents

Solvent	Boiling Point(°C)
Hexane	68
Diethyl ether	34.6
Ethyl acetate	77.1
Ethanol	78.37
Acetone	56
Water	100

After the completion of 20 cycles, the extract was allowed to cool. The extract was dried under reduced pressure using rotary evaporator to obtain the powdered form of extract. Weight of the powdered extract was recorded and was reconstituted. The extraction was carried out in the order of increasing polarity, such that the highly non polar compounds and drawn out at first.

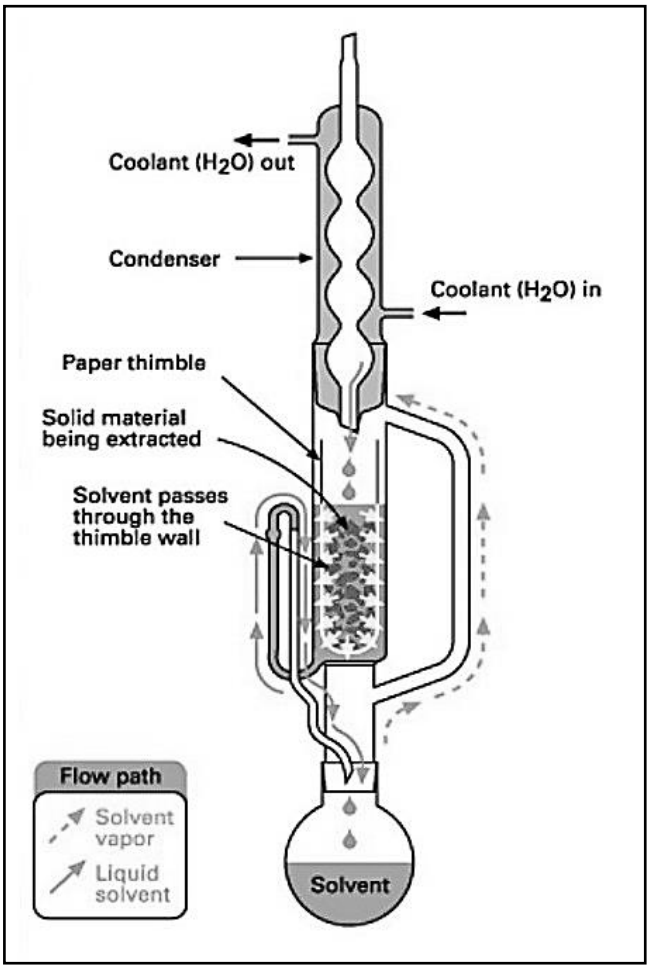


Figure 6: Soxhlet extractor showing flow of solvent during extraction

Rotary evaporation: A rotary evaporator is a device used in chemical laboratories for the efficient for removal of solvents from samples by evaporation under reduced pressure.

The main components of a rotary evaporator are:

1. A motor unit that rotates the evaporation flask or vial containing the user's sample.
2. A vapor duct that is the axis for sample rotation and is a vacuum-tight conduit for the vapor being drawn off of the sample.
3. A vacuum system, to substantially reduce the pressure within the evaporator system.
4. A heated fluid bath (generally water) to heat the sample.
5. A condenser with a coil passing coolant.
6. A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
7. A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

The vacuum system used with rotary evaporators can be as simple as a water aspirator with a trap immersed in a cold bath or as complex as a regulated mechanical vacuum pump with refrigerated trap. Modern equipment often adds features such as digital control of vacuum, digital display of temperature and rotational speed, and vapor temperature sensing.

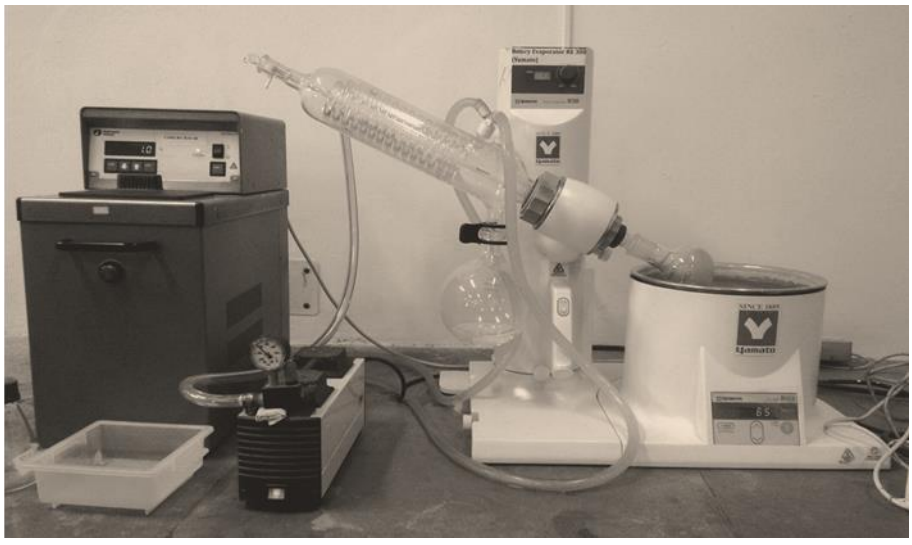


Figure 7: Rotary evaporator in operation

4.3 Maintenance and handling of cell lines

4.3.1 Preparation of media:

Dulbecco's Modified Eagle's Medium (DMEM) has roughly twice the concentration of amino acids and four times the amount of vitamins as EMEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino acids (though not all nonessential amino acids). We have used AT065A is DMEM which has low glucose and is modified for autoclaving.

Media was prepared as per manufacturer instructions. Briefly, 9.6gms of the powdered media was dissolved in 900ml double distilled water with constant, gentle stirring until the powder was completely dissolved. pH of media was adjusted to 4.0 before autoclaving. Volume was adjusted with double distilled water after subtracting the volumes of 4% Sodium bicarbonate solution and 200mM L-glutamine solution to be added after autoclaving from the final volume (1000 ml).Media was autoclaved at 121°C at 15psi for 15minutes. The medium was removed promptly from the autoclave to avoid extended heating or evaporation and allowed the media to cool at room temperature. 26.5 ml of 4% Sodium bicarbonate solution (filtered sterile) and 20ml of 200mM L-glutamine solution (filtered sterile) was added to the final volume of the medium being prepared. The pH of media (if required) was adjusted using sterile 1N NaOH or 1N HCl. Additionally, media was subjected to filtration through 0.22µ filter membrane under vacuum. Sterile media was stored at 2-8°C and in dark till use.

4.3.2 Preparation of PBS:

One litre of 1X PBS was prepared by adding 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 was added in 800 ml of double distilled water. pH was adjusted to 7.4 using HCL or NaOH. Volume was made up to 1 litre by double distilled water. PBS was autoclaved for 20 minutes at 121°C. After autoclaving PBS was filter sterilized and stored at 4°C temperature

4.3.3 Revival of cell lines

Vial containing frozen cell line (Stored at -80^{o}C) was thawed rapidly by agitating gently in water bath at 37 °C ± 1 °C till the complete melting of ice. Immediately added twice volumes of complete growth medium (DMEM containing 10%FBS and antibiotics) aseptically. The vial at centrifuged 1000 rpm for 10 minutes at room temperature and the supernatant was discarded and the pellet was resuspended in 1 ml complete growth medium. Cell counting was done with the

help of hemocytometer using trypan blue stain (0.4 % w/v). Cells were seeded in tissue culture flask (T-25) contain 10 ml complete growth media, labelled and incubated at 37 °C and 5% CO_2. Flask was monitored at regular intervals to check the cell growth, morphology, pH change and contamination.

4.3.4 Passaging and maintenance of cell lines

Anchorage-dependent cell lines growing in monolayers need to be sub-cultured at regular intervals to maintain them in exponential growth. When the cells are near the end of exponential growth (roughly 70% to 90% confluent), they are ready to be sub-cultured. The sub-culturing of cell lines was done as following:

After the cell lines have attained 70-80% confluency, media was removed from flask and rinsed with 4-5 ml of 1X- PBS buffer.4 ml of warm (37°C) 0.25% Trypsin solution was added to cells to disrupt cell layer and incubated at 37^{o}C in CO_2 incubator for 5-7 minute. The homogeneous cell suspension was checked for cell dissociation by microscopy and was taken in a separate vial progress of. As soon as cell layer dislodged completely, trypsin was deactivated by adding twice volumes of complete growth medium (DMEM, supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100µg/ml), amphotericin (2.5 µg/mL) in sterile centrifuge tube. Cells were aspirated gently by pipetting. The cells were centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and the cell pellet was resuspended in 10 ml 1X- PBS buffer and centrifuged at 1000 rpm for 10 minutes. Then the supernatant was discarded and finally the cell pellet was resuspended in 1 ml of complete medium. 10 µl cells were taken in a separate vial and diluted in media and trypan blue. Cells were counted using hemocytometer as described in section 2.4. Cells were seeded in appropriate split ratio in tissue culture flasks (T-25 or T-75) complete growth media (10 ml for T25 flask and 20 ml for T75 flask) and incubated again at 37^{o}C (5% CO_2). Flasks were monitored, microscopically and with naked eye to check the cell growth, morphology, pH change and contamination.

4.3.5 Cell counting

Cell counting is necessary in order to establish or monitor growth rates as well as to set up new cultures with known cell numbers. Hemocytometers are commonly used to estimate cell number and determine cell viability. A hemocytometer is a fairly thick glass slide with two counting

chambers, one on each side. Each counting chamber has a mirrored surface with a 3 × 3 mm grid of 9 counting squares. The chambers have raised sides that will hold a coverslip exactly 0.1 mm above the chamber floor. Each of the 9 counting squares holds a volume of 0.0001 mL.

Hemocytometer and coverslip was cleaned, thoroughly dried, and assembled. A small amount of cell suspension was transferred to the edge of each of the two counting chambers. Cell suspension was allowed to be drawn into the counting chamber by capillary action. The hemocytometer was placed under microscope and viewed the cells at 40× magnification. Cells were counted in each section A, B, C and D (shown in figure 2). Average the number of cells, and multiply by the dilution factor.

$$\textbf{Cell count} = \{(A+B+C+D)/4\} \times \text{dilution factor} \times 10^{-4}$$

where A,B, C and D are the cell counts in chamber A, B, C and D, respectively.

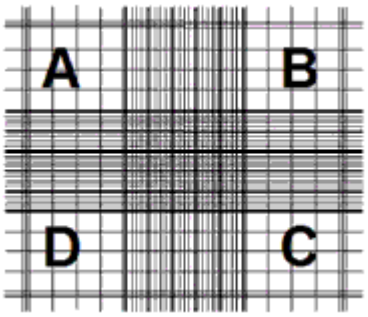


Figure 8: Haemocytometer

4.4. Cytotoxicity assay

4.4.1 Effect of extract on cancer cell line

Two cell lines, MCF-7 (Human Breast cancer) and HeLa (Human cervical cancer) were used to study anti-proliferative activity. The cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Cell lines MCF-7 and HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin and 1ml/L tylosin. Cells were maintained in a humidified incubator with 5% CO_2 for 37°C in T25/T75 culture flasks. *In vitro* response against extracts and standard drug was evaluated by means of a growth inhibition using the 3-(4, 5-

dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT assay). The cells were trypsinised and seeded at a density of ~2×10^4 per well and incubated overnight. After 16 h, sample extracts (10% DMSO + 90% complete DMEM media) and positive control were added well plate into the 96, making total volume 200 µl (cells + media + extract). The assay was carried out in triplicates. After 72 h of incubation, 20 µL of MTT reagent (5 mg/mL) was added to each well and again incubated for 4 h. 160µl of media was removed from each well and purple formazan crystals formed were dissolved in 100µl DMSO in each well and absorbance was recorded at 570 nm, taking 630nm as the reference wavelength. Final O.D. was calculated as difference between both O.D. by micro titter plate reader. Paclitaxel was assayed as a positive control at the concentration of 1 mg/ml (4µl). The resulting data represents the net outcome of cell proliferation and cell death. The cell viability was obtained by comparing the absorbance between the samples and a negative control of DMSO.

Percentage of inhibition was calculated by following equation

$$\frac{OD_{Control} - OD_{test}}{OD_{control}} \times 100$$

4.4.2 Effect of extract on peripheral Blood Mononuclear Cells (PBMCs)

To assess the cytotoxicity of the extracts on primary cells (PBMC's) the MTT assay was used.

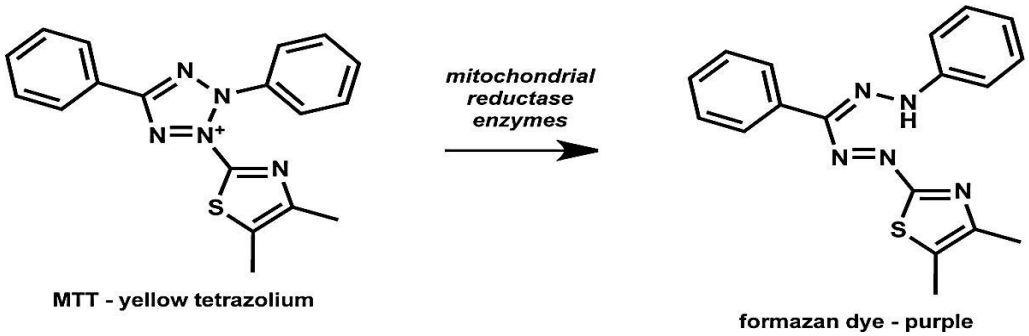
4.4.2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

PBMC isolation was carried out using Histopaque-1077 as per manufacturer's instruction. To a 15-mL conical centrifuge tube, added 3 mL of Histopaque-1077(room temperature). 3mLWhole blood from a healthy donor was carefully layered onto the Histopaque-1077 and centrifuged at 400 x g for exactly 30 minutes at room temperature. After centrifugation, carefully removed the top layer plasma layer(yellow coloured) with a pipette and collected opaque interface containing mononuclear cells (PBMCs) in clean conical centrifuge tube. The cells was washed by adding 10 mL of sterile 1X PBS buffer and centrifuged at 250 x g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended with 5 mL of sterile 1X PBS buffer. Again centrifugation was done at 250 x g for 10 minutes. After discarding the supernatant and finally

cell pellet was resuspneded in 1 mL of cell culture medium. Cells were counting using hemocytometer as discussed in section 4.3.4.

4.4.2.2 Cytotoxicity assay

In vitro response against extracts and standard anticancer drug was evaluated by means of a growth inhibition using the MTT assay. The PBMCs seeded at a density of ~1×10^5 per well and cells were stimulated with pokeweed mitogen 1 µg/ml (4µl). After 16 h incubation of stimulated and unstimulated cells, sample extracts (10% DMSO + 90% complete DMEM media) and positive control were added, making total volume 200 µl (cells + media + extract). The assay was carried out in triplicates. After 72 h of incubation, 20 µL of MTT reagent (5 mg/mL) was added to each well and again incubated for 4 h. The 96 well plate of centrifuged at 2000 rpm for 10 min. 160µl of media was removed from each well and purple formazan crystals formed after 4 h of incubation were dissolved in 100µl DMSO in each well and absorbance was recorded at 570 nm, taking 630nm as the reference wavelength.



CHAPTER 5: RESULTS AND DISCUSSION

5.1 Yield of *Amomum subulatum* crude solvents extracts

Extracts were prepared under optimised conditions of soxhlet extraction using sample of *A. subulatum* procured from Indian Cardamom Research Institute (ICRI), Gangtok, Sikkim. Twenty five grams of *A. subulatum* fruits and 10 g of *A. subulatum* seeds were subjected to Soxhlet extraction in six different solvents based on varying polarity. After extraction respective solvent were removed under reduced pressure by Rota evaporator to get final yield as given in Table 1. All the extracts were reconstituted in DMSO.

Table 4: Extract of 25 g of *A. subulatum* fruit and seed their yield

Solvent	Extraction temperature (°C)	Fruit Yield (mg)	Seed Yield (mg)
Hexane	60	480	440
Diethyl ether	25	370	50
Ethyl acetate	70	320	100
Ethanol	70	290	70
Acetone	45	250	500
Water	80	1.66	-

5.2 Effect of *A. subulatum* fruit extracts on the proliferation of MCF-7 cells

5.2.1 Assay optimization to assess the effect of *A. subulatum* fruit extracts on MCF-7 cells

Each extract was diluted 10 times with complete DMEM media so as to get the final extract in 10% DMSO. The extracts were filter sterilized before usage. To optimize the experimental set up, 20µl and 40µl of three extracts (Hexane, Diethyl Ether and Ethyl acetate) were added in the culture and incubated for 72 h at 37°C and humidified 5% CO_2. MTT assay was done to evaluate the effect of these extracts on MCF-7 cells and then absorbance was recorded at 570nm with reference wavelength of 630 nm. Each experiment was performed in triplicates. Absorbance which is directly proportional to the number of viable cells was analysed for different solvent extracts and control. It was observed that there was inhibition in proliferation as compared to the control as shown in Table2 and Figure 1.

Table2: Assessment of three solvent extracts for activity and assay optimization

Volume (20 µl)	Control 1	Control 2	Hexane	Diethyl Ether	Ethyl acetate
Mean absorbance ±SD	0.686±0.04*	0.656±0.02	0.438±0.04	0.157±0.03	0.026±0.02

Volume (40 µl)	Control 1	Control 3	Hexane	Diethyl Ether	Ethyl Acetate
Meanabsorbance ± SD	0.686±0.04	0.511±0.01	0.174±0.01	0.042±0.06	0.025±0.01

Control 1: Media + Cells, Control 2: Media + Cells + 20 µl (10%DMSO in DMEM)

Control 3: Media + Cells + 40 µl (10%DMSO in DMEM)

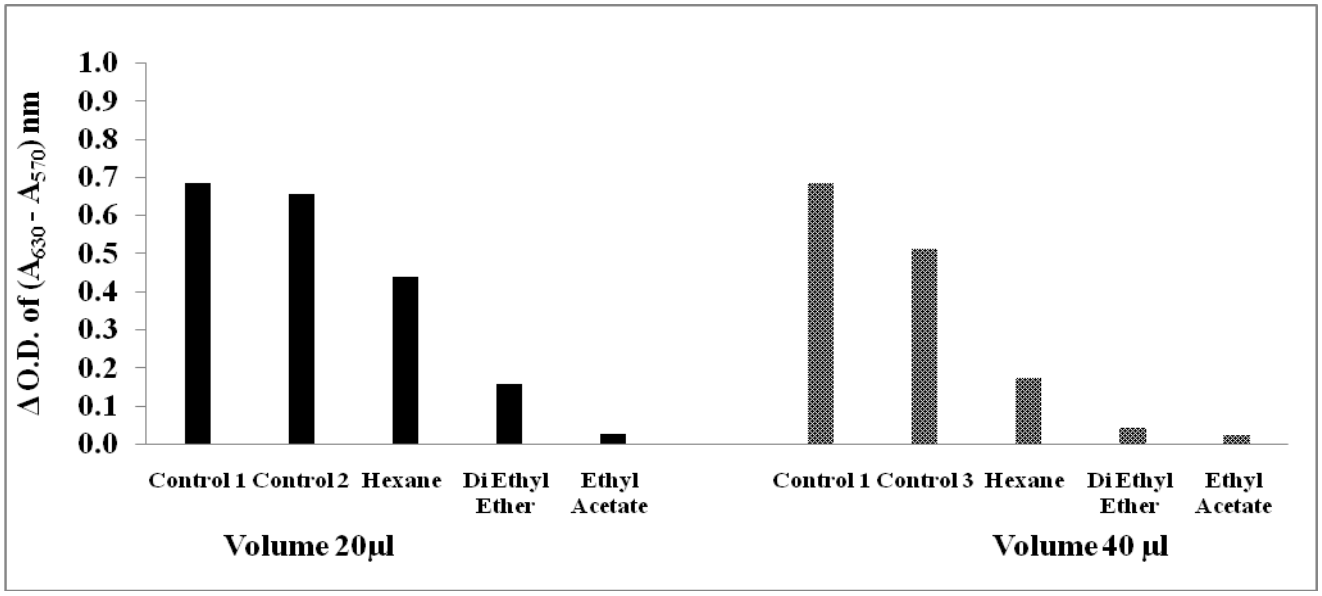


Figure 9: Assessment of 20 and 40 µl of three solvent extracts for activity

5.2.2 Comparison of different solvent extracts for inhibition in proliferation of MCF-7 cells

20 µl each of the six different extracts was tested for their inhibition effect and percentage of inhibition was calculated. Inhibition was observed for all extracts but the most pronounced effect was observed in case of hexane and ethyl acetate (Table3 and Figure2). Ethyl acetate has shown maximum inhibition effect, hence further different volume gradients (5, 10, 15 & 20 µl) were used in order to evaluate the effect. Interestingly, it is observed that with increase in volume of extract, percentage of inhibition has also increased (Table 4 and Figure3).

Table 5: Effect of 20 µl of different solvent extract on the proliferation of MCF-7 cells

Solvent	Exp I	Exp II	Exp III	Mean±SD
Hexane	58.7	72.8	88.1	73.2±14.7
Diethyl ether	39.4	39.2	65.5	48.0±15.1
Ethyl acetate	80.3	88.2	92.5	87.0±6.2
Ethanol	54.7	58.5	74.0	62.4±10.2
Acetone	26.3	43.2	39.8	36.4±8.9
Water	12.9	18.0	15.2	15.4±2.6

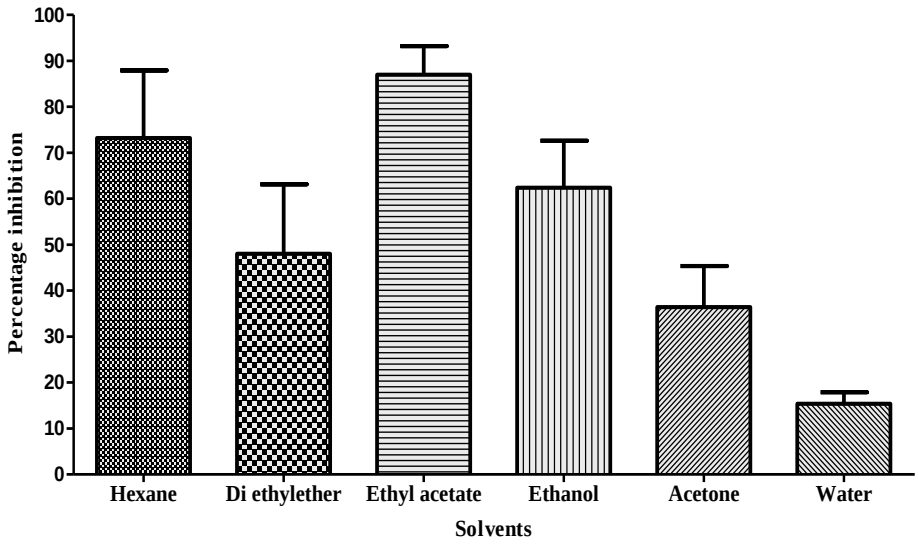


Figure 10: Effect of 20 µl of different solvent extract on the proliferation of MCF-7 cells

Table 6: Effect of increasing volume of ethyl acetate extract on MCF-7 cells

Ethyl Acetate (µl)	Experiment I	Experiment II	Mean±SD
5	20.7	0.0	10.3±14.6
10	66.9	37.1	52.0±21.1
15	85.0	64.0	74.5±14.8
20	93.7	81.2	87.4±8.8

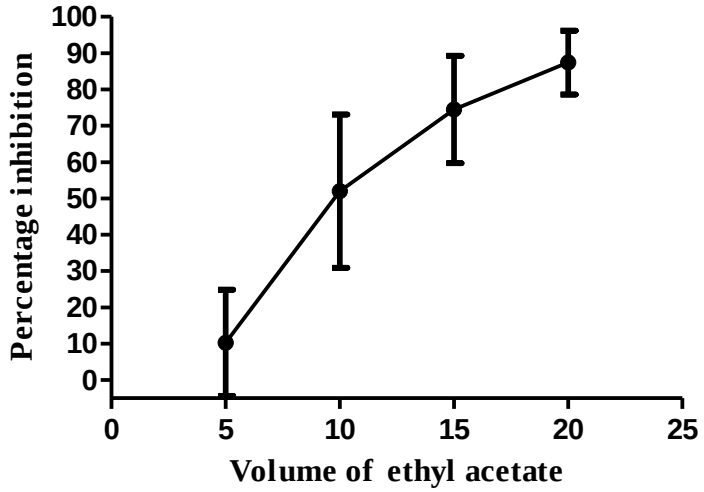


Figure 11: Effect of increasing volume of ethyl acetate extract on MCF-7 cells

5.3 Effect of *A. subulatum* seed extracts on the proliferation of MCF-7 cells

Seeds of *A. subulatum* contain most of the active compound hence for further analysis seed was used for extraction. Since hexane and ethyl acetate extracts of *A. subulatum* fruit has shown most pronounced inhibition effect, hexane and ethyl acetate extracts of seeds were analysed for their cytotoxic effects and inhibitory concentration 50 (IC50) values for each of these extracts was determined against MCF-7cells.

5.3.1 Cytotoxic effect of Hexane extracts in MCF-7 cells:

Different concentrations of hexane **e**xtract (250,500,750 and 1000 µg/ml) were tested for their cytotoxic inhibition against MCF-7 and percentage of inhibition was calculated (Table 5). It was observed that as the concentrations of extract increases, inhibition percentage also increases (Table 5 and Figure 4). The inhibitory concentration 50 (IC50) for hexane extract on MCF-7 cells was found to be 750 µg/ml (Table 5). Further, kinetics (w.r.t. to time) of cytotoxic inhibition by hexane extract and paclitaxel 100 µg/ml was studied for MCF-7 cell line at 750µg/ml (near to IC-50 value) for 24, 48 and 72 h. It was observed that with increase in time there is increase in cytotoxic effect. With these observations, we can say that cytotoxic effect becomes more pronounced with increase in both concentration and time.

Table 7: Percentage of Inhibition of MCF-7 by Hexane extract

Concentration of Hexane extract in (µg/mL)	Experiment I	Experiment II	Experiment III	Mean ± SD
250	18.5	12.8	31.6	21.0 ± 9.6
500	41.6	31.9	52.3	41.9 ± 10.2
750	47.2	41.7	61.6	50.2 ± 10.2
1000	81.5	82.6	99.2	87.8 ± 9.9

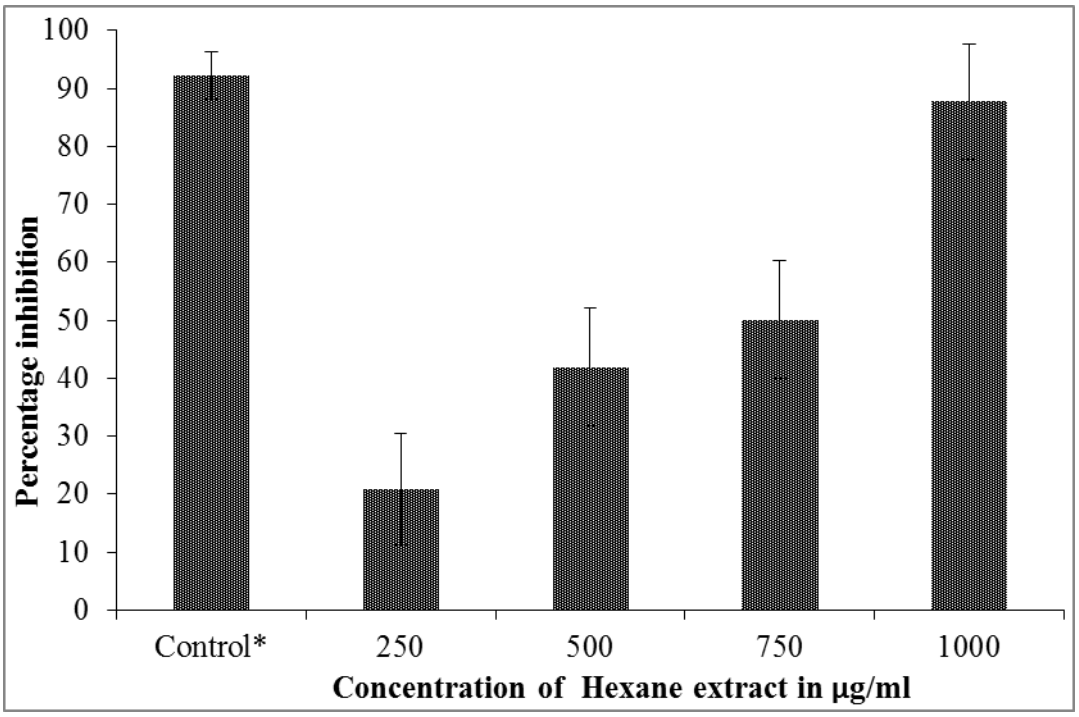


Figure 12: MCF-7 cell proliferation inhibition using different concentration of Hexane extract (*Control - Paclitaxel 100 µg/ml)

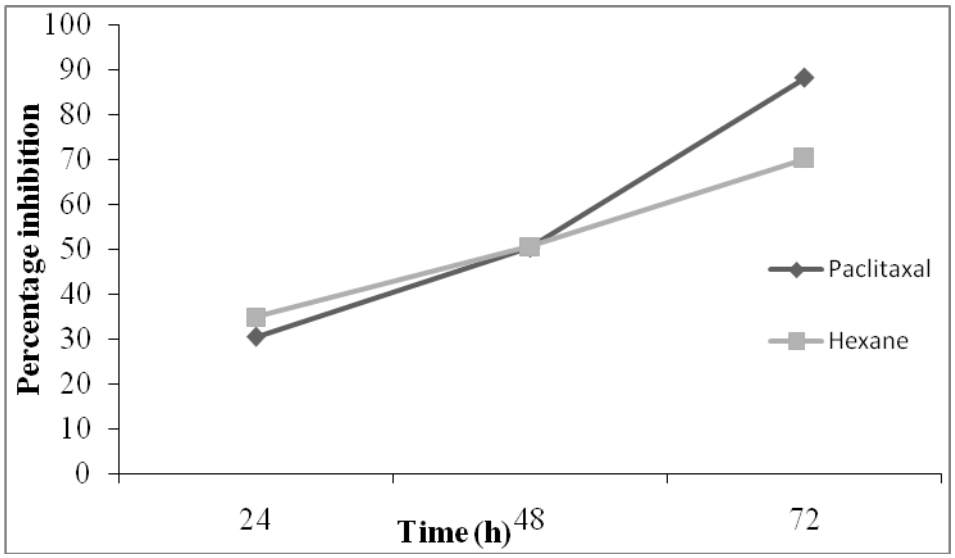


Figure 8: Kinetics of inhibition in MCF-7 cell when grown with Hexane extracts and Paclitaxel

5.3.2 Cytotoxic effect of Ethyl acetate extracts in MCF-7 cells:

Different concentrations of Ethyl acetate (750, 1000, 1250 and 1500 µg/ml) were tested for their cytotoxic inhibition against MCF-7 and percentage of inhibition was calculated (Table 6). It was observed that as the concentrations of extract increases, inhibition percentage also increases (Table6 and Figure6). The inhibitory concentration 50 (IC50) for ethyl acetate extract on MCF-7 cells was obtained to be 870 µg/ml (Table 6). Further, kinetics (w.r.t. to time) of cytotoxic inhibition by ethyl acetate extract and paclitaxel 100 µg/ml was studied for MCF-7 cell line at 1000 µg/mL (near to IC50 value) for 24, 48 and 72 h (Figure7). It was observed that with increase in time there is increase in cytotoxic effect. With these observations, we can say that cytotoxic effect becomes more pronounced with increase in both concentration and time.

Table 9: Percentage of Inhibition of MCF-7 by Ethyl acetate extract

Concentration of Ethyl acetate extract in (µg/mL)	Experiment I	Experiment II	Experiment III	Mean ± SD
750	33.33	33.51	58.65	41.8 ± 14.6
1000	41.08	50.41	79.17	56.9 ± 19.9
1250	56.47	59.1	89.19	68.3 ± 18.2
1500	69.16	67.78	98.8	78.6 ± 17.5

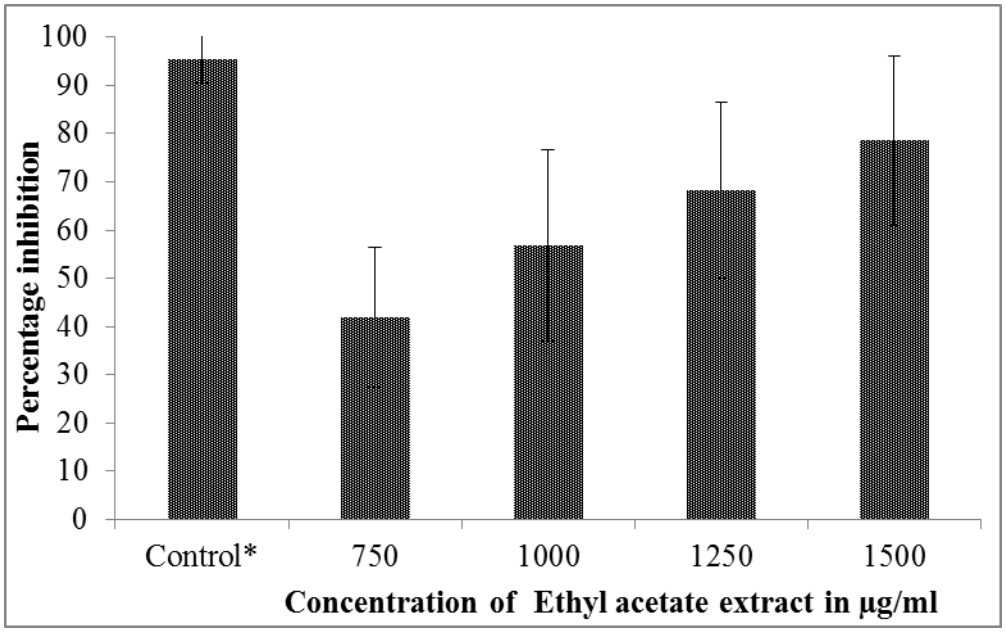


Figure 13: MCF-7 cell proliferation inhibition using different concentration of Ethyl acetate extract (*Control-Paclitaxel)

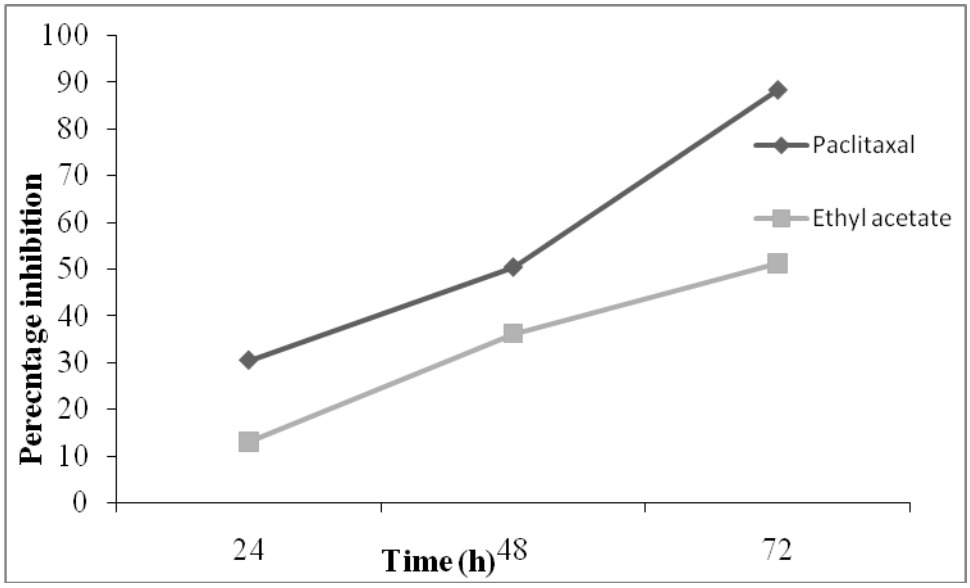


Figure 14: Kinetics of inhibition in MCF-7 cell when grown with Ethyl acetate extracts and paclitaxel

5.3.3 Effect of *A. subulatum* fruit extracts on the proliferation of HeLa cells

20 µl each of all the six different extracts were tested for their inhibition effect and percentage of inhibition was calculated. Inhibition was observed for all extracts but the most pronounced effect was observed in case of hexane and ethyl acetate (Table 7 and Figure 8).

Volume 20 µl	Percentage inhibition
Hexane	42.48
Diethyl ether	23.30
Ethyl acetate	67.55
Ethanol	23.30
Acetone	20.45
Water	9.28

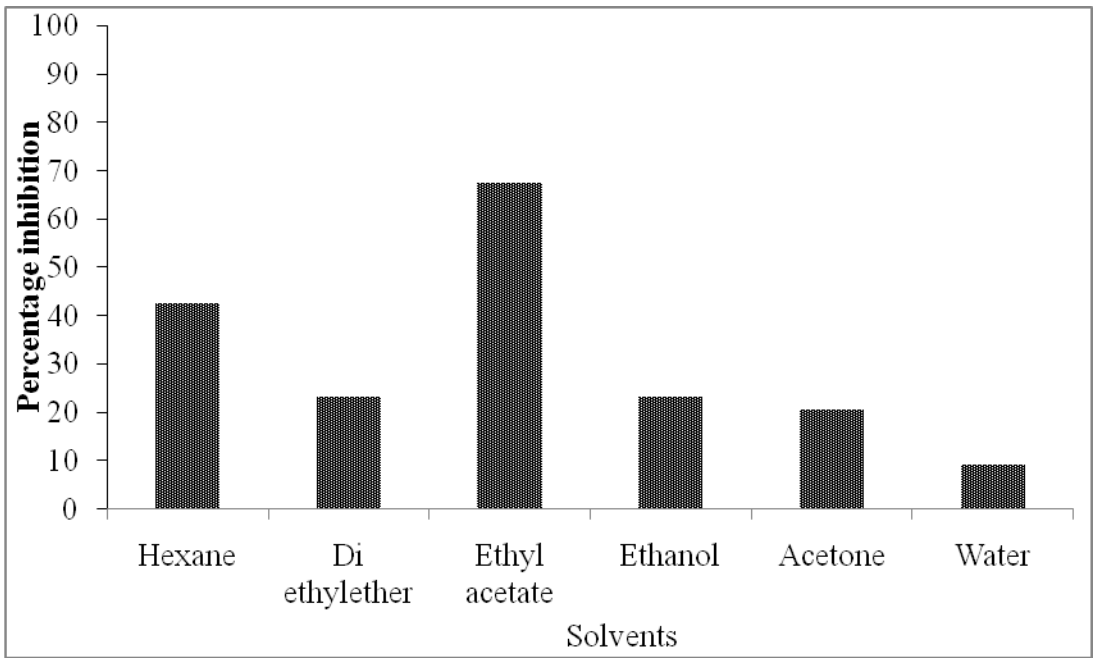


Figure 15: Cytotoxic inhibition by different extract on HeLa cells

5.3.4 Cytotoxic effect of Hexane extracts in HeLa cells:

Different concentrations of hexane **e**xtract (250,500,750 and 1000 µg/ml) were tested for their cytotoxic inhibition against HeLa and percentage of inhibition was calculated (Table 8). It was observed that as the concentrations of extract increases, inhibition percentage also increases (Table8 and Figure9). The inhibitory concentration 50 (IC50) for hexane extract on HeLa cells was found to be 510 µg/ml (Table 8). Further, kinetics (w.r.t. to time) of cytotoxic inhibition by hexane extract and paclitaxel 100 µg/ml was studied for HeLa cells line at 500 µg/mL (near to IC-50 value) for 24, 48 and 72 h (Figure10). It was observed that with increase in time there is increase in cytotoxic effect. With these observations, we can say that cytotoxic effect becomes more pronounced with increase in both concentration and time.

Table 10: Cytotoxic effect of Hexane extracts in HeLa cells

Concentration of Hexane extract (µg/mL)	Experiment I	Experiment II	Experiment III	Mean ± SD
250	26.1	27.9	16.2	23.4 ± 6.3
500	28.7	55.1	60.9	48.2 ± 17.2
750	74.1	89.3	91.7	85.1 ± 9.5
1000	90	94.3	92.1	92.1 ± 2.2

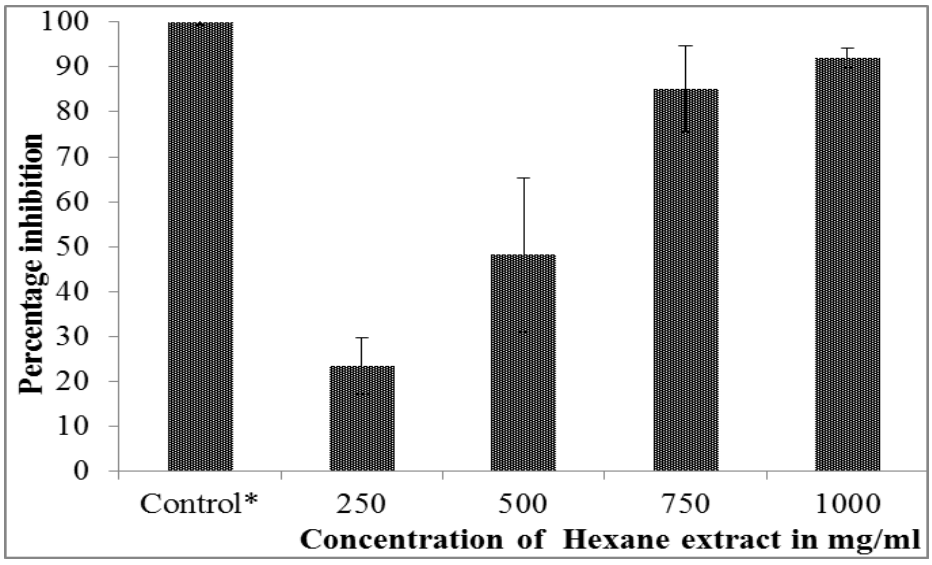


Figure16: HeLa cells proliferation inhibition using different concentration of Hexane extract (*Control - Paclitaxel 100 µg/ml)

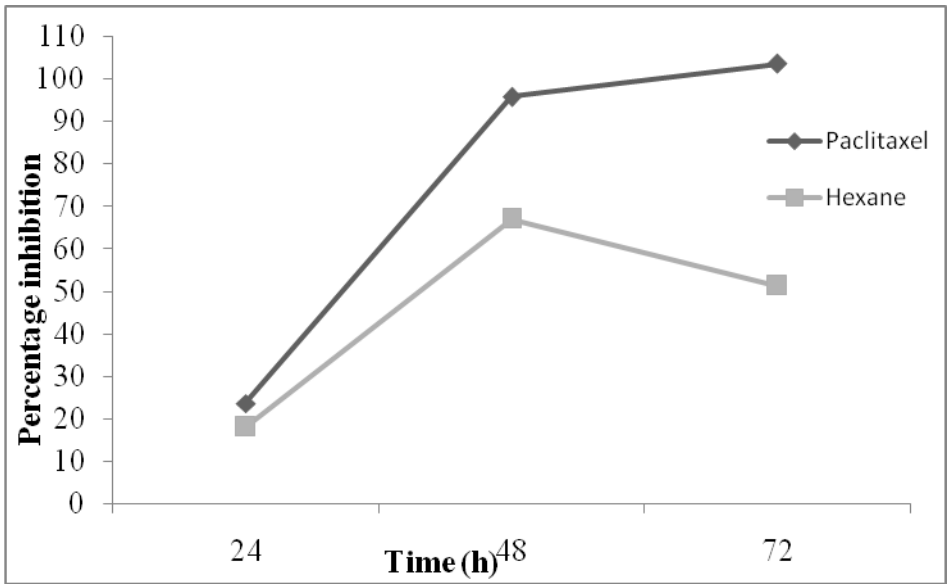


Figure17: Kinetics of inhibition in HeLa cells when grown with Hexane extracts and paclitaxel

5.3.5 Cytotoxic effect of Ethyl acetate extracts in HeLa cells:

Different concentrations of Ethyl acetate (750, 1000, 1250 and 1500 µg/ml) were tested for their cytotoxic inhibition against HeLa and percentage of inhibition was calculated (Table 9). It was observed that as the concentrations of extract increases, inhibition percentage also increases (Table9 and Figure11). The inhibitory concentration 50 (IC50) for ethyl acetate extract on HeLa cells was found to be 875 µg/ml (Table 9). Further, kinetics (w.r.t. to time) of cytotoxic inhibition by ethyl acetate extract and paclitaxel 100 µg/ml was studied for HeLa cells (near to IC-50 value) value for 24, 48 and 72 h (Figure12). It was observed that with increase in time there is increase in cytotoxic effect. With these observations, we can say that cytotoxic effect becomes more pronounced with increase in both concentration and time.

Table 11: Cytotoxic effect of Ethyl acetate extracts in HeLa cells

Concentration of ethyl acetate extracts in µg/ml	Experiment I	Experiment II	Experiment III	Mean ± SD
750	11.4	42.92	47.51	33.9 ± 19.7
1000	58.86	58.87	77.26	65.0 ± 10.6
1250	62.91	43.15	87.91	64.7 ± 22.4
1500	72.62	49.38	99.21	73.7 ± 24.9

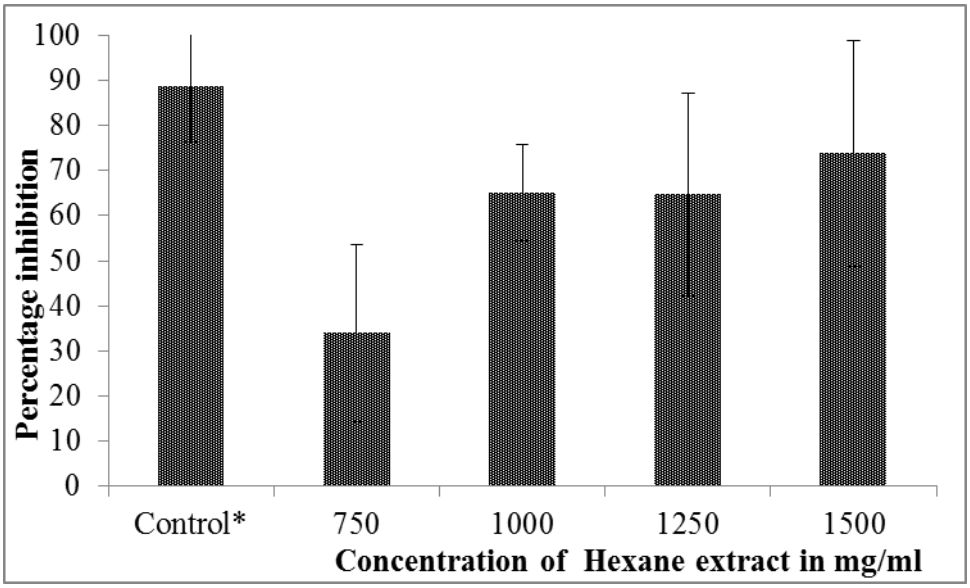


Figure 11: **HeLa cells proliferation inhibition using different concentration of Ethyl acetate extract (*Control- Paclitaxel)**

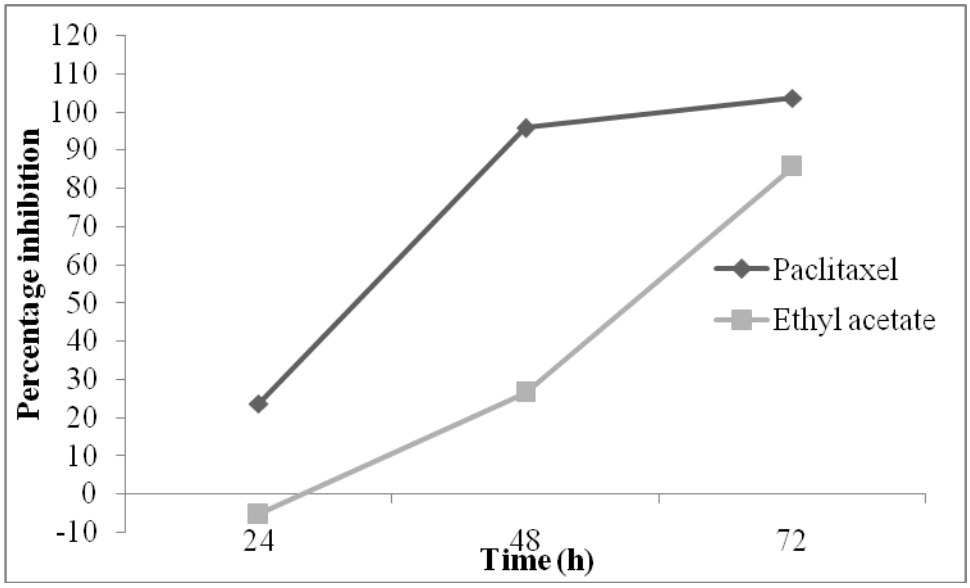


Figure 38: Kinetics of inhibition in HeLa cells when grown with Ethyl acetate extracts and paclitaxel

5.3.6 Effect of *A. subulatum* fruitextracts on Peripheral blood mononuclear cells (PBMC)

3.6.1 Optimization of cell number for the effect of extracts on PBMC proliferation

Different cell count were taken for the effect of ***A. subulatum*** **fruit extracts** on PBMC and incubated with 20 µl of ethyl acetate extract for 72 h. Percentage of inhibition was calculated as given in table 10. Based on this data, we have selected $1x10^5$cells for further experiments.

Table 12: Percentage inhibition of PBMCs

Number of cells	Percentage Inhibition
$20x\ 10^3$	-29.2
$60\ x\ 10^3$	21.6
$100\ x\ 10^3$	71.9
$140\ x\ 10^3$	76.0

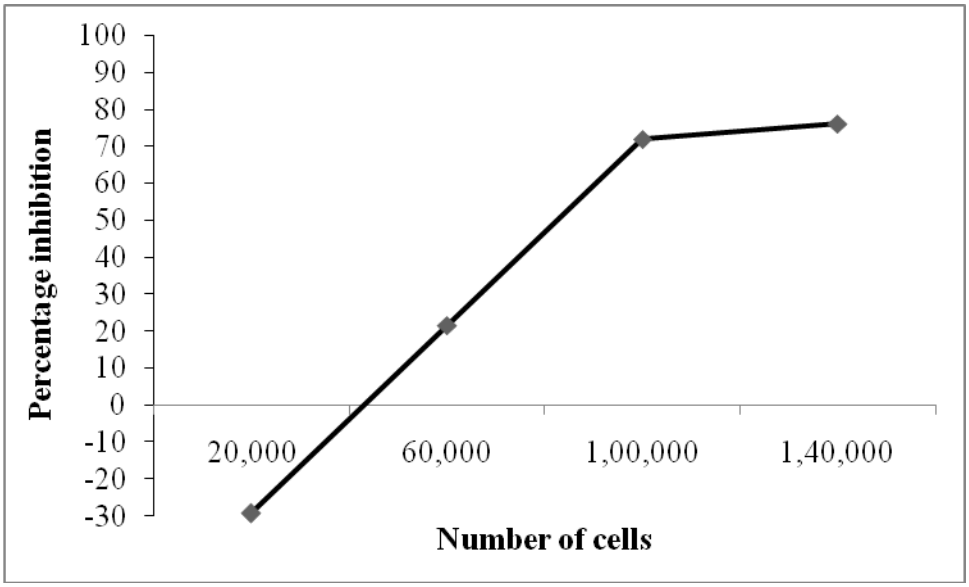


Figure19: Effect of ethyl acetate extract on varying concentration of PBMC

5.3.7 Effect of hexane and ethyl acetate extract on PBMC's

Different concentration (750, 1000 and 1250 µg/ml) of hexane and ethyl acetate extract were tested for their effect on stimulated (Pokeweed mitogen treated) and unstimulated PBMCs in 72 h culture. Paclitaxel (Anticancer drug) is used as control. It appears that most of the stimulated PBMCs and unstimulated died with the effect of hexane extract. In case of ethyl acetate hand, more than 80% inhibition was observed in unstimulated PBMCs. While in stimulated PBMCs, it was observed that with increase in concentration, there is increase in inhibition.

Table13. Percentage inhibition shown by extracts against unstimulated PBMCs

	Concentration µg/ml	Percentage inhibition
PWM	100	-35.5
Paclitaxel	100	107.8

Concentration of extract in µg/ml	Hexane Percentage inhibition	Ethyl acetate Percentage inhibition
750	123	88.8
1000	100.6	82.8
1250	96.4	90.8

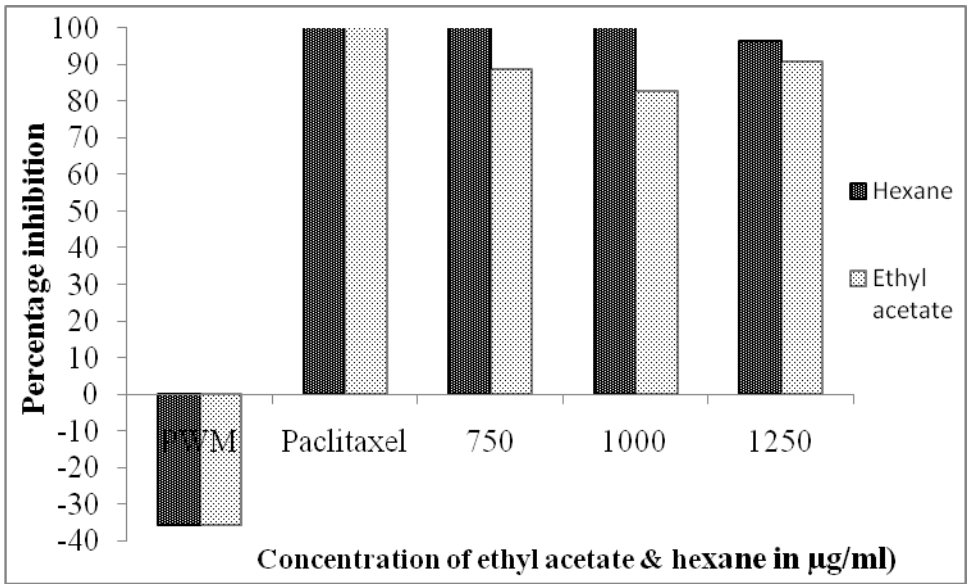


Figure 20: Effect of ethyl acetate & Hexane extract on PBMC

Table14. Percentage inhibition of the Extracts on stimulated PBMCs

		Percentage inhibition
PWM	100	-35.5
Paclitaxel	100	107.8

Concentration of Extract (µg/ml	Percentage inhibition in Hexane extract	Percentage inhibition in Ethyl acetate extract
750	114.6	35.6
1000	99.1	76.7
1250	98.9	87.8

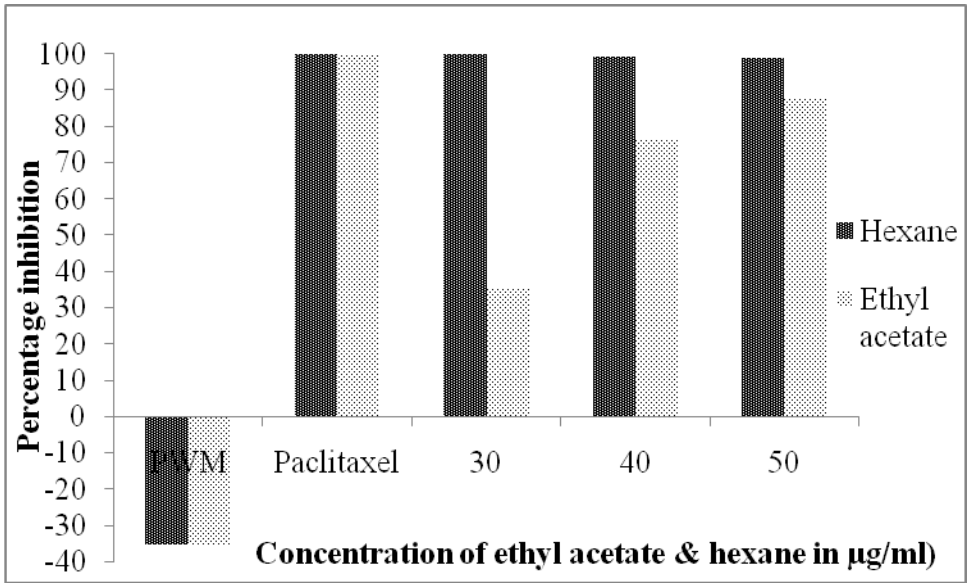


Figure 21: Effect of ethyl acetate & Hexane extract on PBMCs supplemented with PWM

CHAPTER 6: SUMMARY

Breast cancer and cervical cancer is the leading cause of death among women across the globe. Various anticancer drugs are available against cancer but they are associated with high risk of side effects. There is recent trend of alternative medicine with orientation of having lesser side effects. Spices are known to have bioactive properties such anticancer, antimicrobial and antioxidant. *Amomum subulatum* is one of the commonly used spices in Indian cuisines and is commonly used to treat disease like nausea vomiting cough dyspepsia and for throat infect. Hence in the present study, the effect of different solvent extracts of *Amomum subulatum* was assessed on Breast cancer cells (MCF-7), cervical cancer cells (HeLa) and peripheral blood monuclear cells (PBMCs). Six different solvents (Hexane, Diethyl Ether, Ethyl acetate, Ethanol, Acetone and Water) based on varying polarity were used for extraction. These extracts were then tested on cancer cells and immune cells for their effect using MTT assay. Initially, different volume of solvents extracts prepared from fruit of *Amomum subulatum* was used to assess the effect on proliferation on MCF-7 and HeLa cells. It was found that each extract has exhibited inhibition in proliferation of these cancerous cells but hexane and ethyl acetate has shown pronounced inhibition effect. For further experiments, extracts of seed of *Amomum subulatum* was prepared as seed contains most of the bioactive compounds. Hexane and ethyl acetate were used for assessing the cytotoxic effect and to find out the IC_{50} (Inhibitory concentration-50) value. Further cell inhibition kinetics study was also carried out with these two solvent extracts. It has been observed that cytotoxic effect of hexane and ethyl acetate extracts becomes more pronounced with increase in both concentration and time in MCF-7 and HeLa cells. IC_{50} value for hexane and ethyl acetate extracts in MCF-7 was found to be 750µg/ml and 870µg/ml respectively while IC_{50} value of hexane and ethyl acetate extracts for Hela cells was found to be 510µg/ml and 875µg/ml, respectively. Several anticancer drugs also posses the immunosuppressive effect hence these extracts were assessed for their effect on PBMCs. Paclitaxel, an anticancer drug used in this experiment has shown immunosuppressive effect. Hexane and ethyl acetate extracts have also shown inhibition of stimulated (pokeweed mitogen treated) and unstimulated PBMC which shows that these extracts may exhibit immunosuppressive effect. Hence this study shows that the *Amomum subulatum* can be used to obtain anticancer and immunosuppressive agent.

CONCLUSION

The consumption of spices by humans from centuries reveals that there are no considerable side effects on their intake make them ideal agents for research purpose. The result presented in this study shows that extracts of *Amomum subulatum* reduces growth of MCF-7 and HeLa cells. Further experimental analysis would definitely reveal the important chemical constituents responsible for cytotoxicity because the probable cytotoxicity with active biochemical constituents would be higher than the extracts due to the presence of mixture of varied constituents. We have shown that *Amomum* subulatum extracts has potent cytotoxic activities and potentially be a good source for a pharmacologically active product suitable for development as a chemotherapeutic agent.

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