ANTICANCER AND ANTIBACTERIAL ACTIVITIES OF CINNAMON

A Dissertation report
Submitted in partial fulfillment of the requirement for
the award of degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

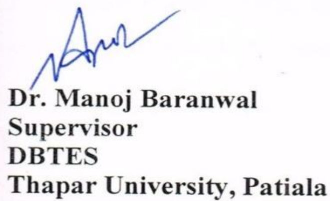
By
RENU
Registration No. 301101027

Under the Supervision of
DR. MANOJ BARANWAL
DR. VIKAS HANDA
(Assistant Professors)
DEPARTMENT OF BIOTECHNOLOGY & ENVIRONMENTAL SCIENCES
THAPAR UNIVERSITY, PATIALA

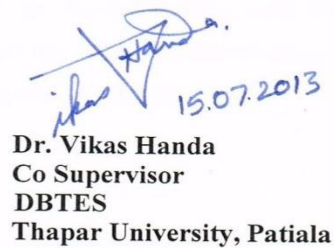
JULY, 2013

CERTIFICATE

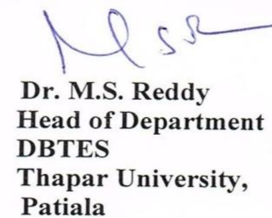
This is to certify that the thesis entitled " **Anticancer and Antibacterial activities of Cinnamon**" submitted by Renu (Roll no: 301101027) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology in the Department of Biotechnology and Environmental Sciences, 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 or institute.



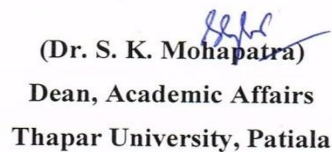
Dr. Manoj Baranwal
Supervisor
DBTES
Thapar University, Patiala



Dr. Vikas Handa
Co Supervisor
DBTES
Thapar University, Patiala



Dr. M.S. Reddy
Head of Department
DBTES
Thapar University, Patiala



(Dr. S. K. Mohapatra)
Dean, Academic Affairs
Thapar University, Patiala

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I hereby declare that the work which is being presented in the dissertation entitled **"Anticancer and Antibacterial activities of Cinnamon"** in partial fulfillment of the requirements for the award of Degree of Master of Sciences in Biotechnology, Thapar University, Patiala, is an authentic record of my own work during a period of six months from January 2013 to June 2013, under the supervision of **Dr. Manoj Baranwal,** Assistant Professor and **Dr. Vikas Handa,** Assistant Professor, Department of Biotechnology and Environmental Sciences, 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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Date: 15/ July /2013

Renu

Roll No. 301101027

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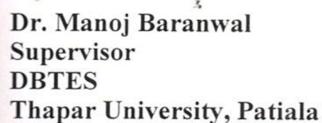

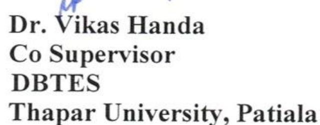

15.07.2013

Dr. Manoj Baranwal	**Dr. Vikas Handa**	**Dr. M.S. Reddy**
Supervisor	**Co Supervisor**	**Head of Department**
DBTES	**DBTES**	**DBTES**
Thapar University, Patiala	**Thapar University, Patiala**	**Thapar University, Patiala**

ABSTRACT

Cinnamon is an evergreen tree that contains several active components such as essential oils (cinnamic aldehyde and cinnamyl aldehyde), tannins, mucus, and carbohydrate, and used as antiemetic, antidiarrheal, antiflatulent, stimulant. Cinnamon contains so many properties that can be helpful in treating various diseases; hence it was interesting to study the anticancer and antibacterial activities of cinnamon bark solvent (ethanol, methanol, acetone, ethyl acetate) extracts. We have found that cinnamon bark extracts have shown strong antibacterial activities against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, and Pseudomonas aeruginosa*. The methanol and acetone extracts of cinnamon have shown anticancer activity at a concentration 250 µg/ml against the MCF-7 breast cancer cell lines. The ethyl acetate extract of cinnamon showed an activity at a concentration of 125 µg/ml, and ethanol extract of cinnamon did not show any activity against the MCF-7 cells. We also observed immunostimulation and immunosuppressive activities in peripheral blood mononuclear cells (PBMCs). Interestingly, we have found anticancer and immunomodulatory activities in different cinnamon extracts. We have performed limited experiments hence to confirm these activities, it is important to carry out more experiments.

KEYWORDS:- Cinnamon, MTT, MCF-7 cells, Extracts

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Place: - Thapar University, Patiala (**Renu)**

Date: -15th July 2013

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ABBREVIATIONS

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

Cinnamon is an evergreen tree that belongs to the family Lauraceae and is a native of Sri Lanka and tropical Asia containing several active components such as essential oils (cinnamic aldehyde and cinnamyl aldehyde), tannins, mucus, and carbohydrate (Kwon *et al.*, 2010). The flowers of cinnamon are arranged in panicles with a greenish color and a distinct odour. The fruit is a purple one-centimeter berry containing a single seed. Its flavor is due to an aromatic essential oil (cinnamic aldehyde and cinnamyl aldehyde) which makes up 0.5 to 1% of its composition. In Ayurvedic medicine, cinnamon bark has been used as an antidiarrheal, antiflatulent, and general stimulant (Krishna *et al.*, 1946, Pao, 2000). Twenty out of 250 species of the genus *Cinnamomum* occur in India. 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. Some of these species are *C. cassia* Presl Cassia (Chinese cinnamon), *C. verum* Presl (syn. *C. zeylanicum* Nees) (commonly known as True or Ceylon cinnamon), *C. burmannii* Blume (also known as Indonesian cassia), *C. loureirii* Nees (Vietnamese cassia), *C. tamala* (Buch.-Ham.) Nees and Eberm. (Indian cassia), *C. cordatum* (Kosterm) are abundantly found in Perak and Pahang in the Malaysian peninsula. Every parts of the tree in all *Cinnamomum sp*. are aromatic and are used in traditional medicine as *medang*, but this plant has not been explored for commercial applications. Cinnamon is known by different names in different languages. Like it is known as Ceylon Cinnamon, True Cinnamon in English, Cannelle in French, Ceylonzimt, Kaneel in German, Cannella in Italian, Canela (Spanish), Yook Gway (Chinese), Dal-chini, Darchini, DhallCheene (Hindi), Kurundu (Sinhalese), Karuvappadai (Tamil).

1.1 *Cinnamomum zeylanicum*

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). Studies have shown the potential

therapeutic activities of cinnamon which can help us in reducing the risk of getting the infection or disease. 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 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). The ethanolic extract (70%) of cinnamon was found effective on acute inflammation in mice. 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. 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.

However, the action mechanism related with cinnamon is not fully understood. The use of different assays could allow for evaluating and obtaining more information about the possible action mechanism of this herbal medicine and spice which would be very important in the nutrition field. Considering the requirements of effectiveness and convenience of the application of natural antimicrobial products, there has been a constant increase in the search of alternative and efficient compounds for food preservation aimed at partial or total replacement of antimicrobial chemical additives. In order to explore the potential usefulness of cinnamon extracts, it is important to know their chemical constituents and understand its effect on phytotoxic and antifungal activity. Hence it is interesting to evaluate the antimicrobial, anticancer and immunomodulatory activities of cinnamon extracts, prepared in different solvents (ethanol, methanol, acetone and ethylacetate).

CHAPTER 2

REVIEW OF LITERATURE

2.1 Antimicrobials

Antimicrobial agents are defined as "Substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors" in the Code of Federal Regulations (CFR) (FDA 2006b). CFR also defined the chemical antimicrobial agents as "Any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oil extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties." Even though salt, sugar, vinegars and spices are not considered chemical preservatives in the CFR, they provide an important synergistic effect for controlling the growth of microorganisms (Naidu, 2000; FDA 2006b).

2.2 Natural antimicrobials

Some of the natural components can exhibit antimicrobial activity in the foods in which they are commonly used as ingredients or may be used as additives in other foods. According to some authors, natural antimicrobials can be grouped in six categories. In the first group there are the state-of-the art-antimicrobials, which have been used for centuries, such as salt, sugar, phosphate salts and chlorine. The other five categories are lacto, ovo, bacto, acid and phyto-antimicrobials which have been isolated from animals, plants or are microbial by-products and have been investigated thoroughly during the last 20 years (Sofos, 1998; Naidu, 2000; Roller, 2003; Davidson, 2005). Plants, flowers, herbs and spices are widely recognized to have antibacterial properties and were used by ancient cultures. Nevertheless most antimicrobial research of herbs and spices has been done recently. Antibacterial compounds of herbs and spices have been isolated and characterized as phenolics and polyphenolics, terpenoids, alkaloids, lectins, and polypeptides which suggested that their commercial use as natural antimicrobials could be expanded.

Some plants, herbs and spices are recognized for delivering health benefits from their natural components. These natural components have the potential ability to decrease blood cholesterol levels and protect against osteoporosis and cancer development. These aforementioned characteristics of plants, herbs and spices have caused them to be defined as nutraceuticals. "A nutraceutical is any

substance, food or part of a food, that provides medical or health benefits, including the prevention and treatment of diseases."

Sanla and coworkers in 2006 showed that cinnamon extracts have strong antibacterial activity than other spices extracts against *Aeromonas hydrophila, Aeromonas salmonicida* and *Edwardsiella tarda*. According to Muthuswamy *et al.* (2008), different extracts of cinnamon have antimicrobial activity against two marker microorganisms, *Escherichia coli* O157:H7 and *Listeria innocua* that helps in extending the shelf life of fresh cut apple slices that means it can be used as preservative for food materials.

According to Mandal and coworkers (2010), ethanolic extracts of cinnamon (*Cinnamomum zeylanicum*; CIN), clove (*Syzygium aromaticum*, CLV) and cumin (*Cuminum cyminum*, CMN) have shown *in vitro* antibacterial activity against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA). They have found that the zone diameter of inhibition (ZDI) obtained due to CIN, CLV and CMN ranged between 22-27 mm, 19-23 mm and 9-15 mm, respectively; while the MICs, for the isolates, were in the range of 64-256, 64-512 and 128-512 µg/ml, respectively. When they tested for their MIC levels the ethanolic extracts of cinnamon (*Cinnamomum zeylanicum*) and clove (*Syzygium aromaticum*) were showing the bactericidal activity after 6h of incubation, while cumin (*Cuminum cyminum)* showed bactericidal activity after 24h. They concluded that *C. zeylanicum* and *S. aromaticum* shows the strongest *in vitro* antibacterial activity followed by *C. cyminum* against MRSA (Methicillin- Resistant *Staphylococcus aureus)*, and such findings could be considered a valuable support in the treatment of infection and may contribute to the development of potential antimicrobial agents for inclusion in anti-*S. aureus* regimens.

Crude extracts of culinary spices like Aniseed (*Pimpinella anisum*), Star anise (*Illicum verum* Hook fruit) and Cinnamon (*Cinnamomum zeylanicum*) also showed the antibacterial activity against common fish pathogens like *Aeromonas hydrophila, Aeromonas salmonicida* and *Edwardsiella tarda.* It was explored by finding the MIC and a 96-well microtitre assay using 20 µl of p-Iodonitrotetrazolium violet (INT) as indicator of formation of formazon crystals. The essential oils of cinnamon *(Cinnamomum sp.*), clove (*Syzygium aromaticum*) and galangal (*Alpinia galangal*) and their principal constituents including cinnamaldehyde, eugenol and geraniol have been investigated for their antimicrobial activity against ten pathogenic and spoilage bacteria (including *Aeromonas hydrophila, Bacillus cereus, Escherichia coli, Escherichia coli O1587:H7, Listeria monocytogenes, Micrococcus luteus, Pseudomonas aeruginosa,*

Salmonella enteridis, Staphylococcus aureus and Enterococcus faecalis and three strains of yeast including *Candida albicans, Saccharomyces cerevisiae, and Zygosaccharomyces rouxii*) using an agar well diffusion assay (Sanla-Ead *et al.,* 2006). The minimum inhibitory concentration (MIC) of these essential oils and their principal constituents were determined using an agar dilution method ranged from 0.78 to 200 µl/ml. The MICs of cinnamon, clove and galangal oils in a range of 6.25-200 µl/ml could inhibit the growth of all test microorganisms except *Pseudomonas aeruginosa*, which was not sensitive to all essential oils at the highest concentration used (200 µl/ml) and *Escherichia coli* and *S. enteridis* which were not sensitive to galangal oil. The principal constituents showed a higher antimicrobial activity than those of essential oils. Cinnamaldehyde possessed the strongest antimicrobial activity whereas galangal oil did not show growth inhibition of most microorganisms. They have also demonstrated the possible application in active packaging as antimicrobial edible films.

Antimicrobial and antioxidant activity of spice essential oils were determined for their antimicrobial and antioxidant activities against *Staphylococcus aureus, Pseudomonas fluorescens*, and *Salmonella rissen* by Suree *et al.,* in 2011**.** Of all, cinnamon oil had the highest antibacterial activity. The most sensitive bacteria were Bacillus cereus (0.5 mg/ml minimum inhibitory concentration, MIC). Two oil combinations: i) cinnamon and mace oils and ii) cinnamon and prikhom oils showed a synergistic effect against *Staphylococcus aureus, Pseudomonas fluorescens,* and *Salmonella rissen* (0.32- 0.38 mg/ml fractional inhibitory concentration index, FICI). These oils contained high amount of total phenolics (51.54- 140.9 µg gallic acid equivalents/mg oil).

Muthuswamy and colleagues in 2008 investigated the potential of different extracts of true cinnamon to use as a food additive to extend the shelf life of fresh-cut apples. Different extracts of cinnamon were tested against two marker microorganisms, *Escherichia coli* O157:H7 and *Listeria innocua.* An ethanolic extract of cinnamon bark (2% w/v) inhibited the growth of *E. coli* and *L. innocua* by 94 and 87%, respectively. The ethanolic extract of cinnamon was incorporated with in a commercial anti-browning dipping solution, FreshExtend^{TM}, and it was found that the cinnamon bark extract (1% w/v) reduced significantly ($P < 0.05$) the microbial growth on apple slices stored for 12 days at 6° C in comparison to the control. The cinnamon extract had no influence on the anti-browning properties of FreshExtend^{TM}.

Saraf *et al.* (2011) have investigated the *in vitro* antibacterial activity of butanolic extracts of two different Cinnamon species (*C. zeylanicum*; Commercial variety and *C. flexuous*; Wild variety). They evaluated this activity on the basis of Zones of Inhibition measurement by "Disc diffusion method". *C. zeylanicum* showed the maximum inhibition against gram positive bacterium-*Staphylococcus aureus*. C.

flexuous but was inactive against *Klebsiella pneumoniae*. The lowest values of MIC obtained for *Staphylococcus aureus* was [MIC-76.25 (C. f.) 78.5 (C. z.) and MBC-112.6 (C. f.) 98.35 (C. z.)]. Thus this bacteria is most inhibited by the extract, whereas *Bacillus subtilis* was least inhibited as indicated by MIC [(98.50 (C. f.) 96.2 (C. z.)] and MBC [169.50 (C. f.) 151.2 (C. z.)] values.
Masih *et al.* (2012) evaluated the *in vitro* antibacterial activities of two spices cinnamon bark (*Cinnamonum zeylanicum)* and Ajowan fruits (*Trachyspermum ammi*) ethanol and acetone extracts against two gram negative food spoilage bacteria *Pseudomonas sp., Escherichia coli* and two gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus.* Disc diffusion method was performed to test the *in vitro* antibacterial activity and it was found that ethanol extract of cinnamon and ajowan has antibacterial activity against *Pseudomonas sp*., whereas acetone extract of spices exhibited highest activity against *Escherichia coli.* Acetone extract of cinnamon and ajowan showed no activity against *Staphylococcus aureus* and *Bacillus subtilis.* This study suggested that the ethanol extract of C*innamonum zeylanicum* and *Trachyspermum ammi* revealed a significant antibacterial activity.

Dilek *et al.* (2011) studied the antimicrobial activities of the ethyl acetate, acetone and methanol extract of 12 plant species (*Capsicum annuum* (red pepper) (fruit) *Zingiber officinale* (ginger) (root), *Cuminum cyminum* (cumin), Alpinia ficinarum (galingale), *Coriandrum sativum* (coriander), *Cinnamomun zeylanicum* Nees (cinnamomun), *Origanum onites* (thyme), *Folium sennae* (senna), *Eugenia caryophyllata* (cloves), *Flos tiliae* (lime), *Folium menthae crispae* (peppermint) and *Piper nigrum* (black pepper) were tested in vitro against 2 fungi and 8 bacterial species by the disc diffusion method, *Klebsiella pneumonia* 13883, *Bacillus megaterium* NRS, *Pseudomonas aeruginosa* ATCC 27859, *Staphylococcus aureus* 6538 P, *Escherichia coli* ATCC 8739, *Enterobacter cloaca* ATCC 13047, *Corynebacterium xerosis* UC 9165, *Streptococcus faecalis* DC 74, *Kluyveromyces marxianus, Rhodotorula rubra* were used in this investigation. The results indicated that extracts of different spices has shown antibacterial activity in the range of 7-24 mm inhibition zone, 7-20 mm inhibition zone, *Capsicum annum* (red pepper) and *Cinnamomun zeylanicum* (cinnamon) bark, 7-8 mm inhibition zone, *Coriandrum sativum* (coriander) to the microorganisms tested.

2.3 Cancer:

Cancer is a disease in which uncontrolled growth and replication of cells or group of cells occurs and invade adjacent cells or tissues and then ultimately spread to other body parts. There are main three types of agents which cause cancer are known as carcinogens. These carcinogens include physical

agents (carcinogens) include ultraviolet and ionizing radiations, chemical carcinogens, include asbestos, components of tobacco smoke, aflatoxin (a food contaminant) and arsenic (a drinking water contaminant), biological carcinogens include infections from viruses, bacteria or parasites.

2.4 Breast Cancer: Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk**.** 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.

2.4a Invasive breast cancer - The cancer cells break out from inside the lobules or ducts and invade nearby tissue.

2.4b Non-invasive breast cancer - This is when the cancer is still inside its place of origin and has not broken out.

2.5 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).

2.5a 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.

2.5b 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.

2.6 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.

2.7 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.

Golamreza *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.

According to Flores *et al.* (2010), phytochemicals are known to modulate immune function and possess antitumor and antimicrobial properties. In this study, aqueous and methanol extracts of *Coriandrum sativum* (leaf and seed), *Piper nigrum* and *Cinnamomum zeylanicum* seeds have *in vitro* antitumor and immunomodulating activities. They observed that aqueous extract of *C. sativum* (leaf), *P. nigrum,* and *C. zeylanicum* caused significant (P<0.05) 24, 39 and 61 percent L5178Y-R lymphoma cells toxicity at 31.2, 31.2 and 7.8 mg/ml (MICs) respectively, whereas the methanol extract of *C. sativum* (seed and leaf), *P. nigrum,* and *C. zeylanicum* caused 40, 31, 26 and 39 percent cytotoxicity at 7.8, 62.5, 15.6 and 7.8 mg/ml (MICs), respectively. Furthermore, all spice aqueous extracts tested were observed to significantly (P<0.01) reduce up to 100% nitric oxide production by LPS-stimulated macrophages.

In this study, Kwon *et al.* (2010), identified that anti-tumor effect of cinnamon extracts is also link with enhanced pro-apoptotic activity by inhibiting the activities NF_{β} and AP1 in mouse melanoma model. They tested 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.

2.8 Immunomodulation

Immunomodulation means that one can modulate immunity using various substances either of natural or synthetic origin. An immunomodulatory agent is a drug that may be an immunosuppressant or an immunostimulator based on its effect on the immune system.

2.8a Immunosuppressants An agent capable of suppressing the body's immune response they are given to patients after organ transplant surgery to prevent organ rejection. They may be applied exogenously or endogenously. Examples of immunosuppressants are corticosteroid hormones in large amounts, cytotoxic drugs, and irradiation.

2.8b Immunostimulator An agent capable of stimulating or increasing the activity of the immune system. An example of immunostimulator is the granulocyte macrophage colony-stimulating factor.

Two major types of immunostimulators are: specific immunostimulator and non-specific immunostimulator.

2.8b.1 Specific immunostimulator:

A type of immunostimulator that provides antigenic specificity in immune response, such as vaccine or any antigen.

2.8b.2 Non- specific immunostimulator:

A type of immunostimulator that augments immune response of other antigen or stimulate the immune system without antigenic specificity, such as adjuvant.

Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defence mechanism has to be activated under conditions of impaired immune response, or when a selective immunosuppression is desired in situations such as autoimmune disorders.

2.9 Natural Immunomodulators: Immunomodulators are natural or synthetic substances that help regulate or normalize the immune system. The natural immunomodulators act to strengthen weak immune systems and to moderate immune systems that are overactive. Natural immunomodulators are less potent than prescription immunomodulators and also less likely to cause side effects. Plant sterols and sterolins are natural immunomodulators found in some raw fruits and vegetables and in the alga, spirulina. Other natural immunomodulators include aloe vera, plumbago indica, aegle marmalos, ginseng root, chamomile tea, reishi mushroom extract, olive leaf extract, N. sativa oil, polysaccharides isolated from *Juniperus scopolorum, Isodon serra* extract, *Ficus carica* leaf extract.

According to Flores *et al.*, 2010 phytochemicals are known to modulate immune function and possess antitumor and immunomodulating properties. In this study, aqueous and methanol extracts of *Coriandrum sativum* (leaf and seed), *Piper nigrum* and *Cinnamomum zeylanicum* seeds have *in vitro* antitumor and immunomodulating activities. They observed that aqueous extract of *C. sativum* (leaf)*, P. nigrum,* and *C. zeylanicum* caused significant ($P<0.05$) 24, 39 and 61 percent L5178Y-R lymphoma cells toxicity at 31.2, 31.2 and 7.8 mg/ml (MICs) respectively, whereas the methanol extract of *C. sativum* (seed and leaf), *P. nigrum,* and *C. zeylanicum* caused 40, 31, 26 and 39 percent cytotoxicity at 7.8, 62.5, 15.6 and 7.8 mg/ml (MICs), respectively. Furthermore, all spice aqueous extracts tested were

observed to significantly (P<0.01) reduce up to 100% nitric oxide production by LPS-stimulated macrophages. They have shown for the first time and in a single study the antitumor potential of *C. sativum*, *P. nigrum* and *C. zeylanicum* aqueous and methanol extracts, and their effect on stimulating lymphoproliferation and suppressing nitric oxide production by macrophages without altering their viability.

As this is the only study that has done on immunomodulatory activity of *Cinnamomum zeylanicum* it would be interesting to explore this activity of *Cinnamomum zeylanicum.*

CHAPTER 3
OBJECTIVES

The main objectives of present study are to explore **the antibacterial and anticancer effect** of different solvent extracts of cinnamon bark.

Work plan of the current study is as follows:

1. Preparation of cinnamon bark extract in different solvents
2. Estimation of antibacterial activity based on agar diffusion assay and determining minimal inhibition concentration of different extracts
3. Determination of anticancer activity in MCF-7 breast cancer cell lines based on MTT assay

CHAPTER 4

MATERIAL AND METHOD

4.1 MATERIAL

Table 4.1: Requirements during the whole project work

REQUIREMENTS	**COMPANY**
DMEM	Himedia
FBS	Himedia
Trypsin	Sigma Aldrich
Sodium bicarbonate	Himedia
L-glutamine	Himedia
Penicillin Sodium G	Himedia
Streptomycin Sulphate	Himedia
Ficoll	Sigma
Trypan blue	Himedia
MTT reagent	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	SRL
Nutrient agar	Himedia
Luria broth	Himedia
Muller Hinton agar	Himedia
Silica gel G	Himedia

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

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

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%
Sodium Chloride (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)	8.01 g/l
KCl (2.7 mM)	0.2 g/l
Na_2HPO_4 (4.3 mM)	1.44 g/l
KH_2PO_4	0.24 g/l
pH	7.4

Table 4.6: 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 (1×10^8 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

4.2 METHOD

4.2a PREPARATION OF EXTRACTS

Cinnamon extracts were prepared in different solvents. Sticks of cinnamon were washed and dried to remove any kind of dust and other materials. The dried sticks of cinnamon were grinded in a grinder to make a fine powder. 30 g of this fine powder was taken and suspended in the flasks containing 100 ml of methanol (99.9% pure), acetone (99%), ethyl acetate (extrapure), and pure ethanol solvent. This suspension of the cinnamon powder and solvents were kept at 37°C for 72 hours at 130 rpm in the shaking incubator and then after incubation, these were filtered by using Whattman filter paper no.1 in separate falcon tubes. Now the filtrate was evaporated at 30°C till the solvent was completely evaporated and we get the dried powder. Now dry mass was calculated using the formula:

Dry mass= initial weight – final weight

The dried mass was dissolved completely in pure DMSO accordingly so that the final concentrations of extracts become 100 mg/ml. These dissolved extracts were now stored in aliquots at 0° C for further use.

4.3 DETERMINATION OF ANTIMICROBIAL ACTIVITY OF CINNAMON

The antimicrobial activity of cinnamon was determined in four different cultures of bacteria which include two gram positive and two gram negative. To find whether these different extracts of cinnamon 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 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 Turbidity standard for inoculums preparation (NCCLS, 1997)

To standardize the inoculum density for susceptibility test, a Barium sulphate ($BaSO_4$) turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent should be used. A $BaSO_4$ 0.5 McFarland standard was prepared as 0.5 ml aliquot of 0.048 mol/l $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) is added to 99.5 ml of 0.18 mol/l H_2SO_4 (1% w/v) with constant stirring to maintain a suspension. The correct density of turbidity standard, it must be verified by using a spectrophotometer with a 1cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm should be 0.144 to 0.146 for the McFarland standards. The Barium sulphate ($BaSO_4$) suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of same size as those used in growing or diluting the bacterial inoculums (NCCLS, 1997).

4.3e Preparation of Mueller-Hinton Agar

Mueller-Hinton Agar was prepared from a HiMedia dehydrated base according manufacturer's instructions. Immediately after autoclaving, it was cooled and dispensed into plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform surface to give a uniform depth of approximately 5 ± 0.5 mm. This corresponds to 25 ml of medium for plates with diameter of 90 mm. The agar medium was allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8° C). Plates were used within seven days after preparation unless adequate precautions, such as wrapping in parafilm, have been taken to minimize drying of the agar. A representative sample of each batch of plates was examined for sterility by incubating at 37ºC for 24 hours or longer.

4.3f Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into two types based on the principle applied in each system. They include:

4.3f.1 Diffusion

Agar well diffusion assay

4.3f.2 Dilution

Minimum Inhibitory Concentration Broth dilution

4.3f.1 Agar- Well Diffusion assay

Initial screening of potential antibacterial activity from cinnamon 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 37° 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 30 µl 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 37° 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.

4.3f.2 Minimum Inhibitory Concentration Broth Micro dilution Assay (Eloff, 1998)

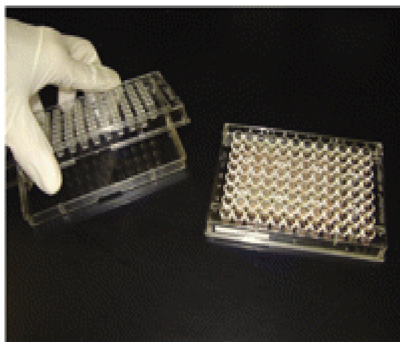


Figure 1: A broth micro dilution 96 titer well plate

Principle:

In minimum inhibitory concentration dilute on a $\log_2$ scale each antimicrobial agent in broth to provide a range of concentrations and to inoculate each tube or, if a micro plate is used, each well containing the antimicrobial agent in broth with a standardized suspension of the microorganism to be tested. The lowest concentration of antimicrobial agent that inhibits the growth of the microorganism is the minimum inhibitory concentration (MIC). The MIC and the zone of inhibition are inversely correlated. In other words, the more susceptible the microorganism is to the antimicrobial agent, the lower the MIC and the larger the zone of inhibition. Conversely, the more resistant the microorganism, the higher the MIC value and the smaller the zone of inhibition. The miniaturization and mechanization of the test by use of small, disposable, plastic "micro dilution" trays has made broth dilution testing practical and popular. Standard trays contain 96 wells, each containing a volume of 0.1 ml that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray (Jorgensen *et al.,* 2007 National Committee for Clinical Laboratory Standards, 2009).

The advantages of the micro dilution procedure include the generation of MICs, the reproducibility and convenience of having prepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test.

The main disadvantage of the micro dilution method is some inflexibility of drug selections available in standard commercial panels.

Procedure:

A suspension of test organism was prepared equivalent to a 0.5 McFarland standard using isolated colonies. 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 were added in other wells. The absorbance of each well was determined using an automatic ELISA tray reader adjusted at 600 nm.10 µl 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 37° 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 ANALYSIS OF COMPONENTS BY TLC (THIN LAYER CHROMATOGRAPHY)

Principle

In TLC, the separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (via capillary action) travel over the surface of stationary phase. During this movement the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture is achieved. Once separation occurs individual components are visualized as spots at respective level of travel on the plate. Their nature is identified by means of suitable detection agents.

Procedure:

50 g of silica gel G was weighed out and shaken to form a homogeneous suspension with 100 ml of distilled water. This suspension was poured into TLC applicator which was adjusted to 0.25 mm thickness. Plates were laid together in a row and coated with silica gel by drawing the applicator. The plates were allowed to dry at room temp and then dried at 110° C for 30 minutes in hot air oven. After drying the plates a thin mark is made at the bottom of the plate with a pencil to apply the sample spots. Then samples are applied on the spots marked on the line at equal distances. The mobile phase is poured into the TLC chamber to a level few centimeters above the chamber bottom. A filter paper moistened in mobile phase is placed on the inner wall of the chamber to maintain equal humidity in the entire chamber. Then the plate prepared with sample spotting is placed in TLC chamber such that the side of the plate with sample line is towards the mobile phase. Then the chamber is closed with a lid and allowed for development of spots. Then the plates are removed and allowed to dry. The sample spots are visualized in suitable UV light chamber or any other methods as recommended for the said sample (Kumar *et al.*, 2012). The solvent systems were used Toluene: Acetic acid (7:3).

The staining of a TLC plate with iodine vapour is among the oldest methods for the visualization of organic compounds. It is based upon the observation that iodine has a high affinity for both unsaturated and aromatic compounds.

A chamber may be assembled as follows:

The wide mouth jar (with cap) is added a piece of filter paper and few crystals of iodine. Iodine has a high vapour pressure for a solid and the chamber will rapidly become saturated with iodine vapour. TLC plate is inserted and allowed it to remain within the chamber until it develops a light brown colour over the entire plate. Commonly, if the compound has an affinity for iodine, it will appear as a dark brown spot on a lighter brown background. Carefully the TLC plate is removed at this point and gently circle the spots with a dull pencil. The iodine will not remain on the TLC plate for long periods of time so circling these spots is necessary if one wishes to refer to these TLC's at a later date.

Ninhydrin is used for the detection of amino acids. Ninhydrin was prepared by dissolving 1.5 g ninhydrin in 100 ml of *n*-butanol and then add 3.0 ml acetic acid.

Vanillin is very good general stain, giving a range of colours for different spots. Vanillin was prepared by dissolving 15 g vanillin in 250 ml ethanol and 2.5 ml concentrated sulfuric acid.

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

4.5a PREPARATION OF POWDERED DMEM MEDIA:

9.6 g 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° C and in dark till use. 10% heat inactivated fetal bovine serum (57° C for 30 minutes) and filter sterilized antibiotics (Streptomycin (10 mg/ml), Penicillin (10^4 IU/ml), Amphotericin (2.5 mg/ml) and Tylosinom (1 ml/l) were added to media before culturing of cells.

4.5b 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 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 4^0 C temperatures.

4.5c REVIVING THE MCF-7 CELL LINES.

Culturing of cell lines was carried out aseptically under BSL-2 and CO_2 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 37^0C and 5% CO_2.

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 CO_2 (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.

d. 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 10^4 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 CO_2 incubator at 37° C and 5% CO_2 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.

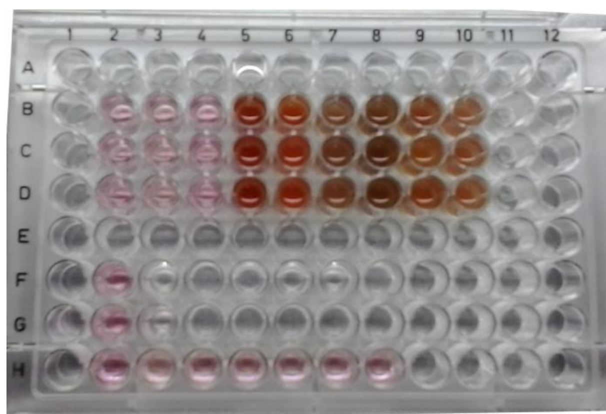


Figure 2: 96 well micro titer plate with different cinnamon extracts

4.6 DETREMINATION OF IMMUNOMODULATORY AVTIVITY

4.6a Preparation of RPMI 1640 Media:

10.3 g of powder RPMI 1640 media was suspended in 900 ml of tissue culture grade water with constant stirring until the powder was completely dissolved. pH was adjusted to 4.0. The pH was adjusted to 0.2-0.3 unit below the desired pH since the pH tends to rise during filtration. The volume was made to 1 liter with tissue culture grade water. Volumes of 7.5% sodium bicarbonate and 200 mM L-glutamine solution were subtracted from the final volume. Sodium bicarbonate and L-glutamine was filter sterilized before use. The medium was autoclaved at 121° C at 15 lbs for 15 minutes. The medium was removed promptly from the autoclave to avoid extended heating or evaporation and allowed to cool at room temperature. 49.3 ml of sodium bicarbonate solution and 20 ml of 200 mM L-glutamine solution was added to final volume of the medium being prepared. 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. Medium was stored at 2-8ºC till use.10% heat inactivated fetal bovine serum (57° C for 30 minutes) and filter sterilized antibiotics (Streptomycin, Penicillin, Amphotericin and Tylosin) were added to media before culturing of cells.

4.6b Isolation of Peripheral Blood Mononuclear Cells from Whole Blood:

Blood was drawn from a healthy human being with the help of vacutainer system (EDTA coated, Becton Dickinson). Blood was diluted in 1:1 ratio with PBS. Now the blood sample was layered carefully layered over equal volume of ficoll and it was centrifuged at 700xg for 40 minutes at 25° C. Plasma layer was removed and then buffy coat layer was taken out with the help of an auto pipette. Buffy coat layer was diluted to 1:1 with PBS ant centrifuged at 400xg for 10 minutes at 25° C. Supernatant was discarded and pellet was again washed with PBS (5ml) and centrifuged at 400xg for 10 minutes at 25° C. Supernatant was discarded and pellet of PBMC was suspended in cell culture medium [(DMEM + 10% FBS + Penicillin (100 IU/ml) + Streptomycin (100 µg/ ml)].

4.6c 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 trypan blue 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:

$$\text{Cell count} = \frac{\text{Total number of cells counted}}{\text{Number of chambers counted}} \times \text{Dilution Factor}$$

Figure 3: Hemocytometer used for cell counting

4.6d MTT Assay:

Cell proliferation was tested using a 3-(4, 5- dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT assay). For the assay, PBMC (2 X 10^5 cells/200 µl) were freshly isolated and 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 CO_2 incubator at 37° C and 5% CO_2 concentration for 48 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 micro titer plate reader.

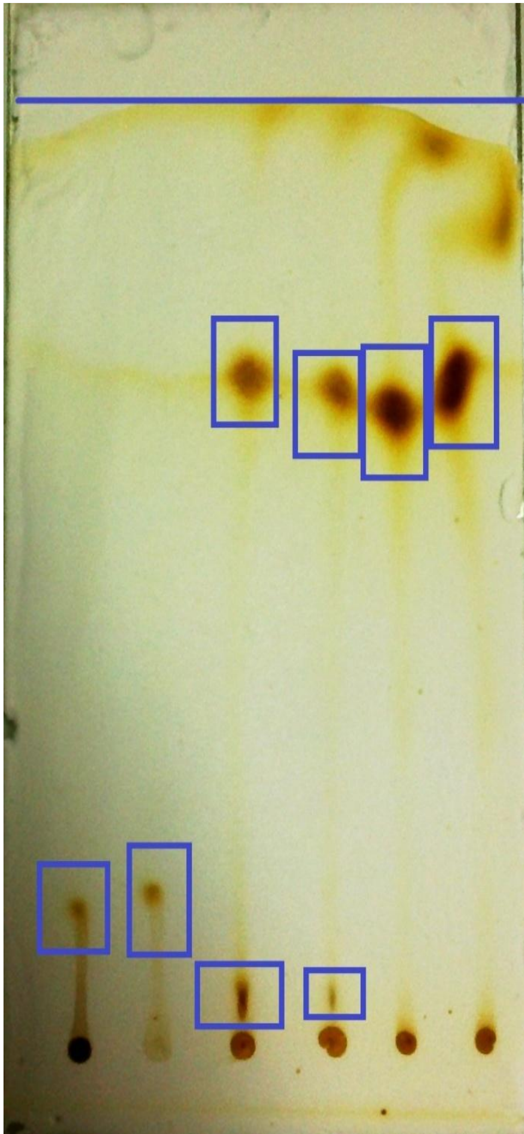
CHAPTER 5

RESULTS AND DISCUSSION

5.1 TLC RESULTS

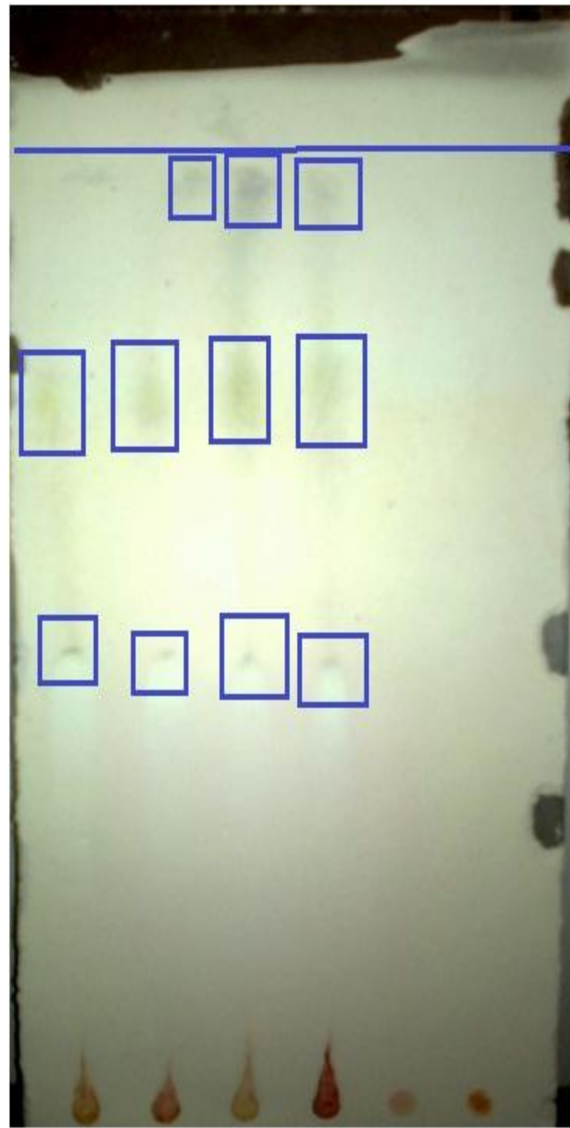
For extraction of soluble components in cinnamon, five different solvents were used i.e. water, ethanol, methanol, ethyl acetate, and acetone. To investigate difference in extraction by the above mentioned solvents, the extracts were analyzed by thin layer chromatography using Toluene: acetic acid (7:3) as mobile phase on silica plates. The separated analytes were visualized by iodine and vanillin staining separately. Ninhydrin staining does not show any bands. The chromatograms exhibited one to three visible bands in different extracts stained by two methods. From these preliminary results it was inferred that methanol and acetone extracts are similar and on the other hand ethyl acetate and ethanol extracts also showed similar patterns of analytes migration. There may be finer differences in these extracts which could not be discerned due to poor sensitivity of the technique. However it was concluded from these results that following studies should be carried out separately with all the extracts.

IODINE



Methanol extract
Acetone extract
Ethyl acetate extract
Ethanol extract
Cold aqueous extract
Hot aqueous extract

VANILLIN



Methanol extract
Acetone extract
Ethyl acetate extract
Ethanol extract
Cold aqueous extract
Hot aqueous extract

5.1 ANTIBACTERIAL ACTIVITY

Antimicrobial activity of the different extracts of cinnamon was determined by using agar well diffusion assay and MIC. We have prepared the different extracts of cinnamon using solvents: methanol, ethanol, acetone and ethylacetate. 30 µl of each extract from the stock concentration of 100 mg/ml was loaded in wells of the bacterial cultures MHA plates and incubated for 24 hours. After incubation, zone of inhibition was observed in different extracts which was noted and mentioned in table 5.1. Interestingly, we have found the inhibition activity of all four extracts of cinnamon in case of all four bacteria (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus cereus)* as shown in figure 4,5,6,7 and 8. Maximum inhibitory activity was observed in case of ethyl acetate extract against *Escherichia coli* and then followed by *Pseudomonas aeruginosa* and *Bacillus cereus*. Hence our results show that cinnamon has strong antibacterial activities.

Table 5.1: Antimicrobial activity of Cinnamon extracts

EXTRACTS OF CINNAMON	ZONE OF INHIBITION IN mm			
	E.coli	Staphylococcus	Pseudomonas	Bacillus
Cinnamon methanol (30%)	20.0	12.3	16.2	16.2
Cinnamon acetone (30%)	27.0	17.7	22.3	22.3
Cinnamon ethylacetate (30%)	30.0	26.0	28.0	28.0
Cinnamon pure ethanol (30%)	30.0	19.0	24.5	24.5
Streptomycin (+ve control)	28.0	23.0	25.5	29.3
DMSO (-ve control)	NI*	NI	NI	NI

*Not inhibited

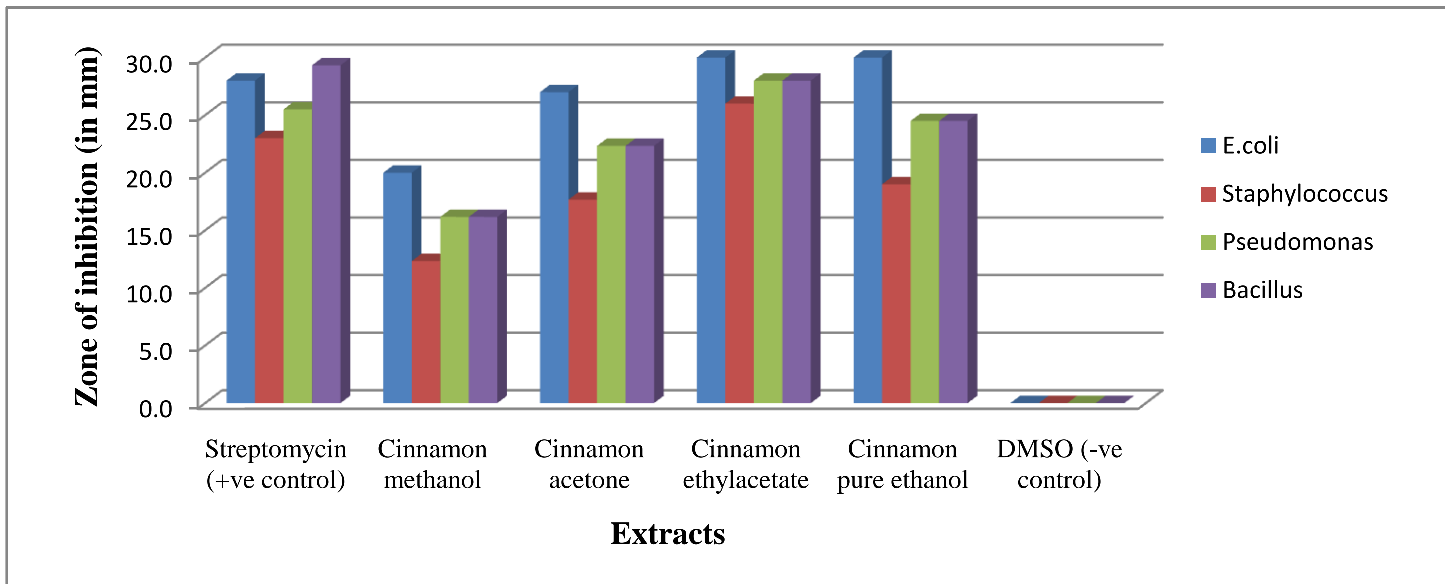


Figure 4: Antibacterial activity of different cinnamon extracts on different bacteria (Agar well diffusion assay)

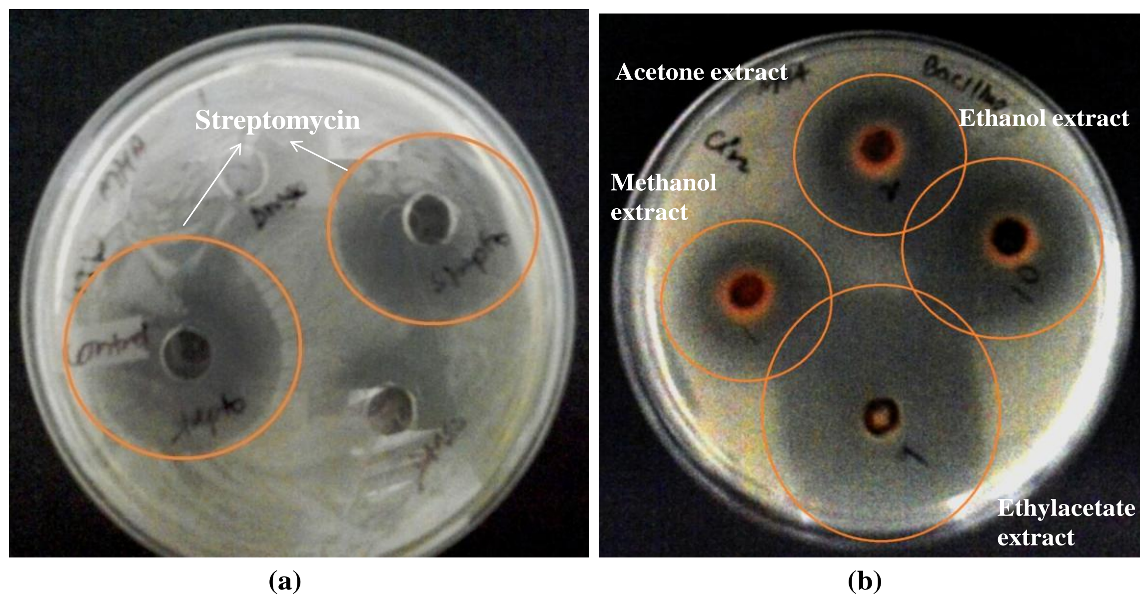
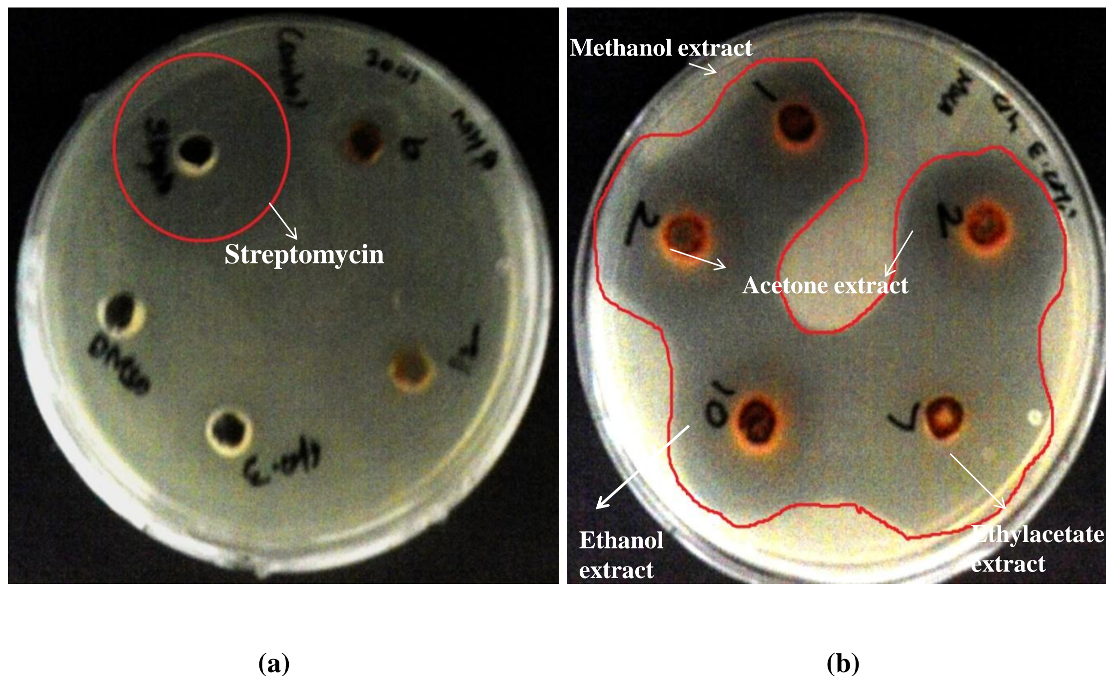
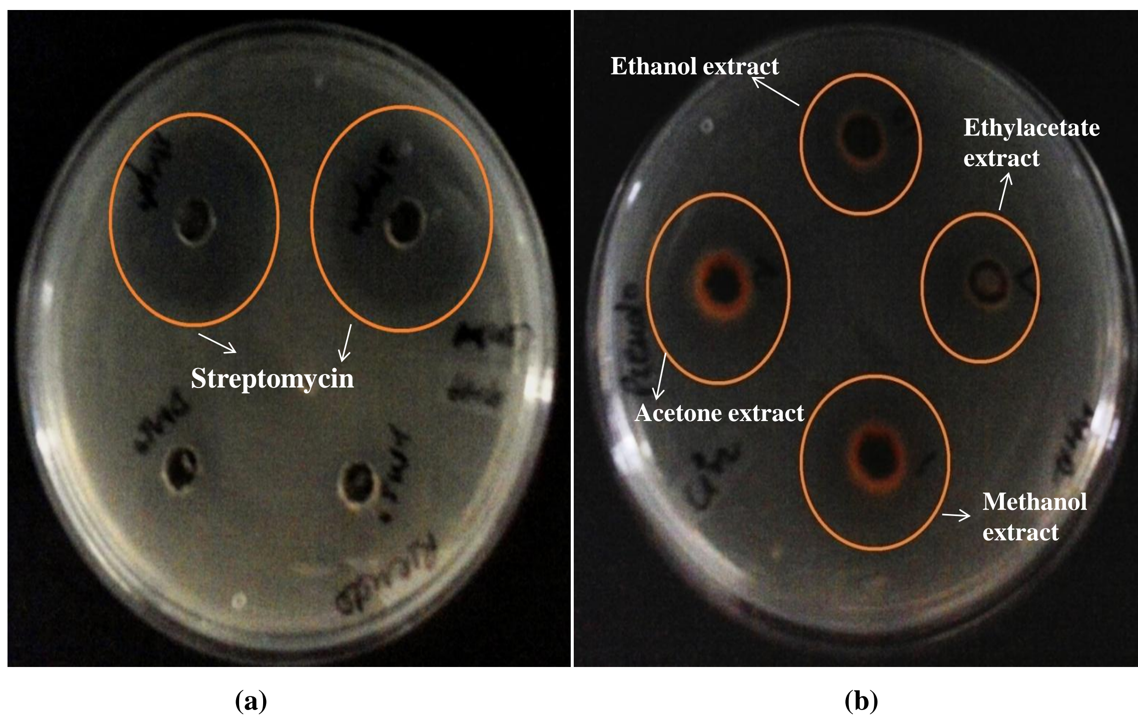


Figure 5 : Antibacterial activity of cinnamon extracts against *Bacillus cereus*



(a) (b)

Figure 6 : Antibacterial activity of cinnamon extracts against *Escherichia coli*



(a) (b)

Figure 7 : Antibacterial activity of cinnamon extracts against *Pseudomonas aeruoginosa*

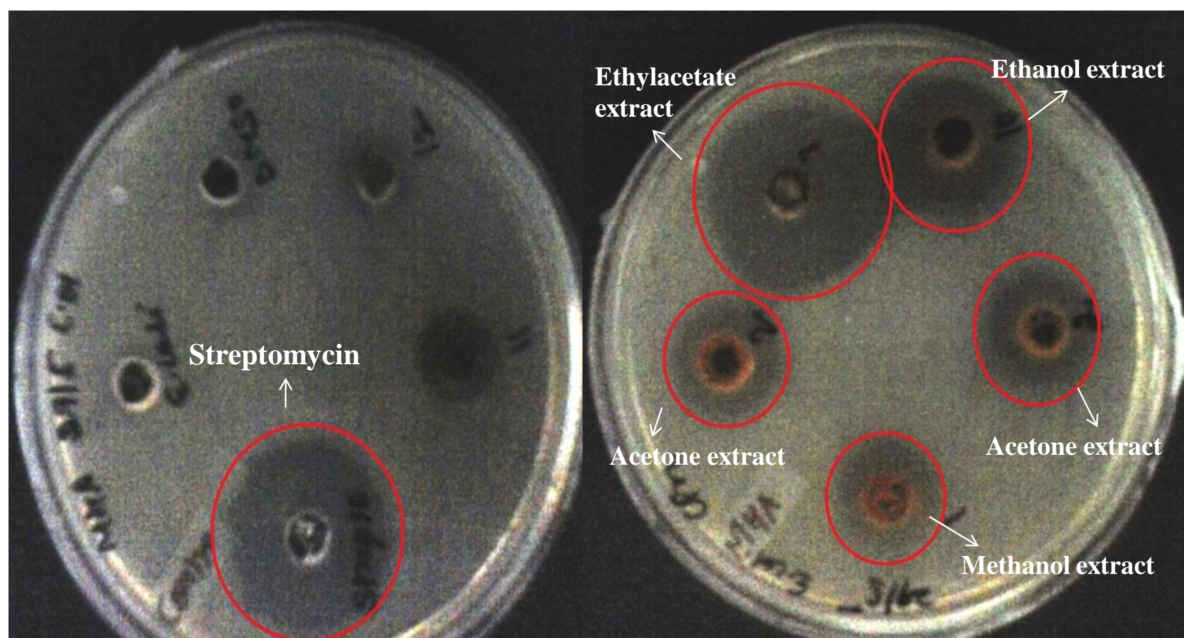


Figure 8 : Antibacterial activity of cinnamon extracts against *Staphylococcus aureus*

5.2 MINIMUM INHIBITORY CONCENTRATION

To find out the Minimum Inhibitory Concentration, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml concentrations of different extracts were used and the reading were taken at 0 hour and 24 hour after incubation. The results which we found after the assay were as follows:

The **ethyl acetate extract** and **acetone extract** of cinnamon showed the minimum inhibitory concentration of 5 mg/ml against *Bacillus cereus* (Figure 9) (Table 5.2).The **methanol** and **ethanol extracts** of cinnamon showed no activity against *Bacillus cereus.*

The **ethyl acetate** and **ethanol extracts** of cinnamon showed the minimum inhibitory concentration of 10 mg/ml against *Escherichia coli* (Figure 10) (Table 5.3). The **methanol extract** and **acetone extract** of cinnamon showed no activity against *Escherichia coli.*

The **methanol extracts, acetone, ethyl acetate, and ethanol extracts** of cinnamon showed a minimum inhibitory concentration at 5 mg/ml against *Pseudomonas aeruoginosa.* (Figure 11) (Table 5.4).

The **methanol extracts** of cinnamon showed a minimum inhibitory concentration at 10 mg/ml against *Staphylococcus aeurus,* whereas it showed no activity in case of **acetone, ethyl acetate and ethanol extracts** of cinnamon. (Figure 12) (Table 5.5).

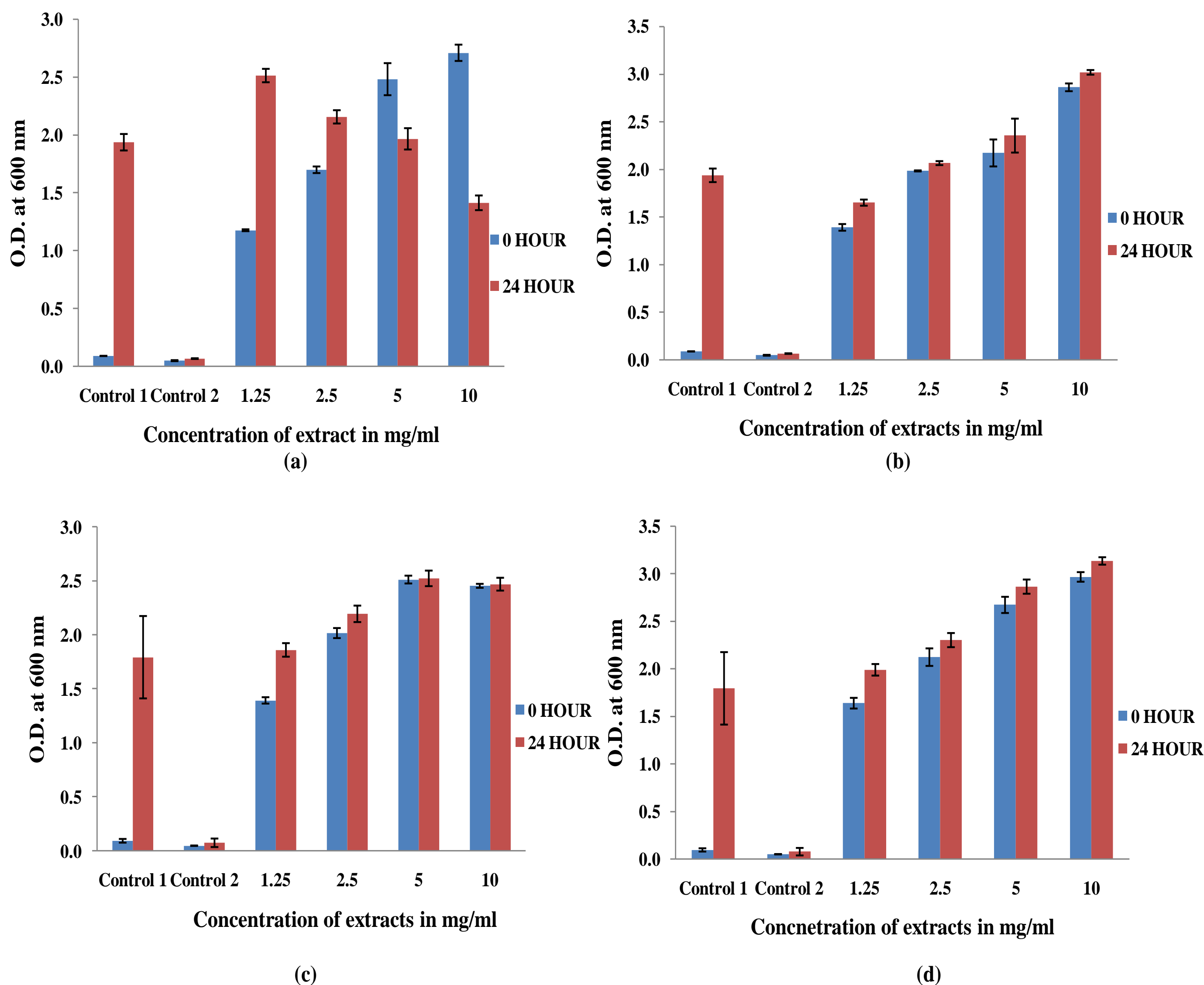


Figure 9 : Antibacterial activity against *Bacillus cereus* (MIC assay), (a) acetone extract, (b) methanol extract, (c) ethylacetate extract, (d) ethanol extract

Control 1= *Bacillus cereus* cells, Control2 = *Bacillus cereus* cells+streptomycin

Table 5.2: Antibacterial activity of cinnamon extracts against *Bacillus cereus*

	Methanol extract		Acetone extract		Ethyl acetate extract		Ethanol extract	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.092	1.939	0.092	1.939	0.094	1.793	0.094	1.793
Control 2	0.051	0.068	0.051	0.068	0.048	0.075	0.048	0.075
1.25mg/ml	1.392	1.652	1.178	1.415	1.394	1.861	1.637	1.988
2.5 mg/ml	1.985	2.067	1.701	1.968	2.017	2.195	2.121	2.301
5 mg/ml	2.173	2.355	2.484	2.158	2.512	2.523	2.671	2.863
10mg/ml	2.862	3.019	2.712	2.515	2.454	2.470	2.965	3.133

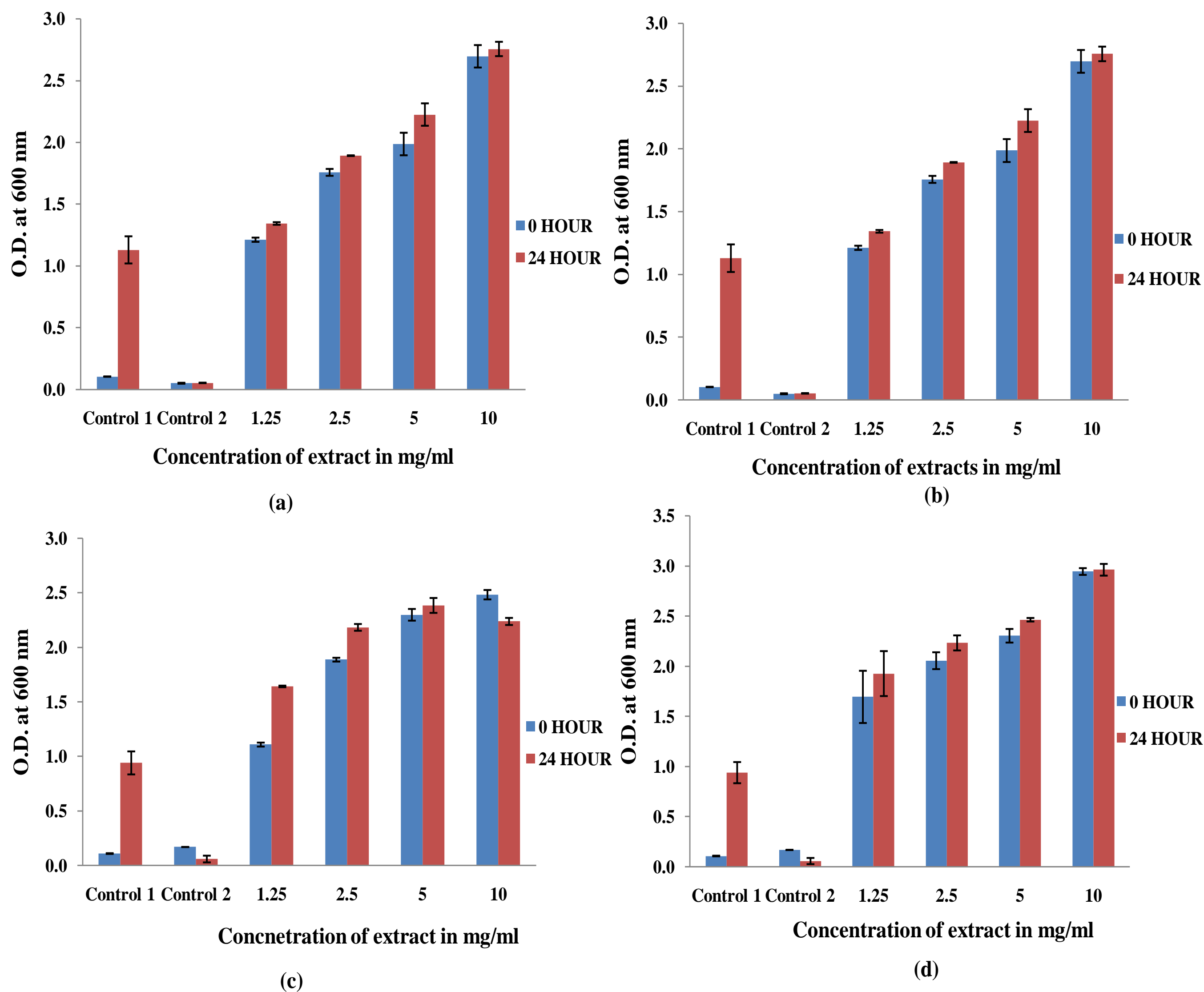


Figure 10 : Antibacterial activity against *Escherichia coli* (MIC assay), (a) acetone extract, (b) methanol extract, (c) ethyl acetate extract, (d) ethanol extract

Control 1= *E.coli* cells, Control2 = *E.coli* cells+streptomycin

Table 5.3: Antibacterial activity of cinnamon extract against *Escherichia coli*

	Methanol extract		Acetone extract		Ethyl acetate extract		Ethanol extract	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.103	1.129	0.103	1.129	0.108	0.939	0.108	0.939
Control 2	0.049	0.052	0.049	0.052	0.058	0.168	0.058	0.168
1.25mg/ml	1.434	1.573	1.212	1.343	1.107	1.641	1.694	1.926
2.5 mg/ml	1.801	1.970	1.757	1.892	1.886	2.182	2.055	2.232
5 mg/ml	1.311	1.694	1.986	2.225	2.297	2.383	2.304	2.463
10mg/ml	2.792	2.867	2.697	2.756	2.482	2.236	2.944	2.962

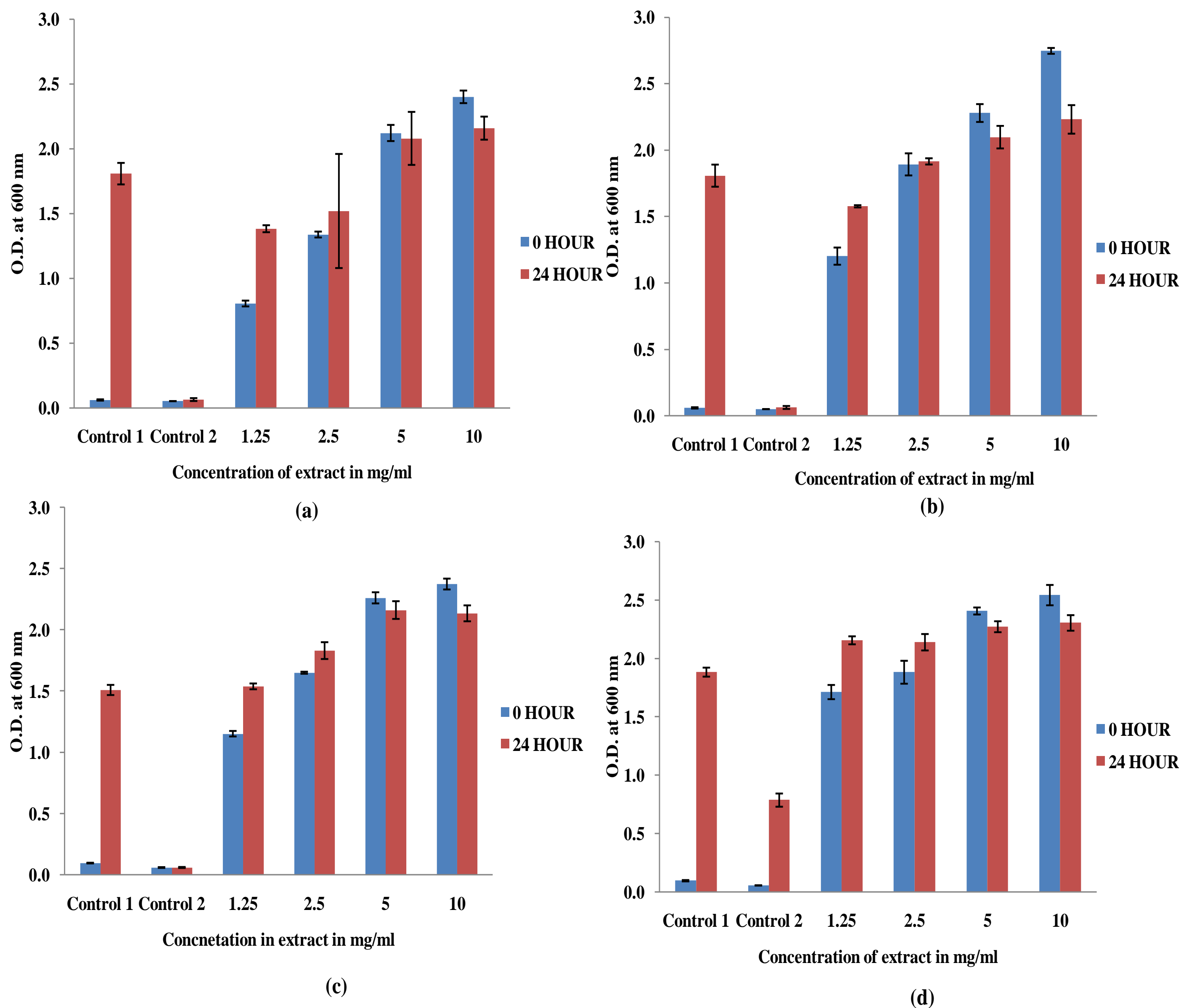


Figure 11 : Antibacterial activity against *Pseudomonas aeruoginosa* (MIC assay), (a) acetone extract, (b) methanol extract, (c) ethyl acetate extract, (d) ethanol extract

Control 1= *P. aeruoginosa* cells, Control2 = *P. aeruoginosa* cells+streptomycin

Table 5.4: Antibacterial activity of cinnamon extracts against *Pseudomonas aeruoginosa*

	Methanol extract		**Acetone extract**		**Ethyl acetate extract**		**Ethanol extract**	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.061	1.808	0.061	1.808	0.095	1.509	0.095	1.882
Control 2	0.051	0.064	0.051	0.064	0.059	0.059	0.055	0.786
1.25mg/ml	1.203	1.577	0.805	1.383	1.152	1.538	1.711	2.154
2.5 mg/ml	1.893	1.915	1.338	1.520	1.650	1.830	1.881	2.138
5 mg/ml	2.279	2.098	2.122	2.081	2.262	2.162	2.406	2.271
10mg/ml	2.747	2.232	2.401	2.160	2.374	2.135	2.541	2.303

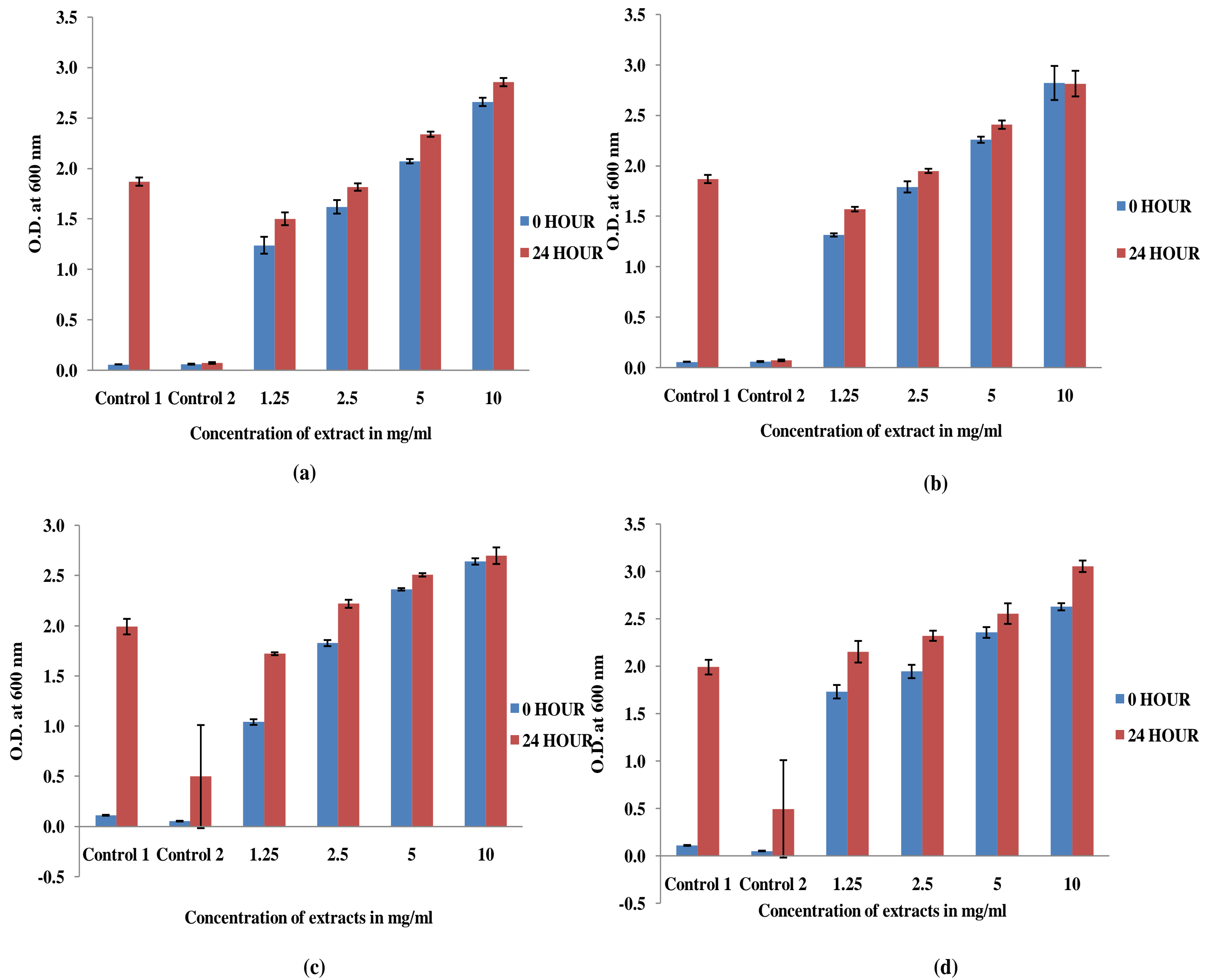


Figure 12 : Antibacterial activity against *Staphylococcus aeurus* (MIC assay), (a) acetone extract, (b) methanol extract, (c) ethyl acetate extract, (d) ethanol extract.

Control 1= *S. aeurus* cells, Control2 = *S. aeurus* cells+streptomycin

Table 5.5: Antibacterial activity of cinnamon extracts of against *Staphylococcus aeurus*

	Methanol extract		Acetone extract		Ethyl acetate extract		Ethanol extract	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.059	1.871	0.059	1.871	0.111	1.990	0.111	1.990
Control 2	0.061	0.073	0.061	0.073	0.052	0.496	0.052	0.496
1.25mg/ml	1.316	1.572	1.240	1.502	1.039	1.720	1.731	2.153
2.5 mg/ml	1.793	1.951	1.620	1.816	1.826	2.218	1.944	2.321
5 mg/ml	2.260	2.410	2.073	2.340	2.362	2.506	2.356	2.555
10mg/ml	2.823	2.817	2.661	2.857	2.639	2.697	2.627	3.054

5.3 ANTICANCER ACTIVITY OF CINNAMON EXTRACTS ON MCF-7 CELLLINE

It was interesting to explore the anticancer property of different extracts of cinnamon bark. We have used MCF-7 breast cancer cell lines to assess the anticancer property. The MTT assay was done to find the anticancer activity using the different concentrations (250 µg/ml, 500 µg/ml, 1 mg/ml) of the methanol and acetone extracts of cinnamon. The methanol and acetone extracts of cinnamon have shown anticancer activity at a concentration 250 µg/ml against the MCF-7 breast cancer cell lines (Figure 13) (Table 5.6). In case of ethyl acetate and ethanol extracts of cinnamon, we have used 125µg/ml, 250 µg/ml, and 500 µg/ml concentrations. The ethyl acetate extract of cinnamon showed an activity at a concentration of 125 µg/ml, and ethanol extract of cinnamon did not show any activity against the MCF-7 cells (Figure 14) (Table 5.7). We observed anticancer activity at lower concentration in different extracts but not at higher concentration which is conflicting our data. One explanation could be that extract is a mixture of different compounds hence the behavior is different. With these results it is difficult to conclude the exact anticancer potential of the cinnamon extract. It would be interesting to vary the concentration and then perform more experiments to confirm the anticancer activities.

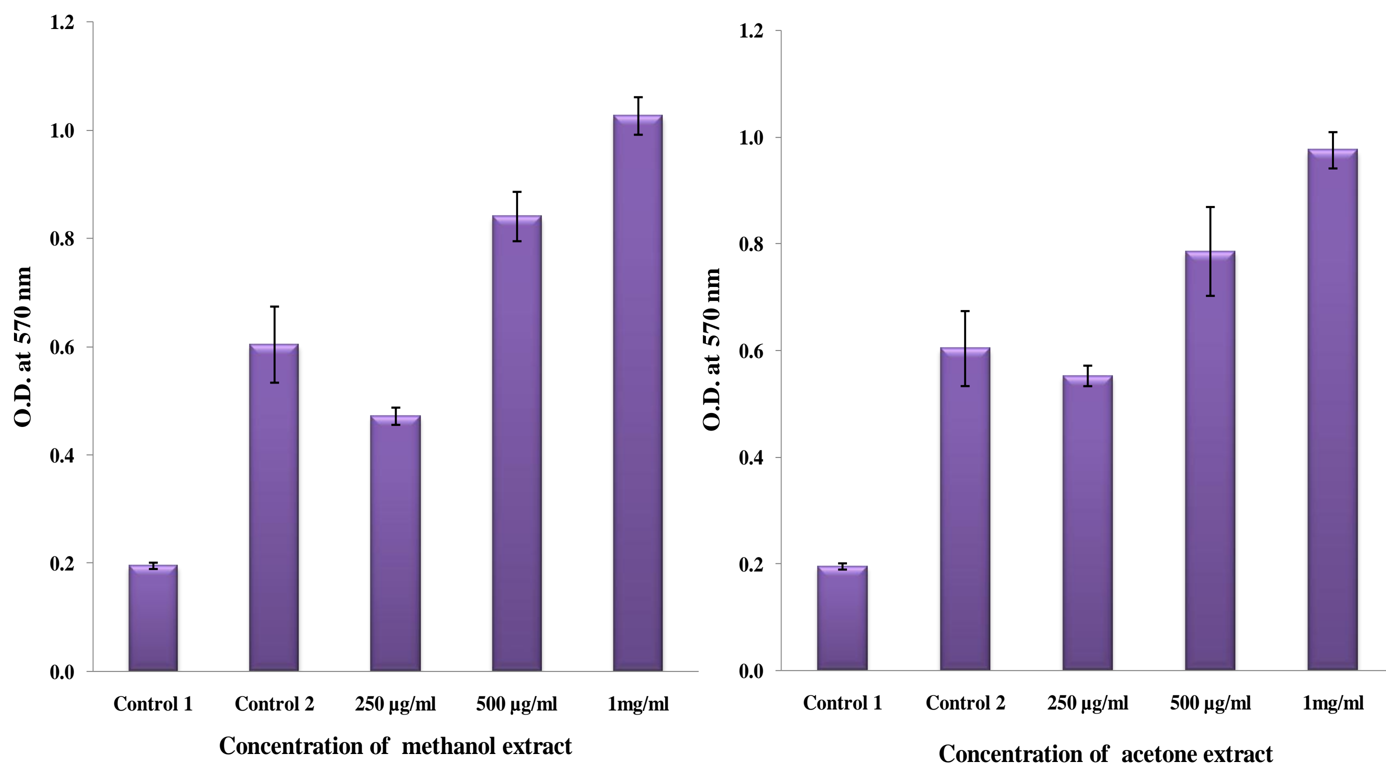


Figure 13 : Antiancer activity of methanol and acetone extracts of cinnamon

Control 1= MCF-7 cells, Control 2 = MCF-7 cells+DMSO

Table 5.6: Anticancer activity of methanol and acetone extracts on MCF-7 breast cancer cell line

	Methanol Extract	Acetone Extract
Control 1	0.194	0.194
Control 2	0.603	0.603
250 µg/ml	0.470	0.551
500 µg/ml	0.839	0.785
1 mg/ml	1.025	0.974

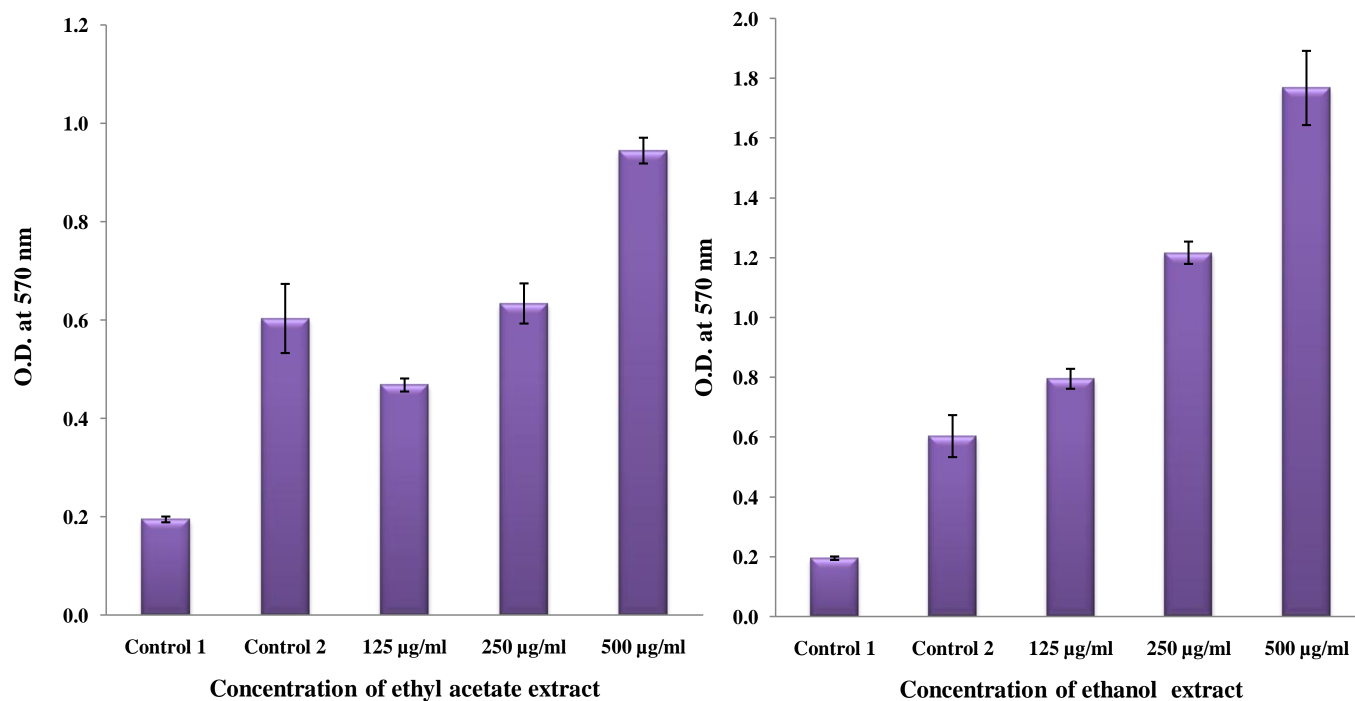


Figure 14 : Antiancer activity of ethylacetate and ethanol extracts of cinnamon

Control 1= MCF-7 cells, Control2 = MCF-7 cells+DMSO

Table 5.7: Anticancer activity of ethyl acetate and ethanol extracts on MCF-7 breast cancer cell line

	Ethyl acetate Extract	Ethanol Extract
Control 1	0.194	0.194
Control 2	0.603	0.603
125 µg/ml	0.944	1.768
250 µg/ml	0.633	1.216
500 µg/ml	0.467	0.795

5.4 IMMUNOMODULATORY ACTIVITY OF CINNAMON EXTRACTS

One of the important aspects of our study was to look for immunomodulatory activity of different extracts of cinnamon. Immunomodulatory activities were determined by lymphocyte proliferation assay. In our experiment, we used three different concentrations (250µg/ml, 500µg/ml and 1mg/ml) of cinnamon extracts and checked their activity in $2*10^5$ cells/200µl PBMCs which were seeded in each well. To check the proliferation, PBMCs were cultures for 72 hours and then MTT assay was done. It appears that cinnamon extract is showing mostly immunostimulation activities after 72 hour culture (Figure 15, 16) (Table 5.8). But it was also observed that 250 µg/ml of methanol, acetone and ethyl acetate extracts of cinnamon has shown immunosuppressive potential (Figure 15). The immunomodulatory behavior of extracts varies in different concentration of extracts like we observed in anticancer experiments. The same explanation could be that it contains mixture of different compounds. It will be interesting to carry out more experiments in order to confirm their immunomodulation activities of these extracts.

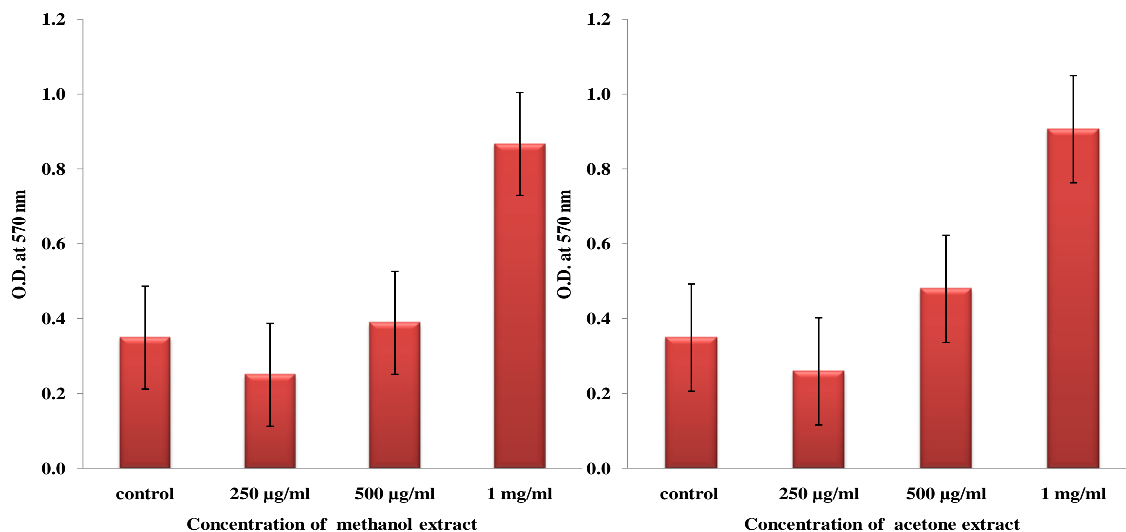


Figure 15: Effect of cinnamon extracts on PBMCs

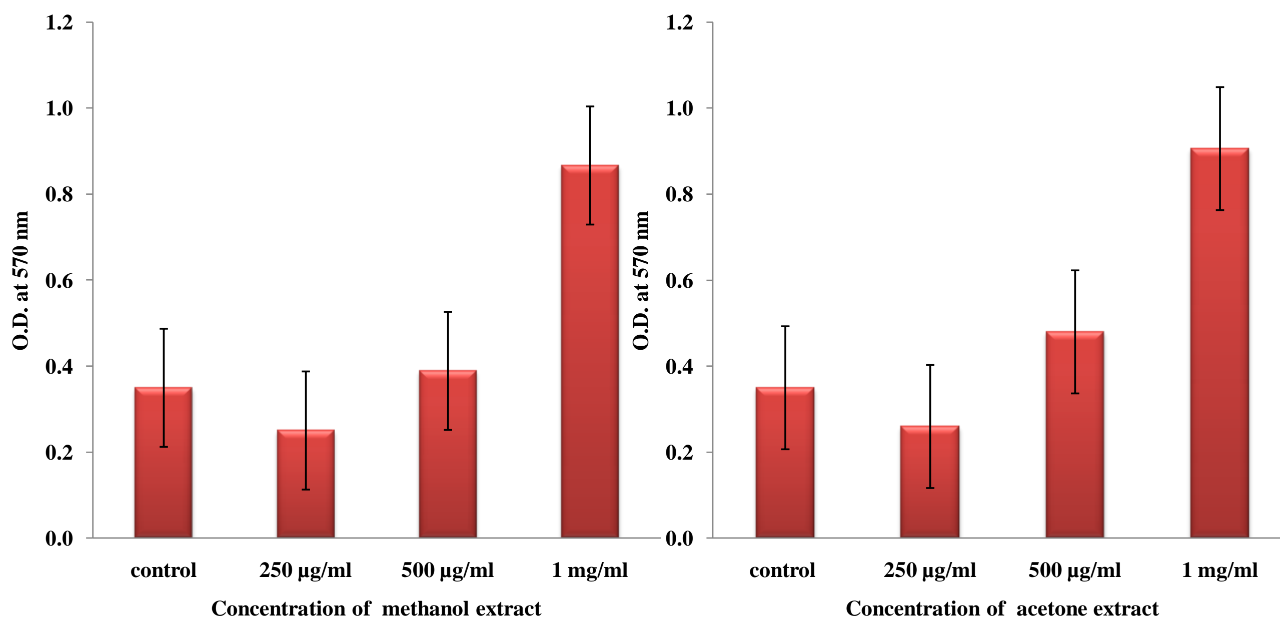


Figure 16: Effect of cinnamon extracts on PBMCs

Control= Lymphocytes

Table 5.8: Effect of cinnamon extracts on PBMCs

	Methanol extract	Acetone extract	Ethyl acetate extract	Ethanol extract
Control	0.349	0.349	1.283	0.349
250 µg/ml	0.250	0.259	1.224	0.487
500 µg/ml	0.389	0.479	1.274	0.708
1 mg/ml	0.867	0.906	1.599	1.279

CHAPTER 6
CONCLUSION

Cinnamon 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. Cinnamon holds promising putative bioactive, which possess valuable health benefits.

We have prepared different cinnamon (*Cinnamomum zeylanicum)* extracts by using ethanol, methanol, acetone, ethyl acetate and water as solvents. Thin layer chromatography was done to find out the different fractions of components present in the different extracts. Vanillin and iodine staining showed one to three bands of differently migrating analytes.

From all the experiments that we have conducted during the whole project it was concluded that cinnamon has strong antibacterial activity as it showed that cinnamon bark ethyl acetate extracts have strong antibacterial activities against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, and Pseudomonas aeruginosa*. We observed minimum inhibitory concentration mostly at 5 mg/ml and 10 mg/ml of different cinnamon extracts.

Cinnamon also shows anticancer activity against MCF-7 breast cancer cell lines. The methanol and acetone extracts of cinnamon have shown anticancer activity at a concentration 250 µg/ml and the ethyl acetate extract of cinnamon showed an activity at a concentration 125 µg/ml, and ethanol extract of cinnamon did not show any activity against the MCF-7 cells.

We also observed immunostimulation and immunosuppressive activities in peripheral blood mononuclear cells (PBMCs). Interestingly, we have found anticancer and immunomodulatory activities in different cinnamon extracts. But we observed discrepancy in these activities as activity is not maintained in higher concentrations. One possible explanation is that cinnamon bark extracts contain a mixture of different compound. We have performed limited experiments hence to confirm these activities, it is important to carry out more experiments.

CHAPTER 7

SUMMARY

Cinnamon 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 cinnamon. In the present work, we have studied the anticancer, antibacterial and immunomodulatory properties of cinnamon bark extracts.

We have prepared different cinnamon (*Cinnamomum zeylanicum)* extracts by using ethanol, methanol, acetone, ethyl acetate and water as solvents. TLC was done to find out the different fractions of components present in the different extracts. Vanillin and iodine staining showed one to three bands of differently migrating analytes.

Agar well diffusion and minimum inhibitory concentration (MIC) assays were performed in two gram positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) and two gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) for antibacterial activity. Agar well diffusion assay has shown antibacterial activity against all the four organisms but strong antibacterial activity was observed against *E.coli* in case of ethylacetate extract of cinnamon.

Based on minimum inhibitory concentration assay the best MIC values were obtained at 5 mg/ml and 10 mg/ml with different extracts. The **ethyl acetate extract** and **acetone extract** of cinnamon showed the minimum inhibitory concentration of 5 mg/ml against *Bacillus cereus.* The **ethyl acetate** and **ethanol extracts** of cinnamon showed the minimum inhibitory concentration of 10 mg/ml against *Escherichia coli.* The **methanol extracts, acetone, ethyl acetate, and ethanol extracts** of cinnamon showed a minimum inhibitory concentration at 5 mg/ml against *Pseudomonas aeruoginosa.* The **methanol extracts** of cinnamon showed a minimum inhibitory concentration at 10 mg/ml against *Staphylococcus aeurus.*

MTT assay was performed to assess the anticancer activity of cinnamon in MCF-7 breast cancer cell lines and found that the highest anticancer activity was shown by 125 µg/ml concentration of ethyl acetate extract. The methanol and acetone extracts of cinnamon showed anticancer activity at a concentration 250 µg/ml whereas ethanol extracts showed no anticancer activity on MCF-7 breast cancer cell lines.

To find out the immunomodulatory activity of cinnamon extracts we have performed experiments on peripheral blood monocyte cells (PBMCs) from whole blood. It appears that cinnamon extract shows mostly immunostimulation activities after 72 hour culture. But 250 µg/ml of methanol and acetone extracts of cinnamon showed immunosuppressive potential.

Interestingly, we have found anticancer and immunomodulatory activities in different cinnamon extracts. But we observed discrepancy in these activities as activity is not maintained in higher concentrations. One possible explanation is that cinnamon bark extracts contain a mixture of different compound.

We have performed only a few experiments with MCF-7 cell lines and PBMCs, hence, it is not possible for us to deduce the actual anticancer and immunomodulation activity of cinnamon extracts. Hence to confirm these activities, it is important to carry out more experiments by varying the parameters.

CHAPTER 8

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