EVALUATION OF CYTOTOXIC AND FREE RADICAL SCAVENGING ACTIVITIES OF *CINNAMOMUM ZEYLANICUM* BARK EXTRACTS

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

Maitri Bhandari

601304008

Under the guidance of

Dr. Manoj Baranwal

Assistant Professor

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA (PUNJAB)-147004

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CERTIFICATE

This is to certify that the thesis entitled "Evaluation of cytotoxic and free radical scavenging activities of *Cinnamomum zeylanicum* bark extracts" submitted by Maitri Bhandari in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology to Thapar University, Patiala, is a record of student's own work carried by her. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute.

Dr. Manoj Baranwal
Assistant Professor, Supervisor
Department of Biotechnology
Thapar University, Patiala

Dr. Dinesh Goyal
Professor and Head
Department of Biotechnology
Thapar University, Patiala

Dr. S.S. Bhatia
Dean, Academic Affairs
Thapar University, Patiala

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled**"**Evaluation of cytotoxic and free radical scavenging activities of *Cinnamomumzeylanicum* bark extracts"in the partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my work during the period of one year from July 2014 to June 2015, under the guidance of Dr. ManojBaranwal, Assistant Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Place:ThaparUniversity,Patiala — Maitri Bhandari

Date:14/07/15 — (601304008)

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Maitri Bhandari

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ABSTRACT

Traditional/Herbal medicines are plant derived products which include crude plant extracts such as fruit, flower, bark, materials which are native to an area. These constitute a major part of Complementary and Alternative Medicines (CAMs) which are being researched thoroughly due to their lower risks of side effects. Cancer is a disease of uncontrolled cell growth and proliferation and the treatments available like Chemotherapy also cause non-specific cytotoxicity to normal cells. Several phytochemicals present in spices have shown a potential to treat cancer. *Cinnamomum zeylanicum*is a commonly used spice with many medicinal properties but not much scientific data is available on its cytotoxic effects. Hence, the present study focuses on evaluation of the cytotoxic effects of *Cinnamomum zeylanicum* bark extracts (Hexane, Dichloromethane, Chloroform and Methanol) on Cancer cell lines HeLa and RAW 264.7 and their free radical scavenging activity. Phenol content was found to be highest in methanol extract. All extracts showed cytotoxic effects but more pronounced effects were seen in the case of hexane followed by DCM for both cell lines. Chloroform and DCM at some concentrations showed proliferative response in both cell lines. All extracts possessed free radical scavenging activity but the maximum was found in methanol extract. This could be attributes to the presence of high phenol content in this extract. Methanol also induced proliferation in case of stimulated PBMCs which may be due to the presence of some compounds. The experimental data thereby support the use of *Cinnamomum zeylanicum*in traditional medicines.

CHAPTER 1: INTRODUCTION

Traditional/Herbal medicines are plant derived products which include crude plant extracts such as fruit, flower, bark, materials such as essential oils, gums, resins or the herbal preparations done through fractionation, extraction, concentration, etc (WHO, 1997).

The potency of many herbs like Garlic, turmeric, Ginger etc have been proved to be effective against a variety of diseases such as cancer, allergies, diabetes etc. They are recently under investigation and have become a major part of the Complementary and Alternative medicines (WHO, 2015). Many spices have curative and disease prevention properties apart from the nutritional benefits. Different spices are used as home remedies in common illness such as cold, cough, fever, influenza, bruises, wounds.

Cinnamon is a common spice which is used around the world for several centuries. It belongs to the family Lauraceae. The most common varieties are:

1) *Cinnamomumzeylanicum*
2) *Cinnamomum cassia*
3) *Cinnamomumverum*
4) Cinnamomumburmannii

The bark of Cinnamon has been widely used as a component of herbal medicine for treating common cold, cardiovascular diseases, and chronic gastrointestinal and gynecological disorders (NCCIH, 2011).Cinnamon bark has been extensively studied and its essential oil and water based extracts have been seen to have possessed pharmacological properties- anti-bacterial (Brindha*et al.*, 2014), anti-inflammatory (Hong *et al.*, 2012) and anti cancer(Kwon *et al.*, 2009). It is used in manufacture of toothpaste, mouthwash, lotion, pharmaceuticals, cosmetics, Chocolates, coffee, candies, tea, alcoholic beverages, and stimulating appetite.

The species *Cinnamomumzeylanicum*originates from Ceylon, being also native to South-East India, are a source of cinnamon bark and leaf and their essential oils. Its sensorial qualities are flavor, slightly sweet, pleasant, warm and bitter,

besides being strongly aromatic (WHO, 1999). Cinnamon provides various kinds of oils, it has been established that the oil extracts from cinnamon possess a distinct antioxidant activity, which is especially attributed to the presence of phenolic and polyphenolic substances (Schmidt E, 2006).

The essential oil from the bark of Cinnamon is found to be a rich monoterpenerich natural source with transcinnamaldehyde (Wong *et al.,* 2014*).* These canbe isolated using Soxhlet operation with the correct choice of solvent and then dried using rotary evaporator.

The GC-MS analysis of the bark extracts of Cinnamon showed to have possessed over 38 compounds (Y.-q. Li *et al*.,2013),Cinnamaldehyde, Eugenol, Linalool, Camphor and Coumarin in major quantities:

- Cinnamaldehyde is the pale yellow, viscous organic compound present in Cinnamon in major quantities and gives cinnamon its flavor and odor. It possesses antibacterial (Gill *et al*., 2004), antiviral (Hayashi *et al.,*2007) and antifungal (Cheng *et al.,* 2008) properties.
- Eugenol, a colorless to pale yellow liquid is used mostly in perfume industries and as local anesthetic and antiseptic. It also possesses antibacterial properties (Gill *et al*., 2004) and antifungal (Cheng *et al.,* 2008) properties.
- Linalool, a terpene alcohol, is majorly used in the perfume industry. It is a suppressant for *Candida albicans, Escherichia coli*, and *Staphylococcus aureus.*

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). There are more than 100 types of cancer. 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*).*Hence, a search for

new plant based, risk free, superior compound with novel cytotoxic activity is the need of the hour.

Reactive oxygen species (ROS) plays a crucial role in the development of ailments like asthma, dementia, and carcinoma (Halliwell*et al.*,1990). Free radicals produced in the body may react with biological molecules- lipids, proteins which eventually results in the imbalance between oxidants and anti-oxidants. Spices have been found to possess phenolic compounds which help the cells against oxidant damage caused by free radicals (Kahkonen*et al.*, 1999).

Ayurveda registers over 20,000 medicinal plants out of which only 7,000 plants have been assessed. Many spices have curative and disease prevention properties apart from the nutritional benefits. Different spices are used as home remedies in common illness such as cold, cough, fever, influenza, bruises, wounds.*Cinnamomumzeylanicum* has many medicinal properties and has been found to be anti-allergic, anti-pyretic and anti-ulcerogenic (Varalakshami*et al.*,2014).

Although the efficacy of *Cinnamomumzeylanicum* is well established in traditional medicines but not much scientific data is available to support its bioactive role. Present study explores the cytotoxic effects of*Cinnamomumzeylanicum*bark extracts on cancer cell lines HeLa and RAW 264.7 and evaluation of their free radical scavenging activity.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Traditional Medicine

Traditional medicine (TM) is well described as a result of different practices, knowledge and beliefs, approaches used to cure or prevent an illness. The therapies may include plant, animals, manual techniques, and spiritual therapies etc which are generally native to a particular area or region (WHO, 2003). Many countries in Asia, Africa and Latin America have been using the traditional herbal preparations for primary health issues. In developed nations, these preparations are often referred as "Complementary and Alternative medicines (CAM) or Non-Conventional medicines. It refers to a broad set of practices which are not a part of a country's tradition and not a dominant practice as well (WHO, 2015).

TM is being used effectively in many countries:

- Herbal Preparations are used around 30-50% of the total medicinal consumption in China and are referred as Traditional Chinese Medicines (NCCIH, 2013).
- More traditional medicines are being added in Malaysia, milk thistle and dandelion were used on a large scale in 2014 as an ingredient for liver tonics(Euromonitor International, 2015).
- In countries like Ghana and Nigeria, 60% people are treated with the use of traditional medicine at home from the fever resulting from malaria.
- Because of the natural origin and negligible side effects, TMs registered positive growth value in Germany, 2014(Euromonitor International, 2015)..
- The global market in US for traditional medicine rose to US $ 593 million in 2014 (Euromonitor International, 2015). Also the demand for analgesics and herbal teas showed a growth of 5% and 10% respectively.
- 75% people living with HIV/AIDS rely on Traditional medicines in London and South Africa (WHO, 2015).

- The Indian sub-continent is a vast repository of medicinal plants, where more than 1.5 million practitioners are using traditional medicine system for healthcare in India(Pandey*et al.*, 2008).

2.2 Herbal Preparations

Herbs are plants known for their scent, flavor or therapeutics effects. Herbal preparations have many advantages over modern medicines, as described in Table 1. For herbal/traditional preparations different parts of plants such as root, flower, fruit, seed, bark etc are used. Gums, resins, dry powders, juices and essential oils extracted from herbs also constitute TMs. These can be obtained by processes such as extraction, purification, concentration, fractionation, heating or soaking etc. Mostly the herbs are administered in the form of fresh juice or powder. Some examples of herbs possessing medicinal properties are- Garlic, Ginger, Tulsi, etc, as mentioned in Table 2.

Table 1: Comparison of herbal preparations and Modern Medicines (Vickers *et al.*, 2001)

Factors	**Modern Medicines**	**Herbal/Traditional Products**
Side-effects	More	Low/negligible
Composition	One or two compounds	Mixture of compounds
Active ingredient	Identified	Uncertain/Not known
Safety/efficacy	New; not used in humans before	Used in humans since ancient times
Clinical/Scientific Data	Available	Usually not available
Knowledge protection	Open access	Closed; patent protected
Quality Control	Relatively easy	Complicated
Cost	Expensive	Inexpensive

Table 2: Some examples of Herbal drugs and their properties(NCCIH, 2011)

Herb		Properties
Allium sativum (Garlic)		Anti-microbial/ anti-cancer
Aloe vera		Anti-inflammatory
Azadirachtaindica (Neem)		Anti-malarial, anti-bacterial
Ocimum sanctum (Tulsi)		Anti-bacterial, anti-fungal, anti-tumor, antiviral
Zingiberofficinale (Ginger)		Relieves cold and cough
Papaver rhoeas (Poppy)		Soothes asthma, bronchitis
Lavundalaangustifolia (Lavender)		Antiseptic
Eucalyptus globules (Eucalyptus)		Analgesic, cold and cough

2.3 Traditional Medicines/Herbs in India

India is well known for the traditional medicinal systems- Ayurveda, Siddha and Unani which are very well documented in the ancient scriptures. The word Ayurveda is derived from "Ayur" meaning longevity and "Veda" meaning science or knowledge (NIH, 2006). Ayurveda originated in the hymns contained in "Atharvaveda" and is one of the oldest organized systems of medicine. Due to the growing awareness in scientific community Ayurveda, Siddha and Unani have entered the mainstream. In Western countries, 40% of the population is now using TMs (Euromonitor, 2015).

India has a vast repository and hence the largest producer of medicinal plants used in Traditional medicines. 70% of the rural population in India depends on the use of these plants to deal with illness. Ayurveda registers over 20,000 medicinal plants out of which only 7,000 plants have been assessed.More than 7800 units are involved in the manufacturing of plant based formulations (Pandey*et al.*, 2008).

Many Indian spices have curative and disease prevention properties apart from the nutritional benefits. Different spices are used as home remedies in common illness such as cold, cough, fever, influenza, bruises, wounds, diabetes (Modak*et al.*, 2007).

2.3.1 Spices and their free radical scavenging activity

Spices generally show medicinal properties and have been playing a critical role in the health care of ancient and modern cultures (WHO, 1993). Ayurveda mainly uses plant based drugs to treat ailments as they contain components of therapeutic value and possess low risks of side effects, are relatively cheaper and non-toxic as compared to modern medicine (Agbor*et al*., 2005). Many spices contain phenolic compounds which protects the cell against Reactive Oxygen species (ROS).

ROS such as hydroxyl radical, hydrogen peroxide and superoxide anion often lead to diseases like arthritis, dementia, asthma, and carcinoma. These free radicals are often produced in the body through aerobic respiration or exogenous sources (Halliwell*et al.,* 1990). They, in turn, react with lipids and

proteins inside body and lead to an imbalance between oxidants and antioxidants.

Spices are seen to have possessed phenolic compounds which help in protecting the cells from the damage caused by free-radicals and thus, are known as Radical scavengers. These phenolic compounds help in terminating the action of free radicals thereby protecting the body from various ailments (Kahkonen*et al.,* 1999).

2.3.2 Spices and 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 is 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.

Natural plant derived agents are being used for the treatment of cancer as they possess lower side effects risks. The spices have been consumes since past many centuries primarily because of their taste and aroma. Recent studies have proved their biological activities and efficacy. Many plant based phytochemicals have been identified to have possessedanti cancer and therapeutic properties, as mentioned in Table 3.

Table 3: Major Spices and their active Phytochemicals (Aggarwal*et al.*,2008)

Spice		Active Phytochemical
Foeniculumvulgare (Fennel)		Anethole
Zingiberofficinale (Ginger)		Gingerol
Curcuma longa (Turmeric)		Curcumin
Cuminumcyminum (Cumin)		Thymoquinone
Crocus sativus (Saffron)		Crocetin
Rosemarinusofficinalis (Rosemary)		Ursolic acid
Origanumvulgare (Oregano)		Quercetin
Piper nigrum (Black pepper)		Piperine

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 (*Elettariacardamomum*) and black pepper (*Piper nigrum*) have been studied for their immunomodulatory activity by Majdalawieh and Carr (2010). Intake of spices is one of the most effective, convenient and economical ways of fortifying oneself against infectious diseases and related cancers (Steinmetz and Potter, 1996).

2.4 Introduction to *Cinnamomumzeylanicum*

Cinnamomumzeylancium(Figure 1) is the oldest herbal medicine known and is even mentioned in the Chinese literature since 4000 years ago. *C.zeylanicum* belongs to the family Lauraceae and its bark and leaves are widely used as spice and flavoring agents. It is also used for medicinal purposes due to its unique properties.

Figure 1: Bark of *Cinnamomumzeylanicum* and its powdered form (Peras Conference, 2014)

Around 300 volatiles have been reported in the essential oil of *C. zeylanicum*. The essential oil is rich in trans-cinnamaldehyde which is a pale yellow, viscous organic compound present in major quantities and gives cinnamon its flavor and odor. Eugenol and Linalool are also found in large amounts in the essential oil and are found to possess antibacterial, antiviral and antifungal properties.

2.5 Biological Activities of *Cinnamomumzeylanicum*

2.5.1 Some reported activities of *C. zeylanicum*

- Antibacterial (Brindha*et al.*, 2014)
- Anti-inflammatory activity (Hong *et al.*, 2012)
- Anti-tumor (Kwon *et al.*, 2009)
- Anti-fungal (Brindha*et al.*, 2014).
- Immunostimulant (Niphade*et al*., 2009)
- Immunosuppressant (Ravindran*et al*., 2004)

2.5.2 Immumodulatory properties of *C.zeylanicum*

Spices have been consumes since past many centuries primarily because of their taste and aroma. Recent studies have proved their biological activities and efficacy.

C.*zeylanicum*was found to enhance cellular immunity as reported by Balekar*et al* (2014). Mice were treated with Sheep Red Blood Cells (SRBCs) as antigen and different concentrations of C.*zeylanicum*aqueous extract (10, 25, 50 mg/kg) were fed to them. Concentrations 25 and 50 mg/kg showed dose dependent increase in primary and secondary antibodies

In vivo studies conducted on mice induced with melanoma have shown that aqueous extract of C.*zeylanicum*potentiates CD8+ T cell activity(Kwon *et al.,* 2009). Also, $CD8^{+}$ T cells were found to express IFN-γ and TNF-α and Granzymes.

In vivo studies in mice treated with Polyphenolic extract of *C.zeylanicum*(25 or 50 mg/kg) showed that the mean number of peritoneal macrophages was found to increase significantly (Balekar*et al*., 2014).

Cytokine analysis was done using RAW 264.7 cells and human PBMCs were incubated with cinnamaldehyde (0–10 μg/ml) in the absence or presence of LPS. Cell free supernatants were taken and checked for IL10 and TNF-α using ELISA. Concentrations higher than 1 μg/ml inhibited cytokine secretion of immunosuppressive cytokines like IL10 as well as of inflammatory cytokines like TNF-α (Kim *et al*., 2010).

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) categorized bioactive 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, as shown in Table 4.

Table 4: Solvents according to their polarity (Poacher,2004)

Relative Polarity	Compound Formula	Group	Representative Solvent Compounds
Nonpolar	H - H	Alkanes	Petroleum ethers, ligroin, hexanes
	Ar - H	Aromatics	Toluene, benzene
	R - O - R	Ethers	Diethyl ether
	R - X	Alkyl halides	Tetrachloromethane, chloroform
	R - COOR	Esters	Ethyl acetate
	R - CO - R	Aldehydes and ketones	Acetone, methyl ethyl ketone
	$R - NH_2$	Amines	Pyridine, triethylamine
	R - OH	Alcohols	Methanol, ethanol, isopropanol, butanol
	$R - COHN_2$	Amides	Dimethylformamide
	R - COOH	Carboxylic acids	Ethanoic acid
Polar	H - OH	Water	Water

Increasing Polarity

2.7 Cell Viability and Cytotoxic 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, as shown in Figure 2

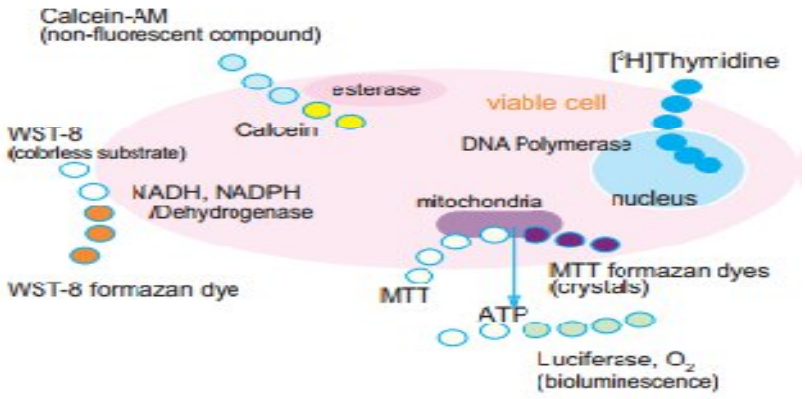

Figure2: Reagents for cell viability detection (dojindo, 2007)

Tritium-Labeled Thymidine Uptake method assay and ^{51}Cr 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.,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

RAW 264.7 and HeLa were used for the cytotoxic studies.

2.8.1 Characteristics of RAW 264.7 cells

RAW 264.7 is an adherent macrophage; Abelson murine leukemia virus transformed cell line (Figure 3). It was isolated in 1978 from the tissue-Abelson murine leukemia virus induced tumor, ascites of an adult male mouse (Raschke, 1978).

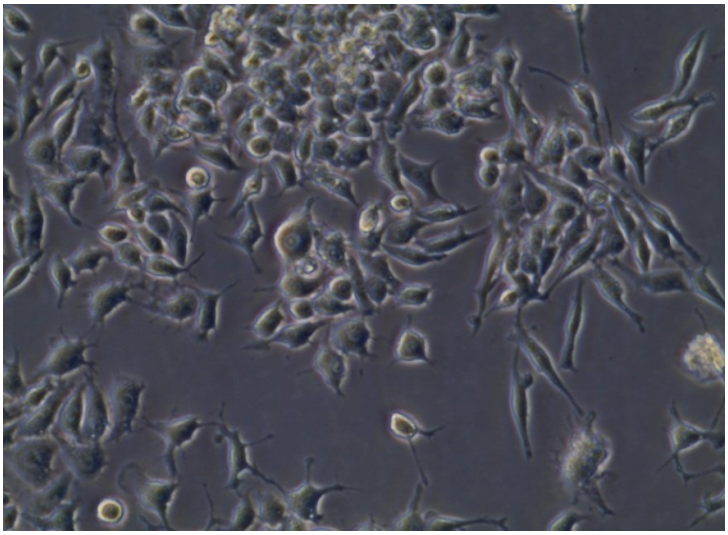

Figure 3: RAW 264.7 cells under light microscope

RAW 264.7 show receptor expression of Complement C_3. It has a doubling time of 12 hours.

2.8.2 Characteristics of HeLa cells

HeLa is the oldest, immortal and most widely used cell lines in research (Figure 4). 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.

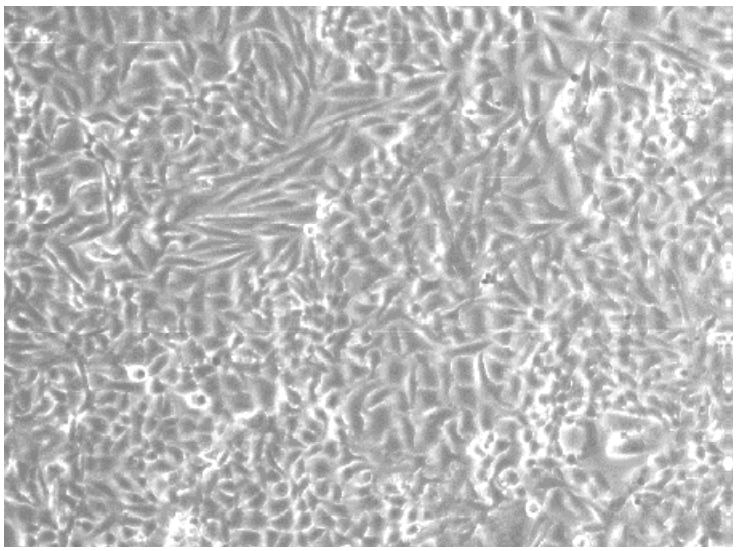

Figure 4: HeLa cells under light microscope

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 grow as an adherent culture with a doubling time of almost 24 hours.

CHAPTER 3: OBJECTIVES

1) Preparation of *Cinnamomumzeylanicum* extracts in different solvents of increasing polarity index and detection of the presence of phenolic content in these extracts.
2) Screening of the solvent extracts for the assessment ofcytotoxic effects on cancer cell lines HeLa and RAW 264.7.
3) Detection of free radical scavenging activity in the solvent extracts of *Cinnamomumzeylanicum*.

CHAPTER 4: MATERIALS AND METHODS

This chapter deals with the materials, methodology and design of experiments undertaken to achieve the objectives discussed in Chapter 3.

4.1 Materials

List of chemicals and reagents required during the project:

Chemicals/Reagents	**Company**
DMEM	Himedia
FBS	Gibco®Life technologies
Streptomycin	Himedia
Penicillin G	Himedia
Amphotericin	Himedia
Tylosin	Sigma-Aldrich
Trypsin	Himedia
Tamoxifen	Sigma-Aldrich
Histopaque 1077	Sigma-Aldrich
Sodium bicarbonate	Himedia
L-Glutamine	Himedia
DMSO	SRL
MTT	Sigma-Aldrich
Trypan Blue	Himedia
Hexane	EMPARTA® Merck
Dichloromethane	EMPARTA® Merck
Chloroform	EMPARTA® Merck
Methanol	EMPARTA® Merck
Paclitaxel	Sigma-Aldrich
Linalool	Sigma-Aldrich
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich
Acetonitrile	EMPARTA® Merck
Concanavalin A	Sigma-Aldrich
Liquid Nitrogen	

4.2 Preparation of *Cinnamomumzeylanicum* extracts

The bark of *Cinnamomumzeylanicum* was taken and made free of impurities by washing with distilled water. It was then dried at 37°C for two days. The dried bark was transferred to a clean, dry porcelain pestle and mortar and was grinded in the presence of liquid nitrogen to fine powder. Powdered bark was subjected to Soxhlet extraction in four different solvents (Hexane, Dichloromethane, Chloroform and Methanol) based on increasing polarity (Table 5) and further removal of solvents was done under reduced pressure using rotary evaporator. The concentrations of the extracts were calculated as:

Concentration of extract (in mg/ml) = (Wet wt. of flask-dry wt. of flask) / Dry weight of flask x __ ml of DMSO

Table 5: Solvents and their polarity index

Solvent	Polarity
Hexane	0.1
Dichloromethane	3.1
Chloroform	4.1
Methanol	5.1

Soxhlet Extraction: Dried and powdered sample of *C.zeylanicum* was weighed and transferred into the extraction thimble and the thimble was placed 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. The whole assembly was placed on heating mantle. Condenser was connected to inlet and outlet pipes for the continuous supply of water, as shown in Figure 5. Temperature of the mantle was adjusted according to the boiling point of solvent (10°C below the boiling point). Twenty cycles of extractions were carried out at a fixed temperature for a particular solvent.

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.

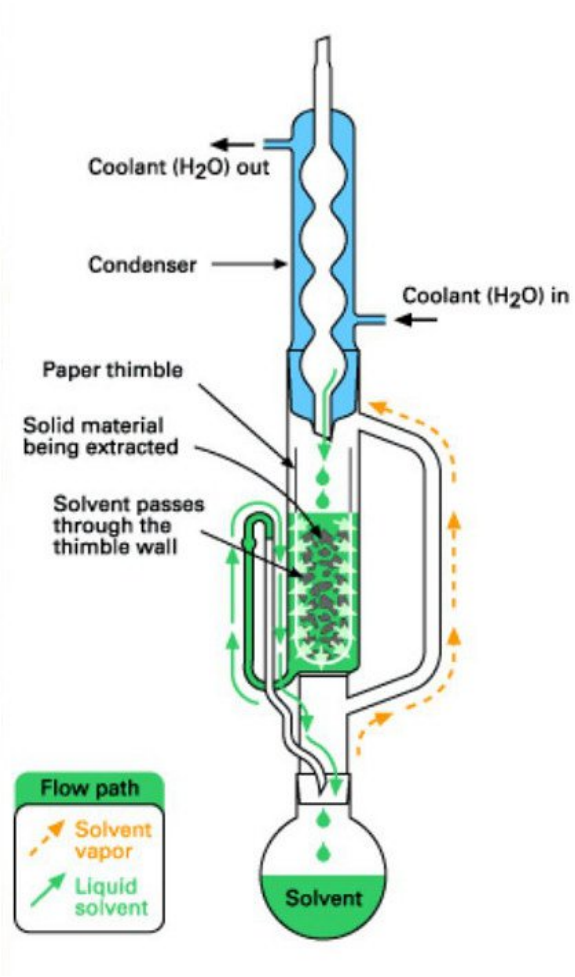

Figure 5:Soxhlet extractor showing flow of solvent during extraction(Nonneon,2013)

Rotary evaporation: A rotary evaporator is a device, as shown in Figure 6, used in chemical laboratories for the efficient removal of solvents from samples by evaporation under reduced pressure.

The main components of a rotary evaporator are:

1. Motor unit- rotates the evaporation flask or vial containing the user's sample.
2.Vapor duct- the axis for sample rotation and is a vacuum-tight conduit for the vapor being drawn off of the sample.
3. Vacuum system- substantially reduces the pressure within the evaporator system.
4. Heated fluid bath (generally water) - heats the sample.
5. Condenser- It is attached with a coil to pass coolant.
6. Condensate collecting flask- It is present at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
7. 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.

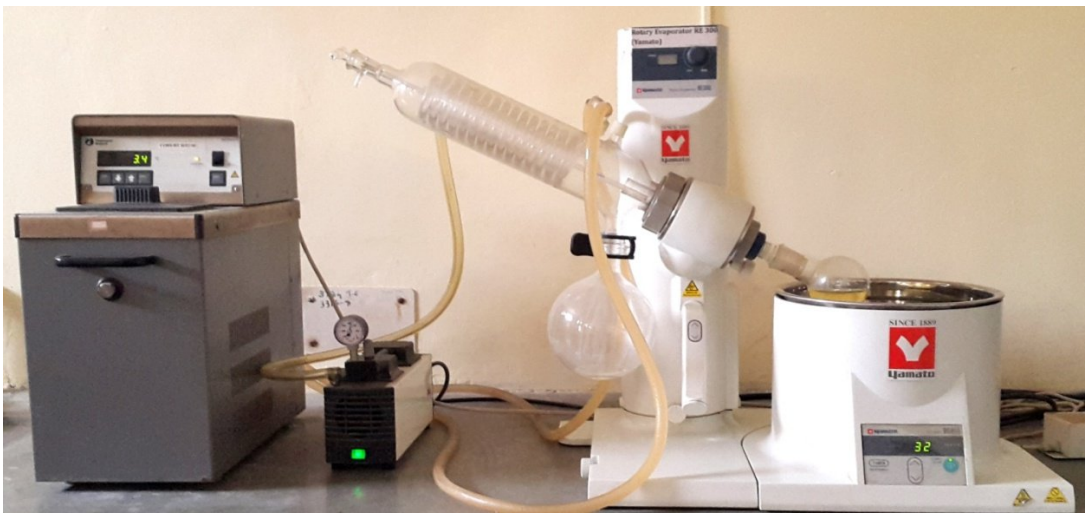

Figure 6: Rotary evaporator during operation

4.2 Maintenance and handling of cell lines

4.2.1 Preparation of media

Dulbecco's Modified Eagle's Medium (DMEM): 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 non-essential amino acids). We have used AT065A DMEM which has low glucose and is modified for autoclaving.

Media was prepared as per manufacturer instructions. Briefly, 9.6 gm 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℃ 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 0.22µm filter membrane under vacuum. Sterile media was stored at 2-8°C and in dark till use.

4.2.2 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 using double distilled water. PBS was autoclaved for 20 minutes at 121°C. After autoclaving, PBS was filter sterilized and stored at 4°C.

4.2.3 Revival of cell lines

Vial containing frozen cell line (Stored at -80°C) was thawed rapidly by agitating gently in water bath at 37°C ± 1°C till the complete melting of ice. Immediatelycomplete growthmedium (DMEM containing 10%FBS and antibiotics) was added twice its volume aseptically. The vial was centrifuged

at 1000 rpm for 10 minutes at room temperature, 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, labeled 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.

4.2.4 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°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 4.2.5. 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°C (5%). Flasks were monitored, microscopically and with naked eye to check the cell growth, morphology, pH change and contamination.

4.2.5 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 (Figure 7). 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 3x3 mm grid of 9 counting squares. The chambers have raised sides that will hold a coverslip exactly 0.1mm above the chamber floor. Each of the 9 counting squares hold volume of 0.0001 ml. Hemocytometer and cover slip were 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 cells are viewed at 40X magnification. Cells were counted in each section A, B, C and D (Figure 7).

Average the number of cell and multiply by the dilution factor.

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

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

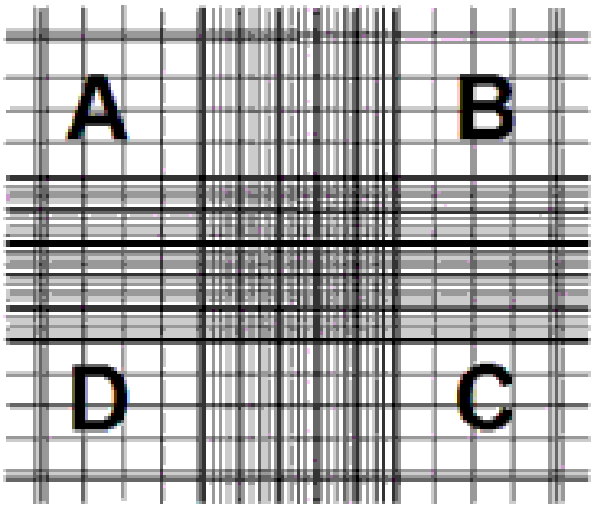

Figure 7: Hemocytometer

4.3 Assessment of phenolic Content

A number of spectrophotometric methods have been developed for the estimation of phenols in the plant extracts. This was determined using Folin-Ciocalteu assay method given by Chang *et al* (2002). The reaction forms a blue chromophore constituted by a phosphotungstic-phophomolybdenum complex, where the maximum absorption of the chromophore depends on the concentration of phenolic compounds (Schofield, 2001).

50 µl of solvent extracts (1mg/ml) and standard solution of gallic acid (6.25, 12.5, 25, 50, and 100) µg/ml prepared in distilled water were added in 96 well plate (Chang *et al*., 2002). 50 µl of distilled water was then added to the plate. Distilled water was used as blank. 50µl of 1M sodium carbonate solution and 50µl of 10% Folin-Ciocalteu's phenol reagent were added to the mixture in a 96 well plate. The plate was incubated in dark for 60 minutes. Absorbance was measured at 750nm with the help of ELISA plate reader. Higher absorbance represented higher phenolic content in the extracts.

4.4 Cytotoxicity assay

4.4.1 Effect of extract on cancer cell lines

Cell Lines such as RAW 264.7 (mouse leukemic monocyte macrophage cell line), Hela (human epitheloid cervix carcinoma) were used to study the effects of extracts on cancer.The cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Cell lines RAW 264.7 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/mlamphotericin and 1ml/ltylosin. 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 × 104 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 (Figure 8 and 9) 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 and Tamoxifen were used 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:

$$\text{Inhibition \%} = \frac{OD_{Control} - OD_{test}}{OD_{Control}} \times 100$$

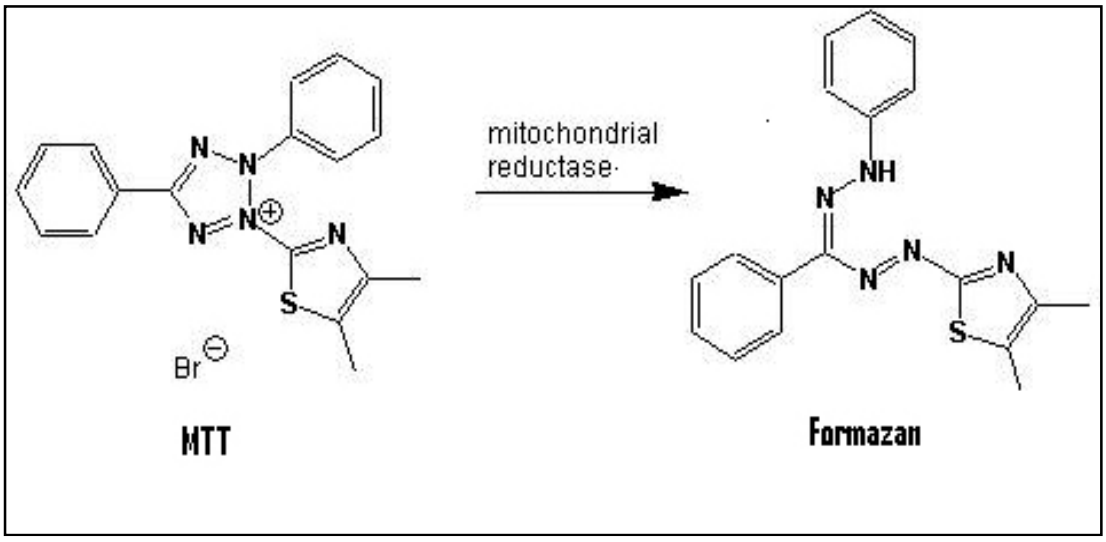

Figure 8: The formation of reduced product formazan (Farahnaz *et al.*, 2014)

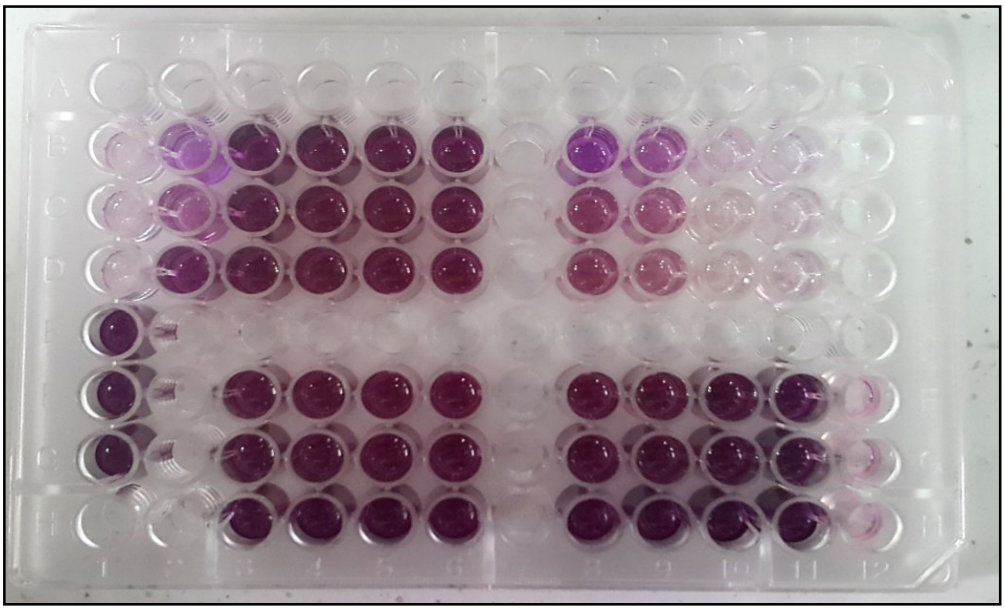

Figure 9: Development of formazan crystals in 96 well plate

4.5 Free radical scavenging activity (Antioxidant Assay)

Free radicals are produced in the biological systems and are found to cause disorders like carcinogenesis, mutagenesis and ageing (Singh and Singh, 2008). Antioxidants are those compounds which have been found to intervene the free radical mediated oxidative process (Cui *et al*, 2004). In order to know the antioxidant capacity of the crude cinnamon extracts, a well known antioxidant assay known as the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed, Figure 10.

When DPPH is mixed with a substance that can donate a hydrogen atom (free radical) then this gives rise to a reduced form (Blois, 1958) and violet color is reduced to pale yellow giving rise to a non radical form, 2,2-diphenyl-1-picrylhydrazyl is reduced to 2,2-diphenyl-1-picrylhydrazine (yellow, non radical).

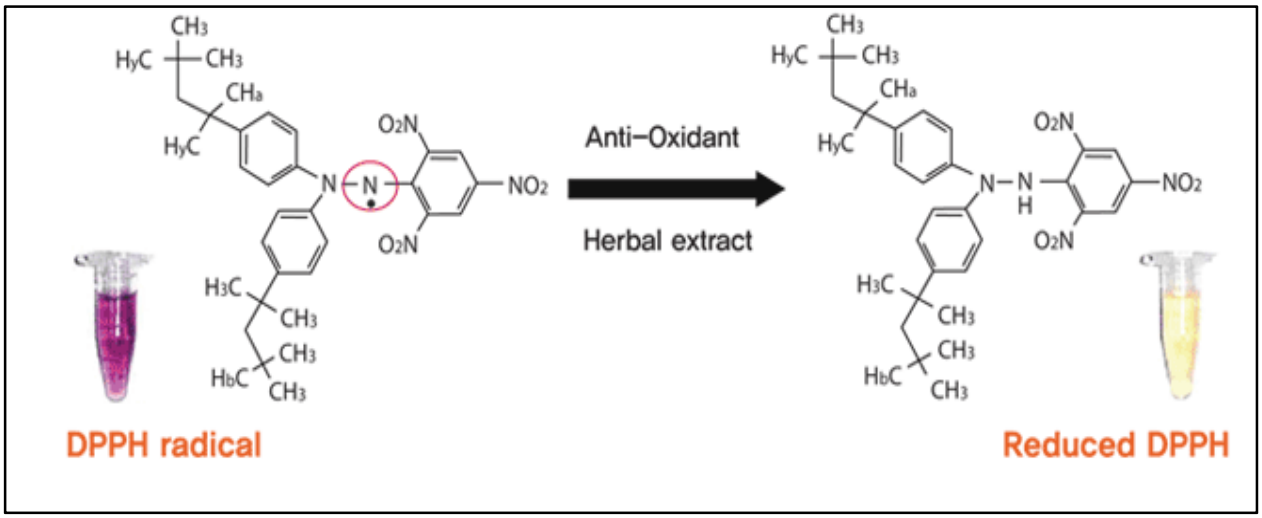

Figure 10: Principle of Antioxidant assay when performed on herbal extracts (Damo.co., 2010)

5 mg/ml of all the solvent extracts were prepares as stock solutions. They were diluted appropriately in methanol to make a final concentration of 250µg/ml and added in 96 well plate. Ascorbic acid (100µM) in distilled water was taken as standard. 150µl of DPPH (100µM) was prepared in methanol and then added in a to the extracts. Appropriate blanks, distilled water, methanol, methanol+DPPH were also run simultaneously with the extracts. Plate was incubated in dark for 45 minutes, after which the absorbance was measured at 517nm in Elisa plate reader.

Scavenging activity was expressed as:

$$\% \text{ Radical Scavenging Activity} = \frac{\text{Control}_{abs} - \text{Sample}_{abs}}{\text{Control}_{abs}} \times 100$$

4.6 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.

4.6.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and seeding

PBMC isolation was carried out using Histopaque-1077 as per manufacturer's instruction. To a 15 ml conical centrifuge tube, 3 mlof Histopaque-1077(room temperature) was added. 3ml whole 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, the top plasma layer(yellow colored) was carefully removed with a pipette and opaque interface was collected containing mononuclear cells (PBMCs) in clean conical centrifuge tube. The cells were 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, cell pellet was resuspended in 1 ml of cell culture medium. Cells were counted using hemocytometer as discussed in section 4.2.5. PBMCs were seeded at a density of $1x10^5$cells per well and solvent extracts were added in varying concentrations. After 2 hours, a mitogen, Concanavalin A (10 μg/ml) was added and then the plate was incubated for 72 hours in CO_2 incubator. MTT assay was performed as discussed earlier.

4.7 High- Performance Liquid Chromatographic (HPLC) Analysis for the detection of Linalool

Linalool is a monoterpene alcohol which occurs naturally in many aromatic plants and is responsible for flavor and fragrance (Bahl, 2002). Recent studies showed that Linalool has anti microbial, anti inflammatory and anti oxidant activities. According to the literature, Linalool is an oily compound and remains as a neutral molecule during separation. Thus, acetonitrile and deionized water were used as the mobile phase in a ratio 55:45 (v/v). The column temperature was set as 25°C and the detection wavelength was taken as 210nm. Working concentration of Linalool was taken as 100μg/ml.

Injection volume for the sample was taken as 20 µl. Chromatograms were analyzed to detect the presence of Linalool in the methanolic extract of *C. zeylanicum.*

CHAPTER 5: RESULTS AND DISCUSSIONS

This chapter will discuss the results of experiments proposed in the methodology (Chapter 4). The results will first focus on the yield of *Cinnamomumzeylanicum*solvent extracts (Hexane, Dichloromethane, Chloroform and Methanol) and presence of phenolic content in these extracts. Cytotoxic effects shown by different concentrations of *Cinnamomumzeylanicum* extracts on HeLa and RAW 264.7 were assessed by MTT assay followed by the evaluation of free radical scavenging activity of these extracts using DPPH. Effect of the solvent extracts on stimulated PBMCs and the results of HPLC analysis are discussed in the subsequent sections.

5.1Yield of *Cinnamomumzeylanicum* solvent extracts(Hexane, Dichloromethane, Chloroform and Methanol)and assessment of their phenolic content

5.1.1 Yield of *Cinnamomumzeylanicum* solvent extracts

Cinnamomumzeylanicum extracts were prepared in soxhlet apparatus using four different solvents, i.e., Hexane, Dichloromethane, Chloroform and Methanol respectively, according to increasing polarity. Twenty grams of powdered *C.zeylanicum*was taken and subjected to soxhlet extraction in four different solvents of varying polarity. Each solvent was run for 18-20 cycles. After extraction, respective solvents were removed under reduced pressure by Rotary evaporator to get final yield as mentioned in Table 6. The dried extract was reconstituted in 40% DMSO.

Table 6: Different extracts of *C.zeylanicum* and their yield

Solvent	Extraction temperature (°C)	Yield (mg/ml)
Hexane	58	35
Dichloromethane	29	45
Chloroform	51	40
Methanol	54	100

5.1.2 Assessment of Phenolic Contentin *Cinnamomumzeylanicum*extracts

Calorimetric assays are widely used for the UV spectrophotometric analysis as they are rapid and easy to perform. Polyphenols in plant extracts react with specific redox reagents such as Folin-Ciocalteau (FC) Reagent to form a blue colored complex which can be visualized under visible light spectrophotometry. The reaction forms a blue chromophore constituted by a phosphotungstic-phophomolybdenum complex, where the maximum absorption of the chromophore depends on the concentration of phenolic compounds (Schofield, 2001).

250 µg/ml of each of the solvent extracts was assessed for phenolic contentpresenceusing FC assay where gallic acid was used as a standard. Graph was plotted between the concentrations of solvent extracts versus the absorbance (750 nm). Higher value of absorbancdepicts higher phenolic content. Methanolic extract showed the highest absorbance value, hence, highest phenolic content followed by DCM and then Chloroform. Hexane showed the least absorbance, therefore, least phenolic content (Figure 11).

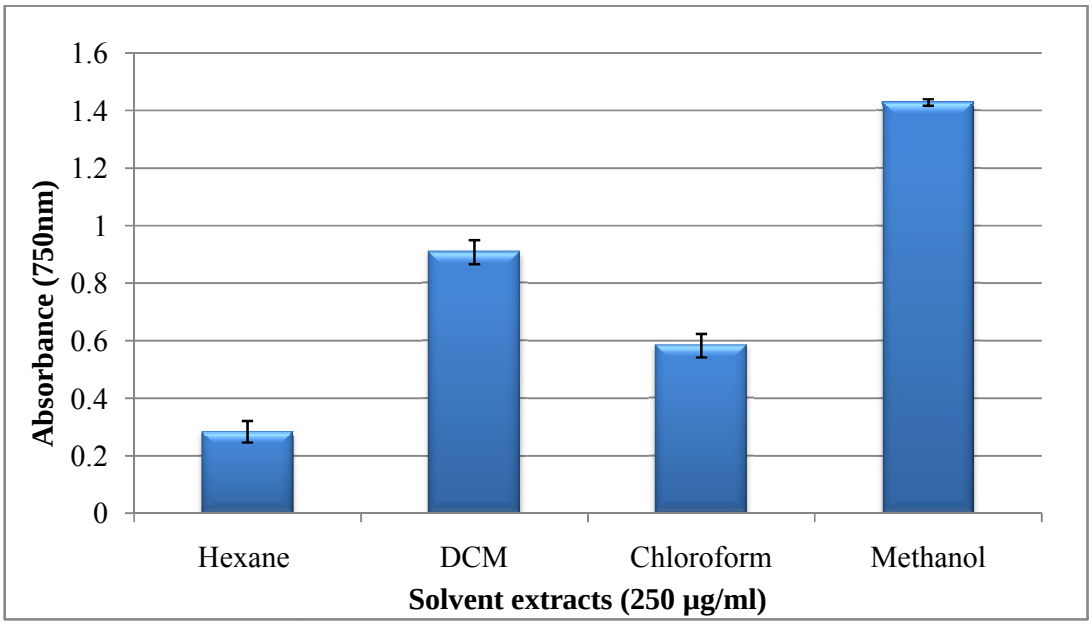

Figure 11: Total phenolic content of solvent extracts(250µg/ml) vs. Absorbance (750nm)

5.2 Effect of *Cinnamomumzeylanicum*extracts on the proliferation of HeLa cells

The effect of all four extracts on the proliferation of HeLa was assessed using MTT assay. A wide range of concentrations were used and percentage inhibition was calculated for each extract.

5.2.1 Effect of different concentrations of*Cinnamomumzeylanicum*extracts on HeLa cells

Each extract was diluted with complete DMEM appropriately so as to bring the final concentration of the extracts to 1mg/ml. The final DMSO concentration in these extracts was 5%. Control solvent was made accordingly using DMEM and DMSO. Different concentrations (50, 100, 150, 200, 250, 300, and 350) µg/ml of all extracts (Hexane, DCM, Chloroform, and Methanol) was added in the culture and incubated for 48 hours at 37°C and humidified 5% CO_2. MTT assay was performed to evaluate the effect of these extracts on HeLa cells. Paclitaxel was used as the positive control.

Variable effects on proliferation of HeLa were observed for different extracts. Hexane extracts showed upto 97% inhibition between concentrations 100-250 µg/ml, DCM extracts showed rise in inhibition with an increase in concentration of the extract and upto 96% inhibition between concentrations 300-350 µg/ml. A maximum inhibition of 47% was observed in Chloroform extract in concentration 250 µg/ml after which proliferation was observed. Methanol extracts showed variable responses. It exhibited an increase in inhibition with the rise in concentration and its maximum activity was observed at 200 µg/ml. Further rise in concentration depicted a proliferative activity in the methanol extracts. 28% proliferation was observed in 350 µg/ml extract. The results are discussed through table 7 and figures 12-16.

Table 7: Assessment of cytotoxic effects indifferent concentrations of all solvent extracts

Concentration (µg/ml)	Inhibition %			
	Hexane	DCM	Chloroform	Methanol
50	78.59	40.14	16.58	4.51
100	96.53	61.36	5.71	11.75
150	97.48	74.62	16.55	34.44
200	97.54	79.73	25.19	47.53
250	96.98	91.92	47.54	-15.98
300	93.32	96.47	-0.71	-16.38
350	94.39	96.70	3.42	-28.62

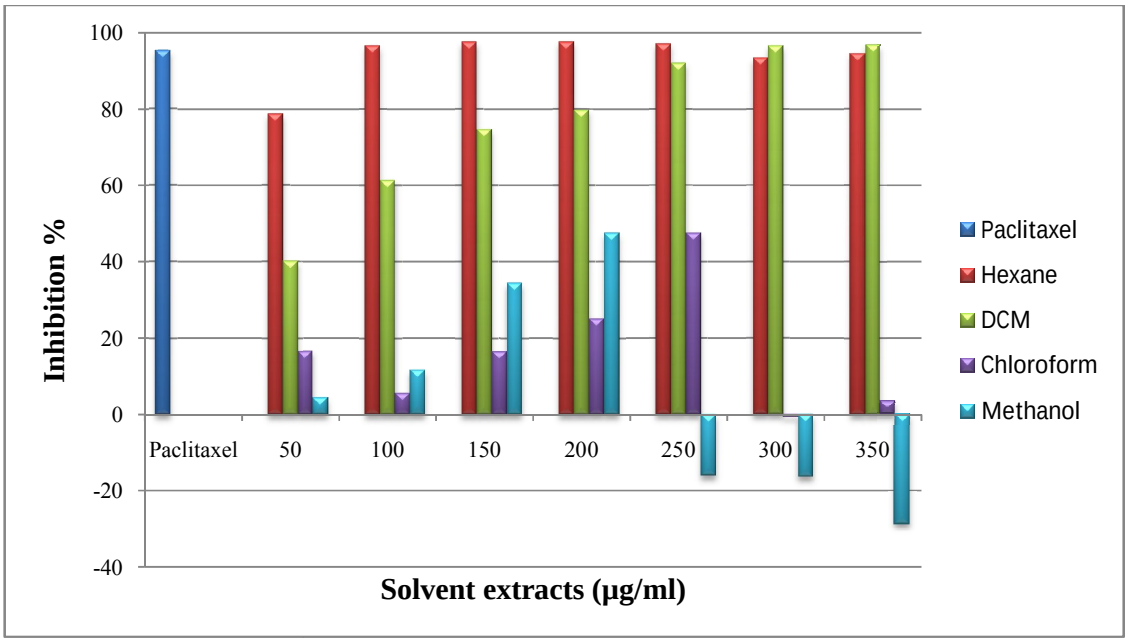

Figure 12: % Inhibition of proliferation in HeLa cells using different concentrations of solvent extracts.

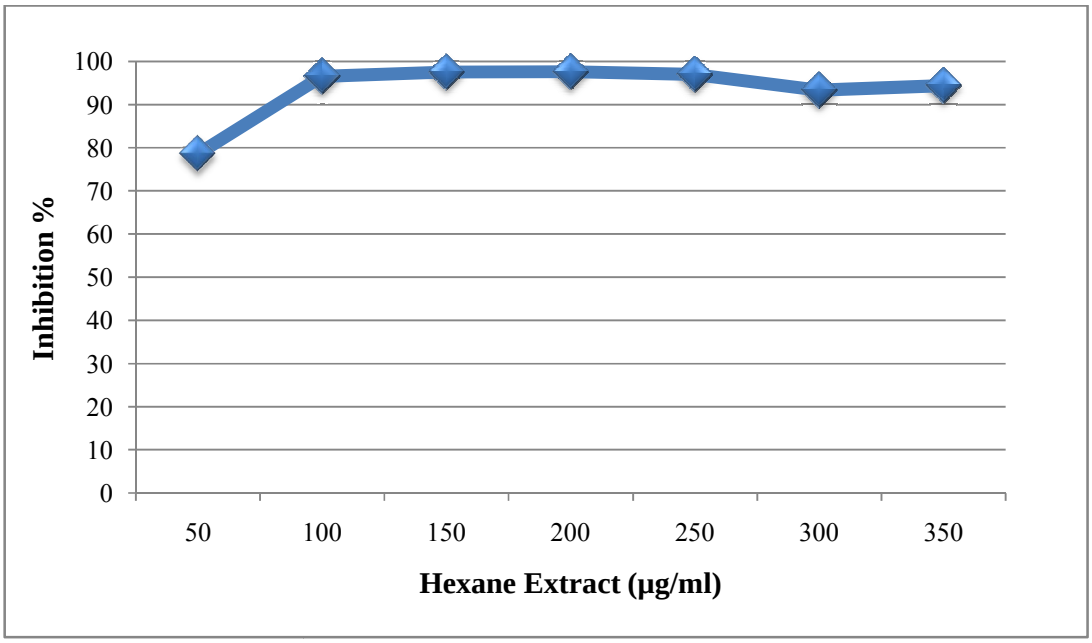

Figure 13: Effect of increasing concentrations of Hexane extract on HeLa cells

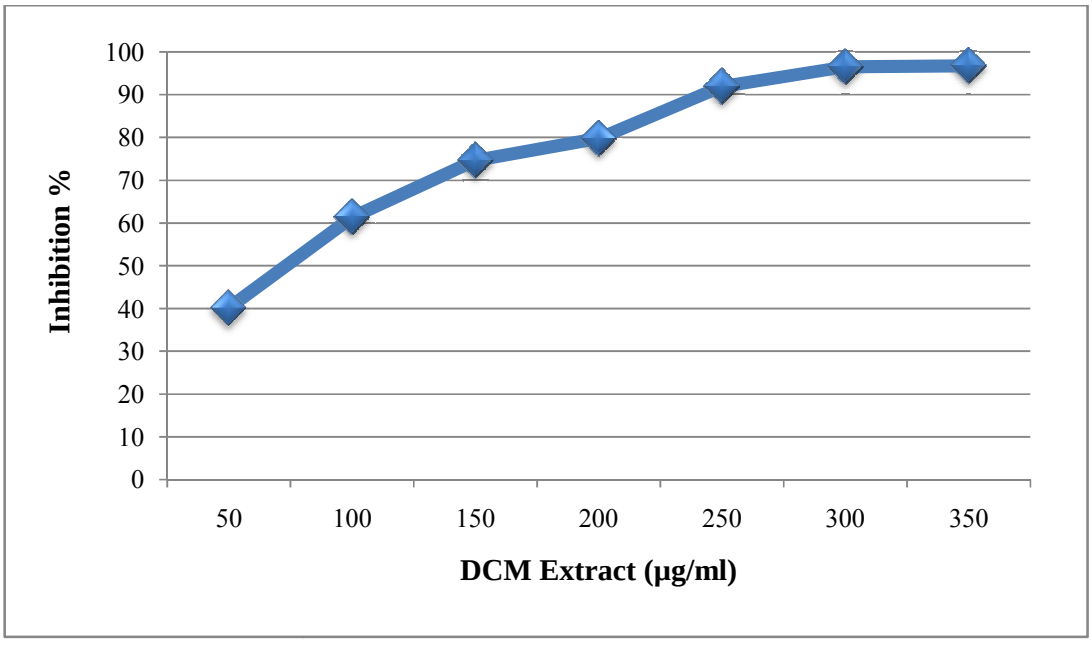

Figure 14: Effect of increasing concentrations of DCM extract on HeLa cells

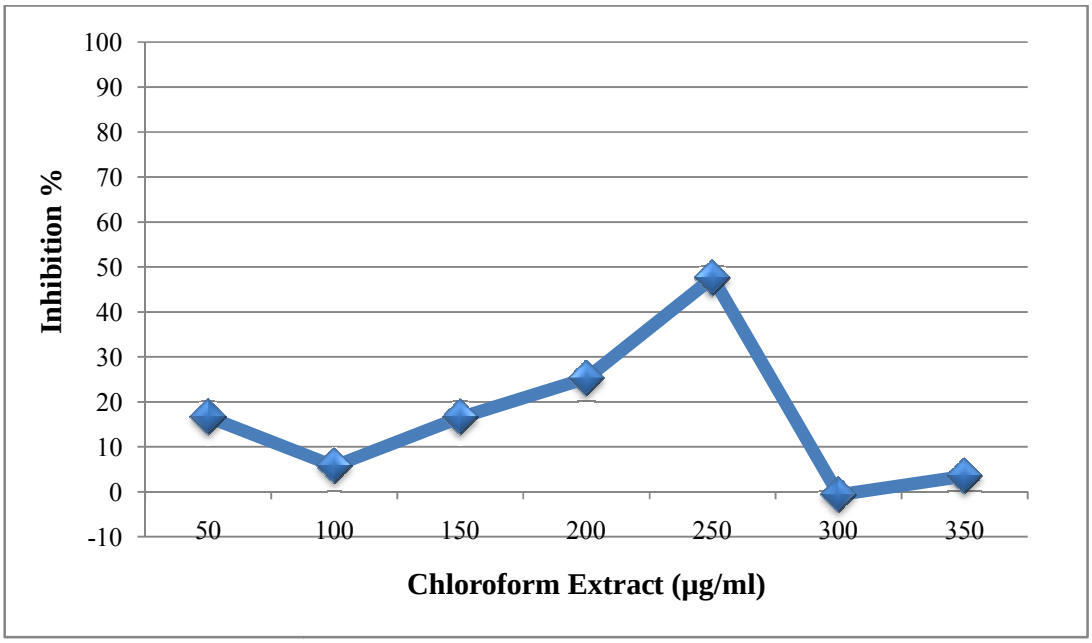

Figure 15: Effect of increasing concentrations of Chloroform extract on HeLa cells

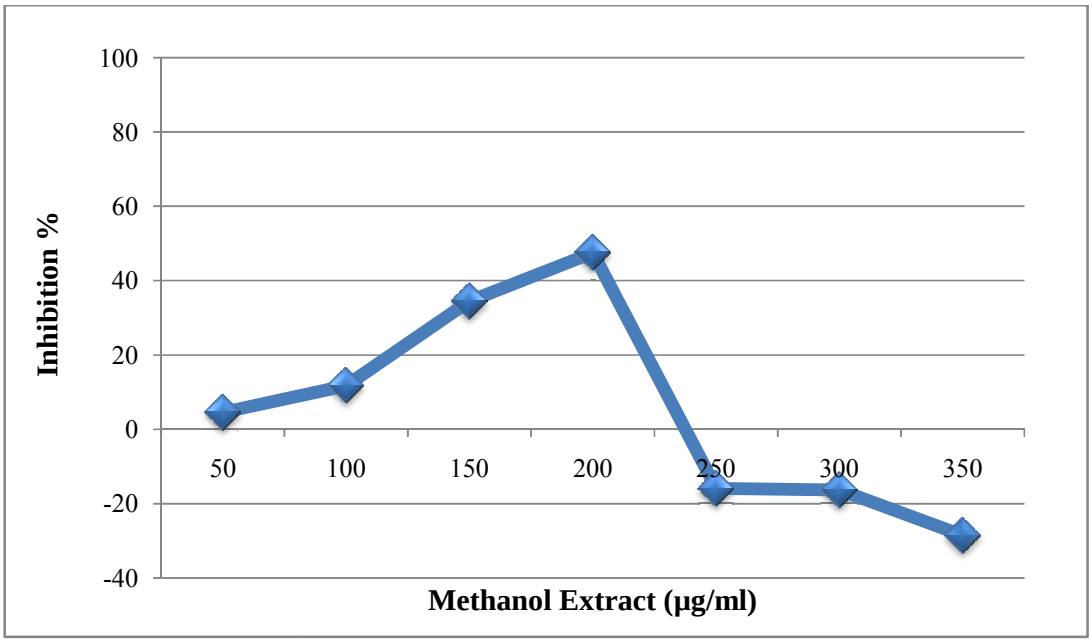

Figure 16: Effect of increasing concentrations of Methanol extract on HeLa cells

5.2.2 Cytotoxic effects of different*C. zeylanicum* solvent extracts onHeLa Cells

To confirm the cytotoxic effects on HeLa, different concentrations of the four extracts (250, 500, and 1000) µg/ml were tested. MTT assay was performed and percentage of inhibition was calculated after 48 hours of the assay.Tamoxifen was used as the positive control. It was found that the most pronounced effect was seen in the case of hexane which showed up to 80-90% inhibition in all three concentrations. DCM showed a rise in % inhibition with the increase in concentration of the extracts and its maximum inhibition was observed at 500 µg/ml. Chloroform and Methanol showed variable effects. Cell growth inhibition was observed in concentrations 250 µg/ml and 1000 µg/mlbut a proliferative response was observed in 500 µg/ml for both extracts. The results are discussed in Table (8-10) and Figure (17-19) given below.

Table 8: Effect of*C.zeylanicum*(250µg/ml) solvent extracts on HeLa cells

Solvents	Inhibition %			Mean ± SD
	Experiment I	Experiment II	Experiment III	
Hexane	80.41	80.92	78.59	79.97 ± 1.22
Dichloromethane	32.22	55.50	31.46	39.72 ± 13.66
Chloroform	18.62	31.59	17.81	22.67 ± 7.73
Methanol	8.53	4.5	35.15	16.06 ± 16.65

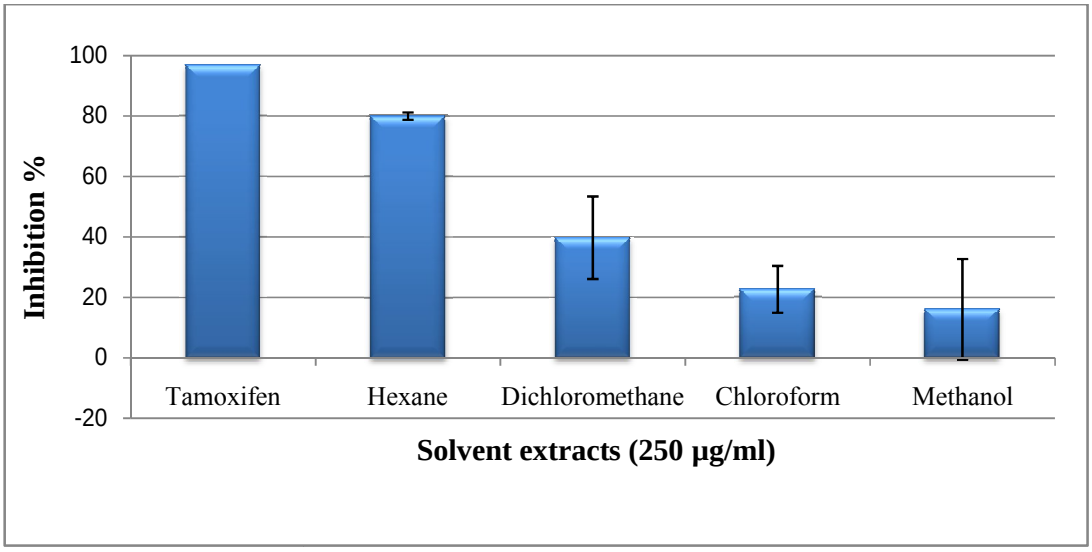

Figure 17: Effect on HeLa cells using different solvent extracts(250µg/ml)

Table 9: Effect of*C.zeylanicum*(500 µg/ml) solvent extracts on HeLa cells

Solvents	Inhibition %			Mean ± SD
	Experiment I	Experiment II	Experiment III	
Hexane	96.53	82.14	89.68	89.45 ± 7.19
Dichloromethane	80.20	72.03	74.53	75.59 ± 4.18
Chloroform	-2.93	-7.20	-4.87	-5.0 ± 2.14
Methanol	-15.81	-13.35	-11.75	-13.63 ± 2.04

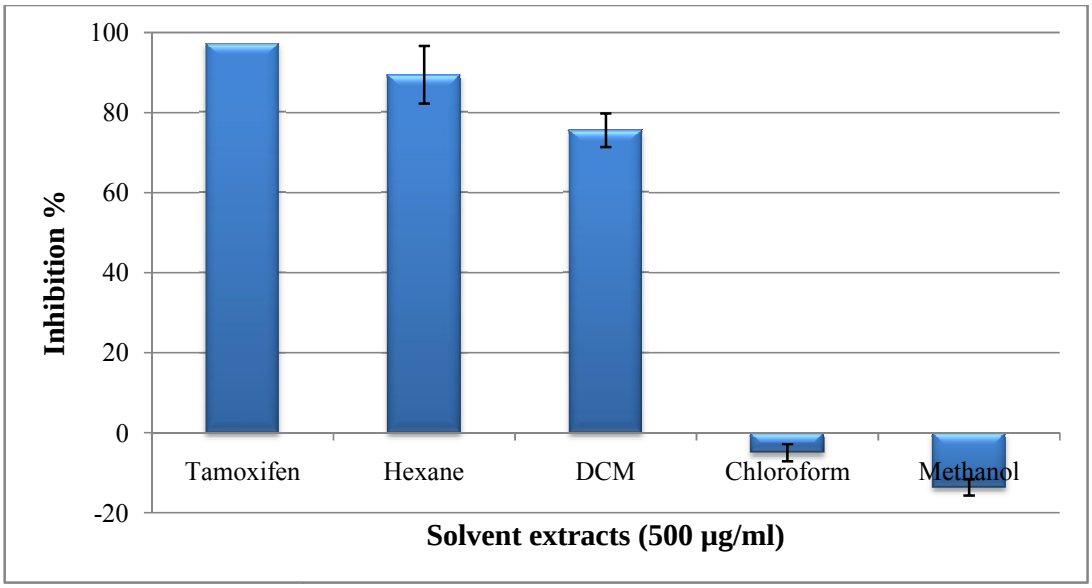

Figure 18: Effect on HeLa cells using different solvent extracts (500 µg/ml)

Table 10: Effect of*C.zeylanicum*(1000µg/ml) solvent extracts on HeLa cells

Solvents	Inhibition %			Mean ± SD
	Experiment I	Experiment II	Experiment III	
Hexane	97.54	92.57	82.85	90.99 ± 7.46
Dichloromethane	70.68	83.65	81.74	78.69 ± 7.00
Chloroform	15.06	22.67	25.74	21.15 ± 5.49
Methanol	32.68	43.09	66.81	47.5 ± 17.48

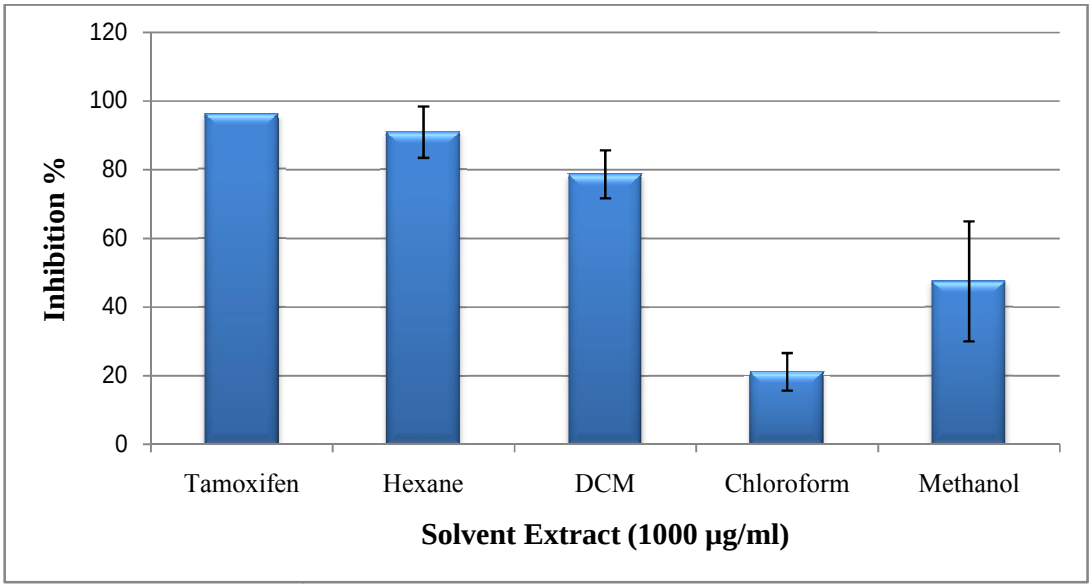

Figure 19: Effect on HeLa cells using different solvent extracts (1000 µg/ml)

5.3 Effect of *C.zeylanicum* extracts on the proliferation of RAW 264.7 cells

Each extract was diluted with complete DMEM appropriately so as to bring the final concentration of the extracts to 5mg/ml. The final DMSO concentration in these extracts was 5%. Control solvent was made accordingly using DMEM and DMSO. Tamoxifen was used as the standard anti-cancer drug. MTT assay was performed to evaluate the effect of these extracts on RAW 264.7 cells. Each experiment was performed in triplicates. Hexane showed maximum inhibition of proliferation followed by DCM and Chloroform. Least inhibition was observed in Methanol extract. This is further shown in Table 11 and Figure 20.

Table 11: Effect of 250µg/ml of different solvent extracts of *C.zeylanicum*

Solvents (250µg/ml)	Inhibition %			Mean ± SD
	Experiment I	Experiment II	Experiment III	
Hexane	83.7	99.8	110.0	97.89 ± 13.24
Dichloromethane	48.6	90.8	66.8	68.78 ± 21.14
Chloroform	43.9	96.0	50.1	68.37 ± 28.44
Methanol	31.4	21.1	17.6	23.41 ± 7.19

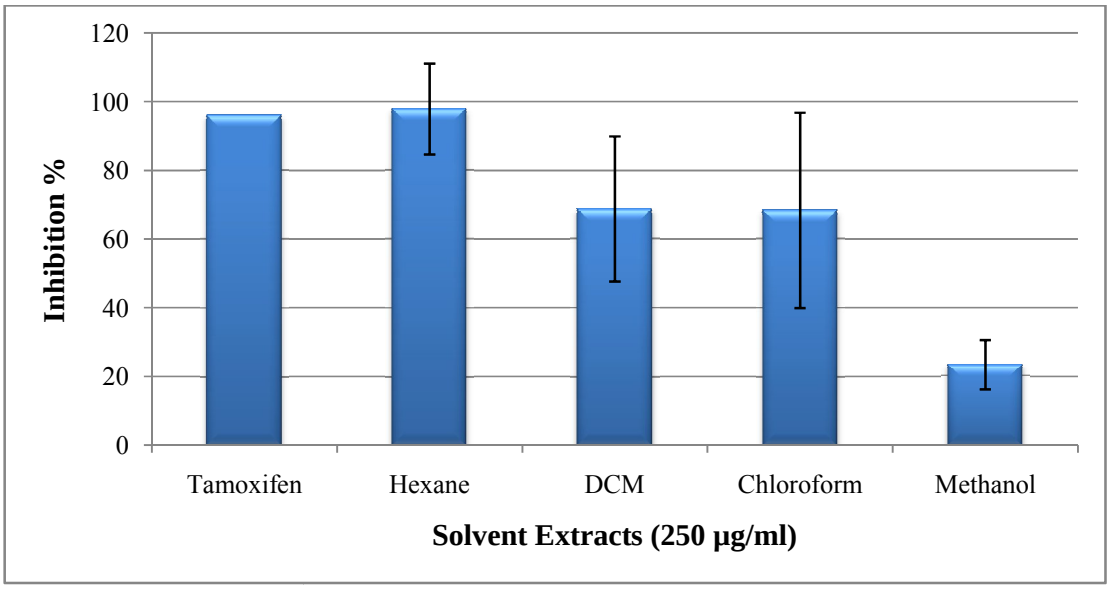

Figure 20: Effect of250µg/ml of different solvent extracts of *C.zeylanicum* on RAW 264.7 cells

5.4 Free radical scavenging activity of *C.zeylanicum* extracts

Free radical Scavenging activity of *C.zeylanicum* extracts was detected through DPPH assay. The experiments were performed in triplicates for 250 µg/ml concentration of the extracts. Ascorbic acid was taken as the positive control.The maximum activity was seen in Methanol followed by Chloroform and DCM respectively. Hexane exhibited the least free radical scavenging activity. The results are shown in Table 12 and Figure 21.

Table 12: Free radical scavenging activity of solvent extracts

Extracts	Radical scavenging activity %			Mean ± SD
	Experiment I	Experiment II	Experiment III	
Ascorbic acid	96.15	95.62	96.54	96.11 ± 0.46
Hexane	46.45	43.46	67.16	52.36 ± 12.90
DCM	88.16	84.74	82.46	85.12 ± 2.86
Chloroform	89.49	90.26	83.99	87.91 ± 3.41
Methanol	91.37	88.57	88.79	89.58 ± 1.55

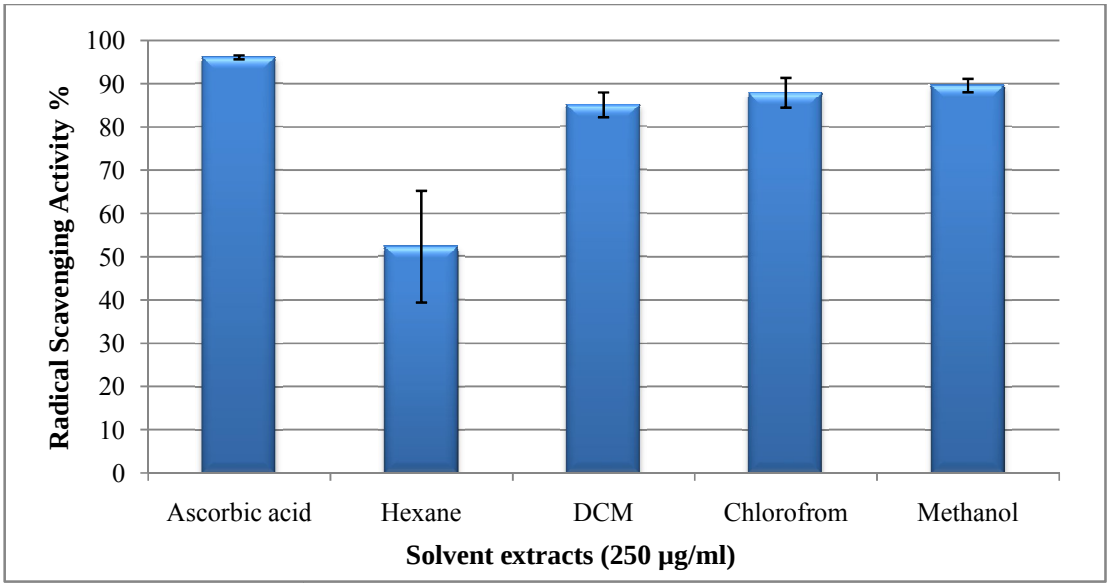

Figure 21: Free radical scavenging Activity of the four extracts(250µg/ml)

5.5Effect of *Cinnamomumzeylanicum* extracts on Stimulated PBMCs

250 µg/ml of each extract (Hexane, DCM, Chloroform and Methanol) was investigated on mitogenic stimulation of T-cell proliferation. Concanavalin A stimulated PBMCs were assessed to test this. Immunosuppressive effect was found in the three extracts (Hexane, DCM, Chloroform) while methanol extract showed a proliferative activity on stimulated PBMCs as shown in Table 13 and Figure 22.

Table 13: Inhibition % of solvents on Stimulated PBMCs

Solvent	Inhibition %
Hexane	58.01
DCM	54.9
Chloroform	55.52
Methanol	-53.66

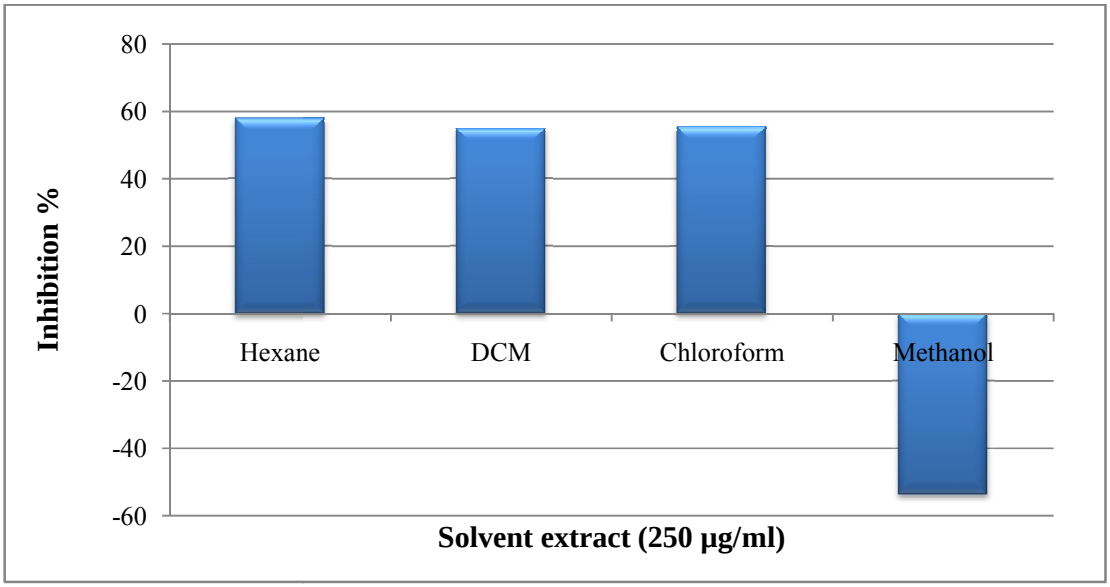

Figure 22: Effect of different solvent extracts (250 µg/ml) on stimulated PBMCs

5.6 High- Performance Liquid Chromatographic (HPLC) Analysis for the detection of Linalool

Methanolic extract of *Cinnamomumzeylanicum* has showed inhibitory as well as proliferative effects at different concentrations in the cancer cell lines Also, it possesses highest phenol content and radical scavenging activity. This may be due to the presence of some compounds present in this extract. Therefore, methanol extract was analyzed for the presence of Linalool (terpene alcohol). Acetonitrile:water (55:45) was used as the mobile phase at a flow rate of 1ml/min. The column temperature was set as 25°C and the detection wavelength was taken as 210nm. Working concentration of Linalool was taken as 100µg/ml. Injection volume for the sample was taken as 20 µl.

The chromatogram of Standard Linalool showed a peak at 12.277 min (Retention time) while a peak was observed in the methanolic extract near to the retention time of linalool, at 12.002 minute, shown in Figures 23 and 24.

This showed the presence of the compound Linalool in the methanolic extract of *C.zeylanicum.*

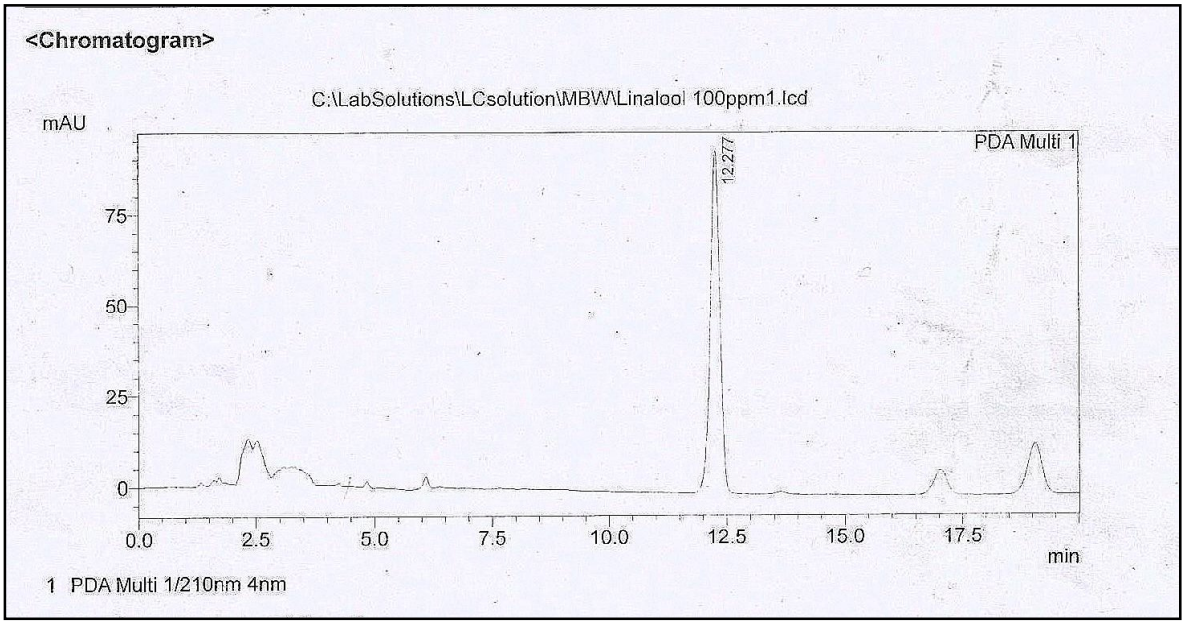

Figure 23: Chromatogram of Standard Linalool

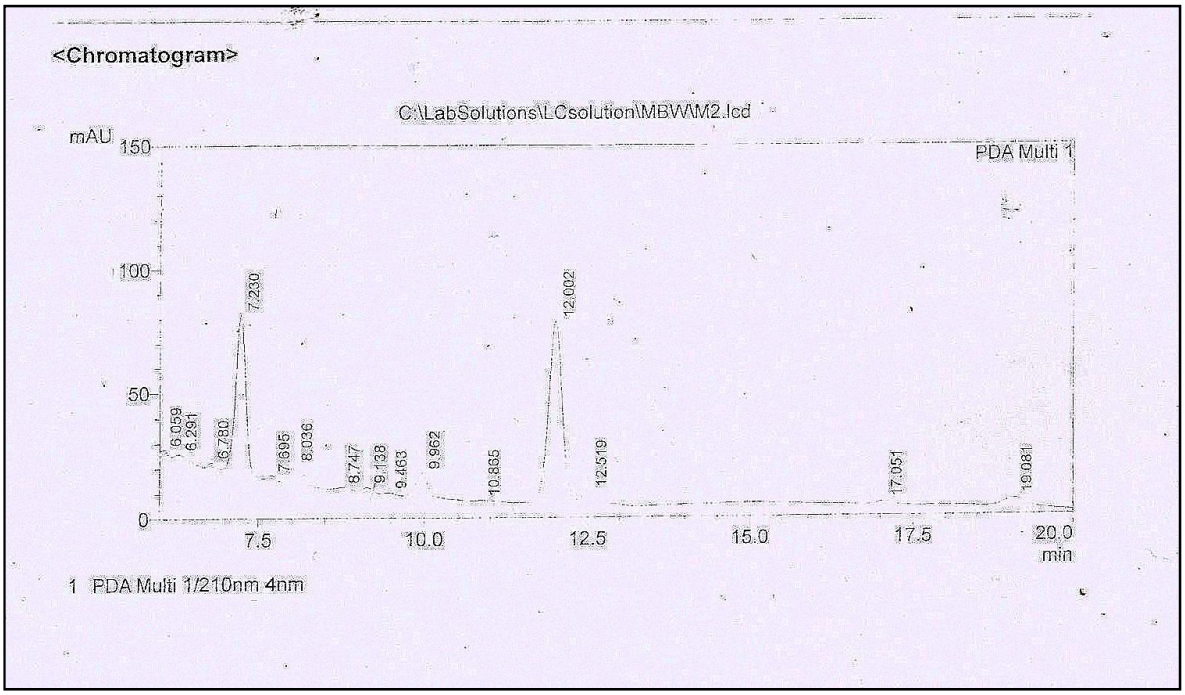

Figure 24: Chromatogram of Methanolic Extract. Peak was observed at 12.002 min

CHAPTER 6: CONCLUSION

Spices have been used since ages in Ayurveda to cure illness. They are known to possess certain phytochemicals which show anticancer, analgesic, antimicrobial, antifungal, antioxidant properties. Also, they are found to have fewer/negligible side effects as compared to their modern medicine counterparts. They come under the category of Traditional Medicines and are being researched and studies due to their many advantages over pharmaceuticals.

Cinnamomumzeylancium is the oldest herbal medicine known and is even mentioned in the Chinese literature since 4000 years ago. It is also used for medicinal purposes due to its unique properties. It is found to ne antimicrobial, anti-inflammatory, immune-suppressant and immune-stimulant. However, less scientific data is available on its cytotoxic effects and other bioactivities. The present study involved the preparation of*Cinnamomumzeylancium*solvent extracts and detection of phenolic content in them, effect of different solvent extracts of on cell lines RAW 264.7 and HeLa and their free radical scavenging activity.Four solvents (Hexane, Dichloromethane, Chloroform and Methanol) were chosen for extraction according to their varying polarity.

Presence of phenolic content for each extract was performed using FC assay where gallic acid was used as a standard.Methanol extract showed highest absorbance as compared to the other extracts and hence, contained more phenolic content followed by Dichloromethane extract.Hexane and Chloroform showed least absorbance.

The extracts were tested for different concentrations on the cell lines using MTT assay. Paclitaxel, an anti-cancer drug was used as a Control in the experiments.Hexane extracts showed up to 97% inhibition between concentrations 100-250 µg/ml, DCM extracts showed rise in inhibition with an increase in concentration of the extract and up to 96% inhibition was seen between concentrations 300-350 µg/ml. A maximum inhibition of 47% was observed in Chloroform extract in concentration 250 µg/ml. Methanol extracts exhibited an increase in inhibition with the rise in concentration and its maximum activity was observed at 200 µg/ml. Further rise in concentration

depicted a proliferative activity in the methanol extracts. 28% proliferation was observed in 350 µg/ml extract.

In case of RAW 264.7 cell lines, 250µg/ml concentration of extract solvents was tested for each solvent using MTT assay. Tamoxifen was used as the Control. Hexane showed approximately 97% of inhibition of proliferation followed by DCM and Chloroform with 68% inhibition. Least inhibition was observed in Methanol extract as 23%

The solvent extracts were further tested for the presence of free radical scavenging activity (antioxidant activity) using DPPH assay. Hexane showed least anti-oxidant activity (52.35%). The anti-oxidant activity was greater for the rest of the extract solventsand was found to be 85.1% for Dichloromethane, 87.9% for Chloroform and 89.5% for Methanol.

PBMCs were isolated and solvent extracts were added to them in a 96 well plate to check the percentage inhibition through MTT assay. The PBMCs were stimulated through a mitogen, Conacanavalin A and the absorbance was measured after 72 hours to check the inhibition of proliferation. It was found that extracts Hexane, DCM and Chloroform showed nearly 50% of inhibition of proliferation while Methanolic extract showed around 53% proliferation in the stimulated PBMCs.

HPLC analysis was performed using standard Linalool, in the methanolic extract of *C.zeylanicum.* A compound was found to elute at 12.002 minutes which was near to the retention time of Standard Linalool. This confirmed the presence of Linalool in the extract.

Methanolic extract of *Cinnamomumzeylanicum* showed an interesting behavior as compared to rest of the extracts. It showed the highest free radical scavenging activity which may be due to the presence of high phenolic content. Also, it exhibited a proliferative effect in PBMCs and for some concentrations in HeLa. Further research is needed on methanolic extract for the detection of compounds which are responsible for variable activities in the extract. Due to the presence of bioactive compounds which give *C.zeylanicum* unique properties, it can be further developed as a chemotherapeutic agent.

APPENDIX I

APPENDIX II

CHAPTER 7: REFERENCES

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