Evaluation of anticancer activity of indigenous plant extract

Thesis submitted for the Partial Fulfillment of the Degree of Masters of Pharmaceutical Technology in the Faculty of Engineering & Technology Jadavpur University 2016

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CERTIFICATE

This is to certify that **Suchandra Mazumder** has carried out the research on the project entitled "**Evaluation of anticancer activity of indigenous plant extract"** under my supervision, in the Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700 032.

She has included her findings into the thesis of the same title being submitted by her in partial fulfillment of the requirement for the award of Degree of Master of pharmaceutical Technology, Jadavpur University. I am satisfied that she has carried out her thesis with proper care and confidence to my entire satisfaction.

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ACKNOWLEDGEMENT

I would like to convey my immense gratitude to my guide **Dr. Pallab Kanti Haldar** for his continuous motivation, innovative ideas and valuable guidance throughout the project. I am also very grateful to him for giving me the scope and facilities to carry out this project successfully.

I am also thankful to **Prof. (Dr.) Biswajit Mukherjee**, the Head of the Department and all other faculty members of the Department of Pharmaceutical Technology, Jadavpur University, who have enriched me with valuable knowledge and suggestions throughout my tenure.

I also express my deepest thanks to my lab members including: **Indrajit Karmakar, Sagnik Haldar, Mainak Chakraborty, Avratanu Das, Poulami Majumder, Sanjay Paria, and Diksha Kumari** who have supported me in my work.

I am also thankful to **AICTE** for giving me scholarship to carry out my
M. Pharm project.
Finally I extend my profound gratitude to my **beloved parents and brother** who deserves to be truly responsible for laying down strong foundation to this course with their divine blessings and love.
Last but not the least I thank **Lord Almighty** for his choicest blessings on me.

Suchandra Mazumder


Dedicated
To
my Family

PREFACE

The present study “Evaluation of anticancer activity of indigenous plant 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 *Quercus lanata* 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.

Suchandra Mazumder

CONTENTS


Introduction

INTRODUCTION:

In spite of good advancements for diagnosis and treatment, cancer is still a big threat to our society (Kotnis *et al*., 2005). This is the second most common disease after cardiovascular disorders for maximum deaths in the world (Jemal *et al.*, 2007). Cancer is a multi-gene, multi-step disease originating from a single abnormal cell with an altered DNA sequence. It is characterized by uncontrolled cellular growth, local tissue invasion and distant metastases (Dashora *et al*., 2010). The four characteristics of cancer cells that distinguish them from normal cells are uncontrolled proliferation, dedifferentiation and loss of function, invasiveness and metastasis. A normal cell turns into cancer cell because of one or more mutations in its DNA, which can be acquired or inherited. These altered cells divide uncontrollably to form lumps or masses of tissue called tumors. Another hallmark of the disease is resistance to apoptosis (programmed cell death), either by inactivation of proapoptotic factors or by activation of antiapoptotic factors. 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 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 blood vessels and lymphatics, where they continue to proliferate. Chemotherapy is now considered as the most effective method of cancer treatment. Unfortunately, currently available cancer chemotherapeutic agents insidiously affect the host cells, especially bone marrow, epithelial tissues, gonads and the reticula-endothelial system (Mascarenhas, 1994). A major challenge for medical oncology is to develop therapeutic modalities that will prevent toxicity induced by antitumor treatments without impairing their antitumor effects (Das *et al*., 2012). Hence, natural products are considered to have exceptional value in the development

of effective anticancer drugs with minimal host cell toxicity and high antioxidant and eradication potential (Gupta *et al*., 2007). Researchers have recently focused on the use of Ehrlich's ascites carcinoma (EAC) cells in the investigation of plants reported to cure cancer locally. The Ehrlich tumor was initially described as a spontaneous murine mammary adenocarcinoma. It is a rapidly growing carcinoma with very aggressive behavior and can grow in almost all strains of mice. In the ascitic form, it has been used as a transplantable tumor model to investigate the antitumor effects of several substances (Segura *et al*., 2000).

Statistics:

There were an estimated 14.1 million cancer cases around the world in 2012, out of these 7.4 million cases were in men and 6.7 million in women. The numbers of deaths were 8.2 million in 2012. This number is expected to increase to 24 million by 2035 (Torre *et al*., 2012).

1. Lung (1.6 million deaths)
2. Liver (745 500 deaths)
3. Stomach (723 100 deaths)
4. Colorectal (693 900 deaths)
5. Breast (521 900 deaths)
6. Esophageal cancer (400 200 deaths)
7. Cervical cancer (265 700 deaths)
8. Urinary bladder cancer (165 100 deaths)
9. Non-Hodgkin lymphoma (199 700 deaths)
10. Cancer of the lip and oral cavity (145 400 deaths)
11. Nasopharyngeal cancer (50 800 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 cancers 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.

Cancer causes:

Cancer is ultimately the result of cells that uncontrollably grow and do not die. Normal cells in the body follow an orderly path of growth, division and death. Programmed cell death is called apoptosis and when this process breaks down, cancer begins to form. Unlike regular cells, cancer cells do not experience programmatic death and instead continue to grow and divide. This leads to a mass of abnormal cells that grows out of control.

It is nearly impossible to prove what caused a cancer in any individual, because most cancers have multiple possible causes. The great majority of cancers, some 90 - 95% of cases, are due

to environmental factors. The remaining 5 - 10% is 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 environmental pollutants.

Lifestyle factors:

Smoking, a high-fat diet and working with toxic 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 death. Physical inactivity is believed to contribute to cancer risk, not only through its effect on body weight but also through negative effects on the immune system and endocrine system.

Hereditary and genetic factors:

It may play an important role in some childhood cancers. It is possible for cancer of varying forms to be present more than once in a family. Less than 0.3% of the populations are carriers of a genetic mutation that has a large effect on cancer risk and these causes less than 3 - 10% of all cancer. Cells can experience uncontrolled growth if there are mutations to DNA and therefore, alterations to the genes involved in cell division. 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, 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.

Cancer occurs when a cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide. Similarly, cancer is a result of mutations that inhibit oncogene and tumor suppressor gene function, leading to uncontrollable cell growth. Certain inherited

mutations in the genes *BRCA1* and *BRCA2* with a more than 75% risk of breast cancer and ovarian cancer.

Carcinogens:

Carcinogens are a class of substances that are directly responsible for damaging DNA, promoting or aiding cancer. Tobacco, asbestos, arsenic, radiation such as gamma and x-rays, the sun and compounds in car exhaust fumes are all examples of carcinogens. When our bodies are exposed to carcinogens, free radicals are formed that try to steal electrons from other molecules in the body. These free radicals damage cells and affect their ability to function normally.

Infections:

A virus that can cause cancer is called an *oncovirus*. These include human papilloma virus (cervical carcinoma), Epstein–Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi's sarcoma herpes virus (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. Epstein-Barr virus and 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 cells.

Hormones:

Some hormones play a role in the development of cancer by promoting cell proliferation. Insulin-like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis.

Hormones are important agents in sex-related cancers, such as cancer of the breast, endometrium, prostate, ovary and testis, and also of thyroid cancer and bone cancer. For example, the daughters of women who have breast cancer have significantly higher levels of estrogen and progesterone than the daughters of women without breast cancer. These higher hormone levels may explain why these women have higher risk of breast cancer, even in the absence of a breast-cancer gene.

Environmental exposures:

Pesticides, fertilizers and power lines have been researched for a direct link to childhood cancers.

Some forms of high-dose chemotherapy and radiation. 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:

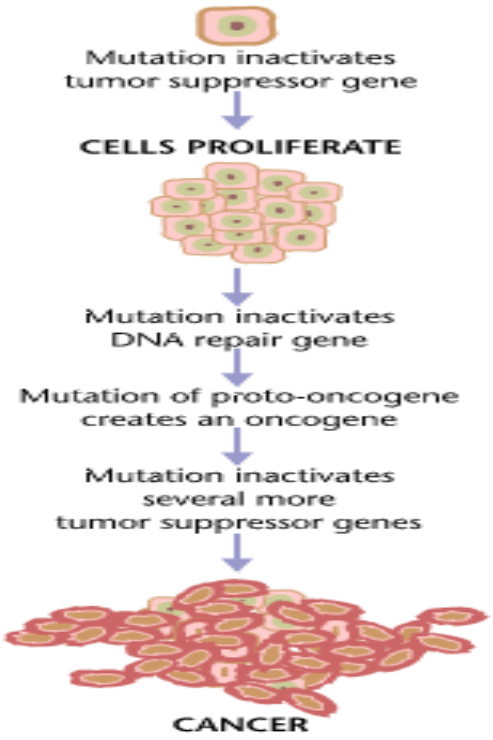

Figure 1: Causes of cancers (mutations).

Cancer mechanism:

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

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

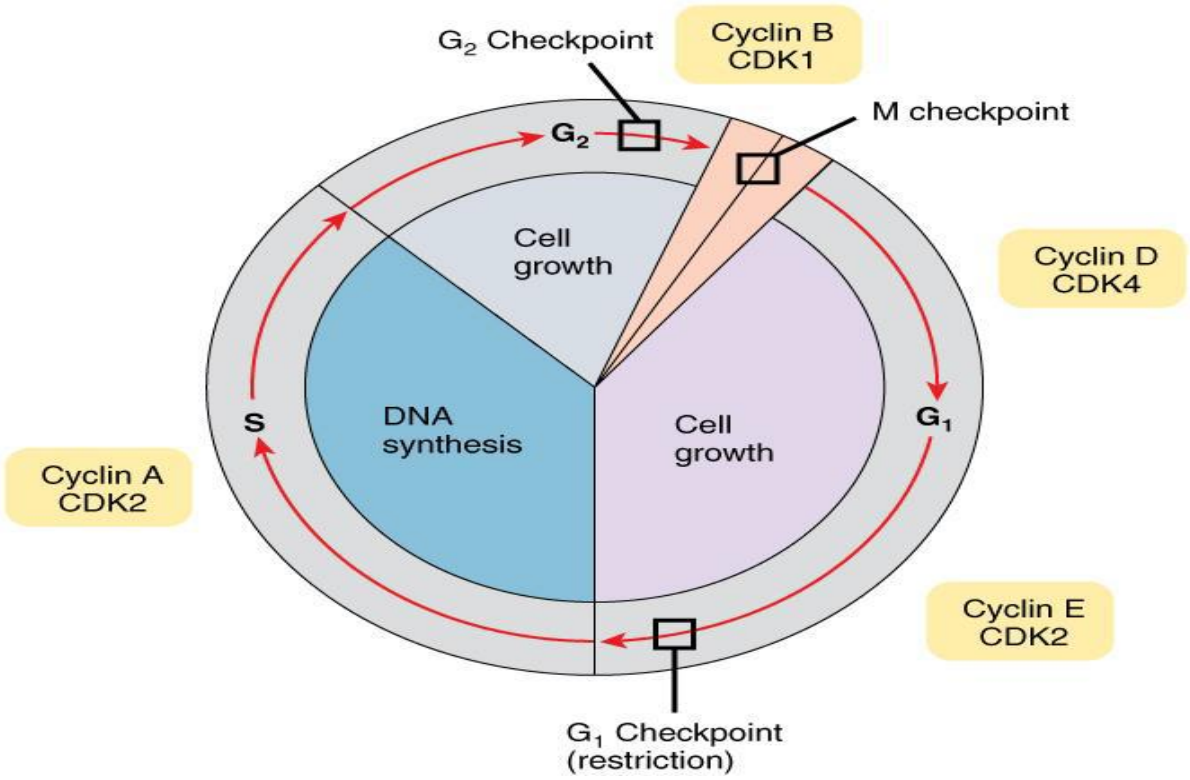

Figure 2: G_1, S, G_2 and M phase.

M is the phase of mitosis.

S is the phase of DNA synthesis.

G_1 is the gap between the mitosis that gave rise to the cell and the S phase; during G_1, the cell is preparing for 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, there is a fifth state, G0 (also known as quiescence) into which the cell may reversibly exit from G1, if it is deprived of the appropriate growth-promoting signals. (Garrett, 2001).

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 or 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 G1/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 G2/M checkpoint, which monitors the fidelity of DNA replication and like the G1/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 to divide. 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 determine progress through the cycle are two families of proteins: 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 two proteins, a kinase and a cyclin.They often contain other proteins with poorly understood functions. These complexes undergo change in the kinase and cyclin components that are believed to drive the cell from one stage of the cell cycle to another (Pardee *et al*., 1989; Xiong *et al*., 1991; Sherr *et al*., 1993; Morgan *et al*., 1995; Hwang *et al*., 1998; Zhan *et al*., 1999; Raleigh *et al*., 2000). 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 (cyclin 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 to 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 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 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 inhibitory proteins, also termed KIP or kinase inhibitory proteins)-p21, p27 and 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. This allows for DNA repair. If the repair is successful, the cycle proceeds past 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 in 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 p53 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 control and transform cells, mainly by eliminating p53 and pRb (reviewed by Sherr, 1996). Defective checkpoint mechanisms in cancers, as well as defective DNA repair, produce further chromosomal aberrations and genomic instability (Nojima, 1997). Thus checkpoint 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 (mutation 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 cells.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 regulate 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. Oncogenes are genes that promote cell growth and reproduction. Tumor suppressor genes are genes that inhibit cell division and survival. Malignant transformation can occur through the formation of novel oncogenes, the inappropriate over-expression of normal oncogenes, or by the under-expression or disabling of tumor suppressor genes. Typically, changes in many genes are required to transform a normal cell into a cancer cell. 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. The activation of anaerobic glycolysis (the Warburg effect), which is not

necessarily induced by mutations in proto oncogenes 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. In 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. Most oncogenes 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 become 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-inducing agent, an oncogene. Example of an oncogene is the Bcr-Abl gene found on the Philadelphia Chromosome, a piece of genetic material seen in Chronic Myelogenous Leukemia caused by the translocation of pieces from chromosomes 9 and 22. The proto-oncogene can become an oncogene by a relatively small modification of its original function. Most oncogenes are dominant mutations; a single copy of this gene is sufficient for expression of the growth trait. This is also a “gain of function” mutation because the cells with the mutant form of the protein have gained a new function 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:

Oncogenesis	Protein functions	Neoplasm
Growth Factors: sis Int2 Trek	Platelet derived growth factor Fibroblast derived growth factor Nerve derived growth factor	Factor fibro sarcoma 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: bcr-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: bcl-2	Anti-apoptotic protein	non-Hodgkin‟ 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 (Weinberg, 2014). The proteins made by tumor suppressor genes normally inhibit cell growth, preventing tumor formation. Mutations in these genes result in cells that no longer show normal inhibition of cell growth and division. The products of tumor suppressor genes may act at the cell membrane, in the

cytoplasm, or in the nucleus. Mutations in these genes result in a loss of function (that is, the ability to inhibit cell growth) so they are usually recessive. This means that the trait is not expressed unless both copies of the normal genes are mutated. Tumor suppressor genes can be grouped into categories including caretaker genes, gatekeeper genes and landscaper genes; the classification schemes are evolving as medicine advances, learning from fields including molecular biology, genetics and epigenetics. Tumor suppressor genes can directly or indirectly inhibit cell growth. Those that directly inhibit cell growth or promote cell death are known as "gatekeepers" and their activities are rate limiting for tumor cell proliferation. Mutations that inactivate one allele of a gatekeeper gene can be inherited through the germ line, which in conjunction with somatic mutation of the remaining allele, leads to cancer predisposition syndromes. For example, mutations of the APC gene lead to colon tumors. Somatic mutations that inactivate both gatekeeper alleles occur in sporadic tumors. . Those tumor suppressor genes that do not directly suppress proliferation, but function to promote genetic stability are known as "caretakers." Caretakers function in DNA repair pathways and elimination of caretaker‟ s results in increased mutation rates. Because numerous mutations are required for the full development of a tumor, elimination of caretaker tumor suppressors can greatly accelerate tumor progression. As with gatekeepers, mutations can be inherited through the germ line and can give rise to cancer predisposition syndromes. An example of a caretaker gene is MSH2, which functions in the mismatch DNA repair system, and inherited mutations in this gene gives rise to the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome.

Table 1.2:

Tumor suppressor gene	Protein function	Neoplasm(s)
APC	Cell adhesion	Colon
BRCA 1	Transcription factor	Ovary and breast
BRCA 2	DNA repair	Ovary and 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

Cancer treatment:

Many treatment options for cancer exist, with the primary ones including surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care. Which treatments are used depends on the type, location and grade of the cancer as well as the person's health and wishes. The treatment intent may be curative or not curative.

Chemotherapy:

Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs (chemotherapeutic agents) as part of a standardized regimen. The term encompasses any of a large variety of different anticancer drugs, which are divided into broad categories such as alkylating agents and antimetabolites. 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.

Hormonal therapy:

Many hormonal antitumor agents are functional agonist or antagonist of the steroid hormone family.

Adrenocorticoids, Antiandrogen, Estrogen, Antiestrogen, Progestins, Aromatase inhibitor, Gonadotropin-releasing hormone agonists, Somatostatin analogues etc.

Biological therapy:

- Immunotherapy: Cytokines, Cellular therapy, Tumor vaccine.
- Hematopoietic growth factors.

Nature as a source of anticancer drugs:

Natural products from plant, animal and minerals have been the basis of the treatment of various human diseases. Current estimate explains 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. The use of herbal medicine is becoming popular due to the high toxicity and side effects of allopathic medicines. The popularity of herbal medicine has been increasing day by day.

There are many traditional systems of medicine in the world, each with different associated philosophies and cultural origins. Some of these, such as Tibetan traditional medicine, remain relatively localised in their country of origin; while others such as 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. India has one of the richest traditions of plant based therapy in the world. There are estimates, to be around 25,000 effective plant based formulations, used in folk medicine and known to the rural communities in India (Kamboj, 2000). In the

last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources (Verma and Singh, 2008).

The use of traditional medicine has been increasing 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).

Plants have a long history of use in the treatment of cancer and it is significant that over 60% of currently used anti-cancer agents have come from natural sources. Naturally occurring drugs that are part of the war against cancer include vinca alkaloids (vincristine, vinblastine, vindesine and vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivative (etoposide, teniposide), camptothecin and its derivatives (topothecan, irinothecan), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin) and others. The approved anti cancer agents in 2002 doxorubicin, in 2002 estradiol, in 2004 cholorophyll and l- aspartic acid and taxol nanoparticles in 2005 (Butler, 2005). Three new drugs also introduced in 2007 originate from microbial sources for the treatment of cancer is marine alkaloid trabectedin, epothilone derivative ixabepilone and temsirolimus (Bailly, 2009). Nature is an attractive source of new therapeutic candidate compounds as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms as potential anti-cancer agent (Newman, 2003).

Plants used for cancer treatment:

India is the largest producer of medicinal plants and is rightly called the "Botanical garden of the World". The history of plant as source of anti-cancer agents started in earnest in the 1950s with the discovery and development of the vinca alkaloids (vinblastine and vincristine) and

the isolation of the cytotoxic podophyllotoxins. Vinca alkaloid was responsible for an increase in the cure rates for Hodgkin's disease and some forms of leukemia (DeVita *et al*., 1970). Vincristine inhibits microtubule assembly, inducing tubulin self-association into coiled spiral aggregates (Noble RL., 1990). Etoposide is an epipodophyllotoxin, derived from the mandrake plant Podophyllum peltatum and the wild chervil Podophyllum emodi (Stähelin H., 1973). It has also significant activity against small-cell lung carcinoma (Harvey AL., 1999). Etoposide is a topoisomerase II inhibitor, stabilizing enzyme-DNA cleavable complexes leading to DNA breaks (Liu LF., 1989). The taxanes paclitaxel and docetaxel has been shown antitumor activity against breast, ovarian and other tumor types in the clinical trial. Paclitaxel stabilizes microtubules and leading to mitotic arrest (Wani *et al*., 1971). In addition, the camptothecin derivatives irinotecan and topotecan have shown significant antitumor activity against colorectal and ovarian cancer respectively (Creemers *et al*., 1996). These compounds were initially obtained from the bark and wood of Nyssacea Camptotheca accuminata and act by inhibiting topoisomerase I (Liu *et al*., 2000). The taxanes and the camptothecins are presently approved for human use in various countries. Rohitukine the plant alkaloid, isolated from the leaves and stems of Dysoxylum binectariferum (Maliaceae) (Harmon *et al*., 1979). Synthetic flavone derived from rohitukine, Flavopiridol representing the first cyclin-dependent kinase inhibitor to enter the clinical trial (Losiewicz *et al*., 1994). The mechanism of action involves interfering with the phosphorylation of cyclin-dependent kinases and arrest cell-cycle progression at growth phase G1 or G2 (Worland *et al*., 1993) Homoharringtonine an alkaloid isolated from the Chinese tree Cephalotaxus harringtonia (Cephalotaxacea) (Powell *et al*., 1970). The mechanism of action is the inhibition of protein synthesis and blocking cell-cycle progression (Zhou *et al*., 1995). It has shown efficacy against various leukemias (Kantarjian *et al*., 1996). A lung-cancer-specific antineoplastic agent 4-Ipomeanol is isolated from the sweet potato Ipomoea batata (Convolvulaceae)

(Rowinsky *et al*., 1993). The mechanism of action is converted into DNA-binding metabolites upon metabolic activation by cytochrome P450 enzymes that are present in cells of the lung (Rehm *et al*., 1993). DNA topoisomerase I inhibitor β-lapachone, that induces cell-cycle delay at G1 or S (synthesis) phase before inducing either apoptotic or necrotic cell death in a variety of human carcinoma cells, including ovary, colon, lung, prostate and breast (Li el al., 1999). Various types of anti-cancer plant are Zedoary (Curcuma zedoaria), Rodent Tuber (Typhonium flagelliforme), God's Crown (Phaleria macrocarpa), Madagaskar Periwinkle (Catharanthus rosens), Artocarpus Integer (Selaginella corymbosa), Bamboo Grass (Loathatreum Gràcies) , handsome (Taraxacum mongolicum), fruit makasar (Brucca javanica), Garlic (Allium sativum), Echo China (Smilax china), Sunflower (Helianthus annus), Leunca (Solanum nigrum), Job's Tears (Coix Lachryma-Jobi), Bamboo Rope (Asparagus cochinchinensis) and others.

Dietary source of anti cancer agents:

Natural dietary agents including fruits, vegetables and spices have drawn a great deal of attention from both the scientific community and the general public owing to their demonstrated ability to suppress cancers.

Table 1.3:

S. No.	Botanical Name	Source	Compound
1	Carica papaya, Family- Caricaceae	Berries	β-Cryptoxanthin
2	Glycyrrhiza glabra; Glycyrrhiza radix; Glycyrrhiza uralensis, Family- Leguminosae	Licorice root	Glycyrrhizin
3	Cannabis sativa, Family- Cannabiaceae	Hemp	Cannabinol
4	Rosmarinus officinalis, Family- Lamiaceae	Rosemary	Carnosol
5	Glycine max, Family- Fabaceae	Soybeans	Genistein
6	Prunus armeniaca, Family- Rosaceae	Apricots	Carotenoids
7	Zingiber officinale, Family- Zingiberaceae	Tuber	Gingerol
8	Lycopersicon esculentum, Family- Solanaceae	Tomato	Lycopene, Lutein, Kaempferol
9	Piper nigrum; Piper longum, Family- Piperaceae	Black pepper	Purpurogallin; Piperine
10	Ocimum sanctum, Family- Lamiaceae	Basil	Ursolic acid
11	Betula alba, Family- Betulaceae	Birch tree	Betulinic acid
12	Crocus sativus, Family- Iridaceae	Saffron	Carotenoids

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


Materials
Procedure
Hypothesis
Conclusion
MARTIN
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PLAN OF WORK:

Objective: Evaluation of anticancer activity of indigenous plant 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 methanol as solvent.
- Acute toxicity study by LD_{50} method as per OECD guidelines.
- Evaluation of free radical scavenging activity of plant extract by *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.
    - Determination of tissue antioxidant parameters.

Literature Review

LITERATURE REVIEW:

Plant Taxonomy:

Kingdom: Plantae

Phylum: Angiospermae

Class: Dicotyledoneae

Order: Fagales

Family: Fagaceae

Genus: Quercus

Species: *Quercus lanata*

Plant Description:

Name: *Quercus lanata*

Family: Fagaceae

Synonyms: *Quercus banga* Buch-Ham. Ex D. Don

Quercus banga Ham. Ex Hook.f.

Quercus lanuginose D. Don

Quercus nepaulensis Desf

Local Name: Ban oak; Woolly oak; Tong maili; Banjh (Nepal)

Parts used medicinally*:* Stem bark, Flower.

Morphology of the plant:

It is a large tree up to 30 m tall, evergreen, twisting trunk. Leaves 10-22 × 3-9 cm; oval oblong, twice or three times as long as wide; glabrous, stiff, rough above, rusty white tomentose beneath, sometimes only along veins; margin entire or slightly toothed in apical half; apex obtuse, more or less acuminate; base rounded or broadly conical; 14-17 parallel vein pairs; petiole 6-25 mm, grey brown tomentose, becoming glabrous. Flowers-pistillate inflorescences 4-14 cm long in June-July. Fruits are acorn 1.1-1.8 cm long, 0.9-1.2 cm wide; ovoid mucronate, glabrous, singly or paired; enclosed 1/3 to ½ by cup, cup sessile, 1 cm in diameter; with small, triangular, appressed

scales; maturing in 1 or 2 years. Barks are thick, brown ash grey, lenticellate, peeling into thin plates; young shoots densely pubescent, becoming partially glabrous.

Geographical distribution:

Quercus lanata or woolly-leaved oak is a species of *Quercus* native to southern and southeastern Asia (Himalayas, Assam, Bhutan, Nepal, Indochina, Vietnam, Myanmar, northern Thailand) and southwestern China.

Ethnomedicinal evidences:

Folk use of wood tea of *Quercus lanata* (Fagaceae) as a laxative may verify the actions of tannin. Tannins reveal activities against central nervous system disorders and inflammation. Further pharmacological evaluation of the extracts of those species which reveal weak pharmacological validities are needed before they can be used as therapeutic potentials. The compounds which contribute to the antioxidative properties are polyphenols, vitamin C, beta carotene, anthocyanins and flavonoids. *Quercus lanata* Hardwood; as tea, laxative and resin; soothing body ache (Manandhar, 2002) dry resin; dysentery (Baral & Kurmi, 2006). The name of the phytoconstituent present is Cyclobalanone, pelagonodin, sitosterol, tannins. Resin and bark tannin is anti-inflammatory. Juice of the stem bark of the plant *Quercus lanata* is taken by the woman after delivery to treat giadriasis and gastritis. The mighty oak tree looms large in both mythology and herbal medicine and has been part of the plant pharmacopoeia for thousands of years. Oak bark is a powerful astringent treatment for throat and mouth infections, bleeding gums and to cure acute diarrhea. Oak bark is a styptic, one of the herbs used to slow bleeding in cuts and wounds. Oak bark tinctures and extracts have been studied for use in kidney infections and kidney stones. Using oak bark externally as an alcohol tincture or cream helps fight staph infections. Use it in a cold compress to treat burns and cuts. It also has a good antidiabetic

activity as well as good anticancer activity it also support the lymph system so that it can better handle the removal of dead cancer cells, or to reduce tumor size more rapidly, it is always good to use.

Rationale for selection of the plant:

Given the wide range of ethnomedicinal uses and also the fact that 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- Bark of *Quercus lanata* plant

B- Whole plant of *Quercus lanata*

C- Leaf of *Quercus lanata*

D- Fruits of *Quercus lanata*

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Collection, extraction and phytochemical screening

COLLECTION AND EXTRACTION:

The dried bark of the plant material (2 kg) was collected from the hilly region of Sikkim in the month of August 2014. Then the dried bark was powdered in a mechanical grinder and the extraction of the powder (200 gm) was carried out in soxhlet apparatus by using methanol (21.5% w/w, yield). The solvent was completely removed under reduced pressure in a rotary vacuum evaporator and 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). Here chemical group tests were performed for Alkaloids, Flavonoids, Saponins, Tannins, Steroids, Glycosides, Proteins and Terpenoids.

Chemical Tests:

Test for Steroid (Zhou *et al*, 2004)

- *Libermann-Burchard Test*

10 mg extract was dissolved in 1 ml chloroform. To this 1 ml acetic anhydride was added with subsequent addition of 2 ml concentrated sulphuric acid. Formation of reddish violet or pinkish colour indicates presence of steroid.

- *Salkowski Test*

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

Test for Alkaloid (Raffauf *et al*, 1962)

- 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.

- 1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added in 2 ml solution of extract in a test tube. Development of orange brown coloured precipitate suggested the presence of alkaloids.
- 2 ml of extract solution was treated with dilute hydrochloric acid and 0.1 ml of Wagner's reagent. Formation of reddish brown precipitate indicated the positive response for alkaloids.
- 2 ml of extract was allowed to react with 0.2 ml of dilute hydrochloric acid and 0.1 ml of Hager's reagent. A yellowish precipitate suggested the presence of alkaloids.

Test for Flavonoid

- *Alkaline reagent test* (Ugochukwu *et al*, 2013)

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

- *Shinoda's Test* (Palinisamy *et al*, 2012)

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

Test for Saponin

- *Foam test* (Bhandary *et al*, 2012)

Test solution was mixed with water and shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result.

- 1 ml extract was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins (Sinha *et al*, 1985).

Test for Tannin (Segelman *et al*, 1969)

- 5 ml extract solution was allowed to react with 1 ml of 5% ferric chloride solution. Greenish black colouration indicates presence of tannin.

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.

- *Borntrager's Test*

A few ml of dilute sulfuric acid added to 1 ml of the extract solution. Boiled, filtered and extracted the filtrate with chloroform. The chloroform layer was treated with 1 ml 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 turned to brick red. It indicates the presence of proteins.

Results:

Extract	Steroid	Alkaloid	Flavonoid	Saponin	Tannin	Glycoside	Terpenoid	Protein
Methanol	+	+	+	+	+	+	-	-

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

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TOXICITY STUDY

TOXICITY STUDIES:

Introduction:

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

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, sub 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 and 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 government's advisory body abolished the requirement for the oral test in 2001. Three alternative methods 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, anaesthesia, tremors, arching and rolling. Alternative methods save numbers experimental animals (Deora et al, 2010).

Fixed Dose Procedure (FDP)-OECD TG420:

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 TG423:

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 TG425:

This method does still use death as end point, but doses animal 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 2000 mg/kg body weight of MEQL with each animal being dosed 48 hours after an animal has been dosed and observed for safety. The animals were scrutinized for increased motor activity, anaesthesia, tremors, arching and rolling, clonic convulsions, tonic extension, lacrimation, Straub reaction, salivation, muscle pain, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia.

Result:

Oral administration of methanol extract of *Quercus lanata* (MEQL) upto 2000 mg/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 MEQL is safe upto dose 2000 mg/kg body weight. Based on this we selected 50 mg/kg body weight and 100 mg/kg body weight of MEQL for our animal experiments.

References:

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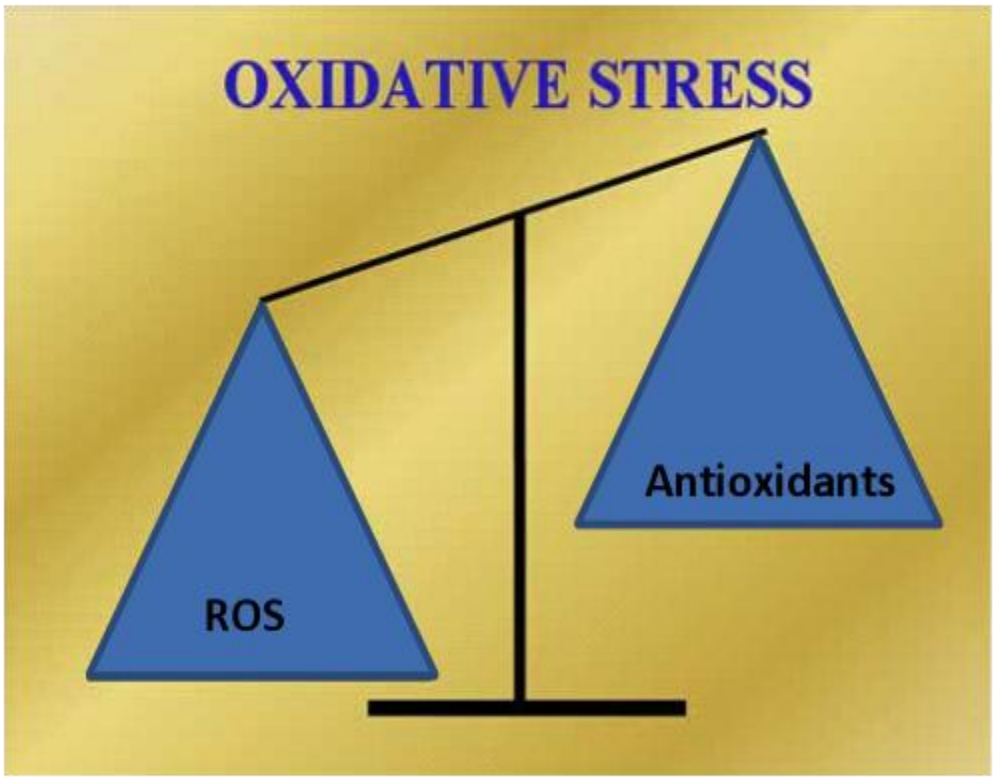
www.frame.org.uk

www.oecd.org

ANTIOXIDANT STUDY



OXIDATIVE STRESS:

Many diseases like diabetes, liver damage, nephrotoxicity, inflammation, cancer, neurological, cardiovascular disorders and aging are associated with oxidative stress caused by free radicals both reactive oxygen species (ROS) and reactive nitrogen species (RNS) which contains one or more unpaired electrons and can donate or receive electrons to become stable. It can propagate and produce further radicals and also can be made inactive by antioxidants (Mirshafiey *et al*, 2008). The oxygen derived molecules (ROS) are superoxide (O_2^-), hydroxyl radical (OH), hydroperoxyl (HO_2), peroxyl (ROO), alkoxyl (RO) as free radical and H_2O_2 oxygen as non-radical. The nitrogen derived oxidant species are mainly nitric oxide (NO), peroxy nitrate (ONOO), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) (Badarinath *et al*, 2010). Free radicals cause lipid peroxidation of the cell membrane and oxidative modification of proteins as well as genomic structures. Each cell in human body takes about 10,000 oxidative hits per second (Mirshafiey *et al*, 2008). Body has its own defense system to scavenge the oxidative free radicals by using reduced glutathione, catalase and superoxide dismutase by scavenging both the ROS and RNS, in the system by donating the hydrogen to the respective radicals (Bala *et al*, 2009). Although these free radicals can be scavenged by the *in vivo* antioxidant component, but this endogenous antioxidants are insufficient to completely remove them to maintain a balance. As a result, dietary antioxidants are required to counteract excess free radicals (Wang *et al*, 2010). Commercially available synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are effective as antioxidants, and currently used in industrial processes. However, since suspected actions as promoters of carcinogenesis and other side effects, their application in food, cosmetic and pharmaceutical products has been declined. 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*, 2011).

The aim of the present study was to evaluate the antioxidant or free radical scavenging activity of methanol extract of *Quercus lanata* by employing DPPH antioxidant assay method.

Materials and Method:

Chemicals:

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. All other reagents 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 2.8 ml test solution or ascorbic acid (in methanol) and 0.2 ml DPPH (100 μM in methanol) were mixed and incubated at 37˚C for 30 minutes. Absorbance was taken after incubation at 517 nm using spectrophotometer.

Percentage of inhibition calculation:

The percentage inhibition was calculated from the following formula

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

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 vs. percentage inhibition using GraphPad Prism software 5.1.

Results:

From the result it was found that methanol extract of *Quercus lanata* showed good antioxidant activity by *in vitro* assay.

DPPH assay proved that the extract showed inhibitory activity on DPPH in a dose dependent manner. The IC_{50} values of MEQL and ascorbic acid was found to be 17.37 ± 1.41 and 7.32 ± 0.52 µg/ml respectively. (Fig.-6.1)

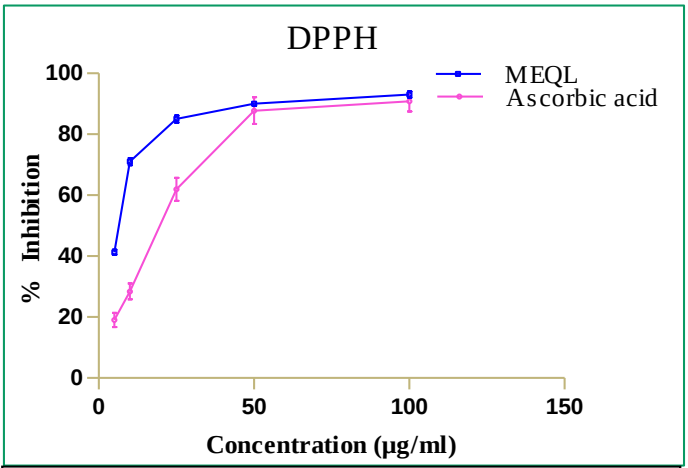


Figure (6.1): 1, 1-diphenyl-2-picrylhydrazil (DPPH) scavenging activity of extracts and ascorbic acid. The data represent the percentage of DPPH inhibition. Each point represents the values obtained from three experiments, (mean ± SEM).

Discussion:

In living system the free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various diseases especially degenerative diseases and also lysis of the cells and tissues. Oxidative stress refers to a situation where in the production of oxidants exceeds the capacity to neutralize them. Different therapeutic approaches can be used to decrease the oxidative stress and include scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or by targeting the signaling routes and expression of molecules involved in the inflammatory

cascade (Sunanda *et al*, 2011). Many synthetic drugs protect against oxidative damage but they have adverse effects. 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 test 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 517 nm.

Conclusion:

In conclusion, it can be concluded that the methanol extract of *Quercus lanata* bark possesses good antioxidant activity which may be potentially responsible for its use as folklore remedies. However, the components responsible for the antioxidant activity are still to be approved. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the 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 as the extract posses considerably good anti-oxidant properties, it may 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 MEQL 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 al*, 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).

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

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-dimethythiazol- 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 37˚ 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 37˚ 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.

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

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. From the graph the IC_{50} value was obtained. The IC_{50} value was found to be 123.70 ± 2.13 µg/ml. This value reflects that the extract has significant cytotoxicity towards EAC cells.

In the MTT assay, the IC_{50} value for 24 and 48 hours incubation were found to be 281.6 ± 1.79 and 195.3 ± 2.25 µg/ml respectively. From these data it may infer that MEQL is cytotoxic to EAC cells and also that it exerts its effect in a time dependent manner i.e. exposure of MEQL for a longer time will bring about more cytotoxicity to the EAC cells.

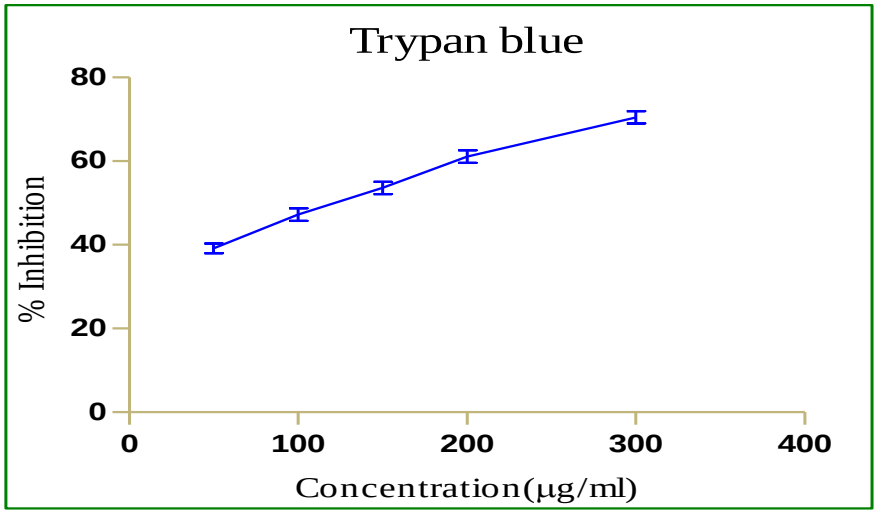

Figure (7.1): Trypan blue exclusion assay of MEQL. Each point represents mean ± SEM obtained from three individual experiments. The IC_{50} value is 123.70 ± 2.13µg/ml.

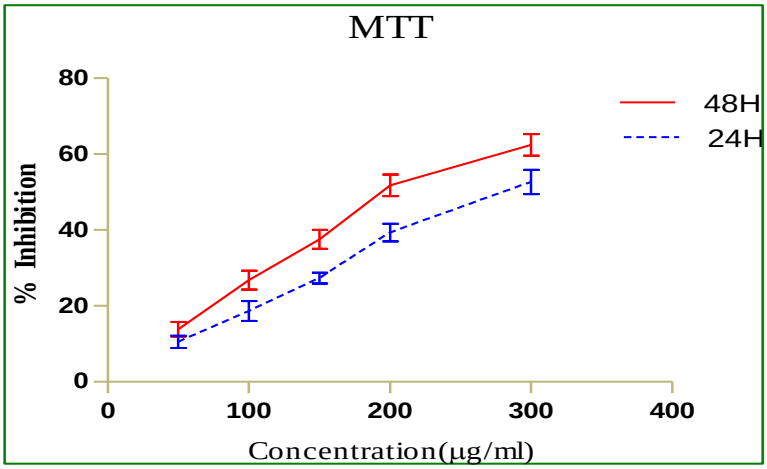

Figure (7.2): MTT assay of MEQL. Each point represents mean ± SEM obtained from three individual experiments. The IC_{50} value at 24 hr is 281.6 ± 1.79 µg/ml and at 48 hr is 195.3 ± 2.25 µg/ml.

Conclusion: The *in vitro* cytotoxicity studies showed that MEQL 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 *Quercus lanata* 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 ± 2° 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. Ascitic 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 ml/kg i.p.) and group-II served as EAC control. 24 h after EAC transplantation, Group-III and IV received methanol extract of *Quercus lanata* (MEQL) at a dose of 50 and 100 mg/kg i.p. for nine consecutive days, respectively. Group-V received reference drug 5-FU (20 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 ascitic 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 ascitic 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 *et al*, 1994).

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

Tumor cell count:

The ascitic 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.

Hematological parameters:

Blood was obtained from cardiac puncture that was used for total count of RBC, WBC and haemoglobin by standard method.

Biochemical parameters:

Collected blood was set aside for 15 minutes to allow it to clot and then centrifuged at 5000 rpm for 10 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).

Preparation of liver and kidney homogenates:

Livers and kidneys collected from the sacrificed animals were homogenized separately in 10 ml of phosphate buffer (20 mM, pH-7.4) and centrifuged at 12000 rpm for 30 min at 4° 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) 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 30 ºC for 90 minutes. The reaction was then stopped by adding 1 ml 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 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, kept on ice bath for 10 minutes and 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 in 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 centrifuged at 3000 g for 10 minutes. 1 ml supernatant was mixed with TBA (1% w/v) and heated for 1 hour or water bath at 95° C until a stable pink color was 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 analysed 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 MEQL to EAC induced group significantly reduced the tumor volume, packed cell volume and the number of viable cells compared to the EAC control group (Table 8.1).

Life Span:

The mean survival time and the percentage of life span increased significantly compared to the EAC control group (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 MEQL 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 MEQL treated groups. The enzymatic and non enzymatic antioxidants like super oxide dismutase and reduced glutathione were found to be restored towards normal levels. (Table 8.4 and Figure 8.2 [a-c]).

Table 8.1:

Groups	Tumor volume (ml)	Packed cell volume (ml)	Cell count($\times 10^7$/ml)		Viable %	Non-viable %	MST (Day)	ILS %
			Viable	Nonviable				
EAC control	1.93 ± 0.12	1.19 ± 0.12	7.68 ± 0.27	0.36 ± 0.08	95.52	4.48	20	00
EAC + 50 mg/kg MEQL	1.03 ± 0.14*	0.69 ± 0.03*	5.28 ± 0.25*	3.06 ± 0.44*	63.34	36.69	31	55
EAC + 100 mg/kg MEQL	0.70 ± 0.15*	0.45 ± 0.06*	3.85 ± 0.32*	3.94 ± 0.15*	49.52	50.60	37.5	87.5
EAC + 5-FU (20 mg/kg)	0.57 ± 0.12*	0.29 ± 0.23*	2.52 ± 0.17*	5.27 ± 0.24*	32.36	67.63	41	105

Shows the effect of MEQL on various parameters such as tumor volume, packed cell volume, % of viable and nonviable cells, mean survival time and the increase in life span. Values are represented as mean ± SEM. *p<0.001 when EAC control compared with treated groups.

Table 8.2:

Parameters	Normal	EAC control	50 mg/kg MEQL	100 mg/kg MEQL	20 mg/kg 5-FU
RBC(cell × 10^6/mm^3)	5.68 ± 0.20	2.69 ± 0.26[a]	4.77 ± 0.16***	5.07 ± 0.07***	5.21 ± 0.06***
WBC(cell × 10^6/mm^3)	4.85 ± 0.45	7.97 ± 0.13[a]	6.54 ± 0.27**	5.28 ± 0.11***	5.12 ± 0.06***
Hb(g/dl)	11.51 ± 1.21	4.33 ± 0.27[a]	7.68 ± 0.51*	9.37 ± 0.50**	10.89 ± 0.66***

Effect of different concentration of the extracts on hematological parameters. Values are represented as mean ± SEM. [a]p< 0.001 when EAC control compared to Normal, *p< 0.05, **p< 0.01 and ***p< 0.001 when EAC control compared with treated groups.

Table 8.3:

Parameters	Normal	EAC control	50 mg/kg MEQL	100 mg/kg MEQL	20 mg/kg 5-FU
TP (g/dl)	11.14 ± 1.18	4.33 ± 0.67#	5.23 ± 0.68	7.13 ± 0.60	9.46 ± 0.90**
SGOT (IU/L)	13.33 ± 1.20	29.00 ± 2.30#	24.96 ± 1.86	17.56 ± 1.00**	15.17 ± 1.69***
SGPT (IU/L)	14.79 ± 1.24	33.67 ± 1.76#	23.00 ± 1.15***	19.69 ± 1.42***	16.66 ± 1.23***
SALP (KA/U)	61.00 ± 2.08	224.3 ± 2.96#	133 ± 2.09***	110.7 ± 2.33***	96.33 ± 3.53***

Effects of different concentrations of the extract on serum parameters. Values are represented as mean ± SEM. #p<0.001 when EAC control compared to normal, **p<0.01 and ***p<0.001 when EAC control compared with treated groups.

Table 8.4:

Groups		Lipid peroxidation (nM MDA/mg protein)	Superoxide dismutase (mU/min/mg protein)	Reduced glutathione (ng/GSH/mg protein)
Normal	Liver	18.58 ± 1.294	188.3 ± 1.091	1620 ± 38.55
	Kidney	25.33 ± 1.205	171.6 ± 3.903	1490 ± 41.80
EAC control	Liver	74.33 ± 2.028#	47.86 ± 5.323#	452.5 ± 30.24#
	Kidney	82.00 ± 2.082#	78.19 ± 1.533#	340.2 ± 24.69#
50 mg/kg MEQL	Liver	53.67 ± 1.764***	69.20 ± 2.958*	850.1 ± 29.36***
	Kidney	63.67 ± 2.186***	119.5 ± 2.516***	627.5 ± 32.72***
100 mg/kg MEQL	Liver	42.33 ± 1.453***	121.7 ± 11.62***	1034 ± 33.69***
	Kidney	50.00 ± 4.041***	138.3 ± 3.237***	852.2 ± 27.37***
20 mg/kg 5-FU	Liver	22.00 ± 2.646***	164.8 ± 3.062***	1356 ± 33.62***
	Kidney	26.86 ± 1.695***	158.7 ± 4.536***	1234 ± 35.30***

Effects of different concentrations of MEQL on different biochemical parameters. The values are represented as mean ± SEM. #p<0.001 when EAC control compared to normal livers and kidneys, *p<0.05 and ***p<0.001 when EAC control compared to EAC livers and kidneys.

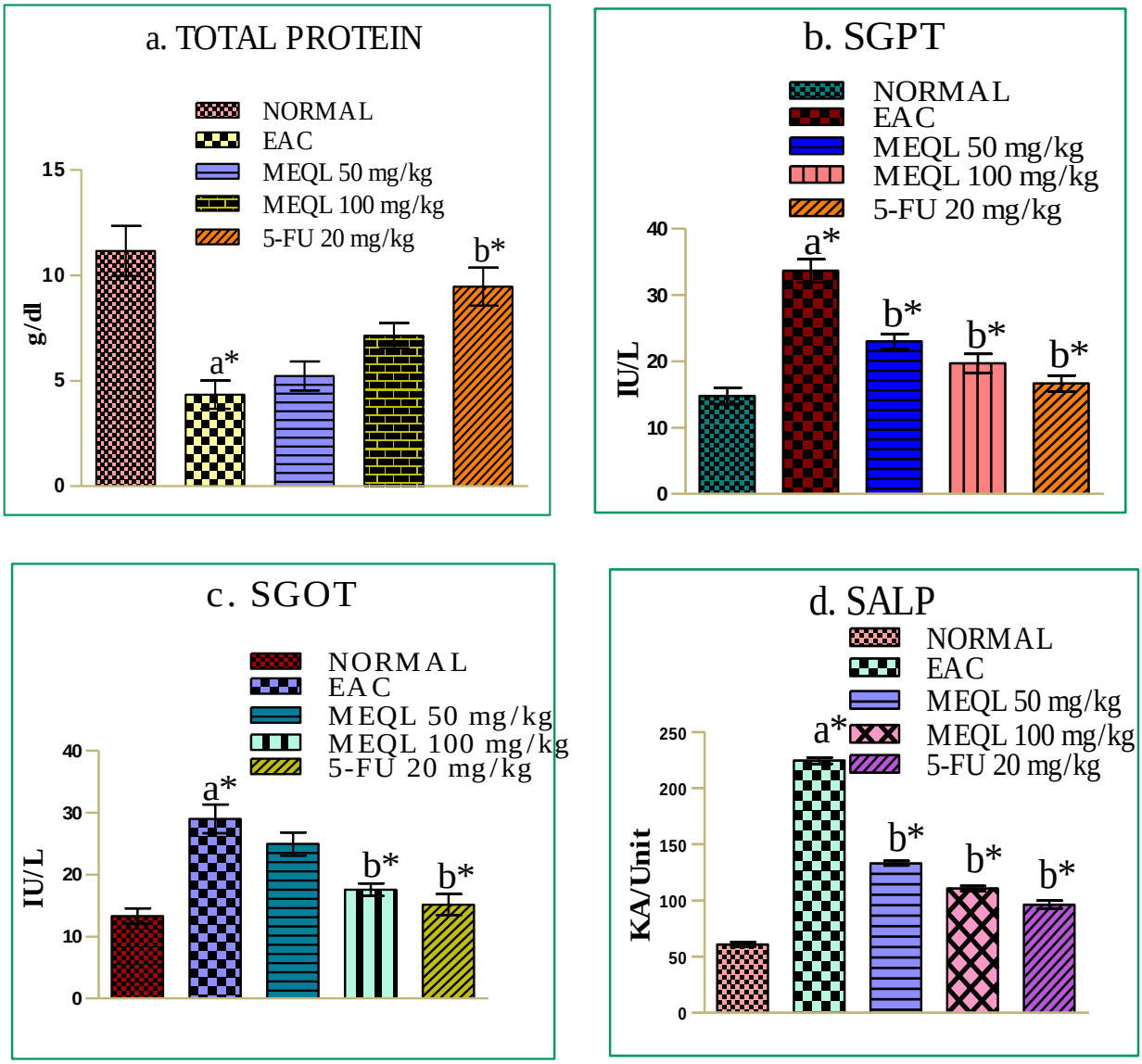

Figure 8.1(a-d): Effects of different concentrations of MEQL on Serum biochemical parameters (a) Total protein (b) SGPT (c) SGOT and (d) SALP. Values are represented as mean ± SEM. a*p<0.05 when EAC control compared to Normal and b*p<0.05 when treated groups compared to EAC Control.

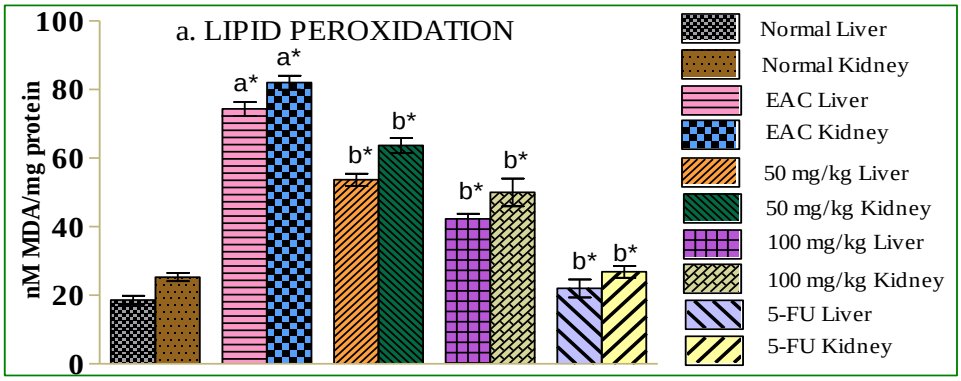

Figure 8.2 (a): Effects of different concentrations of MEQL on lipid peroxidation. The values are represented as mean ± SEM. $^{a*}p<0.05$ when EAC control compared to normal liver and kidney, $^{b*}p<0.05$ when treated groups compared to EAC liver and EAC kidney.

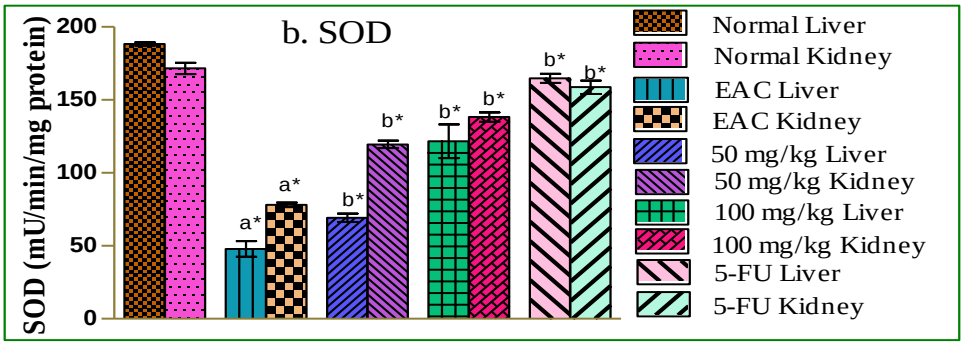

Figure 8.2 (b): Effects of different concentrations of MEQL on SOD. The values are represented as mean ± SEM. $^{a*}p<0.05$ when EAC control compared to normal liver and kidney, $^{b*}p<0.05$ when treated groups compared to EAC liver and EAC kidney.

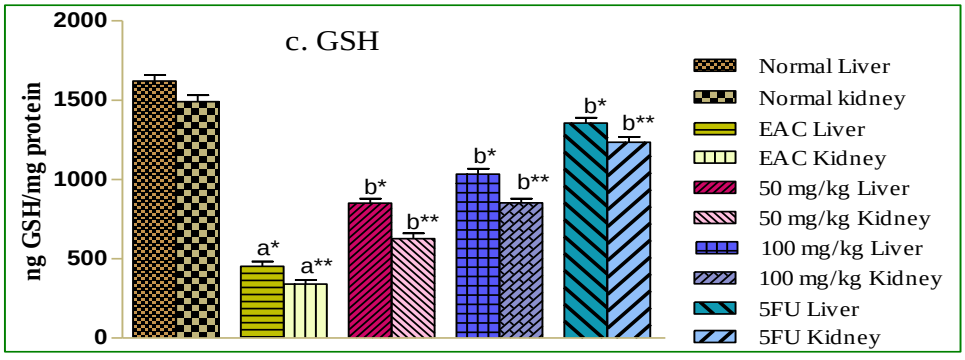

Figure 8.2 (c): Effects of different concentrations of MEQL on GSH. The values are represented as mean ± SEM. $^{a*}p<0.05$ when EAC control compared to normal liver and kidney, $^{b*}p<0.05$ when treated groups compared to EAC liver and EAC kidney.

Histopathology:

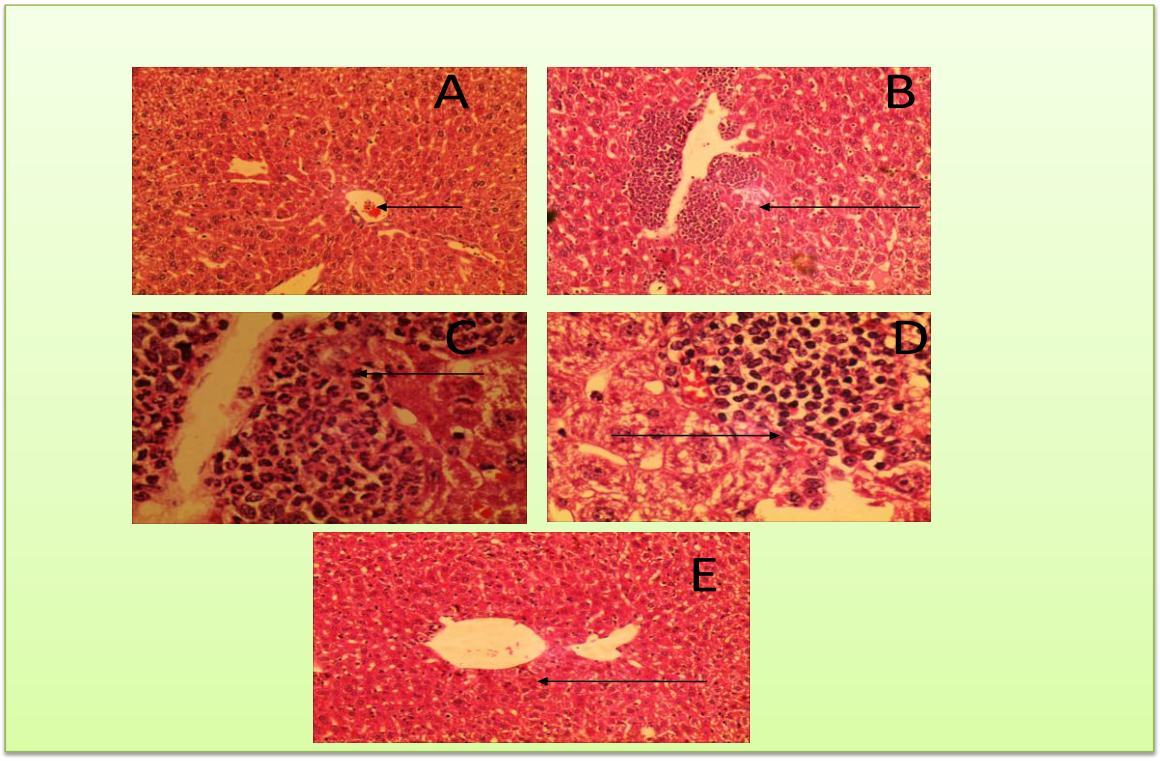

Figure 8.3: A) Normal liver tissue showing prominent sinusoids. B) Predominantly acute inflammation in EAC control. C) Less acute inflammation in MEQL low dose treated group. D) Regeneration of hepatocytes in MEQL high dose treated group. E) Regeneration of functioning sinusoids in 5FU treated group.

Discussion

In the present study EAC cell line was used to evaluate the anticancer activity of MEQL. EAC appeared firstly as a spontaneous breast cancer in a female mouse. It is referred to as an undifferentiated carcinoma, and is originally hyperdiploid, has high transplantable capability, no-regression, rapid proliferation, shorter life span, 100% malignancy and also does not have tumor specific transplantation antigen. 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 ascitic fluid formation and accumulation (Bala *et al*, 2010). The ascitic fluid is essential for tumor growth, since it acts as a direct nutritional source

for tumor cells (Shimizu *et al*, 2004). MEQL 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 ascitic fluid. These results could connote either a direct cytotoxic effect of MEQL 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 MEQL treated group showed enhancement of life span compared to the tumor treated group which indicates that MEQL 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 MEQL 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 MEQL 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). From the experiment it was found that EAC control group exhibited increased levels of liver enzymes such as SGOT, SGPT and SALP while the levels of total protein were decreased due to hepatocellular damages. The MEQL treated group showed restoration of these biochemical parameters to more or less normal levels.

The disbalance between the reactive oxygen metabolites and the antioxidant defence systems leads to “oxidative stress” which deregulates various cellular functions causing pathological conditions (Bandyopadhyay *et al*, 1999 and Adesegun *et al*, 2009). Reactive oxygen species (ROS) formed in

cancer tissues result in lipid peroxidation and subsequent increase in MDA and other TBARS levels. MDA, the end product of lipid peroxidation, a biomarker of oxidative stress, was reported to be higher in cancer tissues than in the non-diseased organ (Yagi *et al*, 1991 and Neilson *et al*, 1997). The present study showed that TBARS level measured as MDA in the EAC bearing liver tissues were higher than those in normal saline treated liver tissues. Treatment with MEQL inhibited hepatic lipid peroxidation as revealed by reduction of MDA levels towards normal levels. This indicated the reduction in free radical generation by MEQL in tumor bearing mice.

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 decreased 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 MEQL was found to increase the GSH content in the liver and kidney as compared to the tumor control group.

The free radical scavenging system SOD is present in all oxygen-metabolizing cells. Its function is to provide a defence against the potentially damaging reactivates of superoxide. Inhibition of SOD activities as a result of tumor growth was also reported and similar findings were obtained in the present investigation with EAC-bearing mice. Treatment with MEQL significantly increased the SOD level indicating antioxidant and free radical scavenging activity of the extract.

Conclusion

The *in vivo* anticancer study delineates that the methanol extract of *Quercus lanata* does have some potential to fight against cancer. Also 100 mg/kg MEQL showed better response compared to 50 mg/kg MEQL 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 still to be identified. Also a thorough mechanistic study remains to be undertaken to establish constituents of MEQL 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 bark of *Quercus lanata* 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 bark of *Quercus lanata* 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 have reported therapeutic benefits against various ailments including cancer. So the alkaloids or 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.