# EFFECT OF *CINNAMOMUM ZEYLANICUM* ON GROWTH OF BREAST CANCER CELLS AND MICROBES

**A Dissertation report**

**Submitted in partial fulfillment of the requirement for**

**the award of degree of**

**MASTER OF TECHNOLOGY**

**IN**

**BIOTECHNOLOGY**

**By**

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I hereby declare that the work which is being presented in the dissertation entitled '**Effect of *Cinnamomum zeylanicum* on growth of breast cancer cells and microbes**' in partial fulfillment of the requirements for the award of degree of Master in Technology in Biotechnology in the Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during a period of six months from January 2014 to June 2014, under the supervision of **Dr. Manoj Baranwal,** Assistant professor, Department of Biotechnology, Thapar University, Patiala, India. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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# ABSTRACT

Cinnamon is a widely used herbal remedy and has many applications in perfumery, flavoring and pharmaceutical industries. The aim of the study is to examine the biological activity of different cinnamon extracts and major active constituent against microbial strains and breast cancer cells. The extracts were obtained from authentic *Cinnamomum zeylanicum* which was given by ICFRE, Dehradun. Five different extracts were obtained by soxhlet extraction: hexane, diethyl ether, ethyl acetate, ethanol, and acetone. The extracts were tested by *in vitro* MTT bioassay. Antifungal activity of *Cinnamomum zeylanicum* hexane extract was evaluated against *Candida albicans*. It was found that at concentration of 2.5 mg/ml, $IC_{50}$ was obtained. Antibacterial activity of *Cinnamomum zeylanicum* hexane extract was also tested on *Staphylococcus aureus, Bacillus cereus, Escherichia coli,* and *Pseudomonas aeruginosa* and results were inferred on the basis of percentage of inhibition. Extracts exhibited significant inhibitory effect on mycelial, bacterial growth and MCF-7 cells proliferation. Interestingly, ethanol and aqueous extract showed more proliferative effect towards MCF-7 cells rather than inhibition. $IC_{50}$ was obtained in case of MCF-7 cells at hexane extract concentration of 125 µg/ml. We could perform only some preliminary MIC assays on microbial strains.

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# ABBREVIATIONS

| | |
|---|---|
| **WHO** | World health Organization |
| **Da** | Dalton |
| **$NF_{\beta}\kappa$** | Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells |
| **AP1** | Activator Protein 1 |
| **%** | Percentage |
| **HER2** | Human Epidermal growth factor Receptor 2 |
| **mTOR** | mammalian Target Of Rapamycin |
| **Bcl-2** | B-cell lymphoma 2 |
| **GST** | Glutathione S-transferase |
| **COX** | Cycloxygenase |
| **LOX** | Lipoxygenase |
| **Cz/CZ** | *Cinnamomum zeylanicum* |
| **µg/µl** | microgram per microliter |
| **$IC_{50}$** | Inhibitory concentration 50 |
| **MCF-7** | Michigan Cancer Foundation-7 |
| **DMEM** | Dulbecco's Modified Eagle Medium |
| **GAP** | GTPase-Activating Protein |
| **FBS** | Fetal Bovine Serum |
| **°C** | Degree Celsius |
| **DMSO** | DiMethyl SulphOxide |
| **Fig** | figure |
| **psi** | pound per square inch |

| | |
|---|---|
| **mM** | millimolar |
| **N** | normal |
| **IU/ml** | International Unit per milliliter |
| **PBS** | Phosphate Buffer Saline |
| **NaCl** | Sodium Chloride |
| **KCl** | Potassium Chloride |
| **$Na_2HPO_4$** | Sodium hydrogen phosphate |
| **$KH_2PO_4$** | Potassium dihydrogen phosphate |
| **$CO_2$** | Carbon dioxide |
| **rpm** | Rotation per minute |
| **O.D.** | Optical Density |
| **nm** | nanometer |
| **CFU** | Colony Forming unit |
| **stdev** | standard deviation |

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# CHAPTER 1 INTRODUCTION

The *Cinnamomum* genus (cinnamon) is a very popular spice throughout the world which belongs to Lauraceae family. The species *Cinnamomum zeylanicum* 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). This cinnamon species are one of the world's finest spices, mainly exported as 'cinnamon quills'.

Cinnamon are recognized for their flavor and aroma in addition to their antimicrobial medicinal applications and are generally recognized as safe(GRAS) natural products by the U.S. Food and Drug Administration (FDA)and it is generally accepted that their volatile compounds are the main reason for their antimicrobial properties (Ayala-Zavala *et al*., 2008; Tzortzakis, 2008).Cinnamon provides various kinds of oils, it has been established that the oils and extracts from cinnamon possess a distinct antioxidant activity, which is especially attributed to the presence of phenolic and poly phenolic substances (Schmidt E, 2006; Muchuweti *et al*., 2007). Recently, natural antioxidants are in high demand because of their potential in health promotion and disease prevention, and their improved safety and consumer acceptability.

*Cinnamomum zeylanicum* provides various types of oils depending on the part of the plant used for distillation. These are evergreen trees and shrubs and many species are aromatic. The leaf and bark of *C. zeylanicum* are used as spices and for the production of volatile oils. Leaves have a spicy odor and a hot taste. Volatile compounds have low molecular weight (<300 Da) and therefore vaporizes readily at room temperature. These aromatic compounds are commonly extracted by steam distillation or solvent extraction because they are largely volatile (Angerosa, 2002).

Cinnamon is widely used for the treatment of diabetes in the traditional systems through its antioxidant and insulin potentiating activities, water soluble polyphenols present in cinnamon are found to be responsible for this biological activity. Due to its antioxidant activity it has been used as a food preservative also. Anti-inflammatory activity of cinnamon has also been reported by Lee *et al.* (2007). Fang and coworkers (2004) showed that cinnamon sp. have a cytotoxic effect of trans-cinnamaldehyde on human cancer cell lines: Human lymphocytic cell line, Jurkat and monocytic cell line, U937.

It has been reported that the extracts of cinnamon prepared in ethanol and acetone shows antibacterial activity against *Pseudomonas* sp. and *Escherichia coli* respectively (Masih *et al.*, 2012 and Muthuswamy *et al.*, 2008). Cinnamon is also directly linked with the enhanced pro-apoptotic activity and inhibition of $NF_{\beta}k$ and AP1 activities in mouse melanoma model which shows the antitumor activity (Kwon *et al.*, 2010). Cinnamon is also used in treating various health problems such as remedy of digestive abnormalities, the excessive gas or flatulence accumulated in the digestive tract components stimulate gastric acid and promote the breakdown of food which aids digestion. The special aroma of cinnamon stimulates the digestion and whets the appetite. Cinnamon possesses anti-platelet, antithrombotic and anti-sclerotic properties, which encourage blood circulation and used to treat abnormalities related to poor circulation. It helps in treating the respiratory infections and mouth ulcers. Cinnamon is a natural astringent and will dry up your bowel very quickly.

There is recent trend to find various anticancer drugs and antibiotics from natural resources with the intention of having lesser side effects. Few reports are there for *Cinnamomum zeylanicum* to have bioactive compounds; hence the present study is focused to study the anticancer and antimicrobial activity.

# CHAPTER 2 REVIEW OF LITERATURE

## 2.1 Cancer

Cancer is a potentially fatal disease caused mainly by environmental factors that mutate genes encoding critical cell-regulatory proteins. The resultant aberrant cell behavior leads to expansive masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs resulting in disseminated disease, commonly a harbinger of imminent patient death.

### 2.1.1 Breast cancer and cinnamon

Breast cancer is a malignant tumor that starts in the cells of the breast .A malignant tumor is a group of cancer cells that can grow into (invade) surrounding tissues or spread (metastasize) to distant areas of the body.

The most common type of breast cancer is ductal carcinoma, which begins in the lining of the milk ducts (thin tubes that carry milk from the lobules of the breast to the nipple). Another type of breast cancer is lobular carcinoma, which begins in the lobules (milk glands) of the breast. Invasive breast cancer is breast cancer that has spread from where it began in the breast ducts or lobules to surrounding normal tissue. Breast cancer occurs in both men and women, although male breast cancer is rare.

An estimated 40,030 breast cancer deaths (39,620 women, 410 men) are expected in 2013. Breast cancer ranks second as a cause of cancer death in women (after lung cancer).Death rates for breast cancer have steadily decreased in women since 1989, with larger decreases in younger women; from 2005 to 2009, rates decreased 3.0% per year in women younger than 50 and 2.0% per year in women 50 and older.

**Current Treatments/ therapies available in market:** Taking into account tumor size, extent of spread, and other characteristics, as well as patient preference, treatment usually involves

- Breast-conserving surgery (surgical removal of the tumor and surrounding tissue)
- Mastectomy (surgical removal of the breast). For women undergoing mastectomy, significant advances in reconstruction techniques provide several options for breast reconstruction, including the timing of the procedure.
- Sentinel lymph node biopsy, a procedure in which only the first lymph nodes to which cancer is likely to spread are removed, has a lower chance of long-term side effects

- Full axillary node dissection, in which many nodes are removed.
- Radiation therapy
- Chemotherapy (before or after surgery)
- Hormone therapy (e.g., selective estrogen response modifiers, aromatase inhibitors, ovarian ablation)
- Targeted therapy(e.g., monoclonal antibodies for HER2 protein, blockage of mTOR)

**2.1.2 Anticancer agents**: Cancer is a major public health burden in both developed and developing countries. Plant derived agents are being used for the treatment of cancer. Several anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world. A number of promising agents such as flavopiridol, roscovitine, combretastatin A-4, betulinic acid and silvestrol are in clinical or preclinical development.

**2.1.3 Natural Anticancer agents:** Natural products discovered from medicinal plants have played an important role in the treatment of cancer. Natural products or natural product derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales (Butlet, 2004). Two plant derived natural products, paclitaxel and camptothecin were estimated to account for nearly one-third of the global anticancer market or about $3 billion of $9 billion in total annually in 2002 (Oberlines and Kroll, 2004). There are more than 270,000 higher plants existing on this planet. But only a small portion has been explored phytochemically. So, it is anticipated that plants can provide potential bioactive compounds for the development of new 'leads' to combat cancer diseases.

Noudeh *et al.* (2010) presented the evidence for tumor inhibition of the essential oils of *Heracleum persicum* and *Cinnamomum zeylanicum.* Methanol and petroleum ether were extracted from *C. zeylanicum* by potato disk method and these fractions showed cytotoxic effects in brine shrimp lethality assay (BSL). They found that both *H. persicum* (57.16%) and *C. zeylanicum* (72.90%) had inhibition effects on *Agrobacterium tumefaciens* which induced crown gall tumor on potato disk. *C. zeylanicum* also inhibited the growth of all tested Gram- positive and Gram-negative strains. In all the findings of this study completely correspond to the results obtained in brine shrimp lethality.

Cinnamon is one of the most widely used herbal medicines with diverse bioactive effects. However, little evidence has been reported about the potential anti-tumor effects of cinnamon. In addition, cinnamon treatment increased the anti-tumor activities of CD8(+) T cells by increasing the levels of cytolytic molecules and their cytotoxic activity. Cinnamon extract has the potential to be an alternative medicine for tumor treatment (Kwon *et al*., 2009; 2010).Anti-tumor activity and elucidated action mechanism of cinnamon water extract using various types of tumor cell lines including lymphoma, melanoma, cervix cancer and colorectal cancer *in vitro* and *in vivo* mouse melanoma model. Cinnamon extract strongly inhibited tumor cell proliferation *in vitro* and induced active cell death of tumor cells by up-regulating pro-apoptotic molecules while inhibiting $NF_{\beta}k$ and AP1 activity and their target genes such as Bcl-2, BcL-xL and survivin. Then they concluded that further elucidation of active components of cinnamon extract could lead to development of potent anti-tumor agent or complementary and alternative medicine for the treatment of diverse cancers.

**2.1.4 Spices and immunomodulation:** Allopathic drugs are available for counteracting the oxidative stress and hence improve immunity, but the side effects and prohibitive cost of these allopathic drugs makes it necessary to search for an alternative. The Ayurvedic system of medicines not only provides that alternative, but also scores over the side effects and cost factor of allopathic medicine. Immunomodulators are becoming very popular (Perera *et al*., 2010; Meera *et al*., 2008) in the worldwide natural health industry as people start to realize the importance of a healthy immune system in the maintenance of health and the prevention of diseases. Although extensive work has been carried out in the field of chemotherapy during this century, it is only in the last two decades that a number of compounds with immunomodulatory activity have been identified from the plant materials. From ancient times, medical treatment in India has relived to a large extent on the use of spices.

Indian spices are a rich source of substances that are claimed to induce paraimmunity, the non-specific immunomodulation of granulocytes, macrophages, natural killer cells and complement function in mammalian models (Balakrishnan *et al*., 2007). In the recent past, scientific studies on spices used in ethno medicine have led to the discovery of many valuable drugs. The current practice of prescribing phytochemicals to support the immune system or to fight infections is based on centuries' old traditions (Vrushali *et al*., 2006).

Research also indicates that spices, or their bioactive components, may act alone or in concert to reduce cancer risk through their anti-microbial, anti-oxidant, and anti-tumorigenic properties, as well as their direct suppressive effect on carcinogen bioactivation.

**Table 2.1: Some of popular Indian spices and their properties**

| SPICE | ACTIVE INGREDIENT(S) | ACTION | REFERENCE |
|---|---|---|---|
| **Cardamom** | ethyl acetate-soluble fraction, containing several phenolic compounds, aqueous cardamom suspensions | antioxidant, decrease azoxymethane-induced colon carcinogenesis by virtue of its anti-inflammatory, antiproliferative and proapoptotic activities, enhance detoxifying enzyme (GST activity) and decrease lipid peroxidation, enhance splenocyte proliferation (along with black pepper) | Kikuzaki *et al*., 2001; Bhattacharjee *et al*., 2007; Majdalawieh and Carr, 2010 |
| **Saffron** | Aqueous saffron preparations | inhibit chemically induced skin carcinogenesis, shift in caspases and an increase in Bax protein | Das and Chakrabarty, 2004; Mousavi et al., 2009 |
| **Turmeric** | Curcumin, a diferuloylmethane | anti-inflammatory activity that could impact on risk for cancer, but in addition for risk of diabetes, obesity, metabolic syndrome, and atheroschlerosis | Goel *et al*., 2008; Bachmeier *et al*., 2010 |
| **Fenugreek** | Diosgenin | suppress the proliferation of human myelogenous leukemia cells, colon cancer cell lines and breast cancer cell lines, inhibits NFκß activity, COX enzymes, suppresses LOX | Raju *et al*., 2007; Shishodia *et al*., 2006; Srinivasan *et al.*, 2009; Raju et al., 2009 |

## 2.2 CINNAMON: *Cinnamomum zeylanicum*

The genus *Cinnamomum* comprises of about 250 species, of which 20 occur in India (Anon., 1950). The most important volatile oils from cinnamon are from *C. zeylanicum* bark and leaf oils, *C. cassia* (cassia oil) and *C. camphora*. However, a number of other *Cinnamomum* species are distilled on a smaller scale and the oils are used either locally or exported to regional markets. The important species are as follows:

- *C. cassia* Presl Cassia, Chinese cinnamon, "Cassia lignea"
- *C. verum* Presl (syn. *C. zeylanicum* Nees) True or Ceylon cinnamon
- *C. burmannii* Blume Indonesian cassia
- *C. loureirii* Nees Vietnamese cassia
- *C. tamala* (Buch.-Ham.) Nees and Eberm. Indian cassia
- *C. cordatum* (Kosterm) abundantly found in Perak and Pahang in the Malaysian peninsula

*Cinnamomum zeylanicum* Blume (synonym *C. verum* J. S.Presl) the cinnamon of commerce, provides various types of oils depending on the part of the plant distilled. These are evergreen trees and shrubs and many species are aromatic. *C. zeylanicum*, the source of cinnamon bark and leaf oils, is an indigenous tree of Sri Lanka, although most oil now comes from cultivated areas. Smaller areas of wild trees are also found in the south-western parts of India. *Cinnamomum cassia*, the source of internationally traded cassia oil, occurs wild as a bush in the mountains of Southern China, but is now cultivated for oil production, mainly in the provinces of Kwangsi and Kwang- tung. The other cassia occurs wild on the islands of Sumatra and Java in Indonesia (*Cinnamomum burmannii*), in Vietnam (*Cinnamomum loureirii*), and India and Nepal (*Cinnamomum tamala*) (The Wealth of India, 1992).

The leaf and bark of *C. zeylanicum* are used as spices and for the production of volatile oils. Leaves have a spicy odor when brushed and have a hot taste. Volatile compounds are low molecular weight compounds (<300 Da) which vaporize readily at room temperature. Some volatile compounds reach the olfactory epithelium, dissolve into the mucus, and may bond with olfactory receptors to give an odor sensation. As these aromatic compounds are largely volatile, they are commonly extracted by steam distillation or solvent extraction (Angerosa, 2002). Three of the main components of the essential oils obtained from the bark of Cz are trans-cinnamaldehyde, eugenol and linalool, which represent 82.5% of the total composition

(Chericoni *et al*., 2005). Trans-cinnamaldehyde, accounts for approximately 49.9–62.8% of the total amount of bark oil (Singh *et al*., 2007; Simic *et al*., 2004). Cinnamaldehyde and eugenol are also the major components of Cz extracts (Usta *et al*., 2003).

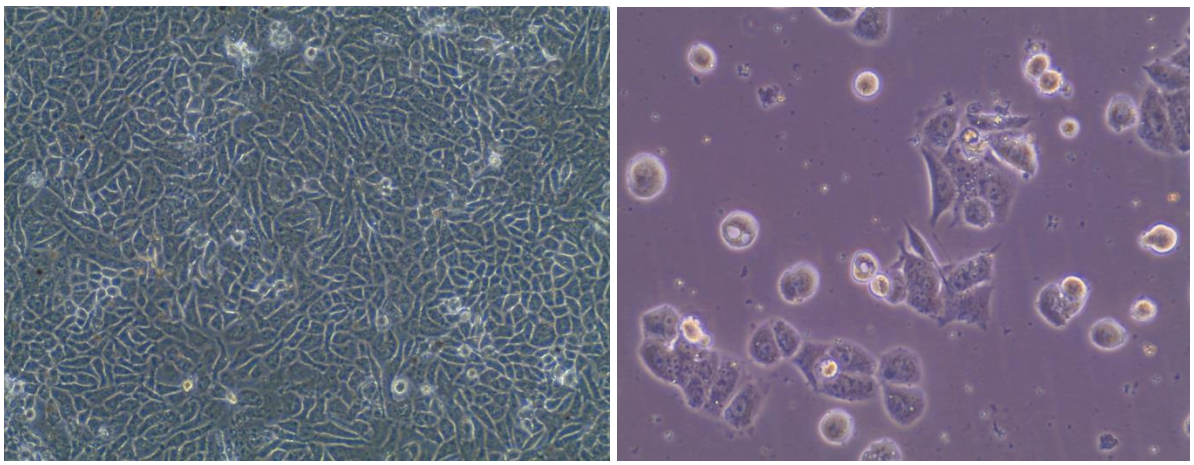
**Table 2.2: Antimicrobial activity of *Cinnamomum zeylanicum* and its oils**

| Organism(s) tested | Main results | References |
|---|---|---|
| *Clostridium perfringens, E.coli, Klebsiella pneumonia, Pseudomonas aeruginosa, S. aureus, Streptococcus faeca* and *Yersinia enterocolitica* | Volatile oils from CZ had significant activity against the growth of food poisoning organisms, food spoilage organisms and organisms of faecal origin | Barattha MT *et al*. ,1998 |
| *Candida albicans* | Among all spices tested CZ inhibited *C. albicans* most effectively (MIC 7.81 µl/ml) | Bhatia M *et al*., 2012 |
| *Salmonella typhi, Staphylococcus aureus, E.coli, Klebsiella pneumoniae* | CZ essential oils inhibited growth of all organisms. Gram-negative organisms were more susceptible than Gram-positive ones | Dubey RC *et al*., 2005 |
| *S. pyogenes, S. agalactiae, S. pneumonia, Klebsiella pneumoniae, H. Influenza* and *S. aureus* | Of the 13 essential oils evaluated CZ and thyme showed the highest activity inhibiting all the strains studied | Fabio A *et al*., 2007 |
| Human rota-virus | CZ leaves and bark was able to inhibit the propagation of human rotavirus 32.4% and 33.9% respectively. | Gonçalves JLS *et al*., 2005 |
| *Trichophyton mentagrophytes, T. tonsurans, T. rubrum, Microsporum canis, M. gypseum, M. audouini, Aspergillus fumigates, Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *Crytococcus neoformans* | Among all the essential oils, the leaf and bark oils of CZ showed the highest activity against all the fungi with MIC values of 0.04 to 0.63 µg µL−1 | Jantan IB *et al*., 2008 |
| *Multi drug resistant (MDR) strains of Escherichia coli, Klebsiella pneumoniae* and *Candida albicans.* | The MDR strains were sensitive to the antimicrobial activity of CZ. | Khan R *et al*., 2009 |
| *Trichophyton mentagrophytes, T. rubrum, Microsporum* | CZ was a moderate inhibitor of all the fungi tested (MIC values 1.26 – 2.51 | Mastura M *et al*., 1999 |

| | | |
|---|---|---|
| *canis,Candida albicans* and *C. glabrata* | µg/µl) | |
| *Escherichia coli, Salmonella typhi, Salmonella paratyphi A/B, Brucella abortus* and *Brucella melitensis* | CZ extract were active only against *Brucella melitensis* | Agasthya AS *et al*., 2009 |
| *Listeria monocytogenes, S. aurus, E. coli, Enterococcus faecalis,Klebsiella pneumoniae, Enterobacter cloacae, Acinetobacter baumannii* | CZ extracts demonstrated significant inhibitory effects on *S. aureus, Enterobacter cloacae, Acinetobacter baumannii* and *Listeria monocytogenes* (MIC 0.4 mg/ml) | Bayoub K *et al*., 2010 |
| *Helicobacter pylori* | CZ exhibited the most inhibitory effect on *H. pylori* and essential oils of CZ with $IC_{50}$=0.3 µl/ml completely inhibited the growth of *H. pylori.* | Hosseininejad Z *et al*., 2011 |
| *S. aureus, Streptococcus pyogenes, S. pneumonia, Enterococcus faecalis, Enterococcus faecium, Bacillus cereus, Acinetobacter lwoffii, Enterobacter aerogenes, E. coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhimurium, Clostridium perfringens, Listeria monocytogenes, Listeria ivanovii, Mycobacterium smegmatis, Candida albicans, Candida parapsilosis ,Candida krusei* | The essential oil of CZ showed strong antimicrobial activity against all microorganisms tested. | Unlu M *et al*., 2010 |
| *Bacillus cereus, B. coaguiaris, B. subtilis, S. aureus, E. coli, Pseudomonas aeruginosa* | All crude extracts of CZ fruits showed antibacterial activity. Ethyl acetate and benzene extracts showed higher activity than methanol and water extract. | Negi PS *et al*., 2007 |

## 2.3 MCF-7 Cell Line

MCF-7 is a human breast cancer cell line that was first established from the pleural effusion from a 69 year female Caucasian suffering from a breast adenocarcinoma isolated in 1970. MCF-7 is the short form of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established. MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen via estrogen receptors. MCF-7 cells are also sensitive to cytokeratin. When grown *in vitro*, the cell line is capable of forming domes and the epithelial like cells grow in monolayers. Growth can also be inhibited using tumor necrosis factor alpha (TNF alpha).



**A** **B**

**Fig 2.1 A shows fully confluent MCF-7 cells grown *in vitro* and Fig 2.1 B shows MCF-7 cells in growing phase in complete DMEM medium *in vitro***

### 2.3.1 Uses for the MCF-7

MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line.

### 2.3.2 Characteristics of MCF-7

In addition to retaining their estrogen sensitivity, MCF-7 cells are also sensitive to cytokeratin. They are unreceptive to desmin, endothelin, GAP, and vimentin. When grown *in vitro*, the cell line is capable of forming domes and the epithelial like cells grow in monolayers. Growth can be inhibited using tumor necrosis factor alpha (TNF alpha), and treatment of MCF-7 cancer cells with anti-estrogens can modulate insulin-like growth factor finding protein's, which ultimately have the effect of a reduction in cell growth.

# CHAPTER 3 OBJECTIVES

The main objectives of the present studies are to explore the effects of different extracts *Cinnamomum zeylanicum* bark on growth of breast cancer cell line (MCF-7) and on different microbes.

Work plan of the current study is as follows:

1. Preparation of *Cinnamon zeylanicum* bark extracts using different solvents of increasing polarity index using soxhlet apparatus and drying these extracts in rotaevaporator.
2. Determination of effect of each of these extracts on proliferation of MCF-7 cell lines using MTT assay and then screen out the best result giving extract.
3. Using this best extract and trying its different gradients, find out $IC_{50}$ i.e. concentration of extract at which 50% of the population of cells are found to be dead using MTT assay.
4. Same extract will be tested on microbes for preliminary MTT assay for its antimicrobial potential.

# CHAPTER 4 MATERIAL AND METHODS

## 4.1 MATERIALS

**Table 4.1: Requirements during the whole project work**

| REQUIREMENTS | COMPANY |
|---|---|
| DMEM powder | Himedia |
| FBS | Himedia |
| Streptomycin | Himedia |
| Penicillin G | Himedia |
| Amphotericin | Sigma Aldrich |
| Tylosin | Sigma Aldrich |
| Trypsin/Accutase | Himedia |
| Sodium bicarbonate | Sigma Aldrich |
| L-glutamine | Himedia |
| DMSO | SRL |
| MTT reagent | Sigma Aldrich |
| Trypan Blue | Himedia |
| PDB | Himedia |
| MHB | Himedia |
| NB | Himedia |
| Agar | Himedia |
| Hexane | Merck |
| Diethyl ether | Loba |
| Ethyl acetate | Loba |
| Ethanol | Loba |
| Acetone | Loba |

## 4.2 OPTIMIZATION OF EXTRACT PREPARATION

OLD METHOD:

Initially, *Cinnamomum zeylanicum* extracts were prepared using following method. 30 g of *C. zeylanicum* barks were cleaned under running water and then dried under sunlight. These were then ground to fine powder using grinder. This sample was then put into extraction chamber of soxhlet apparatus after putting some glass wool in it to avoid leakage of powder into extract collecting flask.150 ml of least polar solvent was poured into a round bottom flask with some glass beads and put on a heating mantle. Soxhlet apparatus was arranged accordingly and heating mantle was turned on at 10-15℃ less than the boiling point of solvent. This was run for about 6 hrs. After cooling down, extract was dried with the help of rotaevaporator and thus, solvent and pure extract were separated. Dried extract was finally dissolved with the help of pure DMSO and DMEM. This was stored at -4°C for further use.

NEW/OPTIMIZED METHOD:

The new method was modified accordingly so as to obtain final concentration in absolute way and thus, experiments performed were also modified respectively. The changes in the old method have been described in this paragraph. 40 g of *C. zeylanicum* was ground it till fine powder using pestle and mortar to avoid temperature effects on final extract. Then we put it into a cellulose thimble (to avoid filtration and any kind of sample leak into extract) .We dried our extract and obtained its concentration by finally dissolving it in 100% DMSO.

$$\text{Concentration of extract (in mg/ml)} = \frac{\text{(Wet wt. of flask-dry wt. of flask)}}{\text{Dry weight of flask}} \times \_\_ \text{ ml of DMSO}$$

We avoided doing rotary evaporation of aqueous extract because it requires very high temperature, due to which our compound gets destroyed. So, due to lack of lyophilizer availability, we omitted preparing aqueous extract and working with it. Rest, all of the crude extracts (along with known absolute concentration) were stored at -4°C for further use.

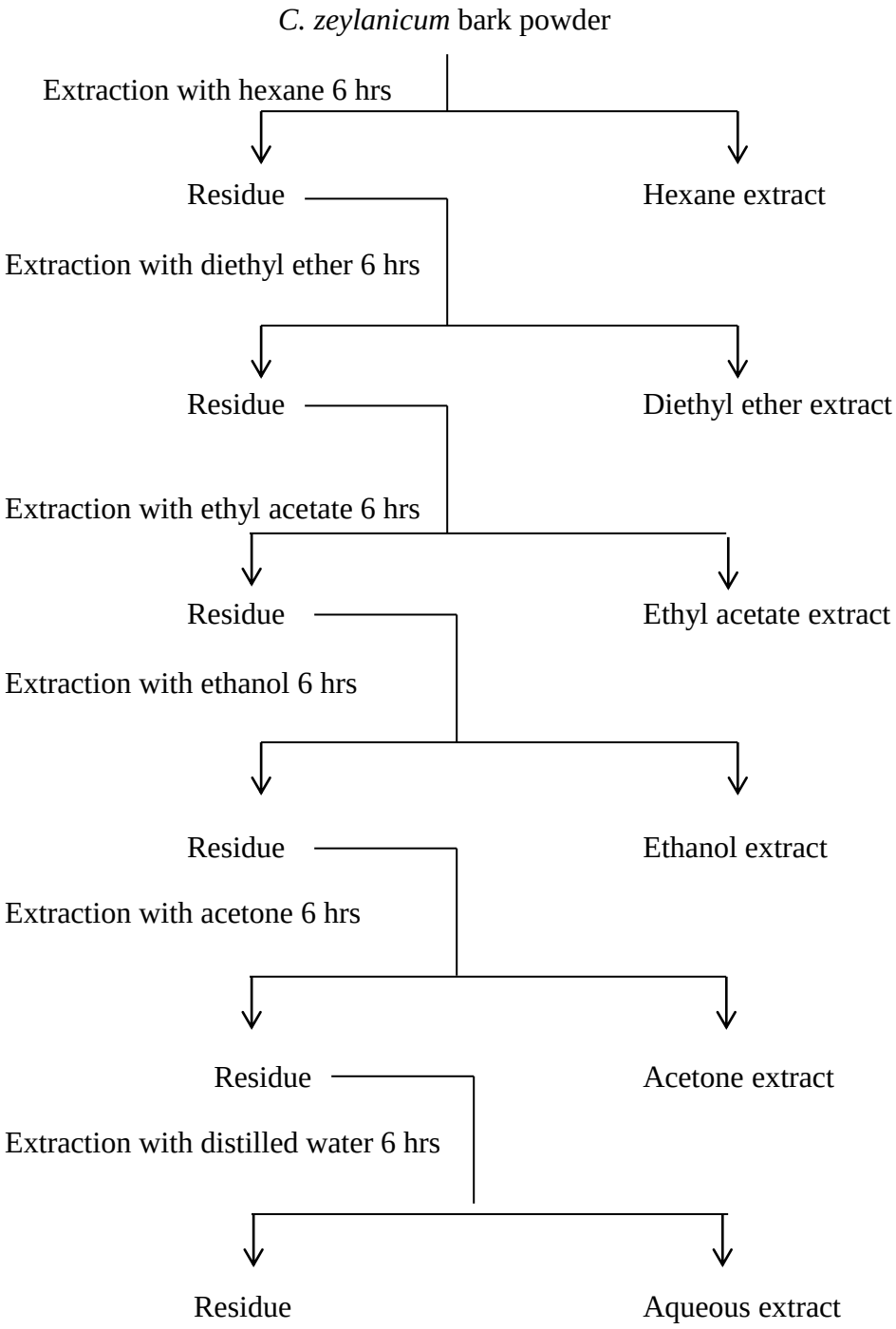



**Figure 4.1: Schematic diagram of Soxhlet operation**

As, first extraction was performed using old method of soxhlet extraction. So, initially concentration of extracts prepared could not be calculated as final extract was dissolved using DMSO + DMEM. But, preliminary experiments were still performed on MCF-7 cell lines. But, after an optimized method, we obtained pure extracts which were dissolved in 100% DMSO to obtain their actual or crude concentration and then, diluting them for further use.

### 4.2.1 SOXHLET EXTRACTION:

A **Soxhlet extractor** is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent. Normally a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The extraction solvent to be used is taken into a distillation flask and the Soxhlet extractor is now placed onto this flask. The Soxhlet is then equipped with a condenser.

The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material.

The chamber containing the solid material is slowly filled with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound.

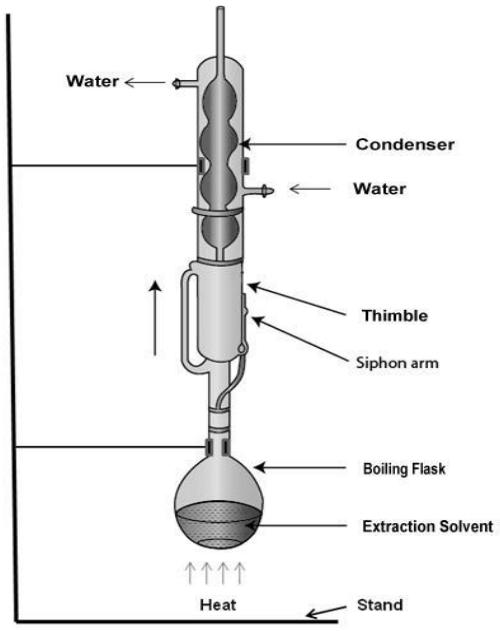



**Fig 4.2: Soxhlet apparatus operation**

### 4.2.2 ROTARY EVAPORATION:

A rotary evaporator is a device used in chemical laboratories for the efficient and gentle 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 either 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.

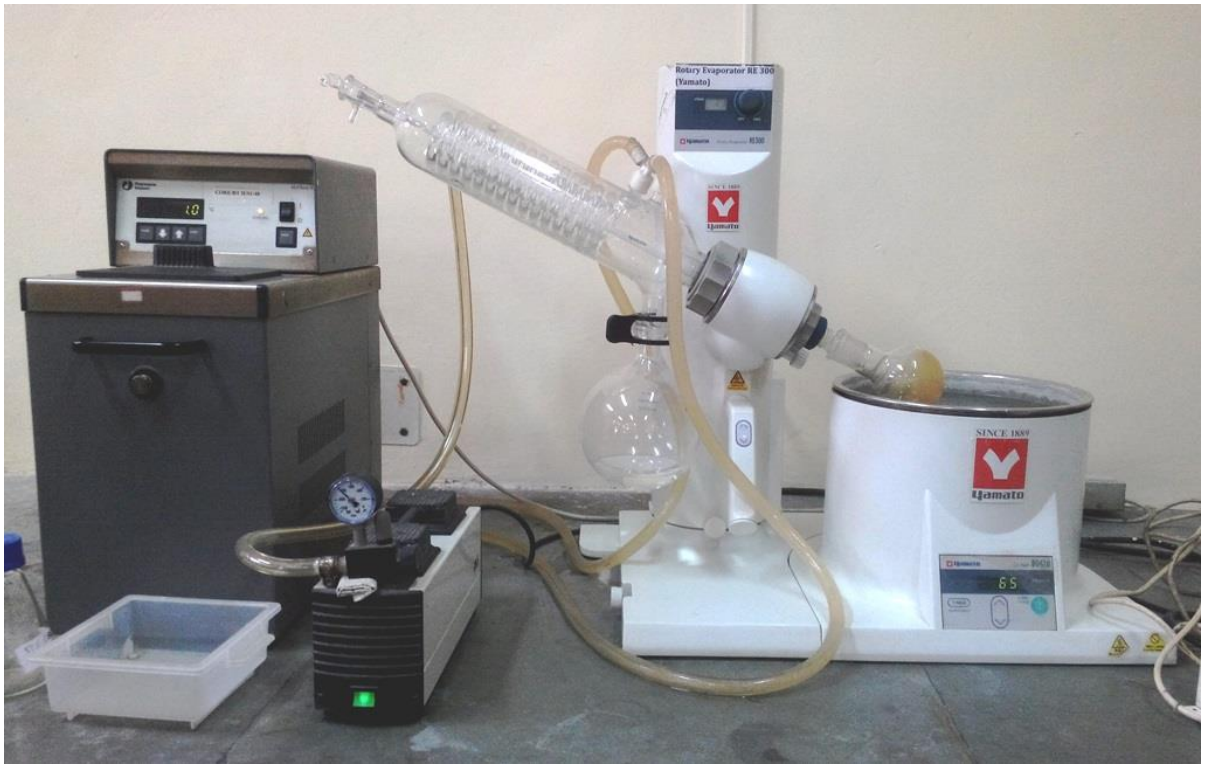



**Fig 4.3: Rotary evaporator in operation**

## 4.3 DETERMINATION OF ANTICANCER ACTIVITY OF *CINNAMOMUM ZEYLANICUM* EXTRACTS IN MCF-7 CELL LINE

### 4.3a PREPARATION OF COMPLETE DMEM LIQUID MEDIUM:

9.6 g of powder DMEM media was suspended in 900 ml tissue culture grade water (or Mili Q water) and constantly, stirred gently until the powder was completely dissolved and autoclaved for 15 minutes at 121°C and 15 psi pressure in an autoclave. After autoclaving allow it to cool to room temperature and then add 49.3 ml of 7.5% sodium bicarbonate solution and 20 ml of 200 mM L-glutamine solution to 1 liter of medium and stirred until dissolved. pH was adjusted to 4.0 using 1N HCl or 1N NaOH pH of the medium was adjusted ±0.2 below the desired pH since the pH tends to rise during filtration. The final volume was made up to 1000 ml with tissue culture grade water. The medium was immediately sterilized by filtering through a sterile membrane filter with porosity of 0.22 micron , using positive pressure rather than vacuum to minimize the loss of carbon dioxide. Liquid medium was stored at 2-8°C and in dark till use.

10% heat inactivated fetal bovine serum and filter sterilized antibiotics: Streptomycin (10 mg/ml), Penicillin ($10^4$ IU/ml), Amphotericin (2.5 mg/ml) and Tylosin (1 ml/l) were added to media before culturing of cells. This is called complete DMEM.

### 4.3b 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.

### 4.3c REVIVING THE MCF-7 CELL LINES:

Culturing of cell lines was carried out aseptically under Bio Safety Level-2 and 5% $CO_2$ incubator set at 37°C. Vertical laminar was used for working with MCF-7 cells. Incubator and lab was regularly maintained by fumigation and by changing of water in incubator to maintain 5 % $CO_2$ level.

**a. Thawing of MCF-7 cells:**

1. Removed the vial containing cells from storage and thawed quickly in a 37°C water bath.

2. Immediately added 2 volumes of complete growth medium to the vial containing frozen and mixed very gently.

3. Centrifuged the cells at 1000 rpm for 10 minute at room temperature.

4. Discarded the supernatant.

5. Gently resuspended the cells in complete growth medium [DMEM supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), Amphotericin (2.5 μg/ml) and Tylosin (1 ml/l)].

6. Plated the cells in tissue culture T-flask and incubated at 37°C and 5% $CO_2$.

**b. Sub culturing of cells:**

1. Used complete DMEM to grow MCF-7 cells in T-flask and maintained the temperature at 37°C in humidified, concentrated $CO_2$ (5%) atmosphere.

2. Once MCF-7 cells reached approximately 80% confluence in plates, removed media from flask and rinsed with 1X PBS.

3. Added 2-3 ml of warm (37°C) 0.25% Trypsin solution to cells to detach the cell layer. Observed under an inverted microscope (Detachment should happen between 5 and 15 minutes). If cells are not detaching properly, place the flask back in 37°C incubation chamber. Do not agitate the cells during dispersal, either by hitting or shaking the flask. This may cause clumping as the cells detach.

4. Once MCF-7 cell layer is dispersed, Trypsin was deactivated by adding same volume of complete growth medium in flask and then, transferring them to tubes or eppendorfs. Aspirate cells by gently retropipetting.

5. Centrifuged cells in growth medium for 10 minutes at 1000 rpm.

6. Removed trypsin growth medium suspension from tube.

7. Resuspended the pellet (MCF-7 cells) in 2 ml PBS and centrifuged at 1000 rpm for 10 minutes.

8. Resuspended the cells in 1 ml of complete medium.

9. Counted the cells using hemocytometer (10 µl cells + 10 µl of trypan blue+ 80 µl complete DMEM).

10. Resuspended the cells in complete DMEM media (5 ml for T25 flask and 15 ml for T75 flask).

11. Observed culture daily by eye and under an inverted microscope to ensure culture is free of contamination and culture has not reached confluence.

In most cases, cultures at a high cell density exhaust the medium faster than those at low cell density as is evident from the change in pH. A drop in pH is usually accompanied by an increase in cell density, which is an indicator to subculture the cells. Cells may stop growing when the pH is between 6-7 and loose viability between 6-6.5. Media in flask turns pale red if there is drop in pH whereas it turns pinkish if pH becomes high.

**4.3d Cell counting and Viability Testing:**

Cell counting was done with the help of hemocytometer using trypan blue as a stain. Trypan blue is a stain that penetrates through the cell wall of dead cells and stains them in blue color while live cells remain unstained. 10 µl of cell suspension, 80 µl of media and 10 µl of 0.4% trypan blue solution (made in 1X PBS) were mixed. Now cell suspension was diluted 10 times to the original cell suspension, and this diluted suspension with trypan blue was loaded on hemocytometer. Hemocytometer was focused on using the 10X objective of the microscope and cells were counted in all 4 sets of squares of hemocytometer using 40 X objective of the microscope.

Cell count was calculated using the formula:

$$\text{Cell count} = \frac{\text{Total number of cells counted}}{\text{Number of chambers counted}} \times \text{Dilution Factor} \times 10^4 \text{ cells/ml}$$

**4.3e MTT Assay:**

Cell proliferation was tested using a 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT assay). For the assay, freshly trypsinized MCF-7 cells were taken (20,000 cells/well) and plated in 96-well flat bottomed microtiter plate along with 80 µl complete DMEM. This plate was pre-incubated at 37°C and 5% $CO_2$ incubator for 12-24 hrs for attachment to surface of plate. Different concentrations of each extracts were added to the wells along with their corresponding controls and final volume was maintained as 200 µl. Plate was incubated in 5% $CO_2$ incubator at $37^0$C for 72 hours. After incubation, 20 µl of MTT reagent (5 mg/ml) was added to each well and incubation of other 4 hours was given for reduction of MTT to formazan. After incubation, 170 µl of supernatant was removed and formazan crystals were dissolved in 100 µl of DMSO. In order to properly dissolve formazan crystals, 10 minutes incubation was given at $37^0$C and O.D. was taken at 570 nm and 630nm as reference O.D. and final O.D. was calculated as difference between both O.D. by micro titer plate reader.

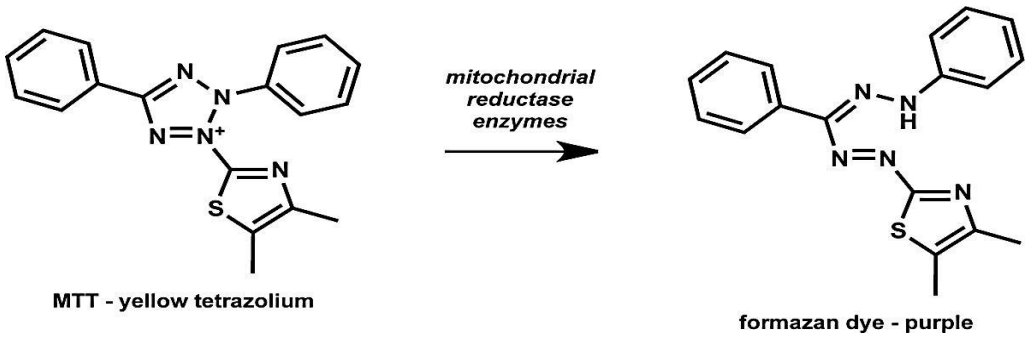



**Fig 4.4**: **Reduction of MTT to formazan**

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. If we get purple or pink color that means cells are viable and might be proliferating too, whereas if color of MTT remains unchanged , it means cells are dead as they lack the mitochondrial reductase for reduction of MTT to formazan, to yield purple color. Percentage of inhibition was calculated by following equation:

$$\frac{\text{OD control} - \text{OD test}}{\text{OD test}} \times 100$$

## 4.4 DETERMINATION OF ANTIMICROBIAL ACTIVITY OF *CINNAMOMUM ZEYLANICUM* HEXANE EXTRACT

The antimicrobial activity of hexane extract of *Cinnamomum zeylanicum* was determined in one **fungus** i.e. *Candida albicans* and four bacteria i.e. **Gram-positive**: *Staphylococcus aureus* and *Bacillus cereus.* **Gram negative**: *Escherichia coli* and *Pseudomonas aeruginosa.* To find whether hexane extract of *Cinnamomum zeylanicum* have any antimicrobial activity, we have done Minimum Inhibitory Concentration (MIC) Broth dilution method.

Fungus was maintained and stored at -4°C in PDB broth, so as to keep the metabolic process of fungus as slow as possible. Similarly, gram positive bacteria were maintained in MHB and gram negative bacteria were maintained in NB.

**Composition:**

**Table 4.2: Composition of Nutrient Broth (NB)**

| | |
|---|---|
| Peptone | 0.5 % |
| Beef extract/yeast extract | 0.3 % |
| NaCl | 0.5% |
| pH | 6.8 |

**Table 4.3: Composition of Muller Hinton Broth (MHB)**

| | |
|---|---|
| Beef infusion | 30.0% |
| Casein hydrolysate | 1.75% |
| Starch | 0.15% |
| pH | 7.0 |

**Table 4.4: Composition of Potato Dextrose Broth (PDB)**

| | |
|---|---|
| Potatoes infusion | 20.0% |
| Dextrose | 2% |
| pH | 5.1±0.2 |

**Table 4.5: McFarland Standards**

| **McFarland Standard No.** | **0.5** | **1** | **2** | **3** | **4** |
|---|---|---|---|---|---|
| 1.0% Barium chloride (ml) | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 |
| 1.0% Sulfuric acid (ml) | 9.95 | 9.9 | 9.8 | 9.7 | 9.6 |
| Approx. cell density ($1\times10^8$ CFU/ml) | 1.5 | 3.0 | 6.0 | 9.0 | 12.0 |
| Absorbance* | 0.146 | 0.210 | 0.449 | 0.661 | 0.850 |

* Wavelength at 600 nm

### 4.4.1 Growth conditions and MIC testing by broth dilution method

Activation of all the cultures was done as following in sterile conditions:

***Candida albicans***: After coming at room temperature, 10 µl of broth was taken and inoculated in 10 ml of PDB and was kept in shaker incubator for 12-16 hrs at 25º C.

**Bacterial cultures:** After coming at room temperature, 10 µl of culture broths were taken and inoculated in 10 ml of respective broths and were kept in shaker incubator for 5-6 hrs at 37º C.

Quantitative antibiotic susceptibility testing would be very useful to clinical microbiology laboratories because pharmacokinetic/pharmacodynamic surrogate parameters can be correctly calculated using MIC values. An accepted and frequently used method for MIC testing is the broth microdilution method described by the National Committee for Clinical Laboratory Standards (NCCLS). This method is convenient to use, can be standardized and can be prepared *in vitro*. However, automation of this method requires sensitive optical devices like spectrophotometers, ELISA readers and/or sophisticated image-processing software are needed for correct automated reading of broth microdilution trays.

For susceptibility testing, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) produces the highest optical signal in the visible spectrum. This tetrazolium derivative can be used for determination of cell growth and viability. Recently, this principle has also been used successfully for susceptibility testing of yeasts and filamentous fungi as well as for bacterial strains too.

A suspension of test organism was prepared equivalent to a 0.5 McFarland standard using freshly grown cultures of different microorganisms. The wells of a 96- well ELISA tray were filled with 100 µl of MHB (for antibacterial testing) or PDB (for antifungal testing) and then 10 µl of culture suspension was added and then hexane extracts in different concentrations for their antimicrobial activity were added, then final volume was made to 220 µl by adding corresponding broths. The plate was incubated at 25°C for 24 h (for fungus) and 37°C 12-16 hrs (for bacteria) and then, MTT assay was performed, same as described in 4.3e. Reference O.D. was taken as 600 nm and test O.D. was taken as 570 nm and the calculated difference was taken into consideration for finding percentage of inhibition of different microorganisms at various concentrations of hexane extract of *Cinnamomum zeylanicum*.

# CHAPTER 5 RESULTS AND DISCUSSION

## 5.1 Preparation of different extracts:

Six different solvents were considered based on different polarity index as mentioned in the table 5.1. Initially, 30gm of *Cinnamomum zeylanicum*, extracts were prepared from different solvents using soxhlet apparatus and finally dried by rotaevaporator to get final yield as mentioned in table 5.1. Aqueous extract was prepared by evaporating the water and its yield was not known. Again, we have prepared new extracts with some modification as discussed in material and methods using 40 gm *Cinnamomum zeylanicum* and the yields are given in table 5.2.

**Table 5.1: Solvent extract of 30 gm of *Cinnamomum zeylanicum* and their yield**

| Extract (in solvent) | Operated temperature(°C) | Yield( in gms) |
|---|---|---|
| **Hexane** | 50 | 0.2 |
| **Diethyl ether** | 25 | 0.06 |
| **Ethyl acetate** | 60 | 0.25 |
| **Ethanol** | 70 | 1.25 |
| **Acetone** | 45 | 0.9 |

**Table 5.2: Solvent extract of 40 gm of *Cinnamomum zeylanicum* and their yield**

| Extract (in solvent) | Operated temperature(°C) | Yield( in x mg/ml) |
|---|---|---|
| **Hexane** | 50 | 100 |
| **Diethyl ether** | 25 | 40 |
| **Ethyl acetate** | 60 | 100 |
| **Ethanol** | 70 | 250 |
| **Acetone** | 45 | 300 |

## 5.2 Effect of solvent extracts of *Cinnamomum zeylanicum* on the growth of breast cancer cell lines (MCF-7)

### 5.2.1 Effect of 20 and 40 µl solvent extracts

Each extract was finally dissolved in 10% DMSO and filtered for assessing their potential of the effect on growth on the MCF-7 cells. 20 µl of each extract were added in the culture and incubated for 72 hrs at 37°C and 5% $CO_2$. MTT assay was done to evaluate the effect of these extracts on MCF-7 cell lines and then readings were taken at 570nm with respect to reference wavelength of 630nm. Optical density which corresponds to cell proliferation was analyzed for different solvent extracts and control (DMEM + MCF-7 cells + DMSO). 20µl of *Cinnamomum zeylanicum* extracts (hexane, diethyl ether, ethyl acetate and acetone) except aqueous and ethanolic extract have shown that the inhibition in proliferation as compared to control (Table 5.3 and Figure 5.1). The experiments were carried out with 40 µl as well, to look for the effect on increasing the volume of the extract. Hexane and acetone extracts have shown the same inhibition effect like 20 µl (Table 5.4 and Figure 5.2). But 40 µl of others extracts (diethyl ether, ethyl acetate, ethanol, aqueous) did not show any inhibition (Table 5.4 and Figure 5.2).

**Table 5.3: Effect of 20µl of different solvent extracts of *Cinnamomum zeylanicum***

| 20µl | Control | Hexane | Diethyl ether | Ethyl acetate | Ethanol | Acetone | Aqueous |
|---|---|---|---|---|---|---|---|
| Exp 1 | 0.1942 | 0.0068 | 0.1010 | 0.1671 | 0.5278 | 0.0768 | 0.2152 |
| Exp 2 | 0.1981 | 0.0072 | 0.0951 | 0.1470 | 0.5171 | 0.1285 | 0.2191 |
| Exp 3 | 0.1563 | 0.0243 | 0.0628 | 0.0745 | 0.1878 | 0.0042 | 0.0952 |
| Exp 4 | 0.1883 | -0.0099 | 0.1088 | 0.1256 | 0.3227 | -0.0085 | 0.2071 |
| AVG | 0.1842 | 0.0071 | 0.0919 | 0.1285 | 0.3888 | 0.0503 | 0.1842 |
| stdev | 0.0190 | 0.0140 | 0.0202 | 0.0398 | 0.1639 | 0.0643 | 0.0595 |

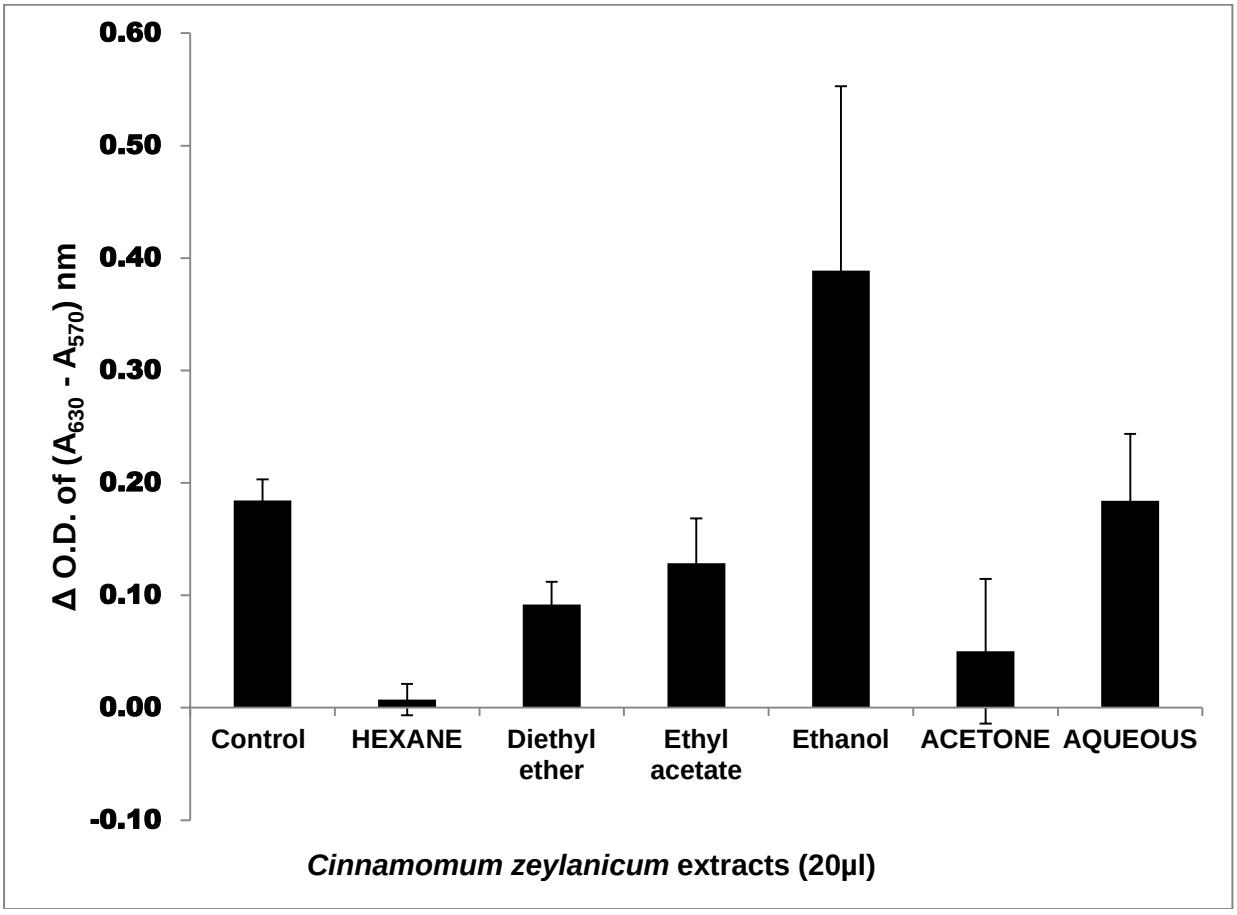



**Fig 5.1: Effect of 20µl of different solvent extracts of *Cinnamomum zeylanicum* to the proliferation of MCF-7 cell lines. Mean and standard deviation of calculated difference in optical density at 630nm and 570nm obtained by quantification of cells after effects of 20µl of different solvent extracts of *Cinnamomum zeylanicum* on MCF-7 cells from four different experiments were plotted in the graph.**

**Table 5.4: Effect of 40µl of different extracts of *Cinnamomum zeylanicum***

| 40µl | Control | hexane | Diethyl ether | Ethyl acetate | Ethanol | acetone | aqueous |
|---|---|---|---|---|---|---|---|
| **Exp 1** | 0.07107 | 0.02603 | 0.18003 | 0.17983 | 0.66460 | 0.00570 | 0.26693 |
| **Exp 2** | 0.04470 | 0.03290 | 0.21663 | 0.15397 | 0.55260 | 0.09963 | 0.36787 |
| **Exp 3** | 0.09857 | 0.03483 | 0.11730 | 0.05927 | 0.25983 | -0.01600 | 0.16670 |
| **Exp 4** | 0.19477 | 0.03190 | 0.14027 | 0.13667 | 0.38017 | -0.00113 | 0.26390 |
| **AVG** | 0.1023 | 0.0314 | 0.1636 | 0.1324 | 0.4643 | 0.0221 | 0.2663 |
| **stdev** | 0.0655 | 0.0038 | 0.0439 | 0.0519 | 0.1796 | 0.0525 | 0.0821 |

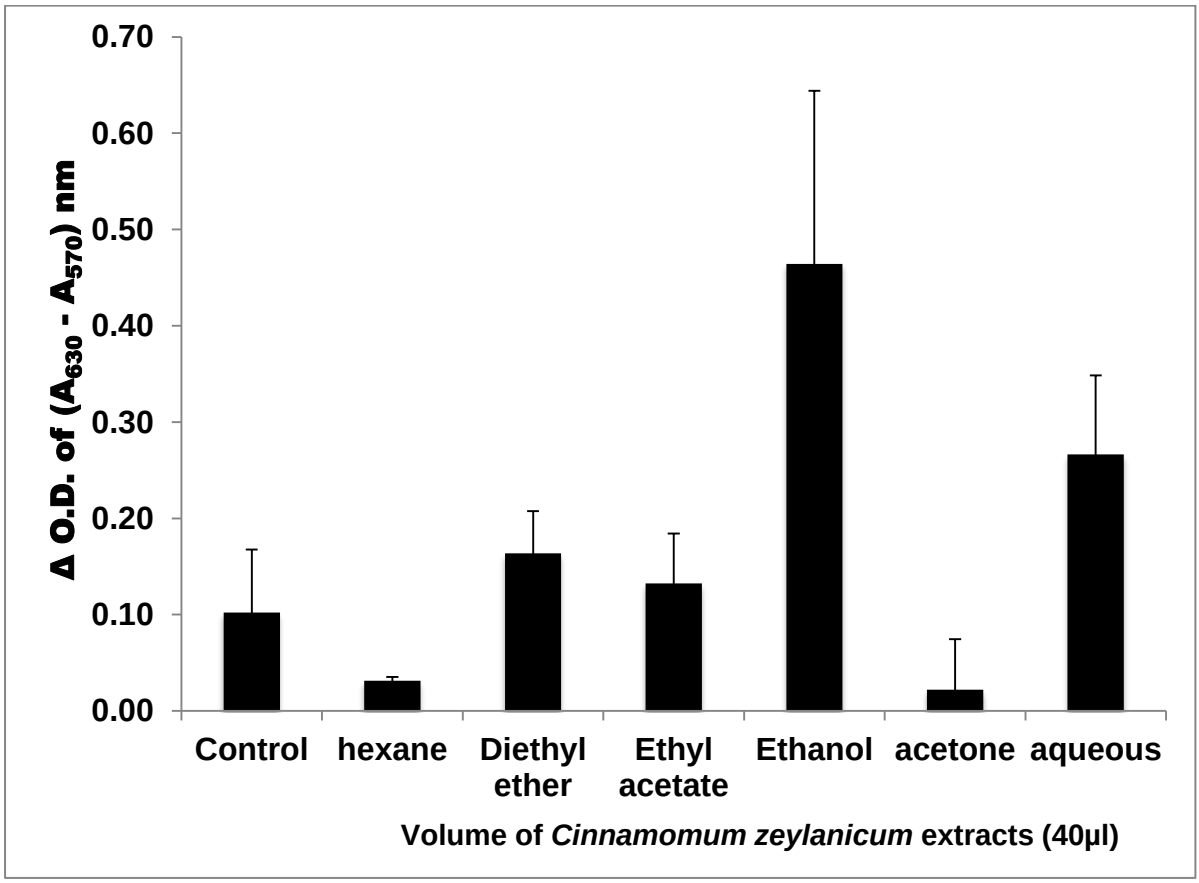



**Figure 5.2: Effect of 40µl of different extracts of *Cinnamomum zeylanicum* to the proliferation of MCF-7 cell lines. Mean and standard deviation of optical density of calculated difference in optical density at 630nm and 570nm obtained by quantification of cells after effects of 40µl of different solvent extracts of *Cinnamomum zeylanicum* on MCF-7 cells from four different experiments were plotted in the graph.**

### 5.2.2 Effect of different volume gradients of solvent extract

We observed variation in proliferation effect with 20 µl and 40 µl, hence we tested the effect with gradients of different volumes. So, firstly we made gradients of our extracts with volume of 10, 20, 30 and 40 µl and then evaluated for the effect. Hexane, diethyl ether and ethyl acetate extract have shown maximum inhibition at volume of 10µl whereas acetone shows maximum inhibition at 30µl volume (Table 5.5 and Figure 5.3). A different volume gradient (0.625, 1.25, 2.5, 5, 10 and 20 µl) were used to analyze the further effect. Maximum inhibition effects were observed at volume of 5µl for hexane, diethyl ether and ethyl acetate while at 20µl volume for acetone (Table 5.6 and Figure 5.4).

Experiment with aqueous extract was carried out with 10, 20, 30 and 40 µl and a mixed pattern was observed were observed. Inhibition in growth observed at 10 and 20 µl while 30 and 40 µl of this extract showed proliferative effect towards MCF-7 cells (Table 5.9 and Figure 5.7). Thus, it could be inferred that increasing volumes of aqueous extracts lead towards proliferation of MCF-7 cells.

Interestingly, in the preliminary experiments ethanolic and aqueous extracts showed proliferative effect rather than inhibition. So, we thought of doing experiments with ethanolic and aqueous extracts as well in from of gradient volumes in same form i.e. with volume of 10 µl, 20 µl, 30 µl, 40 µl (Table 5.7 and Figure 5.5, Table 5.9 and Figure 5.7 respectively). Similarly, another gradient was made with volume of 0.625 µl, 1.25 µl, 2.5 µl, 5 µl, 10 µl, 20 µl and correspondingly graph was plotted (Table 5.8 and Figure 5.6). Thus, from Figure 5.6 we could conclude from graph that as compared to control, the volume of 2.5 µl and 5µl of ethanolic extracts give effect of inhibition on MCF-7 cells. Ethanolic extracts gave kind of mixed effects on MCF-7 cells. No fixed pattern was depicted. Except these two, rest all volumes showed proliferative effect on MCF-7 cells.

**Table 5.5: Percentage of inhibition of different extracts on MCF-7 with their gradient volumes (10-40 µl)**

| | 10 µl | 20 µl | 30 µl | 40 µl |
|---|---|---|---|---|
| **Hexane** | 109.2 | 94.8 | 85.7 | 74.1 |
| **diethyl ether** | 85.0 | 51.0 | 30.0 | 4.5 |
| **ethyl acetate** | 61.3 | 42.8 | 35.3 | 34.9 |
| **acetone** | 68.7 | 100.9 | 126.1 | 108.4 |

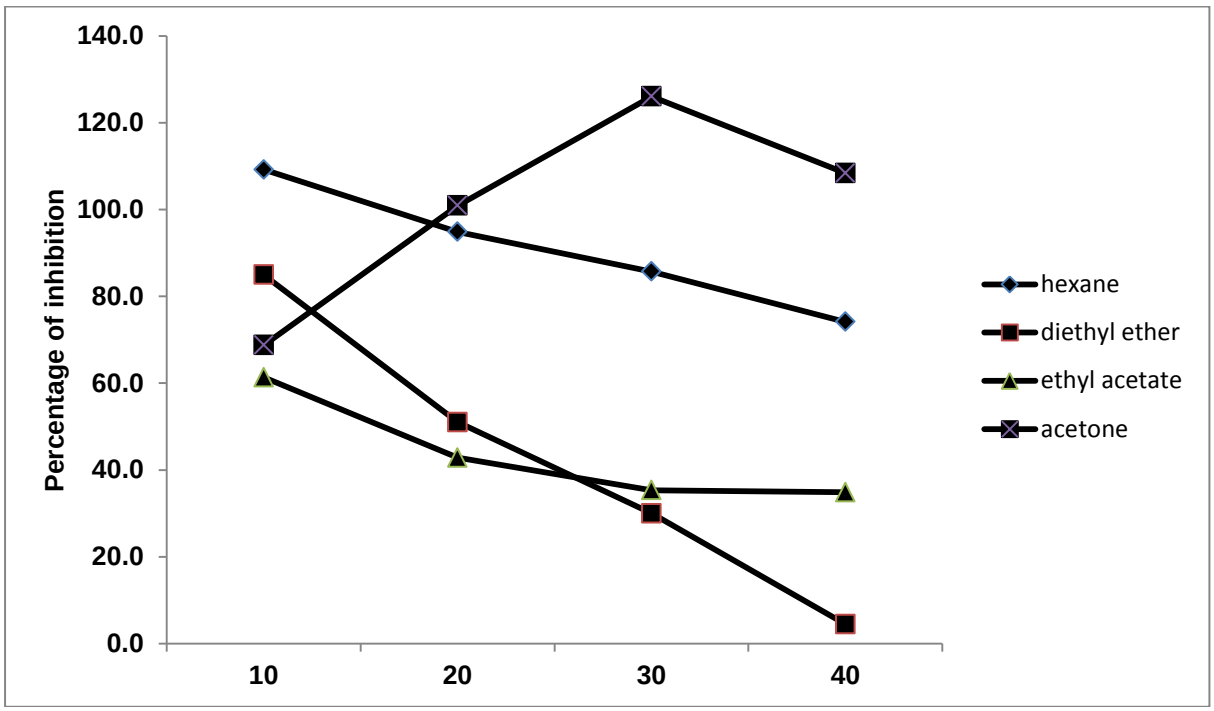



**Fig 5.3: Effect of different volumes of hexane, diethyl ether, ethyl acetate and acetone *Cinnamomum zeylanicum* extracts (10-40 µl) on MCF-7 cells.**

**Table 5.6: Percentage of inhibition of different extracts on MCF-7 with their gradient volumes (0.625-20 µl)**

| | 0.625µl | 1.25µl | 2.5µl | 5µl | 10µl | 20µl |
|---|---|---|---|---|---|---|
| **hexane** | 43.36 | 52.44 | 80.93 | 85.78 | 79.11 | 71.05 |
| **diethyl ether** | 28.80 | 32.55 | 68.13 | 79.09 | 65.63 | 29.71 |
| **ethyl acetate** | 8.56 | 6.41 | 53.39 | 65.80 | 64.94 | 47.47 |
| **acetone** | 34.35 | 31.97 | 44.32 | 15.76 | 35.50 | 102.00 |

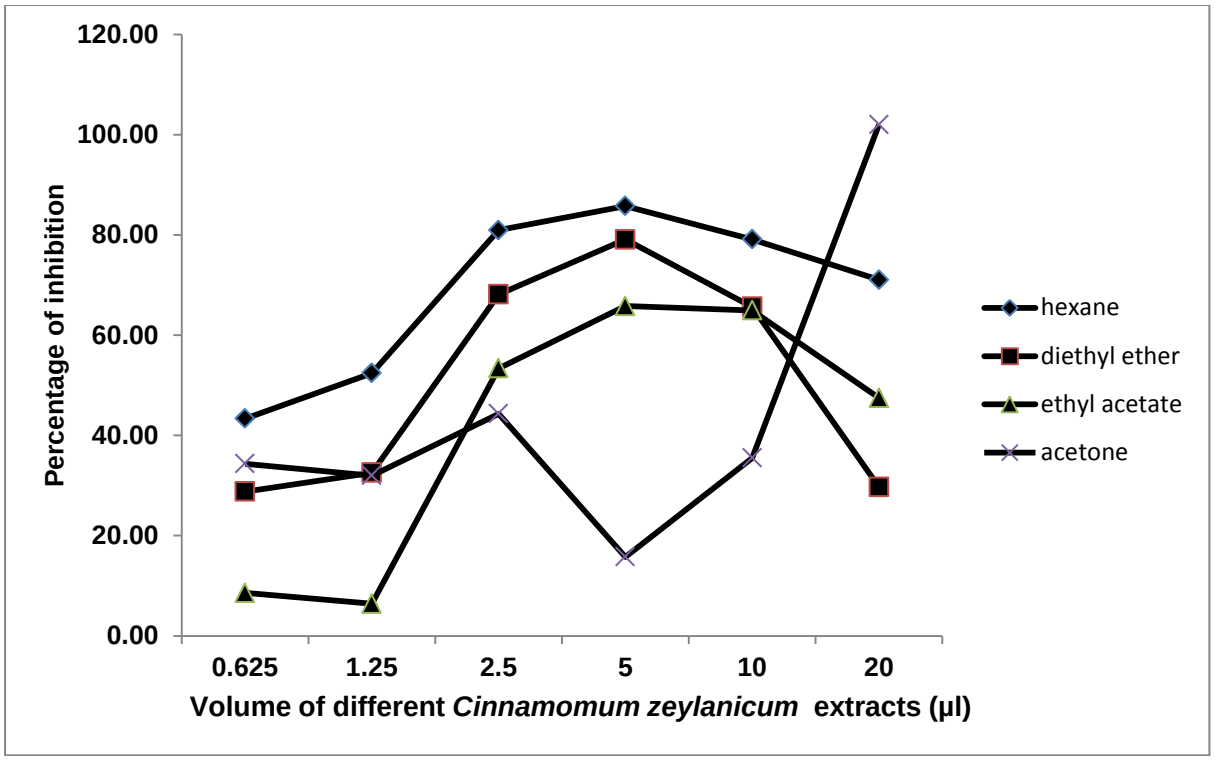



**Fig 5.4: Effect of different volumes of hexane, diethyl ether, ethyl acetate and acetone *Cinnamomum zeylanicum* extracts (0.625-20 µl) on MCF-7 cells.**

**Table 5.7: Effect of ethanolic extracts on MCF-7 with their gradient volumes (10-40 µl)**

| | 10 µl | 20 µl | 30 µl | 40 µl |
|---|---|---|---|---|
| **control** | 0.21 | 0.17 | 0.14 | 0.15 |
| **ethanol** | 0.20 | 0.26 | 0.29 | 0.32 |

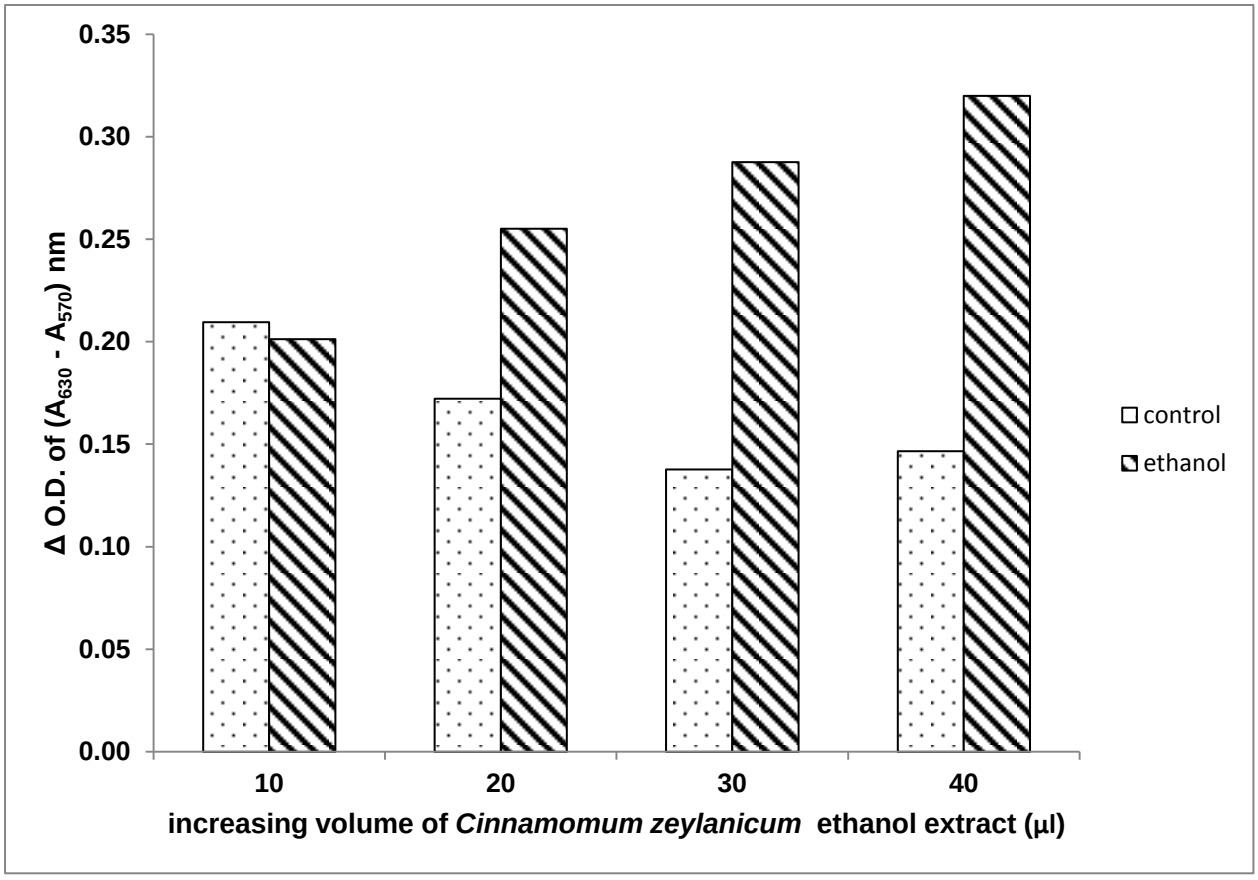



**Fig 5.5: Effect of different volumes (10-40 µl)) of various *Cinnamomum zeylanicum* ethanolic extract on MCF-7 cells.**

**Table 5.8: Effect of ethanolic extracts on MCF-7 cells with their gradient volumes (0.625-20 µl)**

| control | 0.625µl | 1.25µl | 2.5µl | 5µl | 10µl | 20µl |
|---|---|---|---|---|---|---|
| **exp 1** | 0.129 | 0.184 | 0.281 | 0.230 | 0.233 | 0.259 |
| **exp 2** | 0.199 | 0.175 | 0.121 | 0.175 | 0.234 | 0.313 |
| **mean** | **0.164** | **0.180** | **0.201** | **0.202** | **0.233** | **0.286** |
| **stdev** | **0.049** | **0.006** | **0.113** | **0.039** | **0.001** | **0.038** |
| **ethanol** | **0.625µl** | **1.25µl** | **2.5µl** | **5µl** | **10µl** | **20µl** |
| **exp 1** | 0.220 | 0.254 | 0.262 | 0.235 | 0.297 | 0.354 |
| **exp 2** | 0.199 | 0.175 | 0.121 | 0.175 | 0.234 | 0.313 |
| **mean** | **0.209** | **0.215** | **0.191** | **0.205** | **0.266** | **0.333** |
| **stdev** | **0.0153** | **0.0556** | **0.1001** | **0.0429** | **0.0451** | **0.0288** |

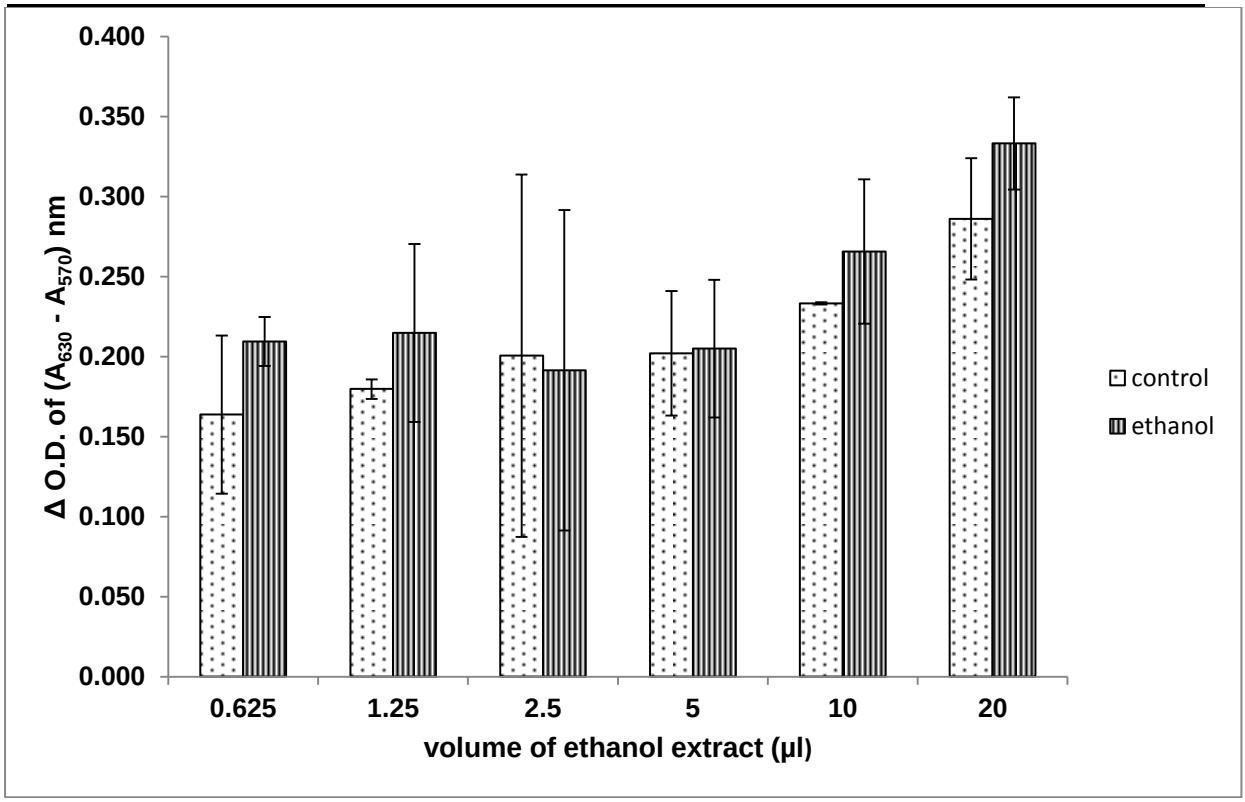



**Fig 5.6: Effect of different volumes (0.625-20 µl) of *Cinnamomum zeylanicum* ethanolic extract on MCF-7 cells. Graph was plotted between difference of O.D. of ($A_{630}$ - $A_{570}$) nm and increasing volume of *Cinnamomum zeylanicum* ethanolic extract used in experiments.**

**Table 5.9: Effect of aqueous extracts on MCF-7 with their gradient volumes (10-40 µl)**

| **control** | **10 µl** | **20 µl** | **30 µl** | **40 µl** |
|---|---|---|---|---|
| **exp 1** | 0.1507 | 0.1563 | 0.117 | 0.098567 |
| **exp 2** | 0.26842 | 0.18832 | 0.15862 | 0.19477 |
| **aqueous** | **10 µl** | **20 µl** | **30 µl** | **40 µl** |
| **exp 1** | 0.0282 | 0.0952 | 0.1342 | 0.1667 |
| **exp 2** | 0.0699 | 0.2071 | 0.2599 | 0.2639 |
| | | | | |
| **mean** | **10 µl** | **20 µl** | **30 µl** | **40 µl** |
| **control** | 0.2096 | 0.1723 | 0.1378 | 0.1467 |
| **aqueous** | 0.0491 | 0.1512 | 0.1971 | 0.2153 |

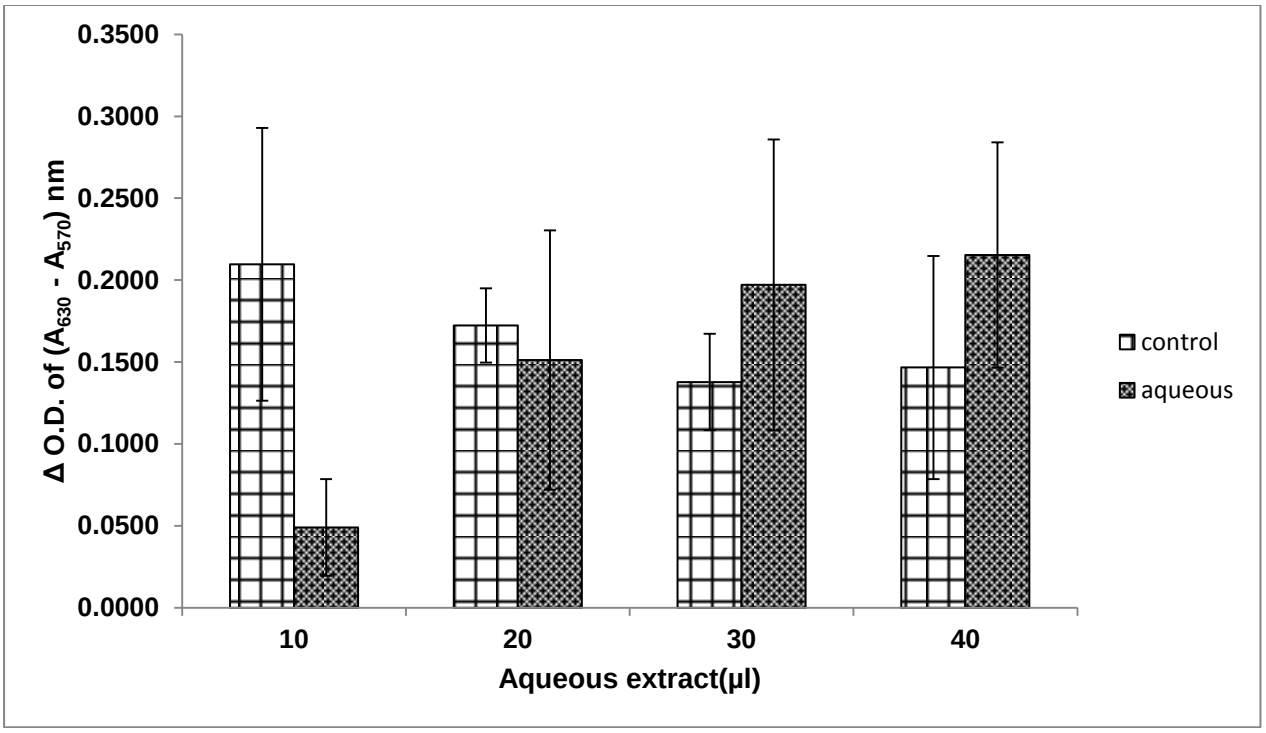



**Fig 5.7: Effect of different volumes of *Cinnamomum zeylanicum* aqueous extract on MCF-7 cells.**

### 5.2.3 Effect of solvent extracts with defined concentration (mg/ml) of *Cinnamomum zeylanicum* on the growth of breast cancer cell lines (MCF-7)

Absolute extracts (dissolved in pure DMSO) were taken and their final concentration was made to 10mg/ml by adding DMEM.

First experiment was designed to check preliminary cytotoxic potential of these prepared extracts on MCF-7 cells. DMSO control (Media + cell + DMSO) was taken was negative control and 0.02 mg/ml of paclitaxel was taken as positive control. Rest 2.5 mg/ml concentration of each extract was added to MCF-7 cells and effect was seen by conducting MTT assay after 72 hrs. Then, difference of O.D. at ($A_{630}$ - $A_{570}$) nm was taken and plotted against different concentrations of various extracts. Hexane has shown the inhibition in proliferation of MCF-7 which appears to cytotoxic effect (Table 5.10 and Figure 5.8). Rest three extracts (ethyl acetate, ethanol and acetone) has shown in increased in proliferation of MCF-7 cells as compared to DMSO control (Table 5.10 and Figure 5.8).

**Table 5.10: Effect of 2.5mg/ml solvent extract of *Cinnamomum zeylanicum* extracts on MCF-7 cells**

| Concentrations (mg/ml) | ΔO.D. |
|---|---|
| **DMSO control** | 0.095 |
| **Paclitaxel (0.02)** | 0.035 |
| **Hexane (2.5)** | 0.025 |
| **Ethyl acetate (2.5)** | 0.434 |
| **Ethanol (2.5)** | 0.433 |
| **Acetone (2.5)** | 0.447 |

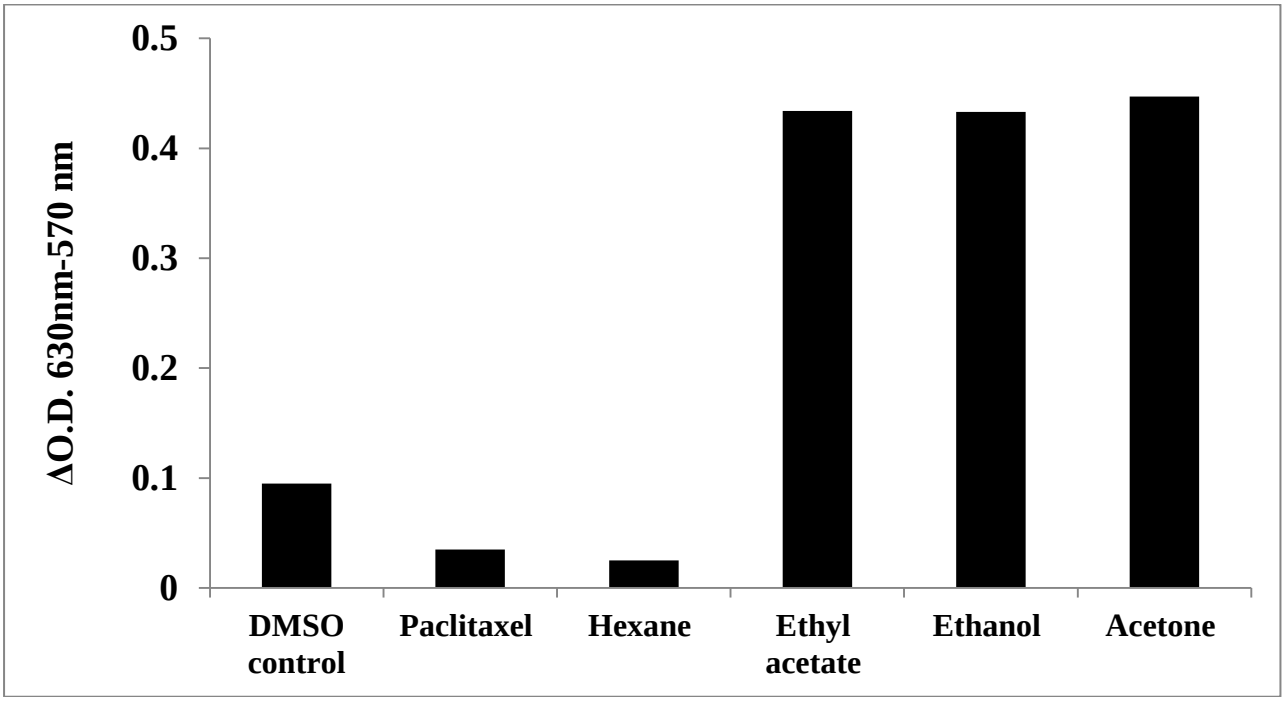



**Fig 5.8: Effect of various solvent *Cinnamomum zeylanicum* extracts on MCF-7 cells. 2.5 mg/ml of various solvent *Cinnamomum zeylanicum* extracts 0.02 mg/ml of pacilitaxel as positive control and DMSO as negative control was used in experiment.**

## 5.3 Cytotoxic effect of hexane extract on MCF-7 breast cancer cell lines

Hexane extract appears to have cytotoxic effect on MCF-7 breast cancer cell lines as observed in the previous effect hence it is considered further to study to see their effect on MCF-7 cells.

Now, the gradients of *Cinnamomum zeylanicum* hexane extract were applied in form decreasing concentrations from 1000 - 62.5 µg /ml so as to obtain range of $IC_{50}$ i.e. inhibitory concentration at which 50% of cells are being suppressed or are dead. Positive control was taken as 20 µg/ml of paclitaxel.

It was observed that $IC_{50}$ could be obtained between the concentrations of 125-62.5 µg/ml. Thus, for our next experiment, we planned to make the gradients from 250-62.5 µg/ml.

From Table 5.12 and Fig 5.10, it could be seen that effective percentage of inhibition is at 125 µg/ml i.e. around 50 % which could be inferred as $IC_{50}$. Thus, we obtained the quantitative concentration of hexane extract which could be used for further isolation of the respective compound responsible for this cytotoxic activity in MCF-7 cells.

**Table 5.11: Percentage of inhibition of *Cinnamomum zeylanicum* hexane extract at concentration gradients ranging from 2-0.25 mg/ml on MCF-7 cells**

| Concentrations | % inhibition |
|---|---|
| **0.02 mg/ml paclitaxel** | 95.63 |
| **2 mg/ml of *Cinnamomum zeylanicum* hexane** | 90.012 |
| **1 mg/ml of *Cinnamomum zeylanicum* hexane** | 93.6164 |
| **0.5 mg/ml of *Cinnamomum zeylanicum* hexane** | 91.7532 |
| **0.25 mg/ml of *Cinnamomum zeylanicum* hexane** | 87.5998 |

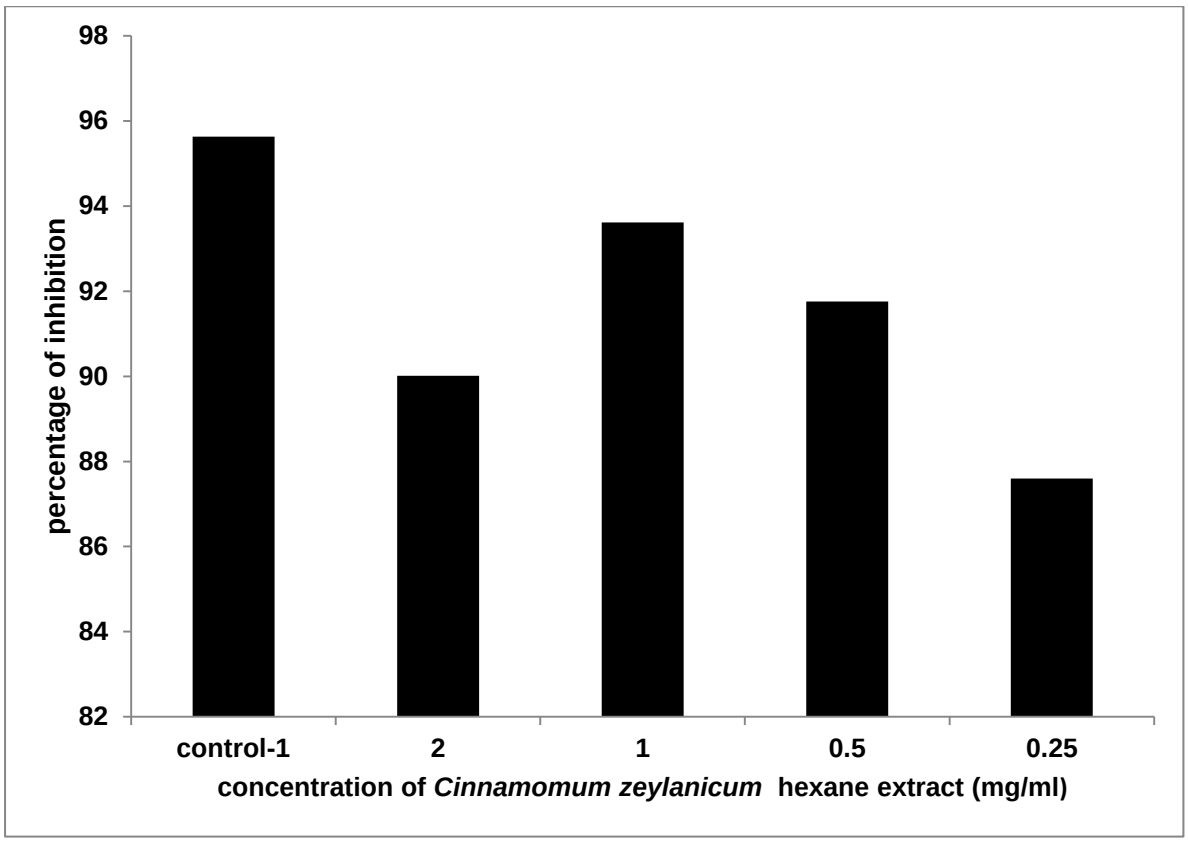



**Fig 5.9: Effect of various *Cinnamomum zeylanicum* hexane extract in decreasing form ranging from 2-0.25 mg /ml concentrations on MCF-7 cells. Graph was plotted between percentage of inhibition and various decreasing concentration gradients of hexane extracts in mg/ml**

**Table 5.12: Percentage of inhibition of *Cinnamomum zeylanicum* hexane extract at concentration gradients ranging from 1000-0.25 µg/ml on MCF-7 cells**

| Concentration of *Cinnamomumzeylanicum* hexane(µg/ml) | Percentage of inhibition |
|---|---|
| 20 paclitaxel | 87.04622 |
| 1000 | 71.37218 |
| 500 | 90.29663 |
| 250 | 91.6793 |
| 125 | 65.81897 |
| 62.5 | 40.44266 |

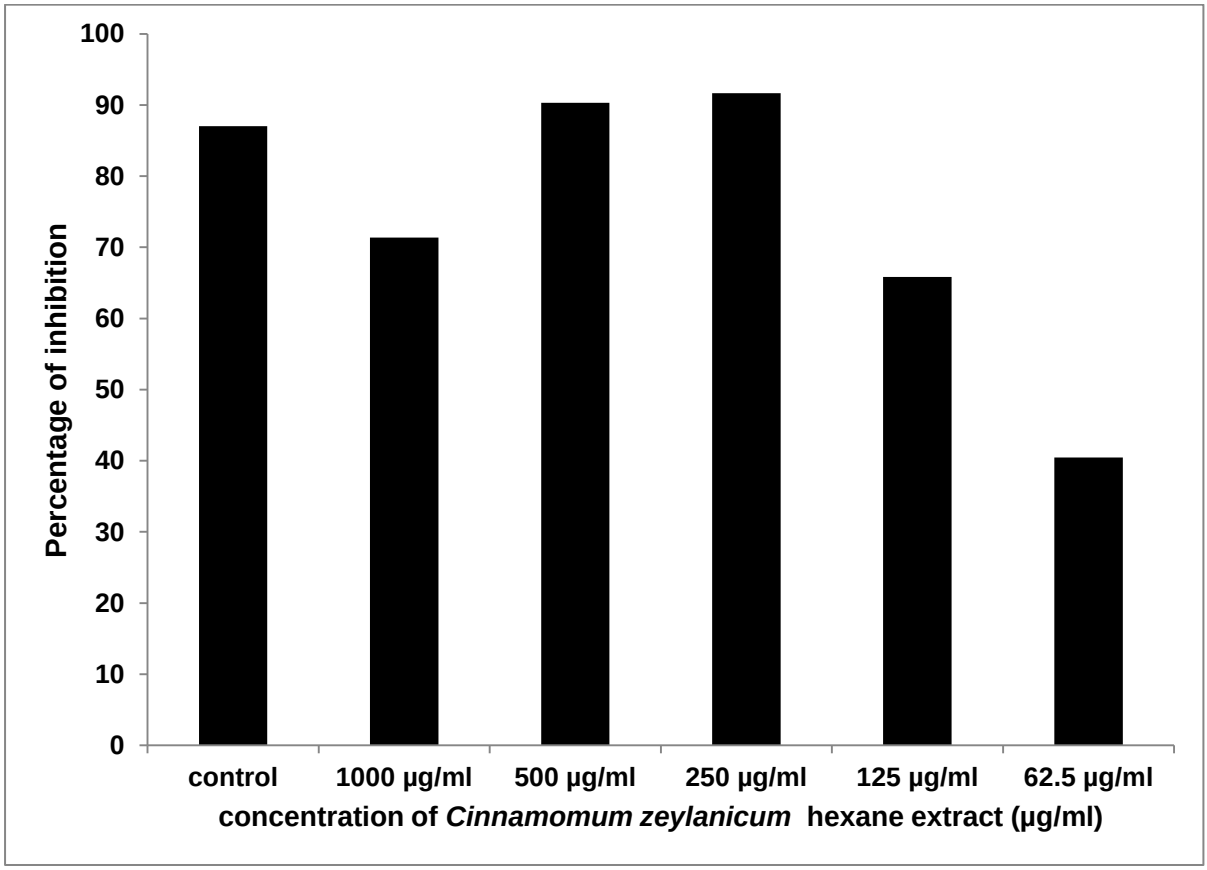



**Fig 5.10: Effect of various *Cinnamomum zeylanicum* hexane extract in decreasing form ranging from 1000-0.25 µg /ml concentrations on MCF-7 cells. Graph was plotted between percentage of inhibition and various decreasing concentration gradients of hexane extracts in µg/ml**

**Table 5.13: Percentage inhibition of *Cinnamomum zeylanicum* hexane extract at concentration gradients ranging from 250-62.5 µg /ml on MCF-7 cells**

| | control | 250 µg/ml | 187.5 µg/ml | 125 µg/ml | 62.5 µg/ml |
|---|---|---|---|---|---|
| **exp 1** | 98.340 | 65.177 | 67.577 | 54.111 | 13.072 |
| **exp 2** | 95.549 | 69.049 | 68.805 | 45.517 | 35.755 |
| **mean** | 96.945 | 67.113 | 68.191 | 49.814 | 24.413 |
| **stdev** | 1.97 | 2.74 | 0.87 | 6.08 | 16.04 |

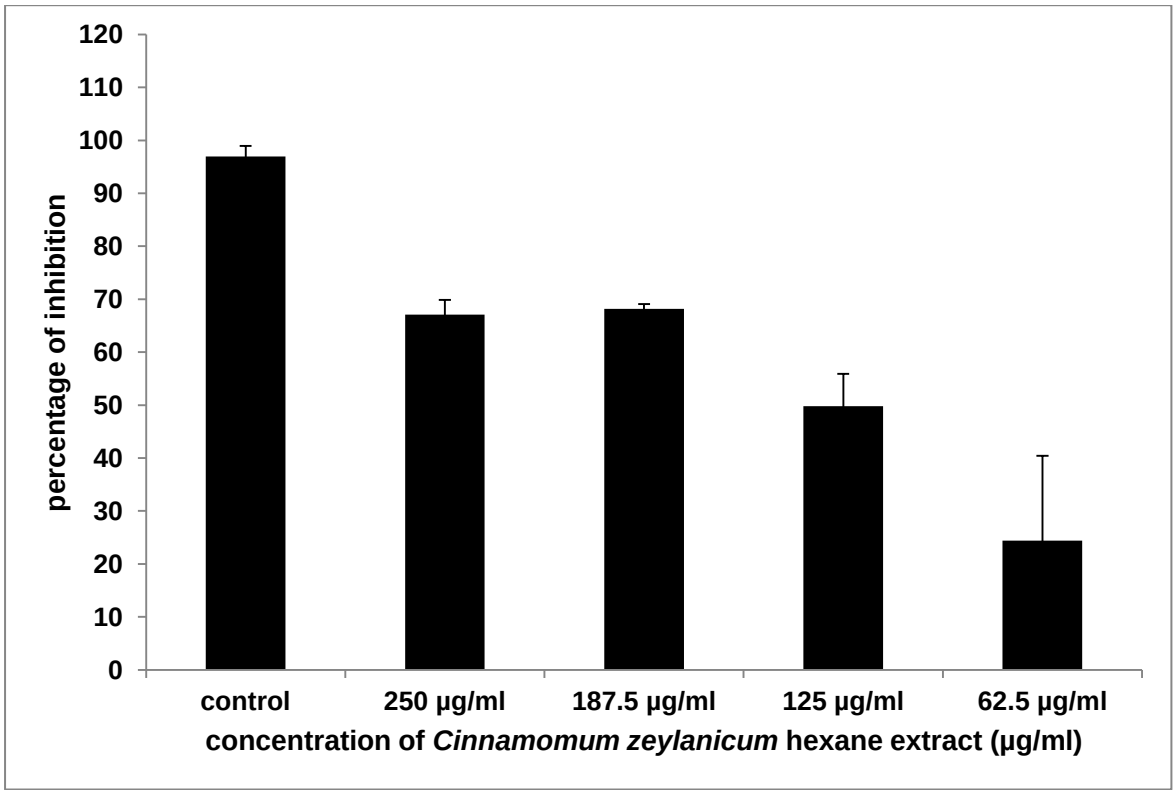



**Fig 5.11: Effect of various *Cinnamomum zeylanicum* hexane extract concentrations in decreasing form ranging from 250-62.5 µg /ml on MCF-7 cells. Graph was plotted between percentage of inhibition and various decreasing concentration gradients of hexane extracts in µg/ml**

## 5.4 Effect of hexane extract of *Cinnamomum zeylanicum* on the growth of different microorganisms

### 5.4.1 Effect of hexane extract on *Candida albicans*

We made 2.5 mg/ml and 5 mg/ml hexane extract of *Cinnamomum zeylanicum* and tested it on *Candida albicans* whose culture was freshly prepared by growing overnight in PDB and had 0.1 optical density at 600 nm which corresponds to 1.5 x $10^8$ CFU/ml as per Mcfarland standards. MIC was done in 96-well titre plate in which 10 µl of cells were added to each well and final volume was made 220 µl by adding PDB and hexane extract. So, final density of fungus going in titre plate was 1 million CFU/ml.

Corresponding controls of same DMSO concentrations were also used as negative controls to check their effect on growth of *Candida albicans.* This plate was incubated for 24 hrs and to do the quantitative analysis, MTT assay was done.

Interestingly, we saw more of decrease in growth of *Candida albicans* at concentration of 2.5 mg/ml rather than 5 mg/ml. This infers that growth of *Candida albicans* is inhibited at lower concentrations of hexane extract rather than higher. Also, we found that $IC_{50}$ was seen at concentration of 2.5 mg/ml of hexane extract (Table 5.14 and Fig. 5.12).

Thus, hexane extract of *Cinnamomum zeylanicum* has a good antifungal effect on growth of *Candida albicans.*

**Table 5.14: Percentage of inhibition of *Cinnamomum zeylanicum* hexane extract at 2.5 mg/ml and 5 mg/ml on growth of *Candida albicans***

| % inhibition | 2.5 mg/ml | 5 mg/ml |
|---|---|---|
| **hexane extract** | 50.16484 | 25.06311 |

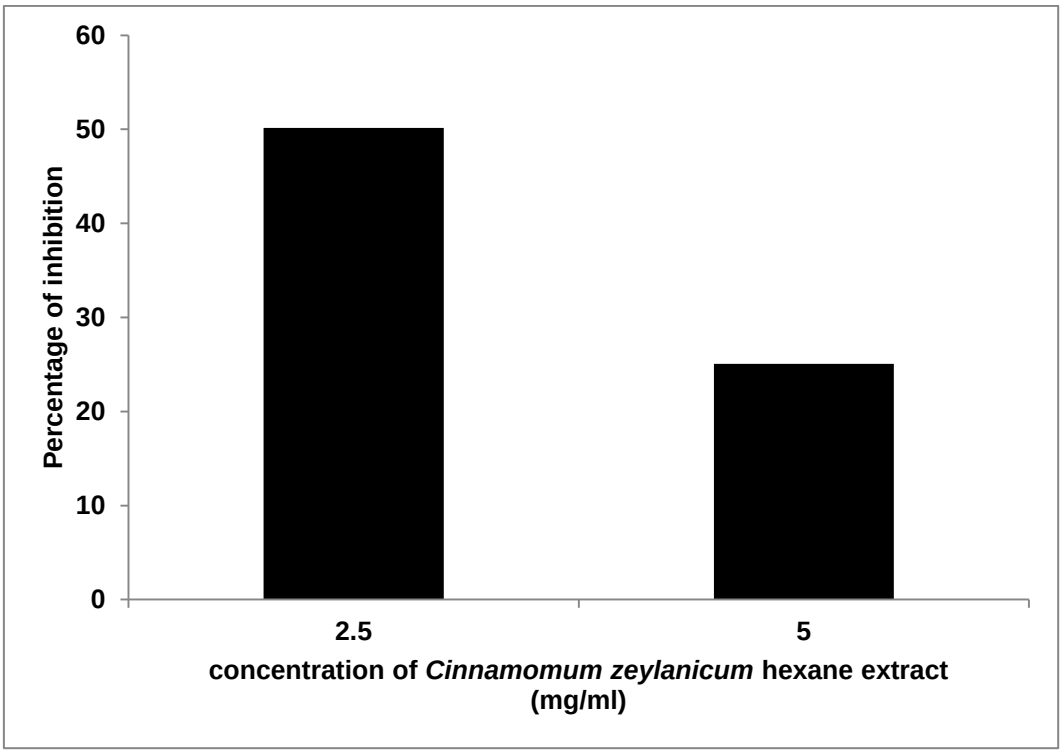



**Fig 5.12: Effect of various *Cinnamomum zeylanicum* hexane extract on growth of *Candida albicans*. Graph was plotted between percentage of inhibition and various concentration gradients of hexane extracts in mg/ml**

### 5.4.2 Effect of hexane extract on various bacterial strains

1 mg/ml concentration of Cz hexane extract was tested for MIC in bacterial strains of

Gram negative: *Pseudomonas aeruginosa, Escherichia coli* (table 5.15 and figure 5.13)

Gram positive: *Staphylococcus aureus, Bacillus cereus* (table 5.16 and figure 5.14)

MIC by broth dilution method and then, MTT assay was carried out using Muller Hinton broth. From table 5.15 and figure 5.13, table 5.16 and figure 5.14, it could be seen that hexane extract has significant amount of inhibition as compared to negative control of DMSO.

Thus, Cz hexane extract at a concentration of 1mg/ml has significantly high percentage of antibacterial activity as inferred by this method.

**Table 5.15: Percentage of inhibition of 1 mg/ml of *Cinnamomum zeylanicum* hexane extract on Gram negative bacteria**

| % inhibition | *Pseudomonas aeruginosa* | *Escherichia coli* |
|---|---|---|
| **control** | 38.492 | 26.416 |
| **hexane extract** | 92.706 | 85.377 |

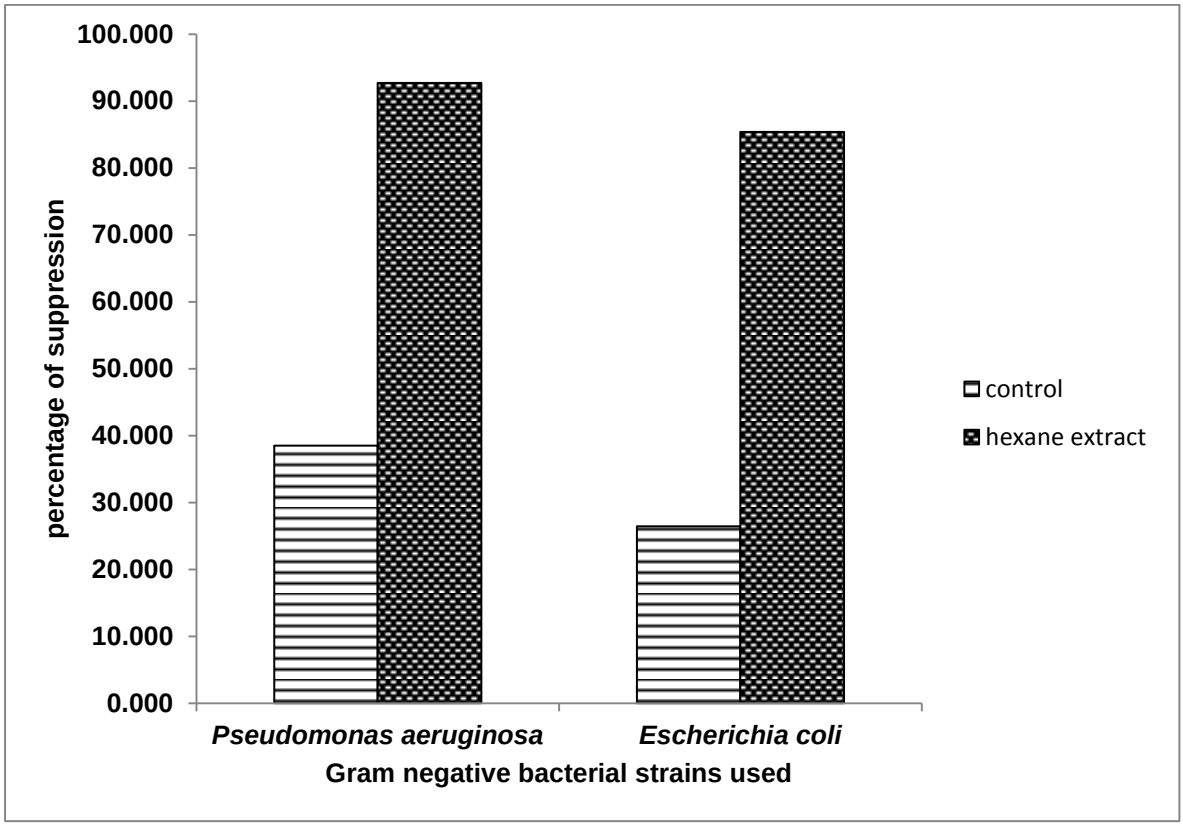



**Fig 5.13: Effect of 1 mg/ml of *Cinnamomum zeylanicum* hexane extract on Gram negative bacterial strains used. Graph was plotted between percentage of inhibition and strains of different Gram negative bacteria used in MIC.**

**Table 5.16: Percentage of inhibition of 1 mg/ml of *Cinnamomum zeylanicum* hexane extract on Gram positive bacteria**

| % inhibition | *Staphylococcus aureus* | *Bacillus cereus* |
|---|---|---|
| **control** | 15.323 | 10.488 |
| **hexane extract** | 85.141 | 83.533 |

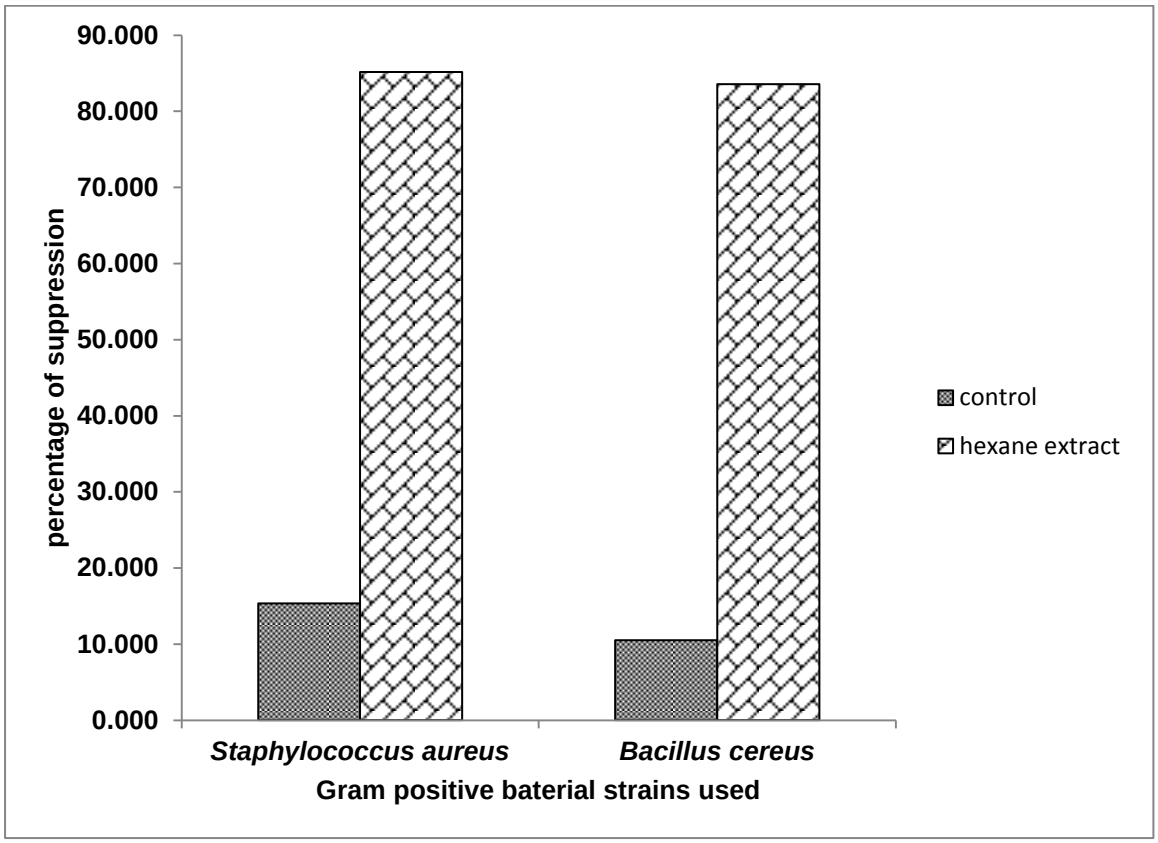



**Fig 5.14: Effect of 1 mg/ml of *Cinnamomum zeylanicum* hexane extract on Gram positive bacterial strains used. Graph was plotted between percentage of inhibition and strains of different Gram positive bacteria used in MIC.**

# CHAPTER 6 CONCLUSION

*Cinnamomum zeylanicum* which is commonly called as Dalchini, is considered as the true species of cinnamon. It is harvested from the evergreen, tropical trees of 'Ceylon' cinnamon and thus, it is one of the most expensive, flavorful and versatile aromatic spices. Sometimes, this species is also called as 'true cinnamon' or *Cinnamomum verum* which is mostly exported as 'quills' and barks.

It's bark contains various bioactive compounds, which are responsible for its aroma and world famous exotic flavor. By doing Soxhlet extraction using various solvents, (according to their polarity index) like hexane, diethyl ether, ethyl acetate, ethanol, acetone and water; we wanted to extract these compounds and test them for their antiproliferative effects on MCF-7 cells and various microorganisms. By conducting optimized soxhlet extraction and drying these extracts using rotaevaporator, we obtained absolute concentration of compound in each solvent by dissolving them in pure DMSO.

After, dilution of these various extracts, we tested them on MCF-7 cells using MTT assay, which is a colorimetric assay and we found out of all, hexane extract was giving best results as compared to others, so, we decided to move on and work with hexane extract itself. Hexane extract of *Cinnamomum zeylanicum* was found to give 50% inhibition at a concentration of 125 μg/ml. Hexane is known to mainly dissolve any fats present in sample; hence, we could conclude that there must be any oily compound present in sample which resulted in inhibition in proliferation of these cancerous cells.

Interestingly, on the other hand, ethanolic extracts were found to have proliferative effect on MCF-7 cells. This was an interesting finding and more work must be carried out to infer these results.

We also tested hexane extract on *Staphylococcus aureus, Bacillus cereus, Escherichia coli,* and *Pseudomonas aeruginosa* which are bacterial strains and on fungus called as *Candida albicans.* We did MIC broth dilution assay along with MTT assay on each one of them and got very interesting results. $IC_{50}$ for *Candida albicans* was found at a concentration of 2.5 mg/ml of Cz hexane extract. For bacteria, we expressed the results in percentage of inhibition at concentration of 1 mg/ml of Cz hexane extract. Due to lack of time, we could not precisely do work with microbes.

# CHAPTER 7 SUMMARY

India is known to be one of the richest sources of exotic spices throughout the world. Various ethnobotanical studies and Vedic systems have been followed in many Asian countries for the use of spices not only in culinary world but also in traditional medicinal use in various forms. So, cinnamon is also such a spice which is known for its various medicinal properties like antioxidant, antidiabetic, antimicrobial, antidiarrheal, antiflatulent etc. Our current project was focused on studying the effect of barks of *Cinnamomum zeylanicum*'s different extracts on breast cancer cells (MCF-7) and on various microbes.

We prepared different extracts of *Cinnamomum zeylanicum* barks in solvents with different polarities (hexane, diethyl ether, ethyl acetate, ethanol, acetone and water) by optimized soxhlet apparatus method and then vacuum evaporation of these solvents (rotary evaporator under reduced pressure). The yield was calculated and then extracts were diluted to 10% DMSO using complete DMEM.

Diluted extracts were initially tested *in vitro* on MCF-7 cells by doing MTT assay using 96-well titer plates. Very good percentage of inhibition was observed in the proliferation of these cancerous cells, when used in high amounts. We also obtained $IC_{50}$ i.e. concentration at which 50% of cell growth is inhibited; in **hexane extract** at a concentration of 125 μg/ml, which was very good.

Due to such a good anticancer activity shown by hexane extract, we continued with same extract in our antimicrobial experiments as well but in different concentrations. MIC broth dilution method along with MTT bioassay was done on on *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa* which are bacterial strains and on fungus called as *Candida albicans.*

$IC_{50}$ for *Candida albicans* was found at a concentration of 2.5 mg/ml of Cz hexane extract. For bacteria, we expressed the results in percentage of inhibition at concentration of 1 mg/ml of Cz hexane extract. Thus, in future we could carefully isolate the respective compound from our crude Cz hexane extract with the help of different methods and we might get a novel bioactive compound which could be helpful in benefit of future mankind.

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