Studies on Antibacterial and Anticancer activities of Black Cardamom

A Dissertation
Submitted in partial fulfillment of the requirement
for the award of degree of
Masters of Science in Biotechnology

Under the guidance of
Dr. Vikas Handa
&
Dr. Manoj Baranwal
Department of Biotechnology and Environmental Sciences

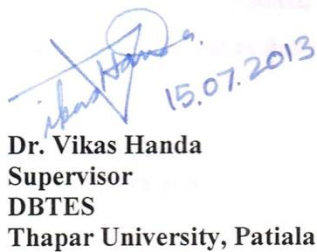
Submitted By:
Gurvinder Kaur
Roll No. 301101012

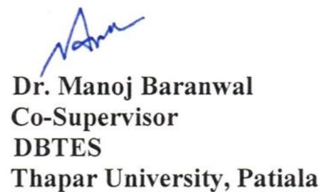
Department of Biotechnology and Environmental Sciences
THAPAR UNIVERSITY
PATIALA-147004
INDIA
July, 2013

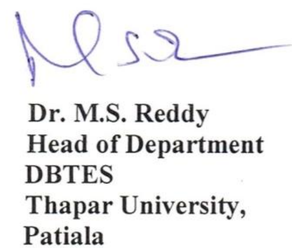
DEDICATED TO MY PARENTS

CERTIFICATE

This is to certify that the thesis entitled **"Studies on Antibacterial and Anticancer activities of Black Cardamom"** submitted by Gurvinder Kaur (Roll no: 301101012) 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.



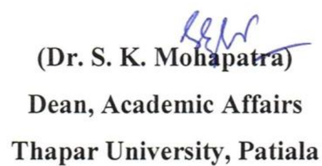




Dr. Vikas Handa
Supervisor
DBTES
Thapar University, Patiala

Dr. Manoj Baranwal
Co-Supervisor
DBTES
Thapar University, Patiala

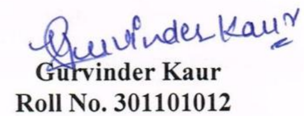
Dr. M.S. Reddy
Head of Department
DBTES
Thapar University, Patiala



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

CANDIDATE'S DECLARATION

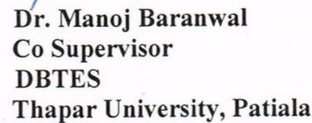
I hereby declare that the work which is being presented in the dissertation entitled **"Studies on Anti bacterial and Anticancer activities of Black Cardamom"** in partial fulfillment of the requirements for the award of Degree of Master of Sciences in Biotechnology, Thapar University, Patiala, is an authentic record of my own work during a period of six months from January 2013 to June 2013, under the supervision of **Dr. Vikas Handa,** (Assistant Professor) and **Dr. Manoj Baranwal,** (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.



Place: Patiala
Date: 15-07-2013

Gurvinder Kaur
Roll No. 301101012

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



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

iv

ACKNOWLEDGEMENT

This is a golden opportunity for me to convey my sincere regards for all those people who enabled me to accomplish my dissertation work successfully.

My utmost gratitude goes to my esteemed advisors **Dr. Vikas Handa,** Assistant Professor, and **Dr. Manoj Baranwal,** Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala for their expertise guidance, kindness, motivation, patience and providing me the opportunity to complete my dissertation work. It is their confidence-imbibing attitude, splendid discussions and endless endeavors through which I have gained a lot and learned a lot building up my future and personality.

I wish to express my deep sense of humbleness to **Dr. M.S Reddy**, Head of the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, Punjab

A special thanks to all faculty members for their constant encouragement and support throughout the project work.

I would like to give my special thanks to Ms. Neha Lohia for her able guidance, constant supervision, keen interest and moral support which she extended to me time to time. Her dedication towards work always inspires me. I am deeply indebted to her as her stimulating suggestions and encouragement helped me all the time of dissertation and writing of thesis.

Life at Thapar University would be unforgettable for me throughtout my life because I was blessed to spend it with friends like Raman, Navpreet, Simran, Arun, Kamal. I thank them all for their great company.

I take pleasure to acknowledge the help given by Renu who was always there when I needed her help.

Words fail me to express my thanks to my parents, my sister, my brother and my grandmother for their selfless sacrifice, encouragement and heart full blessings that continue to enlighten my life.

All these thanks are, however, only fraction of what is due to Almighty for granting me an opportunity and strength to successfully accomplish this assignment

Place: Patiala Gurvinder Kaur

ABBREVIATIONS

$BaSO_4$	Barium sulphate
CFU	Colony forming unit
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
HCl	Hydrogen chloride
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihyrogen Phosphate
LB	Luria Broth
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum inhibitory concentration
MTT	3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NA	Nutrient Agar
NaCl	Sodium Chloride
Na_2HPO_4	Disodium Hydrogen Phosphate
NaOH	Sodium hydroxide
O.D	Optical density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Solution
RPMI 1640	Rosewell Park Memorial Institute
TLC	Thin layer chromatography

S.No.	List of Table	Page No.
Table 5.1	Antibacterial activity of Black cardamom extracts	32
Table 5.2	Minimum inhibitory concentration of seed extracts of black cardamom against *Escherichia coli*	36
Table 5.3	Minimum inhibitory concentration of rind extracts of black cardamom against *Escherichia coli*	37
Table 5.4	Minimum inhibitory concentration of seed extracts of black cardamom against *Staphylococcus aureus*	39
Table 5.5	Minimum inhibitory concentration of rind extracts of black cardamom against *Staphylococcus aureus*	39
Table 5.6	Minimum inhibitory concentration of seed extracts of black cardamom against *Pseudomonas aeruoginosa*	42
Table 5.7	Minimum inhibitory concentration of rind extracts of black cardamom against *Pseudomonas aeruoginosa*	42
Table 5.8	Minimum inhibitory concentration of seed extracts of black cardamom against *Bacillus cereus*	45
Table 5.9	Minimum inhibitory concentration of rind extracts of black cardamom against *Bacillus cereus*	45
Table 5.10	Anticancer activity shown by different extracts of black cardamom seed	48
Table 5.11	Effect of Black cardamom extracts on PBMC (seed and rind)	50

S.No.	List of Figures	Page No.
Figure 5.1	Antibacterial activity of different extracts of black cardamom against gram negative and gram positive bacteria	32
Figure 5.2	Zone of inhibition shown antibacterial activity of black cardamom extracts against *Staphylococcus aureus*	33
Figure 5.3	Zone of inhibition shown antibacterial activity of black cardamom extracts against *Escherichia coli*	34
Figure 5.4	Zone of inhibition shown antibacterial activity of black cardamom extracts against *Pseudomonas aeruginosa*	34
Figure 5.5	Zone of inhibition shown antibacterial activity of black cardamom extracts against *bacillus cereus*	35
Figure 5.6	Minimum inhibitory concentration of seed extract against *Escherichia coli*	37
Figure 5.7	Minimum inhibitory concentration of rind extracts against *Escherichia coli*	38
Figure 5.8	Minimum inhibitory concentration of seed extract against *Staphylococcus aureus*	40
Figure 5.9	Minimum inhibitory concentration of rind extract against *Staphylococcus aureus*	41
Figure 5.10	Minimum inhibitory concentration of seed extract against *Pseudomonas aeruoginosa*	43
Figure 5.11	Minimum inhibitory concentration of rind extract against *Pseudomonas aeruoginosa*	44
Figure 5.12	Minimum inhibitory concentration of seed extract against *Bacillus cereus*	46
Figure 5.13	Minimum inhibitory concentration of rind extract against *Bacillus cereus*	47
Figure 5.14	Anticancer activity of seed extracts of Black cardamom	49
Figure 5.15	Effect of seed extract of black cardamom on PBMC	50
Figure 5.16	Effect of rind extract of black cardamom on PBMC	51

CONTENTS

1. INTRODUCTION 1
2. REVIEW OF LITERATURE 4
3. AIM OF THE STUDY 18
4. MATERIALS &METHODS 19
5. RESULTS & DISCUSSION 30
6. CONCLUSION 52
7. SUMMARY 53
8. REFERENCES 54

Abstract

Black cardamom (*Amomum subulatum*) is commonly known as 'Badi Elaichi' is a tall, perennial, evergreen, herbaceous monocot plant. Black cardamom is well known spice, which has medicinal value. The present study has been designed to explore anticancer and antibacterial activities of black cardamom. Various extracts of seed and rind of black cardamom were evaluated for their antibacterial and anticancer activities. The extracts were tested against gram positive and gram negative bacteria for antibacterial activity and MCF-7 cells for anticancer activity. Antibacterial activity was evaluated by using agar well diffusion assay followed by determination of minimum inhibitory concentration and anticancer activity was evaluated by MTT assay against MCF-7 breast cancer cell line. The results of this study reveals that methanol extract of seed of black cardamom shows maximum inhibition against *Escherichia coli* whereas other extracts of seed show lower inhibition against *Escherichia coli* as well as other bacteria. In general seed extracts of black cardamom show higher inhibition as compare to rind extracts of black cardamom. In case of anticancer activity, all the extracts of black cardamom seed showed anticancer activity but 500 µg/ml concentration showed maximum inhibition of MCF-7 cells in all extracts of black cardamom. With these results, it appears that cardamom can be a good source of anticancer and antibacterial agent.

INTRODUCTION

Black cardamom commonly known as: `Badi Elaichi', Moti Elaichi, large cardamom, greater cardamom, Indian cardamom. It belongs to the family Zingiberaceae (ginger family). Species of black cardamom are *Amomum subulatum, Amomum costatum.* Black cardamom is a tall perennial herbaceous plant with subterranean rhizomes which produces several leafy shoots and panicles. India is the largest producer of black cardamom (*Amomum subulatum* Roxburgh), with an annual production of 4000 MT followed by Nepal and Bhutan (Berrig *et al*., 1993). More than 85 % of the production within India is from Sikkim. The main production centres are the Eastern Himalayas and sub-Himalayan region of West Bengal, Assam and Sikkim.

The fruit is a trilocular many-seeded capsule and roughly 2.5 to 3 cm in length. The pods of black cardamom are dark brown in colour and have a tough, dried skin. Due to its pleasant aroma, it has been used as an essential ingredient in mixed spices for flavourings vegetables, many food preparations and pickles. The major constituent of large cardamom essential oil is 1, 8-cineole (65 – 80 %), while the content of α-terpenyl acetate is low (traces to 5 %). The monoterpene hydrocarbon content is in the range of 5 – 7 %, of which limonene, sabinene, terpinene and pinene are significant components. The seeds of black cardamom are aromatic pungent, stimulant, stomachic, alexipharmic and astringent. The seeds are used to treat stomach pain, indigestion, vomiting, biliousness, throat troubles, and congestion of the lungs, inflammation of the eyelids, digestive disorders, and liver complaints. Large cardamom possesses many medicinal properties few of them are discussed. *Amomum subulatum* (Zingiberaceae), is used to study anti-inflammatory activity of its fruits extracts (methanol and aqueous) at a dose of 250 mg/kg and 500 mg/kg against carrageenan induced paw edema in rats. Both the extracts were able to show a dose dependent anti-inflammatory activity (Kumar *et al*., 2012). The components in the volatile oil of *Amomum subulatum*, e.g. 1, 8-cineole, terpinene, terpiniol, sabinine, α-pinene and limonene, act as a tonic for the heart and liver, an appetizer, promote the elimination of bile and help reduce congestion of the liver (Parmar *et al*., 2010). Crude methanol extract and its different fractions, e.g. essential oil, petroleum ether (60 – 80 °C), ethyl acetate and methanol fractions, were studied in rats for their ability to inhibit gastric lesions induced by aspirin, ethanol and pylorus ligature. These investigations validate the use of large cardamom in gastrointestinal disorders by Unani physicians (Jafri *et al*., 2001). The antimicrobial activity of *Amomum subulatum* fruit extracts were studied against *Streptococcus mutans, Staphylococcus aureus, Lactobacillus acidophilus, Candida albicans* and *Saccharomyces cerevisiae.* The acetone, ethanol and methanol extracts of the selected plants

exhibited antimicrobial activity against all tested microorganism except *L. acidophilus*. The most susceptible microorganism was *Staphylococcus aureus* followed by *Streptococcus mutans, Saccharomyces cerevisiae and Candida albicans* (Joshi *et al*., 2009). Ethanol and aqueous extracts of leaves of *Amomum subulatum* is evaluated for antioxidant activity by the 1, 1-Diphenyl-2-picrylhydrazyle (DPPH) free radical scavenging activity. The ethanol extract showed significant antioxidant activity (Khare *et al*., 2012). Owen *et al*., 2002 studied cardio-protective activity of *Amomum subulatum.* Methanol extract of *Amomum subulatum* displayed strong free radical scavenging activity using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. *Amomum subulatum* were further partitioned into a hexane, chloroform, ethyl acetate and water fraction, retested with DPPH assay. Fractions displaying strongest activity were examined *in vitro* for their ability to protect human low density lipoprotein (LDL) from Cu^{2+} catalyzed oxidation measured using thiobarbituric acid reactive substances production and formation of conjugated dienes. Anti-diabetic activity of *Amomum subulatum* seeds was evaluated in fructose fed metabolic syndrome in rats. *A. subulatum* extracts revealed a significant increment of serum insulin levels and higher reduction in hyperglycemia when compared to the diabetic control rats. The histological studies of the endocrine region of pancreas of diabetic animals revealed that shrinkage of β cells of islets of langerhans (Vavaiya *et al*., 2012)

Out of all these activities we focus mainly on antimicrobial activity because microbes have adverse effect on human beings. They cause infectious diseases to human. Microorganisms are sometimes resistance to antibiotic or chemically synthesized antimicrobial agents, to overcome this problem scientist focus on studying natural synthesized antimicrobial agents because medicinal plants since ancient times have been employed for curative purposes. Traditional healers have used plants or plant products to prevent or cure infectious diseases. Many of these plants have been investigated scientifically for antimicrobial activity, and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. A number of these agents appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross-resistance with agents already in use may be minimal. So, it is worthwhile to study plants and plant products for activity against resistant bacteria.

In our work we also have a focus on two other activities that are anticancer and immunomodulatory activities. Nowadays, most common problem faced by humans is cancer. Cancer is undoubtedly a serious and potentially life-threatening illness. But it is a misconception to think that all forms of cancer are untreatable and lethal. The truth is that there are multiple types of cancer, many of which can today be effectively treated so as to eliminate, reduce or slowed down the impact of the disease. Numerous epidemiological and experimental studies have shown that cancer risk may be modified by

changes in dietary habits. Food flavouring spices are known to possess active compounds with medicinal and therapeutic properties. There has been a growing realization that spices exhibit potent anticancer properties. Also herbal medicines are known to have immunomodulatory properties and generally act by stimulating both specific and non-specific immunity (Sonkar *et al.,* 2011). Many plants and their products used in traditional medicine are reported to have immunomodulatory activities. Some of these plants stimulate immunity including both humoral and cell-mediated immunity while others activate only cellular components of the immune system, i.e. phagocytic function without affecting the humoral or cell- mediated immunity.

With this background, it can be deduced that black cardamom has medicinal value. Antimicrobial activity of black cardamom had been reported but anticancer activity has not been reported till now. So it is interesting to find out antimicrobial, anticancer and immunomodulatory activities of different crude extracts of black cardamom by using different microbial, biochemical, immunological assays.

1. REVIEW OF LITERATURE

1.1 Introduction

Spices are commonly used in food, home remedies and Indigenous system of medicine. *Amomum subulatum* commonly known as Black cardamom is a tall, perennial cash crop and shade-loving crop. It grows under dense (60 – 70 % of full daylight interception) to light shade (26 % full daylight interception) conditions. The plant grows at altitudes between 600 to 2000 m, where rainfall is between 1500 to 3500 mm and the temperature varies from 6 °C to 33 °C (Anon, 1991). India is the largest producer of black cardamom or large cardamom followed by Nepal and Bhutan (Berrig *et al*., 1993). The main production centres are the Eastern Himalayas and sub-Himalayan region of West Bengal, Assam and Sikkim. Other species including *A. subulatum*, or black cardamom grown in northern India and Nepal: *A. aromaticum*, or Bengal cardamom, growing in south-eastern India: *A. krervanth*, or Cambodian cardamom, growing in Thailand, Cambodia, and Vietnam: *A. globosum*, or Chinese cardamom, grown in southern China. (Subba, 1984; Rao *et al*., 1993; Singh and Singh, 1996)

The pods of black cardamom are used as a spice, in a manner similar to the green cardamom pods, but it has a drastically different flavour, so it cannot be substituted in the same recipes, unless a different flavour is acceptable. Its strong, smoky flavour and aroma are derived from the traditional drying procedure, which involves drying over open flames.

2.2 Botany and Uses

Botany

Cultivation of black cardamom is carried out mainly in muddy places along the sides of mountain streams in Nepal, Bengal, Sikkim and Assam (eastern Himalayas). Usually, the plants are grown on the moist and shady sides of mountain streams and along hilly slopes. The plant is a perennial herb having subterranean rhizomes, which give rise to leafy shoots and spikes. Leafy shoots are formed by long, sheath-like stalks encircling one another. The leaves are green or dark green, smooth on both surfaces, with acuminate apex. Inflorescence is a dense spike on a short peduncle bearing 40 – 50 flower buds in an acropetal sequence. Flowers appear during April and May and the capsules mature in September and October.

The fruit is a trilocular many-seeded capsule. The capsule wall is covered with spines and is reddish-brown to dark pink (Rao *et al*., 1993). Dried large cardamom capsules are on average, 25 mm long,

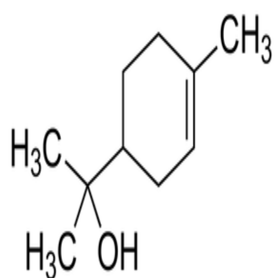
oval shape, greyish-brown to dark reddish-brown. The fruit contains 40 - 50 seeds, held together by a viscous sugary pulp. Though the fruits are clearly identifiable by their larger size and differences in shapes compared with small cardamom, the seeds are of nearly the same size as those of true cardamom.

Uses

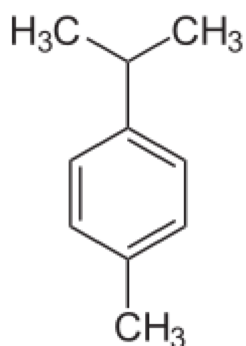
Black cardamom is traditional ingredient of food in Indian subcontinental and is valued for its acceptable taste, flavour and aroma. The spice is used in preparations of many dishes. Large cardamom has a fresh and spicy aroma. The ground seeds are an optional ingredient in mixed preparations and spice masala mixtures, and are also used as a flavouring agent in confectionary, hot or sweet pickles and in beverages. Large cardamom also possesses curative properties in the Ayurvedic and Unani systems of medicine (Mukherjee, 1972; Singh, 1978).

2.3 General Composition

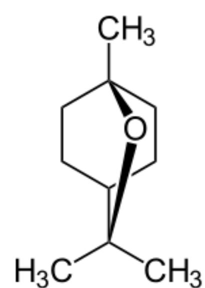
The chemical composition of large cardamom has shown in given below table (Pruthi 1993).The seeds contain 3 % essential oil, which is dominated by 1, 8-cineole (more than 70 %). Smaller and variable amounts of limonene, terpinene, terpineol, terpinyl acetate and sabinene have also been reported. Comparison of chemical analysis of large cardamom and small cardamom seeds are also given below table (Singh, 1978). Structures of main chemical constituents of *Amomum subulatum* is given below in figure 2.1.



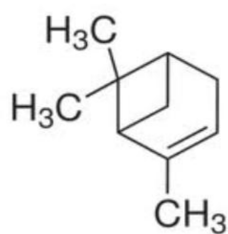
4- Terpineol



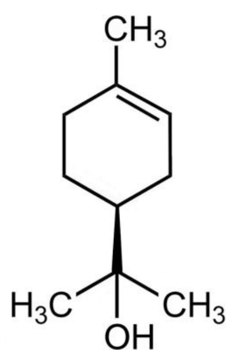
p- Cymene



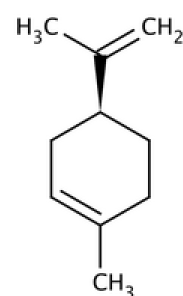
1, 8-Cineolo



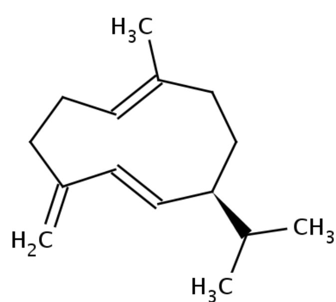
α - Pinene



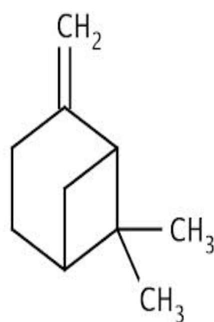
α -Terpineol



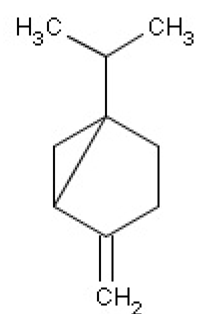
d-limonene



Germacrene



β - pinene



Sabinene

Figure 2.1: Chemical Constituents of *Amomum subulatum*

Table 1.1: General composition of large cardamom

Component	Value (%)
Moisture	8.49
Protein	6.00
Total ash	4.01
Starch	43.21
Crude fibre	22.00
Non-volatile ether extract	2.31
Volatile ether extract	3.00
Alcohol extract	7.02
Volatile extract	2.80
Water-soluble ash	2.15
Alkalinity of water-soluble ash	0.90
Ash insoluble in acid	0.42
Volatile oil	2.80

(Pruthi, 1993)

Table 1.2: Comparison of chemical analysis of large and small cardamom seeds

Character	Large cardamom (average %)	Small cardamom (average %)
Moisture	8.49	8.30
Volatile oil	2.80	8.30
Protein	8.00	10.30
Crude fibre	22.0	9.20
Total ether extract	43.21	45.40
Alcohol extract	7.02	-
Total ash	4.01	5.00

(Singh, 1978)

2.4. Chemistry

Chemistry of volatiles

Volatile oil is the principal aroma-giving compound in the large cardamom. Steam distillation of the crushed seeds gives dark brown oil (2.5 %) with a cineol-like aroma. Due to the presence of 1, 8-cineole black cardamom has pungent smell and because of terpinyl acetate it has pleasant aroma (Karibasappa, 1987). Quantitative chromatographic analysis of the composition of distilled essential oil was reported previously by Nigam and Purohit (1960) and by Lawrence (1970). The major constituent of large cardamom essential oil is 1, 8-cineole (65 – 80 %), while the content of α-terpenyl acetate is low (traces to 5 %). The monoterpene hydrocarbon content is in the range of 5 – 7 %, of which limonene, sabinene, terpinene and pinene are significant components. The terpinols comprise approximately 5 – 7 % of the oil. The high cineole and low terpenyl acetate probably account for the very harsh aroma of this spice in comparison with that of true cardamom (Pruthi, 1993).

Seed oil

The seed oil in *A. subulatum* has been the subject of several investigations. Nigam and Purohit, 1960 obtained 2.5 % oil from the seeds and fractionated the oil into different cineole-rich fractions. Lawrence, 1970 separated the components of the seed oil of *Amomum subulatum* by preparative gas chromatography, identified them by their IR spectra and retention data and found the major component, 1, 8-cineole, in 74 %. Patra *et al.*, 1982 studied the oil by packed column GC and reported that its major components were sabinene (9.1 %), γ-terpinene (16.2 %) and 1, 8-cineole (63.3 %). In another study, Gupta *et al.*, 1984 analysed oils derived from different strains of *A. subulatum* growing wild in Sikkim and found that 1, 8-cineole content varied from 77 to 89 %. The oil and volatile concentrate produced by liquid carbon dioxide extraction of *A. subulatum* were compared by Kaur *et al.*, (1993).

Analysis of the steam-distilled volatile oil of the seeds of the large cardamom using GC-MS identified 25 components, of which 16.3 % was monoterpene hydrocarbons and 75.3 % was oxygenated monoterpenes, and 6.3 % was sesquiterpenes. Its major constituents are 1, 8-cineole (61.3 %), a-terpineol, a- and P-pinene and alloaromadendrene (Gurudutt *et al.*, 1996).

Steam distillation of the crushed seeds of large cardamom yielded 2.5 % dark brown - coloured liquid with the following physical constants: specific gravity (29 °C), 0.9142; refractive index (29 °C), 1.460; optical rotation in chloroform, 18 °C.

Rind oil

The pericarp (husk or rind) of large cardamom yielded 0.18 % volatile oil by the Clevenger hydrodistillation method. This oil was analysed for physical parameters, e.g. specific gravity (0.9148), refractive index (1.4733) and optical rotation (−7.700).

The volatile oil was subjected to GC- MS analysis and 37 compounds were identified, constituting > 98 % of the total oil. The major compounds characterized were 1,8-cineole (38.7 %), β-pinene (13.6 %), α-terpineol (12.6 %), spathulenol (8.3 %), 4-terpineol (4.5 %), germacrene D (3.0 %), α-pinene (2.8 %) and β-selinene (2.7 %). GC and GC-MS data revealed that the 1,8-cineole content was less than 50 % when compared with the seed oil (Naik *et al.*, 2004).

2.5 Antibacterial activity

Antimicrobial agents:

An antimicrobial agent is a substance that kills or inhibits the growth of microorganisms and causes no damage to the host. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. Like antibacterials commonly known as antibiotics which are used against bacteria, antifungals are used against fungi, antiviral are used against viruses and antiprotozoal are used against protozoa. They can also be classified according to their function. Antimicrobials that kill microbes are called microbicidal and those inhibit their growth are called microbiostatic.

Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: antibiotics, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized. A hybrid substance is a semisynthetic antibiotic, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties. Furthermore, some antimicrobial compounds, originally discovered as products of microorganisms, can be synthesized entirely by chemical means. In the medical and pharmaceutical worlds, all these antimicrobial agents used in the treatment of disease are referred to as antibiotics, interpreting the word literally.

The modern era of antimicrobial chemotherapy began in 1928, with Fleming's discovery of the powerful bactericidal substance, penicillin from *Penicillium rubens* fungus and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity. In 1942, penicillin was isolated and purified and injected into experimental animals, where it was found not only to cure infections but also to possess incredibly low toxicity for the animals. It was successfully used to treat

a Streptococcus infection. Since then many other antibiotics and other types of antimicrobial agents have been found and put into use The rapid isolation of streptomycin, chloramphenicol and tetracycline soon followed, and by the 1950's, these and several other antibiotics were in clinical usage.

Natural Antimicrobials:

The traditional medicine system based on natural products continues to play an important role in treatment of many infectious diseases. Natural products and their derivatives have historically been invaluable as a source of therapeutic agents and have contributed to the discovery of natural antimicrobial agents. Hundreds of herbs are known to be used for various diseases including many infectious diseases. Naturally occurring antimicrobial compounds could also be applied as food preservatives to protect food quality and extend the shelf life of foods and beverages (Juneja *et al*., 2012). 25 to 50 % of current pharmaceuticals are derived from plants, and are used as antimicrobials. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties. These plant and plant products have many therapeutic properties.

Tijjani *et al*., 2012 investigated that the efficiency of methanol seed extracts of *Amomum subulatum* against bacteria such as *Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis, Salmonella typhi, Klebsiella pneumoniae, Pseudomonas aerugenosa and Candida albican* was studied by Disc diffusion technique. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts was determined. Results obtained showed considerable inhibition against the bacteria tested except *Salmonella pyrogenes* and *E. coli* which showed considerable resistance at all concentrations of the extract. It was also observed that the extract exhibited greater inhibition on *Klebsiella pneumonia* and *Pseudomonas aerugenosa* (18 ± 0.2 mm and 17 ± 0.3 mm). Both the MIC and MBC of the extracts ranges from 50 to 200 mg/ml. Its further reveals that *Bacillus subtilis, Klebsiella pneumonia* and *Staphylococcus aureus* exhibit broadest activity at MIC and MBC concentrations of 50 mg/ml. While the rest *Salmonella typhii* and *Pseudomonas aerogunosa* and *candida albicans* of the bacteria show negative turbidity. Preliminary screening analysis of the powdered methanol seed extracts showed the presence of Carbohydrate, tannins, cardioactive glycosides, tepenes, flavonoids, alkaloids and saponins.

Joshi *et al*., 2009 investigated that antimicrobial activity of *Amomum subulatum* fruit extracts were studied against *Streptococcus mutans, Staphylococcus aureus, Lactobacillus acidophilus, Candida albicans* and *Saccharomyces cerevisiae*. The acetone, ethanol and methanol extracts of the selected plants exhibited antimicrobial activity against all tested microorganism except *L. acidophilus*. The most susceptible microorganism was *Staphylococcus aureus* followed by *Streptococcus mutans,*

Saccharomyces cerevisiae and Candida albicans. The largest mean zone of inhibition was obtained with the ethanol extract of *A. subulatum* against *Staphylococcus aureus*. Minimum inhibitory concentrations (MIC) of the extracts were also determined. This study depicts that ethanol and acetone extracts of fruits of *Amomum subulatum* can be used as a potential source of novel antimicrobial agents used to cure dental caries.

Agnihotri *et al*., 2010 investigated that methanol extract of fruits of *A. subulatum* shows remarkable antimicrobial activity against *Escherichia coli* whereas in case of other microorganisms used it was found inferior. Methanol extract of rind showed good antimicrobial activity against *Staphylococcus aureus.* It was found that the essential oil isolated was effective against majority of microorganisms used viz. *Bacillus pumilus, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Saccharomyces cerevisiae.*

Sabulal *et al*., 2006 suggested that essential oil from the fruits of *Amomum cannicarpum* (Wight) Bentham ex Baker (*Zingiberaceae*) was hydrodistilled and characterized by gas chromatography-mass spectrometry (GC-MS). Major constituents of the oil were β-pinene (14.00 %), elemol (10.45 %) and α-cadinol (8.50 %). Thirty-three (91.48 %) out of forty-one constituents were identified by GC-MS. Antimicrobial activity of the oil against Gram-positive and Gram-negative bacteria and some fungi was determined by the disc diffusion assay. The oil showed good antibacterial activity against *Salmonella typhi*, *Pseudomonas aeruginosa* and *Proteus vulgaris* and very good antifungal activity against *Candida albicans* and *C. glabrata*.

Afolayan *et al*., 2008 investigated that Acetone, methanol, and water extracts from the leaves and roots of the *Hermannia incana* Cav. (Sterculiaceae) were investigated for antibacterial and antimycotic activities. The methanol extracts of the plant showed moderate activity while on the other hand acetone and water extracts show less activity against Gram-positive and Gram-negative bacteria. All the extracts inhibited the growth of the fungi *Aspergillus flavus, Aspergillus niger,* and *Mucor hiemalis* with growth inhibition based on MIC ranging from 54.31 to 96.67 % at 0.1 - 10 mg/ml. This study has pointed to the potential application of *Hermannia incana,* as a bacteriocide and fungicide.

2.6 Anticancer Activity:

Cancer

Cancer refers to malignant neoplasm which is characterized by indefinite or unregulated cell growth. In cancer, cells divide and grow uncontrollably to form lumps or masses of tissue called tumors. Tumors can grow and interfere with the digestive, nervous, and circulatory systems and they can

release hormones that alter body function. Not all tumors are cancerous. Benign tumors do not grow uncontrollably, do not invade neighboring tissues, and do not spread throughout the body.

Malignant tumors form when a cancerous cell manages to move throughout the body using the blood or lymph systems, destroying healthy tissue in a process called invasion and 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. However depending on the tissue of their origin, three most prevalent types of cancers are Carcinomas, Leukaemias or lymphomas and Sarcomas. Carcinomas account for majority of cancers (> 80 %) and they arises endodermal or ectodermal tissue such as skin or the epithelial lining of internal organs and glands. The majority of cancers of the colon, breast, prostate and lung are carcinomas. The Leukemias and Lymphomas are malignant tumors of hematopoietic cells of the bone marrow and are second most common malignancies. Leukemias proliferate as single cells, whereas lymphomas tend to grow as tumor masses. Sarcomas arise from mesodermal tissues like muscles, connective tissues, bone etc. and have a prevalence rate of about 1%.
Determining what causes cancer is complex. Cancer cells uncontrollably grow and do not die. Normal cells in the body follow an orderly path of growth, division, and death. Programmed cell death is called apoptosis, and when this process breaks down, cancer begins to form. Unlike regular cells, cancer cells do not experience programmatic death and instead continue to grow and divide. This leads to a mass of abnormal cells that grows out of control.
Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests. Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment.
There are over 200 different types of cancer, and each is classified by the type of cell that is initially affected. In our work we discuss about breast cancer because Breast cancer ranks as one of the leading cancer types in the number of new cases diagnosed and is second highest cause death in women. In 2010 the American Cancer Society estimates approximately 209,060 new cases of breast cancer will be diagnosed and 40,230 deaths due to breast cancer will occur in the United States (World Cancer Report, 2008).

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. 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. The vast majority of breast cancer cases occur in female, male breast cancer can also occur. Breast cancer rates are much higher in developed countries compared to developing ones. There are several reasons for this, with possibly life-expectancy being one of the key factors - breast cancer is more common in elderly women. Breast cancer can be of two types:

Invasive breast cancer - the cancer cells break out from inside the lobules or ducts and invade nearby tissue. With this type of cancer, the abnormal cells can reach the lymph nodes, and eventually make their way to other organs (metastasis), such as the bones, liver or lungs. The abnormal (cancer) cells can travel through the bloodstream or the lymphatic system to other parts of the body; either early on in the disease, or later.

Non-invasive breast cancer - this is when the cancer is still inside its place of origin and has not broken out. Lobular carcinoma *in situ* is when the cancer is still inside the lobules, while ductal carcinoma *in situ* is when they are still inside the milk ducts. "*In situ*" means "in its original place". Sometimes, this type of breast cancer is called "pre-cancerous"; this means that although the abnormal cells have not spread outside their place of origin, they can eventually develop into invasive breast cancer.

A considerable part of the current knowledge on breast carcinomas is based on *in vivo* and *in vitro* studies performed with breast cancer cell (BCC) lines. These provide an unlimited source of homogenous self-replicating material, free of contaminating stromal cells, and often easily cultured in simple standard media. Indeed, attempts to culture BCC from primary tumors have been largely unsuccessful. This poor efficiency was often due to technical difficulties associated with the extraction of viable tumor cells from their surrounding stroma. Most of the available BCC lines issued from metastatic tumors, mainly from pleural effusions. Many of the currently used BCC lines were established in the late 1970s. A very few of them are MCF-7, T-47D, and MDA-MB-231.

MCF-7 Cell Line:

MCF-7 is human breast adenocarcinoma cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman. Of the two mastectomies she received, the first revealed the removed tissue to be benign. Five years later, a second operation revealed malignant adenocarcinoma in a pleural effusion from which was taken cells for MCF-7 (Soule *et al.,* 1973). The woman was treated for breast cancer with radiotheraphy and hormonotherapy. MCF-7 is the acronym of Michigan Cancer Foundation - 7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers. The Michigan Cancer Foundation is now known as the Barbara Ann Karmanos Cancer Institute.

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. In addition to retaining their estrogen sensitivity, 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.

Stability of cell line:

Genetically, the MCF-7 line has not been maintained exactly. Originally, it was described as having a karyotype containing 85 chromosomes, which has since been reduced by 16 chromosomes. Today's cell line has a karyotype containing 69 chromosomes.

Anticancer agents:

Natural products have afforded a rich source of compounds that have found many applications in the fields of medicine, pharmacy and biology. Advances in the prevention and treatment of cancer require the continued development of novel and improved chemopreventive and chemotherapeutic agents. Within the sphere of cancer, a number of important new commercialised drugs have been obtained from natural sources, by structural modification of natural compounds, or by the synthesis of new compounds, designed following a natural compound as model. The search for improved cytotoxic agents continues to be an important line in the discovery of modern anticancer drugs. There are number of anticancer agents reported which are obtained from natural products. A few reported anticancer agents derived from natural products that are taxol, betulinic acid, camptothecin, resveratrol, podophyllotoxin and curcumin.

Qiblawi *et al*., 2012 studied the chemopreventive potential of cardamom on 7, 12-dimethylbenz[a]anthracene-initiated and croton oil – promoted mouse skin papillomagenesis. A significant reduction in the values of tumor incidence, tumor burden, and tumor yield and the cumulative number of papillomas was observed in mice treated orally with 0.5 mg of cardamom powder in suspension continuously at pre-, peri-, and post-initiational stages of papillomagenesis compared with the control group. Treatment of cardamom suspension by oral gavage for 15 days resulted in a significant decrease in the lipid peroxidation level of the liver ($P < 0.01$). In addition, the reduced glutathione level was significantly elevated in comparison with the control group ($P < 0.05$)

following cardamom suspension treatment. Taken together, these findings indicate the potential of cardamom as a chemopreventive agent against two-stage skin cancer.

Saha *et al.*, 2012 investigated the efficacy of dietary cardamom against 7, 12-dimethylbenz[a]anthracene (DMBA)-induced skin papillomatogenesis in Swiss albino mice that closely resembles human non melamoma skin cancer. Mice were grouped into normal wild type (untreated), vehicle-treated (acetone), carcinogen-treated (DMBA), and DMBA and cardamom-treated (DMBA + CARD) to delineate the role of cardamom against DMBA-induced papillomatogenesis. Cardamom ingestion in DMBA-treated mice blocked NF-kB activation and down-regulated cyclo-oxygenase-2 expression. As a consequence, both the size and the number of skin papillomas generated on the skin due to the DMBA treatment were reduced in the 'DMBA + CARD' group. Thus, the results from this study suggested that cardamom has a potential to become a pivotal chemopreventive agent to prevent papillomagenesis on the skin.

Unnikrishnan *et al.*, 1990 studied tumour reducing activity of extracts of eight commonly used spices in India in mice transplanted intraperitoneally with Ehrlich ascites tumour. Oral administration of extracts of black pepper, asafoetida, pippali and garlic could increase the percentage of life span in these mice by 64.7 %, 52.9 %, 47 % and 41.1 %, respectively. However intraperitoneal administration of spice extracts did not produce any significant reduction in tumour growth except for sesame (38.8 %). These results indicate the potential use of spices as anti-cancer agents as well as antitumour promoters**.**

Indap *et al.*, 2006 investigated phenolics from different natural products such as ferulic, caffeic, gallic acids and curcumin for their potential anti proliferative and cytotoxic properties in human breast cancer cell line (MCF-7) as well as on a spontaneous mammary adenocarcinoma tumor. Caffeic acid showed substantial growth inhibitory activity.

2.7. Immunomodulatory Activity:

Immunomodulators: Immunotherapy is a medical term defined as the "treatment of disease by inducing, enhancing, or suppressing an immune response". These are biological or synthetic substances, which can stimulate, suppress or modulate any of the immune system including both adaptive and innate arms of the immune response. Clinically immunomodulators can be classified into following three categories:

Immunoadjuvants: These agents are used for enhancing vaccines efficacy and therefore, could be considered specific immune stimulants (Agarwal *et al.*, 1999); example in this regard is of Freund's adjuvant. The immunoadjuvants hold the promise of being the true modulators of immune response. It

has proposed to exploit them for selecting between cellular and humoral, Th1 (helper T1 cells) and Th2, (helper T2 cells) immunoprotective and immunodestructive, and reagenic (IgE) versus immunoglobin G (IgG) type of immune responses, which poses to be a real challenge to vaccine designers (Billiau *et al*., 2001).

Immunostimulants: These agents are inherently nonspecific in nature as they envisaged enhancing body's resistance against infection. They can act through innate immune response and through adaptive immune response. In healthy individuals the immunostimulants are expected to serve as prophylactic and promoter agents i.e. as immunopotentiators by enhancing basic level of immune response, and in the individual with impairment of immune response as immunotherapeutic agents. (Ford *et al*., 2009) There are two main categories of immunostimulants:

1. **Specific immunostimulants** provide antigenic specificity in immune response, such as vaccines or any antigen.
2. **Non-specific immunostimulants** act irrespective of antigenic specificity to augment immune response of other antigen or stimulate components of the immune system without antigenic specificity, such as adjuvants and non-specific immunostimulators.

Immunosuppressants:

These are a structurally and functionally heterogeneous group of drugs, which are administered in combination regimens to treat various types of organ transplant rejection and autoimmune diseases (El-Sheikh ALK 2008). Immunosuppressive drugs are important tools in the management of organ transplantation and autoimmune disease. Immunosuppressive drugs can be classified into five groups. Immune responses depend on lymphocyte proliferation, and cytostatic drugs are immunosuppressive. Glucocorticoids are somewhat more specific inhibitors of lymphocyte activation, whereas inhibitors of immunophilins more specifically target T lymphocyte activation. Immunosuppressive antibodies target an increasingly-broad array of steps in the immune response, and there are still other drugs that modulate immune responses.

Natural immunomodulators:

Immunology is one of the most rapidly developing areas of medical biotechnology research and has great promises with regard to the prevention and treatment of a wide range of disorders. In addition, infectious diseases are now primarily considered immunological disorders, while neoplastic diseases and organ transplantation and several autoimmune diseases are involved in an immunosuppressive state. 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. The benefits of immunomodulators stem from their ability to stimulate natural and adaptive defense mechanisms, such as cytokines, which enables the body to help itself. 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. These natural immunomodulators have been reported.

Chandu *et al*., 2011 studied the immunomodulatory activity of saline extracts of leaves of Aloe vera Linn. (Family: Liliaceae) on the albino mice. The assessment of immunomodulatory activity on specific and nonspecific immunity was studied by administration of test extract. The study demonstrates that A. Vera triggers both specific and non-specific responses to a greater extent.

Majdalawieh *et al*., 2010 the potential immunomodulatory effects of black pepper and cardamom are investigated. Their data show that black pepper and cardamom aqueous extracts significantly enhance splenocyte proliferation in a dose-dependent, synergistic fashion. Enzyme-linked immunosorbent assay experiments reveal that black pepper and cardamom significantly enhance and suppress, respectively, T helper (Th)1 cytokine release by splenocytes. Conversely, Th2 cytokine release by splenocytes is significantly suppressed and enhanced by black pepper and cardamom, respectively.

Gomez-Flores *et al*., 2010 studied *in vitro* antitumor and immunomodulating activities of aqueous and methanol extracts of *Coriandrum sativum* (leaf and seed), *Piper nigrum* and *Cinnamomum zeylanicum.* 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.

Niphade *et al*., 2009 studied the immunomodulatory effect *Cinnamomum zeylanicum* by using different experimental models. The low dose of cinnamon bark (10 mg/kg p.o.) produced only an increase in serum immunoglobulins levels while the high dose of cinnamon bark (100 mg/kg p.o.) increased the phagocytic index in carbon clearance test, increased neutrophil adhesion, increased serum immunoglobulin levels and antibody titer values.

3. AIM OF THE STUDY

The main objective of present study is to explore the antibacterial, anticancer and immunomodulatory effect of different solvent extracts of Black Cardamom (seed and rind).

Work plan of the current study is as follows:

1. Preparation of black cardamom (seed and rind) extracts 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

4. MATERIALS AND METHODS:

4.1. Materials

REQUIREMENTS	COMPANY
Nutrient Broth	Himedia
Luria Broth	Himedia
Muller Hinton Broth	Himedia
Agar	Himedia
Powdered RPMI 1640 media	Himedia
Powdered DMEM media	Himedia
Sodium bicarbonate	Himedia
L-glutamine	Himedia
Fetal bovine serum	Himedia
Penicillin	Himedia
Streptomycin	Himedia
Ficoll	Himedia
Trypan blue	Himedia
MTT reagent	Himedia
Dimethyl sulfoxide (DMSO)	Srl
Sodium Chloride (NaCl)	Himedia
Potassium Chloride (KCl)	Himedia
Disodium Hydrogen Phosphate (Na2HPO$_4$)	Himedia
Potassium Dihyrogen Phosphate (KH_2PO_4)	Himedia
Silica gel G	Loba
Ethanol (Extra pure)	Loba
Methanol (99 %)	Loba
Acetone (99.9 %)	Loba
Ethyl acetate (Extra pure)	Loba
Toluene	Loba
Acetic acid	Loba

Iodine crystals	Loba
Vanillin	SDFCL

Black cardamom purchased from Patiala market

Bacterial cultures – gram positive bacteria (*Baciilus cereus, Staphylococcus aureus*), gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*)

Media- Nutrient agar, Luria broth, Muller Hinton agar, DMEM (Dulbecco's modified eagle's media), RPMI 1640

Composition:

Nutrient Agar (NA): Peptone 0.5 %, Beef extract/yeast extracts 0.3 % Agar 1.5 % NaCl 0.5%, Distilled water pH 6.8

Luria Broth (LB): Tryptone 1.0 %, Yeast Extract 0.5 %, Sodium Chloride (NaCl) 1.0 %, pH 7.0

Muller Hinton Agar (MHA): Beef infusion 30.0 %, Casein hydrolysate 75 %, Starch 0.15 %, Agar 1.7 %, pH 7.0 @ 25 ºC

DMEM (Dulbecco's modified eagle's media): DMEM powder, ANTIBIOTICS: Penicillin 100 unit/ml, Amphotericin 2.5 µg/ml, Streptomycin 100 µl/ml, Tylosin 1ml/litre, Fetal bovine serum 10 %, Sodium bicarbonate, L-glutamine, Distilled water

RPMI 1640 (Rosewell Park Memorial Institute): RPMI 1640 powder, ANTIBIOTICS: Penicillin 100 unit/ml, Amphotericin 2.5 µg/ml, Streptomycin 100 µl/ml, Tylosin 1 ml/litre, Fetal bovine serum 10 %, Sodium bicarbonate, L-glutamine, Distilled water

Apparatus and instruments: Incubator, laminar air flow hood, refrigerator (0 ºC, -20 ºC, -80 ºC), hot air oven, TLC glass plates, petriplates (90 mm), inoculation loop, tissue culture 96 well titre plates, round bottom 96 well titre plates, Whatman filter paper no.1, blotting paper, autopipettes

Chemicals: Silica gel, Benzene, Chloroform, iodine crystals, ninhydrin, vanillin, methanol, ethanol, acetone, petroleum ether, PBS (Phosphate buffered saline), Pure DMSO, 0.5 McFarland standards

4.2. Methods

4.2.1. Preparation of extracts

Black Cardamom extracts were prepared in different solvents. Washed and dried to remove any kind of dust and other materials. Separately, the seed and rind (covering) portion of black cardamom were grinded to obtain a fine powder. Then, 30 g of this fine powder was suspended in the flasks containing 100 ml of respective solvents i.e methanol (99.9 % pure), acetone (99 %), petroleum ether (99 %), pure

ethanol and water. The suspension of the black cardamom powder and solvents were kept at 37 °C for 72 hours at 130 rpm in the shaking incubator. After 72 hours of incubation these prepartion were filtered by using whatman filter paper no.1 in separate falcon tubes. Now the filtrate was evaporated at 30 °C till the solvent was completely evaporated. Now dry mass was weigh. (Subtracting weight of the empty beaker). Dried extract was dissolved completely in pure DMSO accordingly to make the final concentration of 100 mg/ml. These dissolved extracts were now stored as aliquots at 0 °C for further use (Afolayan, 2008).

4.2.2. Analysis of components by TLC (Thin layer chromatography)

TLC is based on the principle of separation. 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 and mixed with 100 ml distilled water to make a homogenous suspension. 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 temperature and then incubated at 110° C for 30 minutes in hot air oven. A known amount of samples were spotted at a distance of atleast ½ inch from edge of plate. The solvent was allowed to evaporate. The silica plates were placed in a closed chamber saturated with appropriate mobile phase. The mobile phase was allowed to travel through the stationary phase till it reaches approximately 10 mm from top of the plate. The sample spots are visualized in suitable UV light chamber or any other methods as recommended for the said sample (Kumar *et al.*, 2012).

Solvent system: Benzene: chloroform (7:3)

Detecting reagent: Iodine vapour, Vanillin, Ninhydrin

Iodine: 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: In a wide mouth jar (with cap) a piece of filter paper and few crystals of iodine were placed. As iodine has a high vapour pressure the chamber will rapidly become

saturated with iodine vapour. TLC plates were incubated in the saturated chamber till the development of light brown colour over the entire plate. The compounds having affinity for iodine appear as a dark brown spot on a lighter brown background. The TLC plates were removed and carefully encircle the spots with a dull pencil.

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 conc. sulfuric acid.

4.2.3. To Determine Antibacterial Activity:

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

4.2.3.1. 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.* The bacterial strains were revived in the following broth: *Escherichia coli* in Luria broth and rest of the strains were revived in Nutrient broth at 37 ºC for 18 - 24 hours at 120 rpm.

4.2.3.2. Maintenance of Microorganisms

The cultures were maintained and stored at 4º C. In order to revive 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.

4.2.3.3. Antibacterial Agent (as control)

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

4.2.3.4. Turbidity standard for inoculums preparation (NCCLS, 1997)

To standardize the inoculum density for susceptibility test, a Barium sulphate ($BaSO_4$) turbidity standard, an equivalent to a 0.5 McFarland standard or its optical equivalent should be used. $BaSO_4$ 0.5

McFarland standard was prepared by mixing 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 recorded at 600 nm should be 0.144 to 0.146 for the 0.5 McFarland standard. 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).

Table 4.1: 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

*at wavelength of 600 nm

4.2.3.5. Preparation of Mueller-Hinton Agar (MHA)

Mueller-Hinton Agar was prepared according manufacturer's instructions. Immediately after autoclaving, it was cooled and poured 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 solidify at room temperature. A representative sample plate was examined for sterility by incubating at 37º C for 24 hours or longer

4.2.3.6. Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into two types which include:

Diffusion

Agar well diffusion assay

Dilution

Minimum Inhibitory Concentration Broth dilution assay

a) Agar- Well Diffusion assay:

Initial screening of potential antibacterial activity from Black cardamom was 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. The agar diffusion test or the Kirby-Bauer disk-diffusion method is a means of measuring the effect of antibacterial agents against bacteria grown in culture. The antibacterial present in the extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimetres.

Procedure:

Well-isolated colonies from an agar plate culture were transferred into flask containing broth medium. The broth culture was incubated at 37 ºC for overnight. The turbidity of growing broth culture was adjusted with sterile saline solution till it achieves the turbidity of the 0.5 McFarland standard (1.5×10^8 CFU/ml). Than 100 µl of respective inoculums was spread on MHA plates and were kept at room temperature for 15 min for absorption to take place. Wells of 6mm size were bored with a sterile borer in the inoculated agar plates and loaded with 80 µl of plant extracts. DMSO was used as a negative control whereas Streptomycin was used as positive control. Prior to incubation at 37 ºC for 24 hrs, the Petri dishes were kept at room temperature for 15 min in order to promote diffusion of the extracts into the agar. All the tests were made in triplicate and the mean diameter of the inhibition zones in millimetre and the standard deviation was calculated (Joshi *et al*., 2009).

b) Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest (i.e minimal) concentration of an antibacterial agent that will inhibit the visible growth of a microorganism after overnight incubation. We determine the concentration by incubating a known quantity of bacteria with specified dilution of antibacterial agent. A lower MIC is an indication of a better antibacterial agent. MIC is generally regarded as the most basic laboratory measurement of the activity of an antibacterial agent against an organism. The method and principles of the microdilution method is essentially the same as the macrodilution method except that the antibacterial dilutions are in 0.1 ml volumes contained in wells of a microdilution tray (usually 96 well trays) (Jorgensen *et al*., 2007 and Clinical and Laboratory Standard 2009).

Procedure:

A suspension of test organism was prepared equivalent to a 0.5 McFarland standard using isolated colonies. Streptomycin was taken as positive control and prepared as stock solutions of 1 mg/ml. The wells of a 96 - well ELISA tray was filled with 100 µl of streptomycin along with 100 µl MHB media as control in triplicates. Similarly, 100 µl of diluted extracts of different concentration from 10 mg/ml to 1.25 mg/ml along with 100 µl of MHB media were added in other wells in triplicates. After that, 10µl of bacterial suspension was added to each well. After plating the microtitre plate absorbance of each well was recorded using an automatic ELISA reader adjusted at 600 nm as 0 hour reading. After taking the reading the plate was incubated at 37 ºC for 24 hour. After incubation absorbance was recorded at 600 nm using ELISA reader. These absorbance values were compared from those obtained (before incubation) at 0 hours. 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.

4.2.4. To determine anticancer activity by using MCF-7 Cell line:

4.2.4.1. Trypsinization

Principle:

Adherent cells attach themselves to surface of tissue culture flasks or dishes using proteins. These proteins, secreted by the cells, form a tight bridge between the cell and the surface. To dislodge cells from the flask, the protein bridges must be broken. Trypsin is a proteolytic (protein degrading) enzyme that will break proteins at specific places. EDTA is often found in trypsin solutions. EDTA allows trypsin to work more efficiently by engaging certain metal ions that may inhibit its activity.

Reagents used for the thawing and subculturing of MCF-7 cells were: DMEM, FBS, PBS, Trypsin, Sodum biocarbonate, L-glutamine, Penicillin, Streptomycin, Ficoll, Trypan blue, MTT reagent, DMSO.

4.2.4.2. Preparation of powdered DMEM media:

9.6 gms of the powder was suspended in 900 ml tissue culture grade water with constant, gentle stirring until the powder is completely dissolved. Adjusted the pH to 4.0 before autoclaving and the volume was made with tissue culture grade water. The volumes of 7.5 % sodium bicarbonate solution and 200 mM L-glutamine solution were subtracted from the final volume. The medium was autoclaved

at 121 °C at 15 psi for 15 minutes. Removed the medium promptly from the autoclave to avoid extended heating or evaporation. Allowed to cool at room temperature. 49.3 ml of 7.5 % Sodium bicarbonate solution and 20 ml of 200 mM L- glutamine solution were added to the medium upto the final volume of the medium being prepared. The liquid medium was stored at 2 - 8 °C and in dark till use.

4.2.4.3. Preparation of PBS (Phosphate Saline Buffer):

For the preparation of 1 litre of 1X PBS weight 8 g of NaCl, 0.2 g of KCl, 1.44 g of $NaHPO_4$, and 0.24 g of KH_2PO_4 were added in 800 ml of distilled water. After that pH was adjusted to 7.4 by using 1N HCl or 1N NaOH. The final volume made upto 1 litre with double distilled water. PBS was autoclaved for 20 minutes at 121 ⁰C. After autoclaving PBS was stored at 4 ⁰C.

4.2.4.4. Reviving the MCF-7 cell lines:

Culturing of cell lines was carried out aseptically under BSL-2 and CO_2 Incubator.

a. Thawing cells:

Removed the vial containing cells from storage (-80 ºC) and thaw quickly in a 37 °C water bath. Immediately added 2 volumes of complete growth medium to the vial containing frozen cells and mixed them very gently. After that centrifuged the cells at 1000 rpm for 10 min at room temperature and discarded the supernatant. Gently resuspended the cells in complete growth medium (DMEM supplemented with 10 %, Streptomycin (10 mg/ml), Penicillin (10^4 IU/ml), Amphotericin (2.5 mg/ml) and Tylosin (1 ml/l). Plated the cells in tissue culture flask and incubated at 37 ^{0}C and 5 % CO_2.

b. Subculturing cells:

Used DMEM, supplemented with 10 % FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), Amphotericine (2.5 μg/ml) and tylosin (1 ml/L). Maintained the temperature at 37 °C in humidified, concentrated CO_2 (5 %) atmosphere. Once MCF-7 cells reached approximately 80 % confluence on plates, removed media from flask and rinsing with 1xPBS. Added 2 - 3 ml of warm (37 °C) 0.25 % Trypsin solution to cells to dispersed cell layer. After adding trypsin incubate flask for 8 to 10 minutes. 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). Once MCF-7 cell layer was dispersed, trypsin was deactivated by adding 2 volumes of complete growth medium in sterile tube. Aspirate cells by gently pipetting. The cells were centrifuged cells in growth medium for 10 minutes at 1000 rpm. Removed trypsin/growth medium suspension from tube.

Resuspended pellet (MCF-7 cells) in 2 ml PBS and centrifuged at 1000 rpm for 10 min. Resuspended the cells in 1 ml of complete medium. Counted the cells using hemocytometer (20 µl cells + 20 µl of trypan blue). Resuspended the cells in complete DMEM media (5 ml for T25 flask and 15 ml for T75 flask). 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 pH 7 to pH 6 and loose viability between pH 6.5 and pH 6.

4.2.4.5. MTT Assay:

Principle:

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. DMSO or 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.

Materials:

PBS, MTT (5 mg/ml in PBS) – filters and kept in dark, DMSO, 96 - well plate (flat bottom)

Procedure:

Cells were plated at a density 2 X 10^4 cells/well (100 µl) in 96 - flat bottomed well plate and treated with 250 µg/ml, 500 µg/ml and 1000 µg/ml of extracts. After 72 hours of incubation at 37 °C and 5 % CO_2 concentration, 10 µl of MTT (5 mg/ml) was added and incubated at 37 °C for 4 hours. Then the purple colored formazon crystals were solubilised in 100 µl DMSO. Absorbance was recorded at 570 nm.

4.2.5. To determine Immunomodulatory activity by PBMC isolation method:

Protocol:

4.2.5.1. Preparation of RPMI 1640 Media:

10.3 gms of RPMI powder was dissolved in 900 ml millipore water, accompanied by constant stirring till the medium was completely dissolved. pH was adjusted to 4.0 before autoclaving. The volume was made up to 963 ml (This volume was derived after subtracting the volume of 7.5 % sodium bicarbonate solution and 200 mM L-glutamine solution from the final volume). This medium was then dispensed in separate bottles and was autoclaved at 121 °C at 15psi for 15 minutes. The medium was promptly removed from the autoclave to avoid extended heating or evaporation and was allowed to cool at room temperature. 26.7 ml of 7.5 % sodium bicarbonate solution and 10.3ml of 200mM L-glutamine solution was added to the final volume of the medium prepared. 10 % of Foetal Bovine Serum and antibiotics were added and then, liquid medium was stored in the dark at 2 - 8 °C**.**

4.2.5.2. Preparation of PBS (Phosphate Saline Buffer):

For the preparation of 1 litre of 1X PBS weight 8 g of NaCl, 0.2 g of KCl, 1.44 g of $NaHPO_4$, and 0.24 g of KH_2PO_4 were added in 800 ml of distilled water. After that pH was adjusted to 7.4 by using 1N HCl or 1N NaOH. The final volume made upto 1 litre with double distilled water. PBS was autoclaved for 20 minutes at 121 ºC. After autoclaving PBS was stored at 4 ºC.

4.2.5.3. Isolation of peripheral blood mononuclear cells from whole blood:

Blood was drawn from a healthy person with the help of vacutainer system (EDTA coated, Becton Dickinson). Blood was diluted in 1:1 ratio with PBS. Then blood sample was layered carefully over Hisep LSM 1073 in the ratio 4:3 and it was centrifuged at 700xg for 40 minutes at 25 ^{0}C. Plasma was removed and then the buffy coat layer was transferred to a clean centrifuge tube with the help of micropipette. Buffy coat layer was washed twice with 3 volumes of PBS by centrifuging at 1000 rpm for 12 minutes at 25 ^{0}C. Supernatant was discarded and pellet of PBMC was suspended in 1 ml of cell culture medium [RPMI + 10 % FBS + Penicillin (100 IU/ml) + Streptomycin (100 µg/ml)].

4.2.5.4. 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 colour 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:

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

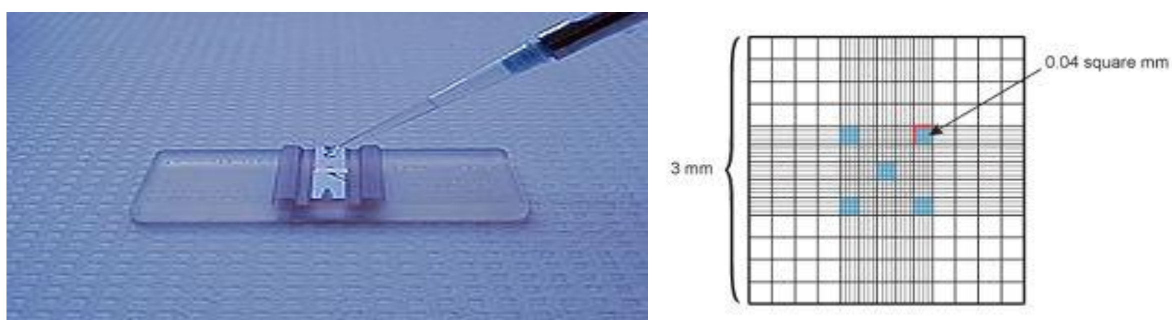


Fig. 4.1. Haemocytometer

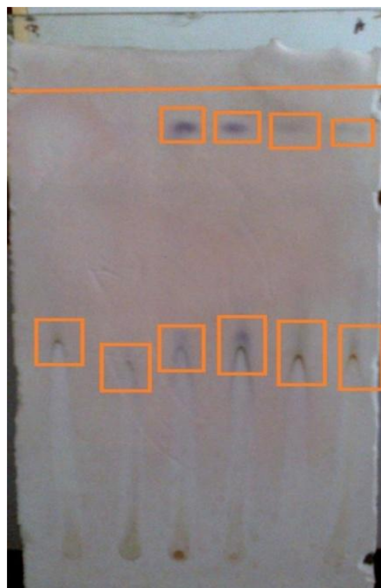
4.2.5.5. MTT assay:

For this assay, freshly isolated lymphocytes (2 x 10^5 cells /200 µl media per well) were seeded in 96-well flat bottom microtiter plate. 20 µl of different concentrations (1 mg/ml, 500 µg/ml and 250 µg/ml) of each extracts were added to the wells. Plate was incubated at 37 ^{0}C and 5 % CO_2 concentration for 72 hours. After 72 hours, 10µl of MTT (5 mg/ml) was added to each well and incubation for another 4 hours was given for reduction of MTT to formazan. Media was removed carefully and purple formazan crystals were dissolved in 100µl of DMSO. Absorbance was recorded at 570 nm by microtiter plate reader.

5. Results and Discussion

5.1. Thin Layer Chromatography:

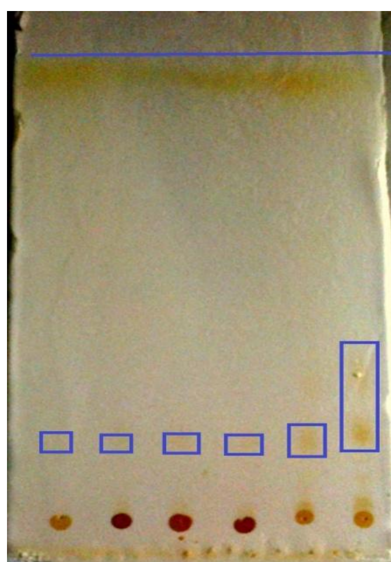
TLC with Vanillin



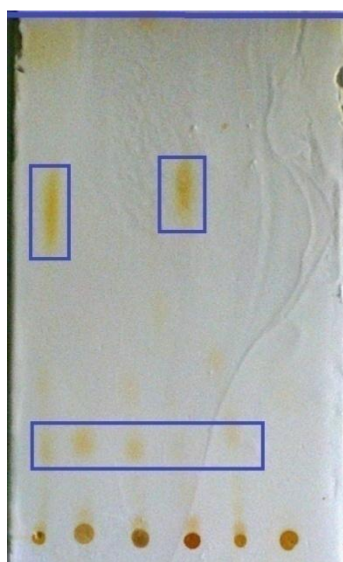
1. Methanol seed
2. Acetone seed
3. Methanol rind
4. Acetone rind
5. P. ether seed
6. Ethanol seed

1. Ethanol rind
2. P. ether rind
3. Cold aq. rind
4. Cold aq. seed
5. Cold aq. seed
6. Acetone seed

TLC with Iodine



1. Cold aq. Seed
2. Hot aq. Seed
3. Cold aq. Rind
4. Hot aq. Rind
5. Methanol rind
6. Ethanol rind



1. Acetone Seed
2. Methanol Seed
3. Ethanol seed
4. Acetone rind
5. P. ether seed
6. P. ether rind

For extraction of soluble components in black cardamom, five different solvents were used i.e. water, ethanol, methanol, petroleum ether and acetone. To investigate difference in extraction by the above mentioned solvents, the extracts were analyzed by thin layer chromatography using Benenze:Chloroform (7:3) as mobile phase on silica plates. The separated analytes were visualized by iodine and vanillin staining separately. Ninhydrin staining did not showed any bands. The chromatogram exhibited one to three visible bands in different extracts stained by the two methods. From these preliminary results it was inferred that in case of vanillin rind extracts of methanol and acetone as well as seed extracts of petroleum ether and ethanol are similar and water extracts showed similar patterns of analytes migration. Similarly, in case of iodine staining one unique band i.e acetone and ethanol extract. 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.

5.2. To determine *in vitro* antibacterial activity:

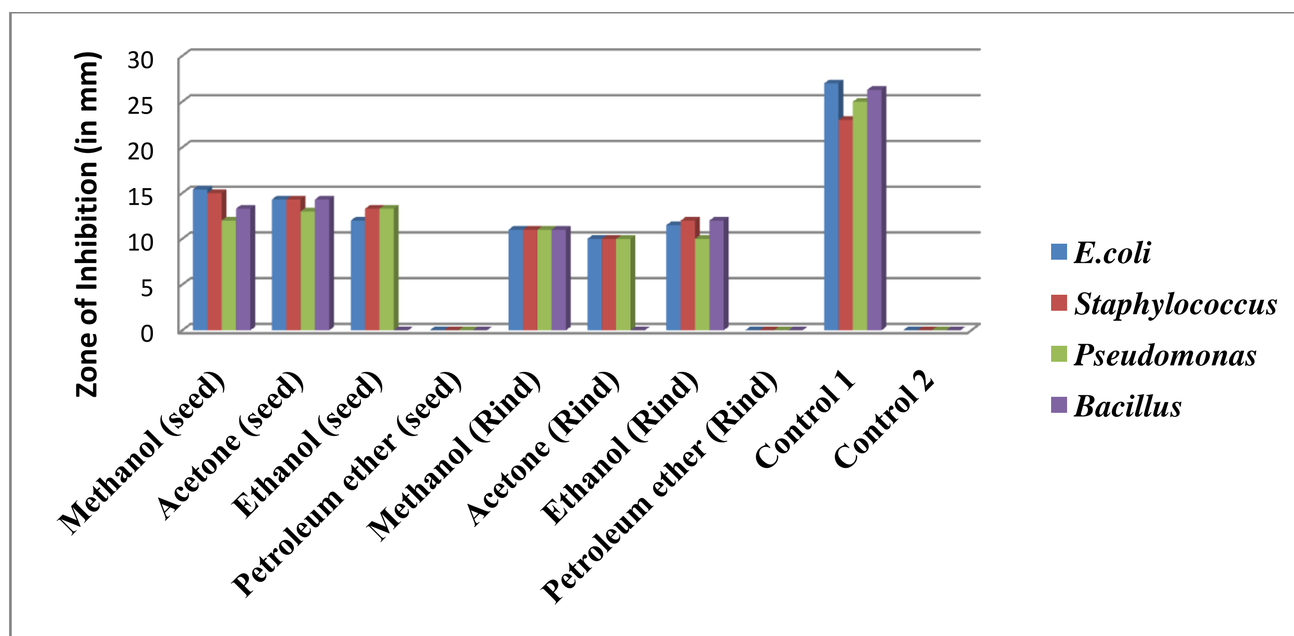
Agar well diffusion assay followed by Minimum inhibitory concentration were done to determine the antibacterial activity of black cardamom (seed and rind). The Extracts of black cardamom were prepared by using acetone, methanol, ethanol and petroleum ether solvents. These extracts were then tested for their antibacterial activity. In agar well diffusion assay, 80 µl of each extract from the stock concentration of 100mg/ml was loaded in wells of bacterial cultures MHA plates. After 24 hrs incubation, zone of inhibition was measured which is mentioned in table 5.1. We found that the acetone, methanol, and ethanol extracts of seed and rind portion of black cardamom showed the zone of inhibition against two Gram positive bacteria *Staphylococcus aureus* and *Bacillus cereus* and two Gram negative bacteria *Escherichia* coli and *Pseudomonas aeruginosa* as shown in figure 5.2 to 5.5. But petroleum ether extracts did not show any kind of inhibition against any bacteria.

Methanol extract of seed showed highest zone of inhibition against *Escherichia coli* and *Staphylococcus aureus* respectively. While in case of *Pseudomonas aeruginosa,* highest zone of inhibition showed by ethanol extract of seed. *Bacillus cereus* showed highest zone of inhibition is shown by acetone extract of seed (Fig 5.1). In case of *B. cereus,* only methanol and acetone extract of seed have shown inhibition while rest of the extracts did not show any kind of inhibition. The methanol, acetone and ethanol extracts of rind showed activity against *E. coli, P. aeruginosa* and *S. aureus* whereas acetone extract of rind showed no inhibition against *B. cereus.* In general seed extracts showed higher inhibition as compared to rind extracts. Hence this study indicated that maximum inhibitory activity was observed in case of seed extracts.

Table 5.1: Antibacterial activity of the Black cardamom extracts

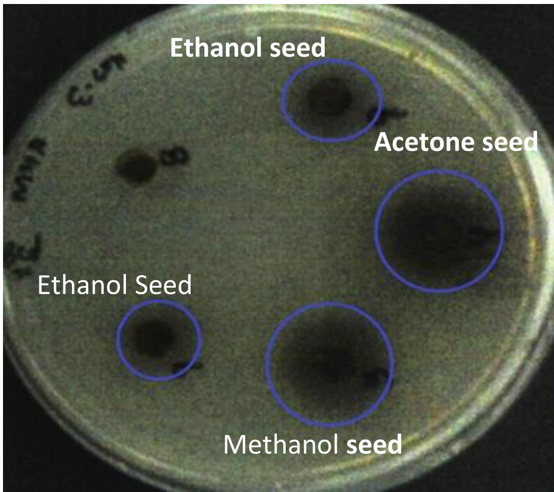
	Zone of Inhibition (Mean diameter in mm)			
EXTRACTS	***E. coli***	***S. aureus***	***P. aeruginosa***	***B. cereus***
Methanol (seed)	15.4	15	12	13.3
Acetone (seed)	14.3	14.3	13	14.3
Ethanol (seed)	12	13.3	13.3	NI
Petroleum ether (seed)	NI*	NI	NI	NI
Methanol (Rind)	11	11	11	11
Acetone (Rind)	10	10	10	NI
Ethanol (Rind)	11.5	12	10	12
Petroleum ether (Rind)	NI	NI	NI	NI
Control 1 (positive)	27.0	23.0	25.0	26.3
Control 2 (negative)	NI	NI	NI	NI

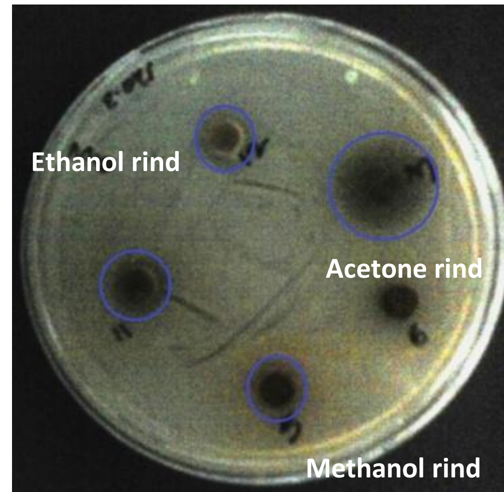
*Not inhibited, Control 1 = Streptomycin, Control 2 = DMSO



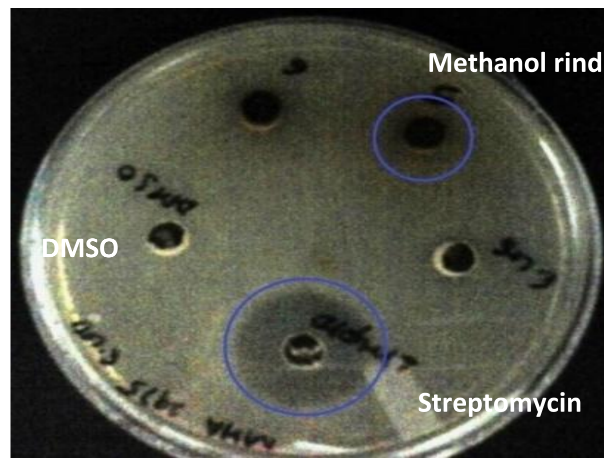
Control 1 = Streptomycin, Control 2 = DMSO

Figure 5.1: Antibacterial activity of different extracts of black cardamom against gram negative and gram positive bacteria



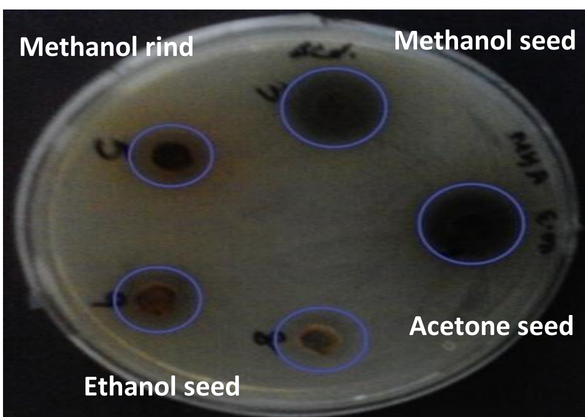


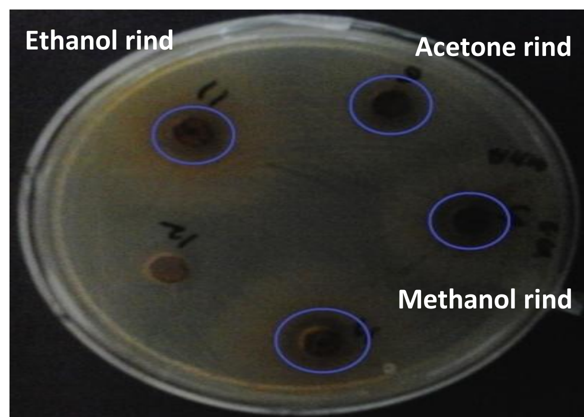
(a) **(b)**



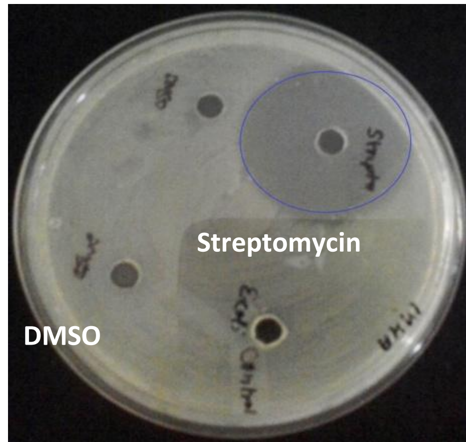
(c)

Figure 5.2: Zone of inhibition shown antibacterial activity of black cardamom extracts against *Staphylococcus aureus*



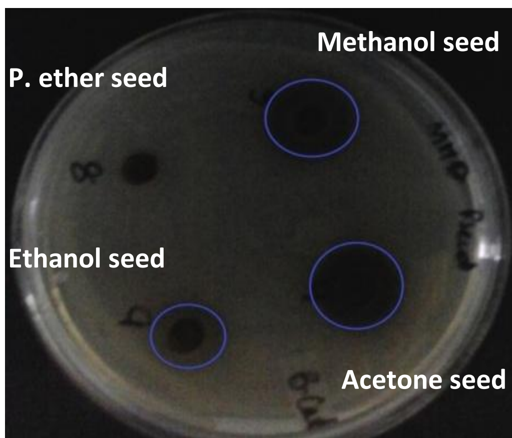


(a) **(b)**

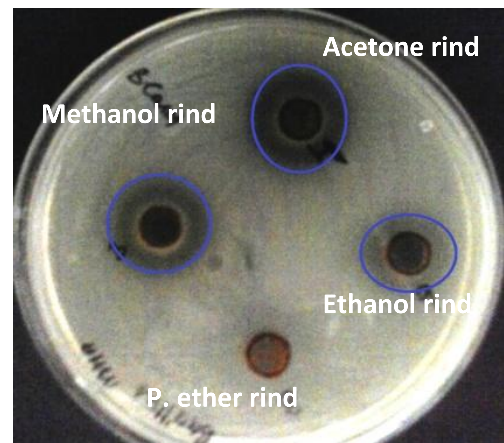


(c)

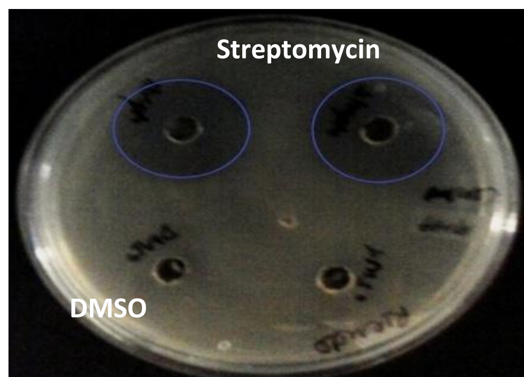
Figure 5.3: Zone of inhibition shown antibacterial activity of black cardamom extracts against *E .coli*



(a)

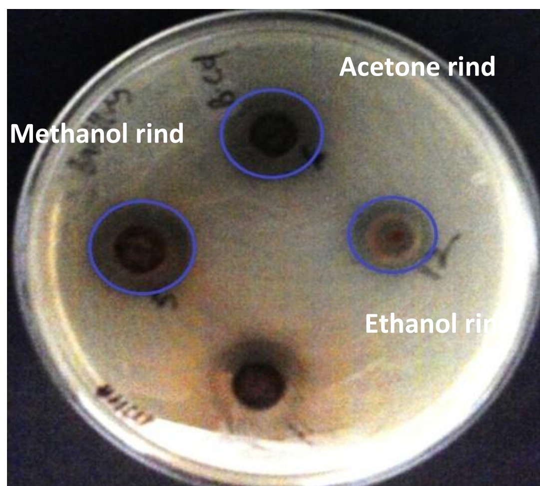


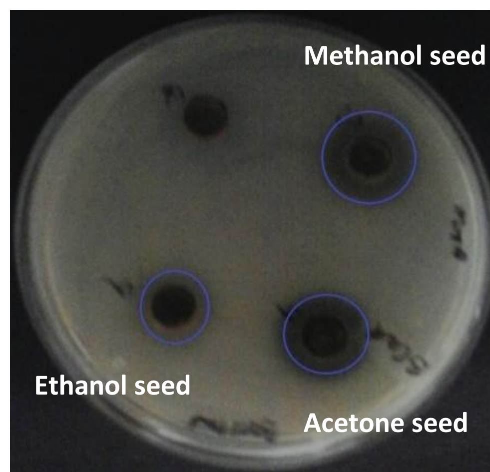
(b)



(c)

Figure 5.4: Zone of inhibition shown antibacterial activity of black cardamom extracts against *Pseudomonas aeruginosa*





(a) **(b)**

Figure 5.5: Zone of inhibition shown antibacterial activity of black cardamom extracts against *bacillus cereus*

Minimum Inhibitory Concentration:

Minimum inhibitory concentration (MIC) was determined by microdilution assay. In this assay we used four different concentrations of extracts i.e 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, and 10 mg/ml. Minimum inhibitory concentration is the lowest or minimum concentration that is used to inhibit the growth of bacteria. We have determined MIC against the four bacteria used in agar well diffusion assay (*E. coli, P. aeruginosa, S. aureus, B. cereus).* In this assay we took absorbance at 600 nm at 0 hour and 24 hour after incubating microtitre plate at 37 ºC. After that we compared the absorbance of 0 hour and 24 hour from where we figure out the MIC of extracts. The methanol extract of seed showed MIC at 5 mg/ml against *E. coli,* 10 mg/ml against *P. aeruoginosa, S. aeurus*, *and B. cereus* (Fig.5.6, 5.8, 5.9, 5.12) (Table 5.2, 5.4, 5.6, 5.8). In case of methanol extract of rind showed MIC at 5 mg/ml against *E. coli* and *P. aeruoginosa,* 10 mg/ml against *S. aeurus*, *and B. cereus* (Fig.5.7, 5.9, 5.11, 5.13) (Table 5.3, 5.5, 5.7, 5.9). The acetone extract of seed showed the MIC of 5 mg/ml against *Pseudomonas aeruoginosa, and E. coli,* 10 mg/ml against *Bacillus cereus,* and *Staphylococcus aeurus* (Fig.5.6, 5.8, 5.9, 5.12) (Table 5.2, 5.4, 5.6, 5.8) while acetone extract of rind showed MIC of 5 mg/ml against E.coli, 10 mg/ml against *P. aeruoginosa, S. aeurus*, *and B. cereus* (Fig.5.7, 5.9, 5.11, 5.13) (Table 5.3, 5.5, 5.7, 5.9). Similarly the ethanol extract of seed showed the MIC of 5 mg/ml against *P. aeruoginosa,* and *E. coli,* 10 mg/ml against *S. aeurus* and no activity against *B. cereus* (Fig.5.6, 5.8, 5.9, 5.12) (Table 5.2, 5.4, 5.6, 5.8). In case of ethanol extract of rind showed the MIC of 10 mg/ml

against *E. coli, P. aeruoginosa, S. aeurus, and B. cereus* (Fig.5.7, 5.9, 5.11, 5.13) (Table 5.3, 5.5, 5.7, 5.9). Petroleum ether extract did not show any activity.

Minimum inhibitory concentration against *E. coli:*

Table 5.2: Minimum inhibitory concentration of seed extracts of black cardamom against *E. coli*

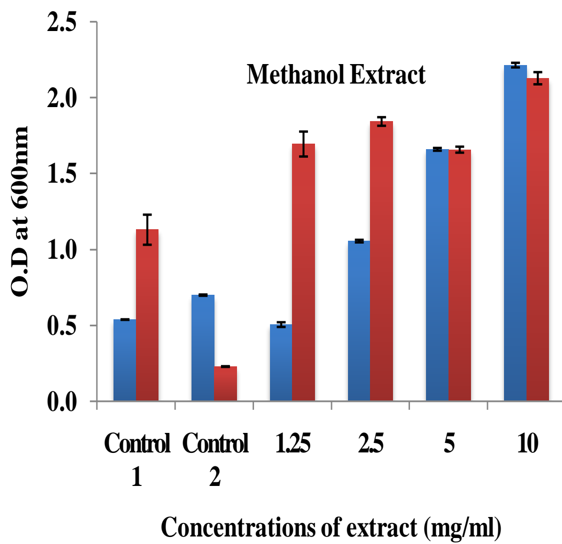
	Methanol extract of seed		Acetone extract of seed		Ethanol extract of seed		Petroleum ether of extract seed	
	0 hour	24 hour	0 hour	24 hour	0 hour	24 hour	0 hour	24 hour
Control 1	0.537	1.129	0.537	1.129	0.508	1.193	0.508	1.193
Control 2	0.698	0.227	0.698	0.227	0.581	0.168	0.581	0.168
1.25 mg/ml	0.504	1.694	0.755	1.640	0.582	1.589	0.598	1.480
2.5 mg/ml	1.054	1.842	0.964	1.880	0.944	1.621	0.410	1.350
5 mg/ml	1.465	1.656	1.845	1.880	2.257	2.201	0.977	1.363
10 mg/ml	2.213	2.227	2.222	1.997	2.435	2.346	1.396	1.589

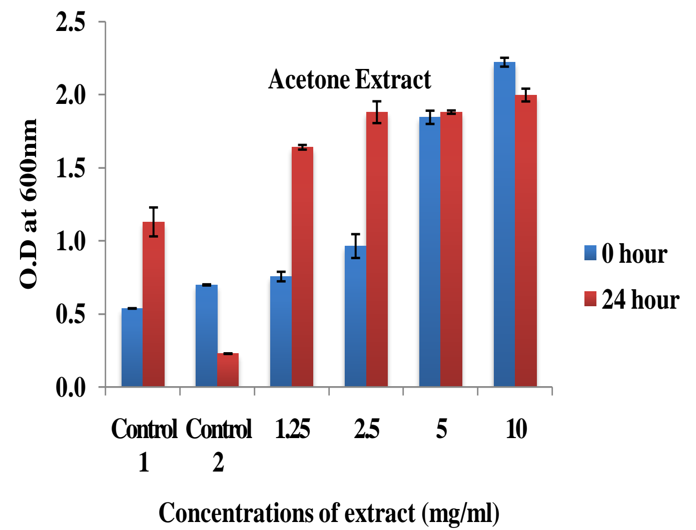
Control 1: *E. coli* cells, Control 2: Streptomycin

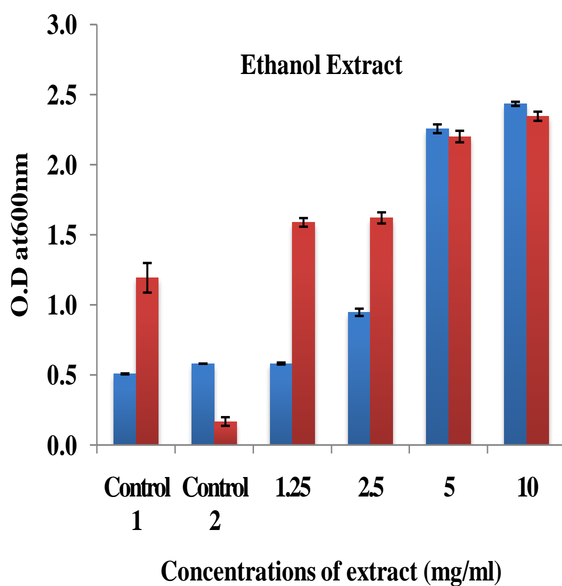
Table 5.3: Minimum inhibitory concentration of rind extracts of black cardamom against *E. coli*

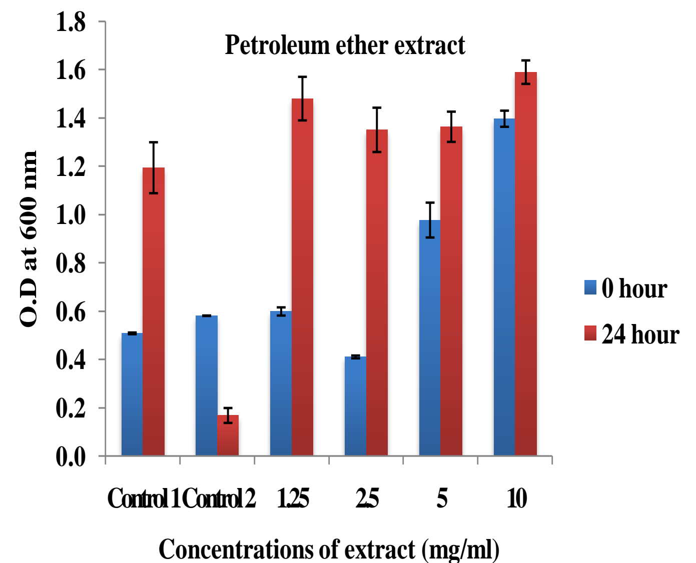
	Methanol extract of rind		Acetone extract of rind		Ethanol extract of rind		Petroleum ether extract of rind	
	0 hour	24 hour	0 hour	24 hour	0 hour	24 hour	0 hour	24 hour
Control 1	0.537	1.129	0.537	1.129	0.508	1.193	0.508	1.193
Control 2	0.698	0.227	0.698	0.227	0.581	0.168	0.581	0.168
1.25 mg/ml	0.557	1.626	0.444	1.268	0.782	1.892	0.161	1.892
2.5 mg/ml	1.294	2.134	1.128	1.930	0.847	1.721	0.166	1.721
5 mg/ml	2.344	2.357	1.694	2.314	2.057	2.401	0.246	2.401
10 mg/ml	2.664	2.465	2.688	2.675	2.245	2.234	0.360	2.234

Control 1: *E. coli* cells, Control 2: Streptomycin



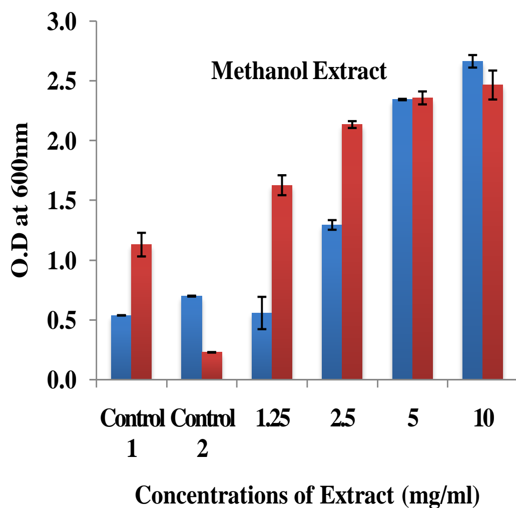


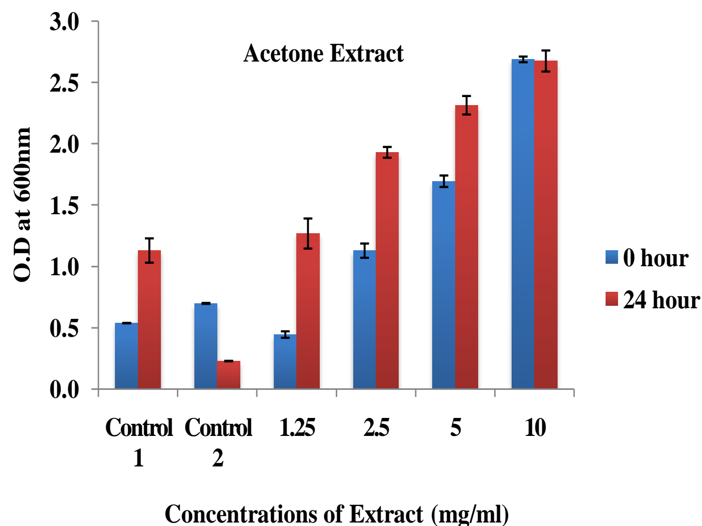


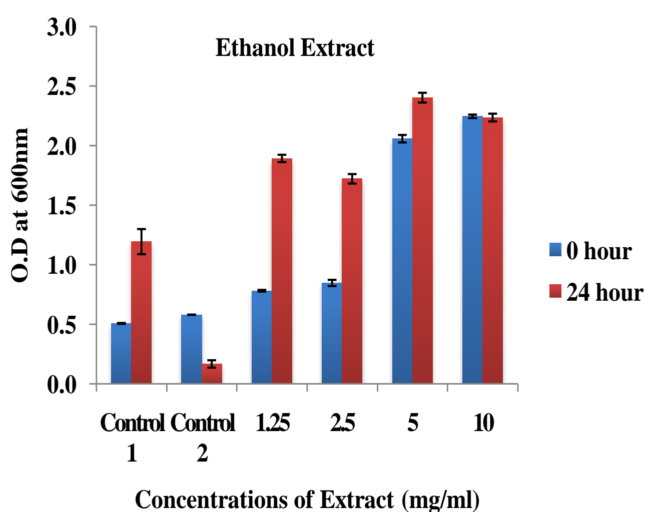


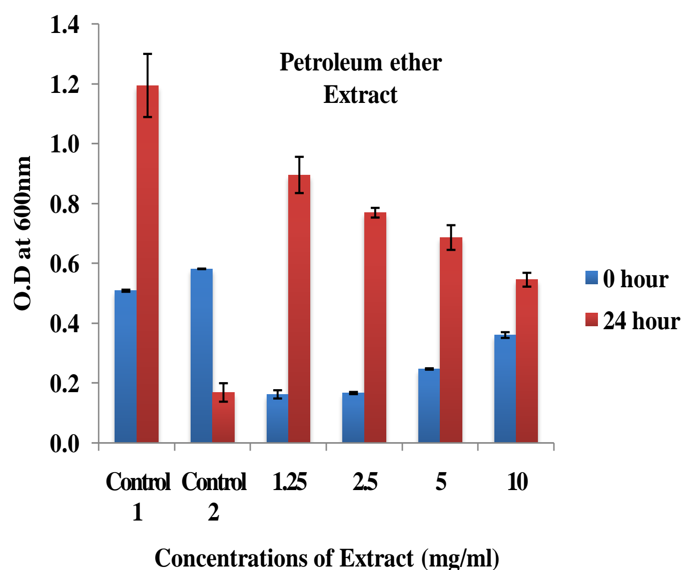
Control 1: *E. coli* cells, Control 2: Streptomycin

Figure 5.6: Minimum inhibitory concentration of seed extract against *E. coli*









Control 1: *E. coli* cells, Control 2: Streptomycin

Figure 5.7: Minimum inhibitory concentration of rind extract against *E. coli*

Minimum inhibitory concentration against *Staphylococcus aureus:*

Table 5.4: Minimum inhibitory concentration of seed extracts of black cardamom against *Staphylococcus aureus:*

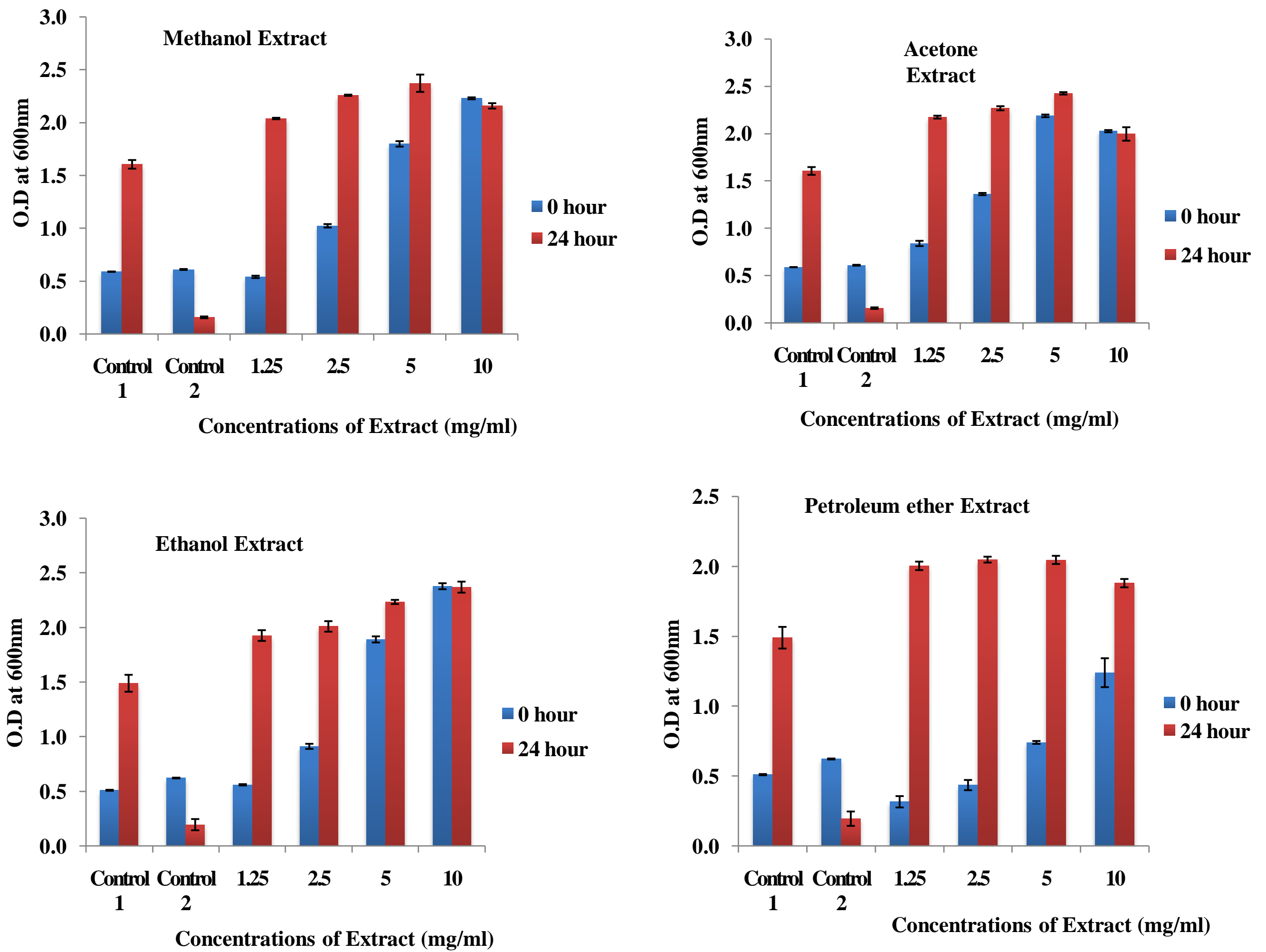
	Methanol extract of seed		Acetone extract of seed		Ethanol extract of seed		Petroleum ether extract of seed	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.589	1.605	0.589	1.605	0.510	1.490	0.510	1.490
Control 2	0.610	0.157	0.610	0.157	0.622	0.196	0.622	0.196
1.25 mg/ml	0.539	2.039	0.840	2.173	0.560	1.926	0.316	2.003
2.5 mg/ml	1.023	2.259	1.361	2.267	0.912	2.010	0.436	2.048
5 mg/ml	1.799	2.373	2.186	2.424	1.891	2.234	0.741	2.046
10 mg/ml	2.2302	2.158	2.025	1.996	2.378	2.370	1.239	1.880

Control 1: *Staphylococcus aureus* cells, Control 2: Streptomycin

Table 5.5: Minimum inhibitory concentration of rind extracts of black cardamom against *Staphylococcus aureus:*

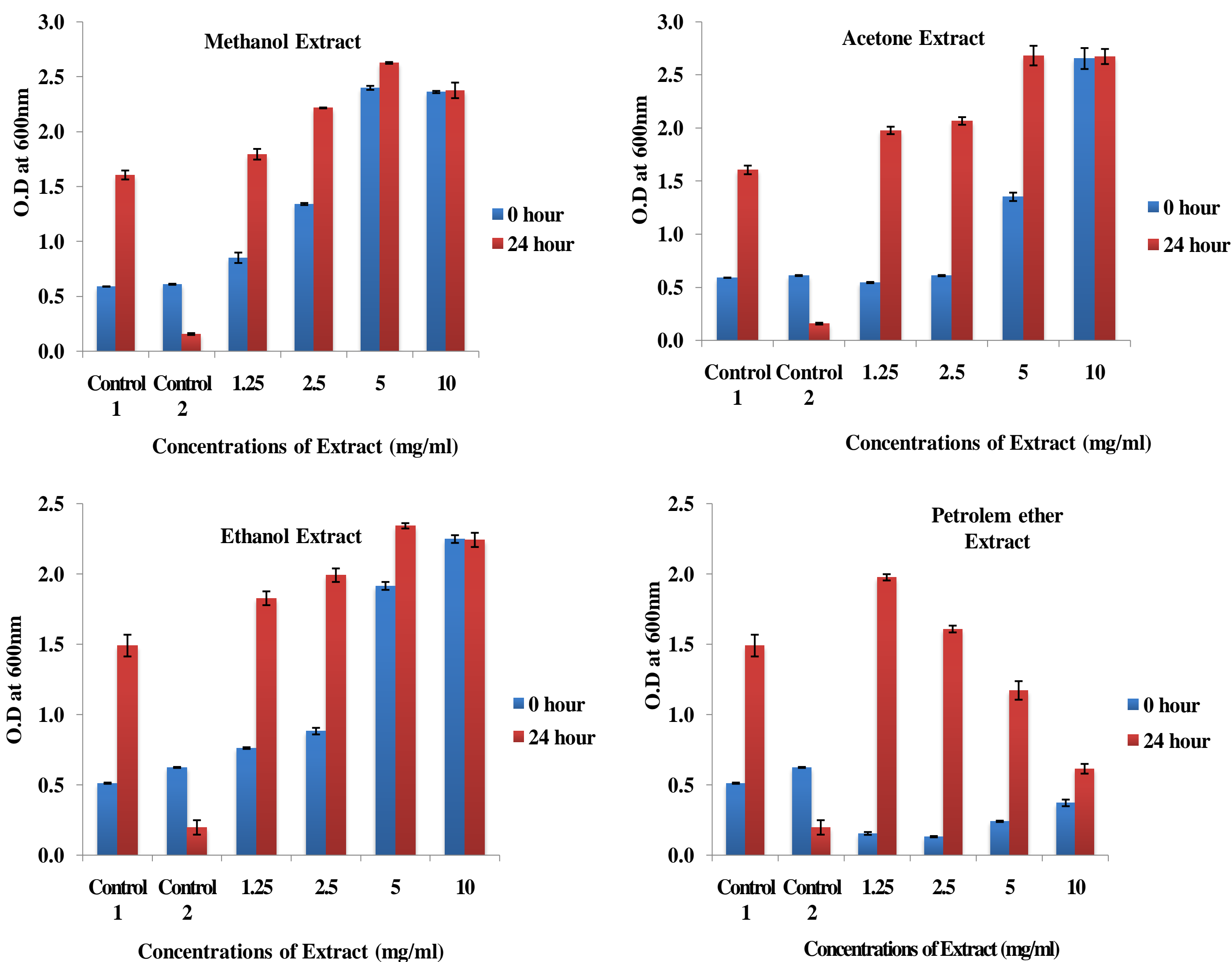
	Methanol extract of seed		Acetone extract of seed		Ethanol extract of seed		Petroleum ether of extract seed	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.590	1.605	0.590	1.605	0.511	1.490	0.511	1.490
Control 2	0.610	0.157	0.610	0.157	0.623	0.196	0.623	0.196
1.25 mg/ml	0.852	1.795	0.544	1.977	0.761	1.827	0.153	1.976
2.5 mg/ml	1.341	2.216	0.610	2.067	0.881	1.991	0.130	1.608
5 mg/ml	2.399	2.626	1.352	2.683	1.915	2.342	0.239	1.171
10 mg/ml	2.361	2.376	2.655	2.674	2.248	2.242	0.371	0.613

Control 1: *Staphylococcus aureus* cells, Control 2: Streptomycin



Control 1: *Staphylococcus aureus* cells, Control 2: Streptomycin

Figure 5.8: Minimum inhibitory concentration of seed extract against *Staphylococcus aureus*



Control 1: *Staphylococcus aureus* cells, Control 2: Streptomycin

Figure 5.9: Minimum inhibitory concentration of rind extract against *Staphylococcus aureus*

Minimum inhibitory concentration against *Pseudomonas aeruoginosa*:

Table 5.6: Minimum inhibitory concentration of seed extracts of black cardamom against *Pseudomonas aeruoginosa:*

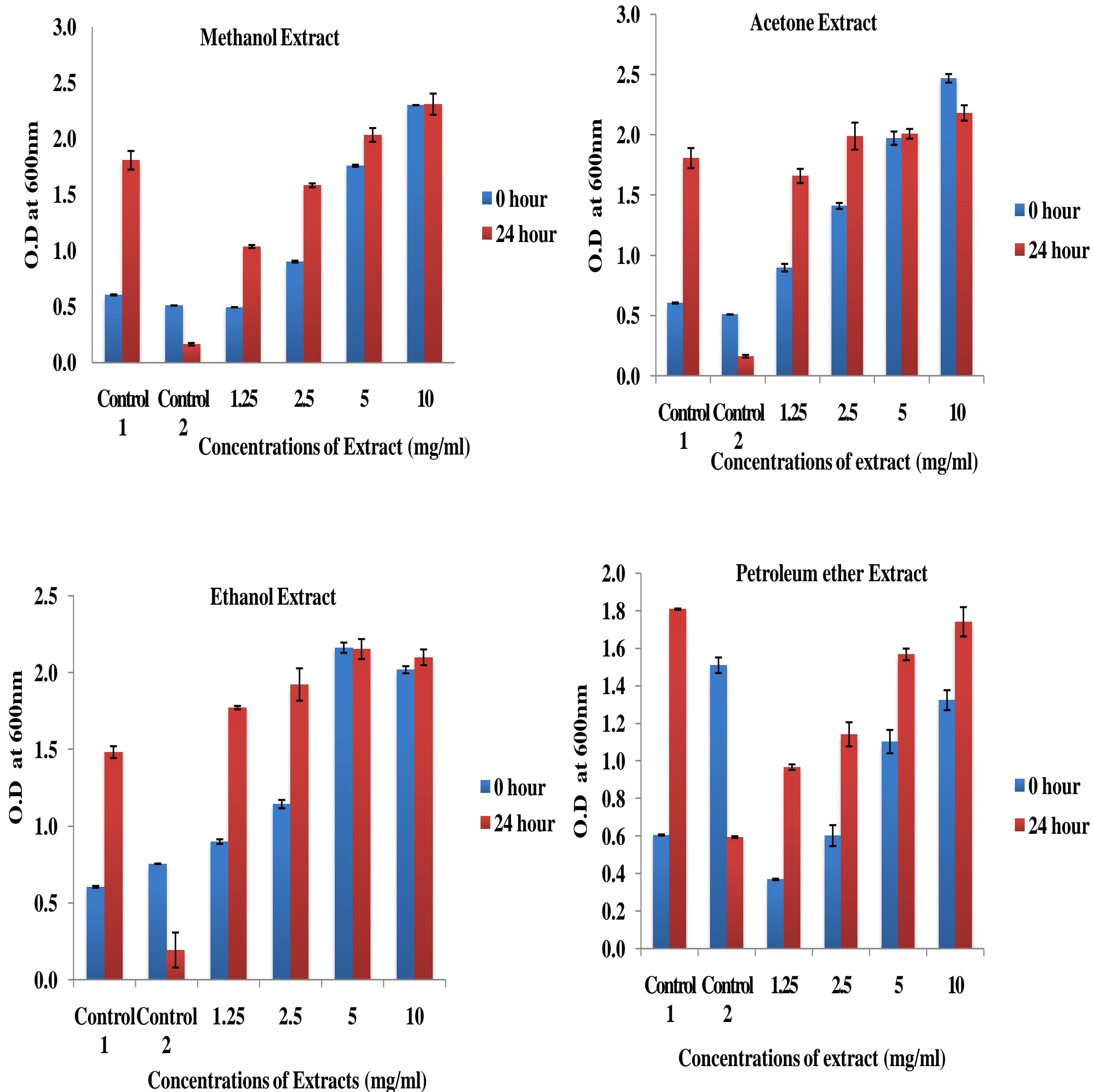
	Methanol extract of seed		Acetone extract of seed		Ethanol extract of seed		Petroleum ether extract of seed	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.605	1.808	0.605	1.808	0.605	1.482	0.605	1.808
Control 2	0.510	0.164	0.510	0164	0.755	0.194	1.509	0.594
1.25 mg/ml	0.495	1.038	0.899	1.660	0.900	1.772	0.369	0.967
2.5 mg/ml	0.903	1.584	1.411	1.991	1.144	1.923	0.602	1.141
5 mg/ml	1.759	2.035	1.973	2.010	2.162	2.154	1.103	1.567
10 mg/ml	2.301	2.310	2.470	2.183	2.019	2.100	1.323	1.741

Control 1: *Pseudomonas aeruoginosa* cells, Control 2: Streptomycin

Table 5.7: Minimum inhibitory concentration of seed extracts of black cardamom against *Pseudomonas aeruoginosa*:

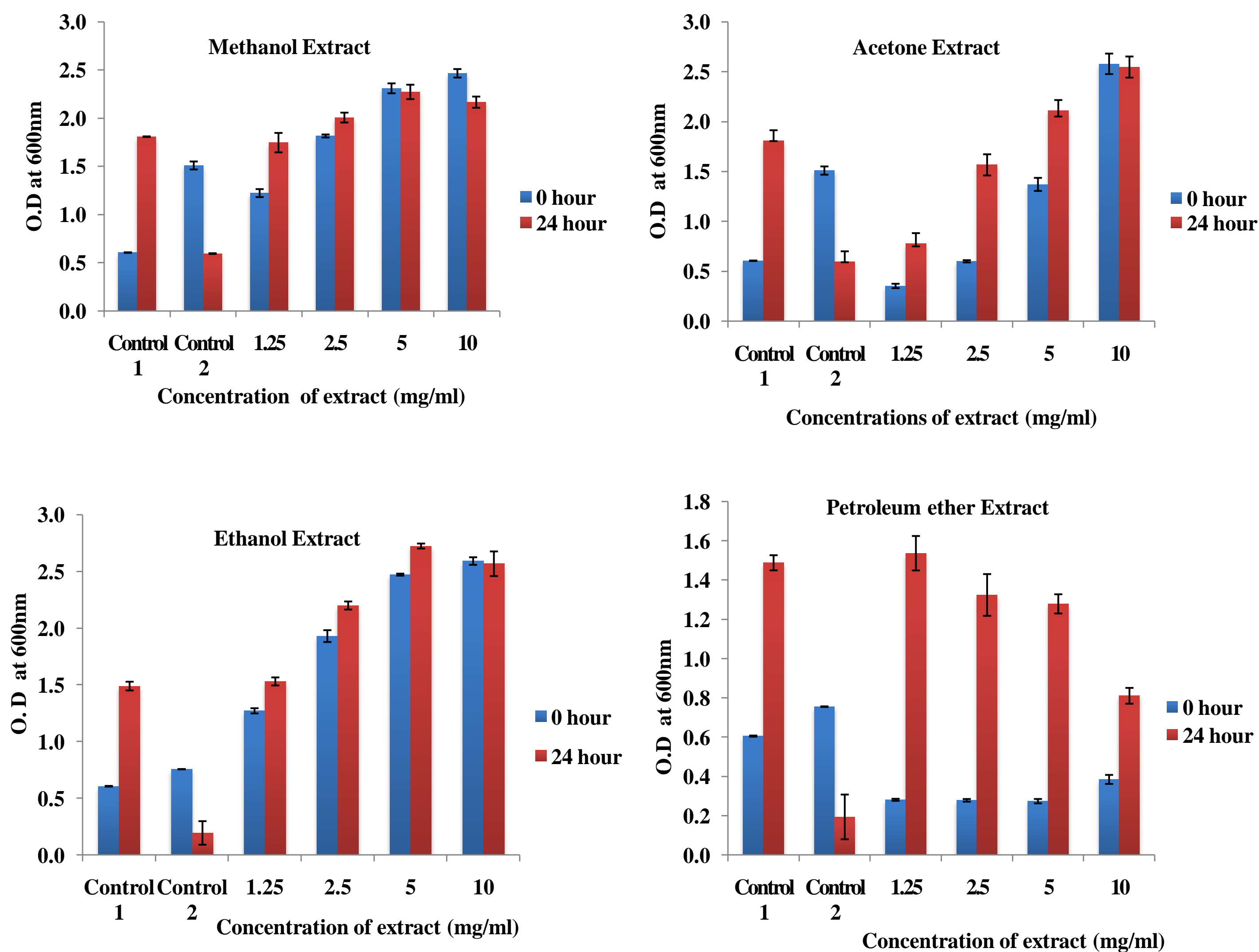
	Methanol extract of rind		Acetone extract of Rind		Ethanol of extract of Rind		Petroleum ether extract of rind	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.605	1.808	0.605	1.808	0.605	1.488	0.605	1.488
Control 2	1.509	0.594	1.509	0.594	0.755	0.194	0.755	0.194
1.25 mg/ml	1.223	1.746	0.353	0.776	1.270	1.529	0.281	1.537
2.5 mg/ml	1.815	2.007	0.600	1.567	1.929	2.198	0.278	1.324
5 mg/ml	2.310	2.273	1.371	2.111	2.471	2.723	0.274	1.279
10 mg/ml	2.466	2.167	2.580	2.547	2.590	2.567	0.385	0.811

Control 1: *Pseudomonas aeruoginosa* cells, Control 2: Streptomycin



Control 1: *Pseudomonas aeruoginosa* cells, Control 2: Streptomycin

Figure 5.10: Minimum inhibitory concentration of seed extract against *Pseudomonas aeruoginosa*



Control 1: *Pseudomonas aeruoginosa* cells, Control 2: Streptomycin

Figure 5.11: Minimum inhibitory concentration of rind extract against *Pseudomonas aeruoginosa*

Minimum inhibitory concentration against *Bacillus cereus:*

Table 5.8: Minimum inhibitory concentration of seed extracts of black cardamom against *Bacillus cereus*:

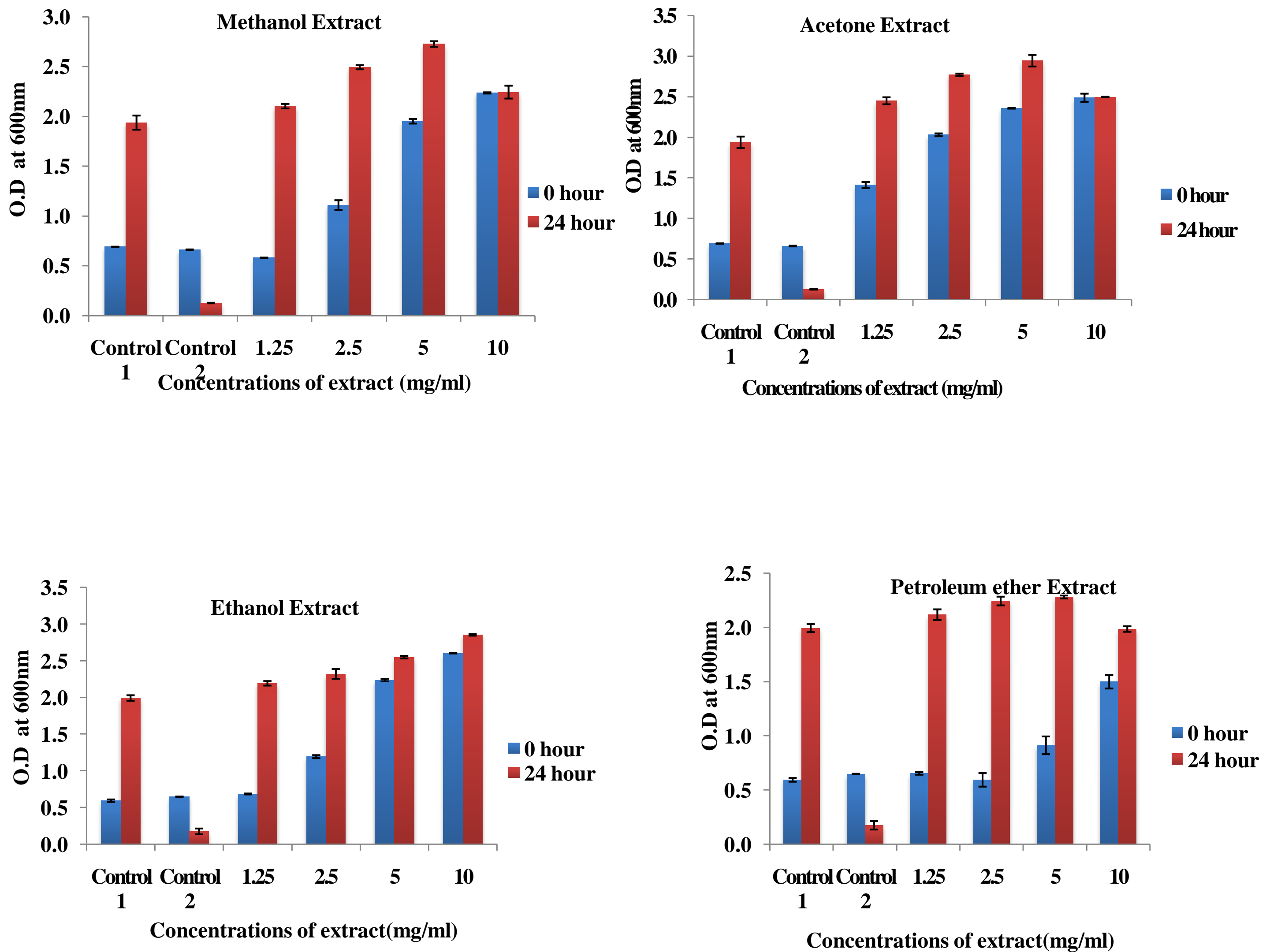
	Methanol extract of seed		Acetone extract of seed		Ethanol extract of seed		Petroleum ether extract of seed	
	0 hour	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.692	1.939	0.692	1.939	0.594	1.993	0.594	1.993
Control 2	0.661	0.127	0.661	0.127	0.648	0.175	0.648	0.175
1.25 mg/ml	0.581	2.104	1.413	2.450	0.685	2.193	0.653	2.117
2.5 mg/ml	1.111	2.495	2.031	2.771	1.194	2.321	0.594	2.243
5 mg/ml	1.952	2.728	2.357	2.944	2.236	2.550	0.912	2.280
10 mg/ml	2.237	2.245	2.488	2.496	2.604	2.854	1.498	1.984

Control 1: *Bacillus cereus* cells, Control 2: Streptomycin

Table 5.9: Minimum inhibitory concentration of rind extracts of black cardamom against *Bacillus cereus*:

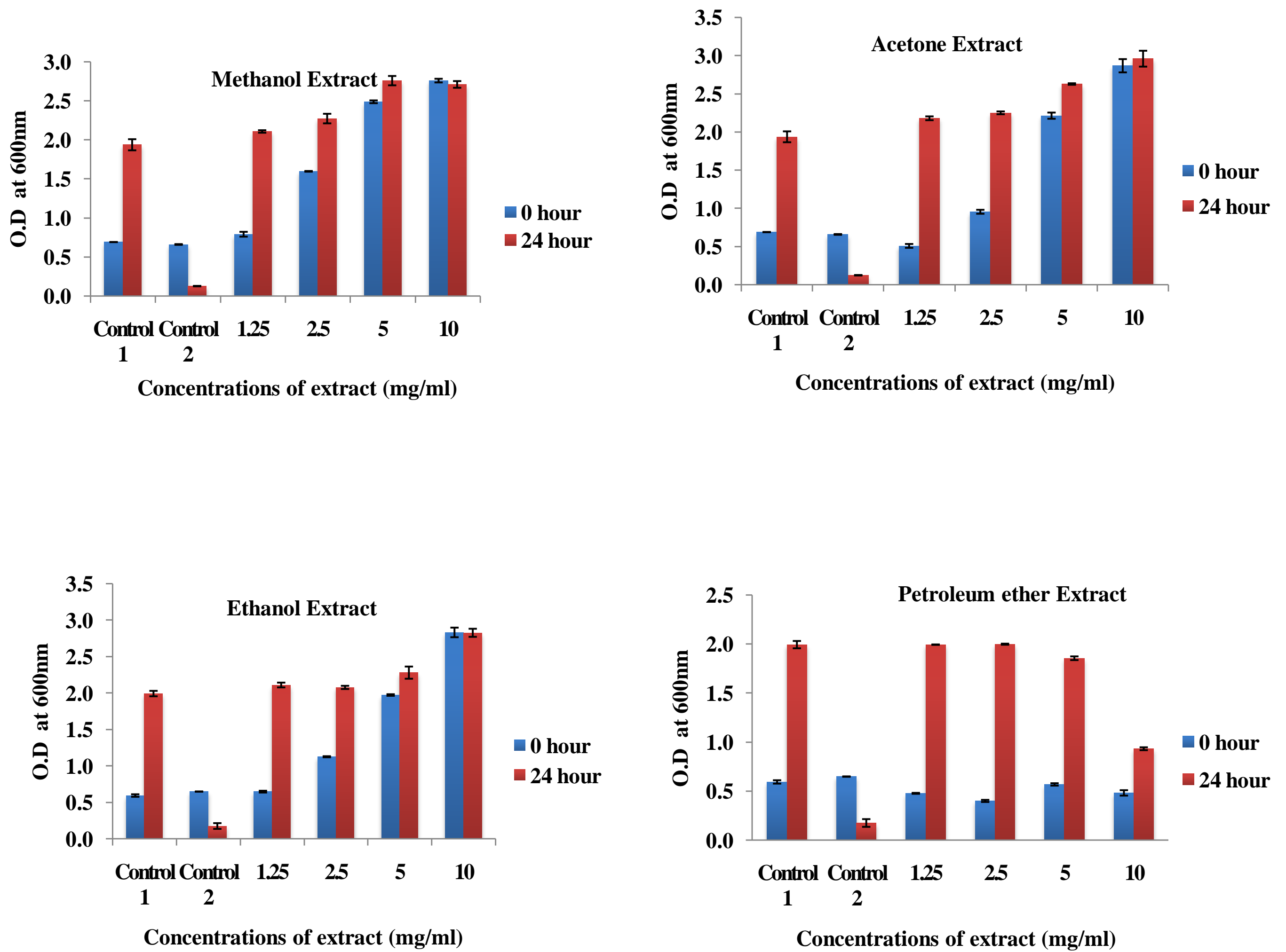
	Methanol extract of rind		Acetone extract of rind		Ethanol extract of rind		Petroleum ether extract of s rind	
	0hour	**24 hour**	**0hour**	**24 hour**	**0 hour**	**24 hour**	**0 hour**	**24 hour**
Control 1	0.692	1.939	0.692	1.939	0.594	1.993	0.594	1.993
Control 2	0.661	0.127	0.661	0.127	0.648	0.175	0.648	0.175
1.25 mg/ml	0.792	2.109	0.511	2.181	0.647	2.111	0.477	1.993
2.5 mg/ml	1.598	2.274	0.957	2.252	1.126	2.078	0.400	1.998
5 mg/ml	2.489	2.760	2.215	2.629	1.974	2.281	0.568	1.855
10 mg/ml	2.760	2.712	2.869	2.961	2.833	2.828	0.482	0.932

Control 1: *Bacillus cereus* cells, Control 2: Streptomycin



Control 1: *Bacillus cereus* cells, Control 2: Streptomycin

Figure 5.12: Minimum inhibitory concentration of seed extract against *Bacillus cereus*



Control 1: *Bacillus cereus* cells, Control 2: Streptomycin

Figure 5.13: Minimum inhibitory concentration of rind extract against *Bacillus cereus*

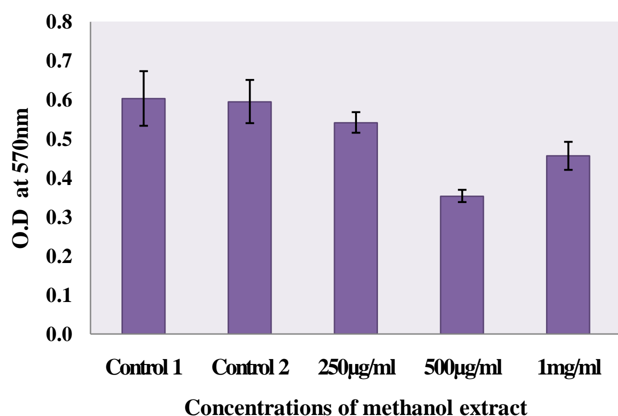
5.3. To determine Anticancer Activity of Black cardamom:

It was interesting to explore the anticancer property of Black cardamom extracts. We have used MCF-7 breast cancer cell lines to assess the anticancer property. MTT assay was done to determined anticancer activity. In MTT assay we used MCF-7 cells and DMSO control. MCF-7 cells were used in the concentration of 2×10^4 cells in each well of microtitre plate. In MTT assay we used three different concentrations of black cardamom extracts (250 µg/ml, 500 µg/ml and 1 mg/ml). All the extracts of black cardamom seed showed anticancer activity but 500 µg/ml concentration showed maximum inhibition of MCF-7 cells in all extracts as compared to other concentrations (Fig.5.14) (Table 5.10). We have found the inhibition in rest of the two concentrations but inhibition was less as compared to 500 µg/ml. Hence, 500 µg/ml was the optimal concentration which showed maximum inhibition. 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 extracts are mixture of different compounds hence the behavior is different. It will be interesting to carry out more experiments in order to confirm the inhibition activity.

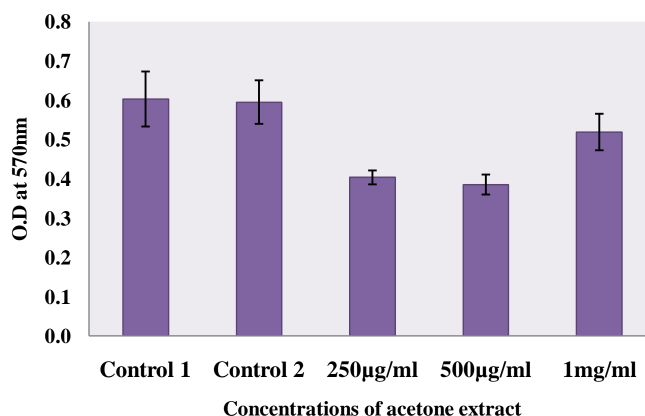
Table 5.10: Anticancer activity shown by different extracts of black cardamom seed

	Cardamom methanol seed	Cardamom acetone seed	Cardamom Ethanol seed	Cardamom Petroleum ether seed
Control 1	0.603	0.603	1.388	1.388
Control 2	0.595	0.595	0.635	0.635
250 µg/ml	0.542	0.404	0.398	0.577
500 µg/ml	0.353	0.386	0.359	0.294
1 mg/ml	0.456	0.519	0.441	0.371

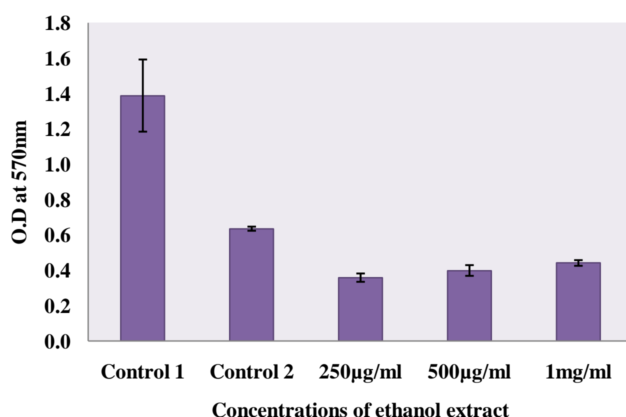
Control 1 = MCF-7 cells, Control 2 = DMSO



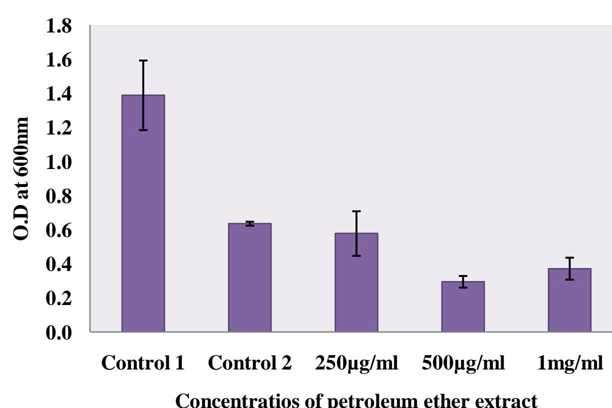
(a)



(b)



(c)



(d)

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

Figure 5.14: Anticancer activity of seed extracts of Black cardamom (a) Methanol extract (b) Acetone extract (c) Ethanol extract (d) Petroleum ether extract

5.4. To determine Immunomodulatory activity of Black Cardamom:

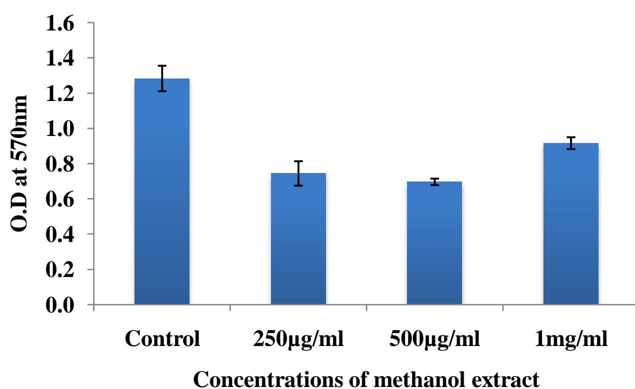
One of the important aspects of our study was to look for immunomodulatory activity in black cardamom because spices contain so many components which show immunomodulatory activity. Immunomodulatory activities were determined by lymphocyte proliferation assay. In our experiment, we used three different concentrations (250 µg/ml, 500 µg/ml and 1 mg/ml) of black cardamom extracts (seed and rind) and checked their activity in 2×10^5 cells/200 µl PBMC which were seeded in each well. To check the proliferation, PBMC was cultures for 72 hours and then MTT assay was done. It appears that there is immunosuppression of all extracts after 72 hours in all three concentrations (Fig. 5.15 & 5.16) (Table 5.11). 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. Since it is result of one experiment hence it will interesting to carry out more experiment to confirm their activity.

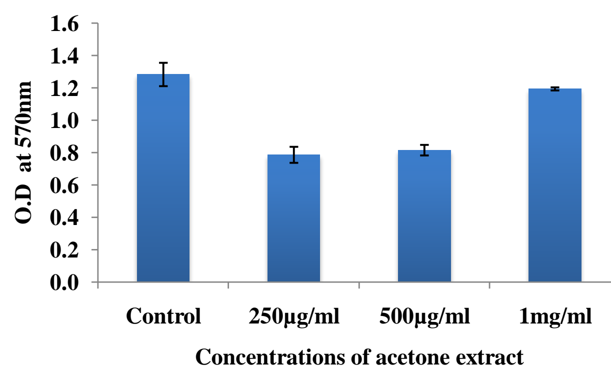
Table 5.11: Effect of Black cardamom extracts on PBMC

	Methanol extract of seed	Acetone extract of seed	Ethanol extract of seed	Petroleum ether extract of seed	Methanol extract of rind	Acetone extract of rind	Ethanol extract of rind	Petroleum ether extract of rind
Control	1.283	1.283	1.283	1.283	0.446	0.446	0.446	0.446
250 µg/ml	0.744	0.786	0.690	0.598	0.209	0.202	0.227	0.304
500 µg/ml	0.696	0.815	0.774	0.512	0.287	0.257	0.286	0.256
1 mg/ml	0.916	1.193	0.598	0.491	0.382	0.350	0.425	0.230

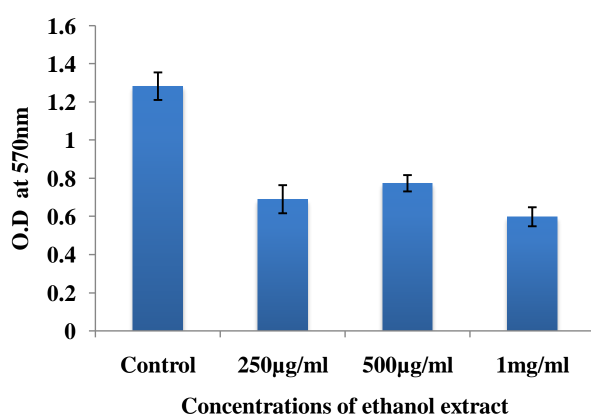
Control = Lymphocytes



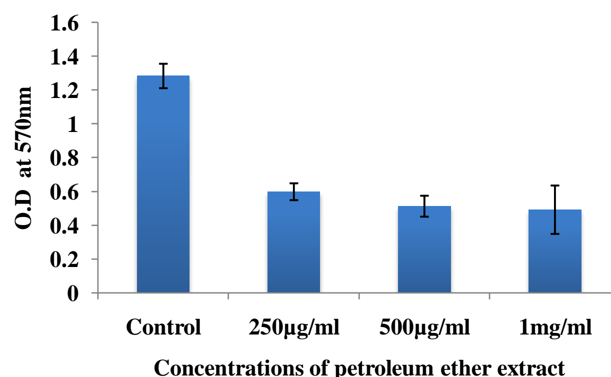
(a)



(b)



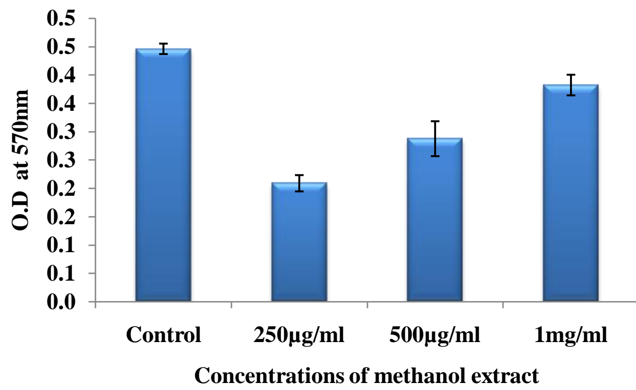
(c)



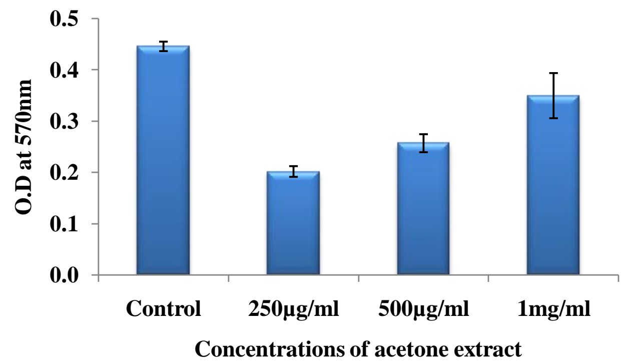
(d)

Control = Lymphocytes

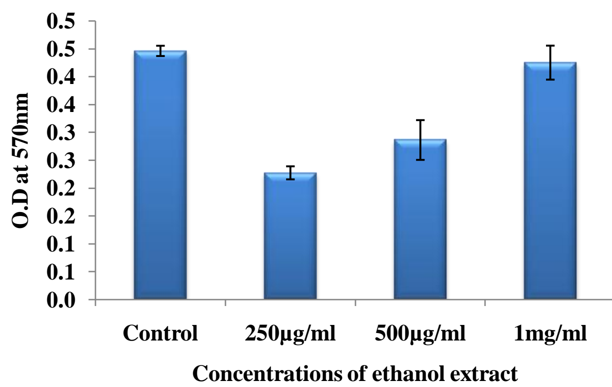
Figure 5.15: Effect of seed extract of black cardamom on PBMC (a) Methanol extract (b) Acetone extract (c) Ethanol extract (d) Petroleum ether extract



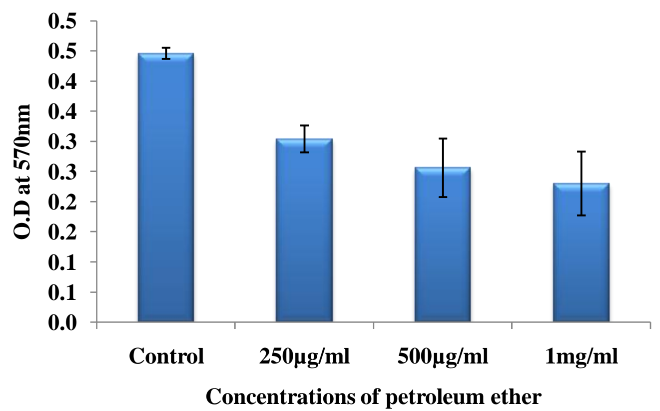
(a)



(b)



(c)



(d)

Control = Lymphocytes

Figure 5.16: Effect of rind extracts of black cardamom on PBMC (a) Methanol extract (b) Acetone extract (c) Ethanol extract (d) Petroleum ether extract

6. Conclusion

Black cardamom (*Amomum subulatum* Roxb, Zingiberaceae) commonly known as 'Badi Ilaichi' is a tall, perennial, evergreen, herbaceous monocot plant. We have prepared different Black cardamom extracts by using ethanol, methanol, acetone, petroleum ether and water as solvents. Thin layer chromatography was done to find out the different components present in the aqueous and organic solvent extracts. Vanillin and iodine staining showed one to three bands of differently migrating analytes while ninhydrin did not show any bands.

From all the experiments that we have conducted during the project we found that Black cardamom extracts show *in vitro* antibacterial, anticancer as well as immunomodulatory activity. The methanol, ethanol and acetone extracts of black cardamom were effective against gram positive and gram negative bacteria i.e *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 black cardamom extracts. Black cardamom also showed anticancer activity against MCF-7 breast cancer cells. 500 µg/ml is the optimal concentration which inhibits MCF-7 cells maximally. The study also demonstrates immunosuppressive activity of PBMC cells isolated from whole blood. Interestingly, we have found anticancer and immunomodulatory activities in different black cardamom extracts. But we observed discrepancy in these activities as activity is not maintained in higher concentrations. One possible explanation is that black cardamom extracts contain a mixture of different compound. We have performed only preliminary experiments with MCF-7 cell lines and PBMC cells and further work is required to confirm these results.

7. Summary

Black cardamom (*Amomum subulatum* Roxb, Zingiberaceae) commonly known as 'Badi Ilaichi' is a tall, perennial, evergreen, herbaceous monocot plant. The seeds are aromatic pungent, stimulant, stomachic, and astringent. In Ayurvedic and Unani medicine, black cardamom is used as preventive as well as curative medicine for congestion of lungs, inflammation and digestive disorders. The seed contains mainly essential oil, flavonoids, carbohydrates and fats. The major constituent of black cardamom essential oil is 1, 8-cineole (65 – 80 %), while the content of α-terpenyl acetate is low (traces to 5 %). The monoterpene hydrocarbon content is in the range of 5 – 7 %, of which limonene, sabinene, terpinene and pinene are significant components.

We have prepared different Black cardamom extracts by using ethanol, methanol, acetone, petroleum ether and water as solvents. Thin layer chromatography was done to analyze components present in the different extracts. Vanillin and iodine staining showed one to three bands of differently migrating analytes while ninhydrin did not show any bands.

To study antibacterial activity we have done Agar well diffusion assay followed by determination of minimum inhibitory concentration. For anticancer activity we used MCF-7 breast cancer cells and the activity was determined by MTT assay. Immunomodulatory activities were determined by lymphocyte proliferation assay.

The results of this study reveals that methanol extract of seed of black cardamom shows maximum inhibition against *Escherichia coli* whereas other extracts of seed show lower inhibition against *Escherichia coli* as well as other bacteria. In general seed extracts of black cardamom show higher inhibition as compare to rind extracts. Based on minimum inhibitory concentration assay the best result was obtained with methanol, acetone and seed extracts of black cardamom where MIC was found to be 5 mg/ml in case of *E. coli, Pseudomonas aeruoginosa,* respectively. Petroleum ether extract did not show any activity.

In case of anticancer activity, all the extracts of black cardamom seed showed anticancer activity but 500 µg/ml is the optimal concentration which showed maximum inhibition of MCF-7 cells in all extracts of black cardamom. The study also showed immunosuppressive activity of PBMC cells isolated from whole blood. But we observed discrepancy in these activities as activity is not maintained in higher concentrations. One possible explanation is that black cardamom extracts contain a mixture of different compound. We have performed only preliminary experiments with MCF-7 cell lines and PBMC cells and further work is required to confirm these results.

Reference

Afolayan AJ, Appidi JR, and Grierson DS (2008). Antimicrobial Activity of *Hermannia incana* Cav. *Pharmaceutical Biology;* 47(7):615-619.

Agarwal S and Singh VK (1999). Immunomodulators: A review of studies on Indian medicinal plants and synthetic peptides. Part 1: Medicinal plants. *Proceedings of the Indian National Science Academy, B 65*; (3-4):179-204.

Agnihotri S and Wakode S (2010). Antimicrobial Activity of Essential Oil and Various Extracts of Fruits of Greater Cardamom. *Indian Journal of Pharmaceutical Sciences* 2010; 72(5):657–659.

Anon. (1991) Wealth of India, *Publications and Information Directorate*, CSIR, New Delhi; Volume 1:227–229.

Berrig, Koechilin R and Tarnutzer A (1993). A preliminary profile with particular emphasis on horticulture and animal husbandry. Study group on institutions. Human Actions and Resource Management, Institute of Geography, University of Zurich, Switzerland; 47–51.

Billiau A and Matthys P (2001). Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *Journal of Leukocyte Biology*; 70:850-860.

Chandu AN, Kumar SC, Bhattacharjee C, Debnat S and Kannan K (2011). Studies on immunomodulatory activity of aloe vera (Linn). *International Journal of applied Biology Pharmaceutical Technology*; 2(1):19-22.

El-Sheikh ALK (2008). Renal transport and drug interactions of Immunosuppressants. Radbound University Nijmegen; 62.

Ford M S and Roach SS (2009). Introductory Clinical Pharmacology 27th edition *Lippincott Williams and Wilkins, USA*; 567-568.

Gomez-Flores R, Hernández-Martínez H, Tamez-Guerra P, Reyes Tamez-Guerra, Ramiro Quintanilla-Licea, Monreal-Cuevas E, and Rodríguez-Padilla C (2010). Antitumor and immunomodulating potential of *Coriandrum sativum*, *Piper nigrum* and *Cinnamomum zeylanicum. Journal of Natural Products*; 3:54-63.

Gupta PN, Naqvi A, Misra LN, Sen T and Nigam MC (1984). Gas chromatographic evaluation of the essential oils of different strains of *Amomum subulatum* growing wild in Sikkim. *Perfume Kosmet*; 65:528–529.

Gurudutt KN, Naik JP, Srinivas, P and Ravindranath B (1996). Volatile constituents of large cardamom (*Amomum subulatum* Roxb.). *Flavour and Fragrance Journal*; 11:7–9.

Indap MA, Radhika S, Motiwale L and Rao KVK (2006). Anticancer activity of phenolic antioxidants against breast cancer cells and a spontaneous mammary tumor. *Scientific Publication of the Indian Pharmaceutical Association*; 68(4):470-474.

Jafri MA, Farah JK and Singh S (2001). Evaluation of the gastric antiulcerogenic effect of large cardamom (fruits of Amomum subulatum Roxb.). J*ournal of Ethnopharmacology;* 75(2–3):89–94.

Jorgensen JH and Turnidge JD (2007). Antibacterial susceptibility tests: dilution and disk diffusion methods; *Manual of clinical microbiolog*y. 9th ed. Washington, *DC: American Society for Microbiology*; 1152-72.

Joshi R and Aneja K.R (2009). Antimicrobial Activity of *Amomum subulatum* and *Elettaria cardamomum* Against Dental Caries Causing Microorganisms Ethnobotanical Leaflets; 13:840-49.

Juneja VK, Dwivedi HP, Yan X (2012). Novel natural food antimicrobials. *Annual Revise Food Science Technology*; 3:380-403.

Karibasappa GS (1987). Post harvest studies in large cardamom (*Amomum sublatum* Roxb.). *Sikkim Science Society Newsletter;* 6(3):2–10.

Kaur S, Naik S N and Maheswari RC (1993). Composition of the essential oil of Amomum subulatum (Roxb.) (Large cardamom) isolated by liquid CO_2 extraction and steam distillation. *Indian Perfumer*; 37(3):249–252.

Khare DP, Kumar B, Hussain A, Verma S and Mishra M. (2012). Evaluation of antioxidant activity of large cardamom (leaves of *Amomum subulatum*). *International Journal of Drug Development & Research;* 4(1):175-179.

Kumar G, Chauhan and Ali M (2012). Anti-inflammatory effect of *Amomum subulatum* roxb. fruits extract. *International Research Journal of Pharmarcy*; 3(12):4722-4724.

Kumar U, Kumar B, Bhandari A and Kumar Y (2010). Phytochemical investigation and comparison of antimicrobial screening of clove and cardamom. *International Journal of Pharmaceutical Sciences and Research*; 1 (12):138-147.

Lawrence BM (1970). Terpenes in two *Amomum* species. *Phytochemistry;* 9:665.

Majdalawieh AF and Carr RI (2010). In vitro investigation of the potential immunomodulatory and anti-cancer activities of black pepper (Piper nigrum) and cardamom (Elettaria cardamomum). *Journal of medicinal food*; 13(2):371-81.

Mukherjee DK (1972). Large cardamom. *World Crops;* 25(1):31–33.

Naik PJ, Rao JM, Kumar TM and Sampathu, SR (2004). Chemical composition of the volatile oil from the pericarp (husk) of large cardamom (*Amomum subulatum* Roxb.). *Flavour and Fragrance Journal;* 19(5):441–444.

NCCLS (1997). Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.

Nigam SS and Purohit RM (1960). Chemical examination of the essential oil derived from the seeds of *Amomum subulatum* Roxb. *Perfumery and Essential Oil Record;* 51(3):121–122.

Niphade SR, Asad M, Chandrakala GK, Toppo E and Deshmukh P (2009). Immunomodulatory activity of *Cinnamomum zeylanicum* bark. *Pharmaceutical Biology;* 47(12):1168-1173.

Owen PL and Joshan T (2002). Antioxidants in medicines and spices as cardio protective agents in tibetan highlanders. *Pharm. Biol.*; 40(5):346-357.

Parmar MY, Shah PA, Gao J and Gandhi TR (2010). Hepatoprotection through regulation of voltage dependent anion channel expression by *Amomum subulatum* Roxb seeds extract. *Indian Journal Pharmacol;* 43(6):671-5.

Parthasarathy VA, Chempakam B and Zachariah TJ (2008). Large Cardamom. *Centre for Agriculture and Biosciences International*; 59-69.

Patra NK, Siddiqui MS, Akhila A, Nigam MC and Naqvi AA (1982). Chemical composition of the volatile oil from the pericarp (husk) of large cardamom (*Amomum subulatum* Roxb.). *Pafai Journal*; 4(4):29–31.

Pruthi JS (1993). Major Spices of India – Crop Management and Post Harvest Technology. *ICAR Publications, New Delhi*; pp.114–179.

Qiblawi S, Al-Hazimi A, Al-Mogbel M, Hossain A, and Bagchi D (2012). Chemopreventive Effects of Cardamom (Elettaria cardamomum L.) on Chemically Induced Skin Carcinogenesis in Swiss Albino. Mic*e journal of medicinal food;* 15 (6):576–580.

Rao YS, Anand K, Sujatha C, Naidu R and George CK (1993). Large cardamom (*Amomum subulatum* Roxb.) – a review. *Journal of Spices and Aromatic Crops* 2(1&2):1–15.

Sabulal B, Dan M, Pradeep NS, Valsamma RK and George V (2006). Composition and antimicrobial activity of essential oil from the fruits of *Amomum cannicarpum. Acta Pharm;* 56:473–480.

Saha T, Das I, Acharya A, Berry DL, Sen S, Williams E, Permaul E, Sengupta A and Bhattacharya S (2012). Antioxidative effects of the spice cardamom against non-melanoma skin cancer by modulating nuclear factor erythroid-2-related factor 2 and NF-*k*B signalling pathways. *British Journal of Nutrition*; 108:984-997.

Singh DB (1978). Large cardamom. *Cardamom* 10(5):3–15.

Singh VB and Singh K (1996). Large Cardamom. Spices. Indian Institute of Plantation Management, Bangalore/New Age International Publishers, New Delhi; pp. 52–57.

Sonkar R and Mishra RN (2011). Immunomodulatory Activity of *Triphala megaext*. *International Journal of Research in Pharmaceutical andBiomedical Sciences*; 2 (2).

Soule HD, Vazguez J, Long A, Albert S, Brennan M (1973). A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute*; 51(5):1409–1416.

Subba JR (1984). Agriculture in the Hills of Sikkim. Sikkim Science Society, Gangtok, India; p. 286

Tijjani MA, Dimari GA, Buba SW and Khan IZ (2012). In-Vitro Antibacterial Properties and Pre-Liminary Phtytochemical Analysis of *Amomum subulatum* Roxburg (Large Cardamom). *Journal of Applied Pharmaceutical Science*; 2(5):69-73.

Unnikrishnan MC and Kuttan R (1990). Tumor reducing and anticarcinogenic activity of selected spices. *Elsevier*; 51(1):85-89.

Vavaiya RB, Patel Amit and Manek R. A (2012). Anti-Diabetic Activity of A*momum Subulatum* Roxb. Fruit Constituents *International journal of research article pharmaceutical innovations;* 2(5).

World Cancer Report (2008). *International Agency for Research on Cancer*. Retrived 2011-02-26.