EVALUATION OF THE ANTICANCER ACTIVITY OF VARIOUS EXTRACTS OF *Evolvulus alsinoides* Linn USING LUNG CANCER CELL LINE(A549) BY INVITRO METHODS

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1. INTRODUCTION

1.1 Cancer:

- Cancer has existed for all of human history. The earliest written record regarding cancer is from circa 1600 BC in the Egyptian Edwin Smith Papyrus and describes cancer of the breast.
- Medically it is known as a malignant neoplasm.
- Cancer is a broad group of diseases involving unregulated cell growth in which cells divide and grow uncontrollably, forming malignant tumors, and invading nearby parts of the body[1].
- The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream.
- Not all tumors are cancerous. Benign tumors do not invade neighboring tissues and do not spread throughout the body[2].
- There are over 200 different known cancers that affect humans.

Six characteristics of malignancies have been proposed[3]:

- ✓ Sustaining proliferative signaling,
- ✓ Evading growth suppressors,
- ✓ Resisting cell death,
- ✓ Enabling replicative immortality,
- ✓ Inducing angiogenesis and

- ✓ Activating invasion and metastasis.

The progression from normal cells to cells that can form a discernible mass to outright cancer involves multiple steps[4].

1.2 Types:

- **Carcinoma:**

A carcinoma is tumor tissue derived from putative epithelial cells whose genome has become altered or damaged to such an extent that the cells become transformed and begin to exhibit abnormal malignant properties[5] [6].

Ex. Lung cancer, Prostate cancer, Breast cancer, Colon cancer, Pancreatic cancer.

- **Sarcoma:**

A sarcoma is a cancer that arises from transformed cells of mesenchymal origin[7].

Ex. Cancers of bone, cartilage, fat and nerve.

- **Lymphoma:**

Lymphoma is a cancer develop in the lymph nodes, spleen and bone marrow[8].

- **Leukemia:**

Leukemia is a cancer of the blood or bone marrow[9].

- **Germ cell tumor:**

Germ cell tumor is a cancer derived from Germ cells(testicle and overy) [10].

- **Blastoma:**

A blastoma is a type of cancer, more common in children that is caused by malignancies in precursor cells[11].

1.3 Causes of cancer:

Cancers are primarily an environmental disease with 90–95% of cases attributed to environmental factors and 5–10% due to genetics[12].

- **Chemical:**

Ex. Alcohol and tobacco smoking.

Tobacco smoking is associated with many forms of cancer[13] and causes 90% of lung cancer[14].

- **Diet and Exercise:**

Diet, Physical activity and obesity are related to approximately 30–35% of cancer deaths[15].

Ex. Betel nut chewing with oral cancer, high salt diet linked to gastric cancer.

- **Infection:**

Worldwide approximately 18% of cancer deaths are related to infectious diseases. This proportion varies in different regions of the world from a high of 25% in Africa to less than 10% in the developed world. Viruses are the usual infectious agents that cause cancer but bacteria and parasites may also have an effect.

A virus that can cause cancer is called an *oncovirus.*

These include human papillomavirus (cervical carcinoma),Epstein–Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi's sarcoma herpesvirus (Kaposi's sarcoma and primary effusion lymphomas), hepatitis B and hepatitis C viruses (hepatocellular carcinoma) and Human T-cell leukemia virus-1 (T-cell leukemias). Bacterial infection may also increase the risk of cancer, as seen in Helicobacter pylori-induced gastric carcinoma. Parasitic infections strongly associated with cancer include *Schistosoma haematobium* (squamous cell carcinoma of the bladder) and the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis* (cholangiocarcinoma) [16] .

- **Radiation:**

Ionizing radiation: Medical imaging and Radon gas

Non-ionizing radiation: Ultraviolet radiation and mobile phone radiation.

Radiation can cause cancer in most parts of the body, in all animals and at any age, although radiation-induced solid tumors usually take 10–15 years and can take up to 40 years, to become clinically manifest and radiation-induced leukemias typically require 2–10 years to appear[17] .

- **Heridity:**

Less than 0.3% of the population are carriers of a genetic mutation which has a large effect on cancer risk and these cause less than 3–10% of all cancer[18] .

Hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) which is present in about 3% of people with colorectal cancer[19] .

- **Physical agents:**

  A prominent example of this is prolonged exposure to asbestos, naturally occurring mineral fibers which are a major cause of mesothelioma, which is a cancer of the serous membrane, usually the serous membrane surrounding the lungs. Other substances in this category, including both naturally occurring and synthetic asbestos-like fibers such as wollastonite, attapulgite, glass wool and rock wool, are believed to have similar effects[20]

- **Hormones:**

  Some hormones play a role in the development of cancer by promoting cell proliferation[21] .

  Hormones are important agents in cancer of the breast, endometrium, prostate, ovary, testis, thyroid cancer and bone cancer[22].

  Insulin-like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis[21].

1.4 Pathophysiology:

Cancer is fundamentally a disease of tissue growth regulation failure. In order for a normal cell to convert into a cancer cell, the genes which regulate cell growth and differentiation must be altered[23].

The affected genes are divided into two broad categories.

1. Ongogenes- Promote cell growth and reproduction
2. Tumor suppressor genes-Inhibit cell division and survival[24].

Both oncogenes and tumour suppressor genes exert their effect on tumour growth through their ability to control cell division (cell birth) or cell death (apoptosis). Normal function of tumour suppressor gene is usually to restrain cell growth and this function is lost in cancer[25].

Genetic alteration:

Genetic changes can occur at different levels and by different mechanisms. The gain or loss of an entire chromosome can occur through errors in mitosis. More common are mutations, which are changes in the nucleotide sequence of genomic DNA.

Large-scale mutations involve the deletion or gain of a portion of a chromosome.

Small-scale mutations include point mutations, deletions and insertions, which may occur in the promoter region of a gene and affect its expression, or may occur in the gene's coding sequence and alter the function or stability of its protein product.

Epigenetic alteration:

Epigenetic alterations refer to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence[26].

Examples of such modifications are changes in DNA methylation (hypermethylation and hypomethylation) and histone modification and changes in chromosomal architecture (caused by inappropriate expression of proteins such as HMGA2 or HMGA1) [27].

P^{53}:

The tumour suppressor, p53, is encoded in human by the *TP53* gene. The gene itself is located on the short arm of chromosome 17 (locus: 17p13.1) and spans 20kb[28].

It plays a significant role in regulation of cell cycle and helps in preventing cancer.

Structurally, p53 is a 393 amino acid long polypeptide with seven domains including an acidic N-terminus transactivation domain, a zinc DNA-binding core domain, and a homooligomerisation domain[29]. p53 also contains a proline-rich and a second transactivation domain which are involved in the pro-apoptotic activity of p53 and its interaction with other proteins[30]. The homo-oligomerisation domain is essential for tetramisation of p53 and subsequent *in vivo* activity[31]. p53 is involved in many cellular functions including apoptosis, cell cycle arrest, DNA repair and senescence. It is modulation of these functions that have led to p53 being labelled 'the 'guardian of the genome'. Furthermore, mutations to *TP53* which result in functionally deficient p53 expression, are present in over 50% of all tumours[32]. Under normal conditions, p53 is maintained at a low level by ubiquitination, which targets the protein for degradation by the proteasome[33]. This is achieved via interaction with the negative regulator of p53, MDM2, an E3 ubiquitin ligase which binds p53 protein and marks it for degradation[34]. MDM2 forms a stable complex with p53 by binding to the N-terminal domain[35]. The C-terminal RING domain of MDM2 is vital to its ability to mark p53 for degradation, as it recruits ubiquitin-conjugating enzyme E3, resulting in polyubiquitination of p53[36].

The p53 transcriptional programme can be activated by a variety of cellular stressors such as DNA damage, oxidative stress, osmotic shock and nucleotide depletion[37] [38] [39] [40]. The first step in p53 activation involves destabilisation of the MDM2-p53 repressor complex, which increases p53 half-life and induces a rapid accumulation of p53 protein. Secondly, p53 is subjected to post-translational modifications such as acetylation and phosphorylation, which activate its transcriptional activity by driving nuclear accumulation of p53[41]. Activation of p53

plays a major role in coordinating cell cycle arrest, DNA repair and apoptosis in response to DNA damage[42].

Apoptosis induced by p53 involves the role of many mediators, important one being bax protein. Bax protein is a member of Bcl-2 protein family. p53 binding site located in the regulatory region of the gene which directly activates transcription of Bax gene and when over expressed Bax induces apoptosis.

Two major events occurring on activation of p53 are

-Half life of p53 is increased, leading to its accumulation in damaged cells

-Serves as a transcription regulator in stressed cells.

In normal humans p53 is continually produced and degraded in cells, which is associated with MDM2 binding, induced by p53 by negative feedback loop. But mutant p53 do not induce MDM2 and starts accumulating at high concentration. Mutant p53 can also inhibit normal p53 protein levels[43].

Bcl2:

Bcl-2 derives its name from *B-cell lymphoma 2*. Bcl2 is a family of regulatory protein that regulates the cell death (apoptosis), by either inducing (pro-apoptotic) it or inhibiting it (anti-apoptotic) [44] [45]. Apoptosis is the process of programmed cell death, when altered gives rise to a number of human diseases like **cancer**, autoimmune disorders and some viral infections.

Anti-apoptotic proteins of this family includes Bcl-2, Bcl-xL, Mcl-1, Bcl-w and A-1 which suppresses the cell death[46] [47].

Pro-apoptotic proteins of this family includes Bax, Bak, Bik, Bad and Bid which causes promotion of cell death[48] [49].

High expression of anti-apoptotic members like Bcl-2 and Bcl-xL which are found in human cancers leads to neoplastic cell expansion by interfering with normal cell death mechanism.

Bcl-2 protein is coded by Bcl-2 gene. **Bcl-2 gene plays a role in number of cancers like lung, breast, prostate and melanoma**[50].

It has been proposed that Bcl-2 functions via intracellularly generated reactive oxygen species (ROS) [51] [52] [53]. Bcl-2, however, prevents apoptosis induced under very low oxygen conditions, suggesting that the mechanism of Bcl-2 action may not necessarily involve the regulation of ROS activity[54]. Therefore, despite progress in defining some of the physiological roles of Bcl-2, the biochemical mechanism of its action remains unknown.

Bax gene was the first pro-apoptotic member of the Bcl-2 family, which promotes apoptosis by competing with Bcl-2. Tumour suppressor protein p53 up regulates the expression of Bax and Bax is proven to be involved in apoptosis mediated by p53.

Majority of Bax is found in cytosol and upon initiation of apoptosis; it undergoes a conformation shift and fits into outer mitochondrial membrane. Further it results in the release of cytochrome c and other pro-apoptotic factors from mitochondria leading to apoptosis[55].

Tumor necrosis factor :

Tumor necrosis factor (**TNF**, **cachexin**, or **cachectin**, and formerly known as **tumor necrosis factor alpha** or **TNFα**) is an adipokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction.

It is produced chiefly by activated macrophages.

It can also produced by other cells such as CD4+ lymphocytes, NK cells and neurons[56].

The primary role of TNF is in the regulation of immune cells.

TNF, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumorigenesis.

Dysregulation of TNF production has been implicated in a variety of human diseases including alzheimer's disease[57], cancer[58], major depression[59] and inflammatory bowel disease (IBD) [60].

TNF can bind two receptors, TNFR1 (TNF receptor type 1) and TNFR2 (TNF receptor type 2). TNFR1 is expressed in most tissues, where as TNFR2 is found only in cells of the immune system[61].

When ligand binds to the TNF receptors, adaptor protein TRADD binds to death domain which enables subsequent protein binding, thereby initiating three pathways, namely NF-κβ pathway, MAPK pathway and by induction of death signaling[62].

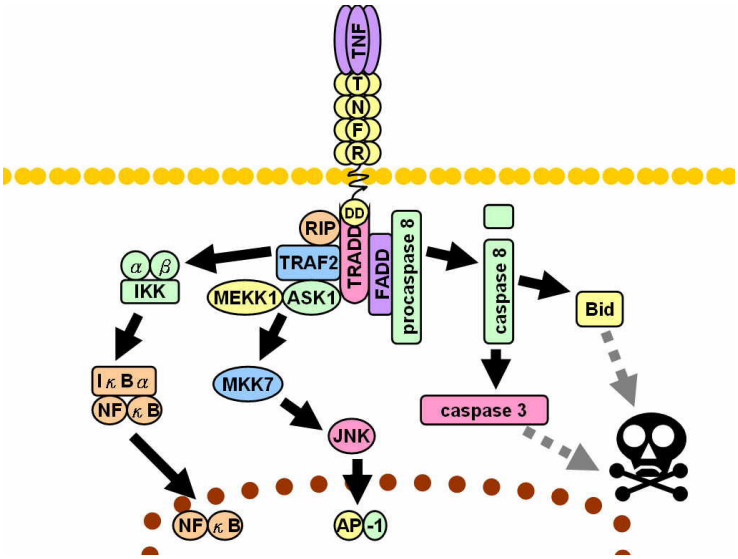


Figure.1.1 Signaling pathway of TNFR1. Dashed grey lines represent multiple steps.

Interleukin:

Interleukins are a group of cytokines that were first seen to be expressed by white blood cells (leukocytes). The function of the immune system depends in a large part on interleukins. The majority of interleukins are synthesized by helper CD4 T lymphocytes, as well as through monocytes, macrophages and endothelial cells[63]. Interleukin 6 (IL-6) is a pro inflammatory cytokine producing multifunctional effects[64].

Interleukin-6 interact with interleukin-6 receptor[65] [66] and glycoprotein-130[67].

De regulation in IL-6 production and signaling are associated with chronic inflammatory diseases, auto immune disorders and **cancer.**

Inhibition of IL-6 production, its receptors or the signaling pathways are current trends of novel therapies for a wide range of diseases. High serum IL-6 levels were detected in patients with lung, breast, prostate, colorectal, gastric, pancreatic, ovarian and renal cell cancers. High serum IL-6 levels were associated with progressive diseases and poor survival[68].

IL-6 stimulates the inflammatory and auto-immune processes in many diseases such as diabetes[69], atherosclerosis[70], major depressive disorder[71], alzheimer's Disease[72], systemic lupus erythematosus[73], multiple myeloma[74], cancer[75], Behçet's disease[76], and rheumatoid arthritis[77].

Advanced/metastatic cancer patients have higher levels of IL-6 in their blood[78].

Hence there is an interest in developing anti-IL-6 agents as therapy against many of these diseases[79] [80].

1.5 LUNG CANCER:

Lungs are located in the chest, when we breathe air goes through our nose, down the windpipe and into the lungs, where it spreads through tubes called bronchi. Most lung cancer begins in the cells that line these tubes. Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue or other parts of the body[86].

Cancers that start in the lungs are known as primary lung cancers are carcinomas that are derived from epithelial cells[86].

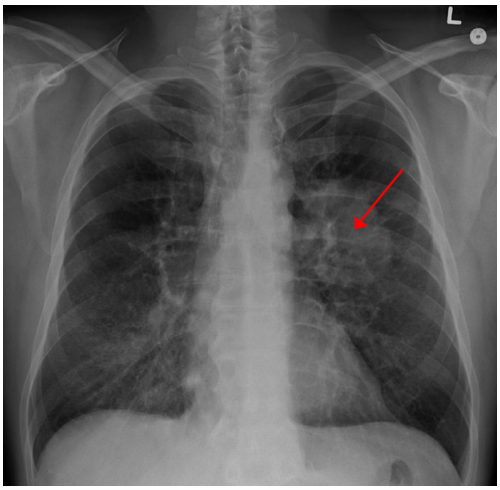


Figure 1.2 A chest X-ray showing a tumor in the lung (marked by arrow)

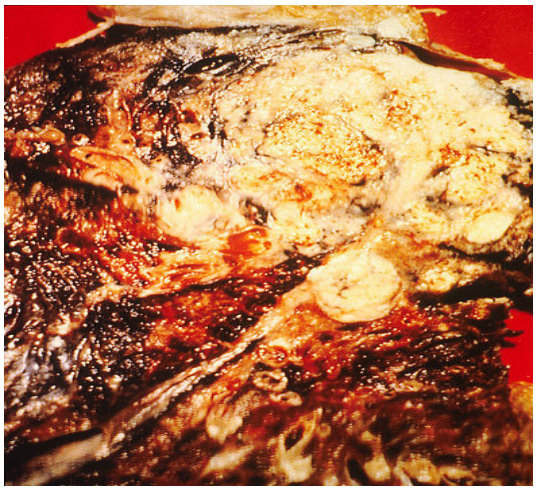


Figure 1.3 Cross section of a human lung: The white area in the upper lobe is cancer; the black areas are discoloration due to smoking.

- The main types of lung cancer are:
  - **Small cell lung carcinoma (SCLC)** also known as 'oat cell carcinoma'
  - **Non small cell lung carcinoma (NSCLC)**.

Small cell lung cancer is a fast growing type of lung cancer and it spreads more quickly than non small cell lung cancer.

Two different types of SCLC are

- Small cell carcinoma (oat cell cancer)
- Combined small cell carcinoma.

Non small cell lung cancer (NSCLC) is the most common type of lung cancer. It usually grows and spreads more slowly than small cell lung cancer.

Three forms of NSCLC are

- Adenocarcinomas are found in the outer area of the lung.
- Squamous cell carcinoma are usually found in the centre of the lung next to an air tube (bronchus).
- Large cell carcinomas can occur in any part of lung where they tend to grow and spread faster than other two types[87].

CANCER- GLOBAL REVIEW:

In 2008 approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers) [81].

In 2010 nearly 7.98 million people died with cancer[82].

Cancers account for nearly **13%** of all deaths each year with most common being

- **Lung Cancer (1.4 million deaths)**
- Stomach Cancer (7,40,000 deaths)
- Liver Cancer (7,00,000 deaths)
- Colorectal Cancer (6,10,000 deaths)
- Breast Cancer (4,60,000 deaths) [83].

Overall men have 44% risk of developing cancer at some time during their lives and women have 38% lifetime risk. In the year 2010 in USA, incidence of lung cancer ranked second after prostate cancer in male and breast cancer in female.

In deaths caused due to cancer, lung cancer outnumbered prostate cancer in male and breast cancer in women to top the chart with 86220 deaths in men and 71080 deaths in women[84] [85].

Lung cancer is the most common cancer and the most common cause of cancer deaths in world.

From the above mentioned data it is quite clear that Lung Cancer is the most deadly disease of all cancers and let's shift our focus purely to lung cancer.

CAUSES:

Smoking:

Smoking, particularly of cigarettes, is the main contributor to lung cancer[88].

Cigarette smoke contains over 60 known carcinogens[89] including radioisotopes from the radon decay sequence, nitrosamine and benzopyrene. Additionally, nicotine appears to depress the immune response to cancerous growths in exposed tissue[90].

Across the developed world, 90% of lung cancer deaths in men during the year 2000 were attributed to smoking (70% for women). Smoking accounts for 80–90% of lung cancer cases[91].

Passive smoking: the inhalation of smoke from another's smoking is a cause of lung cancer in nonsmokers. Studies from the US, Europe, the UK, and Australia have consistently shown a significantly increased risk among those exposed to passive smoke[92].

Passive smoking causes about 3,400 deaths from lung cancer each year in the USA[93].

Radon gas:

Radon is a colorless and odorless gas generated by the breakdown of radioactive radium, which in turn is the decay product of uranium, found in the Earth's crust. The radiation decay products ionize genetic material, causing mutations that sometimes turn cancerous. Radon is the second-most common cause of lung cancer in the USA[93].

The risk increases 8–16% for every 100 Bq/m³ increase in the radon concentration[94].

Asbestos:

Asbestos can cause a variety of lung diseases, including lung cancer. Tobacco smoking and asbestos have a synergistic effect on the formation of lung cancer[95].

Asbestos can also cause cancer of the pleura, called mesothelioma (which is different from lung cancer) [96].

Air pollution[97] and **heredity**[98] are the other factors of lung cancer.

Pathogenesis:

Lung cancer is initiated by activation of oncogenes or inactivation of tumor suppressor genes.[99] Oncogenes are believed to make people more susceptible to cancer. Proto-oncogenes are believed to turn into oncogenes when exposed to particular carcinogens.[100] Mutations in the *K-ras* proto-oncogene are responsible for 10–30% of lung adenocarcinomas.[101] The epidermal growth factor receptor (EGFR) regulates cell proliferation, apoptosis, angiogenesis and tumor invasion. [101]] Mutations and amplification of EGFR are common in non-small-cell lung cancer and provide the basis for treatment with EGFR-inhibitors. Her2/neu is affected less frequently.[101] Chromosomal damage can lead to loss of heterozygosity. This can cause inactivation of tumor suppressor genes. Damage to chromosomes 3p, 5q, 13q, and 17p are particularly common in small-cell lung carcinoma. The *p53* tumor suppressor gene, located on chromosome 17p, is affected in 60-75% of cases. Other genes that are often mutated or amplified are *c-MET*, *NKX2-1*, *LKB1*, *PIK3CA*, and *BRAF*.[101]

Symptoms[102]:

Respiratory symptoms: coughing, coughing up blood, wheezing or shortness of breath

Systemic symptoms: weight loss, fever, clubbing of the fingernails, or fatigue

Symptoms due to local compress: chest pain, bone pain, superior vena cava obstruction, difficulty swallowing

If the cancer grows in the airway, it may obstruct airflow, causing breathing difficulties. The obstruction can lead to accumulation of secretions behind the blockage, and predispose to pneumonia.

Depending on the type of tumor, so-called paraneoplastic phenomena may initially attract attention to the disease. In lung cancer, these phenomena may include Lambert–Eaton myasthenic syndrome(muscle weakness due to autoantibodies), hypercalcemia, or syndrome of inappropriate antidiuretic hormone (SIADH). Tumors in the top of the lung, known as Pancoast tumors, may invade the local part of the sympathetic nervous system, leading to Horner's syndrome (dropping of the eyelid and a small pupil on that side), as well as damage to the brachial plexus.

Many of the symptoms of lung cancer (poor appetite, weight loss, fever, fatigue) are not specific. In many people, the cancer has already spread beyond the original site by the time they have symptoms and seek medical attention. Common sites of spread include the brain, bone, adrenal glands, opposite lung, liver, pericardium, and kidneys.

About 10% of people with lung cancer do not have symptoms at diagnosis these cancers are incidentally found on routine chest radiography[103].

Prevention:

Prevention is the most cost-effective means of decreasing lung cancer development. While in most countries industrial and domestic carcinogens have been identified and banned, tobacco smoking is still widespread. Eliminating tobacco smoking is a primary goal in the prevention of lung cancer, and smoking cessation is an important preventive tool in this process.

Policy interventions to decrease passive smoking in public areas such as restaurants and workplaces have become more common in many countries[104].

Treatment:

Treatment for lung cancer depends on the cancer's specific cell type, its spread and patient's performance status. Most common treatments include palliative care, chemotherapy and radiation therapy.

For non small cell lung cancers, the treatment depends on the stage of cancer.

Surgery is the most common treatment for patients with non small cell lung cancer.

Surgery includes

- One of the lobes of the lung (lobectomy)
- Only small part of the lung (wedge or segment removal)
- Entire lung (pneumonectomy)

Chemotherapy:

In SCLC cisplatin and etoposide are commonly used.

NSCLC is **cisplatin** or **carboplatin** and other drugs are **gemcitabine, paclitaxel, docetaxel, etoposide or vinorelbine**

Adjuvant chemotherapy which refers to use of chemotherapy after apparently curative surgery to improve the outcome.

For potentially curable SCLC cases, chest radiotherapy is often recommended in addition to chemotherapy. Prophylactic cranial irradiation (PCI) is a type of radiotherapy to the brain, used to reduce the risk of metastases. Palliative care or hospice management plays a great role in patients with terminal disease. Chemotherapy may be combined with palliative care in treatment of the NSCLC[105].

LUNG CANCER IN INDIA[106]:

INDIA	MALE	FEMALE	BOTH SEXES
Population (thousands)	610618	570793	1181412
Number of new cancer cases (thousands)	430.1	518.8	948.9
Age-standardised rate (W)	92.9	105.5	98.5
Risk of getting cancer before age 75 (%)	10.2	10.8	10.4
Number of cancer deaths (thousand)	321.4	312.1	633.5
Age-standardised rate (W)	71.2	65.5	68
Risk of dying from cancer before age 75 (%)	8	7.1	7.5
5 most frequent cancers	Lung		
	Breast		
	Cervix uteri		
	Oesophagus		
	Lip, oral cavity		

Table 1.1 shows the burden of lung cancer in India as reported in the Globocan Report 2008.

2. SCOPE OF THE WORK

With cancer being a leading cause of death worldwide, it seems obvious that it would be an important research focus for any medical research institute[107].

Lung cancer is one of the most common cancers we have and a large number of people die of this disease every year. The disease is often discovered in a late stage, but also in earlier stages lung cancer patients have worse outcome than patients with other cancers. Even without spreading to other organs during stage I the survival rate of lung cancer is under 70%. In comparison, for example breast cancer there is 95% survival in stage I and also Lung cancer is the first most common malignancy among population worldwide. With limited success pertaining to available treatment options for lung cancer, alternative and complimentary strategies need to be developed[108].

Several studies have revealed that Epidermal growth factor receptor; a member of receptor tyrosine kinase has been over expressed and identified as a therapeutic target for non small cell lung cancer.

Tyrosine kinase inhibitors like Erlotinib, Gefitinib and monoclonal antibodies have been recently approved in treatment of lung cancer, but the response rates to these drugs are modest and therefore new strategies to treat human lung carcinomas are still a major focus of research.

Even though significant progress has been made in cancer prevention and treatment, the development of effective treatment regimens remains one of the greatest challenges in the area of cancer therapy. Hence there arises a need for continuous search of safer and more effective chemoprevention and treatment to improve the efficacy and reduce the cost of cancer care.

Cancer chemoprevention with natural phytochemical compound is an emerging strategy to prevent, impede, delay or cure cancer[109].

India is one of the earliest civiliza-tions that have recognized the importance of herbal products for disease management, nutrition and beauty enhancement. With the discovery of several new molecules from herbs for treating dreaded diseases like cancer and the relative safety of these products, the global demand for medicinal plant products has increased in recent years.

More than 30% of the pharmaceutical preparations are based on plants (Shinwari and Khan, 1998). An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from medicinal plants. Searching for new drugs in plants implies the screening the plants for the presence of novel compounds and investigation of their biological activities.

Many medicinal plants contain large amount of chemical components having broad spectrum of pharmacological activities. Anticancer activities are mainly due to phenolic acids, flavanoids and phenolic diterpenes. **Natural products** have long been a rich source of cure for cancer, which has been the major cause of death in the past decades[110]

- **Taxol**, one of the most outstanding drug used for the treatment of metastatic ovarian, breast carcinoma and small cell lung cancer have been obtained from bark of western yew tree.
- **Etoposide**, a semisynthetic derivative of podophyllotoxin, a plant glycoside being used in treatment of testicular tumors, lung cancer and bladder cancer.
- **Topotecan** and **irinotecan** are two recently introduced semisynthetic analogues of camptothecin and anti tumor principle obtained from Chinese tree.

- **Vincristine, vinblastine, colchicines** and **ellipticine** are other important molecules from plant source.

There are nearly 250000 species of plants out of which more than one thousand plants have been found to possess significant anticancer properties[111].

Considering the toxicities which arise from cytotoxic drugs like **bone marrow depression, alopecia, lymphocytopenia and occurrence of secondary cancers like leukemia and lymphomas**, the search further intensifies for the toxicity free herbal remedy for cancer, which acts by without interfering with the body's natural healing process[112].

Evolvulus alsinoides L. (Convolvulaceae), commonly known as 'shankhpuspi' in India, Africa and the Philippines, is an important medicinal plant employed for different ailments in India traditionally.

The herb was used to treat dysentery. Mohammedan physicians used the plant as a general tonic to strengthen the brain and memory and to treat fever. *Evolvulus alsinoides* was used to treat bowel problems and to promote conception. The entire plant was considered astringent and useful for treating haemorrhages and there are a variety of other medical applications, including use as an adaptogenic, antiphlogistic, antipyretic, antiseptic, aphrodisiac, febrifuge, stomachic, tonic and vermifuge, in the treatment of asthma, bronchitis, scrofula, syphilis, or in "controlling night emissions" and to promote wound healing.

Its medicinal properties like **anti-inflammatory, antipyretic, antidiarrhoeal, antidiabetic, antioxidant, antifungal, antimicrobial, antidyskinesia and anthelmintic properties** were scientifically evaluated and reported.

Potential effect of *Evolvulus alsinoides* on hair growth also reported.

But there was no report for the evaluation of its anticancer activity focusing on gene expression levels and protein levels in cancerous cells along with apoptosis study.

So the study was carried out to evaluate the anticancer effect of various extracts of leaves of *Evolvulus alsinoides* in **human lung cancerous cell line A549** using various *in vitro* models.

3. OBJECTIVE

- To identify the Phytochemical constituents present in Ethyl acetate, Ethanol and Petroleum ether extract of *Evolvulus alsinoides.*

- To evaluate the cytotoxic activity of Ethyl acetate, Ethanol and Petroleum ether extract of *Evolvulus alsinoides* in human lung cancerous cell line A549 and to establish IC_{50} concentration.

- To evaluate the Apoptotic effect of effective extract of *Evolvulus alsinoides* .

- To evaluate the Protein levels in the effective extract of *Evolvulus alsinoides* treated cancerous cell line.

- To evaluate the Gene expression levels in the effective extract of *Evolvulus alsinoides* treated cancerous cell line.

4. PLANT PROFILE

Figure 4.1. ***Evolvulus alsinoides Linn***

Plant name : *Evolvulus alsinoides*[113]

Kingdom	Plantae
Sub-kingdom	Tracheobionta
Super-division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Asteridae
Order	Solanales
Family	Convolvulaceae
Genus	*Evolvulus*
Species	*Alsinoides*

Vernacular names:

Tamil : Vishnukranti,

Malayalam : krishnakranti,

Telugu : Vishnukranta,

Kannada : Shankhapushpi.

Hindi : Shankhauli,

Sanskrit : Vishnu krantha**,** Harikrantha, Shankha pushpi,

English : Dwarf morning glory,

Distribution:

Widely distributed in tropical and subtropical regions throughout the world. It grows commonly as a weed in open and grassy places throughout India, ascending at 6000ft.

Parts used: whole plant

Description: [114]

Leaves: Leaves are small, entire, elliptic to oblong, obtuse, apiculate, base acute and densely hairy. Petiole is minute or nearly absent. Bracts are linear and persistent.

Flowers: Flowers mostly solitary in upper axils. Corolla blue rotate and broad funnel shaped, Calyx is lobed, lanceolate and the tip acute. Peduncle is long and axillary.

Branches: Its branches are annual, numerous, more than 30 cm long, often prostrate, slender and wiry with long hairs.

Chemical constituents:

The plant contains an alkaloid-shankhapushpine. Fresh plant contains volatile oil and potassium chloride. It also contains a yellow neutral fat, an organic acid and saline substances. Three alkaloids- evolvine, betaine, and an unidentified compound have been isolated.

Uses: [115]

- Nootropic agent,
- Chronic bronchitis.
- Used in fever, nervous debility, loss of memory, also in syphilis and scrofula.
- It is used as Rasayan. Whole plant is used as hepatoprotective

Traditional medicinal uses: [116]

- The whole herb is used medicinally in the form of decoction with cumin and milk in fever, nervous debility, loss of memory and syphilis.
- Decoction of the drug, with Ocimum sanctum is administered in fevers accompanied by indigestion or diarrhea. Decoction was given in cases of malarial fever.
- The root is used by the santals, for intermittent childhood fever,
- The leaves are made into cigarettes and smoked in chronic bronchitis and asthma.
- The oil promotes the growth of hair.

5. REVIEW OF LITERATURE

5.1 WORK DONE IN *Evolvulus alsinoides*:

Adaptogenic, anxiolytic and anti-amnesic activity:

Alok Nahata et al.,2009 Ethanol extract of the aerial parts of the drug was evaluated for CNS activity by using elevated plus maze test, open field exploratory behavior and rota rod performance experiments. The ethanol extract as well as its ethyl acetate and aqueous fractions was tested in experimental models employing rats and mice. The extracts were also studied for their in vitro antioxidant potential to correlate their anxiolytic activity[117].

Kiran Babu Siripurapu et al.,2005 The improvement in the peripheral stress markers and scopolamine-induced dementia by *Evolvulus alsinoides* in the chronic unpredictable stress and acute stress models indicated the adpatogenic and anti-amnesic properties of *Evolvulus alsinoides*, against a well known adaptogen i.e. *Panax quinquefolium*[118].

GuptaP et al.,2007 Phenolics and flavonoids, isolated form bioactivity-guided purification of n-BuOH soluble fraction from the ethanol extract of *Evolvulus alsinoides*, were screened for antistress activity in acute stress models. Stress exposure resulted in significant increase of plasma glucose, adrenal gland weight, plasma creatine kinase and corticosterone levels[119].

Mehta CR et al.,1959 Effects of methanolic extracts of roots of *Evolvulus alsinoides* (MEEA) on acute reserpine-induced orofacial dyskinesia showed increased vacuous chewing frequencies (VCMs) and TPs in acute reserpine-treated animals compared with vehicle-treated

animals. Chronic treatment significantly reversed the reserpine-induced VCMs and TPs in a dose-dependent manner, decreased the locomotor activity as well as the transfer latency in acute reserpine-treated rats[120].

Antioxidant:

Cervenka F et al.,2008 Antioxidant substances were isolated and identified from *Evolvulus alsinoides* by preparing fractions of phenolic and non-phenolic compounds. Results of antioxidant activities of *Evolvulus alsinoides* from 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were not as high as expected. The need of more antioxidant tests with different action mechanisms and also *in-vivo* studies with *Evolvulus alsinoides* were suggested[121].

Ethanolic extracts and water infusion of *Evolvulus alsinoides* were tested for their antioxidant activity in the 2, 2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical cation decolorization assay. Inhibition of lipid peroxidation by plant infusions was carried out using spontaneous lipid peroxidation of rat brain homogenate, and IC50 values were determined. The results from the ABTS assay showed that the ethanolic extract of *Sida cordifolia* was found to be most potent (IC50 16.07 μg/ml), followed by *Evolvulus alsinoides* (IC50 33.39 μg/ml) and *Cynodon dactylon* (IC50 78.62 μg/ml).

Immunomodulatory activity:

Ganju L et al.,2003 The crude extracts of *Emblica officinalis* and *Evolvulus alsinoides* were evaluated for immmunomodulator activity in adjuvant induced arthritic rat model. The anti inflammatory response of both the extracts was determined by lymphocyte proliferation activity and histopathological severity of synovial hyperplasia. Both the extracts showed a marked

reduction in inflammation and edema. At cellular level immunosuppression occurred during the early phase of the disease. There was mild synovial hyperplasia and infiltration of few mononuclear cells in *E.officinalis* or *Evolvulus alsinoides* treated animals. The induction of nitric oxide synthase (NOS) was significantly decreased in treated animals as compared to controls[122].

Evolvine hydrochloride

Krishnamurthy TR et al.,1959 The hydrochloride of alkaloid, evolvine was reported to exhibit lobeline-like action on the cardiovascular system. In cats, the drug demonstrated sympathomimetic activity. The blood pressure remained elevated for a longer duration as compared to adrenaline. Increase in peripheral pressure was observed on local injection of the drug[123].

Antiulcer and anticatatonic activity

Purohit MG et al.,1996 The in vivo evaluation of the alcoholic extract of *Evolvulus alsinoides* revealed its marked antiulcer and anticatatonic activity[124].

Anti Diarrhoeal activity:

Dhana Lekshmi U.M et.al 2011 The extract of E. alsinoides showed dosedependent effects in controlling the diarrhoea. The results are similar to those of the standard drug diphenoxylate with regard to severity of diarrhoea[125].

Antiinflammatory effect

Dhana Lekshmi U.M et.al 2011 Carrageenan-induced rat paw oedema was markedly inhibited by oral pre-treatment with the *Evolvulus alsinoides* extract (250mg/kg and 500mg/kg body weight doses) and indomethacin (20mg/kg body weight dose). In the acute inflammation model, a dose of 250mg/kg body weight of the extract showed significant inhibition ($P < 0.05$) at

3 and 4h, whereas indomethacin and the extract (500mg/kg body weight) exhibited significant inhibition at 2, 3, and 4h[125].

Antipyretic studies

Dhana Lekshmi U.M et.al 2011 The plant extract produced a reduction in hyper pyrexia induced by yeast injection in rats, with activity being pronounced within 90min after administration of the extract. Also, within 2h of administration of the extract, the plant extract was as effective as paracetamol in reducing hyperthermia ($P < 0.05$) [125].

Learning behavior and memory enhancement activity:

Nahata et al., 2010 Two doses (100 and 200mg/kg p.o.) of the ethanol extract and ethyl acetate and aqueous fractions were administered in separate groups of animals. Both doses of all the extracts of EA significantly improved learning and memory in rats[126].

Anthelmintic and Antimicrobial activities:

G. K. Dash et al.,2002 The results of antimicrobial activity revealed that the extract exhibited activity against *Pseudomonas aeruginosa* and *Escherichia coli* but inactive against *Staphyloccus aureus* and *Candida albicans*[127].

Hair Growth:

Bhaiji Amrita et al.,2012 Petrolium ether and methanolic extracts of *Evolvulus alsinoides* has significant hair growth potency in albino rats. The extracts were incorporated into oleaginous base and applied topically on shaved denuded skin of albino rats for 30days. Time required for hair growth initiation and completion was significantly reduced($P< 0.01$) [128].

lipid metabolism:

Duraisami gomathi et al.,2013 The ethanolic extract of *Evolvulus alsinoides* supplementation is useful in hyperlipidemia prevention during diabetes mellitus[129].

Phytochemical analysis:

Omogbai B.A et.al.,2011 showed phytochemical analysis of ethanolic and water extract of *Evolvulus alsinoides* indicated the presence of Glycosides, Alkaloids, Saponins, Tannins, Flavonoids, Volatile oil in ethanolic extract and indicated the presence of Glycosides,alkaloids in water extract[130].

Duraisamy Gomathi et.al 2012 showed phytochemical analysis of ethanol, chloroform, petroleum ether and ethyl acetate extracts of *Evolvulus alsinoides*. The study indicates the difference in the presence of Glycosides, Alkaloids, Saponins, Tannins, Flavonoids, Volatile oil, and Steroids in above extracts[131].

Acute toxicity Studies:

Agarwala N et.al.,1977 showed that the ethanolic (95%) extract of *Evolvulus alsinoides* possess CNS depressant activity with LD50 7500mg/kg[132].

5.2 A549:

A549 cells are adenocarcinomic human alveolar basal epithelial cells which was first developed by D.J. Giard et al., by removing and culturing of cancerous lung tissue in the explanted tumour of a 58year old Caucasian male. When cultured *in vitro* they grow as monolayer cells, adherent to the culture flask. These cells are squamous in nature and are responsible for diffusion substances such as electrolytes and water across the alveoli of lungs.

Membrane phospholipids in cells are maintained by lecithin and high levels of desaturated fatty acids which are synthesized by these cells. A549 can be anchored or suspended in a solution *in vitro.*

A Levy et al.,2011 found that *Cassia alata* leaf extract exhibits cytotoxic activity in A549 lung cancer cells that is dependent on the activation of caspase8. These preliminary results could be justified by the cytotoxic activity of the flavonoid kaempferol which is present in *C alata* species. Similar cytotoxic activity induced by kaempferol has been reported in ovarian cancer cells. Furthermore, kaemp-ferol has been linked to caspase8 and caspase3 activation. Preliminary analysis of the *C alata* extract using HPLC has identified kaempferol as a major constituent in the leaf extract[133].

Qing-Yi Lu et al.,2009 found that the effect of green tea extract on lung cancer (A549) cell line they found that Green tea extract stimulates the expression of lamin A/C in a dose-dependant fashion and that the proteins were present both in nucleoplasm and cytoplasm. Green tea extract induced lamin A/C and annexin I may work together to modulate cell motility[134].

Pourhassan Mohammad et.,al 2011 found the inhibitory effect of *Curcuma longa* extract on telomerase activity in A549 lung cancer cell line[135].

5.3 5-Fluorouracil (5FU) :

Sreelatha et al., 2011 reported the Evaluation of anticancer activity of ethanol extract of *Sesbania grandiflora* (Agati Sesban) against Ehrlich ascites carcinoma in Swiss albino mice. *Sesbania grandiflora* extracts showed significant decrease in ($p<0.01$) tumor volume, viable cell count, tumor weight and elevated the life span of EAC bearing mice. Hematological profile such as RBC, hemoglobin and lymphocyte count reverted to normal level in EESG treated mice. The

extracts significantly ($p<0.05$) decreased the levels of lipid peroxidation and significantly ($p<0.05$) increased the levels of GSH, SOD and CAT. The results showed that the ethanol extract of Sesbania grandiflora was effective in inhibiting the tumor growth in ascitic models and that is comparable to 5-Fluorouracil[136].

M. Thirumal et al., 2012 reported invitro anticancer activity of tecomastans (l.) ethanolic leafextract on human breast cancer cell line (mcf-7). Tecomastans is called as yellow elder in english. Crude ethanolic extract of Tecomastans leaves were examined for their anti cancer activity invitro by testing on MCF-7 breast cancer cell lines using 5-FU as a standard. Activity confirmed by MTT assay[137].

5.4 P53:

Yeung-Leung Cheng et al., 2008 reported The Extract of *Hibiscus syriacus* inducing apoptosis by activating p53 and AIF in Human Lung Cancer Cells. The root bark of *Hibiscus syriacus* has been used as an antipyretic, anthelmintic and antifungal agent in Asia. The antiproliferative effects of *H. syriacus* on human lung cancer cells were evaluated with MTT assay. The apoptotic activity was detected by Hoechst 33342 DNA staining and annexin V staining. The expression of caspases, **p53**, apoptosis induced factor (AIF), Bcl-2 and Bax were evaluated with Western blotting. The *in vivo* anticancer activity was evaluated using A549-xenograft model. The acetone extract of *H. syriacus* (HS-AE) exhibited a better cytotoxic effect on lung cancer cells than its methanol extract (HS-ME) or water extract (HS-WE). The IC_{50} values of HS-AE on A549 (adenocarcinoma), H209 (squamous cell carcinoma) or H661

(large cell carcinoma) lung cancer cells ranged from 14 to 22µg/ml after 48hours of treatment[138].

Jiayu Gatoso et al.,(2011) reported the apoptotic effect of ethanolic extract of *Scutellaria baicalensis* and its active components in lung cancer cell lines A549, SK-LU-1 and SK-MES-1 using 2D gel electrophoresis coupled with protein fingerprinting, flow cytometry and immune blotting analysis. It was observed that following treatment with the extract, increased expression of key proteins like p53 and bax were directly related to the enhancement of apoptotic effect[139].

5.5 Bcl2:

Azizi E et al.,2009 reported the Evaluation of p53 and Bcl-2 genes and proteins expression in human breast cancer T47D cells treated with extracts of Astrodaucus persicus (Boiss.) in comparison to Tamoxifen. since tumorigenesis is thought to result from a series of progressive gene alterations, including activation of oncogenes and inactivation of tumor suppres sor genes, expression of two such genes, p53 and Bcl-2 that are believed to play a crucial role in tumorigenesis and cell death were determined. The results suggest that the methanolic extracts of *Astrodaucus persicus* especially its root extract may contains bioactive compounds, probably coumarins that prevents proliferation of T47D breast carcinoma cells by mechanisms such as apoptosis[140].

Araujo Junior RF et al., 2013 reported *Maytenus ilicifolia* dry extract protects normal cells, induces apoptosis and regulates Bcl-2 in human cancer cells. *Maytenus ilicifolia* is widely used in traditional Brazilian medicine to treat stomach conditions including nausea, gastritis, and

ulcers. The apoptotic effects of a spray-dried extract of *M. ilicifolia* (SDEMI) was evaluated using human hepatocellular cells (HepG2), colorectal carcinoma cells (HT-29) and normal keratinocytes (HaCaT). Positive caspase-3 staining and down-regulation of Bcl-2 were observed, consistent with the induction of cell death detected in these cell lines[141].

5.6 TNF:

Leong, Perng char et al.,2012 has reported that Apigenin, a natural plant flavones induces apoptosis via tumour necrosis factor and Bcl 2 mediated pathways. It has been shown that apigenin inhibits the growth of SCC25 and A431 cells and induces cell cycle arrest in the G2/M phase. It has been reported that apigenin possess antioxidant property and induces cell apoptosis via up regulation of tumour necrosis factor receptor (TNF-R), Bcl 2 mediated caspase dependant cell death pathways in SCC25 cells and TNF related apoptosis inducing lligand receptor (TRAIL-R) [142].

Hasan TN et al.,2011 reported the Anti-proliferative effects of organic extracts from root bark of *Juglans Regia L.* (RBJR) on MDA-MB-231 human breast cancer cells and role of Bcl-2/Bax, caspases and Tp53. The study was undertaken to investigate the effect of root bark of Juglans regia (RBJR) organic extracts on cell proliferation and to determine the molecular mechanism of RBJR-induced cell death by determining the expression of Bcl-2, Bax, caspases, Tp53, Mdm-2 and TNF-alpha in MDA-MB-231 human breast cancer cells. Real Time PCR and western blot analysis revealed that the expression of of Bax, caspases, tp53 and TNF-alpha was markedly increased in MBA-MB-231 cells treated with the RBJR extract[143].

Kim TY et al.,2009 reported the Ethyl alcohol extracts of Hizikia fusiforme sensitize AGS human gastric adenocarcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis[144].

5.7 Interleukins:

Dipak Giri et.al., 2001 reported that Interleukin-6 is an Autocrine Growth Factor in Human Prostate Cancer. They found that interleukin (IL)-6 protein concentrations are increased ~18-fold in clinically localized prostate cancers when compared to normal prostate tissue. The concentration of IL-6 receptor is increased eight fold in the prostate cancer tissues and is increased in the cancer cells by immunohistochemistry. These findings strongly support the hypothesis that IL-6 acts as a significant autocrine growth factor *in vivo* for primary, androgen-dependent prostate cancers. It was concluded that IL-6 antagonist may play a role in treatment of prostate cancer[145].

Sizhi Paul Gao et al.,(2007) reported that tyrosine-phosphorylated STAT3 (pSTAT3) is found in 50% of lung adenocarcinomas and cell lines expressing these persistently activated mutant EGFRs also produced high IL-6 levels. IL-6 expression is substantially elevated in and IL-6 is secreted by, multiple lung cancer cell lines that harbor EGFR mutations. EGFR is a membrane-bound receptor tyrosine kinase and on ligand binding, EGFRs initiate activation of a series of cellular signal transduction pathways that regulate cell proliferation and survival. The EGFR either is mutated or shows altered expression in a variety of human cancers, including carcinomas of the lung, breast, head and neck, ovary and bladder. Blockade of the IL-

6/gp130/JAK pathway led to a decrease in pSTAT3 levels resulting in decreased tumorigenesis[146].

5.8 Plasma C Reactive protein:

According to **Oliveira EB et al.,(1979)** complete amino acid sequence was derived for human c reactive protein and it was found to contain 187 amino acids in a single polypeptide chain. Based on its amino acid composition, minimum molecular weight of 20,946 daltons was calculated for human CRP and it coincides with the molecular weight of 21500 established by gel filteration of CRP in 5.0 M guanidine chloride[147].

Chaturvedi et al., reported the risk of C-reactive protein and its association with lung cancer. A nested case control study of 592 lung cancer patients and 670 controls were carried out and CRP levels were measured. It was reported that increase in CRP levels were associated with increased risk of lung cancer. The levels were reported to be high in case of smokers and in lung cancer patients when compared to former smokers and 2 to 5 years before diagnosis of cancer respectively[148].

5.9 Phosphohistidine Phosphatase:

Xu A et al., (2010) reported that 14 KDa phosphohostidine phosphatase (PHP14) was the first protein histidine phosphatase to be discovered. Its role in lung cancer cell migration and invasion was reported. It was reported that knockdown of PHP14 expression in highly metastatic lung cancer CL 1-5 cells inhibited migration and invasion *in vitro* without altering cell proliferation. Over expression of PHP14 in NCI H1299 cells promoted migration and invasion *in-vitro.* Role of metastatic properties of PHP14 *in vivo* was evaluated by experimental

metastasis assay and it was found that experimental metastasis *in vivo* was extensively inhibited by PHP14 knockdown. The mechanism involved in involvement of PHP14 in cell migration, invasion and metastasis was further examined by proteomics analysis. The results of this study revealed that PHP14 was probably involved in cyto skeletal reorganization and it was supported by actin filament (F actin) staining. It was concluded that PHP14 may be functionally important in lung cancer cell migration and invasion of lung cancer cells mediated through modulation of actin cytoskeleton rearrangement[149].

Su Xia Han et al., (2012) reported that PHP14 is highly expressed in hepatocellular carcinoma (HCC) tissues and cell lines when compared with adjacent non cancerous human liver tissues and cells. Lenti virus mediated delivery of small interfering RNA (siRNA) was used to knockdown the expression of PHP14 in HCC cell line and the effects of PHP14 on cell growth was investigated *in vitro.* It was found that cell proliferation was inhibited and cell apoptosis was markedly increased. Flow cytometry analysis was used to examine how PHP14-si affects cell cycle and it was found that cells after treatment with PHP14 si for 96hours, there was significant increase in G1 phase population in all HCC cells and it indicated that knockdown of PHP14 expression inhibited cell proliferation, which was necessary for entering into S phase. It was concluded that knockdown of PHP14 expression by lenti virus delivered siRNA may be useful in treatment of HCC[150].

5.10 Soxhlet Extraction:

Thendral Hepsibha.B et al.,2010 reported In vitro studies on antioxidant and free radical scavenging activities of *Azima tetracantha*. Lam leaf extracts. The air dried and

powdered leaves were extracted in soxhlet extractor successively with petroleum ether, hexane, ethyl acetate and methanol .The successive extracts were evaporated to dryness and then stored residue was used for analysis. Results of this study shows that *A. tetracantha* leaves are good source of natural phenolic compounds. The methanolic extract of the *A. tetracantha* leaves showed better free radical capacity against different reactive oxygen /nitrogen species, among other extracts although with different efficiencies[151].

S.J.Kulkarni et al., reported Extraction and purification of curcuminoids from Turmeric (curcuma longa L.). Fresh rhizomes were Dried at 50˚c in a hot air oven for six hours. Dried rhizomes were cut in small pieces, powdered by electronic mill. Six gm of sample were taken into a thimble and placed in a Soxhlet apparatus, were set up with various solvent from non polar to polar. 250 ml of solvent was added and extracted according to their boiling point for seven hours. The solvents used were chloroform (B.P.=61˚c), ethyl acetate (B.P.=77˚c), methanol (B.P.=65˚c) and acetone (B.P.=56.53˚c) [152].

5.11 MTT assay:

Nor Azurah Mat Akhir et al., 2011 reported cytotoxicity of aqueous and ethanolic extracts of *Ficus deltoidea* on Human Ovarian Carcinoma Cell Line. MTT assay was used to find the cell growth profile and IC50 concentration of the test compound. MTT assay is a rapid and high accuracy colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to

formazan. IC50 value was 224.39 and 143.03µg/ml for the aqueous and ethanolic extract, respectively[153].

Valko.v et al.,2007 reported anti-proliferative activity of plant extracts from genus *Philadelphus.L.* MTT assay was employed to assess cell viability. The cell proliferation test is based on the ability of the mitochondrial succinate tetrazolium reductase system to convert yellow tetrazolium salt MTT to purple fromazan dye. Effects of extracts were expressed by IC50[154].

5.12 Microscopic Studies:

Aied M Alabsi et al.,2013 reported apoptosis induction, cell cycle arrest and invitro aanticancer activity of Gonothalamin in a Cancer Cell Lines. HeLa cells were quantified using propidium iodide (PI) and acridine-orange (AO) double staining according to standard procedures and examine under fluorescence microscope. After gonothalamin treatment HeLa cell death via apoptosis increased significantly ($*P < 0.05$) in time-dependent manner[155].

Shruti Nair et al.,(2011) reported the anticancer activity of alcoholic and aqueous extracts of *Moringa oleifera* on Hela cells. Anti proliferative effect was assessed by MTT assay and apoptosis of cancer cells were confirmed by DNA fragmentation test and ethidium bromide-acridine orange staining. It was reported that staining the cells which was treated with different concentrations of *Moringa oleifera* aqueous extracts showed viable cells green with intact nuclei and non viable cells with bright orange chromatin[156].

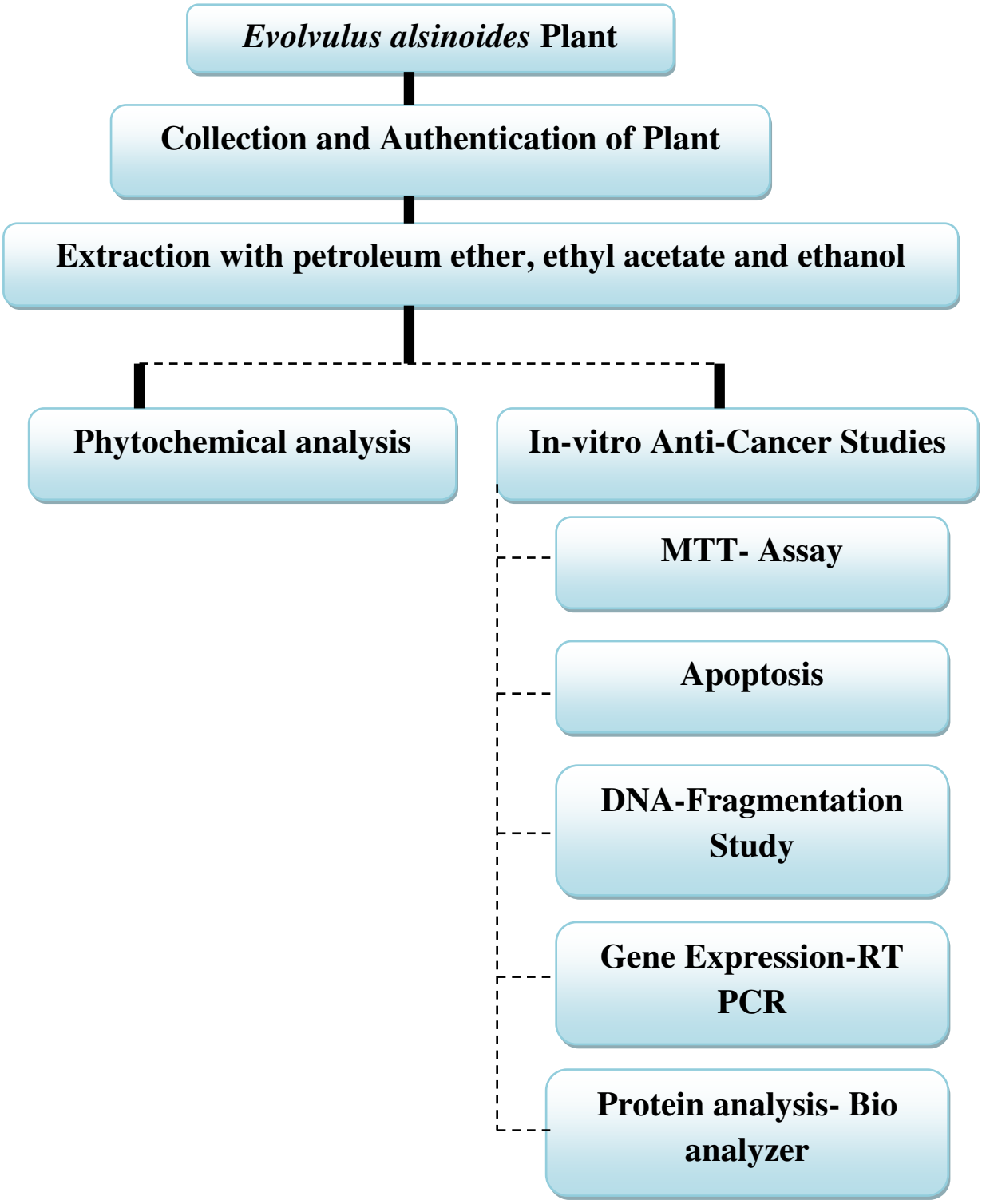
5.13 DNA Fragmentation:

Abhimanyu kumar Jha et al.,(2012) reported the apoptotic effect of ethanolic extract of *Ocimum sanctum, Azadirachta indica* and *Withania somnifera* in SiHa cell line. The IC50 values

of the plant extract was established using MTT assay. It was reported that IC50 concentration of ethanolic extracts of leaves of *Ocimum sanctum* and *Azadirachta indica* and roots of *Withania somnifera* treated in squamous cervical cancer cell line SiHa, resulted in formation of internucleosomal fragments of DNA after 48 hours of treatment[157].

Arulvasu C et al., 2010 reported induction of apoptosis by the aqueous and ethanolic leaf extract of *Vitex negundo L.* in MCF-7 human breast cancer cells . DNA preparation an agarose gel electrophoresis was carried out. Visualization carried by ethidium bromide staining under UV transilluminator. DNA isolated from cells treated with 200µg/ml and 300µg/ml of extract pattern that is characterize the apoptotic cell death after 48hr[158].

PLAN OF WORK:


Evolvulus alsinoides Plant
Collection and Authentication of Plant
Extraction with petroleum ether, ethyl acetate and ethanol
Phytochemical analysis
In-vitro Anti-Cancer Studies
MTT- Assay
Apoptosis
DNA-Fragmentation Study
Gene Expression-RT PCR
Protein analysis- Bio analyzer

6. MATERIALS AND METHODS

6.1 PLANT COLLECTION:

The plant *Evolvulus alsinoides* Linn was collected from Komaneri, Tuticorin district on July 2013 and was identified and authenticated by Professor V. Chelladurai , Research officer-Botany (Retd), Central Council for Research in Ayurveda and Siddha , Government of India.

Instruments used during the course of study:

1. Soxhlet Apparatus
2. CO2 Incubator
3. Cooling Centrifuge
4. Gel Photo Station
5. Deep freezer (-20˚C)
6. Bio Analyzer
7. Real time RT PCR
8. Gel Electrophoresis
9. Inverted Microscope
10. Analytical balance
11. Rotary Evaporator
12. Nano Drop.

Study Centre:

Life teck Research laboratories vadapalani in collaboration with Institute of Pharmacology, Madras Medical College, Chennai-03.

Preparation of Extract:

The Plant were shade dried at room temperature and was subjected to size reduction to a coarse powder by using dry grinder. 50grams of this coarse powder was packed into soxhlet apparatus and was subjected to extraction sequentially with 500ml of petroleum ether, ethyl acetate and ethanol. 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, Chennai. Extracts of ethyl acetate and ethanol were subjected to evaporation by using Rotary evaporator at 60°C.

The percentage of the yield from the plant *Evolvulus alsinoides* using different solvents is given in Table 6.1.

EXTRACT	**Plant material used for Extraction**	**Yield in (gm)**	**Percentage Yield(%)**
Ethanol	50gm	8.6	17.2
Ethyl acetate	50gm	5.7	11.4
Petrolium ether	50gm	1.8	3.6
		TOTAL	32.2

Table 6.1 The percentage of the yield from the plant *Evolvulus alsinoides* using different solvents

6.2 PHYTOCHEMICAL STUDIES:

The freshly prepared extracts of ethyl acetate, ethanol and petroleum ether were subjected to phytochemical screening for the presence or absence of active phytochemical constituents by following methods[130] [131].

Test for alkaloids:

Crude extract was treated with few drops of dilute hydrochloric acid and filtered. The filterate 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 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:

Lead acetate test:

Crude extract was treated with few drops of lead acetate solution. Appearance of yellow colour precipitate indicate 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.

Shinoda test:

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

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 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 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 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 to green indicates the presence of steroidal nucleus.

Test for Terpenoids:

5ml of each extract was mixed in 2ml of chloroform. 3ml of concentrated sulphuric acid was then added to form a layer. A reddishbrown precipitate colouration at the interface formed indicated the presence of terpenoids.

6.3 IN VITRO ANTI CANCER ACTIVITY:

MTT Assay, Fluorescent microscopic studies, DNA fragmentation studies, cell cycle analysis, apoptosis, Gene expression studies including protein assay and RT-PCR were carried out in LIFETECK Research centre Vadapalani, Chennai.

Preparation of Minimum Essential Medium :

Cells when cultured *in vitro* are in need of nutritional requirements to support their growth, maintenance, survival and division. The need for this nutritional requirements for survival of the cells *in vitro* can be fulfilled by provision of Minimum Essential Medium.

This media is prepared by using HIMEDIA protocol.

This is the medium used for passaging and growing of A549 cells throughout the study.

Preparation:

Materials Required:

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

Procedure:

Step1:

Sterilized Millipore distilled water is taken.

Powder form of MEM was dissolved in 1litre of pre sterilized Millipore distilled water, mixed well and closed.

Step2:

Then the medium was sterilized.

Sterilization Duration : 15mins

Sterilization temparature : 121°c

Sterilization Pressure : 15lbs

Step3:

856ml of sterilized MEM was taken in a 1000ml flask and to it each ml of penicillin, streptomycin, phenol red and amphotericin B was added and mixed well.

Step4:

10ml of 3% L-glutamine, 100ml of FBS and 30ml of 7.5% sodium bicarbonate was added to make up the volume to 1000ml.

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.

PASSAGING OF CELL LINES: [159]

Materials Required:

Cell Line:

The cell line chosen for this study is human lung cancerous adherent cell line A549, an adenocarcinomic human alveolar basal epithelial cells. The cell line was procured from National Centre For Cell Science(NCCS), Pune. It was transferred to the laboratory and stored in an incubator at 37°c and 5% CO2, to reach 90% of confluency.

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 5minutes 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 re suspending 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.

6.3.1 MTT Assay: [153] [154]

It is otherwise called as Tetrazolium Salt Assay/ Microculture tetrazolium test.

MTT Assay is an *in vitro* method for anticancer drug screening, which has been internationally accepted.

MTT is a yellow water soluble tetrazolium salt. MTT assay utilizes a colour reaction in measurement of viability of cells.

Principle:

Chemically MTT is 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide which contain tetrazolium ring. MTT salt colour is yellow. Living cells contain Mitochondrial enzyme succinate dehydrogenase that cleves the tetrazolium ring. This converts the MTT to an insoluble purple formazan. Amount of formazan formed is directly proportional to number of viable cells. DMSO has been used as a solubilisation solution, which dissolves serum as well as the purple formazan product. The absorbance at 570nm was measured with a UV-Spectrophotometer to measure the colour intensity.

Advantages:

- ➢ Used for both adherent and suspension cell lines.

- Simple and easy to perform.
- Hundreds of cell samples can be scanned simultaneously, which enables testing of various concentrations of the substance under study.
- Used to determine IC50 of the test substance.

Materials Required:

- 96 well microtitre plates
- Fully grown/confluency reached cells in culture flask
- Minimum essential medium with 10% FBS
- TPVG solution
- Plant extract – Ethyl acetate, Ethanol, Petroleum ether(1000,500,250,125,62.5,31.2,15.6 and 7.8µg/ml)
- Drug – 5-Fluorouracil(1000,500,250,125,62.5,31.2,15.6 and 7.8µg/ml)
- MTT (5mg/ml in PBS - pH 7.4)
- DMSO solution (0.1% v/v)
- Aluminium foil
- Micropipette
- Phenol red free medium
- Tryphan blue
- Bio safety cabinet
- Tissue culture flask
- Reagent bottles
- Inverted microscope

- CO2 incubator
- UV-Chamber
- Neubar chamber

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 5minutes.

After incubation, the TPVG solution containing cells were taken from the culture flask and transferred to the falcon tubes and was subjected to centrifugation at 8000rpm for 5minutes.

After centrifugation, supernatant was discarded and 1ml of medium was added to the pellet of cells which have sedimented at the bottom of the tube and was re suspended well in the medium with the help of micropipette.

10µl of this cell suspension was mixed well with 10µl of tryphan blue in an eppendorf tube for staining the cells and it was counted in a neubar chamber.

Procedure for 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 = 33 and 39 respectively

Numbers of cells in left and right bottom quadrant = 37 and 33 respectively

Total number of cells per ml:

$$= \frac{33+39+37+33 \times 2 \times 10^4}{4}$$

$$= 71 \times 10^4 \text{ cells/ml}$$

71×10^4 cells were present in 1 ml of cell suspension.

Three 96 well plates were taken. Totally 135 wells needs to plate as 45 each of three 96 well plates.

Well Design = 8 concentration for ethanolic extract+ 8 concentration for petroleum ether extract+8 concentration for ethyl acetate extract+8 concentration for 5-FU+3 for control+10 to avoid error. So totally 45 wells are needs to plate. Same procedure continued for remaining two 96 well plates to maintain triplicate.

we need to plate 135 wells each with 100µl containing 10000cells/well for maintaining triplicate condition for control, standard and test extracts.

Therefore, 71×10^4 cells/ml cells were present in 1000µl. 10000cells were present in x µl.

$$X = \frac{10000 * 1000}{71 \times 10^4}$$

= 14.08 µl

14.08µl of cell suspension should be mixed with 85.92µl of medium for plating one well. In order to plate 135wells, 1900.8µl of cell suspension was mixed with 11599.2µl of medium to get a concentration of 10000cells / 100µl for plating the wells.

In order to avoid handling errors cell suspension and medium required for additional 10 wells were calculated and added along with cell suspension and medium for 135wells.

100µl of this cell suspension were seeded into 96 well plate, at a plating density of 10000 cells/ml and the plate was incubated for 24 hours at 37°c, 5% CO2 for attachment of the cells.

After 24hours the seeded cells in the 96 well plate were treated with serial concentration of plant extract and the standard drug 5FU.

Plant extracts of ethyl acetate, ethanol and Petroleum ether and standard drug were initially dissolved in 0.1% v/v DMSO and further diluted in serum free medium to get a desired concentration.

Final volume in each well was 200µl and the plates were incubated again for 48hours.

Triplicate was maintained for all concentrations and the medium without samples were taken as control.

After 48hours of incubation, medium was removed from the 96 well plate and it was replaced with 100µl of phenol red free medium and 15µl of MTT solution was added to the well plates and wrapped with aluminium foil as the dye is light sensitive. The plate was incubated again for 4hours.

After incubation the medium was carefully removed without disturbing the formed formazan crystals and the crystals were solubilised in100µl of 0.1% v/v DMSO and the absorbance was measured at 570nm using UV-Spectrophotometer.

Percentage Cell viability is calculated using the formula

$$\% \text{ Cell Viability} = \frac{\text{Optical density of treated cells}}{\text{Optical density of control cells}} \times 100$$

Figure 6.1 Inverted Microscope

6.3.2 Flourescent microscopic studies: [160]

- 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 IC50 concentration of effective plant extract such that it covers the entire surface area.
- The flask was then incubated for 48 hours at 5% CO2 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.

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

Extraction of DNA, RNA and Protein from the cells which was treated with IC50 concentration of Ethyl acetate extract, IC50 concentration of 5 Fluorouracil and from cells without any treatment 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 IC50 concentration of the effective ethyl acetate extract (IC50 confirmed from MTT Assay)

Flask 3 is treated with IC50 concentration of standard 5 fluorouracil (IC50 confirmed from MTT Assay).

All the three flasks were incubated for 48 hours at 37°c and 5% CO2. 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:

- Chloroform
- Isopropyl Alcohol
- 75% Ethanol
- RNase free water

Steps Involved:

- Homogenisation
- Phase Separation
- RNA Precipitation
- RNA Wash
- Resuspending RNA

Procedure:

Homogenisation:

Cells were pelleted by centrifugation at 1200rpm for 5minutes.

The supernatant was decanted and 1ml of TRI solution was added and mixed by repetitive pipetting.

Phase Separation:

The samples were incubated for 5minutes at room temperature (without exceeding 30°c) to completely dissociate nucleoprotein complex.

0.2ml of chloroform per 1ml of TRI Solution was added, mixed thoroughly and subjected to incubation at room temperature (without exceeding 30°c) for 2-3minutes.

The samples were centrifuged at not more than 12000 rpm for 15minutes at 2-8°c and the mixture thus obtained contained a lower phenol chloroform phase, interphase and colourless upper aqueous phase. RNA was present in upper aqueous phase.

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 1.5ml vial. 0.5ml 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 12000rpm for 10minutes at 2-8°c.

RNA Wash:

Supernatant was decanted and the RNA precipitated as gel like pellet on the sides of 1.5ml 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 10000rpm for 10minutes 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:

- Ethanol (100%)
- 75% Ethanol
- 0.1 M sodium citrate in 10% ethanol.
- 8mM Sodium hydroxide.

- Wash Buffer : 0.1 N Sodium citrate in 10% ethanol.

Steps involved:

- DNA Precipitation
- DNA Wash
- Re suspending the DNA pellet.

Procedure :

DNA Precipitation :

0.3ml 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 2-3minutes.

Samples were centrifuged at 5000rpm for 10minutes at 2-8°c and DNA was obtained as a pellet.

DNA Wash:

DNA pellet obtained was washed with wash buffer per 1ml TRI Solution used.

At each wash pellet was stored in wash solution for 30minutes at room temperature without exceeding 30°c with periodic mixing. Samples were centrifuged at 5000rpm for 10minutes at 2-8°c and the wash step was repeated twice.

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

Re-suspending the DNA Pellet:

DNA pellet was air dried for 5-10minutes. 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 15-20minutes.

Extraction of Protein:

Materials Required:

- Isopropyl alcohol
- Ethanol
- Wash Buffer: 0.3M Guanidium hydrochloride in 95% ethanol.
- 1% Sodium dodecyl sulphate (SDS)

Steps involved:

- Protein precipitation
- Protein wash
- Re-suspending Protein pellet

Procedure:

Protein precipitation:

0.3ml of 100% ethanol was added per 1ml 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 2-3minutes. Samples were centrifuged at 5000rpm for 10minutes at 2-8°c and the supernatant was collected. The supernatant was centrifuged at 12000rpm for 10minutes at 2-8°c.

1.5ml of isopropanol was added to the supernatant per 1.0ml of TRI Solution used for initial homogenization and the samples were stored at room temperature without exceeding 30°c.

Protein Wash:

The pellet obtained was washed with 2ml of wash buffer per 1 ml of TRI Solution used for initial homogenization. The pellets were stored in wash buffer for 20minutes at room temperature without exceeding 30°c and were centrifuged at 10000rpm for 5minutes at 2-8°c.

Wash step was repeated three times and after final wash, protein pellet was vortexed with 2ml of ethanol and stored in ethanol for twenty minutes. Samples were subjected to centrifugation at 10000rpm for 5minutes at 2-8°c.

Re-suspending Protein Pellet:

The pellet was vacuum dried for 5-10minutes and was re-suspended in 1% SDS by incubating at 50°c for 2-3minutes. Insoluble materials were sedimented by means of centrifugation at 10000rpm for 10minutes and the supernatant containing protein were transferred into fresh vial.

6.3.3 DNA FRAGMENTATION: [161]

Apoptosis is 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. This cleavage of DNA or its fragmentation can be visualized by DNA laddering assay.

Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis. 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:

- Agarose
- 5X TAE buffer
- 6X loading dye
- DNA ladder
- Electrophoresis chamber
- Gel casting tray and comb
- Ethidium bromide
- Microwave oven

- Power supply
- Conical flask
- Sterile tips and pipettes

Procedure:

The steps involved are:

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

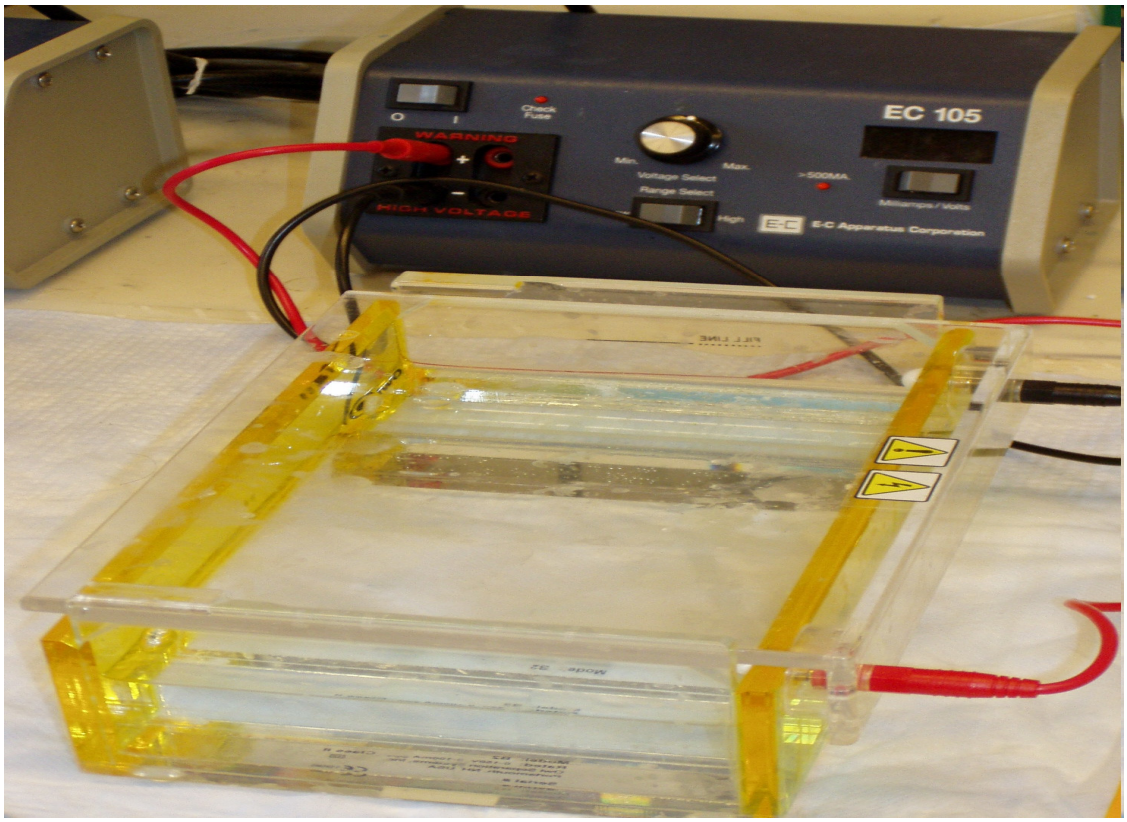


Figure 6.2 Gel Electrophoresis

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. 1.2grams of agarose powder was weighed and transferred to 500ml conical flask containing 100ml 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 2-3mm 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 were quantified by means of nano drop method. Based on the quantification, equal quantity of DNA was taken and mixed with required TAE buffer to make a final volume of 10µl each for a sample. 2µl 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 power supply of about 80 volts were applied. 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.

6.3.4 PROTEIN ASSAY:

Instrument: Agilent high sensitivity protein 250 bio analyzer

Agilent high sensitivity protein 250 bio analyzer was used to identify and quantify the proteins present in cancer cells, without any treatment and in cancer cells after treatment with standard drug and test extract. Proteins isolated from control and treated cancer cells were quantified by nano drop spectrophotometer and equal quantity of protein was subjected to series of processing as described below and was identified and quantified with the help of standard ladder using bio analyzer. The procedure was carried out as per Agilent high sensitivity protein 250 bio analyzer protocol.

Figure 6.3 Agilent Bio analyzer

Materials required:

- Isolated proteins from control and treated cancer cells
- 10X protein 250 standard labeling buffer
- Ethanolamine
- DMSO
- Labeling dye
- Ladder
- Gel matrix

- Destaining solution
- Sample buffer
- Dithiothreitol
- High sensitivity protein chip
- Micropipette and tips.

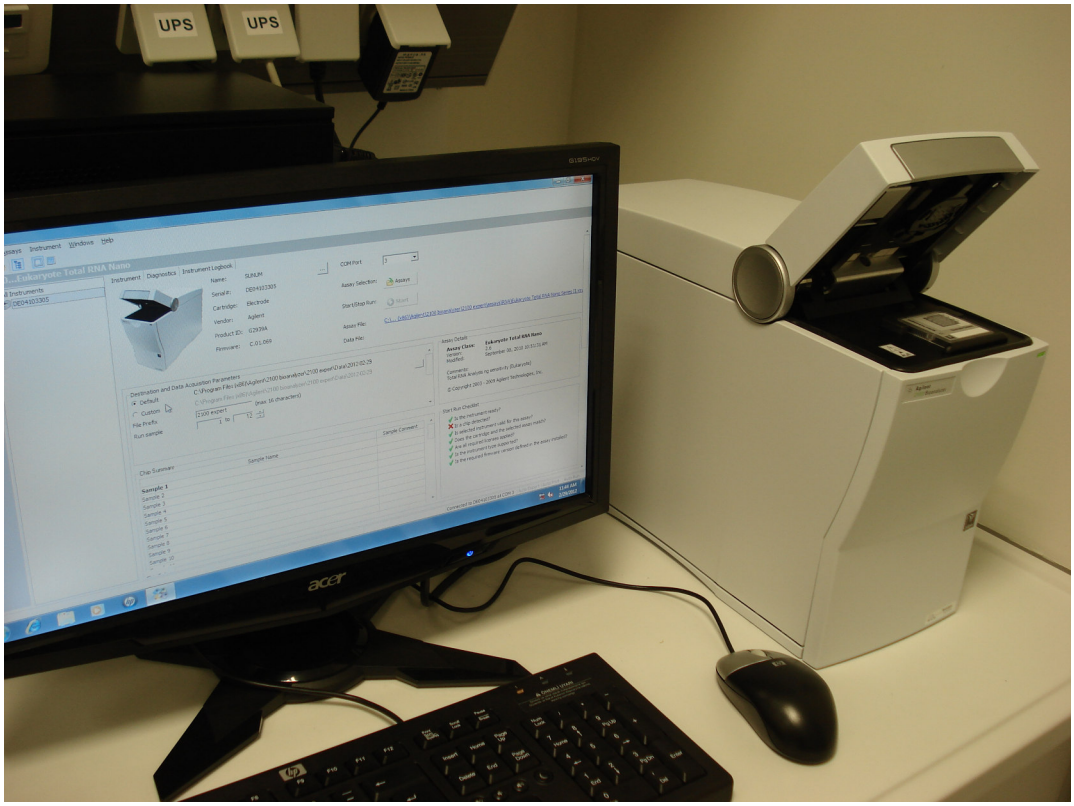


Figure 6.4 Agilent Bio analyzer with complete set up.

Steps involved:

Covalent labeling of proteins with fluorescent dye :

- Preparation of dye
- Preparation of denaturing solution

- Processing of protein
- Labeling of protein/ladder
- Protein and ladder preparation

Separation and detection of labeled proteins with on chip electrophoresis:

- Loading the gel matrix
- Loading the sample
- Inserting a chip in bio analyzer
- Starting the chip run

Procedure:

A. Covalent labeling of proteins with fluorescent dye.

Preparation of dye:

- Fluorescent dye and DMSO was thawed to room temperature.
- After thawing, 50µl of DMSO was added to the dye vial provided in the kit and mixed well.
- Dye solution was stored at - 20°c in amber coloured vial to avoid degradation on light exposure.

Preparation of denaturing solution:

- Denaturing solution was prepared by adding 100µl of sample buffer to 3.5µl of 1M dithiothreitol.

Processing of protein:

- pH of the protein samples were adjusted to 8.0 – 9.0 by adding 10X standard labeling buffer in the ratio 1:9.

Labeling of protein/ladder:

- 0.5µl of prepared dye was added to 5µl of ladder/protein sample and incubated for 30minutes on ice to facilitate uniform labeling.
- After incubation 0.5µl of ethanolamine was added to remove/quench the excess dye and incubated for 10minutes on ice.
- Later, labeled reaction mix was diluted to 200 times (1:200) with water.

Protein and ladder preparation:

- 2µl of denaturing solution was added to 4µl of labeled, diluted protein/ladder and mixed well.
- The reaction mix was heated in a heating block or boiling water bath at 95 to 100°c for 5 minutes.
- Later the samples were cooled to room temperature and spinned for 15seconds and used for analysis of proteins.

B. Separation and detection of labeled proteins with on chip electrophoresis:

Loading the Gel matrix:

- Gel matrix was allowed to equilibrate to room temperature for 30minutes before use.
- High sensitivity protein chip was taken out of sealed bag and placed on chip priming station.
- 12µl of gel was pipetted into the bottom of the well marked **G** as shown in Figure 6.5.
- Plunger was positioned at 1ml and the chip priming station was closed, setting the timer to 90 seconds as shown in figure6.5.
- Plunger of the syringe was pressed down until it was held by the clip and pressure was applied as shown in figure6.5.

- After 90 seconds plunger was released with the clip release mechanism and its movement back to 0.3 ml was inspected visually.
- After 5 seconds plunger was pulled back to 1ml position and the chip priming station was opened.
- 12µl of gel mix was pipetted into each of three wells marked with **G** as shown in figure6.5.
- 12µl of destaining solution was pipetted into the well marked **DS** as shown in figure6.5.

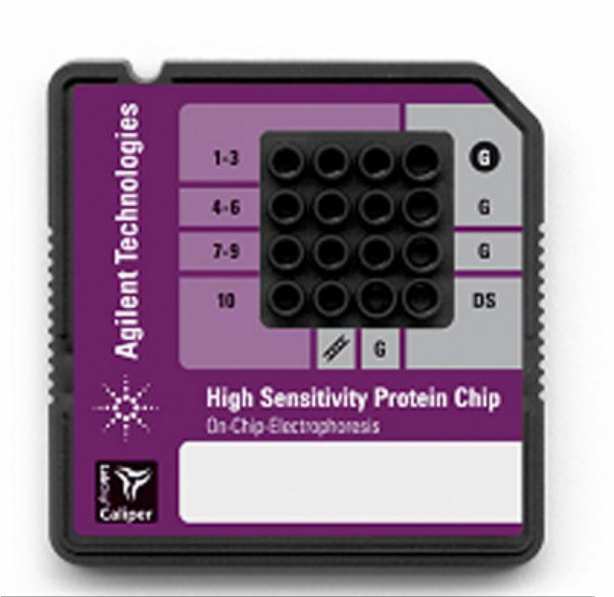


Figure 6.5 High sensitivity Protein Chip

Loading the samples:

- Heat denatured isolated protein samples of control cancer cells and standard and test extract treated cancer cells of volume 6µl was pipetted into the sample wells marked 1 to 3 and the remaining wells were filled with equal volume of water as shown in figure6.5.

- Heat denatured ladder of volume 6µl was pipetted into the well marked with ladder symbol **L** as shown in figure6.5.

Inserting a chip in bio analyzer:

- Lid of the bio analyzer was opened and the electrode cartridge was inserted into the instrument.
- Chips were placed carefully into the receptacle and the lid was closed carefully ensuring the electrodes in the cartilage fit into the wells of the chip.
- Insertion of the chip was confirmed by appearance of chip icon at the top left of the instrument context in the **2100 expert software screen.**

Starting the chip run:

- High sensitivity protein 250 assay was selected from the assay menu in the instrument context.
- File storage location and the number of samples to be analysed was selected.
- Chip run was started by clicking START button in the upper right of the window of 2100 expert software screen.
- After the samples were analysed, the data's were stored in the instrument context and the protein of our interest was studied from the data obtained.

6.3.5 Real time Reverse Transcriptase Polymerase Chain Reaction: [162]

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:

Complementary DNA Synthesis:

Materials Required:

- 5X buffer
- 10mm dNTPs
- Hexamer primer
- Extracted RNA
- Thermal cycler
- Reverse transcriptase
- DTT

Procedure:

- In 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 , 25°c for 5minutes, for binding of hexamer, followed by 42°c for 45minutes for cDNA synthesis, followed by 85°c for 5minutes for denaturation of remaining unconverted RNA's and finally at 4°c for 5minutes.

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.

PRIMER NAME	PRIMER SEQUENCE FROM 5' - 3'
P53	AGGGATACTATTCAGCCCGAGGTG ACTGCCACTCCTTGCCCCATTC
Bcl-2	ATGTGTGTGGAGAGCGTCAACC TGAGCAGAGTCTTCAGAGACAGCC
TNFα	TCTCTAATCAGCCCTCTGGCC TGGGCTACAGGCTTGTCACTC
IL-6	GCCTTCGGTCCAGTTGCCTT GCAGAATGAGATGAGTTGTC
GPDH	ATTGACCACTACCTGGGCAA GAGATACACTTCAACACTTTGACCT

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.

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 1minute

  Step 2: Denaturation at 95˚C for 15seconds

  Step 3: Annealing at 60˚C for 15seconds

  Step 4: Extension at 72˚C for 45seconds Step 2 to step 4 repeated for 40 cycles.
- The relative expression of genes was analyzed and interpreted and by Applied Biosystem Software.

7. RESULTS

7.1 Phytochemical Analysis:

Phytochemical analysis was carried out for petroleum ether, ethyl acetate and ethanol extracts of *Evolvulus alsinoides* and the results are shown in table.

CONSTITUENTS	Petroleum ether Extract	Ethanol Extract	Ethyl acetate Extract
Alkaloids	Absent	**Present**	Absent
Steroids	**Present**	**Present**	**Present**
Flavanoids	Absent	**Present**	**Present**
Tannins/Phenol	Absent	**Present**	Absent
Proteins	Absent	**Present**	Absent
Carbohydrate	Absent	**Present**	Absent
Glycosides	**Present**	**Present**	**Present**
Terpinoids	Absent	Absent	**Present**

Table 7.1 Phytochemical analysis of the various extracts of *Evolvulus alsinoides*

CYTOTOXICITY TEST:

7.2 MTT Assay:

MTT assay was carried out with Petrolium ether, ethyl acetate and ethanol extract of *Evolvulus alsinoides* and with 5-Fluorouracil and the results are shown.

MTT Assay of Ethanolic Extract of *Evolvulus alsinoides*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.09	16.66
2	500	1:01	0.15	27.77
3	250	1:02	0.19	35.18
4	125	1:04	0.23	42.59
5	62.5	1:08	0.27	50
6	31.2	1:16	0.32	59.25
7	15.6	1:32	0.39	72.22
8	7.8	1 64	0.45	83.33
9	Cell control	-	0.54	100

Table7.2 IC50 concentration and %cell viability of ethanolic extract of *Evolvulus alsinoides*

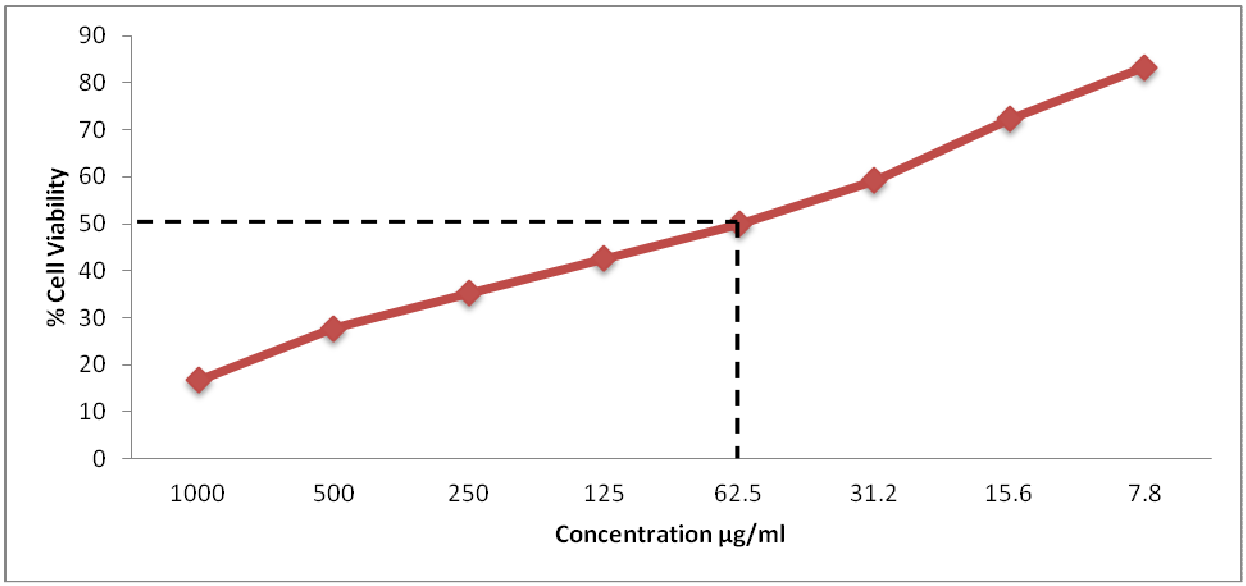


Figure:7.1 Graphical representation of % cell viability vs Concentration in µg/ml of ethanolic extract of *Evolvulus alsinoides*

MTT Assay of Petroleum ether Extract of *Evolvulus alsinoides*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.15	27.77
2	500	1:01	0.19	35.18
3	250	1:02	0.26	48.14
4	125	1:04	0.29	53.70
5	62.5	1:08	0.32	59.25
6	31.2	1:16	0.36	66.66
7	15.6	1:32	0.39	72.22
8	7.8	1 64	0.45	83.33
9	Cell control	-	0.54	100

Table7.3 IC50 concentration and % cell viability of Petroleum ether extract of *Evolvulus alsinoides*.

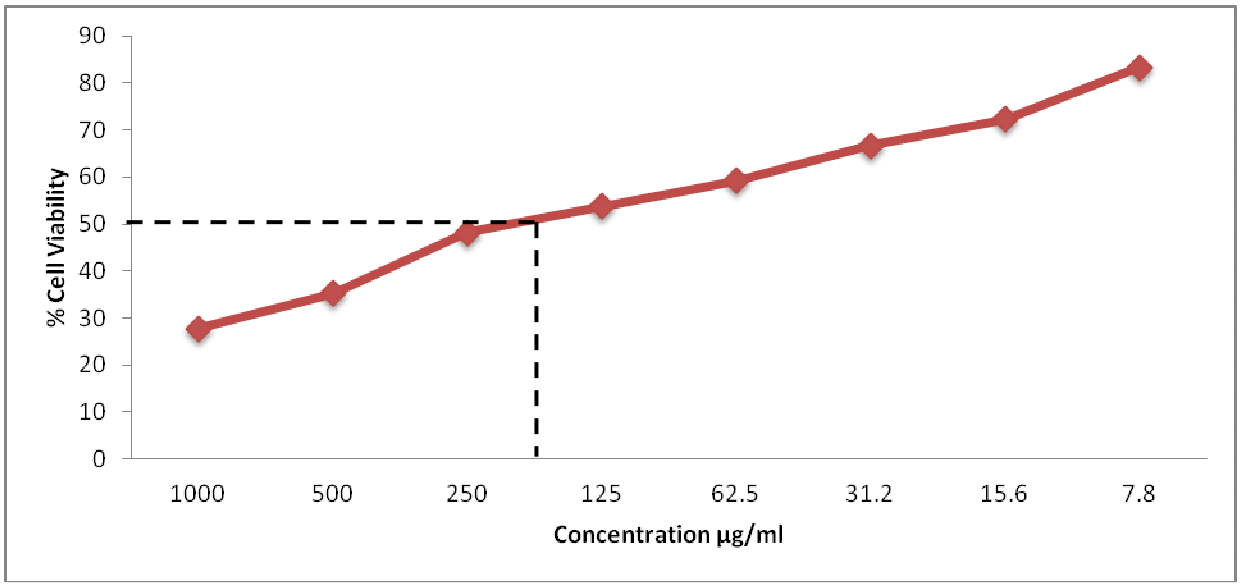


Figure7.2 Graphical representation of % cell viability vs Concentration in µg/ml of Petroleum ether extract of *Evolvulus alsinoides*.

MTT Assay of Ethyl acetate Extract of *Evolvulus alsinoides*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.03	5.55
2	500	1:01	0.08	14.81
3	250	1:02	0.1	18.51
4	125	1:04	0.14	25.92
5	62.5	1:08	0.18	33.33
6	31.2	1:16	0.23	42.59
7	15.6	1:32	0.28	51.85
8	7.8	1 64	0.38	70.37
9	Cell control	-	0.54	100

Table7.4 IC50 concentration and % cell viability of Ethyl acetate extract of *Evolvulus alsinoides*.

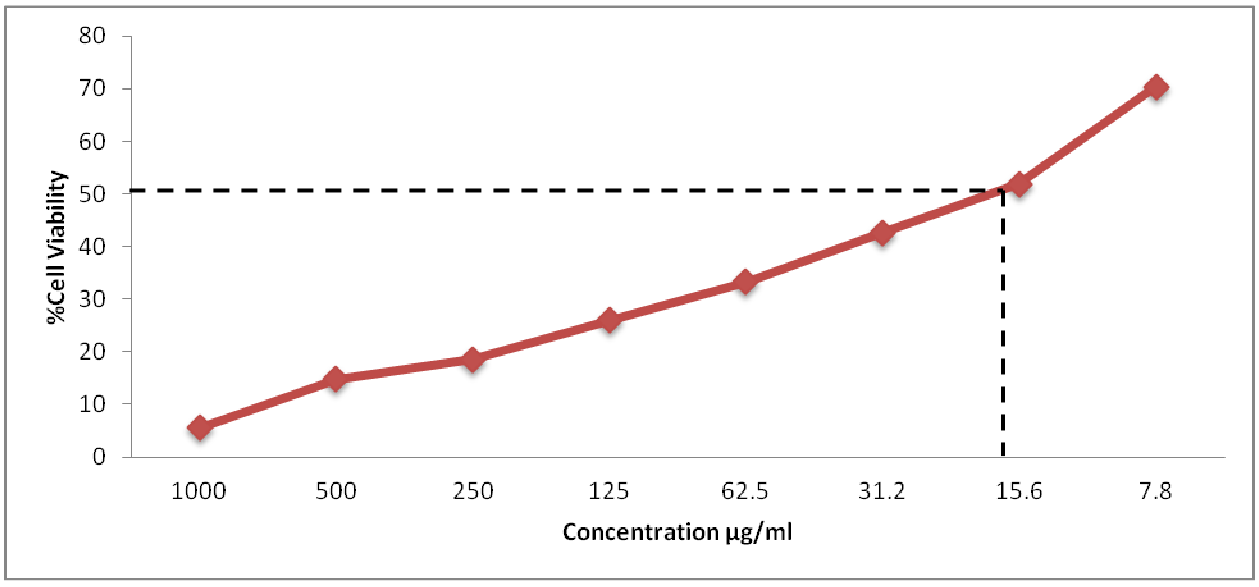


Figure7.3 Graphical representation of % cell viability vs Concentration in µg/ml of Ethyl acetate extract of *Evolvulus alsinoides*.

MTT Assay of 5-Fluorouracil

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.01	1.85
2	500	1:01	0.03	5.55
3	250	1:02	0.06	11.11
4	125	1:04	0.1	18.51
5	62.5	1:08	0.13	24.07
6	31.2	1:16	0.17	31.48
7	15.6	1:32	0.2	37.03
8	7.8	1 64	0.28	51.85
9	Cell control	-	0.54	100

Table7.5 IC50 concentration and % cell viability of 5-Fluorouracil

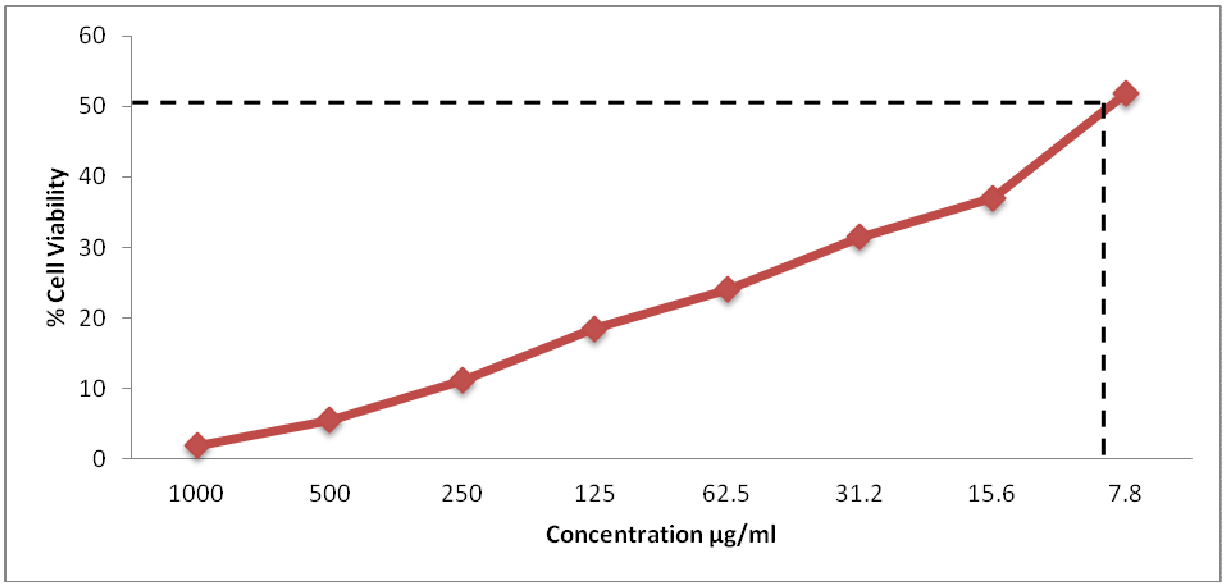
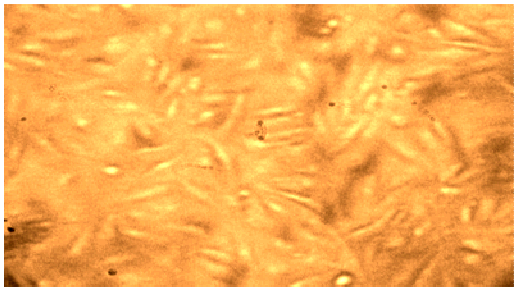
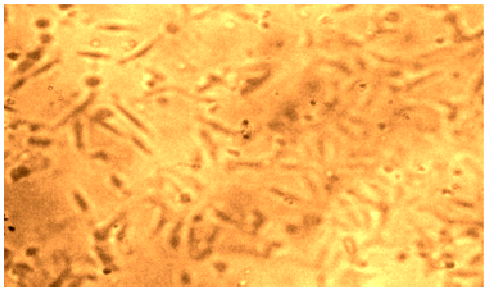


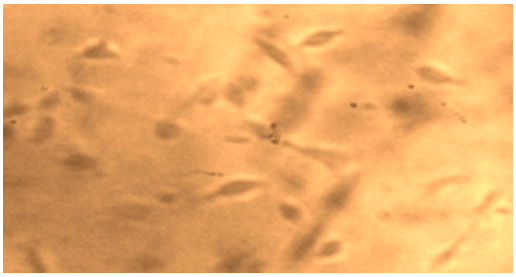
Figure7.4 Graphical representation of % cell viability vs Concentration in µg/ml of 5 Fluorouracil



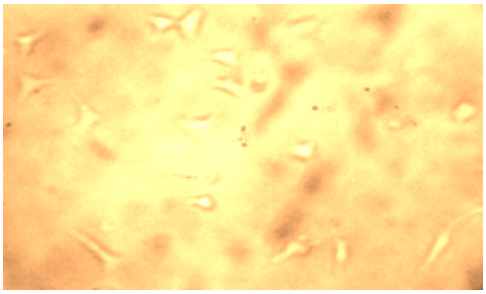
(A)



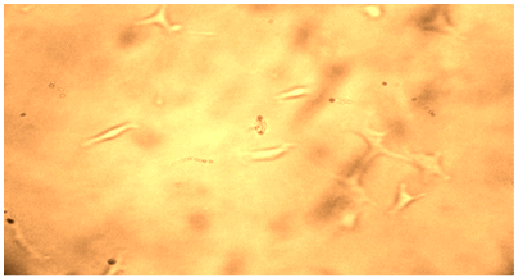
(B)



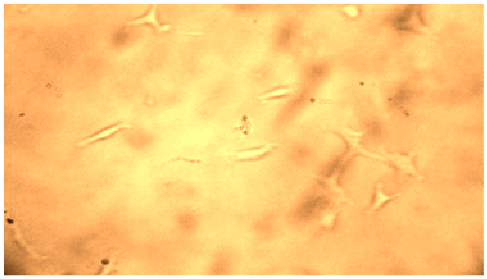
(C)



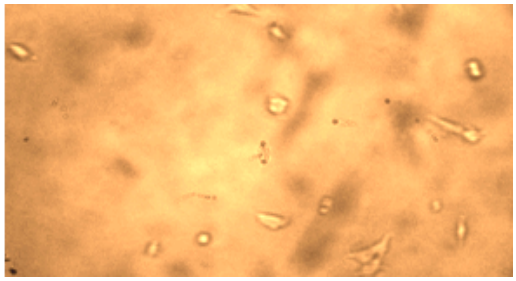
(D)



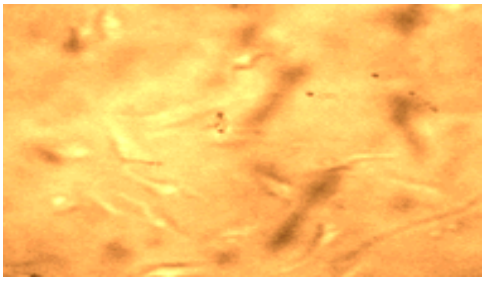
(E)



(F)



(G)



(H)

Figure 7.5 A549 cells treated with Ethyl acetate extracts – (A) Control, (B)7.8 µg/ml, (C)15.6 µg/ml, (D)31.2 µg/ml, (E)62.5 µg/ml , (F)125 µg/ml, (G) 250 µg/ml, (H)1000 µg/ml.

7.3 APOPTOSIS:

Flourescence microscopic observation:

Control cells without any drug or extract treated were bright green in colour.

Cells treated with ethyl acetate extract of *Evolvulus alsinoides* were bright orange in colour with loss of membrane integrity and cytoplasmic contents leaking out of the cell.

The observations of light microscopic studies and fluorescent microscopic studies are depicted in figures

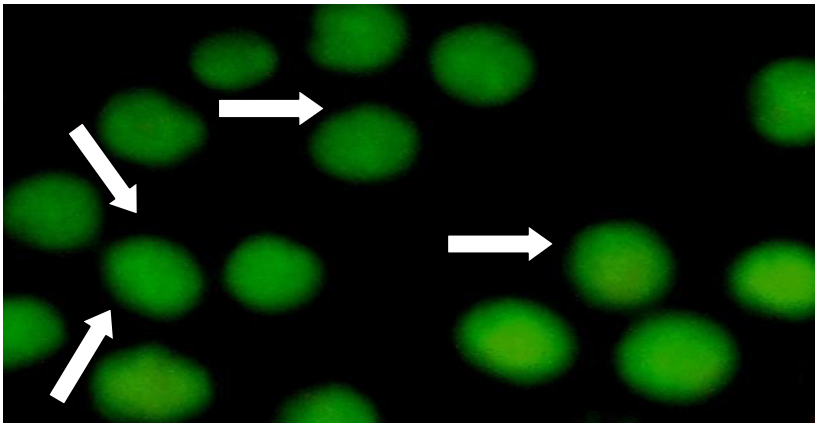


Figure 7.6 A549 cells – Control, indicting viable cells stained green in colour

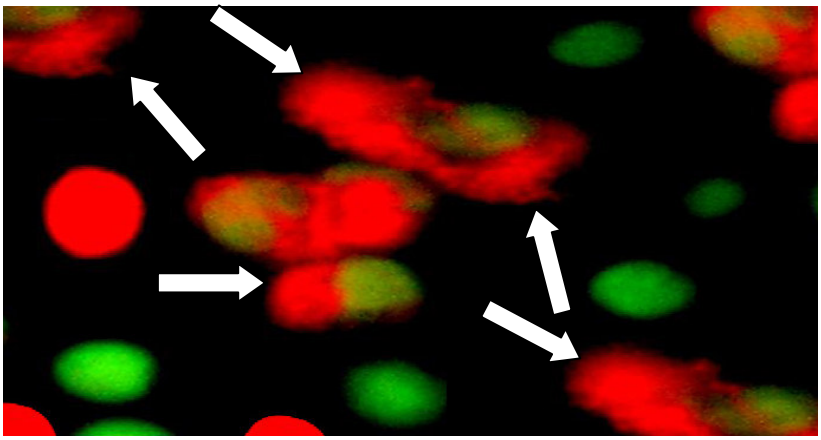


Figure 7.7 A549 cells after treatment with Ethyl acetate extract of *Evolvulus alsinoides* showing dead cells stained orange in colour with loss of membrane integrity and cytoplasmic contents leaking out of the cell.

7.4 DNA FRAGMENTATION:

In the control A549 cells, there was no fragmentation observed in agarose gel. Fragmentation was observed in A549 cell treated with IC50 concentration of standard 5-Fluorouacil and ethyl acetate extract of Evolvulus alsinoides. This Fragmentation of DNA in ethyl acetate extract treated cells indicated the characteristics of apoptotic cells.

Results obtained in fragmentation studies are shown in figure.

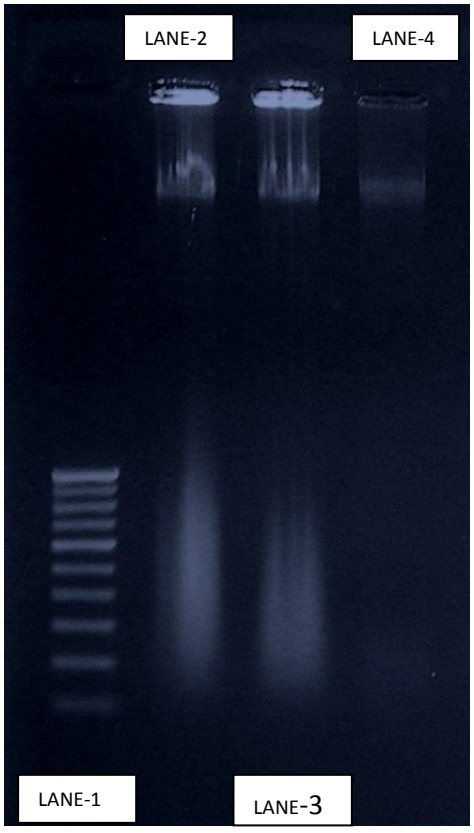


Figure 7.8 . DNA Fragmentation in A549 cells.

Lane 1: 100 base pair DNA marker

Lane 2: A549 cells treated with 5-Fluorouracil

Lane 3: A549 cells treated with ethyl acetate extract of *Evolvulus alsinoides*

Lane 4: A549 cells without any treatment.

7.5 PROTEIN ASSAY:

Quantification of proteins, C reactive protein and Phospho histidine phosphatase present in A549 cells treated with IC50 concentration of ethyl acetate extract of *Evolvulus alsinoides* and 5-fluorouracil and also from cells without any treatment were analysed using Bio analyzer.

The results obtained were given in Table and in Figures.

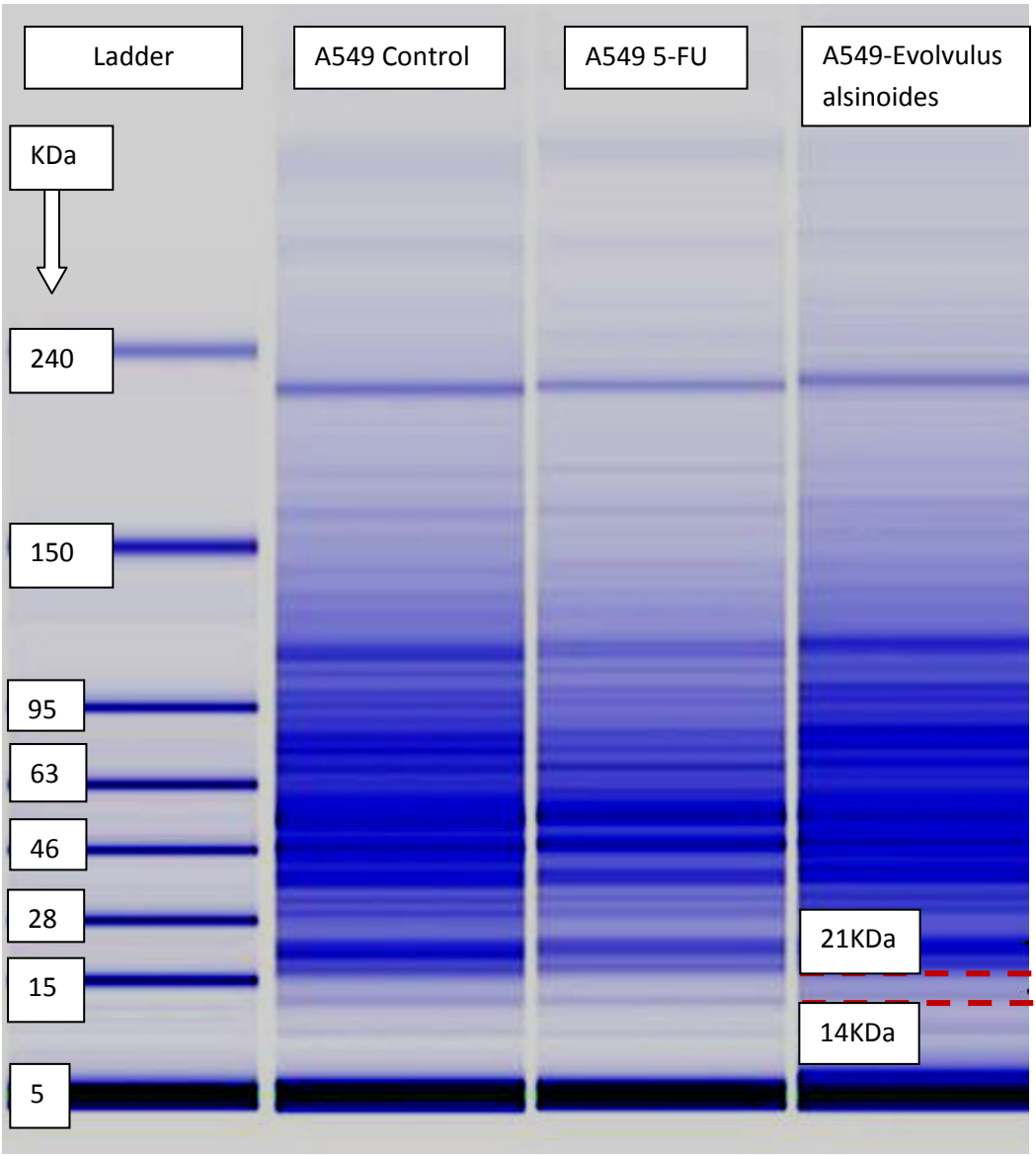


Figure7.9 . Gel Image of Protein analysis

Quantification Of Protein:

C-Reactive Protein:

Protein	Treatment	Size(KDa)	Concentration(pg/ml)
C-Reactive Protein	A549- Control	20.2	420.5
	A549-5-Flourouracil	21.4	330.7
	A549 Ethyl acetate extract of *Evolvulus alsinoides*	20.8	354.6

Table7.6 Quantification of Protein- C-Reactive protein

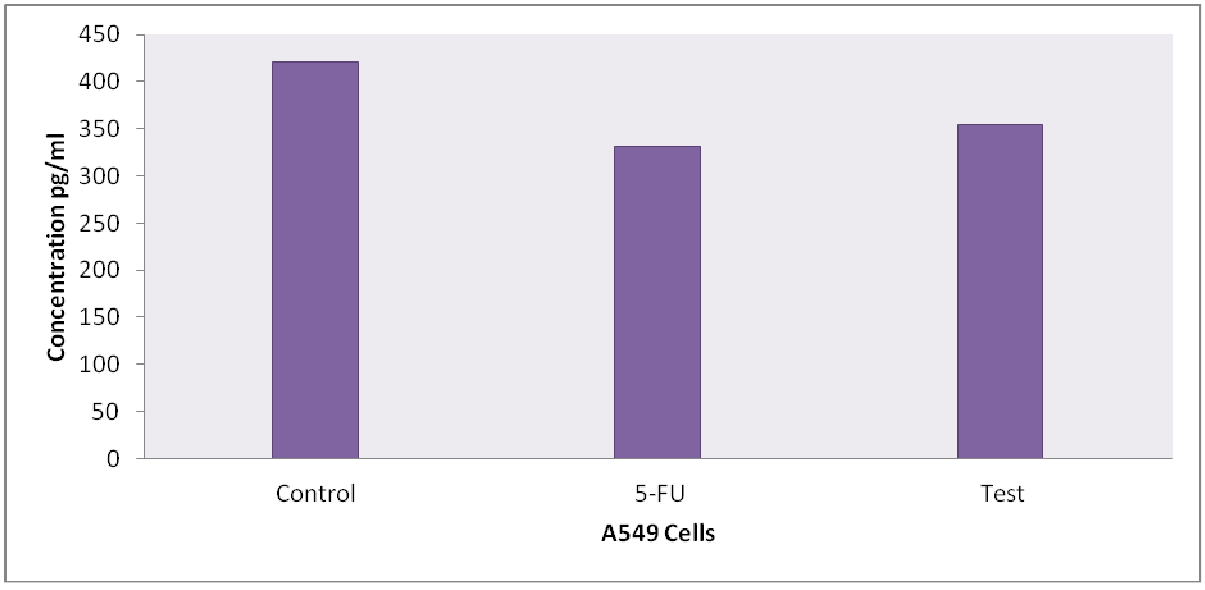


Figure7.10 Graphical representation of concentration of C Reactive Protein in A549 cells before treatment and after treatment with ethyl acetate extract of *Evolvulus alsinoides* and 5 fluorouracil.

Phospho histidine phosphatase:

Protein	Treatment	Size(KDa)	Concentration(pg/ml)
Phospho histidine phosphatase	A549- Control	14.0	128.0
	A549-5-Flourouracil	13.9	65.2
	A549 Ethyl acetate extract of *Evolvulus alsinoides*	13.8	86.4

Table 7.7: Quantification of Protein- Phospho histidine phosphatase

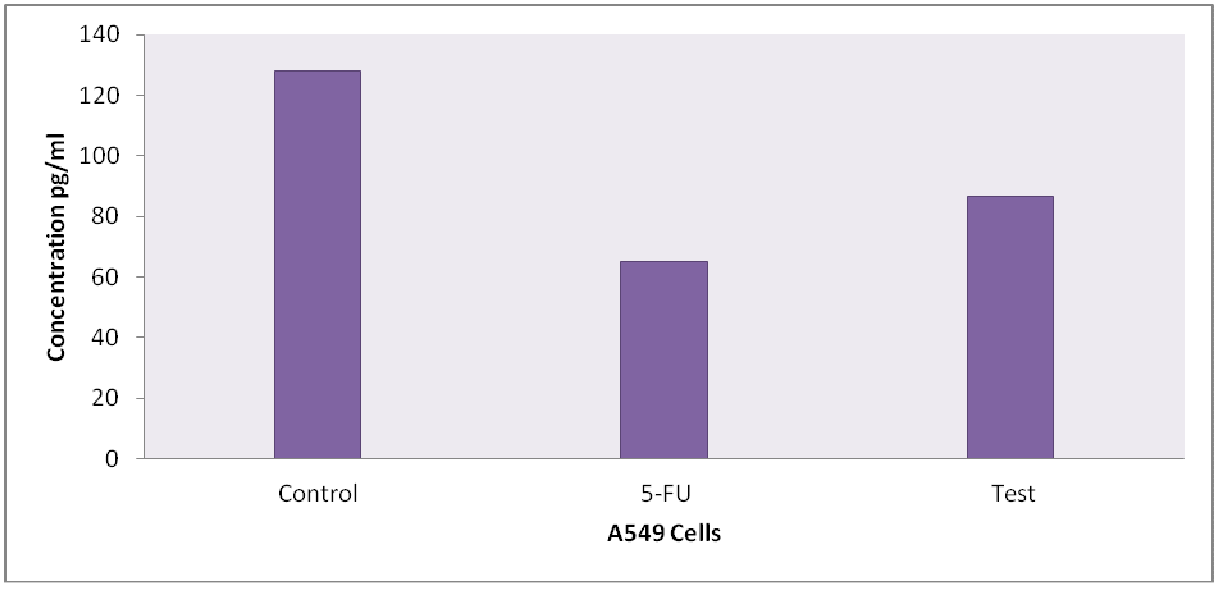


Figure7.11. Graphical representation of concentration of Phospho histidine phosphatase in A549 cells before treatment and after treatment with ethyl acetate extract of *Evolvulus alsinoides* and 5 fluorouracil.

7.6 Gene Expression Studies:

P53 Gene in A549 Cell Line :

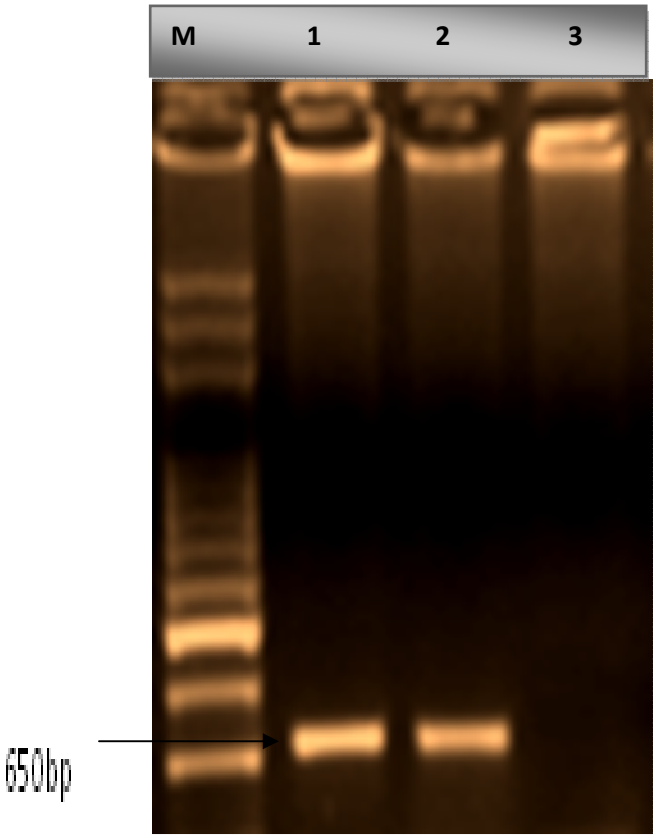


Figure 7.12 p53 Expression

M:Marker- 1 kb DNA Ladder

1: Standard- 5-Flourouracil

2: Test- IC50 concentration of Ethyl acetate extract of *Evolvulus alsinoides*

3: Control- Without any treatment

Expression levels of P-53:

P-53	Relative Quantitation	Standard Error
Standard- 5 Fluorouracil	5.23	0.1
Ethyl acetate Extract	4.62	0.12
Control	1.41	0.03

Table7.8: Expression levels of P-53

Graphical representation of p53 expression

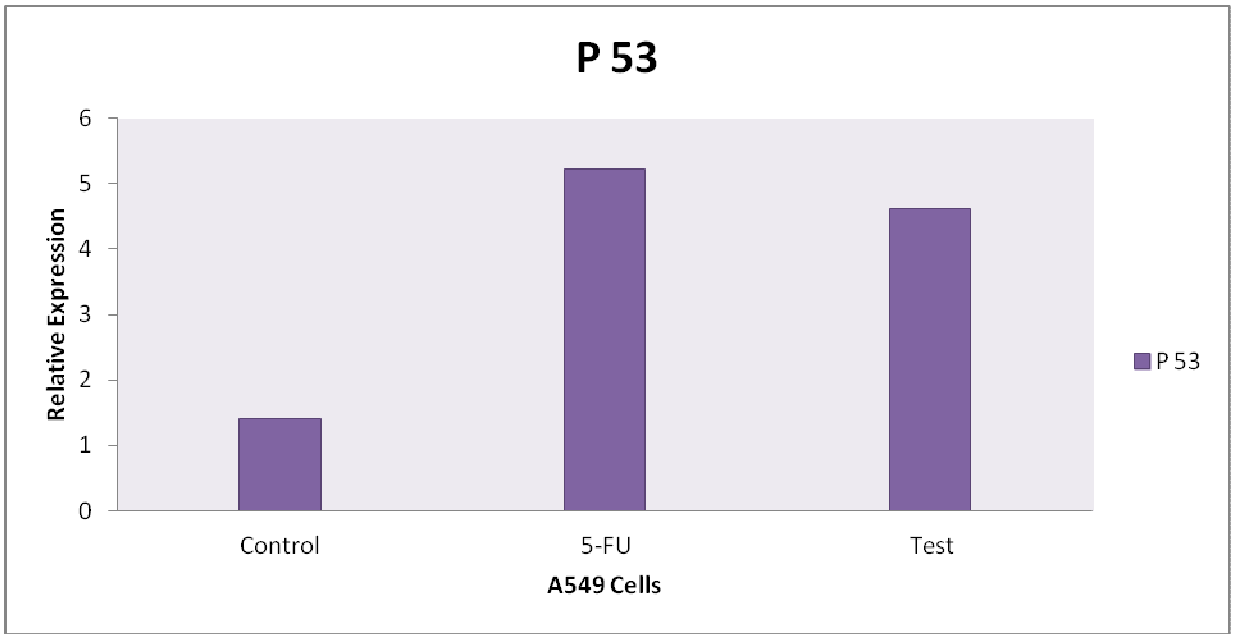


Figure7.13: Graphical representation of p53 expression

Bcl2 Gene in A549 Cell Line :

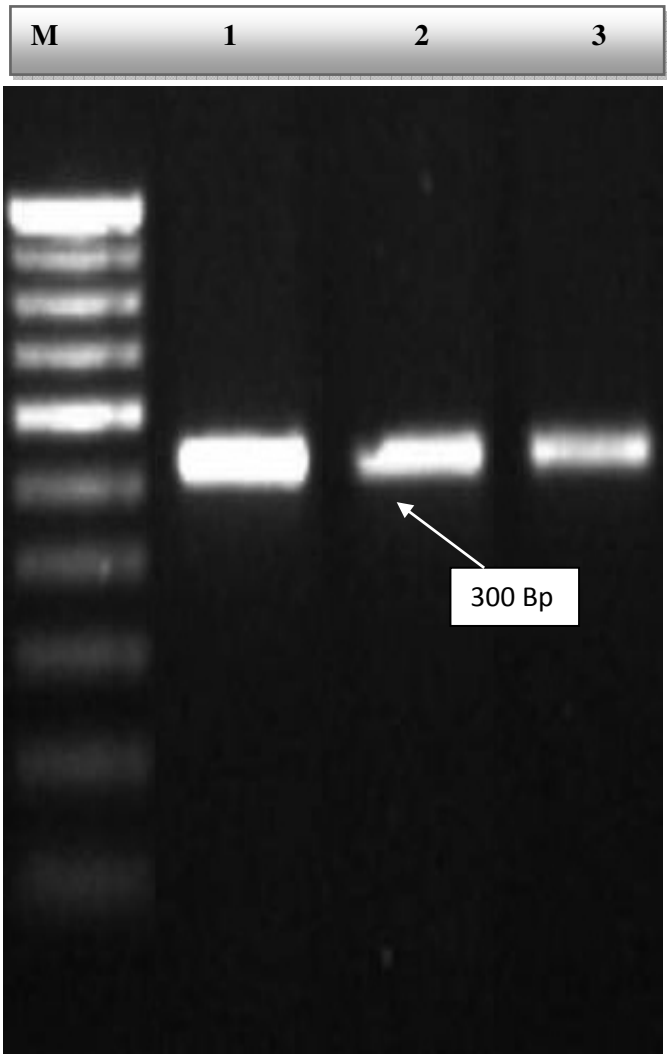


Figure 7.14 Bcl2 Expression

M: Marker DNA

1: Control - Without any treatment

2: Standard- 5-Fluorouracil

3:Test- IC50 concentration of Ethyl acetate extract of *Evolvulus alsinoides*

Expression levels of Bcl2:

Bcl2	Relative Quantitation	Standard Error
Control	6.17	0.12
5-Fluorouracil	3.62	0.106
Ethyl acetate Extract	3.81	0.09

Table7.8: Expression levels of Bcl2

Graphical representation of Bcl2 expression:

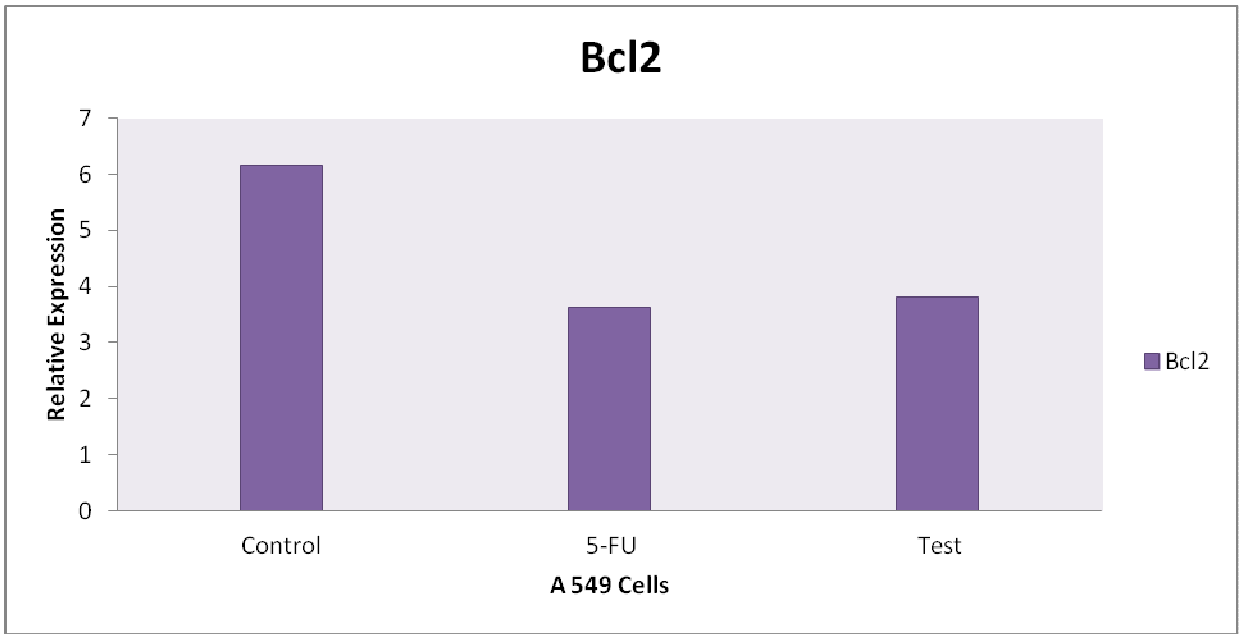


Figure7.15: Graphical representation of Bcl2 expression.

TNF-α Gene in A549 Cell Line :

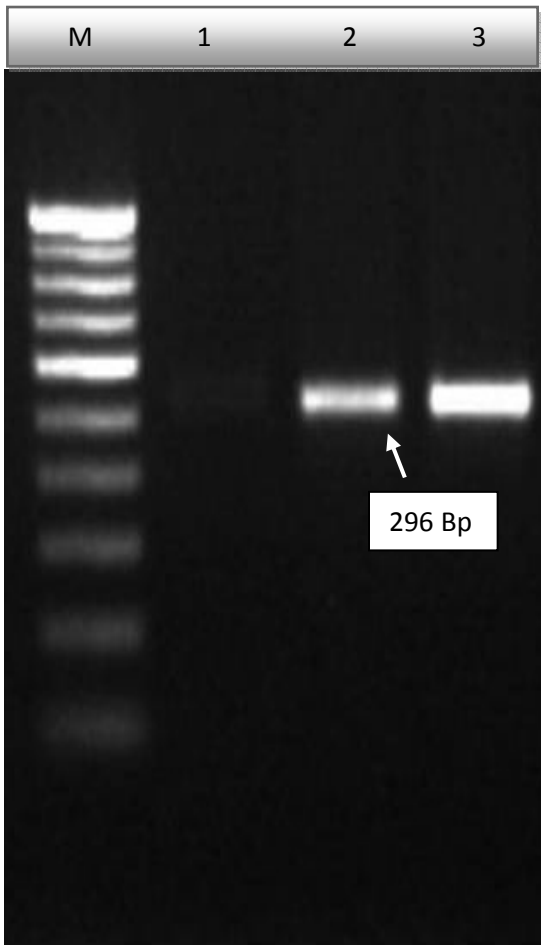


Figure 7.16: TNF alpha Expression

M: Marker DNA

1: Control - Without any treatment

2: Standard- 5-Fluorouracil

3:Test- IC50 concentration of Ethyl acetate extract of *Evolvulus alsinoides*

Expression levels of TNF-α:

P-53	Relative Quantitation	Standard Error
Control	1.21	0.11
5-Fluorouracil	4.12	0.041
Ethyl acetate Extract	3.42	0.03

Table7.9: Expression levels of TNF-α

Graphical representation of TNF-α expression:

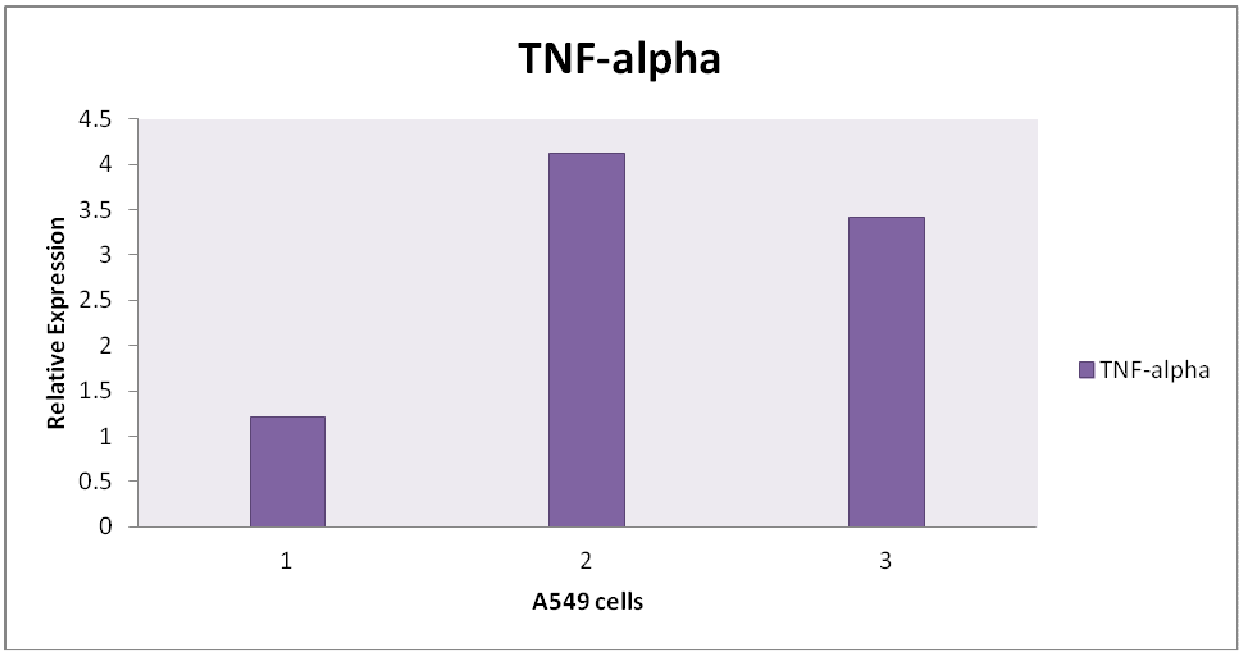


Figure7.17: Graphical representation of TNF-α expression

IL-6 Gene in A549 Cell Line :

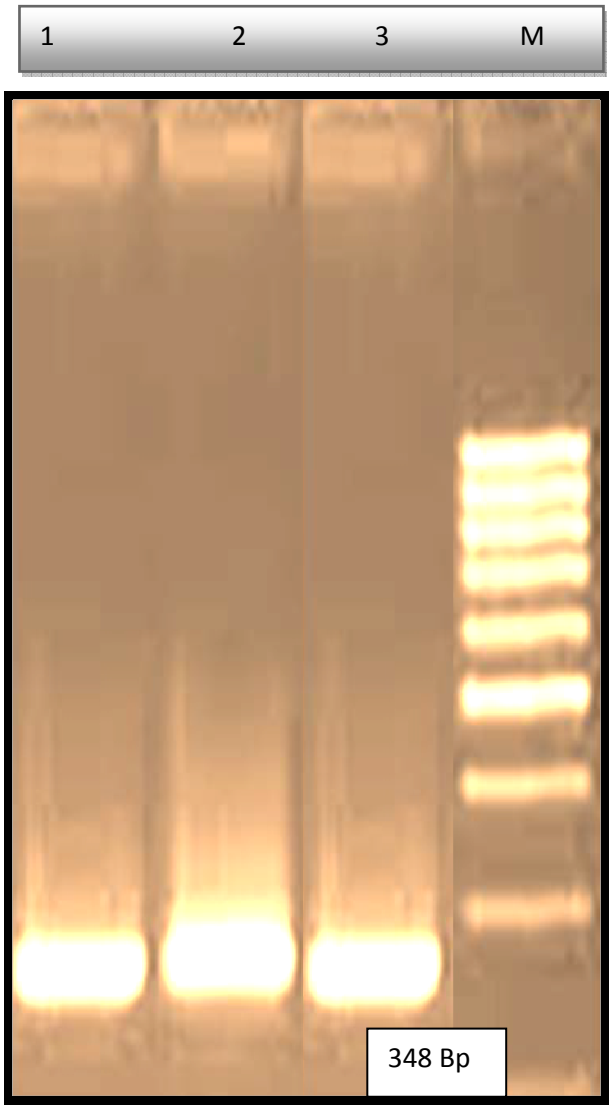


Figure 7.18: IL-6 Expression

M: Marker DNA

1: Standard- 5-Fluorouracil

2: Control - Without any treatment

3:Test- IC50 concentration of Ethyl acetate extract of *Evolvulus alsinoides*

Expression levels of IL-6:

P-53	Relative Quantitation	Standard Error
Control	8.62	0.01
5-Fluorouracil	6.42	0.06
Ethyl acetate Extract	6.12	0.03

Table7.10: Expression levels of IL-6

Graphical representation of IL-6 expression:

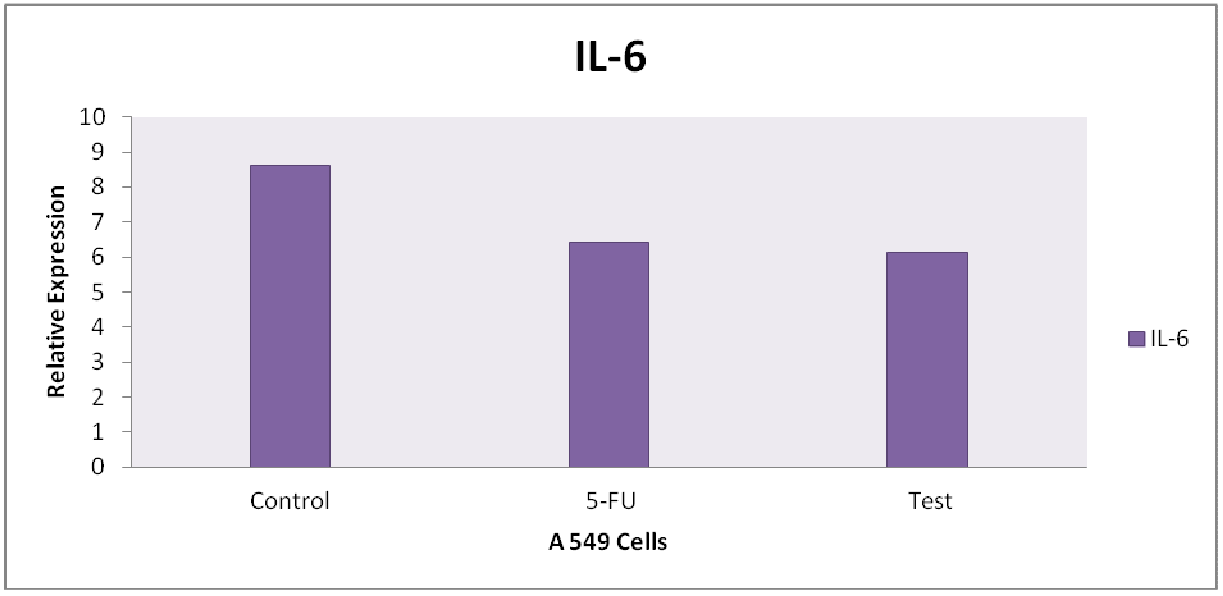


Figure7.19: Graphical representation of IL-6 expression

8. DISCUSSION

Cancer is considered as a serious health problem worldwide. Lung cancer remains a major global health problem and leading cause of cancer mortalities in most of the countries in the world with approximately 1.3 million new cases and 300000 deaths each year estimated by World Health Organisation. Lung cancer has been classified into two groups namely small cell and non small cell lung cancer, with the latter being more prevalent accounting for nearly 80% of lung cancer cases.

With increase in mortality rates among patients suffering from cancer and with limited success being achieved in clinical therapies including radiation, chemotherapy, immune modulation and surgery in treating cancer patients, there arises a need for new way of cancer management.

Natural phytochemicals derived from medicinal plants have attained a greater significance in potential management of several diseases including cancer. Several researches have been carried out in evaluation of plant extracts as prophylactic agents which offer greater potential to inhibit carcinogenic process.

The mechanism of inhibition of tumour progression by natural phytochemicals range from inhibition of genotoxic effects, increased anti inflammatory and anti oxidant effect, inhibition of cell proliferation, protection of intracellular communications to modulate apoptosis and signal transduction pathways.

Discovery of effective herbs and elucidation of their underlying mechanisms could lead to development of an alternative and complimentary method for cancer prevention and treatment.

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around world. Currently more than 3000 plants around world have been reported to possess anti cancer properties.

Screening of different plant components in search of anti cancer drugs is one of the main research activities throughout the world. Vinca alkaloids and cytotoxic podophyllotoxins were discovered in the 1950's as first anticancer agents from plants. In addition to several plants extracted compounds such as camptothecin, vincristine, vinblastine, taxol and podophyllotoxin , many of the natural compounds were structurally modified to result in stronger anti cancer analogues with less adverse effects.

Evolvulus alsinoides plant used traditionally to treat bowel problems and to promote conception. The entire plant was considered astringent and useful for treating haemorrhages, and there are a variety of other medical applications, including use as an adaptogenic, antiphlogistic, antipyretic, antiseptic, aphrodisiac, febrifuge, stomachic, tonic, and vermifuge, in the treatment of asthma, bronchitis, scrofula, syphilis, or in "controlling night emissions" and to promote wound healing.

Phytochemical analysis has revealed the presence of alkaloids, tannins, steroids and flavanoids. It has been reported with adaptogenic, anxiolytic, anti-amnesic, antioxidant, antiulcer, anticatatonic, anti Diarrhoeal, anti-inflammatory, antipyretic and immunomodulatory activity.There was no previous study to prove its anti cancer activity. So the study was carried out to evaluate the anticancer effect of extracts of leaves of *Evolvulus alsinoides* and the gene expression levels playing a role in cancer pathology using *in vitro* models.

Dried whole plant of *Evolvulus alsinoides* were extracted with solvents like petroleum ether, Ethyl acetate and Ethanol. Cytotoxic activity was carried out in lung cancerous cell line A549 with extracts of ethyl acetate, ethanol and was compared with standard drug 5 Fluorouracil. Test for cytotoxicity was carried out by MTT assay and among the three extracts evaluated, the effective extract was found to be ethyl acetate extract with a IC50 value of 15.6µg/ml followed by ethanol and petroleum ether extract with IC50 value of 62.5 µg/ml and 250 µg/ml respectively. IC50 value of 5 Fluorouracil was found to be 7.8 µg/ml. Linearity was expressed with the help of graph plotted in Microsoft excel.

Ethyl acetate extract was found to be more effective of all three extracts by carrying out MTT assay and further studies were carried out with extract of ethyl acetate. Apoptotic study was carried out by Microscopic analysis and DNA fragmentation.

Apoptotic effect of IC50 concentration of ethyl acetate extract of *Evolvulus alsinoides* treated A549 cells were further confirmed with the help of fluorescence microscopy using acridine orange and ethidium bromide. Acridine orange is a vital dye capable of staining both dead and live cells, where as ethidium bromide will stain only cells that have lost their membrane integrity. On examination of cells without any treatment under fluorescent microscope, the cells were stained green in colour representing viable or live cells, whereas examination of cells after treatment with ethyl acetate extract showed reddish or orange colour with loss of membrane integrity and leakage of cytoplasmic contents representing dead cells and and the obtained results were similar to those reported by Shahrul Hisham Zainal Ariffin et al in Hep G2 cells. This led to confirmation that ethyl acetate extract of *Evolvulus alsinoides* showed apoptotic effect in lung cancerous cell line A549.

DNA fragmentation study was carried out by extracting DNA from the cells after treatment with IC50 concentration of ethyl acetate extract of *Evolvulus alsinoides* and standard 5 Fluorouracil for 48 hours and also from cells without any treatment. Apoptosis is characterized by cleavage of chromosomal DNA into oligonucleosomal fragments. Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis and apoptotic cells often produce nucleotide fragments at an interval of 180-200 base pairs, visualized by DNA agarose gel electrophoresis. On examination, DNA fragmentation appeared in A549 cells treated with ethyl acetate extract and 5 fluorouracil when compared to cells without any treatment, which did not show any fragmentation. The results obtained were similar to the results shown by Abhimanyu kumar Jha et al using SiHa cell line. This fragmentation of DNA indicated the characteristics of apoptotic cells. Thus ethyl acetate extract of *Evolvulus alsinoides* causes DNA damage in A549 cells, thereby inducing apoptosis.

Protein levels in cancer cells before and after treatment with extract and 5 Flourouracil were studied using bio analyzer. C Reactive Protein is a 21KDa protein, a systemic biomarker of inflammation whose levels were found to be high in malignancies indicating a close linkage between inflammation and malignancy. It was reported that C reactive protein levels were high in patients suffering from lung cancer and there was a strong association of elevated levels of CRP with tobacco related lung cancer. CRP lowering agents may have promising role in prevention and therapy of malignancies in future. Another protein associated with lung cancer is 14KDa phosphohistidine phosphatase which was reported to play a role in lung cancer cell migration and invasion.

The levels of these two proteins were quantified in A549 cells after treatment with ethyl acetate extract of *Evolvulus alsinoides* and standard for 48 hours. C reactive protein present in A549 cells without any treatment were found to be 420.5 pg/µl, whereas in cells treated with ethyl acetate extract and standard were found to be 354.6 pg/µl and 330.7 pg/µl respectively. Similarly Phospho histidine phosphatase present in A549 cells without any treatment were found to be 128 pg/µl, whereas in cells treated with ethyl acetate extract and standard were found to be 86.4 pg/µl and 65.2 pg/µl. This marked reduction in protein levels which are said to be associated with lung cancer risk in cells after treatment shows that the ethyl acetate extract of *Evolvulus alsinoides* posses the anti cancer effect.

Cancer DNA markers like p53, Bcl2, TNF-α and immune response marker IL-6 plays a major role in cancer pathology and their expression levels determine the progression of the disease. These gene expression levels were studied in cells treated with IC50 concentration of ethyl acetate extract of *Evolvulus alsinoides* and 5-Fluorouracil by RT-PCR methodology using SYBR green.

The expression levels of p53 was found to be increased in cells treated with ethyl acetate extract of *Evolvulus alsinoides* and in cells treated with 5-Fluorouracil when compared to cells without any treatment, indicating the ability of ethyl acetate extract to up regulate p53 and promote apoptosis. The expression levels of TNF-α was found to be increased in cells treated with ethyl acetate extract and in cells treated with 5 Fluorouracil when compared to cells without any treatment, indicating the ability of ethyl acetate extract to induce apoptosis by increasing the expression of TNF-α. The results obtained agreed with those obtained by Azizi et al and Ali Alshehri.

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. The expression levels of Bcl-2 in ethyl acetate extract treated cells and 5 Fluorouracil treated cells was found to be decreased when compared to expression in cells without any treatment which implies that apoptosis in A549 lung cancerous cells may be due to decreased expression of Bcl-2. The results obtained were similar to those reported by Gul Ozcan Arican et al in Hela cells.

Immune response marker IL-6 plays a role in cancer. High serum IL-6 levels were detected in patients with lung, breast, prostate, colorectal, gastric, pancreatic, ovarian and renal cell cancers. High serum IL-6 levels were associated with progressive diseases and poor survival. Expression of IL-6 in control cells were found to be high when compared to cells treated with ethyl acetate extract and cells treated with standard 5 Fluorouracil indicating that apoptosis in cells of A549 may be due to decreased expression of IL-6. These gene expression levels indicates that ethyl acetate extract of *Evolvulus alsinoides* exhibits apoptotic effect by over expression of p53, TNF-α and down regulation of Bcl-2 and immune response marker IL-6.

9. CONCLUSION

- Evaluation of Phytochemical analysis of ethyl acetate, ethanol and petroleum ether extracts of *Evolvulus alsinoides* revealed the presence of proteins, alkaloids, tannins, steroids, phenols and flavanoids.
- In this present study, Ethyl acetate extract of *Evolvulus alsinoides* was found to possess potent cytotoxic activity in human lung cancerous cell line A549 and was compared with standard drug 5 Fluorouracil.
- The Apoptotic effect was confirmed in treated cells by appearance of loss of membrane integrity, leakage of cytoplasmic contents and fragmentation of DNA in treated cells.
- Its anti cancer effect was further confirmed by reduced levels of proteins Phospho histidine phosphatase and C Reactive Protein in ethyl acetate extract of *Evolvulus alsinoides* treated cells, whose elevated levels are said to possess a risk in cancer development.
- Its apoptotic and anti cancer effect may be due to up regulation of genes like p53 and TNF α and down regulation of genes like Bcl-2 and IL-6, which was confirmed by RT-PCR.
- These results show that ethyl acetate extract of *Evolvulus alsinoides* possess anti cancer effect and for future perspective, it can be further confirmed by isolating the compounds responsible for the activity and studying the exact mechanism by which the plant possess this activity and confirm the results using *in vivo* animal models.

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