EVALUATION OF THE ANTICANCER ACTIVITY OF *ADIANTUM INSISUM* EXTRACT

Thesis submitted for the Partial Fulfillment of the Masters of Pharmaceutical Technology in the Faculty of Engineering & Technology Jadavpur University 2019

By

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DECLARATION

I declare that "**EVALUATION OF THE ANTICANCER ACTIVITY OF *ADIANTUM INSISUM* EXTRACT"** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Signatue of the student: ……**.....**

Full name……

Date……..

CERTIFICATE

This is to certify that **Shubhadeep Mondal** has carried out the research on the project entitled **"Evaluation of the anticancer activity of *Adiantum insisum* extract".**
under my supervision, in the Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata – 700032.

He has incorporated his findings into this thesis of the same title being submitted by him in partial fulfillment of the requirement for the award of **Degree of Master of Pharmaceutical Technology, Jadavpur University**. I am satisfied that he has carried out his thesis with proper care and confidence to my entire satisfaction.

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Shubhadeep Mondal

PREFACE

The present study **"Evaluation of the anticancer activity of *Adiantum insisum* extract."** covers original research work conducted by the author for the award of Master of Pharmacy in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata.

It goes beyond saying that today cancer is a leading cause of mortality globally. With the passage of time, theories of cancer are constantly evolving. Various therapeutic attempts to fight cancer have failed mainly owing to the side effects they pose and also due to the high cost associated with them. In this regard it must be mentioned that natural products are safer than their synthetic counterparts and also are less expensive. In fact plant derived molecules like vinblastine, vincristine, paclitaxel etc proved to be effective as chemotherapeutic agent. With a large number of molecules of natural origin already established, yet so many that have passed preliminary screening and there could still be a huge number of them still concealed within nature that could have potential anticancer activities. Hence screening of natural products for efficacy against cancer becomes imperative.

In this work the plant *Adiantum insisum* has been studied preliminarily for its anticancer potentials using EAC model, a popular model in academic research. This plant, obtained from Sikkim is locally an edible plant and hence is likely to be less toxic. Also it has a rich history of traditional use. These reasons justify the choice of the plant for this experimental purpose. Both *in vitro* and *in vivo* studies were conducted and various parameters evaluated.

The thesis covers various aspects of the above study in different chapters in a logical sequence with relevant references annexed to each chapter. The results of different studies are summarized in the form of tables and figures indicating statistical significance levels and conclusions drawn to justify the work scientifically.

Shubhadeep Mondal

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Dedicated
To
My Family
&
Lovely Mentors


Introduction

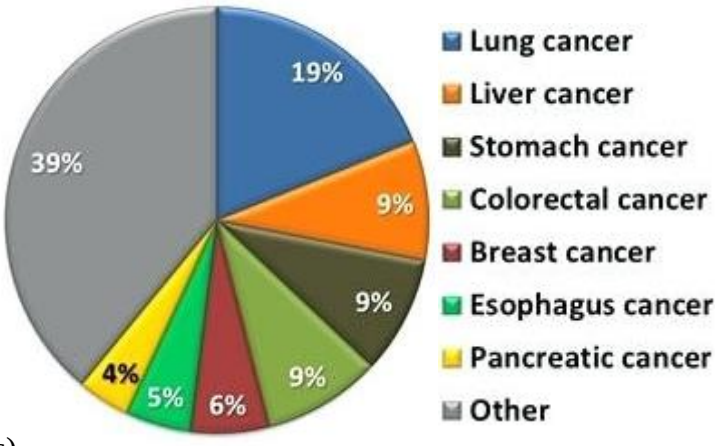
Cancer: A brief Introduction:

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Cancer, the second leading cause of death worldwide next to cardiovascular diseases, is a group of move that 100 different disease, characterized by uncontrolled cellular growth, local tissue invasion and distant metastases. (Dashora NV *et al.*, 2010). In most organs and tissues of a mature animal, a balance is usually maintained between cell renewal and cell death. The various types of mature cells in the body have a given lifespan; as these cells die; new cells are generated by the proliferation of new cells is regulated so that the number of any particular type of cell remains constant. However under certain some abnormal cells (AL-Bala *et* al., 2012) are able to evade this controlled or programmed process of cell death (apoptosis). Its can spread to almost any part of the living system. There are four characteristics of cancer cells that distinguish them from normal cells are uncontrolled proliferation, dedifferentiation and loss of function, invasiveness and metastasis. Altered cells divide uncontrollably to from lumps or masses of tissue called tumors. A tumor that is not capable of indefinite growth and does not invade the healthy surrounding tissue extensively is benign. A tumor that continues to grow and becomes progressively invasive is malignant; the term cancer refers specifically to a malignant tumor. In addition to uncontrolled growth, malignant tumors exhibit metastasis (Kuby *et* al., 2003) in this process, small clusters of cancerous cells from the initial or primary tumor have been released known as secondary tumor, have reached other sites through lymphatic's and blood vessels, and are carried to other tissues, where they continue to proliferate. Radiation and Chemotherapy are the best method of cancer treatment. Chemotherapy is curative for some cancers, such as some leukemia's, ineffective in some brain tumors, and needless in others, such as most non-melanoma skin cancers too. A major challenge for medical oncology is to develop therapeutic treatment that will prevent toxicity induced by antitumor treatments without impairing their antitumor effects (Das *et* al., 2012). In the new way now researchers have recently focused on the uses of Ehrlich's ascites carcinoma (EAC) cells on the local plants which can cure cancer. The Ehrlich tumor was rapidly growing, very aggressive behavior and can grow in almost everywhere in the mice's strains. In the ascetic form, it has been used as a transplantable tumor model which is investigate the effects of several antitumor substances (Segura *et* al., 2000).

Statistics:

In 2018, according to the World Health Organization (WHO) an estimated 17.3 million people death for the cancer. The number of cancer survivors is expected to increase to 27.3 million by 2036.

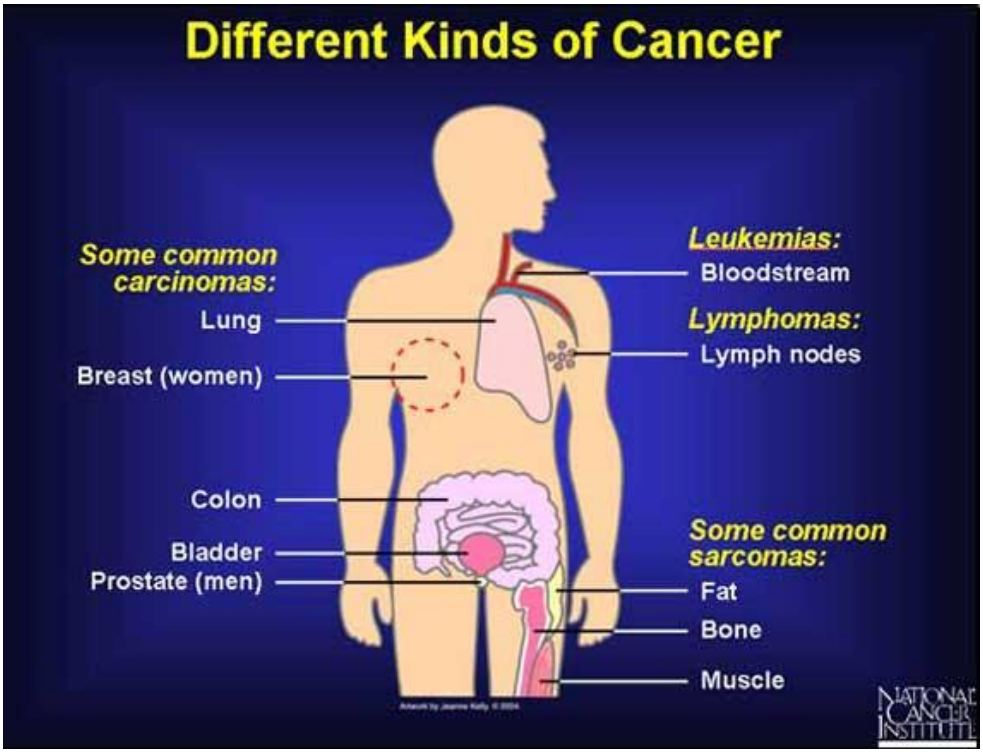
1. Lung (1.63 million deaths)
2. Liver (746 200 deaths)
3. Stomach (723 500 deaths)
4. Colorectal (693 700 deaths)
5. Breast (522 400 deaths)
6. Esophageal cancer (400 225 deaths)
7. Cervical cancer (265 800 deaths)
8. Urinary bladder cancer (165 300 deaths)



Classification:

Cancers are classified by the type of cell that the tumor cells resemble and are therefore presumed to be the origin of the tumor. These types include:

- Carcinoma: Cancers derived from epithelial cells. This group includes many of the most common cancers, particularly in the aged, and includes nearly all those developing in the breast, prostate, lung, pancreas, and colon.
- Sarcoma: Cancers arising from connective tissue (i.e. bone, cartilage, fat, nerve), each of which develops from cells originating in mesenchymal cells outside the bone marrow.
- Lymphoma and leukemia: These two classes of cancer arise from hematopoietic (blood-forming) cells that leave the marrow and tend to mature in the lymph nodes and blood, respectively. Leukemia is the most common type of cancer in children accounting for about 30%.



- Germ cell tumor: Cancers derived from pluripotent cells, most often presenting in the testicle or the ovary (seminoma and dysgerminoma, respectively).
- Blastoma: Cancers derived from immature "precursor" cells or embryonic tissue. Blastomas are more common in children than in older adults.

Causes of Cancer:

There is no single cause of cancer. Scientists believe that, it is interaction of many factors together that produces cancer (Loechler. 2003 and Turker, 2009). The majority of cancers, some 90–95% of cases, are due to genetic mutations from environmental factors. The remaining 5–10% are due to inherited genetics. Environmental, as used by cancer researchers, means any cause that is not inherited genetically, such as lifestyle, economic and behavioral factors and not merely pollution. Common environmental factors that contribute to cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity and pollution.

Lifestyle: A high-fat diet, Smoking and working with chemicals are examples of lifestyle choices that may be risk factors for some adult cancers. Diet, physical inactivity, and obesity are related to up to 30-35% of cancer deaths. In the United States excess body weight is associated with the development of many types of cancer and is a factor in 14-20% of all cancer deaths. Physical inactivity is believed to contribute to cancer risk, not only through is effect on body weight but also through negative effects on the immune system and endocrine system.

Genetic: Some genetic disorders, for example, Wiskott-Aldrich and Beckwith-Wiedemann syndrome are known to alter the immune system. One theory suggests that the cells in the bone marrow, the stem cells, become damaged or defective, so when they reproduce to make more cells, they make abnormal cells or cancer cells. The cause of the defect in the stem cells could be related to an inherited genetic defect or exposure to a virus or toxin. Four key types of gene are responsible for the cell division process: oncogenes tell cells when to divide, tumor suppressor genes tell cells when not to divide, and suicide genes control apoptosis and tell the cell to kill itself if something goes wrong and DNA-repair genes instruct a cell to repair damaged DNA.

Viruses: Worldwide approximately 18% of cancer deaths are related to infectious diseases. A virus called *oncovirus* which is responsible for cancer. This includes Epstein-Barr virus and human papilloma virus. Bacterial infection may also increase the risk of cancer, as seen in Helicobacter pylori-induced gastric carcinoma. HIV the virus that causes AIDS, have been linked to an increased risk of developing certain childhood cancers, such as Hodgkin and non-Hodgkin lymphoma. Possibly, the virus alters a cell in some way. That cell then reproduces an altered cell and, eventually, these alterations become a cancer cell that reproduces more cancer cell.

Environmental exposures: Pesticides, fertilizers and power lines have been researched for a direct link to childhood cancers. Whether infant or infant exposed to these agents causes cancer, or whether it is a coincidence, is unknown. In some cases, children who have been exposed to these agents may develop a second malignancy later in life. These strong anticancer agents can alter cells and/or the immune system. A second malignancy is a cancer that appears as a result from treatment of a different cancer.

Pathophysiology:

In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered. Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide. There are two broad categories of genes which are affected by these changes. Expression of these oncogenes promotes the malignant phenotype of cancer cells. Tumor suppressor genes are genes which inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer-promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell.

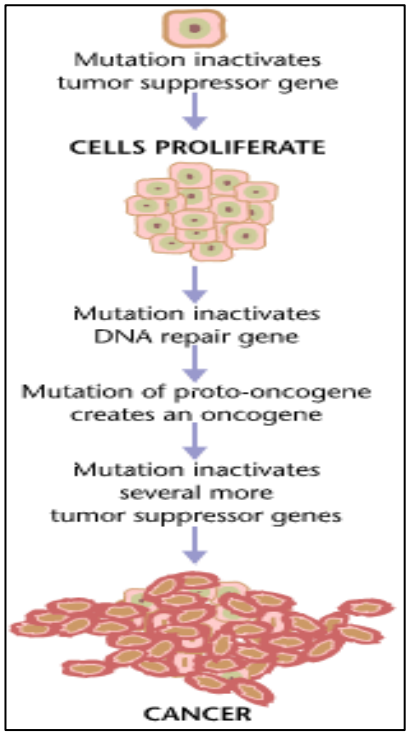
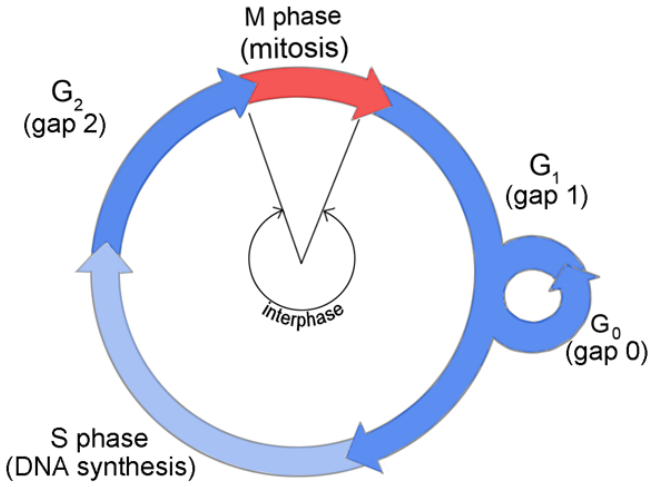


Figure 1: Genesis of Cancer

Mechanism of Cancer:

To understand cancer mechanisms we have to first understand the cell cycle and how it is related to cancer.

The term cell refers to the sequence of events that place within a cell as it tools up for division. The phases of the cell cycle are:



G_1, S, G_2 and M phase.

M is the phase of Mitosis.

S is the phase of DNA synthesis.

G_1 is the gap between the mitosis that give rise to the cell and the S phase; during G_1, the cell is preparing of DNA synthesis.

G_2 is the gap between S phase and the mitosis that will give rise to two daughter cells; during G_2, the cell is preparing for the mitotic division into two daughter cells.

Finally, these is a fifth state, G_0 (also known as quiescence) into which the cell may reversibly exit from G_1, if it is deprived of the appropriate growth-promoting signals. Growth factor action stimulates a quiescent cell-said to be in G_0 (G nought)-to divide, i.e. to start on G_1 phase.

Cell cycle checkpoint:

Cell cycle checkpoint is a mechanism that maintains the observed order of events of each cell cycle (Hartwell and Weinert, 1989). Major function of these checkpoints is to see that the integrity of the genome remains intact throughout the cell cycle. Cell division requires the controlled timing of two critical events of the cell cycle: S phase (DNA replication) and M phase (mitosis). Entry into each of these phases is carefully regulated, and there are thus two 'check points' (restriction points) in the cycle: one at the start of S and one at the start of M.DNA damage results in the cycle being stopped at one of other of these. The integrity of the check points is critical for the maintenance of genetic stability and failure of the check points to stop the cycle when it is appropriate to do so is a hallmark of cancer. The first of these occurs at the G_1/S phase transition and is a major sensor of DNA damage. The cell may also arrest later in S phase due to incomplete DNA replication or again, damage to the DNA. Next is the G_2/M checkpoint, which monitors the fidelity of DNA replication and like the G_1/S checkpoint is an important sensor of DNA damage.

Positive regulator of cell cycle:

The cycle is initiated when a growth factor acts on a quiescent cell, provoking it too divides. One of the main actions of a growth factor is to stimulate production of the cell cycle regulators, which are coded for by the delayed response genes. The main components of the control system that

determines progress through the cycle are two families of protein: cyclins and cyclin-dependent kinases (CDKs).

The heart of the regulatory apparatus during the cell cycle progression is a family of enzymes, called the cyclin dependent kinases (CDKs). The active forms of CDKs are a complex of at least to proteins, a kinase and a cyclin. They often contain other proteins with poorly understood functions. These complexes undergo changes in the kinase and cyclin components that are believed to drive the cell from one stage of the cell cycle to another. According to this paradigm, the cell cycle is determined by the constellation of proteins that are activated or inactivated by phosphorylation, a result of the activity of the CDKs during that stage. In mammalian cells, a succession of kinase Subunits (CDK4, CDK6, CDK2, and CDC2) is expressed along with a succession of cyclins (D, E, A and B), as the cells progress from G1 to mitosis. CDK4 and CDK6 complexed with one of several D-type cyclins functions early in the G1 phase, probably in response to growth factors. CDK2 that complexed with cyclin E, cyclin A, or both is essential for the G1 S transition and DNA replication, Respectively. CDC2 that complexed with cyclin A and cyclin B is essential for mitosis. Each cdk is inactive until it binds to a cyclin, the binding enabling the cdk to phosphorylate the proteins necessary for a particular step in the cycle. It is the cyclin that determines which proteins are phosphorylated. After the phosphorylation event has taken place, the cyclin is degraded by the ubiquitin/protease system.

In quiescent G_0 cells, cyclin D is present in low concentration, and an important regulatory protein- the Rb protein is hypophosphorylated. Hypophosphorylated Rb holds the cell cycle in check at check at check point 1 by inhibiting the expression of several proteins critical for cell cycle progression. The Rb protein accomplishes this by binding to the E2F transcription factors, which control the expression of the genes that code for cyclins E and A, for DNA polymerase, for thymidine kinase, for dihydrofolate reductase, etc. all essential for DNA replication during S phase.

During G_1 the concentration of cyclin D increases and the cyclin D/cdk complex phosphorylates and activates the necessary proteins. In mid-$G_{1,}$ the cyclin D/cdk complex phosphorylates and activates the necessary proteins. In mid-$G_{1,}$ the cyclin D/cdk complex phosphorylates the Rb protein, releasing transcription factor E2F; this then activates the genes for the components specified above that are essential for the next phase-DNA synthesis-namely cyclins E and A, DNA polymerase and so on.

Cyclin E/cdk and cyclin A/cdk regulate progress through S phase, phosphorylating and thus activating proteins/enzymes involved in DNA synthesis.

Cyclin A/cdk and cyclin B /cdk complexes are active during G_2 phase and are necessary for entry into M phase, i.e. for passing check point 2. The presence of cyclin B/cdk complexes in the nucleus is required for mitosis to commence.

Negative regulator of cell cycle:

There are two families of inhibitors:

- The CIP family (cdk inhibitor proteins, also termed KIP or kinase inhibitory proteins)-p21, p27 and0 p57.
- The Ink family (inhibitors of kinases)-p16, p19, and p15.

The p53 gene has been called the 'guardian of the genome'. It codes for a protein transcription factor-the p53 protein. In normal healthy cells, the steady-state concentration of the p53 protein is low. But when there is DNA damage, the protein accumulates and activates the transcription of several genes, one of which codes for p21. Protein p21 inactivates cyclin/cdk complexes, thus preventing Rb phosphorylation, which means that the cycle is arrested at check point 1 into S phase. If the repair is unsuccessful, the p53 gene triggers apoptosis-cell suicide.

The cell cycle and cancer:

The connection between the cell cycle and cancer is obvious: cell cycle machinery controls cell proliferation, and cancer is a disease of inappropriate cell proliferation. Fundamentally, all cancers permit the existence of too many cells. However, this cell number excess is linked in a vicious cycle with a reduction is sensitivity to signals that normally tell a cell to adhere, differentiate, or die. This combination of altered properties increases the difficulty of deciphering which changes are primarily responsible for causing cancer. The hallmark of cancer is deranged growth control (Pardee *et* al., 1978). Checkpoints are defective in cancer cells (Hartwell and Kastan, 1994). Control mechanisms are usually lost by mutation, for example many cancers lose or have mutated p^{53} genes, or have alterations in some components of the Rb pathway. In addition, carcinogenic viruses such as SV40 produce proteins, such as T-antigen, which bypass G1/S controls, limit the appearance of genetic variants and the progression of cancer. The first genetic alterations shown to contribute to cancer development were gain-of-function mutations.

These mutations define a set of oncogenes that are mutant versions of normal cellular proto-oncogenes. The products of proto-oncogenes function in signal transduction pathways that promote cell proliferation. However, transformation by individual oncogenes can be redundant (mutations of one of several genes will lead to transformation) or can be cell type-specific (mutations will transform some cells but have no effect on others). This suggests that multiple, distinct pathways of genetic alteration lead to cancer. Cancer is caused by alterations in oncogenes, tumor-suppressor genes and microRNA genes. A single genetic change is rarely sufficient for the development of a malignant tumor. Most evidence points to a multistep process of sequential alterations in several, often many oncogenes, tumor-suppressor genes, or microRNA genes in cancer cell. Historically, transformation events in cancer have been defined as initiation events (contributing to the early stages of neoplastic transition) or progression events (referring to subsequent transformative processes). Ontogenesis encodes proteins that control cell proliferation, apoptosis, or both. They can be activated by structural alterations resulting from mutation or gene fusion, (Konopka *et* al., 1985), by juxtaposition to enhancer elements, (Tsujimoto *et* al., 1985) or by amplification. Translocations and mutations can occur as initiating events (Finger *et* al., 1986) or during tumor progression, whereas amplification usually occurs during progression (Carlo, 2008).

Genetics:

Cancer is a genetic disease: In order for cells to start dividing uncontrollably, genes that egulate cell growth must be damaged (Vogelstein, Bert and Kinzler, Kenneth, 2004). Cancer is fundamentally a disease of tissue growth regulation failure. In order for a normal cell to transform into a cancer cell, the genes that regulate cell growth and differentiation must be altered. The affected genes are divided into two broad categories. Proto-oncogenes are genes that promote cell growth and mitosis, whereas tumor suppressor genes discourage cell growth, or temporarily halt cell division to carry out DNA repair. Typically, a series of several mutations to these genes is required before a normal cell transforms into a cancer cell. This concept is sometimes termed "oncoevolution". Mutations to these genes provide the signals for tumor cells to start dividing uncontrollably. But the uncontrolled cell division that characterizes cancer also requires that the dividing cell duplicates all its cellular components to create two daughter cells. The activation of

anaerobic glycolysis (the Warburg effect), which is not necessarily induced by mutations in proto-oncogene's and tumor suppressor genes, (Brand and Hermfisse, 1997). Provides most of the building blocks required to duplicate the cellular components of a dividing cell and, therefore, is also essential for carcinogenesis (López – Lázaro, 2010).

Oncogenes:

An oncogene is a gene that has the potential to cause cancer. I tumor cells, they are often mutated or expressed at high levels. Most normal cells will undergo a programmed form of rapid cell death (apoptosis) when critical functions are altered. Activated oncogenes can cause those cells designated for apoptosis to survive and proliferate instead. Mostoncogenes require an additional step, such as mutations in another gene, or environmental factors, such as viral infection, to cause cancer. Oncogenes are derived from normal host genes, also called proto-oncogenes, which deregulated as a consequence of mutation. A proto-oncogene is a normal gene that can become an oncogene due to mutations or increased expression. The resultant protein encoded by an oncogene is termed oncoprotein. Proto-oncogenes code for proteins that help to regulate cell growth and differentiation. Upon activation, a proto-oncogene (or its product) becomes a tumor-including agent, an oncogene. Example of oncogene is the Bcr-Abl gene found on the Philadelpha Chromosome, a piece of genetic material seen in Chronic Myelogenous Leukemia caused by the translocation of pieces from chromosomes 9 and 22. This also a 'gain of function' mutation because the cells with the mutant from of the protein have gained a new functions not present in cells with the normal gene. There are three basic methods of activation:

1. A mutation within a proto-oncogene, or within a regulatory region (for example the promoter region), can cause a change in the protein structure, causing
    - an increase in protein (enzyme) activity
    - a loss of regulation
2. An increase in the amount of a certain protein (protein concentration), caused by
    - an increase of protein expression (through mis regulation)
    - an increase of protein (mRNA) stability, prolonging its existence and thus its activity in the cell.

- gene duplication (one type of chromosome abnormality), resulting in an increased amount of protein in the cell.

3. A chromosomal translocation (another type of chromosome abnormality)

   There are 2 different types of chromosomal translocations that can occur:

   a. Translocation events which relocate a proto-oncogene to a new chromosomal site that leads to higher expression.

   b. Translocation events that lead to a fusion between a proto-oncogene and a 2nd gene (this creates a fusion protein with increased cancerous/ monogenic activity).

Table 1.1

Oncogenes	**Protein functions**	**Neoplasm**
Growth Factors: Sis Int2 Trk	Platelet derived growth factor Fibroblast derived growth factor Nerve derived growth factor	Facor fibrosarcoma Breast Neuroblastoma
Growth Factor Receptors: Erb1/ Erb2/ Her2/ neu Fms Ros	Epidermal growth factor receptor Heregulin Hematopoetic colony stimulating factor Insulin receptor	Squamus Cell Carcinoma Breast carcinoma Sarcoma Astrocytoma
Tyrosine kinases: Ber-abl src and lck	Tyrosine kinase Tyrosine kinase	Chronic Myelogenous Leukemia
Serine- Threonine protein Kinases Raf and mos	Serine-threonine kinase	Sarcoma
Guanine nucleotide binding Proteins H-ras K-ras N-ras	GTPase melanoma GTPase leukemias GTPase	Lung. Pancreas colon, lung, pancreas carcinoma of the genitourinary tract and thyroid; melanoma
Cytoplasmic proteins: Blc-2	Anti-apoptotic protein	non-Hodkin “s B-cell lymphoma
Nuclear proteins: myc Jun Fos	Transcription factor Transcription factor (AP-1) Transcription factor (AP-1)	Burkitt “s lymphoma Osteosarcoma Sarcoma

Tumor-suppressor genes:

A tumor suppressor gene, or antioncogene, is a gene that protects a cell from one step on the path to cancer. When this gene mutates, cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes. The loss of these genes may be even more important than proto-oncogene/oncogene activation for the formation of many kinds of human cancer cells.

Tumor suppressor gene	Protein function	Neoplasm(s)
APC	Cell adhesion	Colon
BRCA 1	Transcription factor	Ovary and breast
BRCA 2	DNA repair	Ovary ad breast
CDK4	Cyclin D Kinase	Melanoma
NF1	GTPase	Neuroblastoma
P53	Transcription factor	Lung, colon, breast
Rb	Cell cycle check point	Retinoblastoma
WT1	Transcription factor	Childhood kidney

TREATMENT OF CANCER:

In the new generation cancer is treated such different ways, with the primary ones including surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care. Surgical procedures may precede or follow other treatment approaches. It is best for the treatment plan either to follow a standard protocol precisely or else to be part of an ongoing clinical research protocol evaluating new treatments. The treatment intent may be curative or not curative.

Chemotherapy: Chemotherapy is the use of any drug to treat any disease. But to most people, the word chemotherapy means drugs used for cancer treatment. Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs as part of a standardized regimen. Traditional chemotherapeutic agents act by killing cells that divide rapidly, one of the main properties of most cancer cells.

Radiation: Radiation therapy involves the use of ionizing radiation in an attempt to either cure or improve the symptoms of cancer. It works by damaging the DNA of cancerous tissue leading to cellular death.

Surgery: Surgery is the primary method of treatment of most isolated solid cancers and may play a role in palliation and prolongation of survival.

Nature as a source of anticancer drugs: Nature is the best source of drugs and due to our interest in the identification of new anticancer natural products that overcome the limitations of cell toxicity and adverse reactions. Current estimate determined that about 80% of people in developing countries still depend on traditional medicine based largely on species of plants and animals for their primary health care. All herbal medicines are becoming popular due to the high toxicity and side effects of allopathic medicines. Suddenly increased the number of herbal drug manufactures (Agarwal, 2005).

Now presents many traditional systems of medicine in the world (Chopra *et* al., 1956), like different philosophies and cultural origins. Ayurvedic and Chinese traditional medicines are increasingly used in many different areas of the world.

The various indigenous systems such as Siddha, Ayurveda, Unani and Allopathy use several plant species to treat different ailments (Rabe and Staden, 1997). In India around 20,000 medicinal plant species have been recorded. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources. The use of traditional medicine has increased in developed countries also, mainly due to the failure of modern medicine to provide effective treatment for chronic diseases and emergence of multi-drug resistant bacteria and parasites. The adverse effects of chemical drugs, questioning of the approaches and assumptions of allopathic medicine, their increasing costs and greater public access to information on traditional medicine has also led to an increase in interest in alternative treatments (WHO 2002). Plant extracts have become a source of hope as a wide group of medicinal plant preparations are available that have been used over the centuries almost exclusively on the basis of empirical evidence. Hence, it has become necessary to revisit the importance of these herbal medicines.

Plants are used for the treatment of cancer: Several anticancer agents including taxol, vinblastine, vincristine, topotecan and irinotecan and etoposide derived from epipodophyllotoxin are in clinical use all over the world. "Botanical garden of the World" is the largest producer of medicinal plants in India. A number of promising agents such as flavopiridol, roscovitine, combretastatin A-4, betulinic acid and silvestrol and in clinical or preclinical development (Mohammad Shoeb, 2006).

Vinca alkaloids, vinblastine and vincristine are isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into clinical use for the treatment of cancer (Cragg and Newman, 2005). The discovery of paclitaxel from the bark of thePacific Yew, Taxus brevifolia Nutt. is another evidence of the success in natural product drug discovery. Various parts of Taxus brevifolia and other Taxus species (e.g, Taxus Canadensis Marshall, Taxus baccata L.) have been used by several Nagative American Tribess for the treatment of some non-cancerous cases while Taxus baccata was reported to use in the Indian Ayurvedic medicine for the treatment of cancer.

The taxanes and the comptothecins are presently approved for human use in various countries. Rohitukine the plant alkaloids, isolated from the leaves and stems of Dysoxylum binectariferum (Maliaceae) (Harmon *et* al., 1979). Synthetic flavones derived from rohitukine, Flavopiridol representing the first cyclin-dependent kinase inhibitor to enter the clinical trial (Losiewicz *et* al., 1994). Epipodophyllotoxin is an isomer of podophyllotoxin which was isolated as the active antitumor agent from the roots of Podophylluam species, Podophylluam peltatum Linnaeus and Podophyllum *emodi* Wallich (Berberidaceae) (Stahelin, 1973). Etoposide and teniposide are two semi-synthetic derivatives of epipodophyllotoxin and are used in the treatment of lymphomas and bronchial and testicular cancers.

Besides these there are huge numbers of reported anticancer activity obtained from plants, vegetables, fruits and plant derived molecules.

Scientific Name	Source	Family	Part of plant used	Compounds
Lycopersicon esculentum	Tomato	Solanaceae	Entire	Lycopene,Lutein,
Viscum album	Mistletoe	Loranthaceae	Leaves	Hydrophilic lectins
Ocimum sanctum	Basil	Lamiaceae	Leaves	Ursolic acid
Leptadenia hastate	Flowering plant	Asclepiadaceae	Bark	α-amyrin, βamyrin, ferulic acid
Crocus sativus	Saffron	Iridaceae	Threads	Carotenoids
Dioscorea collettii	Dioscorea	Dioscoreaceae	Rhizome	Steroidal saponins
Carica papaya	Berries	Caricaceae	Fruit	B-Cayptoxanthin
Cannabis sativa	Hemp	Cannbiaceae	Entire	Cannabinol
Prunus armeniaca	Apricots	Rosaceae	Fruit	Carotenoids
Glycine max	Soyabeans	Fabaceae	Corp, Seed	Genistein
Betula alba	Birch tree	Betulaceae	Shrubs, Bark	Betulinic acid
Alnus japonica	Shrub, Tree	Betulaceae	Wood	Triterpenes
Glycyrrhiza glabra	Licorice	Leguminosae	Root	Glycyrrhizin
Piper nigrum	Black pepper	Piperaceae	Fruit	Purpurogallin, Piperine
Rosmarinus officinalis	Rosemary	Lamiaceae	Entire	Carnosol

Natural products discovered from medicinal plants have played an important role in the treatment of cancer. Natural products or natural product derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales (Butlets, 2004). Two plant derived natural products, paclitaxel and comptothecin were estimated to account for nearly one-third of the global anticancer market or about $3 billion of $9 billion in total annually in 2002 (Oberlines and Kroll, 2004). There are more than 270,000 higher plants existing on this planet. But only a small portion has been explored phytochemical. It is anticipated that plants can provide potential bioactive compounds for the development of new leads to combat cancer diseases.

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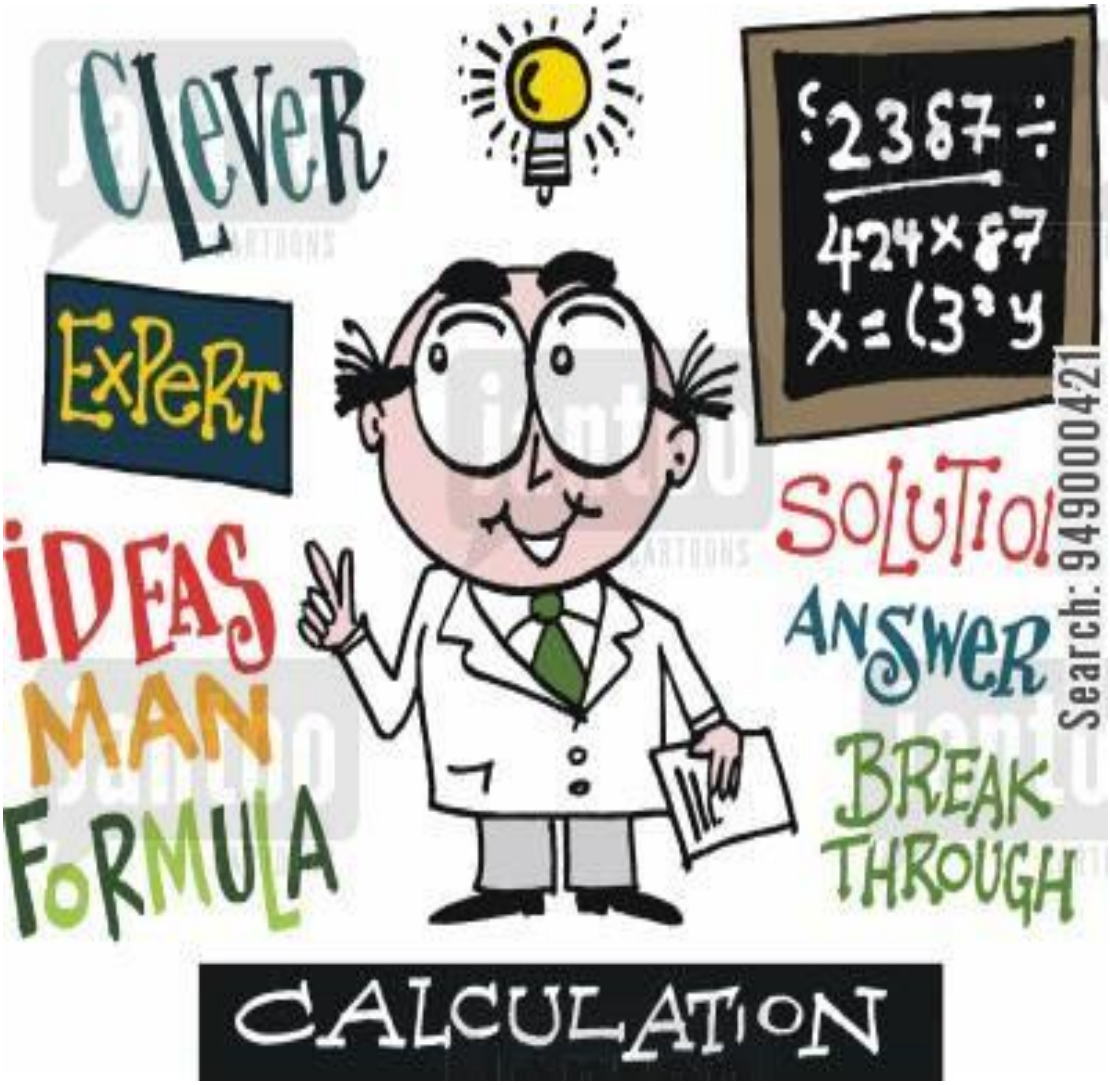
PLAN OF WORK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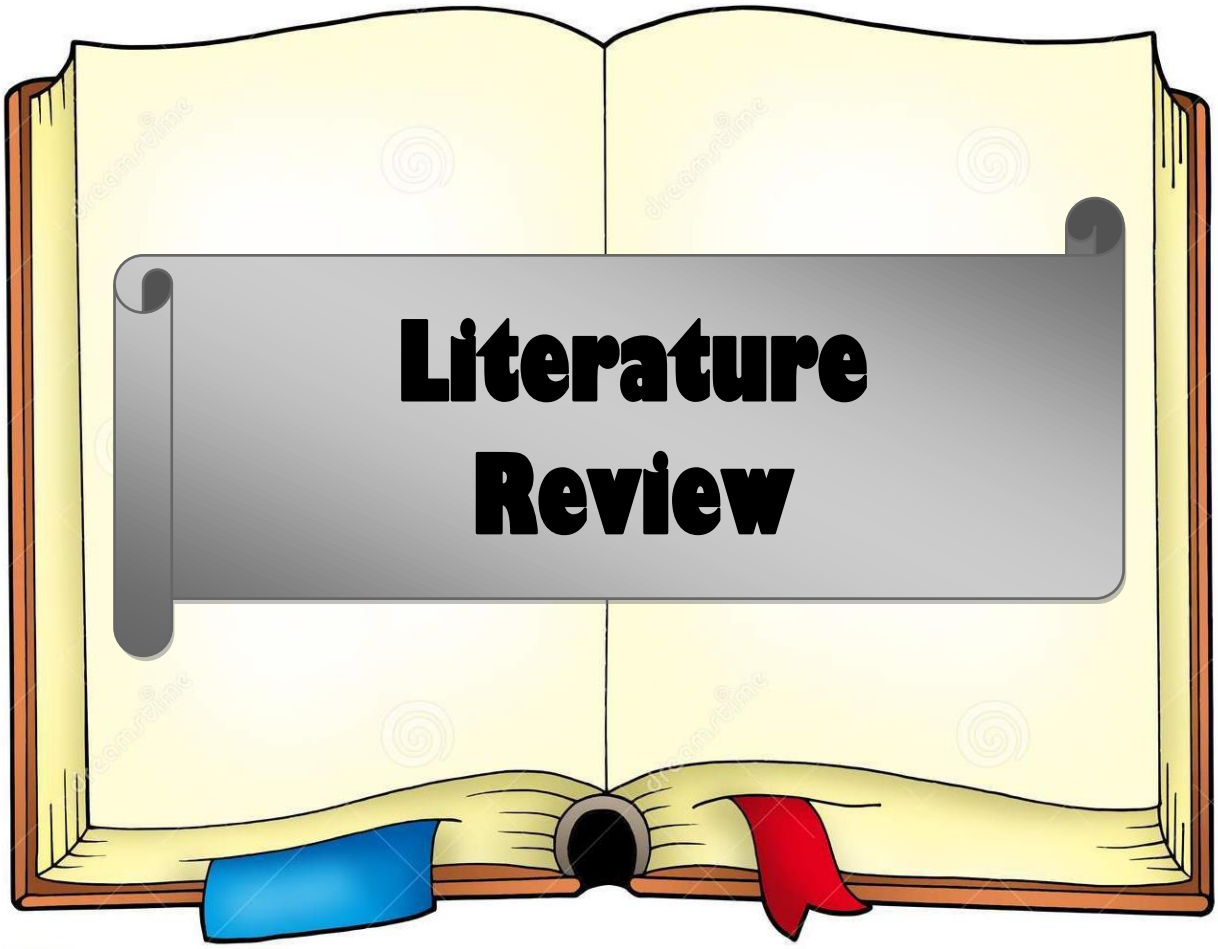
PLAN OF WORK:

Thesis Title

"Evaluation of the anticancer activity of *Adiantum insisum* extract".

Brief outline of the work to be performed:

- Literature survey and plant selection.
- Collection, identification and characterization of the selected plant.
- Extraction by using different solvents.
- Acute toxicity study by LD_{50} method as per OECD guidelines.
- Evaluation of free radical scavenging activity of plant extract by different *in vitro* –
    - DPPH radical scavenging activity
- *In vitro* cytotoxic effect of the plant extract against Ehrlich Ascites Carcinoma cells. The *in vitro* study involves:
    - MTT Assay
    - Trypan blue Assay
- *In vivo* cytotoxic effects of the plant extract against Ehrlich Ascites Carcinoma cells. The *in vivo* study involves:
    - Determination of tumor volume and packed cell volume.
    - Tumor cell count.
    - Percentage increase in life span.
    - Determination of hematological parameters.
- Estimation of biochemical parameters.
    - SGOT (Serum glutamate oxaloacetate transaminase)
    - SGPT (Serum glutamate pyruvate transaminase)
    - ALP (Alkaline phosphatase)
- Determination of tissue antioxidant parameters
    - Lipid Peroxidation
    - Superoxide dismutase
    - Reduced glutathione


Literature
Review

LITERATURE REVIEW

Plant Taxonomy:

Kingdom: Planate - Plant

Subkingdom: Tracheobionta - Vascular plants

Phylum: Pteridophyta

Class: Filicopsida

Family: Pteridaceae - Maidenhair Fern family

Genus:Adiantum L. - Maidenhair fern

Species: *Adiantum caudatum*

Plant Description:

Name: *Adiantum insisum*

Family: Pteridophyta

Synonyms: *Adiantum incisum Forsk.*

Adiantum inoisum Forsk.

Adiantum flagelliferum Wall.

Adiantum hirsutum Bory.

Adiantum capillus-veneris

Local Name: Mayurshikha; Biddapata; Hansraj; Putramchari (Kerala);

Parts used medicinally: Whole plant.

Morphology of the plant:

Rhizome ascending, short and erect with linear scales at the base, 4 to 5 mm long over and 0.2 mm wide, blackish with a narrow margin pale. Fronds linear in outline, 15—30 cm long, simply pinnate, often elongated. Leaflets 1.2—1.8 cm long, nearly sessile. The rachis and stem are dark brown colour. The lower half of the stem is densely hairy, and sometimes wears narrow pale brown scales. Pinnae are 0.7 to 2.3 cm long and 4-11 mm wide, become smaller and smaller at the top. They are alternate, oblong to obliquely triangular, slightly too deeply incised or lobed at the outer and lower margins. Root is cuneiform or rarely rounded, generally sparsely to densely cover with brown hairs. The sori are circular or oblong 1 to 3.5 mm wide Adiantum incisum usually grows on damp rocks and mountain sides in forest areas.

Geographical distribution:

The plains and the lower slopes of the hills in Sikkim, Punjab, Rajasthan, West Bengal, Tamil Nadu and Maharashtra. Also found in Sri Lanka, Malay Peninsula, South China.

Ethnomedicinal evidence:

Adiantum incisum is used in the treatment of chest affections, cough, diabetes, fever and skin diseases. Fresh or dried leaves are ground to make paste or powder. 50 g of this paste powder is mixed in coconut oil 200 ml. This oil is applied on calp to check hair fall (Anupama., 2016).

The Whole and Juice of fronds are used to treat diabetes. The leaves powder used in liver diseases (Sapna Rai., 2016). Leaves are external remedy for skin disease (Ambasta., 1986). The leaves are used in malaria treatment (Chatterjee and Pakrashi., 1991). Large dose of this plant use in emetic. The roots are used in bone fracture. For fever the juice of plant is given with pepper (Anupama., 2016).

Rationale for selection of the plant:

Given the wide range of ethnomedicinal uses and also the fact tat this plant is obtained from high altitude has given us sufficient reasons to select this plant for our study. Moreover, parts of this plant are edible and hence it is likely to pose fewer side effects if used for therapeutic purposes.

A-Whole plant of Adiantum incisum

B- Leaf of Adiantum incisum

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Adiantum caudatum / Synonyms / The Plant List

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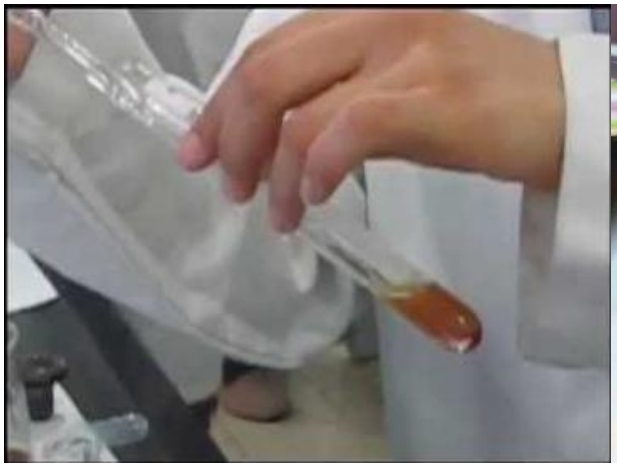
Collection, extraction and phytochemical screening



COLLECTION AND EXTRACTION:

Adiantum insisum dried plants (2 kg) were collected from the hilly region of Majhitar, Sikkim, India. The month of November 2018. Then dried full plant was powdered in a mechanical grinder and the extraction of the powder (300 gm) was carried out in soxhlet apparatus by using methanol (21.6% w/w, yield). The solvent were completely removed under reduced pressure in a rotary vacuum evaporator. The concentrated extracts were stored in vacuum desiccators for further use.

PHYTOCHEMICAL SCREENING:

Preliminary qualitative analysis has been performed to know the type of phytochemicals present in the above extract. Phytochemicals are bioactive secondary metabolites produced naturally in plants (Ugochukwa *et al,* 2013). Various phytochemical molecules present such as vitamins, alkaloids, flavonoids, saponins, tannins, steroids, and others metabolites, which are rich in antioxidant activity (Gracelin *et al,* 2013). Hence determination of the phytoconstituents in a plant material or its extract is of utmost important.

CHEMICAL TEST:

Test for Steroid

- Liebermann- Burchard Test (Zhou et al., 2004)

10 mg of extract was dissolved in 1ml of chloroform. 1ml of Acetic Anhydride was added following the addition of 2ml of concentrated sulphuric acid. Formation of reddish violet or pinkish colour indicated the presence of steroids.

- Salkowski Test (Bosila et al., 2005)

1ml of concentrated sulphuric acid was added to 10 mg of extract dissolved in 1ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by acid layer indicated the presence of steroid.

Test for Alkaloids (Raffauf et al., 1962)

- Mayer's test
- 1.2 ml of extract was taken in a test tube. 0.2 ml of dilute hydrochloric acid and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff coloured precipitate gives positive test for alkaloids.
- Dragendroff's test

0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added in 2ml solution of extract in a test tube. Development of orange brown coloured precipitate suggested the presence of alkaloids.

- Wagner's test

2 ml of extract solution was treated with dilute hydrochloric acid and 0.1 ml Wagner's reagent. Formation of reddish brown indicated the positive response for alkaloids.

Test for Flavonoids

- Alkaline reagent test (Ugochukwu SC et al., 2013)

2ml of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

- Shinoda's test (Palanisamy P et al., 2012)

  Small quantity of the extract was dissolved in alcohol. Two to three piece of magnesium followed by concentrated hydrochloric acid was added and heated. Appearance of magenta colour demonstrates presence of flavonoids.

Test for Saponins (Shinha et al., 1985)

- 1ml solution of the extract was diluted with distilled water to 20 ml and shake in a graduated

cylinder for 15 mins. Development of srable foam suggested the presence of saponins.

- 1 ml extract was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins.

Test for Tannins (Segelman et al., 1969)

- 5 ml of extract solution was allowed to react with 1 ml 5% ferric chloride solution. Greenish
  black coloration indicated the presence of tannins.

- 5 ml of extract was treated with 1ml of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate suggested the presence of tannins.

Test for Glycoside (Salwaan et al., 2012)

- Legal's test

The extract was dissolved in pyridine and sodium nitroprusside solution added to make it alkaline. The formation of pink red to red colour shows the presence of glycosides.

- Brontrager's test

A few ml of dilute sulphuric acid added to 1 ml of the extract solution. Boiled, filtered and extracted the filtrate with chloroform. The chloroform layer was treated with 1ml of ammonia. The formation of red colour shows the presence of anthraquinone glycosides.

Test for Protein (Kumar *et al*, 2012)

- Biuret Test

The extract was treated with 1 ml 10% sodium hydroxide solution and heated. A drop of 0.7% copper sulphate solution to the above mixture was added. The formation of purplish violet colour indicates the presence of proteins.

- Millon Test

3 ml test solutions were mixed with 5 ml Million's reagent separately. White precipitate was formed which on heating tuned to brick red. It indicates the presence of proteins.

Test for Carbohydrate

- Benedict's test (Bhandary et al., 2012)

Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) boiled in water bath, observed for the formation of reddish brown precipitate to show a positive result for the formation of carbohydrate.

- Molish test (Salwaan et al., 2012)

To 2 ml of the extract, added 1ml of α-naphthol solution, and concentrated sulphuric acid through the sides of test tubes. Purple or reddish violet colour at the junction of the two liquid reveals the presence of carbohydrates.

Test for Triterpenoid

- Salkowski test (Nayak et al., 2011)

The test extract was treated with few drops of concentrated sulphuric acid. Formation of yellow colour at the lower layer suggested the presence of triterpenoids.

Results:

S. NO.	PHYTOCONSTITUENT	PRESENCE/ ABSENCE
1.	STEROID	+
2.	ALKALOID	+
3.	FLAVONOID	+
4.	SAPONIN	+
5.	TANNIN	+
6.	GLYCOSIDE	+
7.	PROTEIN	-
8.	CARBOHYDRATE	-
9.	TRITERPENOID	+

'+' indicates the presence of a particular phytoconstituent while **'-'** indicates the absence of the phytoconstituent.

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TOXICITY STUDY

TOXICITY STUDIES:

Introduction:

Toxicology is branch of science that deals with harmful effect of chemicals and drugs or poisons on living system or organism. The term toxicology comes from the word 'toxicon' means poison and 'logos' means science.

Toxicity studies can help to arrive at the following parameters:

1. Establishing the therapeutic dose.
2. To understand the organ-specific harmful effects.
3. To gather information about the mode of toxic action.
4. Establish the toxic substance as a future reference.

Acute Toxicity Study:

Pharmaceutical industries routinely perform toxicity studies in their investigation of new drugs or molecules. The toxicity studies involved are acute and chronic toxicity tests. In the acute toxicity test the main objective is the determination of LD_{50}(the dose which has been lethal to 50% of the animals in the experiment). It is an initial assessment of toxic manifestation of the compound under investigation. It also helps to decide the dose of a test compound to be administered safely in an animal. It was developed in 1920's and called "classical LD_{50}" involved 100 animals for 5 dose-groups, later in 1981 it was modified by the Organization for Economic Co-operation and Development (OECD) and reduced number upto 30 for 3 dose-groups. Methods to calculate LD_{50} values are – Litchfield and Wilcoxson, Reed-Muench, Miller Tainter ad Karber's method. But all these methods require large number of animals. Factors which affect the results of LD_{50} are- Species, Age, Sex, Amount of food, Social environment etc. LD_{50} study has some Limitations and results may vary greatly. Due to excess of animal sacrifice we should go to alternative methods which minimize the number of animals required. FRAME (Fund for the Replacement of Animals in Medical Experiment) believes that the lethal dose test is unnecessarily cruel and scientifically invalid. Several countries, including the UK, have taken steps to ban the oral LD_{50}. The OECD, the international governments' advisory body abolished the requirement for the oral test in 2001. Three alternative methods and these are: Fixed Dose Procedure (FDP)-OECD TG 420, Acute Toxic Class method (ATC)-OECD TG 423, Up-and-

Down Procedure (UDP)-OECD TG 425. These methods only consider signs of toxicity in place of Death. Signs recorded during studies like; increased motor activity, anesthesia, tremors, arching and rolling. Alternative methods save numbers experimental animals (Deora et al. 2010).

Fixed Dose Procedure (FDP)-OECD TG 420

This method does not use death as an end point; instead it uses the observation of clear signs of toxicity developed at one of a series of fixed dose levels to estimate the LD_{50}. Groups of animals of a single sex are dosed in a stepwise procedure using the fixed doses of 5, 50, 300 and 2000 mg/kg. The initial dose level is selected on the basis of a sighting study as the dose expected to produce some signs of toxicity without causing severe toxic effects or mortality.

Acute Toxic Class (ATC) Method- OECD TG 423

This method does not use death as the only end point; it also uses signs of toxicity in its stepwise approach to estimating the LD_{50}. It is based on the Probit model. The ATC method is a sequential testing procedure using only three animals of one sex per step. Depending on the mortality rate three but never more than six animals are used per dose level. This approach results in the reduction of numbers of animals used in comparison to the LD_{50} test by 40-70%.

Up and Down Procedure (UDP)-OECD TG 425

This method does still use death as an end point, but does animals one at a time to see if the dose needs to be put up or down to achieve an estimate of the LD_{50} therefore giving the minimum number of animals a lethal dose of the test substance. In the up-and-down procedure, animals are dosed one at a time. If an animal survives, the dose for the next animal is increased; if it dies, the dose is decreased. Each animal is observed for 1 or 2 days before dosing the next animal. Surviving animals monitored for delayed death for a total of 7 days.

Method of toxicity study:

The method followed for acute toxicity was Up and Down Method following OECD guidelines TG 425. 5 Healthy swiss albino mice were used for the experiment. These pre-acclimatized fasted animals were dosed sequentially with dose of 2000mg/kg body weight of MECA with each animal being dosed 48hrs after an animal has been dosed and observed for safety. The

animals were scrutinized for increased motor activity, anesthesia, tremors, arching and rolling, clonic convulsions, tonic extension, lacrimation, Straub reaction, salivation, muscle spasm, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia.

Result:

Oral administration of methanol extract of Adiantum insisum (MEAI) upto 2000mg/kg body weight did not produce any significant alteration in the behavior, breathing, cutaneous effects, sensory nervous response or gastrointestinal effects. During the toxicity study no deaths occurred inferring that MEAI is safe upto dose 2000mg/kg body weight. Based on this we selected 100mg/kg body weight and 200mg/kg body weight of MEAI for our animal experiments.

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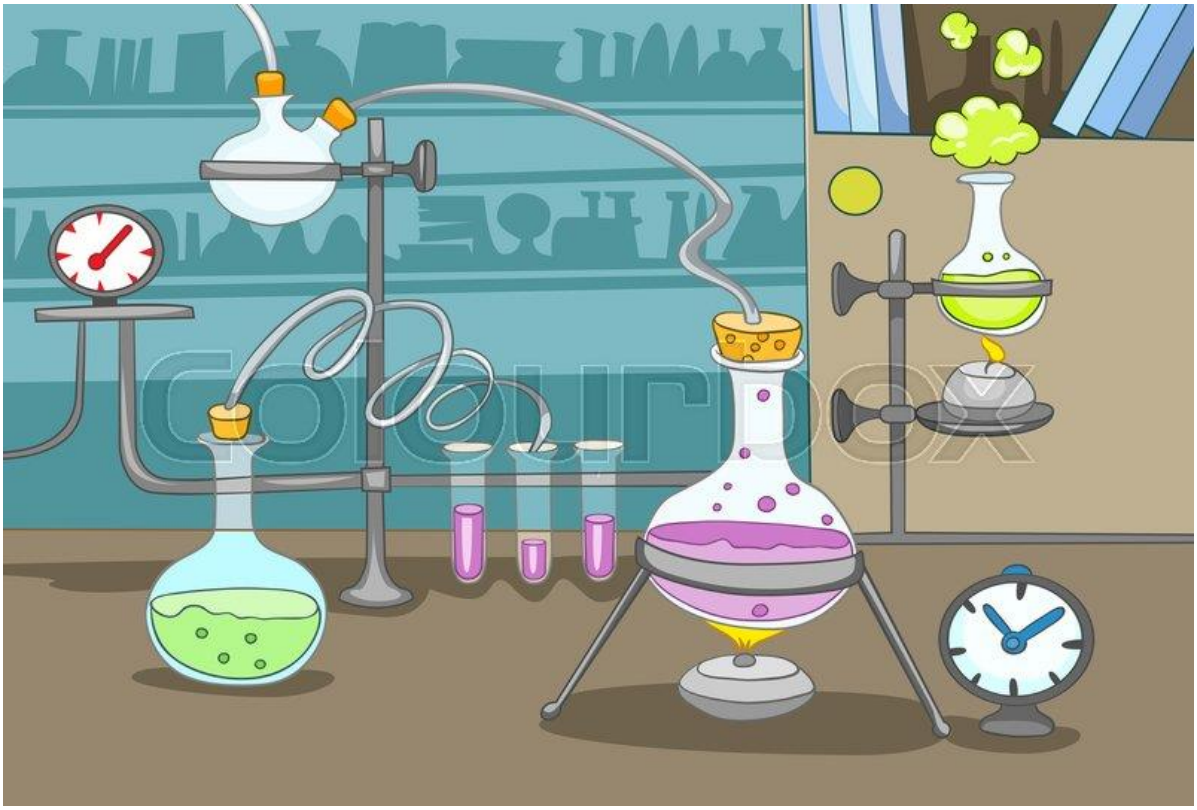
ANTIOXIDANT STUDY



Oxidative Stress: An Overview

The importance of oxidation in the body and in foodstuffs has been widely recognized. Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative changes (Antolovich et al, 2001). Free radicals form when oxygen is metabolized or formed in the body and are chemical species those posses an unpaired electron in the outer (valance) shell of the molecule. This is the reason, why the free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA. These free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the destruction of a living cell. Free radicals may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are $O_2^{\cdot-}$ [superoxide], HO [hydroxyl], HO_2 [hydroperoxyl], ROO [peroxyl], RO [alkoxyl] as free radical and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly NO [nitric oxide], ONOO [peroxy nitrate], NO_2 [nitrogen dioxide] and N_2O_3 [dinitrogen trioxide] (Badarinath et al, 2010).Free radicals, which are generated in several biochemical reactions in the body, have been implicated as mediators of many diseases, including cancer, atherosclerosis and heart diseases (AL-Dabbas et al, 2006). Although these free radicals can be scavenged by the *in vivo* produced antioxidant compounds, the endogenous antioxidants are insufficient to completely remove them and maintain a balance. As a result, dietary antioxidants are requires to counteract excess free radicals (Wang et al, 2010). Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are effective in their role as antioxidants, are commercially available and currently used in industrial processes. However, since suspected actions as promoters of carcinogenesis and other side effects have been reported, their use in food, cosmetic and pharmaceutical products has been decreasing. Thus, there has been an upsurge of interest in naturally-occurring antioxidants from vegetables, fruits, leaves, oilseeds, cereal crops, tree barks, roots, spices and herbs (Peiyuan et al, 2010).

The aim of the present study was to evaluate the antioxidant or free radical scavenging activity of methanol extract of *Adiantum insisum* by employing various antioxidant assay methods.

Materials and Method

Chemicals:

1, 1- Diphenyl-2-picryl-hydrazyl(DPPH) was obtained from Sigma Chemicals, USA. Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), napthyl ethylene diamine dihydrochloride, sodium nitroprusside, ascorbic acid, trichoroacetic acid (TCA), diamine tetra acetic acid (EDTA), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), Thiobarbituric acid (TBA). All reagent used were of high analytical grade.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured using the method given by (Cotelle *et al*., 1996), with some modifications. Different concentrations of 2.8ml test solution or standard ascorbic acid (in methanol) and 0.2ml DPPH (100µM in methanol) were mixed and incubated at 37ºC for 30mins. Care was taken to ensure light protection of the DPPH and the containers containing the reaction mixture. Absorbance was taken after incubation at 517nm using spectrophotometer.

Percentage of inhibition calculation

The percentage inhibition was calculated from the following formula

$$\% \text{ inhibition} = \frac{(\text{absorbance of control}) - (\text{absorbance of test sample})}{\text{Absorbance of control}} \times 100$$

Data Analysis

All the value are given as mean ± SEM. The IC_{50} (50% inhibitory concentration) values were calculated from the graphs plotted between concentrations versus percentage inhibition using GraphPad Prism software 5.1.

Results

Methanol extract of *Adiantum insisum* showed quite good antioxidant activity as was evinced from the results of the vitro antioxidant assays.

DPPH assay proved that the extract showed inhibitory activity on DPPH in a dose depended manner. The IC_{50} values of MEAI and that of Ascorbic acid as obtained from graph for DPPH are 96.7 ± 1.89 µg/ml and 133 ± 2.98 µg/ml respectively. **(Fig 6.1)**

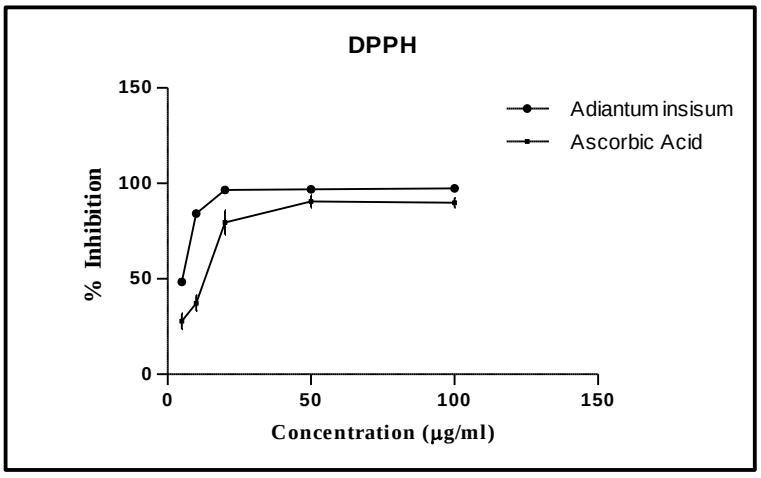


Figure (6.1): 1, 1-diphenyl-2-picrylhydrazil (DPPH) scavenging activity of extracts and ascorbic acid. The data represent the percentage of DPPH inhibition. Each point represents the values obtained from three experiments, (mean ± SEM)

Discussion

In normal physiological and metabolic processes approximately 6% of oxygen gets reduced to oxygen derived free radicals which are capable of attacking and damaging various cells, DNA, lipids, carbohydrates etc. In some conditions our endogenous antioxidant from outside becomes inevitable (Bala et al, 2009). Hence the evaluation of antioxidant potential of a compound or molecule or extract is of utmost importance for it to be established as a good and reliable source of antioxidants. An alternative solution to overcome this problem is to consume natural antioxidants from food supplement as traditional medicines. DPPH is a stable free radical, which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radical reacts with suitable reducing agents and then electrons become paired off and the solution loses colour stoichiometrically with the number of electrons taken up (Dash et al, 2005). Such reactivity has been widely used to rest the ability of the compound or extracts to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517nm.

CONCLUSION:

In conclusion, it can be concluded that the methanol extract of whole plant of *Adiantum insisum* possesses good antioxidant activity which may be potentially responsible for its use as folklore remedies. However, the components responsible for the antioxidant activity are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use. Having gained insight into the antioxidant activities of the extract and finding that it does posses considerably good anti-oxidant properties we can suspect it can have anticancer activities as many literatures suggest that free radical scavenging could be a way to prevent mutations that could lead to cancer.

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IN VITRO STUDY

IN VITRO CYTOTOXICITY METHODS:

In vitro cytotoxicity of Adiantum insisum was performed by trypan blue exclusion method and MTT assay method against Ehrlich Ascites Carcinoma (EAC) cells and further evaluated for *in vivo* anticancer activity on Swiss albino mice using 5-FU as reference standard.

Trypan blue exclusion method:

Principle: Trypan blue is one of the several stains recommended for use in dye exclusion test for viable cell counting. The method is based on the principle that live (viable) cells do not take up certain dyes where as dead (non-viable) cells do. The dye is negatively charged and is selectively excluded by the semi permeable cell membrane in living cells (Tran *et al*, 2011). Moreover it has also been stated that the dye has selective affinity towards serum protein compared to that of the membrane proteins.

Method: 1×10^6 EAC cells were suspended in 0.1 ml of phosphate buffer saline (PBS, 0.2 M, pH 7.4) and mixed with various concentrations of the extract (50, 100, 200 and 300 µg/ml) in PBS and final volume made up to 1.0 ml with PBS. The mixture was incubated at 37°C for 30 minutes. Cell suspension in PBS without extract served as control. After incubation the viability of the cells was determined using 0.4% trypan blue (Boyse *et a*, 1964). After adding trypan blue solution to the cell suspension 5 – 10 minutes were allowed for settling of the mixture. Then the cells were mounted on hemocytometer and viewed under microscope. The percentage of cytotoxicity was determined by calculating percentage inhibition and IC_{50} value (Khanam *et al*, 1997).

$$\text{Cell count} = \frac{\text{(Number of cells} \times \text{dilution factor)}}{\text{Area} \times \text{thickness of liquid flim}}$$

Percentage of inhibition= (Dead cells/ Total cells)× 100

MTT Assay:

Principle: This is a colorimetric assay that measures the reduction of yellow coloured dye, 3-(4,5-dimethytiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase enzyme. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product (Van Meerloo *et al*, 2011). Since reduction of MTT can only occur in metabolically active cells, so that the level of activity is a measure of the viability of the cells.

Method: 30 µl of EAC cells from 10 ml PBS containing 3×10^6 cells were added in a 96 well microplate. 200 µl RPMI media containing adequate penicillin and streptomycin was added in each well. 100 µl of different concentrations of the extract (50, 100, 150, 200 and 300 µg/ml) in PBS was added to this. To the control group 100 µl PBS was added in place of the extract. Thereafter the microplate was incubated in a CO_2 incubator with 5% CO_2 at 37º C for 24 hours. A similar batch was prepared for 48 hours incubation. After 24 hour of incubation, 20 µl MTT solution (5 mg/ml) was added to each well. It was then incubated for 4 hours at 37º C after which the media was removed and 20 µl DMSO added to each well to dissolve the purple formazan formed absorbance was taken at 570 nm to determine percentage of inhibition and IC_{50} value. Similar procedure was followed for the 48 hour batch.

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample} \times 100}{\text{Absorbancer of control}}$$

Data Analysis

All the values are given as mean ± SEM. The IC_{50} (50% inhibitory concentration) values were calculated from the graphs plotted between concentrations versus percentage inhibition using GraphPad Prism software 5.1

Results and Discussion

In the trypan blue exclusion method, the cells that took up the dye and appeared blue under microscope were the dead cells while those that did not take up the stain were the viable cells. The number of each of these (viable and nonviable cell) was calculated and subsequently inhibitory concentrations of extract were calculated. Form the graph the IC_{50} value was obtained. The IC_{50} value was found to be 112 ± 1.92 µg/ml. This value reflects that the extract has significant cytotoxicity towards EAC cells. **(Fig 7.1)**

In the MTT assay, the IC_{50} value for 24 and 48 hours incubation were found to be 242 ± 1.71 and 181 ± 2.57. From these data it may infer that MEAI is cytotoxic to EAC cells and also that it exerts its effect in a time dependent inner i.e. exposure of MEAI for a longer time will bring about more cytotoxicity to the EAC cells. **(Fig 7.2)**

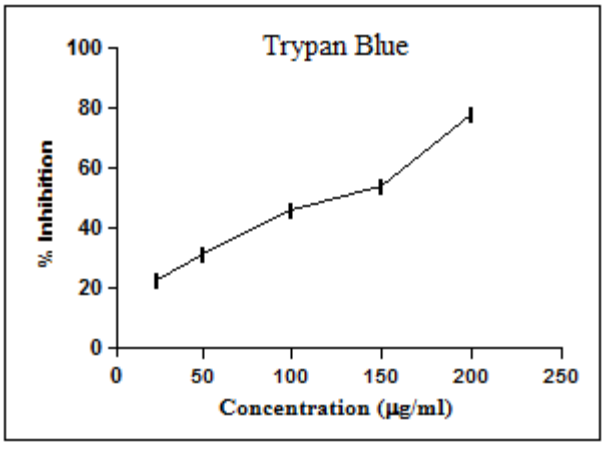

Figure (7.1): Trypan blue exclusion assay of MEAI. Each point represents mean ± SEM obtained from three individual experiments. The IC_{50} value is 112 ± 1.92 µg/ml.

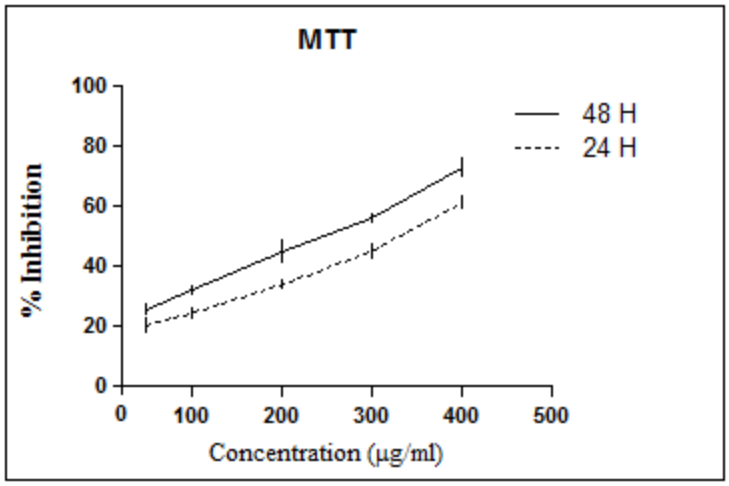

Figure (7.2): MTT assay of MEAI. Each point represents mean ± SEM obtained from three individual experiments. The IC_{50} value at 24 hr is 242 ± 1.71 and at 48 hr is 181 ± 2.57 µg/ml.

Conclusion: The *in vitro* cytotoxicity studies showed that MEAI has significant cytotoxic effect. The results are inspiring to further investigate the extract for its anticancer potential. Hence, *in vivo* antitumor studies were conducted in the next stage.

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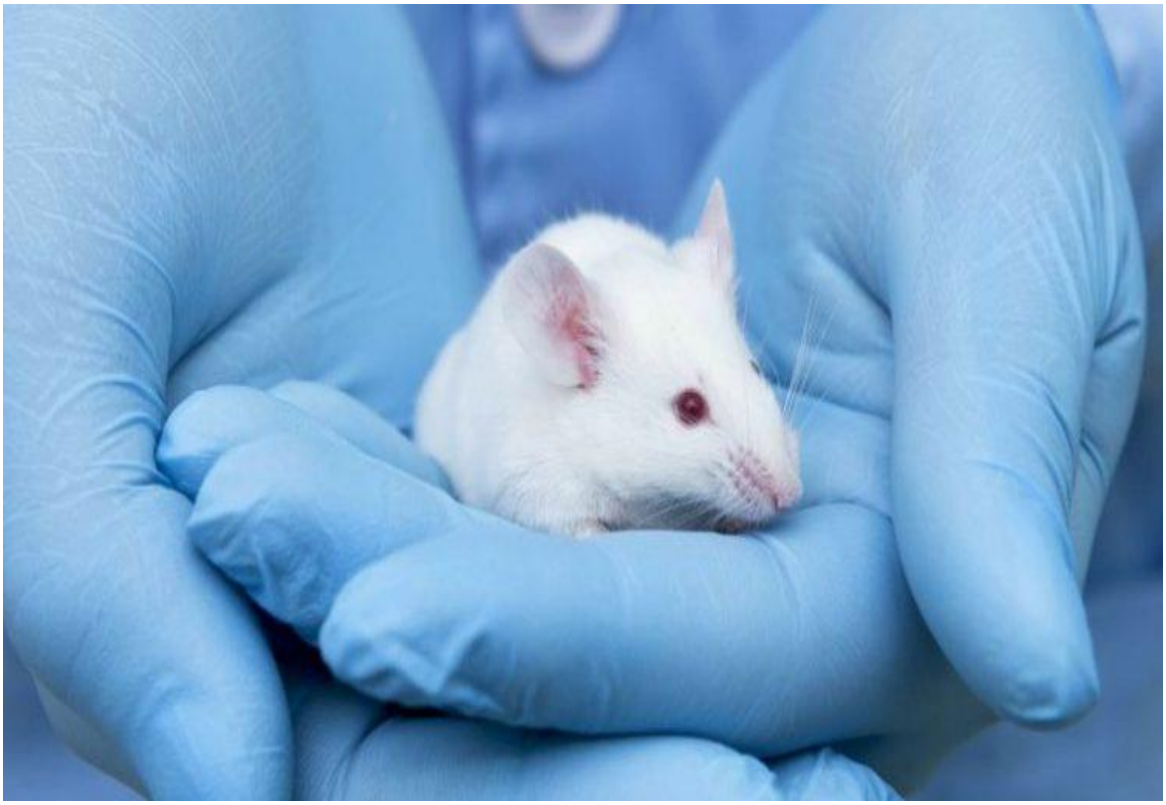
In Vivo Study



IN VIVO ANTICANCER STUDIES:

The aim of the present study was to evaluate antitumor effect of the methanol extract of *Adiantum insisum* against Ehrlich Ascites Carcinoma (EAC) in Swiss albino mice.

Materials and method:

Chemicals:

Chemicals used for the study were Sodium Chloride (NaCl), Trypan blue, 5-fluorouracil (MERCK Limited, Mumbai, India). Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), dithionitro benzene (DTNB) were obtained from Sigma chemicals. All other chemicals or reagents used for the experiment were of highest analytical grade.

Animals:

Healthy male Swiss albino mice weighing 22 - 25 g were taken. They were obtained from Chakraborty Enterprise, Kolkata, India. The mice were grouped and housed in poly acrylic cages (38× 23× 10 cm) with not more than 6 animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2º C and dark/ light cycle 14/10 h). They were allowed free access to standard dry pellet diet and water *ad libitum.* The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All procedures described were reviewed and approved by the University Animal Ethical Committee (ACE/PHARM/1502/10/2015).

Transplantation of tumor:

EAC cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascetic fluid was drawn out from EAC tumor bearing mouse at the log phase (day 7 - 8 of tumor bearing) of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally.

Treatment schedule:

30 Swiss albino mice were divided into five groups (n= 6). All the animals in each groups except Group-I received EAC cells (2×10^6 cells/mouse i.p.). This was taken as day "0". Group-I served as normal saline control (5 mg/kg i.p.) and group-II served as EAC control. 24 h after EAC transplantation, Group-III and IV received methanol extract of *Adiantum insisum* (MEAI) at a dose of 50 and 100 mg/kg i.p.) for nine consecutive days (Bala *et al*.,2010). Twenty four hours from the last dose and 18 hr of fasting, 3 animals of each group were sacrificed by cervical dislocation to measure antitumor, hematological and biochemical parameters and rest of the animals were kept with food and water *ad libitum* to check percentage increase in life span. The following parameters were studied from the sacrificed animals.

Tumor parameters:

The ascetic fluid was withdrawn from the peritoneal cavity of the animals and the volume was measured by taking this fluid in a graduated tube. The packed cell volume was estimated by centrifuging the ascetic fluid at 3000 rpm and noting the volume of the precipitate.

Mean survival time:

The mean survival time of a particular group was estimated as (first death + Last death) ÷ 2

Percentage increase in life span

The effect of MEHS on percentage increases in life span was calculated on the basis of mortality of the experimental mice (Sur P *et al*., 1994).

ILS (%) = [(Mean survival time of the treated group ÷ Mean survival time of the control group)-1] × 100

Tumor cell count:

The ascetic fluid was taken in a WBC pipette and diluted 20 times with WBC fluid. Then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 (4 × 16) small squares were counted.

Viable/ nonviable tumor cell count:

The viability and nonviability of the cell was checked by trypan blue assay. The cells were stained with trypan blue (0.4 % in normal saline) dye. The cells that didn't take up the dye were viable and those that took the dye were nonviable. These viable and nonviable cells were counted according to the following formula:

Cell count = Number of cells × dilution factor / area × thickness of liquid film.0

Hematological parameters:

Blood was obtained from cardiac puncture during the sacrifice of animals. For the total count, blood was drawn into RBC and WBC pipette, diluted with the respective diluting fluids and counted in Neubauer counting chamber. Sahil's hemoglobinometer was used to determine the hemoglobin concentration content. RBC, WBC and Hemoglobin count were determined for normal, EAC control and treated animal groups.

Biochemical parameters:

A part of the blood collected through cardiac puncture was taken to determine serum parameters. Blood was set aside for 15 minutes to allow it to clot and then centrifuged at 5000 rpm for 100 minutes. The supernatants (serum) were collected and total protein, SGOT, SGPT and SALP were determined using the diagnostic reagent kit (Span Diagnostics Ltd, Surat, India) and taking the help of a spectrophotometer.

Preparation of liver and kidney homogenates:

Liver and Kidneys collected from the sacrificed animals were homogenized separately in 10 of phosphate buffer (20 mM, pH-7.4) and centrifuged at 12000 rpm for 30 min at 4º C. The supernatants were collected and used for the following experiments as described below:

SOD assay:

The SOD activity was measured by following the method of Kakkar et al, 1984. About 100 µl tissue homogenate (liver and kidney) were mixed with PMS (186 mM), NADH (780 mM), phosphate buffer saline (100 mM,pH-7.4) and NBT (300 mM). It was then incubated at 30º C for 90 minute. The reaction was then stopped by adding 1ml glacial acetic acid and absorbance of chromogen formed was measured at 560 nm. One unit of SOD activity is defined as the enzyme

concentration required for the inhibition of chromogen production by 50% in one minute under the assay condition.

GSH assay

GSH level was measured by the method of Ellman et al, 1959. About 100 was measured by the method of Ellman et al, 1959. About 100 µl of tissue homogenate and EDTA (0.02 M) were mixed and kept on ice bath for 10 minutes. Then 1 ml distilled water and TCA (50%) were added and again kept on ice bath for 10 minutes. After that mixture was centrifuged at 3000 g for 15 minutes. To 1 ml of supernatant, 0.4 M triss buffer (pH-8.9) followed by DTNB (0.01 M) were added and the absorbance was measured at 412 nm. A standard graph was drawn using different concentration of a standard GSH solution (1 mg/ml). With the help of the standard graph, GSH contents in the liver homogenates of the experimental animals were calculated.

Lipid peroxidation

Degree of lipid peroxidation n tissue homogenate was determined in terms of thiobarbituric acid reactive substances (TBARS) formation. (Ohkawa et al, 1979). About 500 µl of each tissue homogenate was mixed with PBS (0.02 M, pH-7.4) and TCA (10% w/v) and kept at room temperature for 30 minutes. Then the mixture was centrifuge at 3000 g for 10 minutes. 1 ml supernatant was mixed with TBA (1% w/v) and heated for 1 hour at 95º C until a stable pink color formed. The absorbance of the sample was measured at 535 nm using a blank containing all the reagents except the tissue homogenate. As 99% of the TBARS is malondialdehyde (MDA), TBARS concentrations of the samples were calculated using the extinction coefficient of MDA (1.56×10^5 M^{-1} cm^{-1}).

Data Analysis:

All the data are given as mean ± SEM of values obtained from three individual experiments. Data of all the experiments were analyzed using analysis of variance (ANOVA) and group means were compared by Dunnett's by GraphPad Prism software version 5.1.

Results:

Tumor Parameters:

Administration of MEAI to EAC induced group significantly reduced the tumor volume, packed cell volume and the number of EAC cells compared to the EAC control group. The values are given in **(Table 8.1).**

Life Span:

The mean survival time and the percentage of life span increased significantly compared to the EAC control group. The values are given in **(Table 8.1).**

Hematological parameters:

WBC count was found to be reduced in treated group as compared to the EAC control group while RBC and hemoglobin levels were significantly elevated and restored towards normal levels **(Table 8.2).**

Serum Parameters

Among the different serum parameters studied it was found that total protein content increased in the MEAI treated groups compared to the EAC control group. SGOT, SGPT and SALP values were significantly restored towards the normal levels compared to the EAC control group **(Table 8.3** and **Figure 8.1 [a-d]).**

Biochemical Parameters:

The level of malondialdehyde decreased in the extract treated groups compared to the EAC group thereby indicating the decrease in lipid peroxidation in the MEAI treated groups. The enzymatic and non enzymatic antioxidants like the super oxide dismutase and reduced glutathione were found to be restored normal levels. **(Table 8.4** and **Figure 8.2 [a-c]).**

Groups	Tumor Volume (ml)	Packed Cell Volume (ml)	Cell count (x 10^7/ml)		Viable %	Non-viable %	MST (Day)	ILS %
			Viable	Non Viable				
EAC Control	**3.98 ± 0.12**	**1.24 ± 0.11**	**7.88 ± 0.27**	**0.52 ± 0.08**	**93.80**	**6.19**	**19**	**00**
EAC+50 mg/kg MEAI	**1.79 ± 0.27***	**0.55 ± 0.08***	**5.30 ± 0.22***	**2.28 ± 0.21***	**69.92**	**30.07**	**29**	**52.63**
EAC+100 mg/kg MEAI	**1.09 ± 0.07***	**0.35 ± 0.13***	**3.65 ± 0.18***	**3.26 ± 0.36***	**52.82**	**47.17**	**36**	**89.47**
EAC+5-FU (20mg/kg)	**0.65 ± 0.05***	**0.22 ± 0.02***	**2.71 ± 0.11***	**5.31 ± 0.13***	**33.04**	**64.75**	**42**	**121.05**

Table 8.1: Table shows the effect of MEAI on various parameters such as tumor volume, packed cell volume, cell count, % of viability, % of nonviable cells, Mean survival time and the increase in life span. Values are represented as mean ± SEM. EAC control vs Treated group $^{*}p<0.05$.

Parameters	Normal	EAC Control	50mg/kg MEAI	100mg/kg MEAI	20mg/kg 5-FU
RBC	**5.72 ± 0.23**	**2.69 ± 0.17**[a]	**4.77 ± 0.12****	**5.14 ± 0.06****	**5.33± 0.07****
WBC	**4.91 ± 0.30**	**7.88 ± 0.11**[a]	**6.67 ± 0.19***	**5.47 ± 0.10****	**5.21 ± 0.06****
Hb	**11.60 ± 0.57**	**4.43 ± 0.11**[a]	**7.58 ± 0.60***	**9.37 ± 0.52***	**10.65 ± 0.42****

Table 8.2: Effects of different concentrations of the extract on hematological parameters. Values are represented as mean ± SEM. $^{a}p<0.05$ when compared to normal, $^{*}p<0.05$ and $^{**}p<0.05$ when compared with EAC control group.

Parameters	Normal	EAC Control	50mg/kg MEAI	100mg/kg MEAI	20mg/kg 5-FU
Total protein	**10.22 ± 0.60**	**3.65 ± 0.48#**	**4.83 ± 0.22***	**7.05 ± 0.55***	**8.34 ±0.38***
SGOT	**107.1 ± 4.70**	**241.3 ± 11.60#**	**171.1 ±10.29***	**121.0 ± 8.76***	**91.33 ± 2.96***
SGPT	**33.60 ± 1.09**	**76.72 ± 2.38#**	**46.18 ± 3.26***	**42.41 ± 6.87***	**35.67 ± 2.96***
SALP	**66 ± 3.21**	**182 ± 6.36#**	**131 ± 4.93***	**93.33 ±8.45***	**75.33 ±2.90***

Table 8.3: Effects of different concentrations of the extract on serum parameters. Values are represented as mean ± SEM. $^{\#}p<0.05$ when compared to Normal and $^{*}p<0.05$ when compared to EAC Control.

Groups		Lipid peroxidation	Superoxide Dismutase	Reduced Glutathione
Normal	**Liver**	**683.2 ± 7.59**	**268.8 ± 7.19**	**1307 ± 59.33**
	Kidney	**736.9 ± 61.95**	**201.0 ± 6.94**	**1442 ± 29.47**
EAC	**Liver**	**832.2 ± 52.16#**	**57.40 ± 6.23#**	**438.4 ± 21.34#**
	Kidney	**1098 ±39.80#**	**40.99 ± 2.02#**	**333.7 ± 23.30#**
50mg/kg MEAI	**Liver**	**318.7 ± 14.46****	**79.34 ± 4.68***	**842.9 ± 16.40****
	Kidney	**444.5 ± 12.24****	**64.87 ± 7.25***	**637.4 ± 24.77****
100mg/kg MEAI	**Liver**	**279.4 ± 15.84****	**119.8 ± 8.85***	**1057 ± 23.99****
	Kidney	**303.6 ± 8.70***	**114.7 ± 9.52***	**851.4 ± 17.38****
5-FU	**Liver**	**122.8 ± 1.49***	**201.9 ± 6.23***	**1341 ± 27.63****
	Kidney	**136.3 ± 8.25***	**196.3 ± 7.52***	**1245 ± 16.95****

Table 8.4: Effects of different concentrations of MEAI on different biochemical parameters. The values are represented as mean ± SEM. $^{\#}p<0.05$ when EAC control compared to normal livers, and kidney, $^{*}p<0.05$ and $^{**}p<0.05$ when EAC control compared to EAC livers and kidneys.

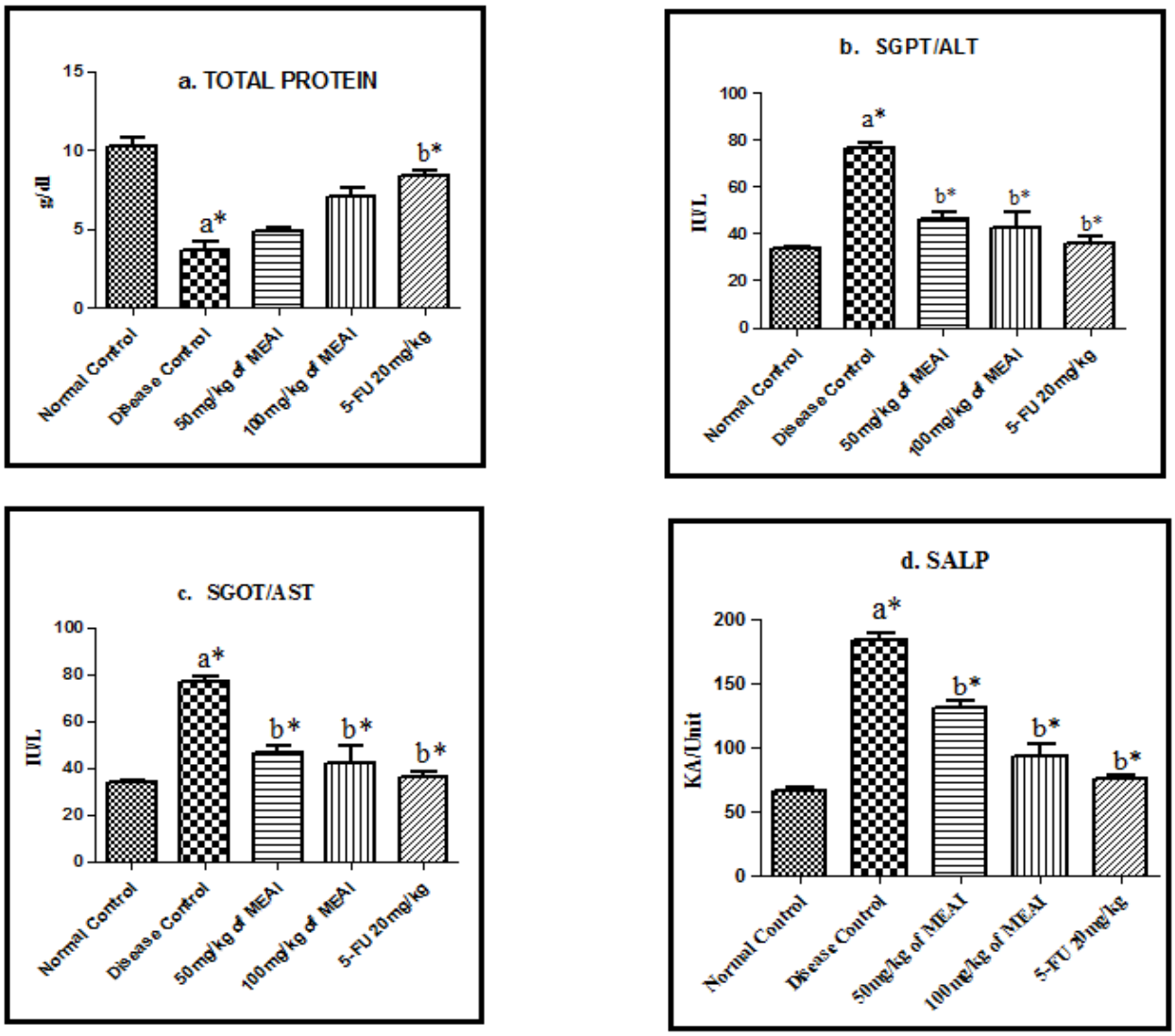


Figure 8.1(a-d): Effects of different concentrations of MEAI on Serum biochemical parameters (a) Total protein (b) SGPT (c) SGOT and (d) SALP.

Values are represented as mean ± SEM. a*$p<0.05$ when Disease Control compared to Normal and b*$p<0.05$ when treated groups compared to Disease Control.

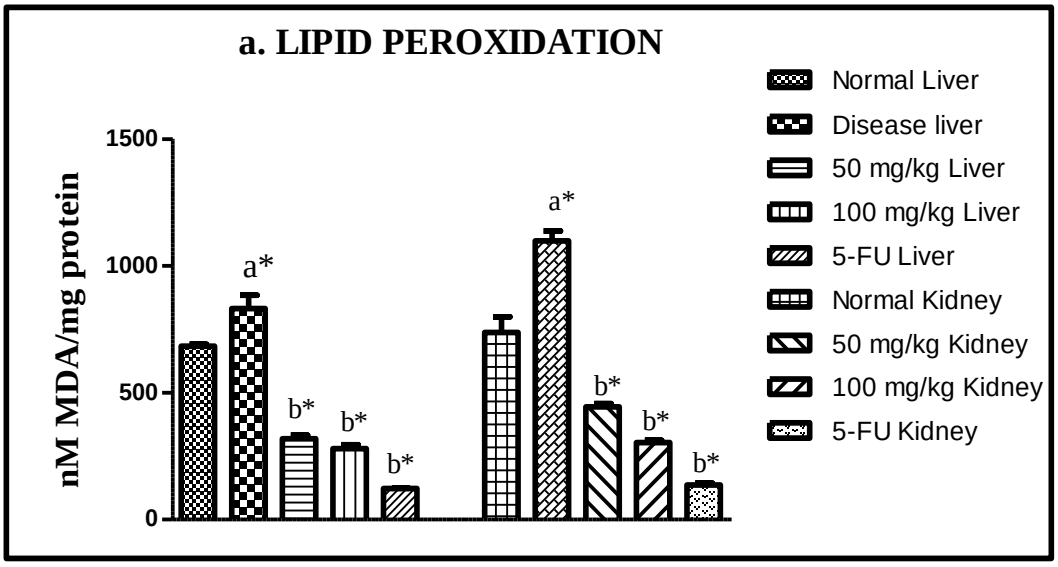


Figure 8.2 (a): Effects of different concentrations of MEAI on Lipid peroxidation. The values are represented as mean ± SEM. a*p<0.05 when Disease control compared to normal liver and kidney, b*p<0.05 when treated groups compared to Disease control liver and kidney.

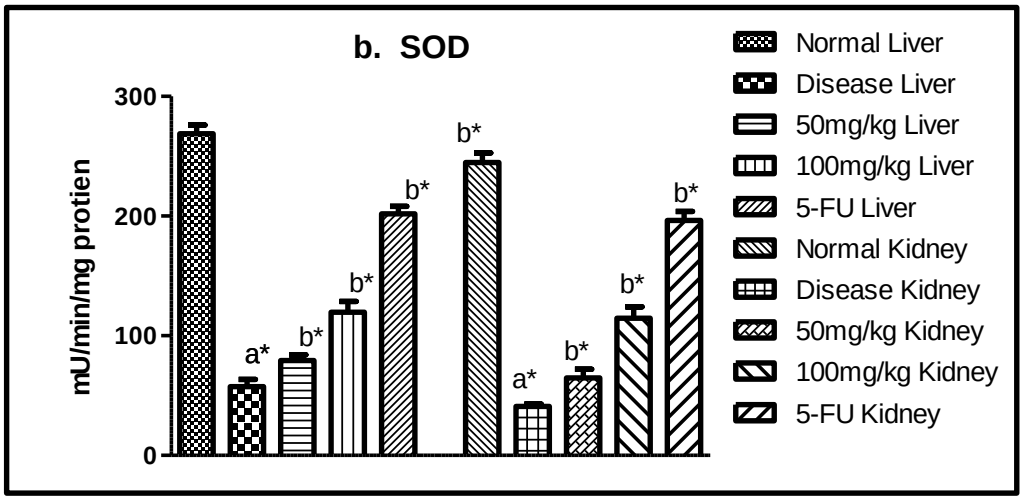


Figure 8.2 (b): Effects of different concentrations of MEAI on SOD. The values are represented as mean ± SEM. a*p<0.05 when Disease control compared to normal liver and kidney, b*p<0.05 when treated groups compared to Disease control liver and kidney.

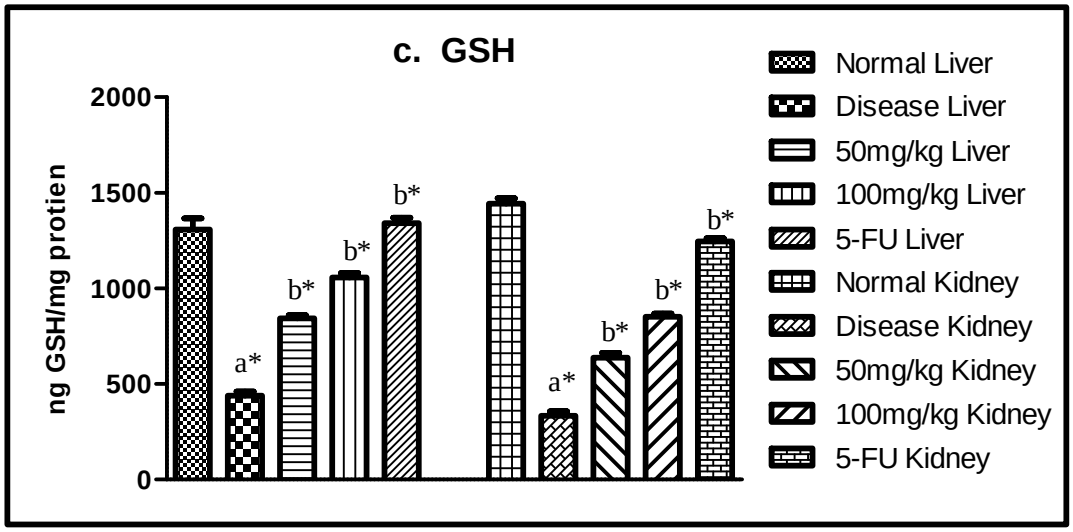
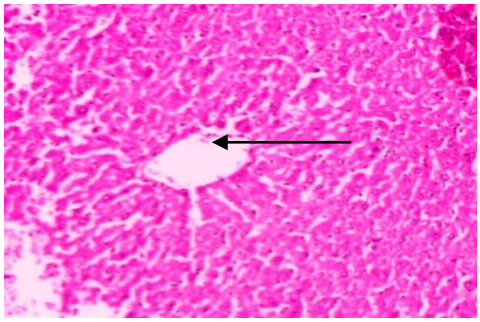
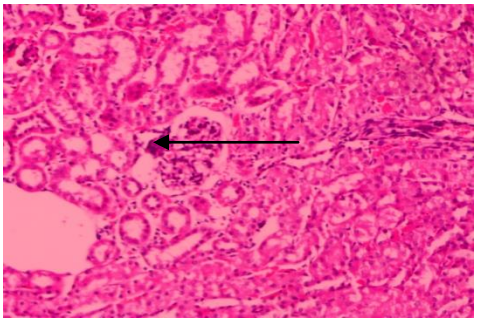


Figure 8.2 (c): Effects of different concentrations of MEAI on GSH. The values are represented as mean ± SEM. $^{a*}p<0.05$ when Disease control compared to normal liver and kidney, $^{b*}p<0.05$ when treated groups compared to Disease control liver and kidney.

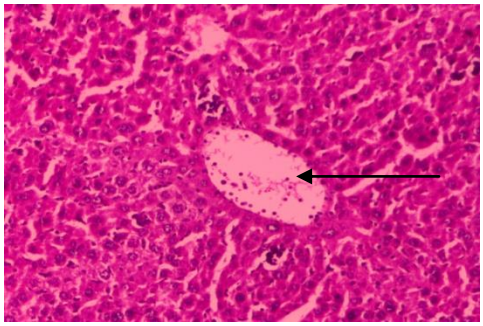
Histopathology:



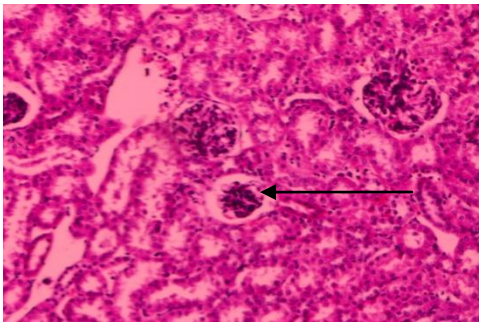
Normal Control Liver



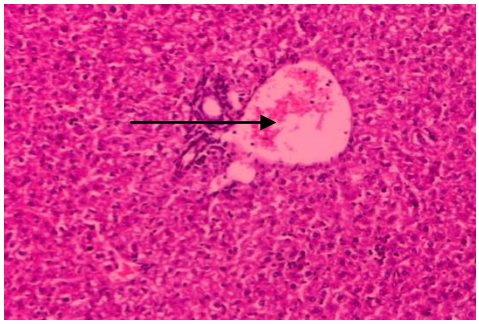
Normal Control Kidney



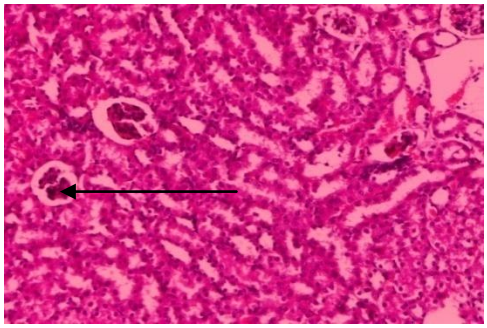
Disease Control Liver



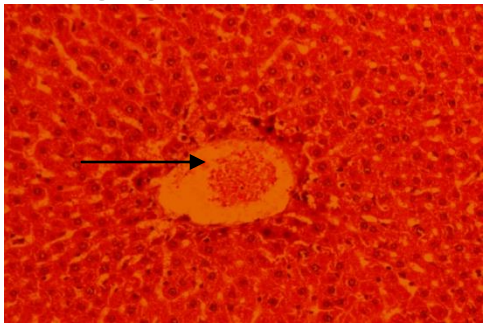
Disease Control Kidney



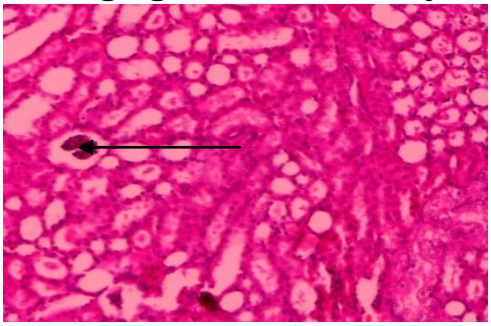
50 Mg/kg of MEAI Liver



50 Mg/kg of MEAI Kidney



100 Mg/kg of MEAI Liver



100 Mg/kg of MEAI Kidney

Discussion:

In the present study EAC cell line was used to evaluate the anticancer of MEAI. EAC or Ehrlich Ascites Carcinoma cells are spontaneous murine mammary rapidly growing adenocarcinoma cells which are highly aggressive in nature and can affect almost all strains of mice (Dolai *et al*., 2012). The Ehrlich Ascitic tumor implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascetic fluid formation and accumulation (Bala *et al*., 2010). The ascetic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells (Shimizu *et al*., 2004). MEAI treatment was able to reduce tumor volume, packed cell volume, viable cell count when compared to the tumor control group probably due to its potency in inhibiting the peritoneal ascetic fluid. These results could connote either a direct cytotoxic effect of MEAI on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition (Bala *et al*., 2010). Increase in life span of the treated animals is a reliable criterion for judging the value of any anticancer drug (Gupta *et al*., 2004). The MEAI treated group showed enhancement of life span compared to the tumor treated group which indicates that MEAI could certainly be a potential candidate for anticancer drug. The major problems faced during cancer chemotherapy are myelosuppression and anemia (Price and Greenfield, 1958). Anemia occurs due to reduction in RBC or destruction of hemoglobin. Treatment with MEAI significantly restored RBC and hemoglobin towards their respective normal levels as compared to the tumor control group. Also the WBC cell count which elevated in the tumor induced group was brought down to near normal levels following MEAI treatment. Enzymes in serum have been studied for many years as possible early indicators of neoplasia and as aids in following the progression and regression of disease (Kathiriya *et al*., 2010). Hepatotoxicity may occur due to cytotoxic agent itself or due to its toxic metabolites. In certain circumstances they can be carcinogenic (Dolai *et al*., 2012). Form the experiment we found that EAC control group exhibited increased levels of liver enzymes such as SGOT, SGPT and ALP while the levels of total protein were decreased due to hepatocellular damages. The MEAI treated group showed restoration of these biochemical parameters to more or less normal levels. The disbalance between the reactive oxygen metabolites and the antioxidant defense systems leads to ‘oxidative stress’ which deregulates various cellular functions causing pathological conditions (Bandyopadhyay *et al*., 1999 and Adesegun *et al*,

2009). The oxidative stress may lead to the damage of the macromolecules such as lipids and can induce lipid peroxidation in vivo (Yagi *et al*., 1991). In EAC bearing mice the level of lipid peroxide in liver and kidney was significantly elevated, which was however reduced to near normal level in the MEAI treated group animals. This reflects the ability of the extract to decrease free radical production and to subsequently reduce the oxidation stress. Glutathione (GSH), a potent inhibitor of neoplastic proliferation process, plays a crucial role as an endogenous antioxidant system. It was found particularly in high concentration in liver and is known to have a key function in the protective process (Haldar *et al.*,2010). The level of reduced glutathione (GSH) was reduced in cancer bearing mice which may be due to its utilization by the excessive amount of free radicals generated in the disease state. Treatment with MEAI was found to increase the GSH content in the liver as compared to the tumor control group. The free radical scavenging system, SOD, and catalase are present in all oxygen-metabolizing cells, and their function is to provide a defence against the potentially damaging reactivates of superoxide and hydrogen peroxide. Inhibition of SOD and CAT activities as a result of tumor growth were also reported. Similar findings were obtained in the present investigation with EAC-bearing mice. Treatment with MEAI significantly increased the SOD and CAT levels indicating antioxidant and free radical scavenging activity of the extract.

Conclusion:

The in vivo anticancer study delineates that he methanol extract of Adiantum insisum does have some potential to fight cancer. Also 100mg/kg MEAI showed better response compared to 50mg/kg MEAI suggesting a possible dose dependent influence of the extract on cancer. However the compound or molecules in the extract that might be responsible for its anticancer activities are compound or molecules in the extract that might be responsible for its anticancer activities are still to be identified. Also a through mechanistic study remains to be undertaken to establish constituents of MEAI as potential candidates for cancer therapy.

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Conclusion

CONCLUSION:

This thesis is a pioneer of scientific investigations on methanol extract of the whole plant of *Adiantum insisum* in the research field. The introductory part of the thesis is designated by the concept of cancer, natural products in cancer treatment, description and literature review of the investigated plant. After that the collection, extraction and chemical characterization has been mentioned in the next chapter. Then acute toxicity study has been written in the next chapter. Then the antioxidant studies have been mentioned in the next chapter. Finally the *in vitro* cytotoxicity and *in vivo* anticancer activity has been written in last two chapters. Considering the *in vitro* and *in vivo* results it can be concluded that the methanol extract of the whole plant of *Adiantum insisum* does have some anticancer properties. The results may be attributed to the phytoconstituents present in the plant as mentioned in the thesis in an earlier chapter. Phytoconstituents like alkaloids and flavonoids of reported therapeutic benefits against various ailments including cancer. So the alkaloids of flavonoids or both present in the extract could be responsible for its activity. The antioxidant studies elucidated that the extract has fairly good free radical scavenging activities which could also be a probable reason for its anticancer activities because free radicals are a major cause of mutation which could subsequently lead to cancer and quenching of these free radicals could counteract such ill effects.

However vague speculations asserting phytoconstituents to be responsible for the extract's anticancer activities are not enough and extensive isolation and characterization techniques must be employed to find out the molecule or molecules in the extract actually responsible for such activities. The anticancer study was based on EAC cells for both the *in vitro* and *in vivo* experiments. Studies on other cell lines especially human cancer cell lines and also other *in vitro* and *in vivo* cancer models need to be conducted to truly confirm its anticancer potentials.

Thank You