EVALUATION OF ANTICANCER ACTIVITY OF VARIOUS EXTRACT OF SEEDS OF *Trachyspermum ammi (L.)Sprangue* USING BREAST CANCER CELLL LINES BY *INVITRO* METHODS

A dissertation submitted to

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Submitted by

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CERTIFICATE

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Dedicated to my beloved Parents

LIST OF ABBREVATIONS

5-FU	5-Fluorouracil
ALL	Acute Lymphocytic Leukemia
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BMI	Body Mass Index
bp	base pairs
BRCA1	Breast cancer susceptibility protein
cDNA	Complementary DNA
DBD	DNA Binding Domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

IC50	Median inhibition concentration
MCF-7	Michigan Cancer Foundation-7
MEM	Minimum Essential Medium
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
NaOH	Sodium Hydroxide
p53	Protein 53
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
qPCR	quantitative or real time Polymerase chain reaction
RBC	Red Blood Cells
RNA	Ribo Nucleic Acid
Rpm	revolutions per minute
RT-PCR	Reverse transcriptase Polymerase chain reaction
SDS	sodium dodecyl sulphate
SiRNA	Small interfering RNA
SYBR	Synergy Brands, Inc.
TAE	Tris-acetate EDTA
Th1	T helper cells 1
TLC	Thin layer chromatography

TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TP53	Tumor Protein 53
TPVG	Trypsin, PBS, Versene/EDTA, Glucose
WBC	White Blood Cells
WHO	World Health Organisation

1. INTRODUCTION

As the world marches towards more advancement in technology, the incidence of newer face of the diseases are also unmasked. This is mainly due to environmental factors like gradual depletion of ozone layer, pollution, unhealthy food etc.

Among the various diseases, carcinoma is the most dreadful disease whose incidence is on increase and has become the greatest enemy to mankind. Cancer is leading cause of death worldwide and accounted for 7.6 million deaths in 2008. In females, among various types of cancer, breast cancer is the most commonly occurring cancer which is rare in male. In India, breast cancer is the second most common cancer with an estimated 115,251 new diagnoses and 53,592 deaths in 2008. (1)

Many modes of treatments are available for breast cancer, which includes surgery, radiation therapy and chemotherapy. The chemotherapeutic drugs approved by FDA for breast cancer are Cyclophosphamide, Methotrexate, 5-fluro uracil, Paclitaxel, Docetaxel, Doxorubicin etc.These drugs belongs to different chemical groups, each exerting their anti cancer activity with different mechanism of action. Although these drugs have remarkable cure rate, many of them have high incidence of adverse effects like bone marrow depression, alopecia etc. Although these drugs offer high success rate, the amount of adverse effects produced are also high. So there is always a search for newer drugs with less adverse effects, but more beneficial effects. (2)

Indigenous systems of medicine like Ayurveda, Siddha and Unani, mainly use medicinal plants for treatment of various ailments of the human beings and animals. With the development of these systems, the herbal plants are being sought after, both by patients and by clinicians in search for cure of diseases. Herbal medicine is a form of

complementary and alternative medicine and is becoming increasingly popular in both developing and developed countries.[3]

WHO has described traditional medicine as one of the surest means to achieve total health care coverage of the world's population. In pursuance of its goal of providing accessible and culturally acceptable health care for the global population, WHO has encouraged the rational use of traditional plant based medicines by member states and has developed technical guidelines for the assessment of herbal medicines.[4,5]

The herbal drugs have been used throughout the world and have raised greater attention in recent times, because of their diverse nature of curing diseases, safety and well tolerated remedies compared to the conventional medicines. More over the herbs with natural combinations of constituents as a whole, are naturally occurring remedies which have proved to be more effective and safer than conventional medicines. [6]

Trachyspermum ammi is a profusely branched annual herb mainly used as digestive aid. From the pharmacological investigation it has been proved to possess anti microbial, anti hypertensive, hepatoprotective, anti inflammatory effects etc.

In *invivo* study, anticancer activity is done by inducing tumor in animals by various methods. Due to ethical issues, the use of animals is much reduced to investigate anticancer activity. Though animal models provide more predictable results, *invitro* evaluation is still preferred.

Invitro methods have an edge over *invivo* methods since they are,

- Less time consuming
- More cost effective
- Large number of compounds can be tested with small amount of sample
- Easier to manage

Because of these advantages, the *invitro* methods have been adopted to assess the anticancer property of a compound at various research laboratories. [7]

As anticancer activity of *Trachyspermum ammi* is not proved, the present study has been taken to investigate *invitro* anticancer activity of various extracts of *Trachyspermum ammi* using MCF-7 cell lines which is universally used for breast cancer.

2. OBJECTIVES OF THE STUDY

- To evaluate Phyto chemical properties of the various extracts of seeds of Trachyspermum *ammi.*
- To evaluate the IC_{50} concentration of ethanolic extracts of *Trachyspermum ammi.*
- To determine the apoptotic activity of ethanolic extracts of *Trachyspermum ammi.*
- To determine the gene expression of cancer DNA markers in MCF-7 Cell line treated with ethanolic extracts of *Trachyspermum ammi*.

3. REVIEW OF LITERATURE

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. These are two types of cancers.

- **Benign tumors** aren't cancerous. They can often be removed, and, in most cases, they do not come back. Cells in benign tumors do not spread to other parts of the body.
- **Malignant tumors** are cancerous. Cells in these tumors can invade nearby tissues and spread to other parts of the body. The spread of cancer from one part of the body to another is called as metastasis.

3.1 TYPES OF CANCER

- **Carcinoma** - cancer that begins in the skin or in tissues that line or cover internal organs. There are a number of subtypes of carcinoma, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma.
- **Sarcoma** - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- **Lymphoma and myeloma** - cancers that begin in the cells of the immune system.
- **Central nervous system cancers** - cancers that begin in the tissues of the brain and spinal cord. [8]

3.2 HISTORY

The Edwin Smith Papyrus, which is the oldest written description of cancer known to exist, describes eight cases of breast tumors or ulcers in Egypt that were treated with cauterization. However, the document also states that there is no treatment for cancer. The original document, written in 3000 BC, was acquired in 1862 by Edwin Smith at Luxor, Egypt.

400 B.C. Hippocrates was the first to use the words "carcinos" and "carcinoma" to describe tumors, and hence the term "cancer" was coined. "Cancer" is derived from the Greek word "karkinos," or crab, which is thought to reference the appearance of blood vessels on tumors resembling a crab's claws reaching out. [9] [10] [11]

168 A.D. Galen: a Roman physician, he believed cancer to be curable in early stages, and that advanced tumors should be operated upon either by cutting around the affected area or by cauterization.

1761 Giovanni Morgagni: began performing autopsies to relate illness to pathological findings,. This helped to set a foundation for the study of cancer. [9] [10] [11]

When the modern microscope was invented in the 19th century, it allowed scientists to study cancer with the unaided eye and the modern pathologic study of cancer was born. [12]

The first use of drugs to treat cancer in the early 20th century was Mustard gas, but its side effect decreasing WBC count was unacceptable. So, researchers looked for other substances and discovered Mustin. Since then, several drugs have been developed to treat cancer. [13]

3.3 What causes cancer?

Cancer arises from one single cell. The transformation from a normal cell into a tumor cell is a multistage process, typically a progression from a pre-cancerous lesion to malignant tumors. These changes are the result of the interaction between a person's genetic factors and three categories of external agents, including:

- ✓ physical carcinogens, such as ultraviolet and ionizing radiation;
- ✓ chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin (a food contaminant) and arsenic (a drinking water contaminant);
- ✓ biological carcinogens, such as infections from certain viruses, bacteria or parasites.

Ageing is another fundamental factor for the development of cancer. The incidence of cancer rises dramatically with age, most likely due to a buildup of risks for specific cancers that increase with age. The overall risk accumulation is combined with the tendency for cellular repair mechanisms to be less effective as a person grows older. [14]

3.4 BREAST CANCER

Definition

Breast cancer is a malignant neoplasm of breast tissue, either in cells that line the ducts that carry milk to the nipple (ductal cancer) and or in cells and or in cells that line the lobules that involved in the milk production (lobular cancer).[15] Breast cancer may be benign or malignant. Benign tumor is not life threatening and non invasive but, malignant tumor is life threatening and invasive. [16]

Types of breast cancer [17] [18]

1. Ductal carcinoma in situ (DCIS) –

This is the earliest form of breast cancer. In DCIS, cancer cells are in the ducts of the breast, but they haven't started to spread into the surrounding breast tissue.

2. Lobular carcinoma in situ (LCIS)

In LCIS, there are changes to the cells lining the lobes, which slightly increases the risk of developing breast cancer later in life. Most women with LCIS never develop breast cancer.

3. Invasive breast cancer –

If cancer cells have spread outside the lining of the ducts or lobules into surrounding breast tissue, it's called invasive breast cancer. There are different types of invasive breast cancer.

a) **Invasive ductal breast cancer**

Invasive ductal breast cancer occurs when cancer cells lining the duct have spread into surrounding breast tissue. It's the most common type of breast cancer – 4 out of 5 breast cancers (80%) are this type.

b) **Invasive lobular breast cancer**

Invasive lobular breast cancer develops from the cells that line the lobes of the breast and can sometimes be difficult to diagnose on a mammogram. About 1 in 10 breast cancers (10%) are this type

3.5 RARE TYPES

Inflammatory breast cancer, triple negative breast cancer and medullary breast cancer are the other types of breast cancers.

3.6 SYMPTOMS OF BREAST CANCER

a) a lump in the breast

b) a change in the size or shape of the breast

c) dimpling of the skin or thickening in the breast tissue

d) a nipple that's turned in (inverted)

e) a rash (like eczema) on the nipple

f) discharge from the nipple

g) Swelling or a lump in the armpit.

h) Pain in either of the breast or armpit not related to menstrual cycle.

i) Pus formation and foul odor

j) Unusual weight loss.

3.7 RISK FACTORS

- **Age and gender** -- Your risk of developing breast cancer increases as you get older. Most advanced breast cancer cases are found in women over age 50. Men can also get breast cancer. But they are 100 times less likely than women to get breast cancer.[19] [20]

- **Family history of breast cancer** -- You may also have a higher risk of breast cancer if you have a close relative who has had breast, uterine, ovarian, or colon cancer. About 20 - 30% of women with breast cancer have a family history of the disease.[21]

- **Genes** -- Some people have genetic mutations that make them more likely to develop breast cancer. The most common gene defects are found in the BRCA1 and BRCA2 genes. These genes normally produce proteins that protect you from cancer. If a parent passes you a defective gene, you have an increased risk of breast cancer. Women with one of these defects have upto an 80% chance of getting breast cancer sometime during their life.[22]
- **Menstrual cycle** -- Women who got their periods early (before age 12) or went through menopause late (after age 55) have an increased risk of breast cancer.[23]

Other risk factors include:

- **Alcohol use** -- Drinking more than 1 - 2 glasses of alcohol a day, may increase your risk of breast cancer.[24]
- **Childbirth** -- Women who have never had children or who had them only after age 30 have an increased risk of breast cancer. Being pregnant more than once or becoming pregnant at an early age reduces your risk of breast cancer.[22]
- **DES** -- Women who took diethylstilbestrol (DES) to prevent miscarriage may have an increased risk of breast cancer after age 40. This drug was given to the women in the 1940s - 1960s.
- **Hormone replacement therapy (HRT)** -- You have a higher risk of breast cancer if you have received hormone replacement therapy with estrogen for several years or more.[22]
- **Obesity** -- Obesity has been linked to breast cancer, although this link is not completely understood. The theory is that obese women produce more estrogen. This can fuel the development of breast cancer.[25]
- **Radiation** -- If you received radiation therapy as a child or young adult to treat cancer of the chest area, you have a very high risk of developing breast cancer.

The younger you started such radiation and the higher the dose, the higher your risk. This is especially true if the radiation was given during breast development.[26]

3.8 PATHOPHYSIOLOGY

The cells of breast tissue undergo abnormal proliferation and differentiation which is due to factors like, predisposing factors (gender, age, and genetics), precipitating factors (diet, contraceptives, and immune suppression) and unknown etiology. It leads to the following,[27]

- ❖ Genetic mutation of cellular DNA e.g. p53 (apoptotic protein), BRCA1 & BRCA2 (tumor suppressor protein), which leads to activation of oncogenes. Inactivation of tumor suppressor gene, which control apoptosis leads to proliferation and differentiation of cancer cells in the milk duct and growth of malignant tumor.
- ❖ Rapid growth of malignant tumor
- ❖ Change in shape of breast
- ❖ Rapid multiplication of cancer cells, leads to an increase in metabolism in the cancer cells .So the normal cells will become deprived of nutrients leading to unusual loss of weight and fatigue.
- ❖ Obstruction of the milk duct cause rupture of it, so there is release of inflammatory mediators such as bradykinin, prostaglandins cause transient vasoconstriction.
- ❖ Compression of blood and lymphatic vessels which leads to decreased blood supply to breast tissue causing ischemia followed by necrosis.

- ❖ Swelling of breast tissue will compress the nerve endings and produce pain and accumulation of the fluid in the cells, cellular debris, live and dead lymphocytes will form pus followed by tissue decay and foul odor will be produced.
- ❖ Cancer cells begin to spread locally via lymphatic vessels and form tumor in axillary lymph nodes and supra clavicular lymph nodes which are immovable, hard and painless nodes.

Mutation of BRCA gene:

Those individuals inheriting defects in DNA and gene or mutation of genes like BRCA1, BRCA2 are having more chances to develop breast cancer.

BRCA1, it is tumor suppressor gene, which produces a protein, called breast cancer type susceptibility protein. The protein encoded by the BRCA1 gene combines with the other tumor suppressors , DNA damage sensors and signal transducers to form a large multi subunit protein complex known as BRCA1- associated genome surveillance complex (BASC). It is found in cells of breast and other tissue, where it helps repair of DNA double strand, or induces apoptosis.

BRCA2 is also a tumor suppressors gene, it is involved in the repair of chromosomal damage with an important role in the error –free repair of DNA double strand breaks

Suppression of apoptotic signals:

(a) **p53** is a protein, encoded by TP53 gene[28,29,30] and having tumor suppressing function. p53 can activate the DNA repair to repair the damaged DNA, if fail to repair the damaged DNA, p53 will induce apoptosis. p53 can arrest the cell cycle by holding at G1/S phase. It plays major role in apoptosis and inhibition of angiogenesis. In cancer,

mutation occurs at DNA binding domain (DBD) so the availability of protein to bind to its target DNA sequence is inhibited, and thus prevents transcriptional activation of genes.[31]

(b) **TNFα,** the primary role of TNF is in regulation of immune cells. TNF was thought to be produced primarily by macrophages,[32] but it is also produced by broad variety of cell types including lymphoid cells, endothelial cells, cardiac myocytes , adipose tissue and neurons. TNF, being an endogeneous pyrogen, is able to induce fever, apoptotic cell death, sepsis (through IL1 and IL6 production), inflammation and inhibit tumorigenesis and viral replication. Dysregulation of TNF production is associated with a variety of human diseases including Alzheimer's disease, [32] cancer, [33] major depression[34] and inflammatory bowel disease.[35]

TNF can bind to two receptors such as TNF-R1and TNF-R2. TNF-R1 found in most tissues where as TNF-R2 found only in immune cells. [37]

Over expression of anti apoptotic gene:

Bcl2 (B cell lymphoma 2) is a anti apoptotic gene plays important role in regulating apoptosis. The Bcl2 gene is involved in many cancers like melanoma, breast, prostate, leukemia and lung carcinoma. Mutation in Bcl2 gene decreases the propensity of the cells for undergoing apoptosis.[38, 39, 40] Bcl-2 plays a major role in cancer and its resistance thereby interfering with therapeutic action of chemotherapeutic drugs. High expression of anti-apoptotic members like Bcl-2 found in human cancers leads to neoplastic cell expansion by interfering with normal cell death mechanism. Decrease in expression of Bcl-2 leads to apoptosis.

3.9 BREAST CANCER TREATMENT

Based upon the tumor size, location, involvement of lymph nodes and metastasis, risk and stages of the disease, appropriate treatment will be given.[41] [42]

SURGERY: Remove cancerous tissue –

- **Lumpectomy** removes the breast lump;
- **Mastectomy** removes all or part of the breast and possible nearby structures

RADIATION THERAPY: To destroy cancerous tissue.

CHEMOTHERAPY:

It is systemic therapy, used to either shrink the tumor or to reduce the risk of reccurence.

A. **Drugs acting directly on cells**

Alkylating agents

- **Nitrogen mustards:** Mecholrethamine, Cyclophosphamide,

Antimetabolites

- **Folate antagonist:** Methotrexate
- **Purine antagonist:** 6-Mercaptopurine, **6-**Thioguanine, Azathioprine, Fludarabine.
- **Pyrimidine antagonist:** 5-Flurouracil, Cytarabine.
- **Taxanes :** Paclitaxel, Docetaxel
- **Antibiotics:** Actinomycin D, Doxorubicin, Daunorubicin, Mitoxantrone, Bleomcins, Mitomycin C.

B. Drugs altering hormonal milieu

Hormone therapy is prescribed to women with ER-positive breast cancer to block certain hormones that fuel cancer growth.

- **Selective estrogen receptor modulators**: Tamoxifen, Toremifene.
- **Selective estrogen receptor down regulators**: Fulvestrant
- **Aromatase inhibitors**: Letrozole, Anastrozole.

3.10 MECHANISM OF ANTICANCER DRUGS

Block nucleic acid (DNA, RNA) biosynthesis [43]

- **Folic Acid Antagonist:** inhibit dihydrofolate reductase (methotrexate)
- **Pyrimidine Antagonist:** inhibit thymidylate synthetase (fluorouracil) ; inhibit DNA polymerase (cytarabine)

Influence the Structure and Function of DNA

- **Alkylating Agent:** cyclophosphamide
- **Antibiotic:** bleomycin and mitomycin C

Interfere Protein Synthesis

- **Antitubulin:** Taxanes (paclitaxel and docetaxel) Bind tubulin, destroy spindle to produce mitotic arrest

Influence hormone homeostasis

These drugs bind to hormone receptors to block the actions of the sex hormones which results in inhibition of tumor growth.

- **Selective estrogen receptor modulators**: Tamoxifen, Toremifene.
- **Selective estrogen receptor down regulators**: Fulvestrant
- **Aromatase inhibitors**: Letrozole, Anastrozole

3.11 MCF CELL LINES

MTT ASSAY

Vidhya and deveraj et al.(2001) reported the anti prolerave action of eugenol treated MCF-7 cell line by MTT assay. The percentage of cell viability was calculated based on absorbance read in elisa reader at 570nm.[44]

Khaghanini et al. (2011) studied the cytotoxicity of aqueous extract of Hibiscus sabdariffa against MCF-7 cell line by MTT assay. To determine the IC50 the absorbance was taken at 540nm spectrometrically. [45]

APOPTOSIS

Ahmad bustmam et al. (2009) examined the morphological changes and probable mode of cancer death induced in G.umbrosus treated MCF-7 celll line. The changes such as memdrane blebs, DNA condensation and fragmentation as sign of apoptosis were observed by inverted and fluorescent microscope used acridine orange and propidium iodide as staining media. Endonucleosomal fragmentation action in treated cells was studied by DNA laderring assay. [46]

Vidhya and deveraj et al. (2001) studied the induction of apoptosis by eugenol in human breast cancer cell lines. Used various staining technique for observe apoptosis such as giemsa stain for morphological alteration, fluorescence microscopy analysis of cells using acridine orange and ethidium bromide and quantification of DNA fragments.[44]

Khaghanii et al. (2011) studied the induction of apoptosis in Hibiscus sabdariffa treated MCF-7 cell lines. The pattern of DNA cleavage as sign of apoptosis was analysed by agarose gel electrophoresis method. [45]

Expression of cancer DNA markers

Ali A Alshawti et al. (2010) reported the expression of mRNA levels of TP53 and caspases using reference gene GAPDH in catechin hydrate treated MCF-7 cells by RT-PCR.[47]

FIGURE-1: Seeds of *Trachyspermum ammi*

3.12 PLANT PROFILE [48, 49, 50, 51]

Botanical name : *Trachyspermum ammi*

Synonym : Carum copticum

Family : Apiaceae

Common names

Tamil : Omam

English : Bishop’s weed

Hindi : Ajwain, Jewain

Sanskrit : Yamini, Yaminiki

Parts used : Seeds

Description

Trachyspermum ammi is a profusely branched annual herb, 60-90cm tall. Stem is striated; inflorescence compound umbel with 16 umblets, each containing upto 16 flowers; flowers are actinomorphic. Leaves are pinnate, with a terminal and 7 pairs of lateral leaflets. Fruit is aromatic, consists of 2 mericarps, grayish brown ovoid, compressed about 2mm long and 1.7 mm wide, consists of 5 ridges and 6 vittae in each mericarps, usually separate.

Habitat

Trachyspermum ammi is widely grown in arid and semi arid regions where soil contains high levels of salt. It is a native of Egypt and it is cultivated in Iran, Iraq, Pakistan and India. In India, it is cultivated in Madhya Pradesh, Uttar Pradesh, Gujarat, Rajasthan, Bihar and West Bengal.

Therapeutic uses [52, 53, 54]

- ✓ The roots are diuretic in nature.
- ✓ The seeds posses digestive, carminative, laxative properties. It also cures abdominal pain and piles.
- ✓ The fruit act as stimulant and antispasmodics.

3.13 Pharmacological investigation

Antihypertensive, antispasmodic and broncho-dilating activity

Gilani AH et al. (2005) reported the antihypertensive effect of *T. ammi* administered intravenously *in vivo*, and the antispasmodic and broncho-dilating actions *in vitro* showed that calcium channel blockade has been found to mediate the spasmolytic effects of plant materials and it is being considered that this mechanism contributed to their observed result and supported the traditional use of *T. ammi* in hyperactive disease states of the gut such as colic and diarrhea as well as in hypertension.[55]

Hepatoprotective activity

The research work done by Gilani AH et al. (2005) on the hepatoprotective actions *in vivo* showed that *T. ammi* was 80% protective in mice against a normally-lethal dose of paracetamol (1 g/kg), it prevented the CCl_4-induced prolongation of pentobarbital sleeping time in mice, and it tended to normalize the high serum levels of liver enzymes caused by CCl_4-induced liver damage in rats.[55]

Antilithiasis and diuretic activity

Ashan SK et al. (1990) reported antilithiasis and diuretic actions *in vivo* of *T. ammi* on inhibiting oxalate urolithiasis induced in rats were also studied. In a further study of a possible diuretic effect, it was found that *T. ammi* was not effective in

increasing the 24-hr urine production. The results concluded that the traditional use of *T. ammi* in the treatment of kidney stones was not supported by their experimental evidence. [56]

Antiplatelet-aggregatory

Srivatsava KC et al. (1988) reported the Antiplatelet-aggregatory experiments *in vitro* with blood from human volunteers and he showed that a dried ethereal extract of *T. ammi* seeds, inhibited aggregation of platelets, induced by arachidonic acid, collagen and epinephrine. Research study was intended to support the traditional use of *T. ammi* in women post parturition. [57]

Anti-inflammatory potential

Thangam C et al. (2003) reported the anti-inflammatory potential of the total alcoholic extract (TAE) and total aqueous extract (TAQ) of the *T.ammi* seeds. TAE and TAQ exhibited significant (P<0.001) antiinflammatory activity in both the animal models. The weights of the adrenal glands were found to be significantly increased in TAE and TAQ treated animals. TAE and TAQ extracts from the *T.ammi* seeds exhibit significant anti-inflammatory potential. [58]

Antitussive effects

Antitussive effects of aerosols of two different concentrations of aqueous and macerated extracts and carvacrol, codeine, and saline were tested by Boskabady MH et al.(2005) by counting the number of coughs produced. The results showed significant reduction of cough number obtained in the presence of both concentrations of aqueous and macerated extracts and codeine ($P < 0.001$ for extracts and $P < 0.01$ for codeine).[59]

Detoxification of aflatoxins

Vijayanandraj S et al. (2010) after his research on the seed extract of *T. ammi*, showed the maximum degradation of aflatoxin G1 (AFG1). The aflatoxin detoxifying activity of the seeds extract was significantly reduced upon boiling. Significant levels of degradation of other aflatoxins viz., AFB1, AFB2 and AFG2 by the dialyzed seeds extract were also observed. Time course study of AFG1 detoxification by dialyzed *T. ammi* extract showed that more than 91% degradation occurred at 24 h and 78% degradation occurred within 6 h after incubation.[60]

Ameliorative effect

Effects of *T. ammi* extract on hexachlorocyclohexane (HCH)-induced oxidative stress and toxicity in rats were investigated by Anilakumar KR et al.(2009). Pre-feeding of ajwain extract resulted in increased GSH, GSH-peroxidase, G-6-PDH, SOD, catalase, glutathione S-transferase (GST) activities and decreased hepatic levels of lipid peroxides. It was concluded that HCH administration resulted in hepatic free radical stress, causing toxicity, which could be reduced by the dietary *T. ammi* extract.[61]

Antimicrobial actions *in vitro*

Antimicrobial actions of *T. ammi*, in the protection of foodstuffs against microbial spoilage, conducting laboratory assays of antimicrobial efficacy *in vitro*, and its use as antimicrobials in humans, were also investigated by Caccioni DLR et al.(2000) The active principles thought to be responsible for the antimicrobial activity of *T. ammi* were reported to be carvacol and thymol. ‘Thymol kills the bacteria, resistant to even prevalent third generation antibiotics and multi-drug resistant microbial pathogens and thus works as a plant based 4th generation herbal antibiotic formulation. Antifungal action of volatile constituents of *T. ammi* seeds on ten fungi (*Acrophialophora fusispora,*

Curvularia lunata, Fusarium chlamydosporum, F. poae, Myrothecium roridum, Papulaspora sp., Alternaria grisea, A. tenuissima, Drechslera tetramera,., and *Rhizoctonia solani*) was tested and found to inhibit the growth of all test fungi by 72-90%. Phenolic compounds, such as thymol and carvacol, are known to be either bactericidal or bacteriostatic agents depending on the concentration used.[62]

Hypolipidemic action *in vivo*

Javed IMS et al. 2002, reported the antihyperlipidemic effect of *T. ammi* seed in albino rabbits. It was assessed that *T. ammi* powder at a dose rate of 2 g/kg body weight and its equivalent methanol extract were extensively effective in lipid lowering action by decreased total cholesterol, LDL-cholesterol, triglycerides and total lipids.[63]

Digestive stimulant actions *in vivo* and *in vitro*

Platel k et al. (2001) showed that *T. ammi* would increase the secretion of gastric acid and he also proved that the addition of *T. ammi* to the infusion increased the amount of gastric acid. Gastric acid secretion was increased nearly four-fold by *T. ammi*. In experimental rats *in vivo*, the addition of *T. ammi* to the diet reduced food transit time and also enhanced the activity of digestive enzymes and/or caused a higher secretion of bile acids.[64]

Anthelmintic activity

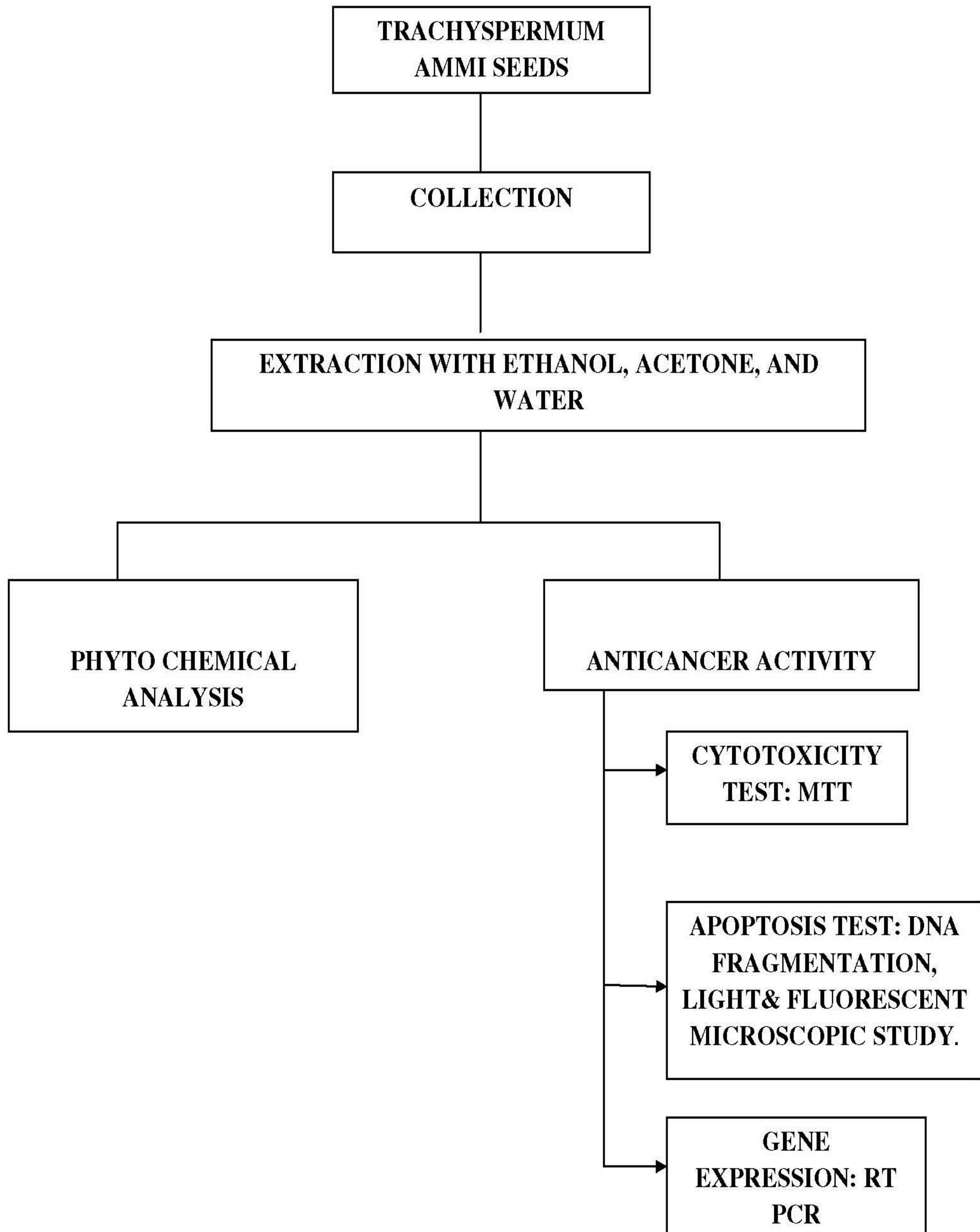
Jabar et al. (2006) reported anthelmintic activity of *T. ammi* and showed its effect against specific helminths, e.g. *Ascaris lumbricoides* in humans and *Haemonchus contortus* in sheep.[65] Anthelmintic activity of *T. ammi* was proved to be exerted by interference with the energy metabolism of parasites through potentiation of ATPase activity and thus loss of energy reserves.[66, 67] The plant has also been reported to possess cholinergic activity with peristaltic movements of the gut, thus helping in

expulsion of intestinal parasites which might also be a contributory factor to its anthelmintic activity.[68]

Nematicidal activity

The nematicidal activity of T.ammi was demonstrated by Kwon I et al. (2006) Pine Wilt disease is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. Nematicidal activity of T.ammi oil (ajwain oil) against PWN may be because of the constituents (camphene, pinene, myrcene, limonene, terpinene, terpinen- 4-ol, thymol and carvacrol) .[69] PWN bodies are treated with the muscle activity blockers levamisole hydrochloride and morantal tatrate.[70] Amino and hydroxyl groups have been hypothesized as target sites of methyl isothiocyanate in nematodes.[71] Some essential oils have been reported to interfere with the neuromodulator octopamin[72] or GABA-gated chloride channels of insect pests.[73] Thymol and carvacrol are very effective against PWN. These studies confirm that the nematicidal activity of ajwain oil is mainly attributed to the activity of thymol and carvacrol.[74] Nematicidal activity of ajwain essential oils LC_{50} values was 0.431 mg/ml.[65]

STUDY DESIGN



4. METHODOLOGY

4.1 PLANT PART USED:

The seeds of *Trachyspermum ammi* were used for extraction which was collected in month of July 2013 at Tirunenveli. It was examined and authenticated by Mr. V. Chelladurai, officer- Botany (scientist retd.), Central Council for Research in Ayurveda & Siddha, Govt. of India.

4.2 EXTRACTION

It is defined as separation of medicinally active parts of plant using selective solvents through standard procedure.

4.3 SOLVENTS USED:

Based on polarity various solvents used such as ethanol, acetone, and water.

4.4 TYPE OF EXTRACTION

Hot continuous (soxhlet) extraction method.

4.5 PRINCIPLE

It is a process of continuous extraction method in which the solvent can be circulated through the extractor for several times. The vapours of solvent are taken to the condenser and the condensed liquid is returned to the extract for continuous extraction.

4.6 PROCEDURE

50 grams of seeds was packed into soxhlet apparatus and was subjected to extraction sequentially with 500ml of ethanol, acetone, and water. The extraction was continued until the colour of the solvent in the siphon tube became colourless.

Extraction procedure was carried out in Institute of Pharmacology, Madras Medical College, and Chennai. Extracts of acetone and ethanol were subjected to evaporation at room temperature till a semisolid mass was obtained. Aqueous extract was subjected to lyophilisation with the help of lyophiliser to a semisolid mass.

4.7 PHYTOCHEMICAL ANALYSIS [75, 76, 77]

The freshly prepared extracts of actone, ethanol and water were subjected to phytochemical screening for the presence or absence of active constituents by following the methods.

Test for Steroids:

Salkowskis test: Crude extract was mixed with 2ml of chloroform. Then 2ml of conc. Sulphuric acid was added carefully and shaken gently. Appearance of reddish brown colour ring indicated the presence of steroids

Test for Flavanoids:

Shinoda test: Crude extract was treated with 5 ml 95% ethanol, few drops concentrated hydrochloric acid and 5 grams magnesium turnings and appearance of pink colour indicated the presence of steroids

Lead acetate test: Crude extract was treated with few drops of lead acetate solution. Appearance of yellow colour precipitate indicates the presence of flavanoids

Alkaline reagent test: Crude extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

Test for Proteins:

Millions test: Crude extract was mixed with 2ml of Millions reagent. Appearance of white precipitate which turns red on gentle heating, indicates the presence of proteins

Test for Glycosides:

Liebermann's test: Crude extract was mixed with 2ml of chloroform and 2ml of acetic acid. Mixture was cooled in ice and conc. sulphuric acid was added. Colour change from violet to blue and then to green indicates the presence of steroidal nucleus

Test for Carbohydrates:

Fehling's test: Crude extract was treated with equal volume of Fehling A and Fehling B reagents and mixed together and gently boiled. Appearance of brick red precipitate at the bottom of the test tube indicate the presence of reducing sugars

Test for phenols and tannins:

Crude extract was mixed with 2ml of 2% solution of ferric chloride. Appearance of violet colour indicate the presence of phenolic compounds and tannins. Crude extract was dissolved in water and treated with 10% of lead acetate solution, appearance of white precipitate indicate the presence of tannins and phenolic compounds.

Test for alkaloids:

Crude extract was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was tested with various alkaloidal reagents such as,

Mayer's reagent – Cream precipitate

Dragendroff's reagent – Orange brown precipitate

Wagner's reagent– Reddish brown precipitate

Test for terpenoids:

5 ml of each extract was mixed in 2 ml of chloroform. 3 ml of concentrated H2SO4 was then added to form a layer. A reddish brown precipitate colouration at the inyerface is formed indicates presence of terepenoids.

4.8 PREPARATION OF MEDIA

Definition

Media is a nutritional supplementation essential for maintenance, growth and division of cells in-vitro.

Materials required

1. Minimum essential medium (MEM) in powder form.
2. Penicillin (100 IU/ml)
3. Streptomycin (100 µg/ml)
4. Phenol red
5. Amphotericin B
6. L-glutamine (3%)
7. Foetal bovine serum (FBS) 10%
8. Sodium bicarbonate (7.5 %)

Procedure:

- ❖ Powder form of MEM was dissolved in one liter of pre sterilized Millipore distilled water, mixed well and closed.
- ❖ The medium was sterilized for 15 minutes at 121°c.
- ❖ 856 ml of sterilized MEM was taken in a 1000 ml flask and to it one ml of penicillin, streptomycin, phenol red and amphotericin B was added and mixed well.

- ❖ 10 ml of 3% L-glutamine, 100 ml of FBS and 30 ml of 7.5% sodium bicarbonate was added to make up the volume to 1000 ml.
- ❖ pH was adjusted to 7.2 – 7.4 and the medium was stored for two days at 37°c and the pH was checked and transferred to the refrigerator.

4.9 CELL LINES AND CULTURE

The Human Breast Cancer cell line, MCF-7 was obtained from Tamil Nadu Veterinary College, Chennai

They were cultured in complete essential growth medium in T25ml cultured vented flask and incubated at 37°C in 5% CO_2 incubator. After 3 days, about 80-90% confluent monolayer (adherent) formation was confirmed by inverted microscope and used for study.

4.10 SUB-CULTURING ADHERENT CELLS [78]

Materials Required:

1. Incubator (37°c, 5% CO_2)
2. Laminar flow hood
3. Minimum essential medium
4. TPVG solution (Trypsin 2%, PBS – pH 7.4, Versene/EDTA- 0.2%, glucose10%)
5. Tissue culture treated culture flask
6. Micropipette
7. Falcon tubes- conical
8. Inverted microscope

Procedure:

- The culture flask containing the adherent cells (primary culture) was removed from the incubator.
- The culture flask was transferred aseptically to the bio safety cabinet along with minimum essential medium and TPVG solution after confirming its 90% confluency with the help of inverted microscope.
- The primary culture was passaged /sub cultured to required number of culture flasks for further study.
- The cap of the culture flask was opened and with the help of micropipette the medium was aspirated from the culture flask and discarded.
- 200µl of TPVG solution was added to the culture flask and rinsed well to remove any adhering medium and later the solution was aspirated and discarded.
- 500-1000µl of TPVG solution was again added to the culture flask and the solution was spread evenly to cover the entire surface of the culture flask.
- The flask was then incubated for 5 minutes for detachment of adhered cells from the monolayer (cell detachment can be viewed and confirmed under microscope after 5 minutes of incubation).
- After detachment of cells from the monolayer, the culture flask was transferred back to the bio safety cabinet. The detached cells were suspended completely in the TPVG solution by resuspending it with the help of micropipette.
- The TPVG solution containing the detached cells were aspirated from the culture flask and distributed into 7 new tissue culture treated culture flasks for further studies.

- 5ml of fresh minimum essential medium was added to all 7 culture flask containing the TPVG solution with suspended cells, such that it covers the entire surface of the flask .

4.11 CYTOTOXICITY STUDY

MTT ASSAY[44,79]

It is a universally accepted *invitro* method for screening the drugs having cytotoxic activity. It was described by Mosmamm (1983) & Monks (1991). This assay is used to determine the IC50 of drugs or extracts.

PRINCIPLE

The Tetrazolium salt, 3,-(4,5- dimethyl thiazol-2-yl)-2,5,diphenyl tetrazolium bromide is reduced into blue formazan product by the mitochondrial dehydrogenase enzyme of live or metabolically active cells.

The intensity of blue or purple colored formazan produced is directly propotional to cell viability.

Materials required

1. **Plant extracts**

    Ethanol (1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8)

2. **Reagents**

    DPBS

    TPVG

    MEM

    Trypan blue

    MTT dye(5mg/ml)

DMSO(0.1% v/v)

3. **Other materials**

MTT plate with plate with lid

Sterile pipette, tips, glove, mask

Aluminium foil

Incubator

Inverted microscope

Falcon tubes

Haemocytometer, cover slip.

PROCEDURE

- Culture flask containing the adherent cells were taken from the incubator and checked for its confluency.
- After confirming the confluency, the adherent monolayer cells were detached to form a cell suspension by adding an ml of TPVG solution and incubating it for 5 minutes.
- After incubation, the TPVG solution containing cells were aspirated from the culture flask and transferred to the falcon tubes and was subjected to centrifugation at 10000 rpm for 5 minutes.
- After centrifugation, supernatant was discarded and 1 ml of medium was added to the pellet of cells which have sedimented at the bottom of the tube and was resuspended well in the medium with the help of micropipette.
- 10µl of this cell suspension was mixed well with 10µl of tryphan blue.

Cell counting:

- After placing the cell suspension along with tryphan blue in haemocytometer, the numbers of cells in four corner quadrants were counted.

- Numbers of cells in left and right top quadrant were found to be 255 and 241 respectively. Numbers of cells in left and right bottom quadrant were found to be 335 and 297 respectively.

$$\text{Average no of cells} = \frac{225+241+335+297}{4} = 274.5 \text{ cells/10µl}$$

$$\text{No of cells per ml} = 549\times 2\times10^4 \text{ cells/ ml}$$

- 549×10^4 cells were present in 1 ml of cell suspension; we need to plate 26 wells each with 100 µl containing 10000 cells/well for maintaining triplicate condition for control, standard and test extracts.
- Therefore, 274.5×10^4 cells were present in 1000 µl. 10000 cells were present in x µl.

$$X = \frac{10000 \times 1000}{549\times10^4} = 1.82 \text{ µl}$$

- 1.82 µl of cell suspension should be mixed with 98 µl of medium for plating one well. In order to plate 26 wells, 47.32 µl of cell suspension was mixed with 2548 µl of medium to get a concentration of 10000 cells / 100 µl for plating the wells.
- 100 µl of this cell suspension were seeded into 26 wells of 96 well plate, and the plate was incubated for 24 hours at 37°c, 5% CO_2 for attachment of the cells.
- After 24 hours the seeded cells in the 96 well plate were treated with serial concentration of plant extract and the standard drug 5FU.
- Plant extracts of ethanol and standard drug were initially dissolved in 0.1% v/v DMSO and further diluted in serum free medium to get a desired concentration.
- 100 µl of each plant extract and standard concentration were added to the 96 well plates to get a final concentration of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 62.5 µg/ml, 31.2 µg/ml and 15.6 µg/ml for plant extracts and 1000 µg/ml, 500 µg/ml, 250 µg/ml, 62.5 µg/ml, 31.2 µg/ml and 15.6 µg/ml for standard drug.

- Final volume in each well was 200 µl and the plates were incubated again for 48 hours.
- Triplicate was maintained for all concentrations and the medium without samples were taken as control.

4.12 Extraction of DNA, RNA and Protein using Genei TRI Solution:

Extraction of DNA, RNA and Protein from the cells which was treated with IC_{50} concentration of Ethanolic extract, IC_{50} concentration of 5 Fluorouracil and control were extracted as per Genei TRI Soln Protocol. Fully grown or 90% confluency reached 3 subcultured or passaged flask is taken. The medium inside the flask is aspirated and decanted while cells adhered to the monolayer was kept intact and the flasks were subjected to drug treatment.

- Flask 1 is replaced with the fresh medium without any test or standard drug and it served as Control.
- Flask 2 is treated with IC_{50} concentration of the effective ethanolic extract (IC_{50})confirmed from MTT Assay)
- Flask 3 is treated with IC_{50} concentration of standard 5 fluorouracil (IC_{50} confirmed from MTT Assay).

All the three flasks were incubated for 48 hours at 37°c and 5% CO_2. After the incubation period, the medium inside the flasks were removed and the adhered cells were disturbed by treatment with TPVG solution and the TPVG solution containing the suspended cells of three flasks were stored in refrigerator for further processing

Extraction of RNA :

Materials Required:

1. Chloroform
2. Isopropyl Alcohol
3. 75% Ethanol
4. RNase free water

Steps Involved:

1. Homogenisation
2. Phase Separation
3. RNA Precipitation
4. RNA Wash
5. Resuspending RNA

Procedure:

Homogenisation:

- ❖ Cells were pelleted by centrifugation at 1200 rpm for 5 minutes.
- ❖ The supernatant was decanted and 1 ml of TRI solution was added and mixed by repetitive pipetting

Phase Separation:

- ❖ The samples were incubated for 5 minutes at room temperature (without exceeding 30°c) to completely dissociate nucleoprotein complex.
- ❖ 0.2 ml of chloroform per 1 ml of TRI Solution was added, mixed thoroughly and subjected to incubation at room temperature (without exceeding 30°c) for 2-3 minutes.

- The samples were centrifuged at not more than 12000 rpm for 15 minutes at 2-8°c and the mixture thus obtained contained a lower phenol chloroform phase, interphase and colourless upper aqueous phase.
- Upper aqueous phase will contain RNA.
- Volume of aqueous phase obtained was about 60% of volume of TRI Solution reagent used for homogenization.

RNA Precipitation:

- Aqueous phase was transferred to fresh 1.5 ml vial.
- 0.5 ml of isopropanol alcohol per 1 ml of TRI Solution was used for initial homogenization.
- Samples were incubated for 10 minutes at room temperature and were subjected to centrifugation at 12000 rpm for 10 minutes at 2-8°c.

RNA Wash:

- Supernatant was decanted and the RNA precipitated as gel like pellet on the sides of 1.5 ml vial.
- RNA pellet was washed once with 75% ethanol, at least 1ml of 75% ethanol was used per ml of TRI Solution reagent used for initial homogenization.
- Solution was centrifuged at 10000 rpm for 10 minutes at 2-8°c

Resuspending RNA:

- RNA pellet was air dried and re-suspended in100µl RNase free water by passing solution few times through pipette tip gently

Extraction of DNA:

Materials required:

1. Ethanol (100%)
2. 75% Ethanol
3. 0.1 M sodium citrate in 10% ethanol.
4. 8mM Sodium hydroxide.
5. Wash Buffer : 0.1 N Sodium citrate in 10% ethanol.

Steps involved:

1. DNA Precipitation
2. DNA Wash
3. Re suspending the DNA pellet.

Procedure :

DNA Precipitation :

- 0.3 ml of 100% ethanol was added per 1 ml of TRI Solution used for homogenization to the inter phase and organic phase which was obtained during phase separation in RNA extraction.
- Samples were stored at room temperature without exceeding 30°c for 2-3 minutes.
- Samples were centrifuged at 5000 rpm for 10 minutes at 2-8°c and DNA was obtained as a pellet.

DNA Wash:

- DNA pellet obtained was washed with wash buffer per 1 ml TRI Solution used.
- After each wash pellet was stored in wash solution for 30 minutes at room temperature without exceeding 30°c with periodic mixing.

- Samples were centrifuged at 5000 rpm for 10 minutes at 2-8°c and the wash step was repeated twice.
- Following wash, DNA pellet was suspended in 2 ml of 75% ethanol and kept for twenty minutes at room temperature and mixed periodically.
- Samples were centrifuged at 5000 rpm for 10 minutes at 2-8°c.

Re-suspending the DNA Pellet:

- DNA pellet was air dried for 5-10 minutes.
- DNA pellet was re-suspended in 300µl to 600µl of 8mM sodium hydroxide so that the concentration of DNA was 0.2 - 0.3µg/ml and samples were incubated at room temperature for 15-20 minutes

4.13 MICROSCOPIC STUDIES:[44, 79]

Light microscopic study

Fluorescent microscopic study

Light microscopic studies:

- Morphological changes in the cancer cells before and after drug treatment can be studied with the help of Light microscope.
- Fully grown or 90% confluency reached subcultured or passaged flask was taken.
- The morphology of the cancer cells in the flask before drug treatment were observed under inverted microscope and photographed.
- The medium inside the flask was aspirated and decanted without disturbing the adhered monolayer of cells.
- The culture flask was then treated with IC_{50} concentration of effective plant extract such that it covers the entire surface area

- The flask was then incubated for 48 hours at 5% CO_2 and 37°c.
- After incubation period, the flask was taken and observed under inverted microscope and the morphological changes were studied and photographed.
- The morphological changes of the cancer cells before and after drug treatment were evaluated.

Flourescent microscopic studies:

- Fluorescent microscopy was used to study the viability of cells as well as nuclei and chromatin condensation with the help of fluorescent binding dye.
- Fully grown or 90% confluency reached two subcultured or passaged flask was taken.
- Flask 1 was used for studying the control cancer cells and flask 2 was used for studying the extract treated cancer cells.
- From flask 1 the adhered cells were detached with the help of TPVG solution and the TPVG solution containing the cells were centrifuged and the cells were pelleted.
- The pellet of cells were then resuspended in phosphate buffer saline of pH 7.4. 100 µl of this cell suspension was introduced into microscopic slide along with equal mixture of acridine orange and ethidium bromide for staining.
- The cells were then viewed under fluorescent microscope and the viability and nuclear changes were studied and photographed.
- In flask 2, the medium was aspirated and decanted without disturbing the adhered monolayer of cells.
- Flask was then treated with IC_{50} concentration of effective plant extract such that it covers the entire surface area.
- The flask was then incubated for 48 hours at 5% CO_2 and 37°c.

- After incubation period, the flask was taken and the cells were pelleted and the same procedure was followed as in flask 1.

4.14 APOPTOTIC DNA FRAGMENTATION[45,80]

Apoptosis is a programmed cell death characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Irregularities in apoptosis have paved way for many diseases like cancer, autoimmune disease and neuronal degeneration. Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis. This cleavage of DNA or its fragmentation can be visualized by DNA laddering assay. This fragmentation of DNA in cancer cells after treatment with standard drug or test extract can be studied with the help of Agarose Gel Electrophoresis. Electrophoresis is a method of separating substances based on the rate of movement under the influence of electric field.

Materials required:

1. Agarose
2. 5X TAE buffer
3. 6X loading dye
4. DNA ladder
5. Electrophoresis chamber
6. Gel casting tray and comb
7. Ethidium bromide
8. Microwave oven
9. Conical flask
10. Sterile tips and pipettes

Procedure:

The steps involved are:

- Preparation of Agarose gel slab
- Loading of samples
- Running the gel

Preparation of Agarose gel slab:

- Gel casting tray and comb was wiped with ethanol and dried. The two open ends of the tray were sealed with tape.
- Place the combs in the gel casting tray.
- 1.2 grams of agarose powder was weighed and transferred to 500 ml conical flask containing 100 ml of TAE buffer.
- Agarose was melted using microwave oven until the solution became clear.
- The solution was cooled to about 50 - 55°c by swirling the flask occasionally or placing it in water bath.
- 3µl of ethidium bromide was added to the solution and mixed well.
- The gel casting tray along with comb was kept horizontally on a flat even surface.
- Agarose solution was poured evenly on the casting tray such that it covers the entire surface evenly without any bubble formation and was allowed to cool and solidify into a gel slab.
- After solidification, remove the tapes from both ends of the tray and place the gel in electrophoresis chamber and add enough TAE buffer so that there is 2-3 mm of buffer over the gel and carefully pull out the comb out of the tray to form wells.

Loading of samples:

- DNA which was extracted from the control, standard and extract treated cancer cells was taken.
- DNA was taken and mixed with required TAE buffer to make a final volume of 10µl each for a sample.
- 2 µl of 6X loading dye was added to the DNA sample and mixed well.
- 10µl of 100 base pair standard DNA ladder and 12µl of DNA samples were loaded carefully in the submerged wells of gel slab in the electrophoresis chamber

Running the Gel:

- Lid was placed on the gel box and the electrodes were connected.
- Electrode wires were connected to power supply and about 80 volts of current were passed.
- Power supply to the electrophoresis chamber was ensured until the blue dye approached the end of the gel.
- Later power supply was cut off, wires were disconnected and the lid was removed.
- Gel tray was carefully taken out and the gel was placed inside the Gel photostation and photographed in presence of UV light

4.15 Real time Reverse Transcriptase Polymerase Chain Reaction:[47]

Real time reverse transcriptase polymerase chain reaction is abbreviated as qRT-PCR. It is a technique where expression of RNA is studied by converting it into cDNA with the help of enzyme reverse transcriptase and quantitatively measuring the amount of amplified target sequence from entire cDNA using fluorescent dye SYBR green in real time.Upon binding with DNA, SYBR green dye used will emit fluorescence and the fluorescence intensity is directly proportional to number of DNA copies or expression produced. The fluorescence which is emitted is analysed by detector with the help of LED source and it gives the relative expression of genes. The procedure was carried out as per Step 1 plus ABI protocol.

Procedure:

A) Complementary DNA Synthesis:

Materials Required:

1. 5X buffer
2. 10mm dNTPs
3. Hexamer primer
4. Extracted RNA
5. Thermal cycler
6. Reverse transcriptase
7. DTT

Procedure:

- ❖ In a 200µl eppendorf tubes, 5µl of 5X buffer, 2µl of 10mm DNTPs and 1.5µl of hexamer primer was added.

- ❖ Later 15µl of extracted RNA was added and the eppendorf tubes were kept in thermal cycler at 70°c for 5 minutes to separate the false double stranded RNA.
- ❖ The tubes were taken out and immediately cooled with ice to prevent binding of false double stranded RNA again.
- ❖ 1.5µl of Reverse transcriptase and 1µl of DTT was added to the tube and spun for few seconds.
- ❖ It was then placed in thermal cycler , at 25°c for 5 minutes for binding of hexamer, followed by at 42°c for 45 minutes for cDNA synthesis, followed by at 85°c for 5 minutes for denaturation of remaining unconverted RNA's and finally at 4°c for 5 minutes

B) Primer synthesis: The primers synthesized were P53, Bcl2, TNF α and IL – 6 along with house keeping gene GPDH. The primers were synthesized by Geno Rime with the help of Primer express software with the available primer sequence.

The concentration of primers synthesized were 100pM/µl. It was diluted in the ratio of 1: 10 with water to get a concentration of 10pM/µl.

C) Real time PCR:

The materials required are

25µl of SYBR green

RT mix

5µl of cDNA

2µl of 25 pM/µl forward primer

2µl of 25 pM/µl reverse primer

16µl PCR grade water.

- For a total of 50 µl reaction, the above mentioned mixtures were added into eppendorf tubes and they were placed in real time PCR instrument and the program was set as follows:
    - ✓ Step 1: Pre denaturation at 95˚C for 1 minute
    - ✓ Step 2: Denaturation at 95˚C for 15 seconds
    - ✓ Step 3: Annealing at 60˚C for 15 seconds
    - ✓ Step 4: Extension at 72˚C for 45 seconds
    - ✓ Step 2 to step 4 repeated for 40 cycles.
- The relative expression of genes was analyzed and interpreted by Applied Biosystem Software.

5. RESULTS

TABLE-1 : Phytochemical Analysis

Constituents	Ethanolic extract	Acetone extract	Aqueous extract
Proteins	Absent	Present	Absent
Flavanoids	Present	Absent	Absent
Alkaloids	Present	Present	Absent
Glycosides	Present	Present	Absent
Steroids	Present	Absent	Absent
Carbohydrates	Present	Present	Absent
Phenols and tannins	Present	Absent	Absent
Terpenes	Present	Absent	Absent

Phytochemical analysis was carried out for ethanolic, acetone and aqueous extracts of seeds of *Trachyspermum ammi* and the results are shown in the above TABLE-1

- Phyto chemical analysis was performed with ethanol, acetone, aqueous extract of ***Trachyspermum ammi***
- Ethanolic extract showed presence of Flavanoids, Alkaloids, Glycosides, Steroids, Carbhohydrates, Phenols, Tannis and Terpenes except Protein.
- Acetone extract showed presence of Proteins, Glycosides, Carbhohydrates.
- Aqueous extract did not show presence of any constituents.

TABLE-2 : Percentage yield of extracts

EXTRACT	% YIELD
1. Ethanol	4.98
2. Acetone	2.09
3. Aqueous	7.05

Percentage yield of various extracts are shown in above TABLE-2

The percentage yield was high in aqueous and low in acetone extract.

2. CYTOTOXICITY TEST

TABLE-3 MTT assay of ethanolic extract.

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.09	14.28
2	500	1:1	0.17	26.98
3	250	1:2	0.26	41.26
4	125	1:4	0.32	50.79
5	62.5	1:8	0.37	58.73
6	31.2	1:16	0.40	63.49
7	15.6	1:32	0.43	68.25
8	7.8	1:64	0.46	73.01
9	Cell control	-	0.63	100

TABLE-3 shows IC_{50} concentration and percentage of cell viability of Ethanolic extract

MTT assay with extract of Trachyspermum *ammi* was performed in various concentrations.

- ✓ Cell viability of 50.79% was observed in concentration 0f 125 µ/ml which has taken as 50% of inhibition of cells (IC_{50})

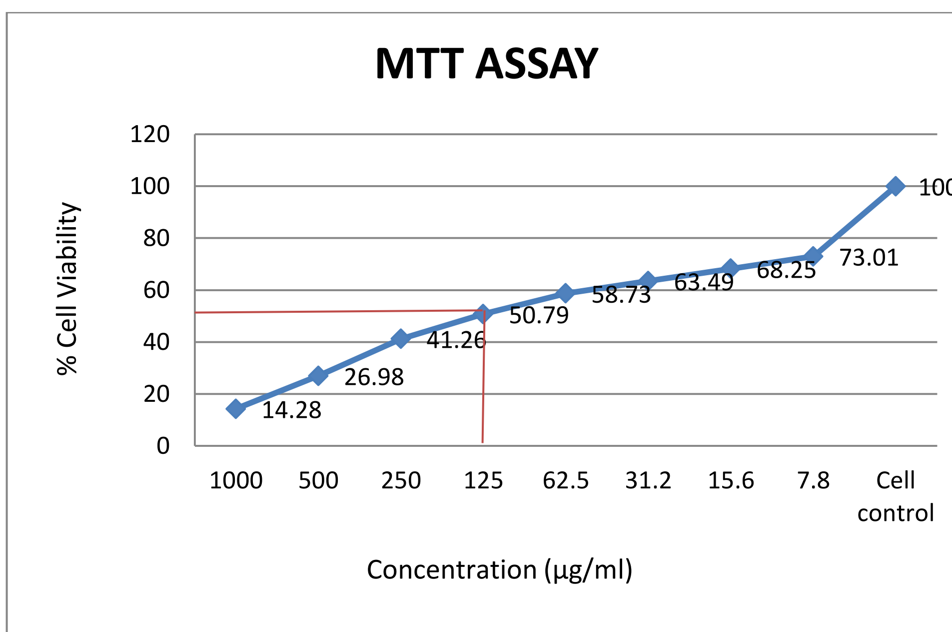
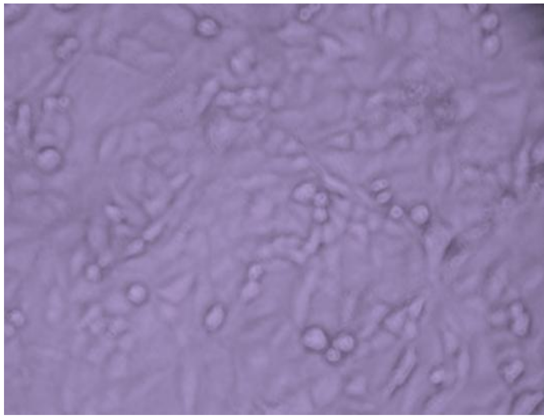


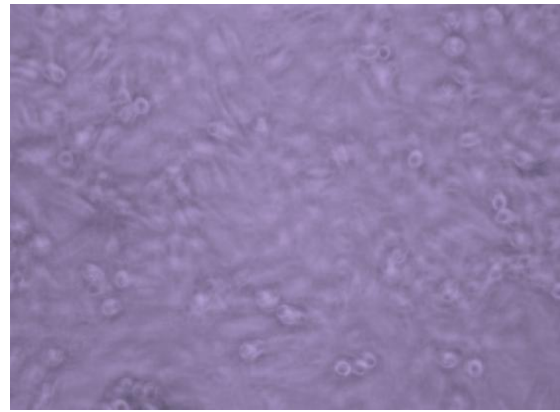
FIGURE- 2. Shows graphical representation of

% Cell viability vs conc (µg/ml) in of Ethanolic extract

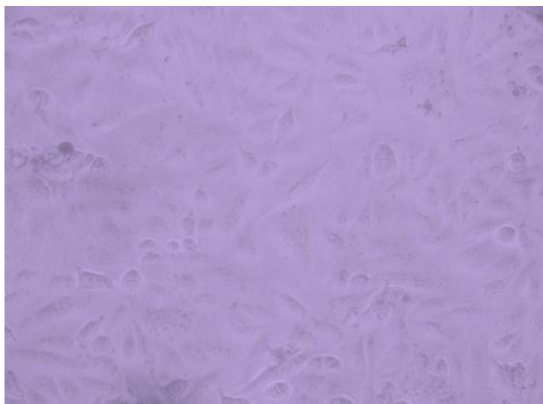
MTT ASSAY



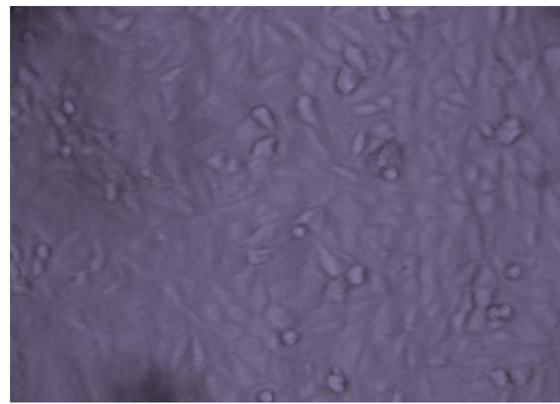
(a)



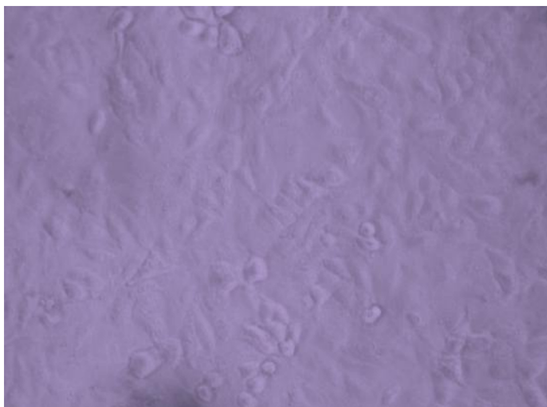
(b)



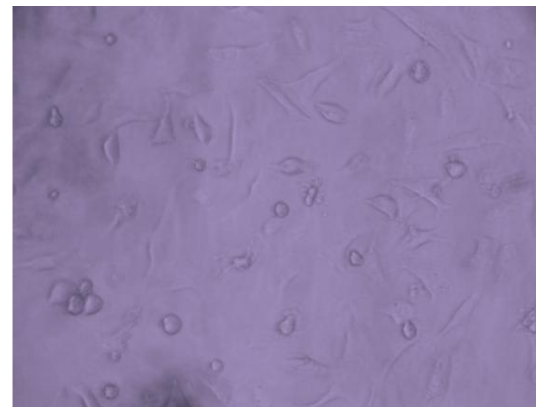
(c)



(d)



(e)



(f)

FIGURE -3 shows the MCF-7 cells treated with various concentration of Ethanolic extracts

a.1000 µg/ml **c.250 µg/ml** **e.62.5 µg/ml**

b.500 µg/ml **d.125 µg/ml** **f.31.2 µg/ml.**

TABLE -4 MTT Assay of 5 Fluorouracil

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.01	1.85185185
2	500	1:01	0.03	5.55555556
3	250	1:02	0.06	11.1111111
4	125	1:04	0.1	18.5185185
5	62.5	1:08	0.13	24.0740741
6	31.2	1:16	0.17	31.4814815
7	15.6	1:32	0.2	37.037037
8	7.8	1 64	0.28	51.8518519
9	Cell control	-	0.54	100

TABLE-4 IC50 concentration and % cell viability of 5 Fluorouracil

Cell viability of 51.85 % was observed in concentration of 7.8 µg/ml which has taken as 50% of inhibition of cells (IC_{50}).

FIGURE-4

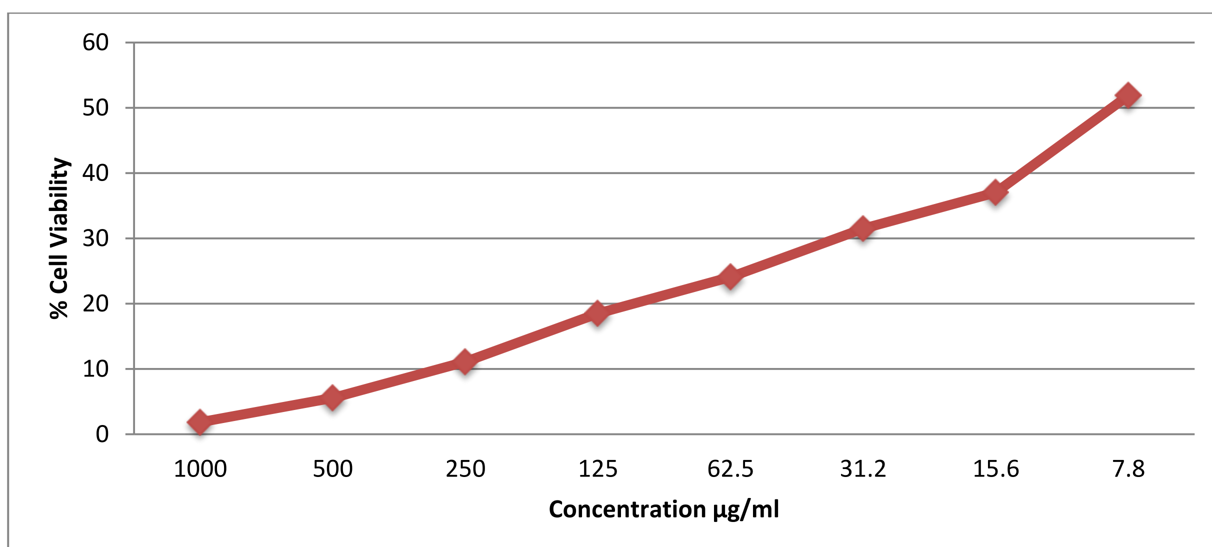


FIGURE-4 is a graphical representation of % Cell viability vs Concentration in µg/ml 5- Fluorouracil

3. Apoptotic activity of ethanolic extracts of *Trachyspermum ammi.*

Microscopic Studies:

Light microscopic observation:

There was no destruction of cells and cell morphology was well maintained in the control group. **(FIGURE-5),** but the cells treated with with IC_{50} concentration of ethanolic extract of *T.ammi* showed-

- ✓ Reduction and detachment of dead cells
- ✓ Loss of integrity of the membrane resulting in crooked and vesicle shape of the membrane
- ✓ Reduction in cell volume
- ✓ Cell shrinkage and chromatin condensation (**FIGURE-6**)

Flourescence microscopic observation:

- Control cells were bright green in colour. (**FIGURE-7**)
- Cells treated with ethanolic extract of *Trachyspermum ammi were* bright orange in colour with loss of membrane integrity and cytoplasmic contents leaking out of the cell. (**FIGURE-8**)

Light Microscopic study images

A) MCF-7 cells – Control

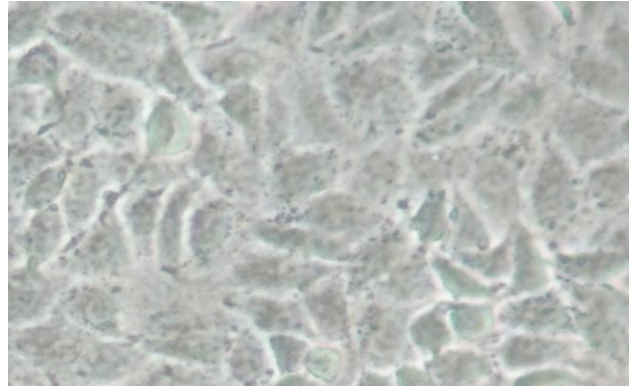


FIGURE-5 shows control cell lines with normal morphology.

B) MCF-7 cells after treatment with ethanolic extract of *Trachyspermum ammi*

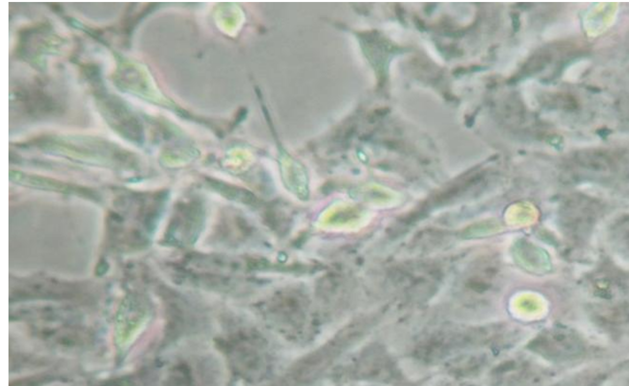


FIGURE-6 shows decrease in cell population, chromatin condensation and destruction of cells.

Flourescent Microscopic Images

A) MCF-7 cells – Control

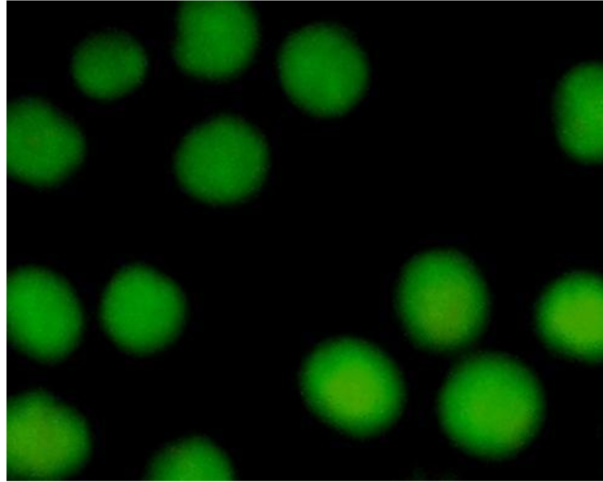


FIGURE -7 shows viable cells stained green in colour

B) MCF-7 cells after treatment with Ethanolic extract of *Trachyspermum ammi*

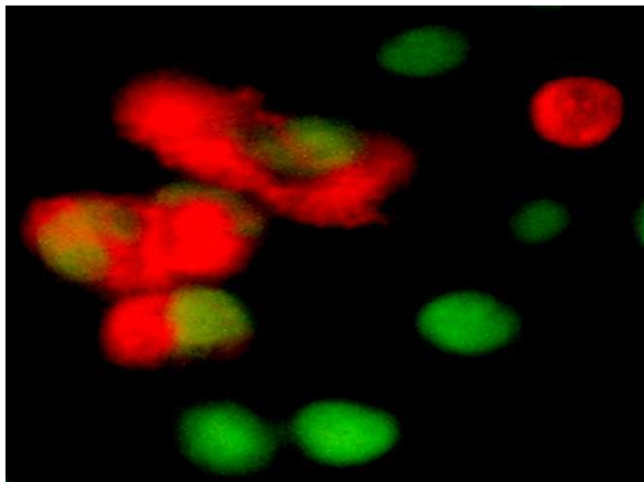


FIGURE-8 shows dead cells stained orange in colour with loss of membrane integrity and cytoplasmic contents leaking out of the cell.

DNA Fragmentation: (Agarose Gel Electrophoresis)

- ✓ Control MCF-7 cells, there was no fragmentation.
- ✓ MCF-7 cells treated with IC_{50} concentration of standard 5 fluorouracil and ethanolic extract of *Trachyspermum ammi* fragmentation was observed
- ✓ This fragmentation of DNA in ethanolic extract treated cells indicates the characteristics of apoptotic cells.
- ✓ Results obtained in fragmentation studies are shown in **FIGURE -9**

Agarose Gel Electrophoresis

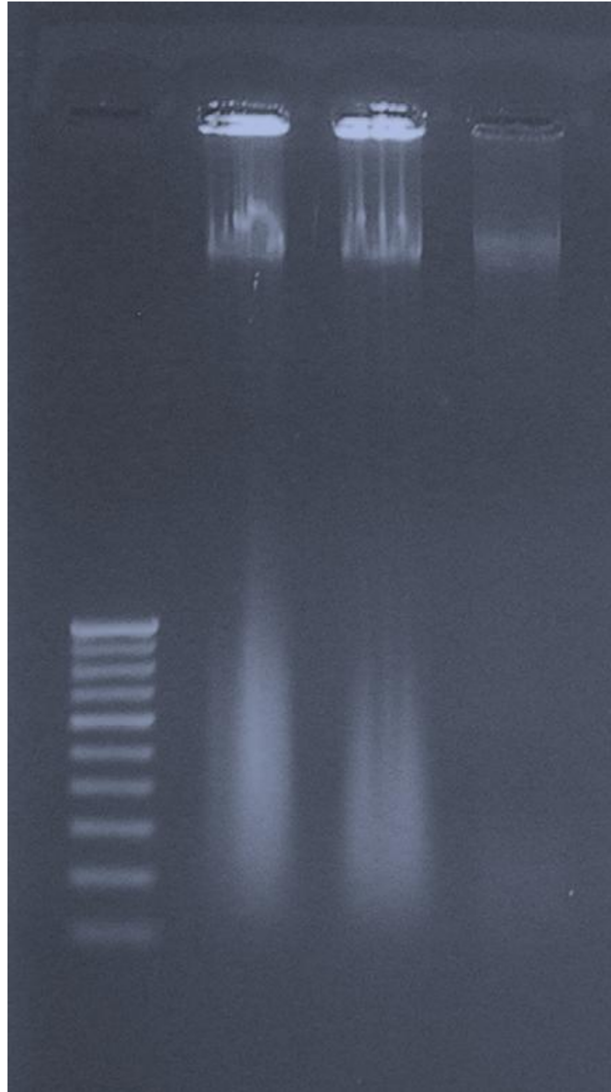


FIGURE 9. DNA Fragmentation in MCF-7

Lane 1: 100 base pair DNA marker

Lane 2: MCF-7 cells treated with 5 Fluorouracil

Lane 3: MCF-7 cells treated with ethanoic extract of *Trachyspermum ammi*

Lane 4: MCF-7 cells without any treatment.

4. Real time PCR:

The expression levels of p53, Bcl-2 were studied using RT-PCR and the results are shown in Table 6 & 7 and figures 10, 11,12 & 13.

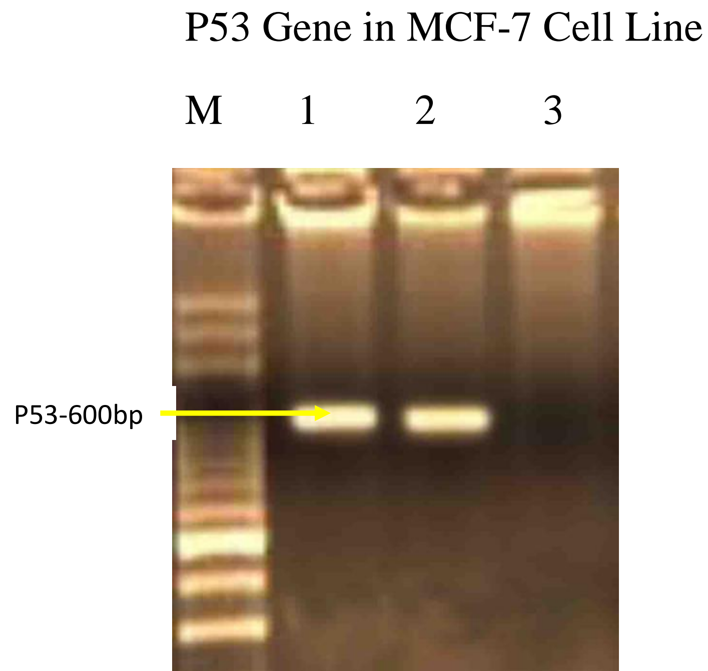


FIGURE 10. P53 gene expression

M- Marker- 1 kb DNA Ladder.

Lane 1: Standard

Lane 2: Sample

Lane 3 : Control.

TABLE-5 QUANTIFICATION OF p53

	RELATIVE QUATIFICATION	**STANDARD ERROR**
CONTROL	2.06	0.1
5-FU	6.45	0.12
TEST	4.5	0.11

TABLE-5 shows expression levels of p53

- ✓ On comparing with control both standard 5 fluorouracil and ethanolic extract of *Trachyspermum ammi* expression levels of p53 was higher.
- ✓ Expression levels of p53 was observed to be maximum with standard 5 fluorouracil(6.45) followed by ethanolic extract of *Trachyspermum ammi* (4.5).

FIGURE- 11 Graphical representation of levels of p53

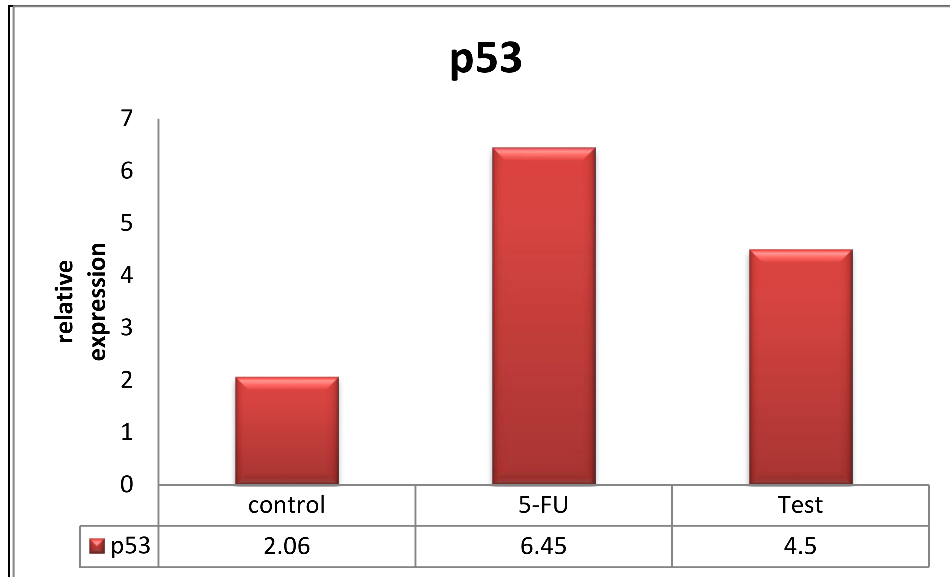


FIGURE- 11 shows expression levels of p53

BCl2 Gene in MCF-7 Cell Line

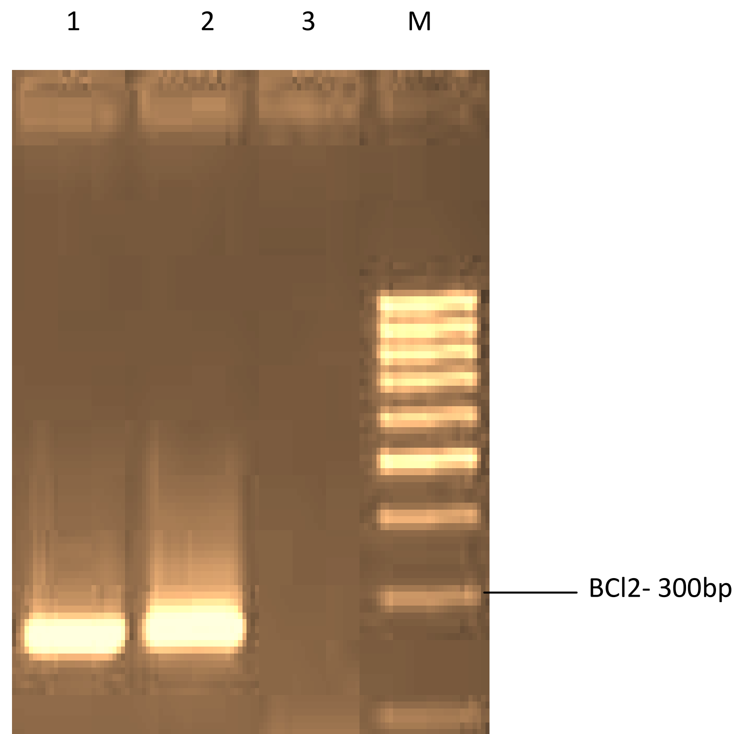


FIGURE 12. Bcl2 gene expression

.

Lane 1: Sample

Lane 2: Sample

M- Marker- 100 bp DNA Ladder

TABLE-6 Quantification of Bcl2

	RELATIVE EXPRESSION	STANDARD ERROR
CONTROL	5.21	0.123
5-FU	2.97	0.09
TEST	2.47	0.106

TABLE-6 shows expression levels of Bcl2

- ✓ On comparing with control both standard 5 fluorouracil and ethanolic extract of *Trachyspermum ammi* expression levels of Bcl2 was lower.
- ✓ Expression levels of Bcl2 was observed to be maximum with of standard 5 fluorouracil(2.97) followed by ethanolic extract of *Trachyspermum ammi* (2.47).

FIGURE- 13 Graphical representation of levels of Bcl2

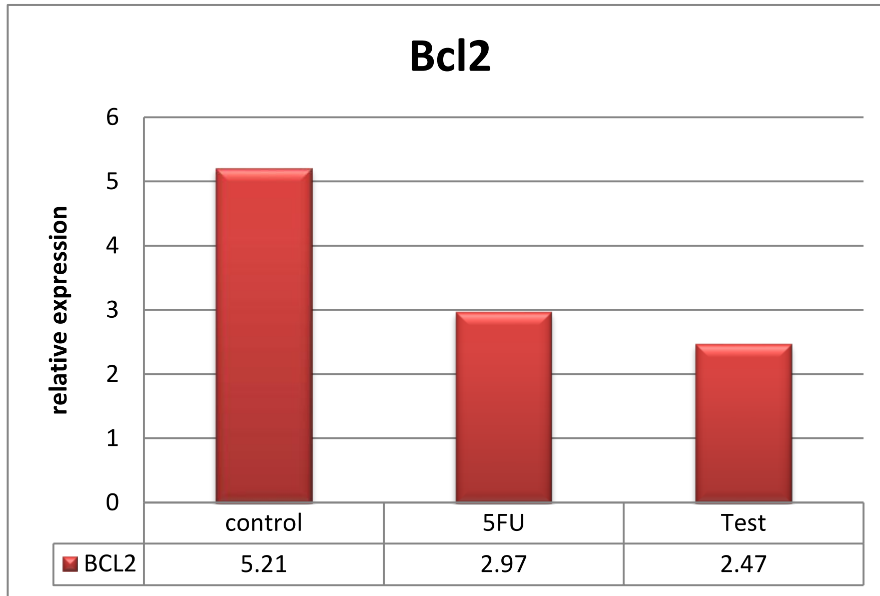


Figure -13 shows relative expression of Bcl2

6. DISCUSSION

Breast cancer is malignant neoplasm occurring in females. It accounts for 23% of newly occurring cancer worldwide and represents 13.7% of all cancer deaths. In both developed and developing countries, breast cancer mortality and incidence rates are projected to rising continuously.

In spite of advancing different types of treatments, the rate of breast cancer has not decreased yet. The report, by NCEPOD has shown that more than 1 in 4 patients died from the side effect rather than from cancer and patients are suffering from treatment related toxicity. Because of dangerous side effects and high cost of chemotherapy, usage of herbal medicine is on increased in large number of population.

In search of new compound to treat breast cancer with fewer side effects, researchers have been evaluating number of medicinal plants. Since the medicinal plants have phyto constituents, which protects the plant from oxidative damages and the same phytoconstituents which when used in humans may have the same role. They have wide range of action such as antitumor, antiviral, antibacterial, antimutagenic etc and they may act in different stage of malignant tumor by protecting the DNA from oxidative damages. They inactivate carcinogen by inhibiting the expression of mutagenic genes; they also inactivate the enzymes with activate the systems responsible for the detoxification of xenobiotics (Bravo, 1998).[81]

Trachyspermum ammi has been reported to show a number of biological activities in traditional medicine and it has been used as digestive, carminative, laxative, diuretic, stimulant and antispasmodics. Several investigation in the seeds of *Trachyspermum ammi* have been indicated to posses many activities like potent antibacterial, antimicrobial, anthelmintics, hypolipidemic, etc.

Phyto Chemical Analysis

The phyto chemical analysis of ethanolic extract of seeds of *T. ammi* revealed the presence of flavanoids, terepenoids, tannis, alkaloids, steroids, glycosides, carbhohydrates. The acetone and aqueous extracts didn't show the presence of flavanoid and terepenoids. The ethanolic extract revealed the presence of flavanoids and terepenoids which shows that it may posses anticancer activity and destroy cancer cells. [82]

MTT Assay

The MTT assay was carried out to determine the IC_{50} concentration of ethanolic extract of *Trachyspermum ammi*.The effective IC_{50} of ethanolic extract was found to be 125 µg/ml.

The effective IC_{50} concentration of ethanolic extract of *Trachyspermum ammi* and standard 5-fluorouracil was chosen for further studies.

Light Microscopy

Control did not show apoptosis, but cells treated with of ethanolic extract of *Trachyspermum ammi* showed typical morphological features of apoptosis like destruction of cells, reduction of MCF-7 cell population, reduction of cell volume, loss of integrity of membrane which resulted in crooked and vesicle shape of the membrane and chromatin condensation.

Fluorescence Microscopy

Apoptotic effects of IC_{50} concentration of ethanolic extract of Trachyspermum ammi treated MCF-7 cells were further confirmed with the help of fluorescence microscopy. Control did not reveal any apoptotic activity. Cells treated with of ethanolic

extract of *Trachyspermum ammi* showed apoptotic activity. The presence of apoptotic activity shows ethanolic extract of *Trachyspermum ammi* may have anticancer activity.

On comparing with control group, both 5 Fluorouracil and ethanolic extract of *Trachyspermum ammi* has produced DNA fragmentation in MCF-7 cells. The fragmentation of DNA indicates that apoptotis has occured. This shows ethanolic extract of *Trachyspermum ammi* posses apoptotic activity.

Gene Expression

Cells treated with 5 Fluorouracil, expression levels of p53 was found to be increased and similar increase was observed in cells treated with ethanolic extract of Trachyspermum ammi.

Expression levels of Bcl-2 were decreased in cells treated with 5 Fluorouracil. Cells treated with ethanolic extract of *Trachyspermum ammi* shows a decrease in expression levels of Bcl-2 slightly lower than 5 Fluorouracil.

Expression levels of p53 and Bcl-2 indicated that ethanolic extract of *Trachyspermum ammi* has anticancer activity.

7. CONCLUSION

From the present study it may be concluded that,

- The phyto chemical investigation revealed the presence of flavanoids, tannins, trepenoids, steroids, glycosides, alkaloids.
- By MTT assay, The IC_{50} value of ethanolic extract of *Trachyspermum ammi* was found to be 125µg/ml.
- The ethanolic extract of *Trachyspermum ammi* posses apoptotic activity.
- The gene expression of cancer DNA markers in MCF-7 Cell line treated with ethanolic extracts of *Trachyspermum ammi* showed increase in p53 expression and decrease in Bcl2 expression.

BIBILOGRAPHY

1. FerlayJ SH, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 vl.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. 2010. Available from: http://alobocan.iarc.fr
2. Advani S. Partner profile: cancer in India. INCTR News 2004; 5.
3. Eisenberg D M, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, Kessler Rc. Trends in alternative medicine use in United States, 1990 -1997: Results of a follow-up national survey. J.Amer. Med. Assoc.1998; (280): 1569-1575.
4. WHO, Regulatory situation of herbal medicine: A world wide review. Geneva: World health organization. 2007.
5. WHO, General guidelines for methodologies on research and evaluation of traditional medicines. . Geneva: World health organization. 2000.
6. Narayanan D B A, Katayar C K; Brindavan N B. Original System Search, research, IDMA Bulletin 1998; 29 (17): 413-416.
7. Drug Screening methods 2nd edition, Jaypee Brothers medical publishers by S.K. Gupta.
8. Varricchio, Claudette G. A cancer source book for nurses. Boston: Jones and Bartlett Publishers; 2004. p. 229.
9. Morton, Leslie T., and Moore, Robert J. A Chronology of Medicine and Related Sciences. Aldershot, England: Scholar Press, 1997
10. The American Cancer Society Inc., "The History of Cancer." 25 Mar. 2002. 13-17 Accessed October 2010 [http://www.cancer.org/Cancer/CancerBasics/TheHistoryofCancer/the-history-of-cancer-cancer-causes-theories-throughout-history]
11. Udwadia, Farokh Erach. Man and Medicine: A History. Oxford: Oxford University Press, 2000.
12. (irange JM, Stanford JL, Stanford CA. Campbell De Morgan's 'Observations on cancer', and their relevance today. J R Soc Med 2002; 95 (6): 296-9.

13. Goodman, Louis S., et al. "Landmark Perspective: Nitrogen Mustard Therapy."*JAMA*. 251 (1984): 2255-2261. [PUBMED]

14. Globocan 2008, IARC, 2010

15. Chalasani P, Downey L, Stopeck AT. Caring for the breast cancer survivor: a guide for primary care physicians. *Am J Med.* 2010;123(6):489-95.

16. SchottenfieldDaF, Jr., Joseph F., editor. Cancer Epidemiology and Prevention, Second Edition. New York: Oxford University Press; 1996.

17. Gunnell D, Okasha M, Smith GD, Oliver SE, Sandhu J, Holly JM. Height, leg length, and cancer risk: a systematic review. Epidemiol Rev 2001; 23:313-42.

18. *Early and localised breast cancer: Diagnosis and treatment.* February 2009. National Institute for Health and Clinical Excellence (NICE).

19. Ursin G, Ma H, Wu AH, et al. Mammographic density and breast cancer in three ethnic groups. Cancer Epidemiol Biomarkers Prev 2003; 12:332-8.

20. Travis LB, Hill D, Dores GM, et al. Cumulative absolute breast cancer risk for young
women treated for Hodgkin lymphoma. J Natl Cancer Inst 2005; 97:1428-37

21. Joany Salayo. Pathophysiology of breast cancer [Internet]. 2012 Nov 24. Available from http://www.slideshare.net/ioanvsalavo/pathophysioloRv-of-breast-cancer-2

22. Matlashewski G, Lamb P, Pirn D, Peacock J, Crawford L, Benchimol S. Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. EMBO J. 1984;3(13):3257-62.

23. Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *N Engl J Med.* 2011;364(25):2381-91.

24. Cuzick J, DeCensi A, Arun B, et al. Preventive therapy for breast cancer: a consensus statement. *Lancet Oncol.* 2011;12(5):496-503.

25. Goss PE, Ingle JN, Alés-Martínez JE, et al. Exemestane for breast-cancer prevention in postmenopausal women. *N Engl J Med.* 2011;364(25):2381-91.

26. Katz VL,Dotters D. Breast diseases: diagnosis and treatment of benign and malignantdisease.In: Lentz GM, Lobo RA,Gershenson DM, Katz VL, eds.

Comprehensive Gynecology. 6th ed. Philadelphia, PA: Elsevier Mosby; 2012:chap 15.

27. Olszewski et al., TNF Trafficking to Human Mast Cell Granules: Mature Chain-Dependent Endocytosis,77ie Journal of Immunolog. 2007;178: 5701 5709
28. Swardfager W, Lanctot K, Rothenburg L, Wong A, Cappell J, Herrmann N. A metaanalysis of cytokines in Alzheimer's disease. Biol Psychiatry 2010; 68 (10): 930-941.
29. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctot KL. A metaanalysis of cytokines in major depression. Biol Psychiatry 2010; 67 (5): 446-457.
30. Brynskov J, Foegh P, Pedersen G, Ellervik C, Kirkegaard T, Bingham A, Saermark T. Tumor necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. Gut 2002; 51 (1): 37-43.
31. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ. 2003; 10(1): 45 65.
32. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (November 1984). Cloning of the chromosome breakpoint of neoplastic B cells with the (14:18) chromosome translocation. Science. 226 (4678): 1097-99.
33. Cleary ML, Smith SD, Sklar J (October 1986). Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the (14:18) translocation. Cell. 47 (1): 19-28.
34. Vaux DL, Cory S, Adams JM (September 1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature.335 (6189): 4402.
35. Sah NK, Khan Z, Khan GJ, Bisen PS (December 2006). Structural, functional and therapeutic biology of survivin. Cancer Lett. 244 (2): 164-71.
36. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs.Cancer Res. 1998;58 (23): 5315-20.

37. Warner E. Clinical practice. Breast-cancer screening. N Engl J Med. 2011 Sep 15;365(11):1025-32.

38. Smolen JS, Maini RN. Interleukin-6: a new therapeutic target. Arthritis Res. Ther.% Suppl 2 (Suppl 2): 2006;S5.

39. Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA. The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. N. Engl. J. Med. 1997;337 (24): 1720-5.

40. Abeloff MD AJ, Lichter AS, et al, eds., editor. Clinical Oncology. 4th ed. Philadelphia, PA: Elsevier; 2008

41. National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines): Breast cancer. Version3.2012. Available at: http://www.nccn.org/professionals/physician_gls/pdf/breast.pdf. Accessed November 16, 2012

42. *Surgical guidelines for the management of breast cancer, Association of Breast Surgery at BASO 2009.* European Journal of Surgical Oncology. 2009. S1–S22.

43. Principle of pharmacology 2nd edition paras medical publishers HL Sharma, KK Sharma.

44. N Vidhya& S Nirajali Devaraj, Induction of eugenol in human breast cancer cells. Indian Journal of Experimetnal Biology, 2011; 49, 871-878.

45. Shahnaz Khaghani, Farideh Razi, Mohsen Mohammadian Yajloo, Malihe Paknejad, Ahmad Shariftabrizi, Parvin Pasalar. Selective Cytotoxicity and Apoptogenic Activity of Hibiscus Sabdariffa Aqueous Extract against MCF-7 Human Breast Cancer Cell Line. J Can Ther.2011;2:394-400.

46. Sidddig Ibrahim Abdel Wahab, Ahmed Bustamann Abdul, SyamMurali Mohan, Abel Sharaf Al-Zubairi and Manal Mohamed Elhasaan. Oncolysis of Breast, Liver and Leukemia Cancer cells using Ethyl acetate and Methanolic extract of Goniothalamus umbrosus. Res J Biol Sci. 2009;4(2):209-215

47. Ali A Alshatwi. Catechin hydrate suppresses MCF-7 proliferation through TP53/Caspase-med\ated apoptosis. J Exp & Clin Cancer Res. 2010, 29:167

48. Ayurvedic Pharmacopoeia of India. Government of India, Ministry of Health and Family Welfare Department of Ayush. Part 1. 1999-2011;1:170–1.

49. Available from: http://plants.usda.gov/java/profile?symbol=TRAM13

50. Joshi SG. Medicinal Plants. New Delhi, India: Oxford and IBH Publishing Co. Pvt. Ltd; 2000.

51. Joy PP, Thomas J, Mathew S, Jose G, Joseph J. Aromatic plants. In: Bose TK, Kabir J, Das P, Joy PP, editors. Tropical Horticulture. Vol. 2. Calcutta: Naya Prokash; 2001. pp. 633–733.

52. Sivropoulou A, Papanikolaou E, Nilolaou C, Kokkini S, Lanaras T, Arsenakis M. Antimicrobial and cytotoxic activities of origanum essential oils. J Agric Food Chem. 1996;44:1202–5.

53. Bentely R, Trimen H. Medicinal Plants. New Delhi: Asiatic Publishing House; 1999. pp. 107–15.

54. Krishnamoorthy V, Madalageri MB. Bishop weeds (*Trachyspermum ammi*): An essential crop for north Karnatka. J Med Aromat Plant Sci. 1999;21:996–8.

55. Gilani AH, Jabeen Q, Ghayur MN, Janbaz KH, Akhtar MS. Studies on the antihypertensive, antispasmodic, bronchodilator and hepatoprotective activities of the Carum copticum seed extract. Journal of Ethnopharmacol. 2005;98:127–35. [PubMed]

56. . Ahsan SK, Shah AH, Tanira MO, Ahmad MS, Tariq M, Ageel AM. Studies on some herbal drugs used against kidney stones in Saudi folk medicine. Fitoterapia. 1990;61:435–8.

57. Srivastava KC. Extract of a spice-omum (*Trachyspermum ammi*)-shows antiaggregatory effects and alters arachidonic acid metabolism in human platelets. Prostaglandins Leukot Essent Fatty Acids. 1988;33:16. [PubMed]

58. Thangam C, Dhananjayan R. Antiinflammatory Potential Of The Seeds Of Carum Copticum Linn. Indian J Pharmacol. 2003;35:388–91.

59. Boskabady MH, Jandaghi P, Kiani S, Hasanzadeh L. Antitussive effect of Carum copticum in guinea pigs. J Ethnopharmacol. 2005;97:79–82. [PubMed]

60. Velazhahan R, Vijayanandraj S, Vijayasamundeeswari A, Paranidharan V, Samiyappan R, Iwamoto T, et al. Detoxification of aflatoxins by seed extracts of the medicinal plant, *Trachyspermum ammi* (L.) Sprague ex Turrill Structural

analysis and biological toxicity of degradation product of aflatoxin G1. Food Control. 2010;21:719–25.

61. Anilakumar KR, Saritha V, Khanum F, Bawa AS. Ameliorative effect of ajwain extract on hexachlorocyclohexane-induced lipid peroxidation in rat liver. Food Chem Toxicol. 2009;47:279–82. [PubMed]

62. Caccioni DL, Guizzardi M, Biondi DM. Relationships between volatile components of citrus fruit essential oil and antimicrobial action on Penicillium digitatum and Penicillium italicum. Int J Food Microbiol. 2000;88:170–5. [PubMed]

63. Javed IM, Akhtar T, Khaliq MZ, Khan G, Muhammad M. Antihyperlipidaemic effect of *Trachyspermum ammi* (Ajwain) in rabbits. In: Faisalabad: Proc 33rd All Pakistan Science Conference University of Agriculture. 2002:80–1.

64. Platel K, Srinivasan K. Studies on the influence of dietary spices on food transit time in experimental rats. Nutr Res. 2001;21:1309–14.

65. Kwon Park Il, Junheon K, Sang-Gil L. Nematicidal Activity of Plant Essential Oils and Components From Ajwain (*Trachyspermum ammi*), Allspice (Pimenta dioica) and Litsea (Litsea cubeba) Essential Oils Against Pine Wood Nematode (Bursaphelenchus Xylophilus) J Nematol. 2007;39:275–9. [PMC free article] [PubMed]

66. Kostyukovsky M, Rafaeli A, Gileadi C, Demchenko N, Shaaya E. Activation of octopaminergic receptors by essential oil constituents isolated from aromatic plants: Possible mode of action against insect pests. Pest Manage Sci. 2002;58:1101–6. [PubMed]

67. Tamurab T, Iwamoto H. Thymol: A classical small molecule compound that has a dual effect (potentiating and inhibitory) on myosin. Biochem Biophys Res Commun. 2004;18:786–91. [PubMed]

68. Jabbar A, Iqbal Z, Khan MN. *In vitro* anthelmintic activity of *Trachyspermum ammi* seeds. Pharmacogn Mag. 2006;2:126–9.

69. . Pelczar MJ, Chan EC, Krieg NR. Control of microorganism by physical agents, in microbiology. New York: Mcgraw Hill International; 1988. pp. 469–509.

70. . Murthy PS, Borse BB, Khanum H, Srinivas P. Inhibitory effects of Ajwain

(*Trachyspermum ammi*) ethanolic extract on A. ochraceus growth and ochratoxin production. Turk J Biol. 2009;33:211–7.

71. Singh G, Maurya S, Catalan C. Chemical, antifungal, antioxidative studies of Ajwain oil and its acetone extract. J Agric Food Chem. 2004;52:3292–6. [PubMed]

72. Choi IH, Shin SC, Park IK. Nematicidal activity of onion (Allium cepa) oil and its components against the pine wood nematode (Bursaphelenchus xylophilus) Nematology. 2007;9:231–5.

73. Kong J, Lee SM, Moon YS, Lee SG, Ahn YJ. Nematicidal activity of plant essential oils against Bursaphelenchus xylophilus. J Asia Pac Entomol. 2006;9:173–8.

74. Wright DJ. Nematicides: Mode of action and new approaches to chemical control. In: Zukerman, Rhode, editors. Plant Parasitic Nematodes. Vol. 3. New York: Academic Press; 1981. pp. 421–49.

75. Wallis TE. Textbook of Pharmacognosy. New Delhi: CBS Publisher & Distributors; 1985 p. 133-248.

76. Khandelwal KR, Practical Pharmacognosy. Pune: niraliprakashan; 1998.p. 20-42

77. Lala PK. Practical Pharmacognosy. Lina; 1981.p. 30-40.

78. Guide to sub culturing cell line monolayer. 2010. Available from http://www.atcc.org

79. Drug screening methods 2nd edition, Jaypee Brothers medical publishers by S.k. Gupta.S

80. Sarveswaran Sivalokanthan, Marati Radhakrishnan Vijayababu, Maruthaiveeran Periyasamy Balasubramanian. Effects of *Terminalia arjuna* bark extract on apoptosis of Human Hepatoma cell line HepG2. *World Journal Of Gastroenterology*. 2006; 129(7), 1081-1024.

81. Bravo L (1998). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev*. 56:317-33.

82. Klaus W.J wahle,* Dino Rotondo, Iain Brown Ana Steven D. Heys. Plant Phenolics in the Prevention and Treatment of Cancer. Bio- farms for

Nutraceuticals: Functional Food and Safety Control by Biosensors. 2009.

83. Yadav AK, Tandon V, Roa HSP (1992). In vitro anthelmentic efficacy of fresh tuber extract of Flemingiavesttia against Ascarrisuum. Fitoterapia, 63: 395-398.

84. Yang XK, Zheng F, Chen Jh, Goa QL, Lu YP, Wang SX, Wang CY, Ma D(2002). Relationship between expression of apoptosis – associated protein ana capase- 3 activity in cisplatin- resistant human ovarian cancer cell line.Ai zheng, 21: 1288-1291.

85. Leung LK, Wang TT (1999). Differential effects of chemotherapeutic agents on Bcl2/bax apoptosis pathway in human breast cancer cell line MCF-7. *Breast Cancer Res.Treat*. 55: 73-83.

86. Tudor G, Aguleria A, Halverson DO, Laing ND, Sausiville EA (2000). Susceptibility to drug- induced apoptosis correlates with differential modulation of Bad, Bcl2 and Bcl2-xl protein levels. *Cell Death Diff*. 7:574-586.