Effect of *Syzygium aromaticum* on the growth of cancer cells and microbes

A Dissertation report

Submitted in partial fulfillment of the requirement for

the award of degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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JULY, 2014

Certificate

This is to certify that the project entitled — **Effect of *Syzygium aromaticum* on the growth of cancer cells and microbes** being submitted by Pankaj Sharma, Roll No.301201013 in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a bonafide work carried out under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled — **Effect of *Syzygium aromaticum* on the growth of cancer cells and microbes** in partial fulfillment of the requirements for the award of Master in Science in Biotechnology, Department of Biotechnology and Environmental Sciences, 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 and Environmental Sciences, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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

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ABSTRACT

Clove, the sun-dried unopened flower bud from the plant *Syzygium aromaticum* is a commonly used spice and food flavor. Clove oils, may play important roles in prevention and treatment of various cancers. The main constituents in clove are polypropenoids and include thymol, carvacol, cinnamaldyhyde, and eugenol. Eugenol, which accounts for the majority of the essential oil of the plant, has been well proven to possess antioxidant effects. clove has shown promise as chemo preventative and therapeutic agents in cancer. *In vitro* and *in vivo* study has reported to have anti-inflammatory and antitumorigenic properties in *Syzygium aromaticum*. Different solvents (Hexane, Diethyl ether, Ethyl acetate, Ethanol, Acetone and aqueous) extracts were prepared based on varying polarity index. Each of these extracts have shown anticancer activity, but most pronounced effect was observed in hexane. The Hexane extract of clove showed an activity against cancer cells at a minimum concentration of 125µg/ml, and anticancer activity at a concentration 350µg/ml showed 50% inhibition (IC_{50}) against MCF-7 cells. Clove buds extracts have shown strong antibacterial activities against *Staphylococcus aureus, Bacillus cereus, Escherichia coli,* and *Pseudomonas aeruginosa* and similarly shown a very good antifungal activity against *Candida albicans* (agar-well diffusion and minimum inhibitory concentration tests).

KEYWORDS:- Clove, Eugenol, PBMCs, MTT, MCF-7 cells, Extracts

CONTENTS

List of Abbreviations

NCBI	National Council of Biological Information
PBMC	Peripheral Blood Mononuclear Cell
WHO	World Health Organization
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffer Saline
EDTA	Ethylenediaminetetraaceticacid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
ELISA	Enzyme-linked immunosorbent assay
Da	Dalton
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
AP1	Activator Protein 1 (AP-1)
MCF-7	Michigan Cancer Foundation-7
MTT	3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
FDA	Food and Drug Administration
CFR	Code of Federal Regulations
µg/ml	microgram per milliliter

INT	p-iodonitrotetrazolium violet
MIC	Minimum Inhibitory Concentration
µl/ml	microliter per milliliter
w/v	weight/volume
ATCC	American Type Culture Collection
FBS	Fetal Bovine Serum
NCCLS	National Committee on Clinical Laboratory Standards
BSL-2	Biosafety Level -2
rpm	Rotation per minute
mol/l	Moles per liter
IU/ml	International Unit per milliliter
mM	milliMolar
RPMI	Rosewell Park Memorial Institute
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffer Saline

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CHAPTER 1 INTRODUCTION

Clove, the sun-dried unopened flower bud from the plant *Syzygium aromaticum* is a commonly used spice and food flavor. Clove oils, may play important roles in prevention and treatment of various cancers. The objective of the present study is to compare the in vitro anticancer activities of different extracts of Clove (*Syzygium aromaticum*), against different kinds of cancer cell line. The main constituents in clove are polypropenoids and include thymol, carvacol, cinnamaldyhyde, and eugenol. Eugenol, which accounts for the majority of the essential oil of the plant, has been well proven to possess antioxidant effects. clove has shown promise as chemo preventative and therapeutic agents in cancer. In vitro and in vivo, each of these compounds has demonstrated potent anti-inflammatory and antitumorigenic properties.

1.1 *Syzygium aromaticum*

Syzygium aromaticum is an evergreen tree from the family Myrtaceae. The tree is cultivated in most tropical countries including Malaysia, Indonesia, Zanzibar, India, and Sri Lanka. The essential oil obtained from the dried flower buds of clove has been used since ancient times in traditional medicine, perfume industry and in flavorings. Traditional uses of clove oil include treating burns, dental care to relieve pain and to treat gum infections when used at high concentrations , and to treat respiratory and digestive problems . Phytochemical studies showed eugenol, eugenol acetate, caryophyllene, and sesquiterpenes as the main constituents of clove essential oil.

The clove tree is an evergreen that grows to a height ranging from 8–12 m, having large square leaves and sanguine flowers in numerous groups of terminal clusters. The flower buds are at first

of a pale color and gradually become green, after which they develop into a bright red, when they are ready for collecting. Cloves are harvested when 1.5–2 cm long, and consist of a long calyx , terminating in four spreading sepals , and four unopened petals which form a small ball in the center.

The volatile clove oil containing up to 85% eugenol and acetyl eugenol, methyl salicylate, pinene, and vanillin. It contain gum, tannins, flavonoids and sterols.

Eugenol comprises 72-90% of the essential oil extracted from cloves, and is the compound most responsible for the cloves' aroma. Other important essential oil constituents of clove oil include acetyl eugenol, beta-caryophyllene and vanillin ;crategolic acid; tannins, gallotannic acid , methyl salicylate; the flavonoids eugenin , kaempferol , and eugenitin ; triterpenoids like oleanolic acid , stigmasterol and campesterol ; and several sesquiterpenes.

This study was undertaken to investigate the antibacterial , antifungal and anti cancer effect of six extracts (Hexane, Diethyl ether, Ethyl acetate, Ethanol, Acetone and Aqueous) from the buds of *S. aromaticum.*

CHAPTER 2 REVIEW OF LITERATURE

2.1 Antibacterial

The antibacterial properties of "*Syzygium aromaticum*" commonly known as "Clove" tested against food borne pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*). Agar diffusion susceptibility test revealed inhibition zone of clove sample. Compare to ethanolic extract, methanolic extract was showing best result against gram positive culture *Staphylococcus aureus* and two gram negative cultures *Pseudomonas aeruginosa* and *Escherichia coli* . The MIC value was determined by using broth dilution methods. Methanolic extract of clove was subjected to get the MIC against test organisms and it was found to be 2.31 mg/ml for *E. coli*, 0.385 mg/ml for *Stapylococcus aureus* and 0.01 mg/ml for *Pseudomonas aeruginosa* (Pandey *et al* 2011).

2.2 Natural antimicrobial agents

A study was carried out in the comparative analysis of ethanolic extract (50%) of clove & clove oil as natural antimicrobial agents on some food spoilage bacteria. The antibacterial activity of clove oil & its extract (50% ethanol) was tested against ten bacteria (seven Gram positive & three Gram negative) & seven fungi by agar well diffusion assays. The clove oil was found to be better antagonistic agent as compared to its extract counterpart by inhibiting both bacteria & fungi. The oil was found to be very effective with a lowest minimum inhibitory concentration (MIC) of 2.5% (v/v) against *Staphylococcus epidermidis* & *Staphylococcus sp*. amongst the fungi, *Aspergillus niger* was found to be highly sensitive to the oil. Sodium propionate (standard food preservative) was used as a positive control. Clove oil was found to be more effective as

compared to both clove extract & sodium propionate. This study shows the potential of clove oil to be used as food bio-preservative.

A crude MeOH extract of *Syzygium aromaticum* (clove) exhibited preferential growth-inhibitory activity against Gram-negative anaerobic periodontal oral pathogens, including *Porphyromonas gingivalis* and *Prevotella intermedia*. By means of bioassay-directed chromatographic fractionation, eight active compounds were isolated from this extract and were identified as 5,7-dihydroxy-2-methylchromone 8-C-beta-D-glucopyranoside, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid, and oleanolic acid, based on spectroscopic evidence. The antibacterial activity of these pure compounds was determined against *Streptococcus mutans*, *Actinomyces viscosus*, *P. gingivalis*, and *P. intermedia.* The flavones, kaempferol and myricetin, demonstrated potent growth-inhibitory activity against the periodontal pathogens *P. gingivalis* and *P. intermedia*.

2.3 Antifungal

Antifungal activities of clove essential oil and its volatile vapour against dermatophytic fungi including *Candida albicans*, *Epidermophyton floccosum*. *Microsporum audouinii*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* were investigated. Both clove essential oil and its volatile vapour strongly inhibit spore germination and mycelial growth of the dermatophytic fungi tested. The volatile vapour of clove essential oil showed fungistatic activity whereas direct application of clove essential oil showed fungicidal activity

Antifungal properties of some essential oils have been well documented. Clove oil is reported to have strong antifungal activity against many fungal species. In this study we have evaluated antifungal potential of essential oil of *Syzygium aromaticum* against some common fungal

pathogens of plants and animals namely, *Fusarium moniliforme*, *Fusarium oxysporum*, *Aspergillus* sp., *Mucor* sp., *Trichophyton rubrum* and *Microsporum gypseum*. All fungal species were found to be inhibited by the oil when tested through agar well diffusion method. Minimum inhibitory concentration (MIC) was determined for all the species. Column chromatography was performed to separate the eugenol rich fraction from clove oil. Out of seven fractions maximum activity was obtained in column fraction II. TLC and HPLC data confirmed presence of considerable Eugenol in fraction II and clove oil. Microscopic study on effect of clove oil and column fraction II on spores of *Mucor* sp. and *M. gypseum* showed distortion and shrinkage while it was absent in other column fractions. So it can be concluded that the antifungal action of clove oil is due to its high eugenol content.

This study was carried out in order to investigate the potential of using plant oils derived from *Leptospermum petersonii* Bailey and *Syzygium aromaticum* L. Merr. Et Perry as natural antifungal agents. The antifungal effects of essential oils at concentrations of 0.05, 0.1, 0.15, and 0.2 mg/ml on the dermatophytes *Microsporum canis*, *Trichophyton mentagrophytes* , *Trichophyton rubrum*, *Epidermophyton floccosum*, and *Microsporum gypseum* were evaluated using the agar diffusion method. The major constituents of the active fraction against the dermatophytes were identified by gas chromatography-mass spectrometry and high-performance liquid chromatography analysis. The antifungal activities of S. aromaticum oil (clove oil) against the dermatophytes tested were highest at a concentration of 0.2 mg/ml, with an effectiveness of more than 60%. Hyphal growth was completely inhibited in *T. mentagrophytes, T. rubrum*, and *M. gypseum* by treatment with clove oil at a concentration of 0.2 mg/ml. Eugenol was the most effective antifungal constituent of clove oil against *the dermatophytes T. mentagrophytes* and *M. canis*. Morphological changes in the hyphae of *T. mentagrophytes,* such as damage to the cell

wall and cell membrane and the expansion of the endoplasmic reticulum, after treatment with 0.11 mg/ml eugenol were observed by transmission electron microscopy (TEM). At a concentration of 0.2 mg/ml, *L. petersonii* oil (LPO) was more than 90% effective against all of the dermatophytes tested, with the exception of *T. rubrum*. Geranial was determined to be the most active antifungal constituent of *L. petersonii* oil. Taken together, the results of this study demonstrate that clove and tea tree oils exhibited significant antifungal activities against the dermatophytes tested in this study

2.4 Cancer

Cancer is a class of diseases characterized by out-of-control cell growth. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected.

Cancer harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumors (except in the case of leukemia where cancer prohibits normal blood function by abnormal cell division in the blood stream). Tumors can grow and interfere with the digestive, nervous, and circulatory systems, and they can release hormones that alter body function. Tumors that stay in one spot and demonstrate limited growth are generally considered to be benign.

More dangerous, or malignant, tumors form when two things occur:

1. a cancerous cell manages to move throughout the body using the blood or lymph systems, destroying healthy tissue in a process called invasion
2. that cell manages to divide and grow, making new blood vessels to feed itself in a process called angiogenesis.

When a tumor successfully spreads to other parts of the body and grows, invading and destroying other healthy tissues, it is said to have metastasized. This process itself is called metastasis, and the result is a serious condition that is very difficult to treat.

2.4a Carcinogens

Carcinogens are a class of substances that are directly responsible for damaging DNA, promoting or aiding cancer. Tobacco, asbestos, arsenic, radiation such as gamma and x-rays, the sun, and compounds in car exhaust fumes are all examples of carcinogens. When our bodies are exposed to carcinogens, free radicals are formed that try to steal electrons from other molecules in the body. Theses free radicals damage cells and affect their ability to function normally.

2.4b Cancer cell types

Cancers are often referred to by terms that contain a prefix related to the cell type in which the cancer originated and a suffix such as -sarcoma, -carcinoma, or just -oma. Common prefixes include:

- Adeno- = gland
- Chondro- = cartilage
- Erythro- = red blood cell
- Hemangio- = blood vessels
- Hepato- = liver
- Lipo- = fat
- Lympho- = white blood cell
- Melano- = pigment cell

- Myelo- = bone marrow
- Myo- = muscle
- Osteo- = bone
- Uro- = bladder
- Retino- = eye
- Neuro- = brain

2.5 Breast Cancer

Breast cancer is a kind of cancer that develops from breast cells. Breast cancer usually starts off in the inner lining of milk ducts or the lobules that supply them with milk. A malignant tumor can spread to other parts of the body. A breast cancer that started off in the lobules is known as *lobular carcinoma*, while one that developed from the ducts is called *ductal carcinoma*.

Breast cancer is the most common invasive cancer in females worldwide. It accounts for 16% of all female cancers and 22.9% of invasive cancers in women. 18.2% of all cancer deaths worldwide, including both males and females, are from breast cancer.

2.6 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 acronym 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).

2.6a 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.

2.6b 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.

2.7 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.

2.8 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.

Spices, active ingredients of Indian cooking, may play important roles in prevention and treatment of various cancers. The objective of the present study is to compare the in vitro anticancer activities of three different extracts of Clove (Syzygium aromaticum L), a commonly used spice and food flavouring agent, against different kinds of cancer cell lines of various anatomical derivations. Water, ethanol and oil extracts were screened for anti proliferative activity against HeLa (cervical cancer), MCF-7 (ER + ve) and MDA-MB-231 (ER – ve) breast cancer, DU-145 prostate cancer and TE-13 esophageal cancer cell lines, along with normal human peripheral blood lymphocytes. Inhibition of cell proliferation was assessed using MTT assay as a vital stain. In the examined five cancer cell lines, the extracts showed different patterns of cell growth inhibition activity, with the oil extract having maximal cytotoxic activity. Morphological analysis and DAPI staining showed cytotoxicity to be a result of cell disruption with subsequent membrane rupture. Maximum cell death and apoptotic cell demise occurred in TE-13 cells within 24 hours by clove oil at 300µl/ml with 80% cell death whereas DU-145 cells showed minimal cell death. At the same time, no significant cytotoxicity was found in human

PBMC's at the same dose. (Miler, 2001; Das, 2002; Hou et al., 2003; Lin et al., 2003; Kim et al., 2005; Mori et al., 2006; Su et al., 2006)

Cloves (*Syzygium aromaticum*) have been used as a traditional Chinese medicinal herb for thousands of years. Cloves possess antiseptic, antibacterial, antifungal, and antiviral properties, but their potential anticancer activity remains unknown. In this study, we investigated the in vitro and in vivo antitumor effects and biological mechanisms of ethyl acetate extract of cloves and the potential bioactive components responsible for its antitumor activity. The effects of clove oil on cell growth, cell cycle distribution, and apoptosis were investigated using human cancer cell lines. The molecular changes associated with the effects of clove oil were analyzed by Western blot and (qRT)-PCR analysis. The in vivo effect of clove oil and its bioactive component was investigated using the HT-29 tumor xenograft model. We identified oleanolic acid as one of the components of EAEC responsible for its antitumor activity. Both clove oil and oleanolic acid display cytotoxicity against several human cancer cell lines. Interestingly, clove oil was superior to oleanolic acid and the chemotherapeutic agent 5-fluorouracil at suppressing growth of colon tumor xenografts. Clove oil promoted G0/G1 cell cycle arrest and induced apoptosis in a dose-dependent manner. Treatment with clove oil and oleanolic acid selectively increased protein expression of p21(WAF1/Cip1) and γ-H2AX and downregulated expression of cell cycle-regulated proteins. Moreover, many of these changes were at the mRNA level, suggesting transcriptional regulation by clove oil treatment. Our results demonstrate that clove extract may represent a novel therapeutic herb for the treatment of colorectal cancer, and oleanolic acid appears to be one of the bioactive components.

CHAPTER 3 OBJECTIVES

The main objectives of present study are to explore the anticancer, antibacterial and antifungal effect of different solvent extracts of clove buds.

Work plan of the current study is as follows:

1. Preparation of *Syzygium aromaticum* extract in different solvents
2. Determination of anticancer activity in MCF-7 breast cancer cell based on MTT assay
3. Estimation of antifungal activity based on MTT assay and agar diffusion assay
4. Estimation of antibacterial activity based on MTT assay

CHAPTER 4 MATERIAL AND METHOD

4.1 MATERIAL

Table 4.1: Requirements during the whole project work

REQUIREMENTS

- DMEM
- FBS
- Trypsin
- Sodium bicarbonate
- L-glutamine
- Penicillin Sodium G
- Streptomycin Sulphate
- Ficoll
- Trypan blue
- MTT reagent
- Dimethyl sulfoxide (DMSO)
- Nutrient agar
- Luria broth
- Muller Hinton agar
- Potato dextrose broth
- Phosphate buffer saline
- Silica gel G

Cultures:

Bacterial cultures:

– gram +ve bacteria (*Bacillus cereus, Staphylococcus aureus*)

– gram -ve bacteria (*Escherichia coli, Pseudomonas aeruginosa*)

Fungal culture:

Candida albicans

Animal cell culture:

MCF-7 (Breast cancer cell line)

PBMC

Composition:

Table 4.2 : Composition of Nutrient Agar

Peptone	0.5%
Beef extract/yeast extract	0.3%
Agar	1.5%
NaCl	0.5%
Distilled Water	
pH	6.8

Table 4.3 : Composition of Luria Broth

Tryptone	1.0%
Yeast extract	0.5%
NaCl	1.0%
pH	7.0

Table 4.4 : Composition of Muller Hinton Agar

Beef infusion	30.0%
Casein hydrolysate	1.75%
Starch	0.15%
Agar	1.7%
pH	7.0

Table 4.5 : Composition of PBS (Phosphate Buffered Saline)

NaCl (137mM)	8.01g/l
KCl (2.7mM)	0.2g/l
Na2HPO4 (4.3 mM)	1.44g/l
KH2PO4	0.24g/l
pH	7.4

Table 4.6 : Composition of PDA (Potato Dextrose Broth)

Water	~1000litre
Potatoes	200gram
Dextrose	20gram
Agar	20gram

4.2 METHOD

4.2a PREPARATION OF EXTRACTS

Clove extracts were prepared in different solvents. Buds of clove were washed and dried to remove any kind of dust and other materials. The dried buds of clove were grinded in a grinder to make a fine powder. 25 gram of this fine powder was taken and used different solvent for extract making. All these solvents were taken according to the polarity index , firstly taken non polar solvent and gradually goes to polar solvents. Six different solvents were used for such purpose and all these extract were made with Soxhlet apparatus.

Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. During each cycle, a portion of the non-volatile compound dissolves in the solvent. 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.

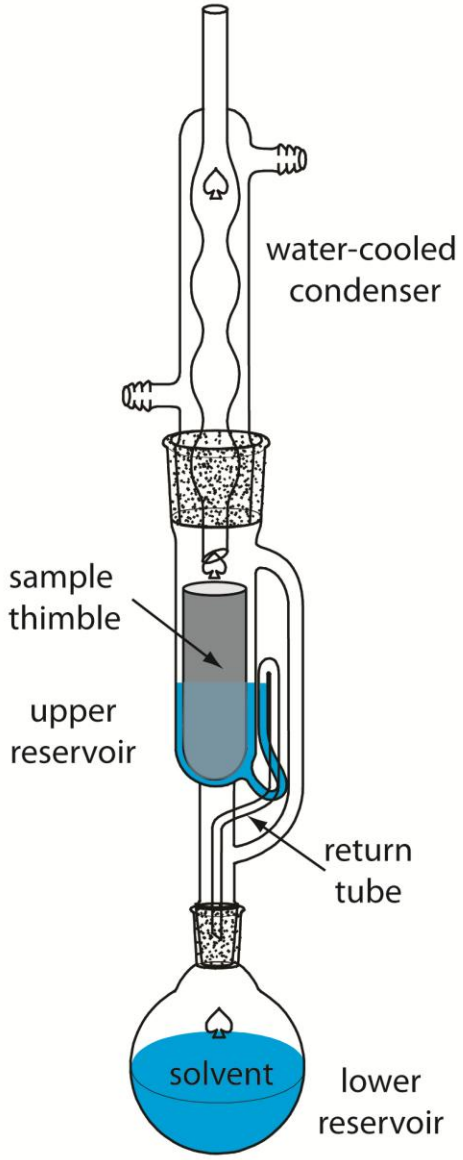

Figure 4.1 : Soxhlet extractor

These extracts were filtered by using Whatman filter paper no.1 in separate falcon tubes. Now the filtrate was evaporated with the help of Rotary vacuum evaporator and we get the dried powder.

Figure 4.2 : *Rotary vacuum evaporator*

Now dry mass was calculated using the formula:

Dry mass = initial weight – final weight

The dried mass was dissolved completely in pure DMSO . These dissolved extracts were now stored in aliquots at 0° C for further use.

4.3 DETERMINATION OF ANTIBACTERIAL ACTIVITY OF CLOVE

The antimicrobial activity of clove was determined in four different cultures of bacteria which include two gram positive and two gram negative. To find whether these different extracts of clove have any antimicrobial activity we have done agar well diffusion assay and Minimum Inhibitory Concentration Broth dilution.

4.3a Tested microorganism and growth conditions

The following bacteria strains were employed in the screening:

Gram-positive: *Staphylococcus aureus* and *Bacillus cereus*.

Gram negative: *Escherichia coli* and *Pseudomonas aeruginosa.*

Escherichia coli were activated in Luria broth and rest of the strains in Nutrient broth at 37º C for 18-24 hours at 120 rpm.

4.3b Maintenance of Microorganisms

The cultures were maintained and stored at 4ºC. Activation of the culture were carried out by streaking culture on to selective media and then incubating them overnight at 37º C. A single colony was picked from this plate and transferred to its respective broth and then incubated for 18-24 hours at 37º C prior to the test.

4.3c Antimicrobial Agent (as control)

The antimicrobial agent used in the present study was Streptomycin (1 mg/ml)

4.3d Estimation of antibacterial activity based on MTT assay

Procedure:

A suspension of test organism was prepared and made a standardized concentration at 600nm absorbance on spectrophotometer. Streptomycin was prepared as stock solutions of 1 mg/ml. The wells of a 96- well ELISA tray were filled with 100 µl of nutrient broth and 100 µl of extracts . The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm. 10µl of bacterial suspension was added to each well. And again absorbance of each well was recorded at same wavelength this was considered as 0 hour reading. The plate was incubated at 37o C for 24 h, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. This procedure eliminated the interference of the tested substance. All tests were performed in triplicate. The MICs value for a test extract was expressed as the lowest concentration that inhibits the bacterial growth.

4.4 DETERMINATION OF ANTIFUNGAL ACTIVITY BASED ON AGAR-WELL DIFFUSION AND MTT ASSAY

4.4a Agar - Well Diffusion assay

Initial screening of potential antibacterial activity from clove may be performed with crude extracts. Agar well assay is popular pre-screen assay used by the clinical microbiologists and phytochemists to check the potential antimicrobial activity of plants and their use in traditional medicines for treatment of infectious diseases (Navarro et al., 1996).

Procedure

The well-isolated colonies of the bacterial cultures from an agar plate culture were transferred into flask containing broth medium. The broth culture was incubated at 37º C for overnight and the turbidity of growing broth culture was adjusted to 0.5 McFarland standards with sterile saline solution. Then 100 µl of the bacterial inoculums was spread on agar plate by using the cotton swab method, and then prepared 5 wells of 6 mm each using a sterile cork borer under aseptic conditions. A fixed volume of extracts was then introduced into the bored agar well. The antimicrobials present in the extracts were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organism. These plates were then incubated at 37º C for duration depending on the test microorganism. After incubation a zone of inhibition was measured to the nearest whole millimeter. The resulting zones of inhibition were uniformly circular as there was a confluent growth of bacterial colonies called lawn of bacteria. The test was carried out in triplicates to reduce the chances of error.

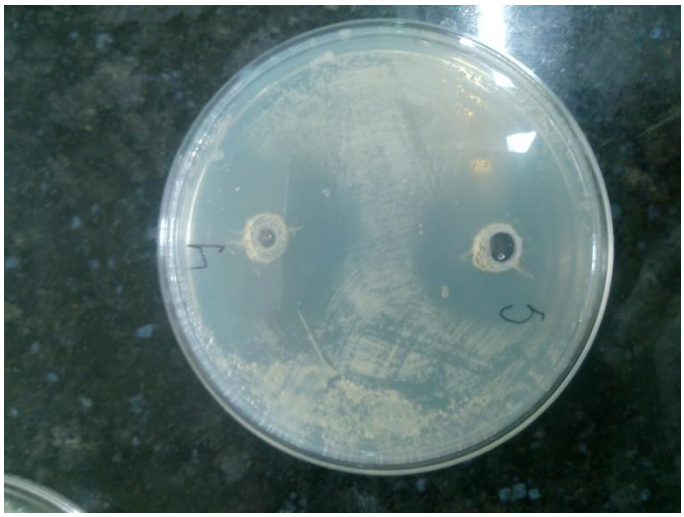

Figure 4.3 : Effect of *Syzygium aromaticum* extract on *Candida albicans*

4.4b Minimum Inhibitory Concentration Broth Micro dilution Assay

Procedure:

A suspension of test organism was prepared and made a standardized concentration at 600nm absorbance on spectrophotometer. Streptomycin was prepared as stock solutions of 1 mg/ml. The wells of a 96- well ELISA tray were filled with 100 µl of nutrient broth and 100 µl of extracts . The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm. 10µl of bacterial suspension was added to each well. And again absorbance of each well was recorded at same wavelength this was considered as 0 hour reading. The plate was incubated at 37o C for 24 h, agitated and the absorbance was read again in the reader at the same wavelength. These absorbance values were subtracted from those obtained before incubation. This procedure eliminated the interference of the tested substance. All tests were performed in triplicate. The MICs value for a test extract was expressed as the lowest concentration that inhibits the bacterial growth.

4.5 DETERMINATION OF ANTICANCER ACTIVITY OF CLOVE IN MCF-7 CELL LINE

PREPARATION OF POWDERED DMEM MEDIA

9.6 gram of powder DMEM media was suspended in 900 ml distilled water and constantly, stirred gently until the powder was completely dissolved and autoclaved for 15 minutes at 121°C and 15 lbs 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 mom L-glutamine solution to 1 liter of medium and stirred until dissolved. pH was adjusted to 4.0 using 1N Hal 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 upto 1000 ml with double distilled water. The medium was immediately sterilized by filtering through a sterile membrane filter with porosity of 0.22 micron or less, using positive pressure rather than vacuum to minimize the loss of carbon dioxide. Liquid medium was stored at 2-8^{0}C and in dark till use. 10% heat inactivated fetal bovine serum (57^0 C for 30 minutes) and filter sterilized antibiotics (Streptomycin (10 mg/ml), Penicillin (104 IU/ml), Amphotericin (2.5 mg/ml) and Tylosinom (1 ml/l) were added to media before culturing of cells.

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 Na2HPO4, 0.24 g of KH2PO4 was added in 800 ml of distilled water. pH was adjusted to 7.4 using HCL and NaOH. Volume was made up to 1 litre by distilled water. PBS was autoclaved for 20 minutes at 121 0 C. After autoclaving PBS was stored at 40 C temperatures.

REVIVING THE MCF-7 CELL LINES

Culturing of cell lines was carried out aseptically under BSL-2 and CO2 Incubator.

a. Thawing 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. 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 with10% 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 flask and incubated at 370C and 5% CO2.

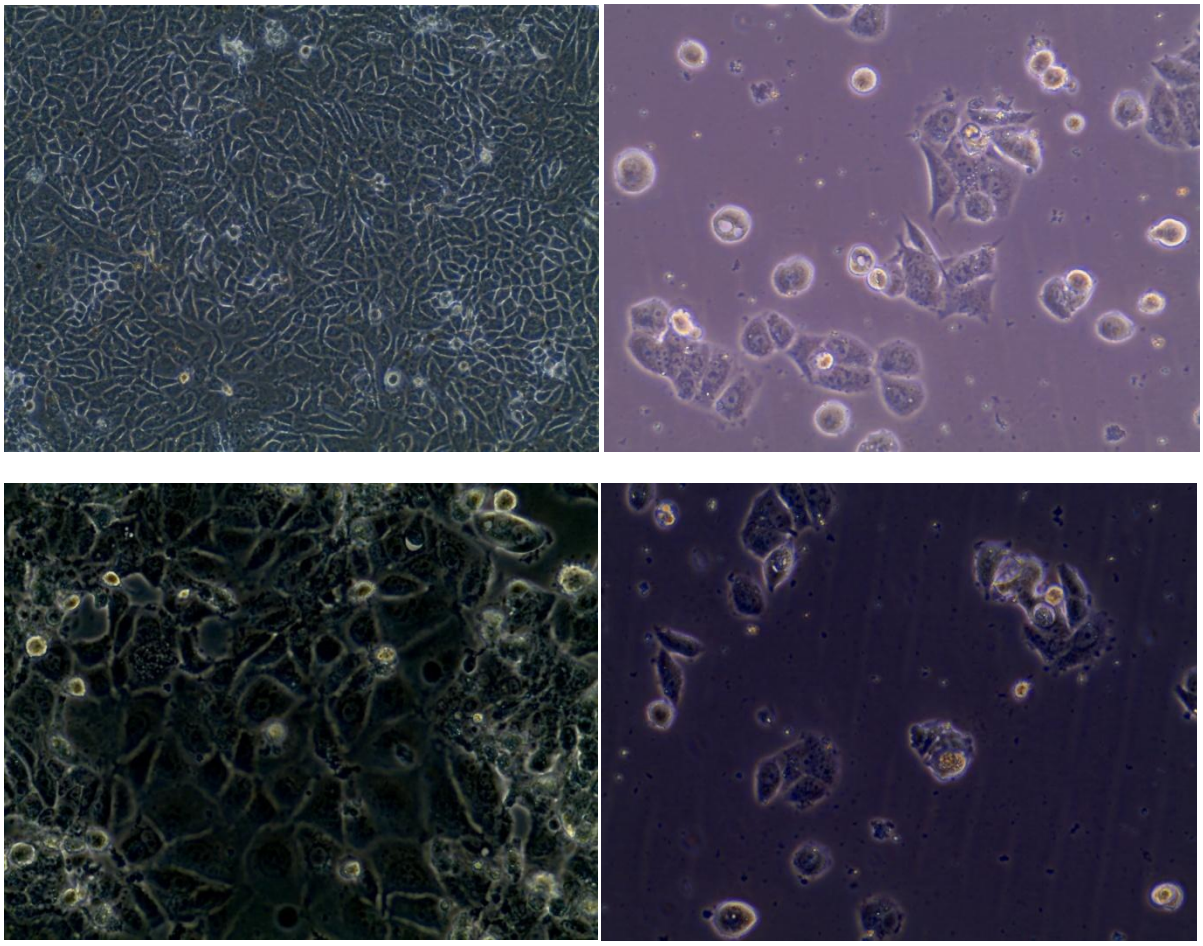

Figure 4.4 : MCF-7 cells shown under Inverted microscope

b. Sub culturing cells:

1. Used DMEM, supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml), Amphotericin (2.5 µg/ml) and tylosin (1ml/l). Maintained the temperature at 37°C in humidified, concentrated CO2 (5%) atmosphere.

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

3. Added 2-3 ml of warm (37°C) 0.25% Trypsin solution to cells to disperse cell layer. Observed under an inverted microscope (Dispersal should happen between 5 and 15 minutes. If cells are not detaching properly, place 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, deactivated Trypsin by adding 2 volumes of complete growth medium in sterile tube. Aspirate cells by gently pipetting.

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 2ml 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 (20 µl cells + 20 µl of trypan blue).

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.

c. MTT assay:

Principle of assay:

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

Procedure:

Cell proliferation was tested using a 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT assay). For the assay, MCF-7 cells (2 X 104 cells/200 µl) were plated in 96-well round bottom microtiter plate containing 100 µl of cell culture medium [(DMEM + 10% FBS + Penicillin (100 IU/ml) + Streptomycin (100 µg/ml)]. 20 µl of different concentrations (1 mg/ml, 500 µg/ml, 250 µg/ml) of each extracts were added to the wells. Plate was incubated in CO2

incubator at 37^0 C and 5% CO2 concentration for 72 hours. After incubation 10 µl of MTT was added to each well and incubation of other 4 hours was given for reduction of MTT to formazon. After incubation formazon crystals were dissolved in 100 µl of DMSO and O.D. was taken at 570 nm by microtiter plate reader.

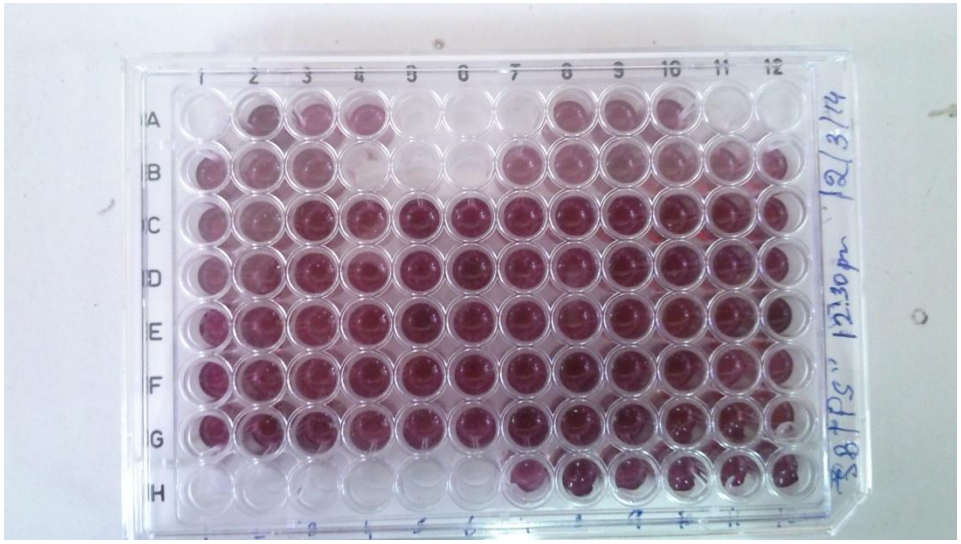

Figure 4.5 : 96 well micro titer plate with different clove extracts

CHAPTER 5 RESULTS AND DISCUSSION

5.1 Preparation of different extracts of *Syzygium aromaticum*

Six different solvents were considered based on different polarity index as mention in the table 1. Initially, 25gram of *Syzygium aromaticum,* extracts were prepared from different solvent using soxhlet apparatus and finally dried by rotaevaporator to get final yield as mention in table 5.1

Table 5.1 : Solvent extract of 25 gram of *Syzygium aromaticum* and their yield

Extract (in solvents)	**Operated temperature(°C)**	**Yield (in gram)**
Hexane	55	1.42
Diethyl Ether	25	0.87
Ethyl Acetate	65	0.61
Ethanol	65	1.27
Acetone	45	0.70
Water	85	4.2

5.2 Anticancer activity of different clove extracts on MCF-7 breast cancer cell line (MTT assay)

5.2.1 Effect of 20 and 40 µl solvent extract

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 reference wavelength of 630nm. Optical density which corresponds to cell proliferation is analyzed for different

solvent extracts control (Media+ cell + DMSO). 20µl of *Syzygium aromaticum* extracts have shown the suppression (Table 5.2b and Figure 5.2b). The experiments were carried out with 40µl as well to look for the effect on increasing the volume of the extract and this also shown the suppression (Table 5.2c and Figure 5.2c). Similarly, the suppression shown by 50µl extract of *Syzygium aromaticum* (Table 5.2d and Figure 5.2d). A fixed concentration of 200 µg/ml of each extract was also used to observe the effect. It was found that each extract at this concentration is showing inhibition in growth of MCF-7 cells (Table 5.2e and Figure 5.2e). Hexane has shown maximum inhibition in most of the cases. Diethyl ether and ethyl acetate has also shown pronounced inhibition. Hence for further experiments, these three extracts were used.

Table 5.2a : Effect of 20µl of different solvent extracts of *Syzygium aromaticum*

20µl Extracts	Control1	Control2	Hexane	Diethyl ether	Ethyl acetate	Ethanol	Acetone	Water
Experiment No. : 1	0.518	0.517	0.019	0.357	0.069	0.276	0.155	0.255
Experiment No. : 2	0.465	0.439	0.000	0.106	0.056	0.173	0.093	0.193
Mean	0.492	0.478	0.009	0.231	0.063	0.224	0.124	0.224
Standard Deviation	0.038	0.055	0.013	0.178	0.009	0.073	0.044	0.044

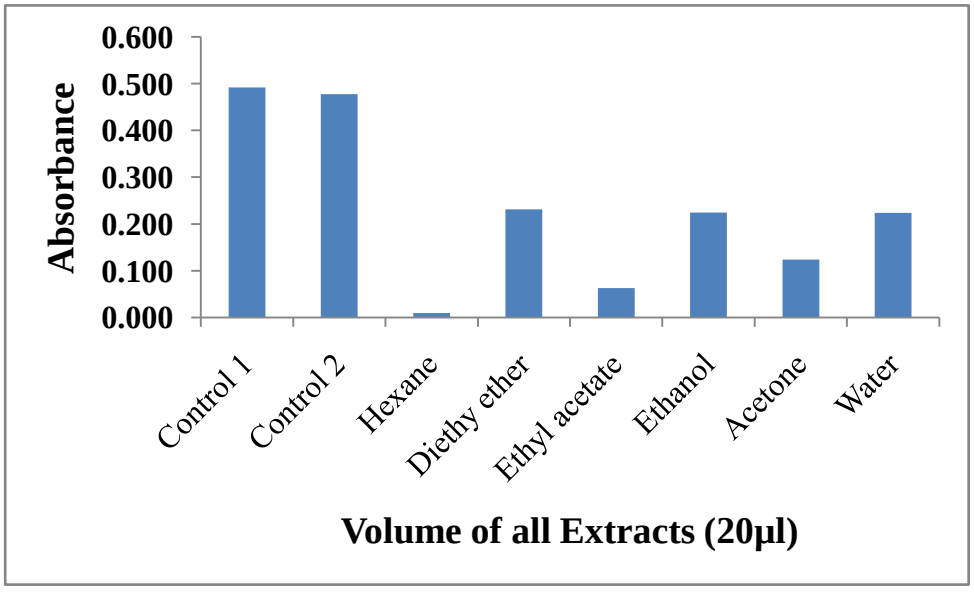

Figure 5.5a : Effect of 20µl of different solvent extracts of *Syzygium aromaticum*

Control 1 = media + cells,**Control 2 =** media + cells + 2%DMSO (20µl)

Table 5.2b : Percentage inhibition shown by 20µl of different solvent extracts of *Syzygium aromaticum*

Percentage of suppression For 20µl Extracts	Hexane	Diethyl ether	Ethyl Acetate	Ethanol	Acetone	Water
Experiment No. : 1	96.36	30.95	86.56	46.69	69.94	50.75
Experiment No. : 2	100.00	75.94	87.15	60.64	78.88	56.01
Mean	98.32	53.44	86.85	53.66	74.41	53.38
Standard Deviation	2.76	31.81	0.42	9.87	6.33	3.72

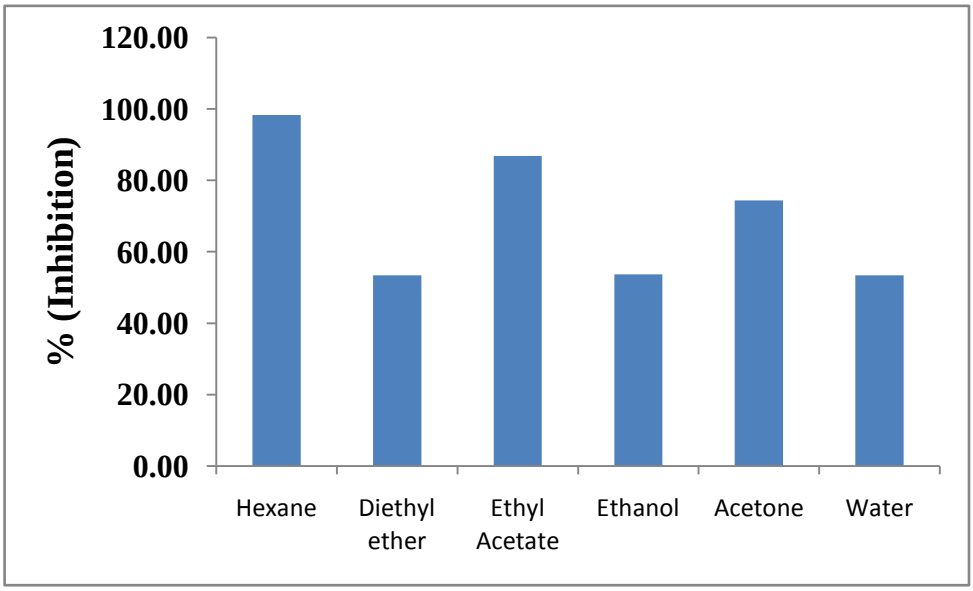

Figure 5.2b : Percentage inhibition shown by 20µl of different solvent extracts of *Syzygium aromaticum*

Table 5.2c : Percentage inhibition shown by 40µl of different solvent extracts of *Syzygium aromaticum*

40µl Extracts		Hexane	Diethyl Ether	Ethyl Acetate	Ethanol	Acetone	Water
Experiment No. : 1		79.96	38.52	76.31	45.27	58.19	53.25
Experiment No. : 2		100.00	87.15	74.80	38.88	52.36	56.89
	Mean	90.56	62.83	75.56	42.08	55.27	55.07
	Standard Deviation	14.99	34.39	1.07	4.52	4.12	2.57

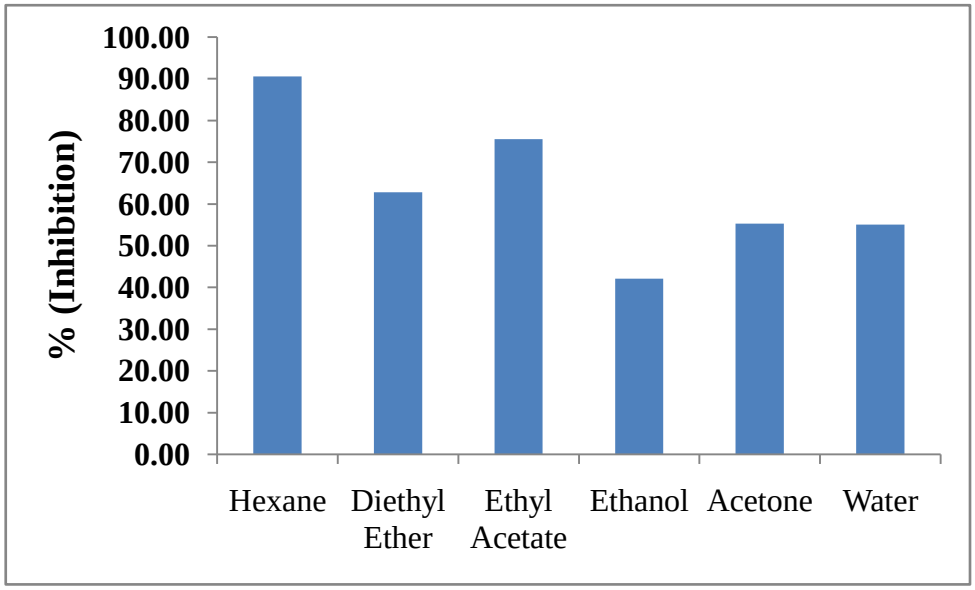

Figure 5.2c : Percentage inhibition shown by 40µl of different solvent extracts of *Syzygium aromaticum*

Table 5.2d : Percentage inhibition shown by 50µl of different solvent extracts of *Syzygium aromaticum*

50µl Extracts	Hexane	Diethyl Ether	Ethyl Acetate	Ethanol	Acetone	Water
% Inhibition	75.73854	53.9502	54.12564	6.757217	35.7442	13.4352

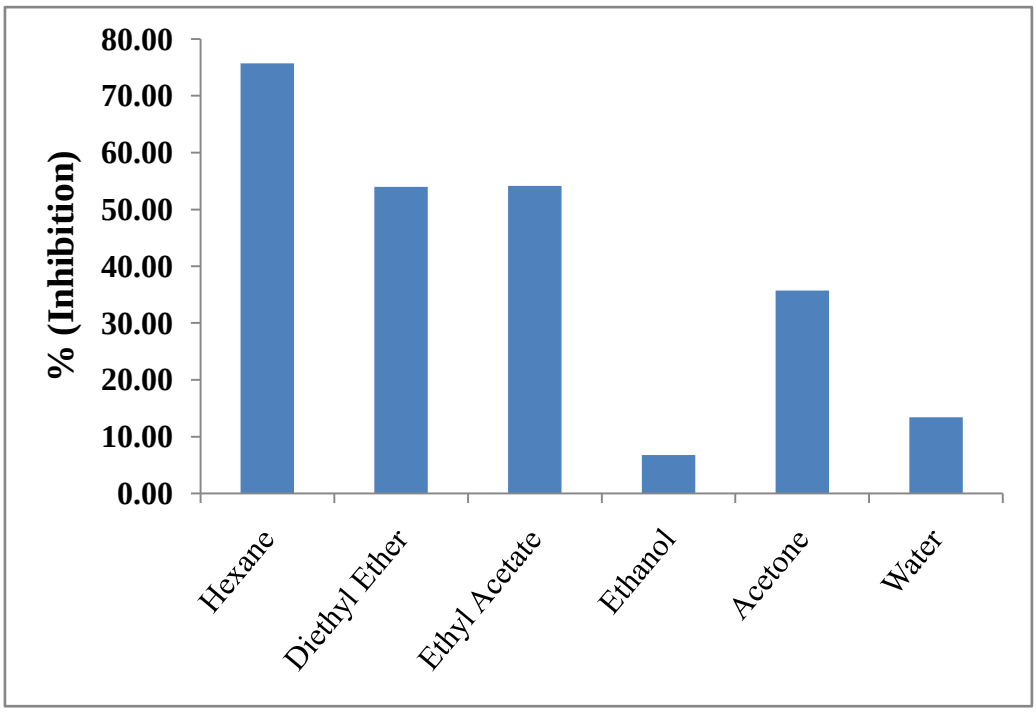

Figure 5.2d : Percentage inhibition shown by 50µl of different solvent extracts of *Syzygium aromaticum*

Table 5.2e : Percentage inhibition shown by 200µg/ml of different solvent extracts of *Syzygium aromaticum*

		Hexane	Diethyl Ether	Ethyl Acetate	Ethanol	Acetone	Water
Experiment No. : 3		42.04	54.20	39.67	16.03	38.80	19.51
Experiment No. : 4		20.15	16.72	2.90	-2.69	17.99	7.35
	Mean	31.10	35.46	21.28	6.67	28.40	13.43
	SD	15.48	26.50	26.00	13.23	14.71	8.60

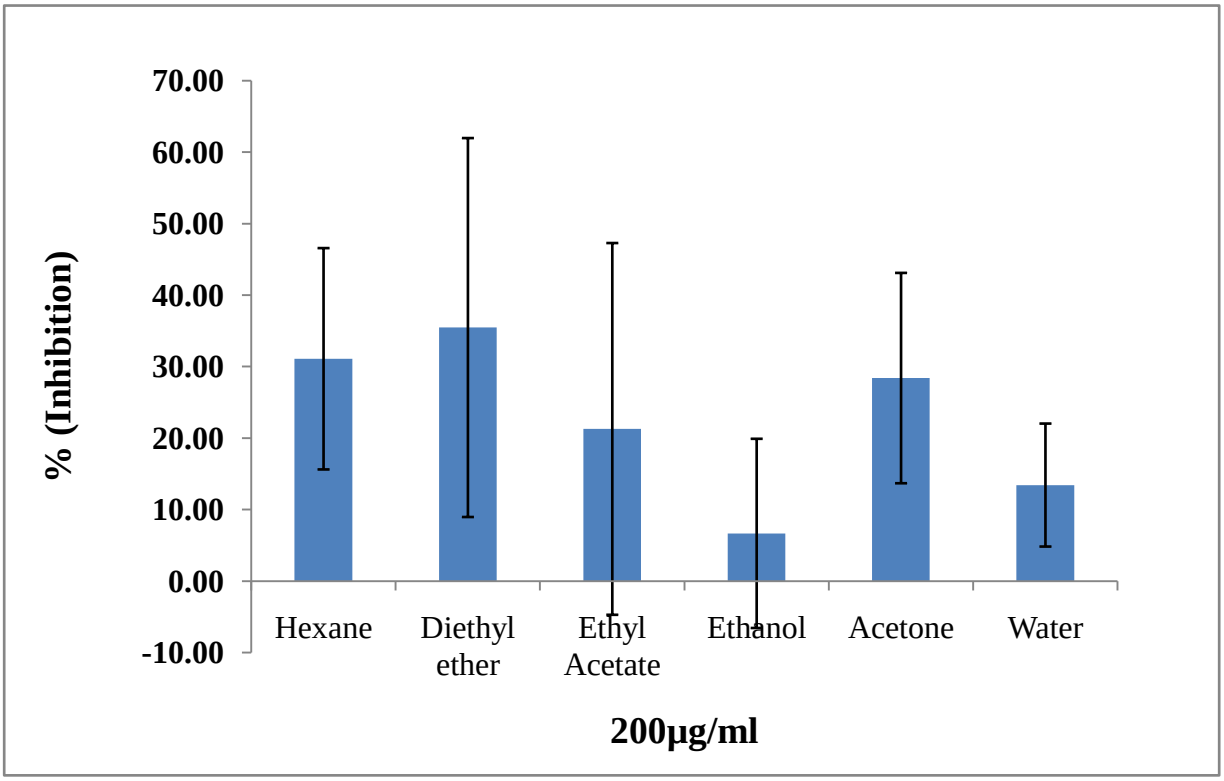

Figure 5.2e : Percentage inhibition shown by 200µg/ml of different solvent extracts of *Syzygium aromaticum*

5.2.2 Effect of hexane, diethyl ether and ethyl acetate extracts of *Syzygium aromaticum* on the growth of MCF-7 breast cancer cells

This time we have taken a fixed concentration of different solvent extract against MCF-7 cell line and we have founded hexane extract shown a very good suppression against this MCF-7 cancer cell line followed to ethyl acetate extract.

Table 5.2f : Percentage inhibition shown by 1250µg/ml of different solvent extracts of *Syzygium aromaticum*

	Hexane	**Diethyl Ether**	**Ethyl Acetate**
% Inhibition	80.04	21.68	64.53

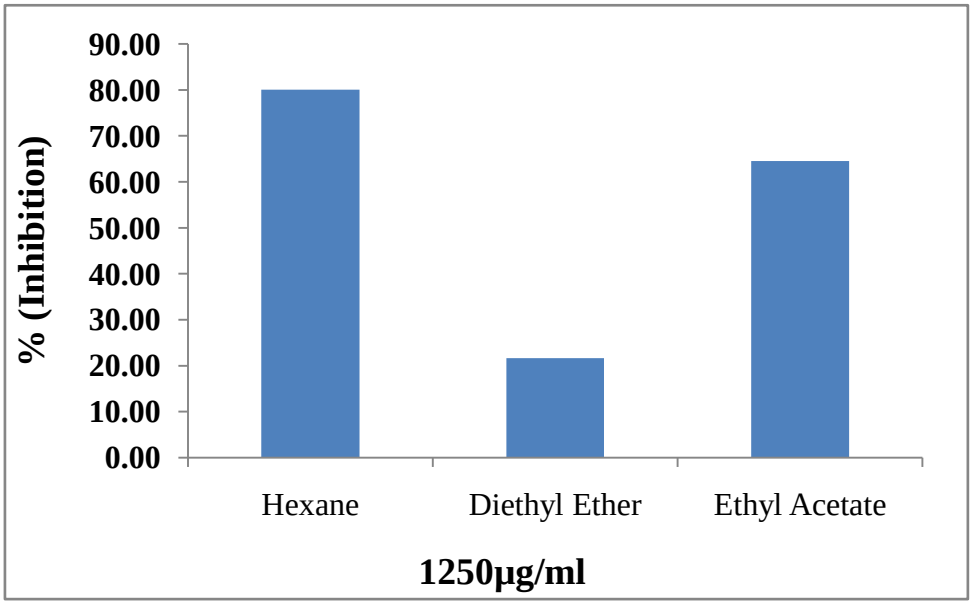

Figure 5.2f : Percentage inhibition shown by 1250µg/ml of different solvent extracts of *Syzygium aromaticum*

5.2.3 Cytotoxic effect of Hexane extracts of *Syzygium aromaticum*

It was observed from the previous experiments that hexane extract has shown maximum inhibition effect. So finally we have decided to work with hexane extract and we have taken different concentration of hexane extract against MCF-7 cell line. We have used minimum concentration 125µg/ml to upto1mg/ml. It was noticed percentage of suppression increases with increase in concentration (Fig. 5.2g to Fig. 5.2j). The IC_{50} value of this extract was found to be at 350µg/ml (Fig. 5.2j).

Table 5.2g : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

		Hexane Extract			
Concentration(µg/ml)		**125**	**250**	**500**	**1000**
Experiment No. : 7		3.09	18.58	19.19	77.20
Experiment No. : 8		1.67	22.48	33.72	95.66
	Mean	2.38	20.53	26.46	86.43
	Standard Deviation	1.01	2.76	10.27	13.06

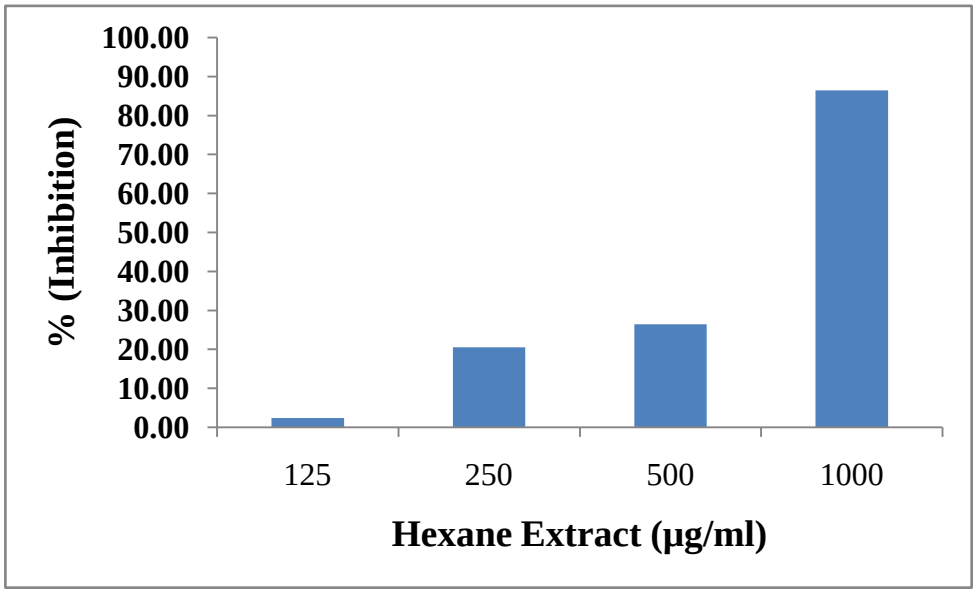

Figure 5.2g : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

Table 5.2h : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

		Hexane	Extract	
Concentration(µg/ml)	625	750	875	1000
% inhibition	94.73	94.24	100	100

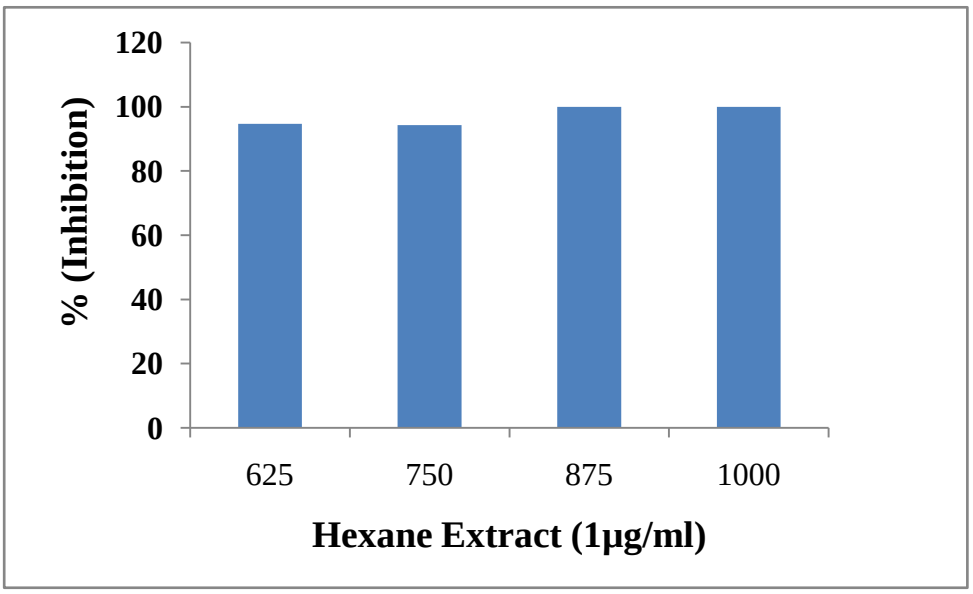

Figure 5.2h : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

Table 5.2i : Percentage inihbition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

Conc. (µg/ml)		500	550	600	650	700
Experiment No. : 7		94.15	96.98	97.12	95.94	95.03
Experiment No. : 8		82.87	98.59	98.24	97.52	94.95
	Mean	88.51	97.79	97.68	96.73	94.99
	Standard Deviation	7.98	1.14	0.79	1.11	0.06

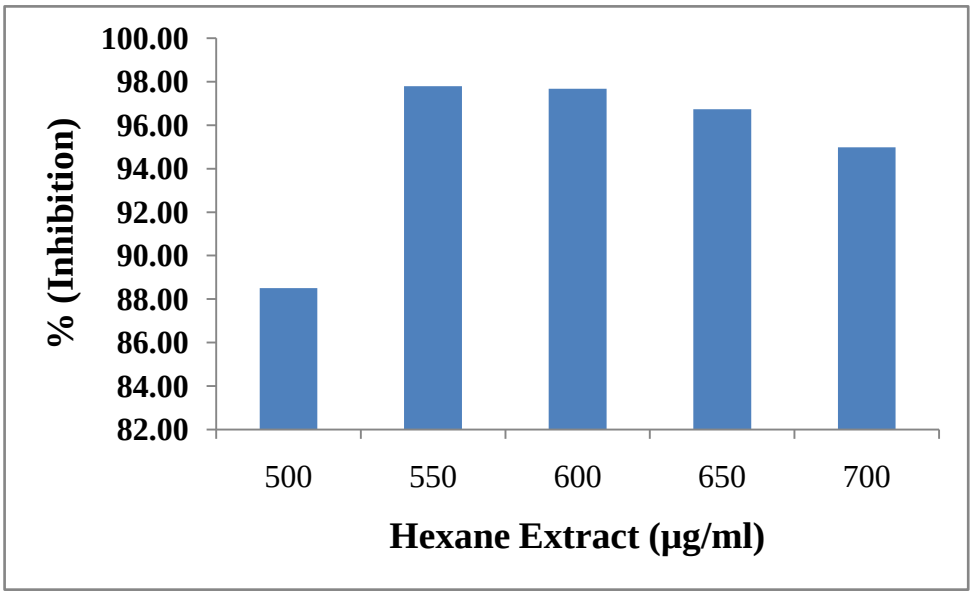

Figure 5.2i : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

Table 5.2j : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

			Hexane	Extract		
Concentration(µg/ml)	**125**	**250**	**300**	**350**	**400**	**450**
% Inhibition	11.10	38.41	47.98	50.51	62.35	64.74
			Hexane	**Extract**		
Concentration(µg/ml)		**500**	**550**	**600**	**650**	**700**
% Inhibition		65.97	64.89	73.05	76.60	99.45

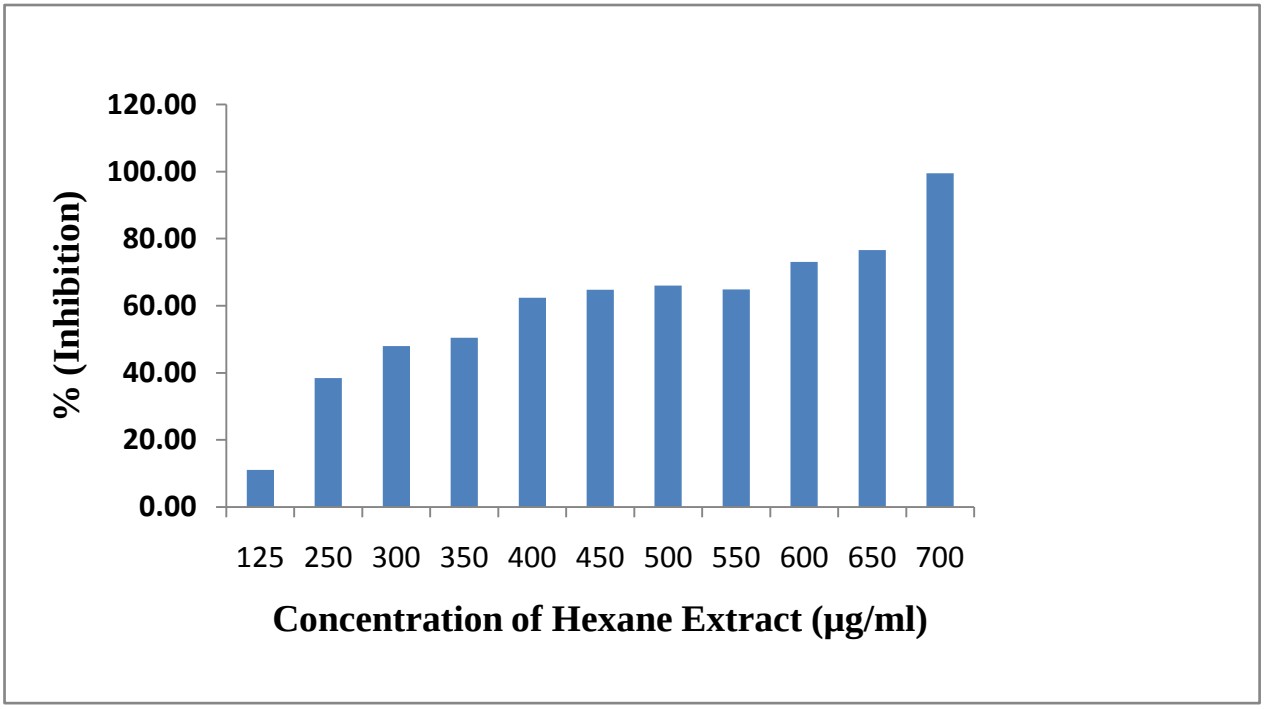

Figure 5.5j : Percentage inhibition shown by different concentration of Hexane extracts of *Syzygium aromaticum*

5.3 Effect of different concentration of Hexane extract of *Syzygium aromaticum* on PBMC

Hexane extract of *Syzygium aromaticum* is also tested on Peripheral blood mononucleated cells (PBMC). With the results it was that determined this hexane extract suppressed the proliferation of stimulated PBMC (Concavillina A treated) and unstimulated PBMC (Table5.3a and Fig 5.3a).

Table 5.3a : Percentage suppression shown by different concentration of Hexane extract of *Syzygium aromaticum* on PBMC

	Hexane	**Extract**
Concentration (µg/ml)	375µg/ml	500µg/ml
without ConA	17.83	55.51
With ConA	43.52	53.81

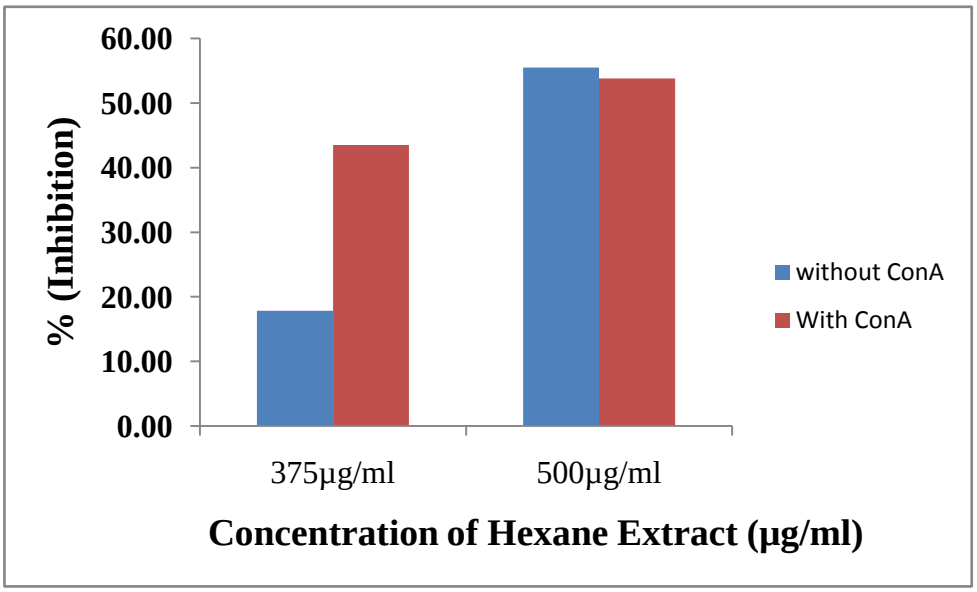

Figure 5.3a : Percentage inhibition shown by different concentration of Hexane extract of *Syzygium aromaticum* on PBMC

5.4 Antifungal activity of Hexane extract on *Candida albicans* (MTT assay)

For antifungal test we taken a different concentration of hexane extract and we have founded as we increased the concentration of an extract antifungal activity decreased. This means that as we increased the concentration of an extract lower the value for suppression against this fungus has been shown.

Table 5.4a : Effect of Hexane extract on *Candida albicans*

control1	control2	control3	control4	control5	250µg/ml	500µg/ml	750µg/ml	1000µg/ml
0.190	0.084	0.072	0.066	0.055	0.022	0.030	0.037	0.045

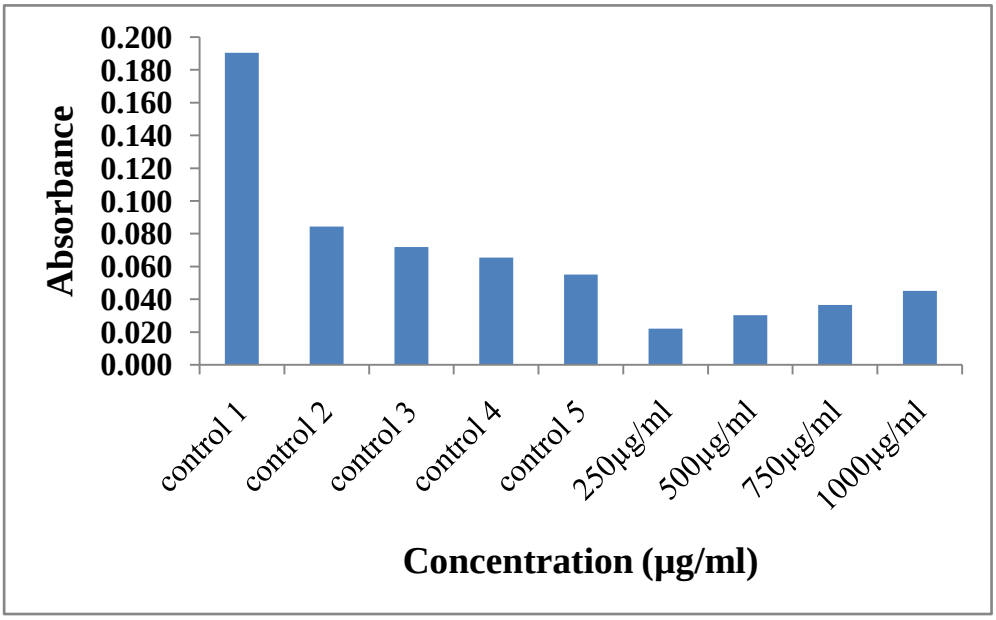

Figure 5.4a : Antifungal activity of Hexane extract on *Candida albicans*

Control 1 = media + cells

Control 2,3,4,5 = media + cells + DMSO

Extract = media + cells + Hexane Extract

Table 5.4b : Percentage suppression shown by Hexane extract on *Candida albicans*

		Hexane	Extract	
Concentration (µg/ml)	**250**	**500**	**750**	**1000**
% Inhibition	73.77	57.82	44.55	17.82

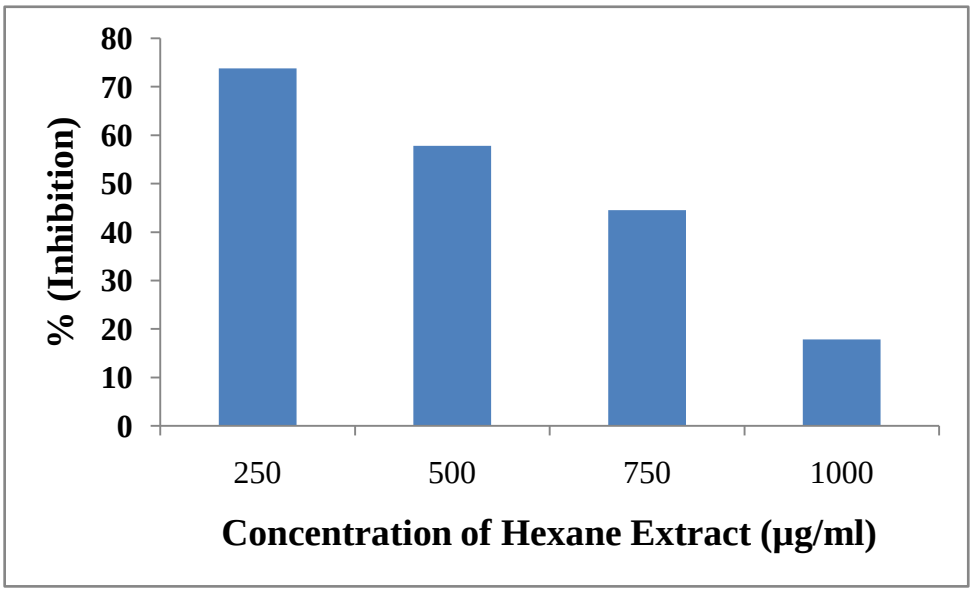

Figure 5.4b : Percentage inhibition shown by Hexane extract on *Candida albicans*

5.5 Antifungal activity of Hexane extract on *Candida albicans* (Agar Plate Diffusion)

Antifungal test has been done by using a agar – well diffusion method. Test has been done by growing fungus *Candida albicans* in an agar plate (PDA) and by making wells on agar plate different concentration of hexane extract has been placed on wells. After 12–24 hours incubation result were analyzed and founded a very well zone of inhibition. Here also we have founded as we increased the concentration of the extract, it also shown a good zone of inhibition. Here we have taken 1mg/ml, 2mg/ml and 4mg/ml concentration of an extract and it shown

approximate 20mm, 25mm and 30mm respectively diameter zone of inhibition against this fungal species.

Table 5.5a : Antifungal activity of Hexane extract on *Candida albicans* (Agar Plate Diffusion)

		Hexane	**Extract**	
Concentration (mg/ml)	DMSO	1mg/ml	2mg/ml	4mg/ml
Diameter in mm	0	21.75	24.75	31.25

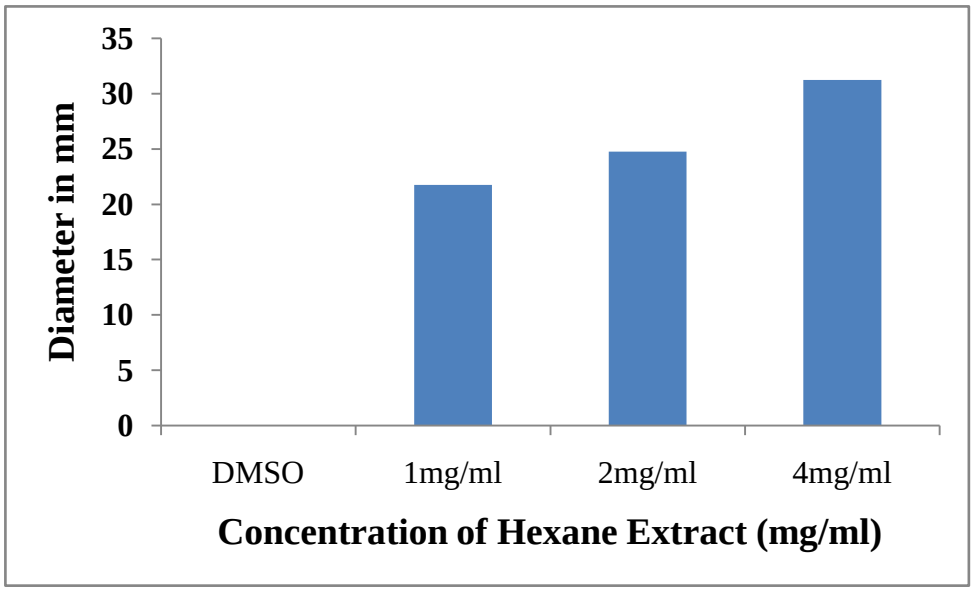

Figure 5.5a : Antifungal activity of Hexane extract on *Candida albicans* (Agar Plate Diffusion)

5.6 Antibacterial activity of Hexane extract (1mg/ml) on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus cereus* (MTT assay)

Antibacterial activity of hexane extract has been tested on different bacteria that includes two gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and two gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) using MTT assay. Test has been done in a 96-wells plate and all the test has been done in triplicates. Absorbance was measured at 600nm with the help of ELISA reader. A fixed concentration (1mg/ml) of hexane extract has been used and it shows very good percentage of suppression against all bacteria (Table 5.6a and figure 5.6a).

Table 5.6a : Effect of Hexane extract (1mg/ml) on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus cereus*

Different Bacteria	***Escherichia coli***	***Bacillus cereus***	***Pseudomonas aeruginosa***	***Staphylococcus aureus***
% Inhibition	86.92	87.46	87.47	91.29

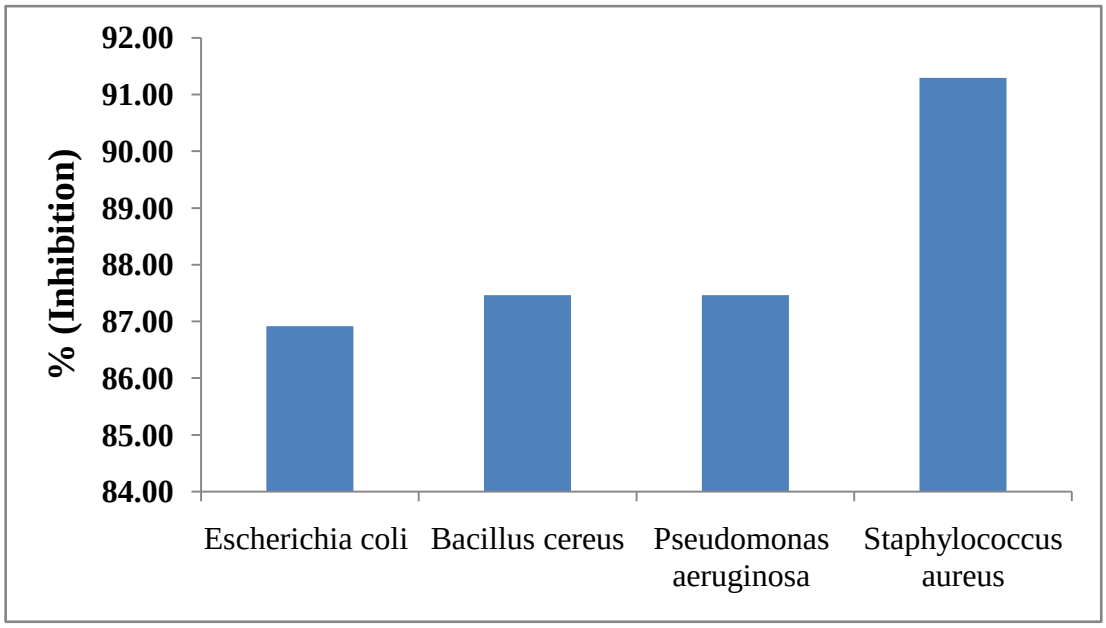

Figure 5.6a : Antibacterial activity of Hexane extract (1mg/ml) on *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus cereus*

CHAPTER 6 CONCLUSION

Clove is an evergreen tree that contains many active components which are having antiemetic, antidiarrheal, antiflatulent, stimulant, and many other therapeutic activities that can help us in reducing the risks of getting the infection or disease. Functional foods and nutraceuticals are often patterned with bioactive compounds. Clove holds promising putative bioactive, which possess valuable health benefits. We have prepared different clove (*Syzygium aromaticum*) extracts by using hexane, diethyl ether, ethanol, acetone, ethyl acetate and water as solvents.

From all the experiments that we have conducted during the whole project it was concluded that clove has strong antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Escherichia coli,* and *Pseudomonas aeruginosa*. Clove shows antifungal activity against *Candida albicans.* The hexane extracts of clove have shown antifungal activity at a concentration 250µg/ml. we have found if we increased the concentration of hexane extract above 250µg/ml, the antifungal activity against *Candida albicans* deceases.

Clove also shows anticancer activity against MCF-7 breast cancer cell lines. The hexane extracts of clove have shown anticancer activity at a concentration 125µg/ml and at a concentration of 350µg/ml, hexane extract shown 50% suppressive effect against MCF-7 cells and similarly at a concentration of ~700µg/ml, hexane extract of clove shown ~90% suppressive effect against MCF-7 cells.

CHAPTER 7 SUMMARY

Clove is an evergreen tree that contains many active components which are having antiemetic, antidiarrheal, antiflatulent, stimulant, and many other therapeutic activities that can help us in reducing the risks of getting the infection or disease. There were some reports which have shown antioxidant, antimicrobial, anti-inflammatory activity of clove. In the present work, we have studied the anticancer, antibacterial and immunomodulatory properties of clove buds extracts.

We have prepared different clove (*Syzygium aromaticum*) extracts by using ethanol, hexane, diethyl ether, acetone, ethyl acetate and water as solvents..

Agar well diffusion and minimum inhibitory concentration (MIC) assays were performed in fungal species named as *Candia albicas* for antifungal activity. Agar well diffusion assay has shown antifungal activity against *Candida albicans*.

Based on minimum inhibitory concentration assay the best MIC values were obtained at 250μg/ml with hexane extracts.

Antibacterial activity were performed in two gram positive bacteria (*Staphylococcus aureus, Bacillus cereus*) and two gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) for antibacterial activity using MTT assay.

The hexane extract of clove has shown strong antibacterial activity on two gram positive bacteria (*Staphylococcus aureus, Bacillus cereus*) and two gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*).

MTT assay was performed to assess the anticancer activity of clove in MCF-7 breast cancer cell lines and found that the anticancer activity was shown by 125 µg/ml concentration of hexane extract. The hexane extracts of clove showed 50% anticancer activity at a concentration 350 µg/ml and showed ~90% anticancer activity at ~700µg/ml.

Hexane extract of *Syzygium aromaticum* is also tested on Peripheral blood mononucleated cells (PBMC). With the results it was that determined this hexane extract suppressed the proliferation of stimulated PBMC (Concavillina A treated) and unstimulated PBMC

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