PHYTOCHEMICAL ANALYSIS AND PHARMACOLOGICAL SCREENING OF *QUERCUS INFECTORIA*

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

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In partial fulfillment of the award of the degree of

MASTER OF PHARMACY

IN

Branch-II- PHARMACEUTICAL CHEMISTRY

Submitted by

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TAMILNADU

OCTOBER – 2018

CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"PHYTOCHEMICAL ANALYSIS AND PHARMACOLOGICAL SCREENING OF *QUERCUS INFECTORIA*"** submitted by the student bearing **Reg. No: 261615201** to "**The Tamil Nadu Dr.M.G.R. Medical University - Chennai**", in partial fulfilment for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Chemistry** was evaluated by us during the examination held on.....

Internal Examiner **External Examiner**

CERTIFICATE

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I do hereby declared that the dissertation **"PHYTOCHEMICAL ANALYSIS AND PHARMACOLOGICAL SCREENING OF *QUERCUS INFECTORIA*"** submitted to "**The Tamil Nadu Dr.M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of **Master of Pharmacy** in **Pharmaceutical Chemistry,** is a bonafide research work has been carried out by me during the academic year 2017-2018, under the guidance and supervision of **Dr. M. VIJAYABASKARAN, M.Pharm., Ph.D.,** Professor & HOD, Department of Pharmaceutical Chemistry, J.K.K.Nattraja College of Pharmacy, Komarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place: Komarapalayam **Mr. R. ANANDARAJ**

Date: **Reg.no.261615201**

Dedicated to

Parents,

Teachers &

My Family

ACKNOWLEDGEMENT

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PHYTOCHEMICAL ANALYSIS AND PHARMACOLOGICAL SCREENING OF *QUERCUS INFECTORIA*

CONTENTS

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Disease always co-existed with livings, detecting their remedies also always continuing, going through the commencement of drug therapy for disease, drugs comes to the force in sudden, in the ancient time human knowledge found the absence of some food forms the base for the development of some disease, they were tried to use the same food material for curing that particular disease and they got success in that work. This motivates the plant researchers to use different plants, plant parts for different ailments.

In olden days they used plants as such and then made into different formulations for their expediency such as powder, juice, decoction, extracts etc. Our traditional system of medicine siddha categorized nearly 5000 plant species and their usage. Later on the allopathic system of medicine comes to force and dominate the siddha and due to the fast relieving nature it reached the world as quickly and diminished the usage of plant medicine as maximum. But allopathic system cannot provide ultimate solution to some disease and also their side effect in particularly the long term therapy, limits their usage still the plant medicine is recommended and used in such cases. This suggests the plant medicine to researchers as and scientific world as alternate to allopathic system of medicine. The world health organization recognize and motivate the plant researchers, hence the plant medicine now considered being an alternative system of medicine.

Even usage of plants are known, since plant species consists of mixture of compound, isolating the single compound and identifying the component is responsible for that particular activity is a major question in front of plant researcher and also it is very difficult to say only these are all the compounds available from particular plant.

Nowadays due to the development of science and technology such as chromatographic technique and spectroscopic technique it is possible to isolate almost all the components of plant and characterize them. Isolation and characterization are very important to improve the effectiveness, minimizing the dose and on set of action.

Now this study is considered as a separate discipline called “photochemistry” defined as a branch of science somewhere in between natural product organic chemistry

and plant biochemistry concerned with organic substances accumulated by plants and deals with the chemical structure of these substances, their biosynthesis, turnover, metabolism, their natural distribution and their biological function. Since detecting the compound responsible for the particular activity, isolating, characterizing the compound and monitoring the activity is of prime importance, the basic requirement needed for the medicinal world, it is the duty of the chemist to do this works.[1]

HERBAL MEDICINES

Medicinal plants are the oldest known health care source. Their importance is still growing although it varies depending on the ethnological, medical and historical background of each country. Medicinal plants are also importance for pharmacological research and drug development, not only when plant constituents are used directly as basic materials for the synthesis of drugs or as models for pharmacologically active compounds.

The World Health Organization (WHO) estimates that billion people, 80 percent of the world population, presently use herbal medicine for one or the other aspect of primary health care. Herbal medicine is a major component in all people's indigenous traditional medicine and a common element in Ayurvedic, Homeopathic, Naturopathic, Traditional oriental and Native American Indian Medicine. Plants have provided the lead molecules for a large number of diseases. During the past 40 years numerous novel compounds have been isolated from plant sources and many of this substance have been demonstrated to possess interesting biological activities.

APPROACHES TO NATURAL PRODUCT RESEARCH AND DRUG DISCOVERY

Different approaches to drug discovery from plants can be enumerated as: random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethno-medical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched/standardized extracts (herbal product development), use

of a plant product, biologically potent but beset with other issues, as a lead for further chemistry, and single new compounds as drugs. The objective of the later approach is the targeted isolation of new bioactive plant products, i.e. lead substances with novel structures and novel mechanisms of action. This approach has provided a few classical examples, but the problem most often encountered here is not enough availability. The problem of availability can be overcome by semi synthesis/ synthesis or using tissue-culture techniques (by genetically modifying the biosynthetic pathway of the compound of interest).

OLDER APPROACH

- Focused on chemistry of compounds from natural sources, but not on activity.
- Straightforward isolation and identification of compounds from natural sources followed by testing of biological activity in animal model.
- Chemotaxonomic investigation.
- Selection of organisms primarily based on ethno pharmacological information, folkloric reputations, or traditional uses.

MODERN APPROACH

- Bioassay-directed (mainly *in vitro*) isolation and identification of active lead compounds from natural sources.
- Production of natural products libraries.

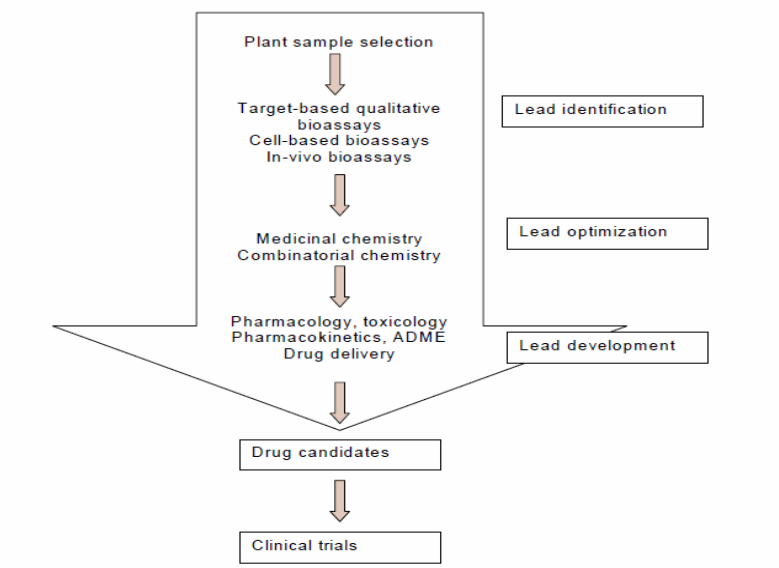

Figure 1: Steps involved in drug discovery from plants

- Production of active compounds by cell or tissue culture, genetic manipulation, natural combinatorial chemistry and so on.
- More focused on bioactivity.
- Introduction of the concepts of de-replication, chemical fingerprinting, and metabolomics.
- Selection of organisms based on ethno pharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.[2]

CHALLENGES IN DRUG DISCOVERY FROM MEDICINAL PLANTS

In spite of the success of drug discovery programs from plants in the past 2-3 decades, future endeavors face many challenges. Natural products scientists and pharmaceutical industries will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts. The process of drug discovery has been estimated to take an average period of 10 years and cost more than 800 million dollars.[3] It is estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be approved for use. In the drug discovery process, lead identification is the first step. Lead optimization (involving medicinal and combinatorial chemistry), lead development (including

pharmacology, toxicology, pharmacokinetics, ADME and drug delivery), and clinical trials all take considerable time.

As drug discovery from plants has traditionally been time-consuming, faster and better methodologies for plant collection, bioassay screening, compound isolation and compound development must be employed· Innovative strategies to improve the process of plant collection are needed, especially with the legal and political issues surrounding benefit-sharing agreements.[4]

The design, determination and implementation of appropriate, clinically relevant, high throughput bioassays are difficult processes for all drug discovery programs. The common problem faced during screening of extracts is solubility and the screening of extract libraries is many times problematic, but new techniques including pre-fractionation of extracts can alleviate some of these issues. Challenges in bioassay screening still remain an important issue in the future of drug discovery from medicinal plants. The speed of active compound isolation can be increased using hyphenated techniques like LC-NMR and LC-MS. Development of drugs from lead compounds isolated from plants, faces unique challenges. Natural products, in general, are typically isolated in small quantities that are insufficient for lead optimization, lead development and clinical trials. Thus, there is a need to develop collaborations with synthetic and medicinal chemists to explore the possibilities of its semi-synthesis or total synthesis.[5] One can also improve the natural products compound development by creating natural products libraries that combine the features of natural products with combinatorial chemistry.

OPPORTUNITIES IN DRUG DISCOVERY FROM MEDICINAL PLANTS

Bio prospecting demands a number of requirements which should be co-coordinated, such as team of scientific experts (from all the relevant interdisciplinary fields) along with expertise in a wide range of human endeavors, including international laws and legal understanding, social sciences, politics and anthropology. In our context, Ayurveda and other traditional systems of medicine, rich genetic resources and associated ethno medical knowledge are key components for sustainable bio prospecting and value-

addition processes.

For drug-targeted bio prospecting an industrial partner is needed, which will be instrumental in converting the discovery into a commercial product. Important in any bio prospecting is the drafting and signing of an agreement or Memorandum of Understanding that should cover issues on access to the genetic resources (biodiversity), on intellectual property related to discovery, on the sharing of benefits as part of the process (short term), and in the event of discovery and commercialization of a product (long term), as well as on the conservation of the biological resources for the future generations. When ethno botanical or ethno pharmacological approach is utilized, additional specific requirements that relate to prior informed consent, recognition of Indigenous Intellectual Property and Indigenous Intellectual Property Rights as well as short- and long-term benefit sharing need to be taken into account.

In order to screen thousands of plant species at one go for as many bioassays as possible, we must have a collection of a large number of extracts. Globally, there is a need to build natural products extract libraries. The extract libraries offer various advantages, such as reduction in cost and time for repeat collection of plants and availability of properly encoded and preserved extracts in large numbers for biological screening in terms of high-throughput screenings and obtaining hits within a short period. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays.[6]

DRUG DISCOVERY FROM NATURAL SOURCES

For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases and disorders. The importance of natural products in modern medicine has been discussed in recent reviews and reports. The value of natural products in this regard can be assessed mainly by using three criteria:

1. The rate of introduction of new chemical entities of wide structural diversity, including serving as lead molecule for semi-synthetic and total synthetic modification

2. The number of diseases treated or prevented by these substances, and

3. Their frequency of use in the treatment of disease.

An analysis of the origin of the drugs developed between 1981 and 2003 showed that natural products or natural product-derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market. In addition, about 24% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products. This combined percentage suggests that the natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug discovery and development.[7]

CANCER

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. These contrast with benign tumors, which do not spread to other parts of the body. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss, and a change in bowel movements. While these symptoms may indicate cancer, they may have other causes. Over 100 types of cancers affect humans.[8]

Tobacco use is the cause of about 22% of cancer deaths. Another 10% are due to obesity, poor diet, lack of physical activity, and excessive drinking of alcohol. Other factors include certain infections, exposure to ionizing radiation and environmental pollutants. In the developing world, 15% of cancers are due to infections such as Helicobacter pylori, hepatitis B, hepatitis C, human papilloma virus infection, Epstein–Barr virus and human immunodeficiency virus. These factors act, at least partly, by changing the genes of a cell. Typically many genetic changes are required before cancer develops[9]. Approximately 5–10% of cancers are due to inherited genetic defects from a person's parents. Cancer can be detected by certain signs and symptoms or screening tests. It is then typically further investigated by medical imaging and confirmed by biopsy.[10]

Many cancers can be prevented by not smoking, maintaining a healthy weight, not

drinking too much alcohol, eating plenty of vegetables, fruits and whole grains, vaccination against certain infectious diseases, not eating too much processed and red meat, and avoiding too much sunlight exposure. Early detection through screening is useful for cervical and colorectal cancer. The benefit of screening in breast cancer is controversial. Cancer is often treated with some combination of radiation therapy, surgery, chemotherapy, and targeted therapy. Pain and symptom management are an important part of care. Palliative care is particularly important in people with advanced disease. The chance of survival depends on the type of cancer and extent of disease at the start of treatment. In children under 15 at diagnosis the five-year survival rate in the developed world is on average 80%. For cancer in the United States the average five-year survival rate is 66%.[11]

In 2015, about 90.5 million people had cancer. About 14.1 million new cases occur a year (not including skin cancer other than melanoma). It caused about 8.8 million deaths (15.7% of deaths). The most common types of cancer in males are lung cancer, prostate cancer, colorectal cancer and stomach cancer. In females, the most common types are breast cancer, colorectal cancer, lung cancer and cervical cancer. If skin cancer other than melanoma were included in total new cancers each year, it would account for around 40% of cases. In children, acute lymphoblastic leukemia and brain tumors are most common except in Africa where non-Hodgkin lymphoma occurs more often. In 2012, about 165,000 children under 15 years of age were diagnosed with cancer[12]. The risk of cancer increases significantly with age and many cancers occur more commonly in developed countries. Rates are increasing as more people live to an old age and as lifestyle changes occur in the developing world. The financial costs of cancer were estimated at $1.16 trillion USD per year as of 2010.

TYPES OF CANCER

Our bodies are made up of billions of cells. The cells are so small that they can only be seen under a microscope. These cells are grouped together to make up the tissues and organs of our bodies. These cells are basically the same, but they vary in some ways. This is because the body organs do very different things.[13] For example; nerves and

muscles do very different things. So nerve and muscle cells have different structures.

Cancers can be grouped according to the type of cell they start in. There are 5 main categories

1. Carcinoma – cancer that begins in the skin or in tissues that line or cover internal organs. There are a number of subtypes, including adeno carcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma

2. Sarcoma – cancer that begins in the connective or supportive tissues such as bone, cartilage, fat, muscle, or blood vessels

3. Leukemia – cancer that starts in blood forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and go into the blood

4. Lymphoma and myeloma – cancers that begin in the cells of the immune system

5. Brain and spinal cord cancers – these are known as central nervous system cancers

Cancers can also be classified according to where they start in the body, such as breast cancer or lung cancer.[14]

SIGNS AND SYMPTOMS[15]

Roughly, cancer symptoms can be divided into three groups.

LOCAL SYMPTOMS

Unusual lumps or swelling (tumour), hemorrhage (bleeding), pain and / or ulceration. Compression of surrounding tissues may cause symptoms such as jaundice

SYMPTOMS OF METASTASIS (SPREADING)

Enlarge lymph nodes, cough and haemoptysis, hempatomegaly (enlarged liver), bone pain, fracture of affected bones and neurological symptoms. Although advanced cancer may cause pain, it is often not the first symptom.

SYSTEMIC SYMPTOMS

Weight loss, poor appetite and cachexia (wasting), excessive sweating (night sweats), anaemia and specific para-neoplastic phenomena, i.e., specific conditions that are due to an active cancer, such as thrombosis or hormonal changes.

CANCER CELL CYCLE[15]

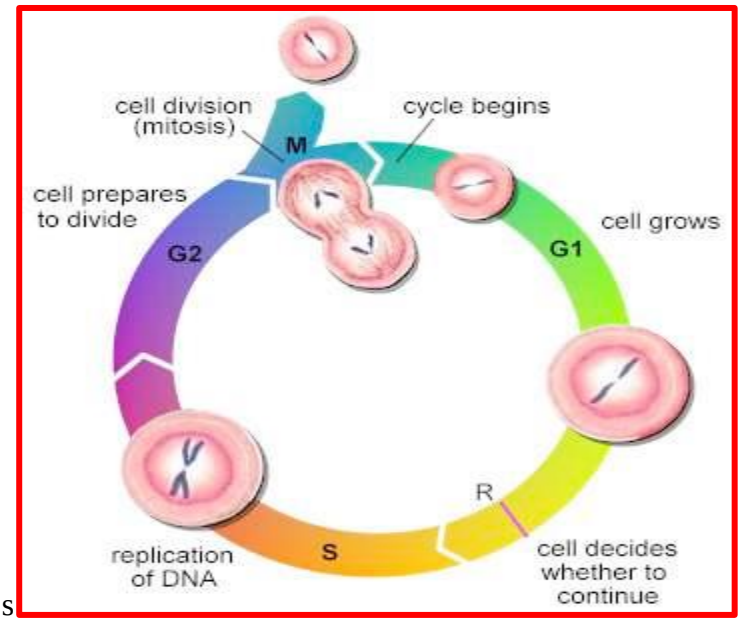

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Figure 2: CANCER CELL CYCLE

Cellular multiplication involves passage of the cell through a cell cycle. The various phases of the cell cycle are characterized as: (i) the interval follow cell division to the point where DNA synthesis starts, known as the pre-synthetic phase (G1). The variability in the length of the cell cycle between rapidly and slowly replicating cells is accounted by the differences in the length of (G1) phase; (ii) after mitosis some of the daughter cells pass into a resting phase or non-proliferative.

Phase (G0), and do not re-enter the cell cycle phase G1 immediately. They may enter the G1 phase later. The G0 phase is the sub phase of G1; (iii) the DNA synthesis(s) occurs; (iv) the pre mitotic or post synthetic (G2) phase follows. In the phase RNA and protein synthesis take place, and it is shorter than the S phase; and (v) lastly mitotic (M0

phase follows, in which the synthetic activity of the cell is low the chromosomes separate in two daughter cells through the sub phase-prophase, metaphase, anaphase and telo phase. These daughter cells have the option of either entering the G1 phase or the G0 sub phase of G1 phase.

Cancers are caused by a series of mutations. Each mutation alters the behaviour of the cell somewhat. Carcinogenesis, when means the initiation or generation of cancer, is the process of derangement of the rate of cell division due to damage to DNA. Proto-oncogenes are genes which promote cell growth and mitosis, a process of cell division, and tumour suppressor genes discourage cell growth, or temporarily halt cell division in order to carry out DNA repair. Typically, a series of several mutations to these genes are required before a normal cell transforms into a cancer cell.

Proto-oncogenes promote cell growth through a variety of ways. Many can produce hormones, a "chemical messenger" between cells which encourage mitosis, the effect of which depends on the signal transduction of the receiving tissue or cells. Some are responsible for the single transduction system and signal receptors in cells and tissues themselves, thus controlling the sensitivity to such hormones.

They often produce mitogens, or they are involved in transcription of DNA in protein synthesis, which creates the proteins and enzymes responsible for producing the biochemical cells use and interact with Mutation sin proto-oncogenes can modify their expression and function, increasing the amount or activity of the product protein. When this happens, they become oncogenes, and thus cells have a higher chance to divide excessively and uncontrollably. The chance of cancer cannot be reduced by removing proto oncogenes from the genome as they are critical for growth, repair and homeostasis of the body.

CHEMOTHERAPY[16, 17]

Chemotherapy plays a significant role in the treatment of early stage disease, in the pro-operative period and as adjuvant therapy for the treatment of micro metastasis. As knowledge has been accumulating in the area of pharmacology, tumour biology,

cytokine ties and resistance, therapeutic strategies have been developed that maximize the tumour-cell kill, decrease resistance and enhance the potential for cure by chemotherapy. The antineoplastic armamentarium currently contains over 30 drugs, with many additional agents under investigation. Human pituitary growth hormone, prostaglandins, cyclin-AMP, RNA-dependent DNA polymerase, etc also show promising results.

Since the differences between normal and neoplastic human cells are merely quantitative rather than qualitative, most antineoplastic drugs are associated with certain side effects. The toxicity usually involves attack of drugs on rapidly proliferating normal body tissues such as bone marrow, hair follicles and intestinal epithelium. In addition, individual drug may produce its own distinctive toxic effects on heart, lungs, kidneys and other organs. Hence with some exceptions it can be said that the antineoplastic agents are generally palliative and not curative.

Many anti-cancer drugs (a) have a very narrow therapeutic index (b) is highly unable (c) are effective at very low concentration and (d) having unusual metabolic pathways.

DRUG DESIGNING FOR CANCER[18]

In designing specific regimens for clinical use, a number of factors must be taken into account. Drugs are generally more effective in combination and may be synergistic through biochemical interactions. These interactions are useful designing new regimens. It is more effective to use drugs that do not share common mechanisms of resistance and that do not overlap in their major toxicities. Drugs should be used as close as possible to their maximum individuals does. And, finally, drugs should be used as close as possible to discourage tumour growth and maximize does intensity (the does gives per unit time, a key parameter in the success of chemotherapy). Based on experimental tumour models, it is necessary to eradicate all tumour cells. The fraction of cells killed with each treatment cycle is constant, with re growth between cycles. Thus, it is desirable to achieve maximal cell kill with each cycle, using the highest drug does possible, and to repeat does ad frequently as tolerated. Since the tumour cell population in patients with visible disease exceeds 1gm, or 10^9 cells, and since each cycle of therapy kills less than

99% of the cells, it is necessary to repeat treatment in multiple cycles to kill all the tumour cells.

THE MECHANISM ON CANCER THERAPY[18]

1. Inhibiting cancer cell proliferation directly by stimulating macrophage phagocytosis, enhancing natural killer cell activity.
2. Promoting apoptosis of cancer cells by increasing production of interferon, interleukin-2 immunoglobulin and complement in blood serum.
3. Enforcing the necrosis of tumor and inhibiting its translocation and spread by blocking the blood source of tumor tissue.
4. Enhancing the number of leukocytes and platelets by stimulating the hemopoietic function.
5. Promoting the reverse transformation from tumor cells into normal cells.
6. Promoting metabolism and preventing carcinogenesis of normal cells.
7. Stimulating appetite, improving quality of sleep, relieving pain, thus benefiting patient's health.

PLANTS: TREATMENT OF CANCER[19]

In the face of failure to fine synthetic drugs against cancer, thousands species of plants have been screened since a long time, for anti-neoplastic activity, in the hope of discovering effective natural products. Compounds have been evaluated. Such work is still going on in several laboratories throughout the world.

The Natural Product Drug Development Program of the U.S. National Cancer Institute has identified about 3000 species of plants and animals as useful in dealing with one or the other aspect of cancer management. Based on *in vitro* data, a large number of species have been identified to be of promise and taken to clinical trials.

However, products of hardly a handful of plant species, such as the Vinca alkaloids, Taxol, Camptothecin, Podophyllotoxin, etc., have passed through the rigorous tests to be officially used against certain types of cancer and are now available in the market. Yet there are severe problems associated with the use of even these largely

'successful' drugs, which are among the most expensive plant products. This reflects the complexity of the scenario of cancer drugs in general and plant base.

EVALUATION OF ANTICANCER STUDIES[19]

Anticancer drugs can be evaluated by *in vitro* and *in vivo* methods.

IN VITRO METHODS: CYTOTOXICITY

There is much pressure, both human and economic, to perform at least part of cytotoxicity testing *in vitro.* Currently it is difficult to monitor systematic and physiological effects *in vitro*, so most assay determine effects at cellular level, or cytotoxicity broadly involve the metabolic alternation of the cells, including the death of cells as a result of toxic effects of the compounds.

The choice of assay will depend on agent on study, the nature of response, and the particular target cell. In anticancer research, the *in vitro* screening involves estimation of cytotoxicity of the drug by different methods. The commonly used methods of studying cytotoxicity are.

DETERMINATION OF CELL VIABILITY BY TRYPHAN BLUE DYE EXCLUSION METHOD

In this method, viability dyes such as tryphan blue is used to determine membrane integrity. Staining for viability assessment is more suited to suspension culture than to monolayer, because dead cells detach from the monolayer and are therefore lost from the assay. The method has been applied with equal success to solid tumor, effusions and haematological malignancies.

DETERMINATION OF CELL VIABILITY BY UPTAKE OF NEUTRAL RED DYE BY THE LYSOSOME IN NEUTRAL RED ASSAY

The uptake of neutral red by lysosome and Golgi bodies has been used to quantitative cell number. The satin appears to be specific for viable cells, but the main limitation of the method is the difference in uptake between cell types. Thus some cell

types. Thus some cell types, such as activated macrophages and fibroblast take up amount very rapidly where as others, such as lymphocytes, show negligible staining.

DETERMINATION OF CELL METABOLIC FUNCTION BY PROTEIN ESTIMATION

Several methods are available for measuring the protein contents of cell monolayers. These include the use of Folin-ciocatechu reagent according to the method of Lowry and amino black.

DETERMINATION OF QUANTITATIVE VALUE FOR THE LOSS OF CELL VIABILITY BY MEASUREMENT OF LACTATE DEHYDROGENASE ACTIVITY BY LDH ASSAY

The measurement of lactate dehydrogenase in culture supernatant gives a quantitative value for the loss of cell viability.

Pyruvate + NADH + H + $\Leftrightarrow$ NAD^+ + lactate

The activity of LDH can be measured as the reduction of Pyruvate to lactate. The reduction is coupled to the oxidation of NADH to NAD, which is followed spectrophotometrically at 340nm.

SULPHOROMAMINE B (SRB) ASSAY

SRB is a pink amino xanthenes dye with two sulfonic groups. Under mild acidic condition, SRB binds to protein basic amino acid residue in (trichloroacetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude. Colour development of SRB assay is rapid, stable, and visible. The developed colour can be measured over a broad range of visible wavelength in 90-microliter plate readers.

CYTOTOXICITY BY MICRO CULTURE TETRAZOLIUM (MTT) ASSAY

The ability of the cell to survive a toxic insult has been the basis of most Cytotoxic assays. This assay is based on the assumption that dead cell or their products do not reduce Tetrazolium salt (3-(4, 5 dimethyl thiazole – 2yl) –2, 5 – diphenyl Tetrazolium bromide) into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase. The numbers of cell are found to be proportional to the extent of formazan production by cells used

IN VIVO METHODS

- Fibro sarcoma solid tumour model.
- EAC model (Ehrlich ascites carcinoma).
- Chemically induced cancer model.
- Virus induced cancer model.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

Julalak Chokpaisarn et al (2018)., studied the *Quercus infectoria* inhibits Set7/NF-κB inflammatory pathway in macrophages exposed to a diabetic environment.[20]

Ahmed A Tayel et al (2018)., reported the application of *Quercus infectoria* extract as a natural antimicrobial agent for chicken egg decontamination.[21]

Gamboa-Gómez et al (2017)., was studied the *in vitro* and *in vivo* assessment of anti-hyperglycemic and anti-oxidant effects of oak leaves (*Quercus convallata* & *Quercus arizonica*) infusions and fermented beverages.[22]

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Savitri Shrestha et al (2014)., has been reported pharmacognostic studies of insect gall of *Quercus infectoria* Olivier (Fagaceae).[25]

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Rehman M et al (2012)., reported chemoprevnetive effect of *Quercus infectoria* against chemically induced renal toxicity and carcinogenesis.[29]

Lodhi G et al (2012)., studied the hepatoprotective effects of *Quercus infectoria* gall extract against carbon tetrachloride treated liver injury in rats.[30]

Nagesh et al (2012)., reported the antibacterial potential of gall extract of *Quercus infectoria* against *Enterococcus faecalis*-an *in vitro* study. Methanol extract of galls of *Quercus infectoria* was found to possess antibacterial property against *Enterococcus faecalis*. [31]

Hermizi Hapidin et al (2012)., had evaluated the potential role of *Quercus infectoria* gall extract on osteoblast function and bone metabolism.[32]

Leela T & Satirapipathkul C (2011)., studied the antibacterial activity of *Quercus infectoria* Galls[33]

Nuntana Aroonrerk & Narisa Kamkaen (2009)., investigated in the anti-inflammatory activity of *Quercus infectoria, Glycyrrhiza uralensis, Kaempferia galanga* and *Coptis chinensis*, the main components of Thai herbal remedies for aphthous ulcer.[34]

Aivazi AA & Vijiyan VA (2009)., studied the larvicidal activity of oak *Quercus infectoria* Oliv. (Fagaceae) gall extracts against *Anopheles stephensi* Liston.[35]

Umachigi SP et al (2008)., studied wound healing properties of *Quercus infectoria*.[36]

Chusri S & Voravuthikunchai SP (2008)., studied the *Quercus infectoria Olivier (nutgalls)* as an alternative treatment for methicillin-resistant *Staphylococcus aureus* infections.[37]

Jae-Kwan Hwang et al (2004)., has been reported as anti-carcinogenic activity of some tropical medicinal plants against s*treptococcus mutans*.[38]

Kaur G et al (2004)., reported the anti-inflammatory evaluation of alcoholic extract of galls of *Quercus infectoria*.[39]

Dar M & Ikram M (1979)., studied the *Quercus infectoria*; Isolation of syringic acid and determination of its central depressive activity.[40]

CHAPTER 3

PLANT PROFILE

3. PLANT PROFILE

Figure 3: Seeds of *Quercus infectoria*

Taxonomical Classification

Kingdom	:	Plantae
Subdivision	:	Angiospermae
Class	:	Eudicots
Sub lass	:	Asterids
Family	:	Fagaceae
Genus	:	*Quercus*
Species	:	*Quercus infectoria*

Vernacular Names

Tamil	:	Masikkai, Machakai
English	:	Oak gall, Magin gall, Dyers' Gall Magic Nut
Telugu	:	Mashikaya
Malayalam	:	Manja-kani
Sanskrit	:	Machika
Hindi	:	Machika

MORPHOLOGY

The plant *Quercus infectoria* (Family-Fagaceae) grows as a shrub or small tree, diclinous and monoecious. It is about 2.5 m in height with many spreading branches. Bark is slightly greyish in colour. Leaves are rigid, glabrescent with spinous teeth measuring 4-6 cm in length; acorns cylindrical. They are alternate, short petiolate, elongate, sinuate, roughly thorny-tipped serrate. Flowers are unisexual. The male flowers are tangled into hanging, axillary catkins, with 6-8 tepaledperigone and 6 to 10 stamens. The female flowers are single or in small groups in the leaf axils of dropping stipules.

The perigone is 6-tipped with an inferior 3chambered ovary surrounded by an initially inconspicuous and then later cup shaped cupula. Fruits are globose, 0.63 cm, lemon in colour and tingled yellow.

This tree grows in Greece, Asia Minor, Syria and Persia. Usually it does not grow in India and are imported but they may be found in Garhwal Himalayas and Nepal .The various *Quercus* species are originated in Iran, Iraq and Turkey, but are now widespread and particularly common in Asia Minor, Europe and North Africa. They are found throughout the Northern Hemisphere, in woods, forest and long hedgerows. There are various trade names of galls which are exported to India and other countries like Aleppo Gall, Mecca Gall, Turkey Gall, Levant Gall, Smyrna Gall, Syrian Gall etc. These all varieties vary in their general appearance, colour and size. Aleppo galls from Asia Minor, is considered to be of best quality and have highest tannin content. The galls obtained from Eastern Europe are commonly known as Knoppers or Acorn Galls and are mainly formed on *Quercus* arbour and other species. They are quite different in appearance from those of Aleppo or Turkish galls. The Basso rah Galls are collected from Tigris or Euphrates rivers in Iraq and shipped frequently to Mumbai are sometimes re-exported as Bombay or Indian Galls. Some galls which are employed in India may be collected from indigenous oaks are found in Kumauni, Garhwal and Bijnor forests.

Chemical Constituents:

The galls from *Quercus infectoria* contain the highest naturally occurring level of tannin, approx. 50–70%, syringic acid, β-sitosterol, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, methyl oleanate and hexagalloyl glucose. They also

contain 2-4% each of gallic and ellagic acid that are polymerized to make tannins. Tannins have been used for hundreds of years for medical purposes and are currently indispensable in dermatology and have been used for tanning of leather.

Medicinal uses

The galls are considered as an astringent, acrid, cooling, haemostatic, constipating, vulnerary, expectorant, digestive, febrifuge, trichogenous and tonic. It is much revered for its strong astringent properties and is used mainly to treat conditions where this property is advantageous.

The powdered gall mixed with alum and tied in a muslin gauze used to be inserted into the vagina to help regression of prolapsed uterus. It is also used in the form of a vaginal douche to treat leucorrhoea, atonic menorrhagia and prolapsed of the uterus. An ointment applied in the vaginal canal was used to treat vaginal laxity.

As a strong astringent the decoction is used to treat diarrhoea and dysentery per oral. It is also used to treat all degrees of haemorrhoids, anal fissures and prolapsed rectum whence the decoction is applied locally as washings or incorporated in sitz's bath. There are also ointments prepared with powders of Oak galls incorporated into it as a local application for these anal diseases.

Oak gall has been incorporated into dental powders. This helps in strengthening gums and teeth. For throat infection like tonsillitis and stomatitis effectively a gargle was prescribe made from decocting oak galls. Decoction of *Q. infectoria* gall is used as an

antidote to various plants poisons especially those due to alkaloids e.g. Opium, nux-vomica, aconite. It is also used in cases of poisoning by emetine or tartar emetic.

As a strong astringent the Oak Gall has many applications in skin problems. It has been used to promote healing of wounds from cuts, skin infections. It also treats impetigo, eczema, hyper hydroid and chapped nipples. Ringworm and alopecia was treated by Unani physicians by making use of oak gall soaked in vinegar.[20-40]

CHAPTER 4

AIM AND PLAN OF WORK

4. AIM AND PLAN OF WORK

Quercus infectoria is a well known Siddha medicine, the roots, leaves and the galls are considered as an astringent, acrid, cooling, haemostatic, constipating, vulnerary, expectorant, digestive, febrifuge, trichogenous and tonic.

It is much revered for its strong astringent properties. As a strong astringent the decoction is used to treat diarrhea and dysentery per oral. It is also used to treat all degrees of hemorrhoids, anal fissures and prolapsed rectum.

Oak gall has been incorporated into dental powders. This helps in strengthening gums and teeth. Looking to the scope of herbal drug and increasing demand especially in case of diseases like liver disorders, hypertension, diabetes, cancer, diarrhoea, arthritis and skin diseases, etc., it was planned to study a plant like *Quercus infectoria*, which is having a variety of traditional uses.

This plant is selected for present study based on its easy availability; degree of research work which is not done. The literature survey revealed that some amount of pharmacological work has been carried out on *Quercus infectoria*.

The present study was aimed to carry out phytochemical analysis and pharmacological screening of seeds of *Quercus infectoria*. So in order to explore the activities and phytoconstituents present in the seeds of *Quercus infectoria*, planned to go for the following studies.

PLAN OF WORK

The work was planned as under mentioned

PHYTOCHEMICAL STUDIES

- Collection and authentication of plant material.
- Extraction of the plant material.
- Preliminary phytochemical screening of the extract.
- Thin layer chromatography of the extract.
- Isolation of plant constituent by column chromatography.
- Characterization of isolated compound by IR, NMR, Mass Spectra.

PHARMACOLOGICAL STUDIES

- *In vitro* anti-cancer activity

CHAPTER 5

MATERIALS AND METHODS

5. MATERIALS AND METHODS

Table 1: Chemicals

S. No.	Chemicals	Supplier/ manufacture
1.	Petroleum ether (60 - 80 ºC)	Loba chemical pvt.ltd., Mumbai
2.	Ethanol (95 %)	Merck-Schuchardt, Mumbai
3.	Silica gel-G (TLC grade)	Loba chemical pvt.ltd., Mumbai
4.	Silica gel G (60 - 120 mesh)	E merck- Germany
5.	Hexane	Loba chemical pvt.ltd., Mumbai
6.	Ethyl acetate	Loba chemical pvt.ltd., Mumbai

Table 2: Instruments and Equipments

S. No.	Equipments/Instruments	Model	Manufacture
1.	Hot air oven	Geninuine	Shivani Scientific industries
2.	Electronic weighing Balance	BT 2245	Surtorius
3.	Melting point apparatus	MRVIS	Lab India
4.	IR Spectrophotometer	Bruker optik GMBH	Germany
5.	NMR- Spectrophotometer	Bruker AVIII Avance FT NMR	Germany
6.	Mass spectrophotometer	JEOL GCmate	Germany
7.	Soxhlet apparatus	Borosil	Chennai

COLLECTION OF PLANT

The seeds of *Quercus infectoria* was collected from Siddha Medical Shop, Madurai, Tamilnadu, India and the specimen was preserved in our Pharmacognosy Lab, JKKN College of Pharmacy, Komarapalayam for further reference.

AUTHENTICATION OF PLANT

The plant was authenticated by Dr. D. Stephen, M.Sc, Ph.D., Professor, Department of Botany, The American College, Madurai-625002, Tamilnadu.

EXTRACTION PROCEDURE

The seeds of *Quercus infectoria* were dried under shade and then made in to a coarse powder with a mechanical grinder. The powder was passed through sieve no. 40 and stored in an airtight container for further use. The dried powder material (200 gm) was extracted with ethanol (95 % v/v) in a soxhlet extractor for 72 hr. The solvent was then distilled off and the resulting semisolid mass was dried in a vacuum evaporator and yield was calculated.

PRELIMINARY PHYTOCHEMICAL ANALYSIS[41-44]

The extract of *Quercus infectoria* was subjected to qualitative tests for the identification of various phytoconstituents.

1) TEST FOR ALKALOIDS

(a) Dragendorff's test: 1 ml of the extract was added with 1 ml of Dragendorff's reagent (potassium bismuth iodide solution). An orange red precipitate indicates the presence of alkaloids.

(b) Mayer's Test: 1 ml of the extract was added with 1 ml of Mayer's reagent (potassium mercuric iodide solution). Whitish yellow colored precipitate indicates the presence of alkaloids.

(c) Hager's Test: 1 ml of the extract was added with 3 ml of Hager's reagent (saturated aqueous solution of picric acid), yellow colored precipitate indicates the presence of alkaloids.

(d) Wagner's Test: 1 ml of the extract was added with 2 ml of Wagner's reagent (Iodide in potassium Iodide), formation of reddish brown precipitate indicates the presence of alkaloids.

(e) Tannic acid Test: 1 ml of the extract was added with 1 ml of 10% tannic acid solution, buff colored indicates the presence of alkaloids.

2) TEST FOR SAPONINS

(a) Foam Test: The extract was diluted with 20 ml of distilled water and shaken in a graduated cylinder for 15 min lengthwise. A 1 cm layer of foam indicates the presence of Saponins.

(b) Lead acetate Test: 1 ml of sample solution was treated with 1% lead acetate solution, formation of white precipitate indicate the presence of saponins.

(c) Hemolytic Test: The extract or dry powder was added one drops of blood placed on glass slide. If hemolytic zone appears shows the presence of saponins.

3) TEST FOR GLYCOSIDES

(a) Legal's Test: Dissolved the extract in pyridine and added sodium nitroprusside solution to make it alkaline. The formation of pink red to red color shows the presence of glycosides.

(b) Baljet Test: 1 ml of the test extract was added with 1 ml of sodium picrate solution and the yellow to orange color shows the presence of glycosides.

(c) Keller- Killiani Test: The ethanol extract 0.5 ml of strong solution of lead acetate was added and filtered. The filtrate is shaken with 5 ml of chloroform. The chloroform layer is separated in a porcelain dish and removes the solvent by gentle evaporation. Dissolve the cool residue in 3 ml of glacial acetic acid containing 2 drops of ferric chloride solution. Carefully transferred this solution to the surface of 2 ml of concentrated sulphuric acid. A reddish brown layer forms at the junction of the two liquids and the upper layer slowly becomes bluish green, darkening with standing.

(D) Borntrager's Test: Added a few ml of dilute sulphuric acid to 1 ml of the extract solution. Boiled filtered and extracted the filtrate with chloroform the chloroform layer was treated with 1 ml of ammonia. The formation of red color of the ammonical layer shows the presence of anthra quinone glycosides.

4) TEST FOR CARBOHYDRATES AND SUGARS

(a) Molisch's Test: 2 ml of the extract was added with 1 ml of α- napthol solution was added and also added concentrated sulphuric acid through the side of the test tube.

Reddish violet color at the junction of the two liquids indicates the presence of carbohydrates.

(b) Fehling's Test: 1 ml of the extract was added with equal quantities of Fehling solution A and B were added, upon heating formation of a brick red precipitate indicates the presence of reducing sugars.

(c) Benedict's test: 1 ml of extract was added with 5 ml of benedicts' reagent, was added and boiled for 2 min. and cool. Formation of red precipitate shows presence of sugars.

(d) Tollen's Test: 1 ml of extract was added with 2 ml of tollen's reagent was added and boiled. A silver mirror is obtained inside the wall of the tube which indicates the presence of aldose sugar.

(e) Seliwanoff's Test: The extract was treated with hydrochloric acid and resorcinol and heated. Formation of red color shows presence of glucose.

(f) Bromine water Test: The little quantity of test extract, bromine water was added. Bromine water decolourization indicates the presence of aldose sugar.

5) TEST FOR TANNINS

(a) Gelatin Test: 1 ml of extract was added with 1% gelatin solution containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannins.

(b) Ferric chloride Test: 1 ml of extract was added with 1ml ferric chloride solution, formation of dark blue or greenish black product shows the presence of tannins.

(c) Vanillin hydrochloride Test: 1 ml of extract was added with vanillin hydrochloride. Formation of purplish red color indicates the presence of tannins.

(d) Lead acetate Test: Taken a little quantity of test solution was taken and mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of tannins.

(e) A little quantity of test extract was treated with potassium ferric cyanide and ammonia solution. A deep red color indicates the presence of tannins.

(f) P**otassium dichromate Test**: The sample solution was treated with 1ml of 10% Potassium dichromate solution gives yellowish brown precipitate indicates the presence of tannins.

6) TEST FOR FLAVONOIDS

(a) Shinoda's Test: The extract solutions, few fragments of magnesium ribbon was added and add concentrated HCL drop wise gives cherry red color appears after few minutes, shows the presence of flavanoids.

(b) Alkaline reagent Test: The extract was treated with sodium hydroxide; formation of yellow color indicates the presence of flavanoids.

(c) Little quantity of extract was treated with lead acetate, a yellow color solution formed, disappears on addition of an acid indicates the presence of flavanoids.

(d) The extract was treated with concentrated sulphuric acid, formation of yellow or orange color indicates the presence of flavanoids.

7) TEST FOR STEROIDS

(a) Libermann- Bur chard's Test: 2 ml of extract was added with chloroform solution, 1-2 ml of acetic anhydride and 2 drops of concentrated sulphuric acid was added along the sides of the test tube. Appearance of bluish-green color shows the presence of steroids.

(b) Salkowsky's Test: Dissolve the extract in chloroform solution, 2 ml conc. sulphuric acid was added. If chloroform layer appear red color indicate the presence of steroids.

8) TEST FOR PROTEINS AND AMINO ACIDS

(a) Biuret Test: 1 ml of the extract was treated with 4% NaOH and few drops of $CuSO_4$ solution, Formation of purple violet color indicate the presence of proteins.

(b) Ninhydrin Test: 1 ml of the extract was treated with 3 drops of 5% Ninhydrin solution in boiling water bath for 10 min; formation of purplish or bluish color appearance indicates the presence of proteins, peptides or amino acid.

(c) Xanthoproteic Test: 1 ml of the extract was treated with 1 ml of concentrated nitric acid. A white precipitate formed, it was boiled and cooled. Then 20% of sodium hydroxide or ammonia is added. Orange color indicates the presence of amino acids.

(d) Millon's Test: 1 ml of the extract was treated with millon's reagent (mercuric nitrate in HNO_3) white precipitate turns to brick red indicate the presence of proteins.

9) TEST FOR TRITERPENOIDS

(a) Knoller's Test: Dissolved 2 or 3 granules of tin metal in 2 ml thionyl chloride solution. Then added 1 ml of the extract into the test tube and warm, the formation of pink color indicates the presence of Triterpenoids.

10) TEST FOR FIXED OILS AND FATS

(a) Spot Test: Pressed a small quantity of extract between two filter papers, the stain on the filter paper indicates the presence of fixed oils.

(b) **Saponification Test:** Added a few drops of 0.5 N of alcoholic potassium hydroxide to small quantity of various extract along with a drop of phenolphthalein separately and heat on water bath for 1 to 2 hrs. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

11) TEST FOR GUMS AND MUCILAGE

10 ml of extract was slowly added 25ml of absolute alcohol with constant stirring, filtered the precipitate and dried in air. The precipitate for its swelling property indicates the presence of carbohydrates.

THIN LAYER CHROMATOGRAPHY[45, 46]

Thin layer chromatography as a procedure for analytical adsorption chromatography was first introduced by Stahl (1958) who was mainly responsible for bringing out standard equipment for preparing thin layers. It is not present an important

analytical tool for qualitative and quantitative analysis of a number of natural products, for separation and estimation of different components.

The principle of separation is adsorption. One or more compounds are spotted on a thin layer of adsorbent coated on TLC plate. The mobile phase flows through because of capillary action (against gravitational force). The component moves according to their affinity towards the stationary phase. The component with lesser affinity towards the stationary phase travels faster and vice versa thus leading to the separation of components. The information provided by a finished chromatography includes the "migrating behavior" of the separated substances. It is given in the form of R_f value (relative to front).

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

R_f value usually lies in the range of 0.1 – 1.

PROCEDURE

1. Preparation of plate

The silica gel G (60-120 mesh) (Fischer & Co) was utilized for the preparation of TLC plates. Silica gel G was mixed with sufficient quantity of water and triturated will to make slurry. The prepared slurry was spread on the meticulously cleaned and scratch free glass plates of definite dimension by using a TLC spreader. The thickness of the absorbent was adjusted to 2 mm throughout the plate. Then the prepared plates were

allowed to set for 15-30 minutes. The activated plates were stored in a vacuum desiccator for future use. Also prior to use the plates were once again activated.

2. Development of Chromatogram

The saturation of atmosphere of TLC chamber by mobile phase before the start of the experiment is almost important to avoid the flawed results due to tailing effect. The sample was spotted on the plate 2 cm away from the bottom. The plate was then developed in the chamber by allowing the plate to run up to ¾ th distance of the plate. The plate was then removed dried up to room temperature and sprayed with suitable spray reagent or kept in iodine chamber for identification of spots of TLC pattern of 95% v/v of ethanol extract of *Quercus infectoria* (EEQI).

COLUMN CHROMOTAGRAPHY [47-49]

The column chromatographic technique is widely used for separation, isolation, and purification of the natural products. The principle underlying the separation of the compounds is adsorption at the solid liquid interface. For the solid support and the interaction between adsorbent and component must be reversible. As the adsorbent is washed with fresh solvent the various components will therefore move down the column until, ultimately, they are arranged in order of their affinity for the adsorbent. Those with least affinity move down the column at a faster rate than, and are eluted from the end of the column before, those with greatest affinity for the adsorbent. By changing the polarity of the mobile phase, the separation can be achieved by column chromatography.

Characterization of the isolated compounds can be carried out by analytical techniques, like IR, NMR and Mass spectroscopy.

Details of Column Chromatography

On the basis of phytochemical screening and TLC study, the EEQI and the solvent system were selected for column chromatography by isocratic elution method.

Details of column chromatography

Adsorbent	- Silica gel for column chromatography (60-120# mesh)
Solvent system	- Toluene: Ethyl acetate (9 : 1)
Length of column	- 60 cm
Diameter of column	- 2 cm
EELA used	- 5 gm
Rate of elution	- 20 drops per minute
Fraction collected	- 40 fractions each of 25 ml
Method of column packing	- Wet packing
Technique	- Isocratic elution

Procedure for preparation of column:

1. The silica gel 60-120 mesh was made into slurry with selected solvent system (Toluene: Ethyl acetate (9 : 1), The silica gel previously activated by heating in hot air oven at 100 ºC for 1hr.

2. The bottom of the column was plugged by cotton and then the silica gel slurry was poured into the column which was filled with solvent system up to 40 cm height, after that it was set aside for 10 minutes and allowed to settle.

3. EEQI was mixed with small amount of silica gel and wetted with solvent system and mixed well and allowed to evaporate the solvent to set the dry residue.

4. Then the dry residue was charged on column with the help of solvent system, after that cotton was placed over it, in order to avoid the disturbance of the top layer of the adsorbent as fresh mobile phase was added to the column.

5. The column was eluted with the selected solvent system by Isocratic method and the fractions were collected in clean 100 ml beaker up to 25 ml with the speed of drops was 20 drops/minute.

Each collected fraction was tested for the presence of various constituents by TLC for the number of types of constituent and similar fractions were pooled together.

SPECTRAL CHARACTERIZATION

FT-IR Spectroscopy

Isolated compound was analysed by IR spectral studies by using KBr pellet technique. In this method, the drug and KBr were mixed at the ratio of 1:100. Then these mixtures were pressed in to a pellet. The FT-IR spectra were recorded for isolated compounds, using KBr pellet method in the region of 4000-400 cm^{-1}.

NMR Spectroscopy

NMR is used to elucidate the structure of an unknown compound, there are three pieces of information which should be considered, the position of resonance of the peak (or chemical shift), the number of hydrogen atoms causing the signal (integration) and the number of peaks constituting the signal (multiplicity). NMR data to solve the structure but it is equally acceptable to use MS or IR data to solve the unknown.

MASS Spectroscopy

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios.[50, 51]

PHARMACOLOGICAL STUDIES

***IN VITRO* ANTICANCER ACTIVITY**

The human breast cancer cell line (MCF-7) and cervical cancer cell lines (HeLa) were obtained from National Centre for Cell Science (NCCS), Pune. The (MCF-7) cells were grown in Dulbecco's modified eagles medium (DMEM) and HeLa cells were grown in Eagles minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37 ^{o}C, 5% CO_2, 95% air and 100% relative humidity.

Maintained cultures were passaged weekly and the culture medium was changed twice a week.

CELL TREATMENT PROCEDURE

The monolayer cells were detached with trypsin – ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a haemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 ^oC, 5% CO_2, 95% air and 100% relative humidity.

After 24 hr the cells were treated with serial concentrations of the 95% v/v of ethanol extract of *Quercus infectoria* (EEQI). They were initially dissolved in dimethylsulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred microlitres per well of each concentration was added to plates to obtain final concentrations of 10, 20, 50, 100, 200 µg/ml. The final volume in each well was 200 µl and the plates were incubated at 37^oC, 5% CO_2, 95% air and 100% relative humidity for 48 hr. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

MTT ASSAY

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an

insoluble purple formazan. Therefore,the amount of formazan produced is directly proportional to the number of viable cells.

After 48 hr of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{o}C for 4 hr. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) X 100.

Nonlinear regression graph was plotted between percent cell inhibition was determined using Microsoft Excel software.[52, 53]

CHAPTER 6

RESULTS AND DISCUSSION

6. RESULTS AND DISCUSSION

The seeds of *Quercus infectoria* belonging to family Fagaceae has been examined in a organized way covering phytochemical analysis and pharmacological screening. Litrerature survey revealed that not much work is done on this plant. Therefore, it was thought worthwhile to carry out the phytochemical analysis and pharmacological screening on this plant.

PHYTOCHEMICAL STUDIES

Extraction of *Quercus infectoria* seeds

Dried crushed seeds of *Quercus infectoria* were extracted with ethanol (95% v/v) continuously with soxhlet apparatus and the result was tabulated in Table 3.

Table 3. Data showing the extractive values of seeds of *Quercus infectoria*

S.No	Extract	Colour/ Physical nature	Percentage yield (% w/w)
1	Ethanol (95% v/v)	Brownish yellow/ Solid	5.09

Preliminary Phytochemical Screening of *Quercus infectoria* seeds

95% v/v of ethanol extract of *Quercus infectoria* (EEQI) was subjected to qualitative phyto chemical screening to identify the phyto constituents present and the results were expressed in Table 4.

Table 4. Preliminary Phytochemical Screening of EEQI

S. No.	Chemical Test	Ethanol (95% v/v) extract
1.	**Alkaloids**	
a.	Mayer's Test	-
b.	Dragendroff's Test	-
c.	Wagner's Test	-
d.	Hager's Test	-
2.	**Carbohydrates**	
a.	Molisch's Test	+
b.	Fehlings Test	+
c.	Barfoed's Test	+
d.	Benedict's Test	+
3.	**Glycosides**	
a.	Legal's Test	-
b.	Keller-Killiani Test	-
c.	Borntrager's Test	-
4.	**Fixed Oil & Fat**	
a.	Stain Test	-
b.	Saponification Test	-
5.	**Tanins & Phenolics**	
a.	Ferric Chloride Test	+
b.	Lead Acetate Test	+
c.	Gelatin Solution Test	+
6.	**Steroids**	
a.	Salkowsky's Test	+
b.	Libermann Burchard's Test	+
7.	**Saponins**	
a.	Foam Test	+
8.	**Proteins**	
a.	Million's Test	-
b.	Ninhydrin Test	-
9.	**Flavonoids**	
a.	Aqueous NaOH Test	+
b.	Conc.Sulphuric Acid Test	+
c.	Shinoda Test	+
10.	**Gum & Mucilage**	-
11.	**Triterpenoids**	
a.	Knoller's Test	+

+ Present - Absent

Thin Layer Chromatography (TLC)

TLC was performed with various solvent systems based on the polarity by trial and error method. EEQI was subjected to thin layer chromatography on silica gel G which had shown good resolution of solvent system Toluene: Ethyl acetate (8: 2). Two spots were identified by means of corresponding detecting agent and R_f values were calculated and showed in Table 5 and Figure 4.

Figure 4. Thin Layer Chromatography of EEQI

Table 5. Thin Layer Chromatography of EEQI

Solvent system	No. of spots	Spray reagent	R_f Values
Ethyl acetate : Chloroform (6:4)	1	Iodine vapour	0.56
Chloroform: Methanol (6:4)	1	Iodine vapour	0.59
Hexane: Ethyl acetate (8: 2)	1	Iodine vapour	0.60
Toluene: Ethyl acetate (8: 2)	2	Iodine vapour	0.62 0.70

Column Chromatography

On the basis of phytochemical screening and TLC study, isolation of active constituents of EEQI was done by column chromatography through isocratic elution technique with the help of solvent system Toluene: Ethyl acetate (8: 2) was shown in Table 6.

Table 6. Column Chromatography of EEQI

Fraction No.	Nature of Residue	Analysis by TLC	Colour of the spot	R_f Value
1-4	No residue	---	---	---
5-8	No residue	---	---	---
9-12	Brown	---	---	---
13-16	Brown	----	---	---
17-20	Light Brown	2 spots with tailing effect	Yellow Brown	0.69 0.72
21-24	Brownish yellow	----	---	---
25-28	Light yellow	2 spots with tailing effect	Yellow Brown	0.69 0.72
29-32	No residue	---	---	---
33-36	Brown	1	Pale brown	0.62
37-40	No residue	---	---	---

The isolated compound from the Fraction 33-36 obtained by column chromatography was named as **EEQI-1**.

Analysis of the isolated compound

Thin Layer Chromatography of the Isolated Compound EEQI-1

The isolated compound EEQI-1 obtained from column chromatography was analyzed by chemical tests, thin layer chromatography using the solvent system Toluene: Ethyl acetate (8: 2) and detected by iodine vapours. EEQI-1 showed the presence of one yellow colour spot with R_f value of 0.62 was shown in Figure 5.

Characterization of EEQI-1

Table 7. Chemical tests for EEQI-1

Chemical Tests	EEQI-1
Ferric Chloride Test	Positive
Lead Acetate Test	Positive

Table 8. Physical Characters of EEQI-1

Parameters	EEQI-1
Yield	42 mg
Physical state and colour	Pale brown amorphous powder
Solubility	Water, Acetone and DMSO
Melting point	258ºC
TLC solvent system	Toluene: Ethyl acetate (8: 2)
R_f value	0.62

Figure 5. Thin Layer Chromatography of EEQI-1

The isolated compound from the seeds of *Quercus infectoria* was tested and it showed the positive test for phenolic compounds. The physical characters of the compound were tabulated above.

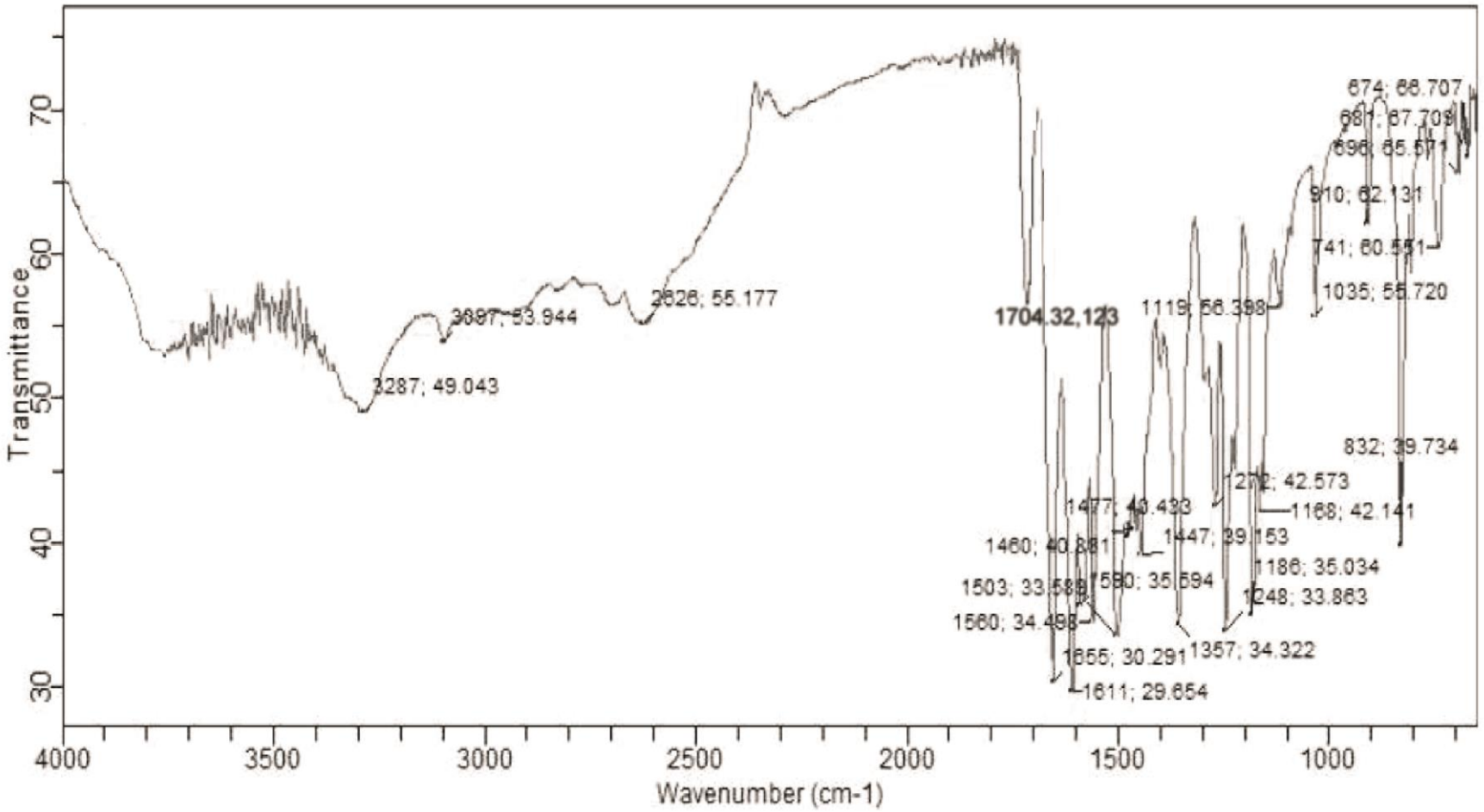


Figure 6. IR Spectrum of isolated compound EEQI-1

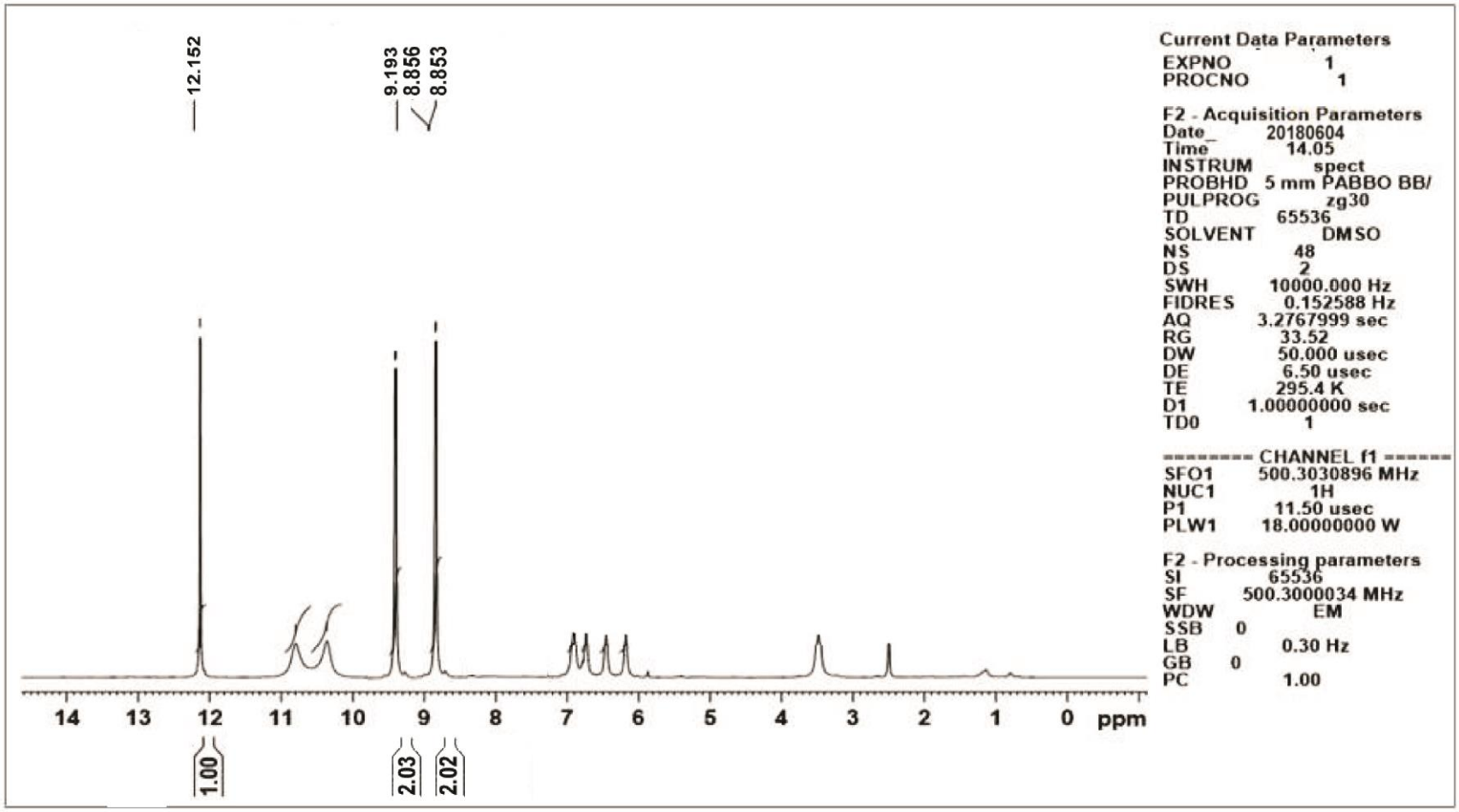


Figure 7. ^{1}H NMR Spectrum of isolated compound EEQI-1

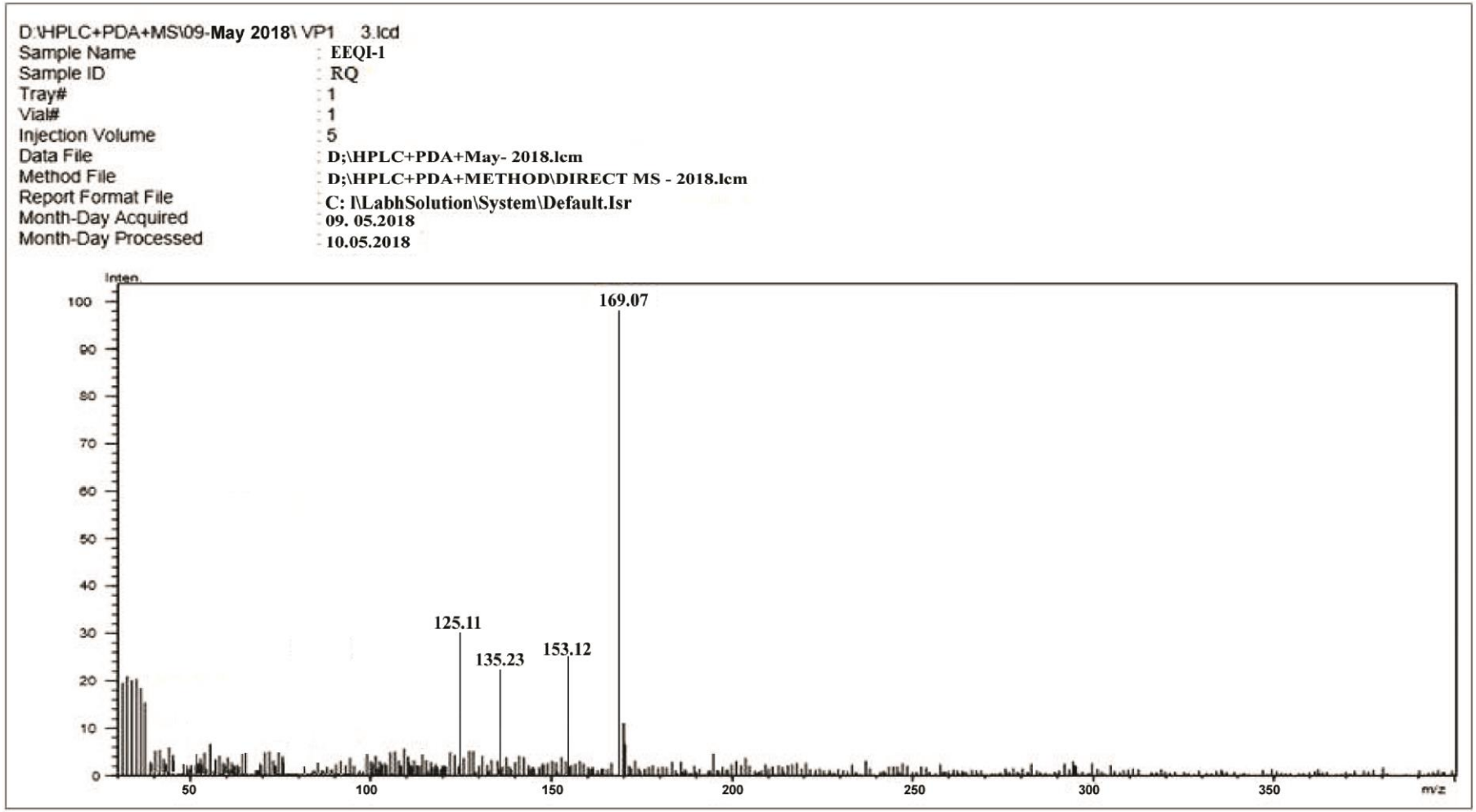
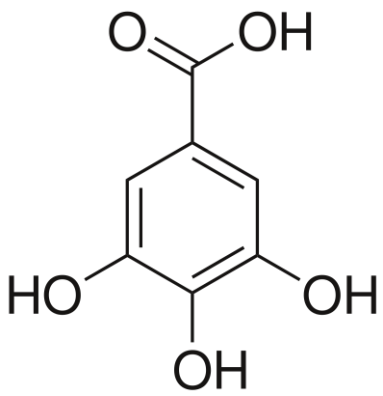


Figure 8. Mass Spectrum of isolated compound EEQI-1

The qualitative analysis of the ethanol extract of seeds of *Quercus infectoria* was summarized in Table 4. Preliminary phytochemical screening revealed that EEQI showed the presence of carbohydrates, steroids, tannins & phenolic compounds, flavonoids, triterpenoids. The TLC study was carried out and the results were summarized in Table 5. From the TLC study of the EEQI, the presence of two spots were observed as maximum number of spots with Toluene: Ethyl acetate (8: 2) using iodine vapours as detecting agent. The R_f values of the spots were calculated and found to be 0.62, 0.70 respectively. The results of column chromatography of the ethanol extract of seeds of *Quercus infectoria* was summarized in Table 6. Phytochemical analysis of the isolated compound EEQI-1 has shown positive result for phenolic compounds.

Compound EEQI-1 was obtained as pale brown amorphous powder and its molecular formula was established as $C_7H_6O_5$ from its mass spectral data that showed [M-H]- ion at m/z 169.07, 153.12, 135.23 & 125.11. The molecular formula EEQI-1 was further supported by its IR, ^{1}H NMR spectral data's. The IR spectra exhibited characteristic bands at the strong and broad band between 3600 and 2500 cm^{-1}, the strong and narrow peak at 1704 cm^{-1}could be assigned to the stretching vibration of OH group and carbonyl group, several peaks in 1310-1026 cm^{-1} region that could be assigned to the stretching vibration of C-O group and bending vibration of OH bond. The ^{1}H NMR spectrum of EEQI-1 also showed the presence of characteristic hydroxyl protons at δ 8.85, δ 9.19 ppm and the acid group at δ 12.15 ppm. The hydroxyl and carboxylic protons have broad signals in the upper spectra characteristic for 3, 4, 5-trihydroxybenzoic acid. Thus, according to the data and by comparison with the literature, the compound was identified as **Gallic acid** (Figure 9).

Figure 9: Structure of Gallic acid



PHARMACOLOGICAL SCREENING

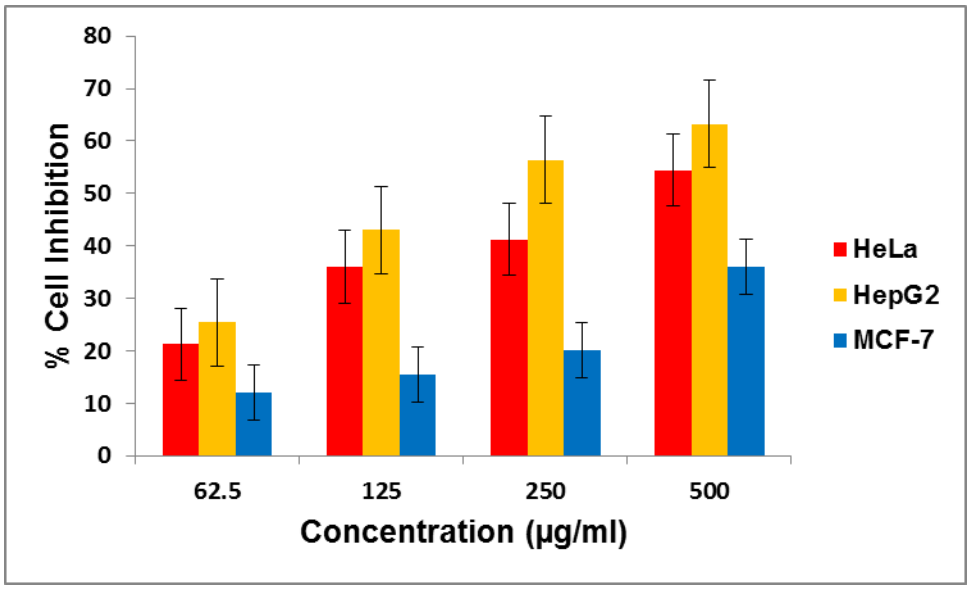
***In vitro* Anticancer Activity**

The anticancer activity of ethanol extract of *Quercus infectoria* was studied on the various cell line such as cervical cancer cell lines (HeLa), human liver cancer cell line) (Hep G2) and human breast cancer cell lines (MCF-7) using MTT assay method. The EEQI at doses of 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml produced a significant anticancer activity against HeLa, HepG2 and MCF-7 cancer cell lines. On comparison between the percentage cell inhibition of HeLa, Hep G2 and MCF-7, the HepG2 showed higher inhibition and significant activity than the HeLa & MCF-7 which was mentioned in table 9 & figure 10.

Table 9. % Cell Inhibition of EEQI in HeLa, Hep G2 and MCF-7 Cell Lines

Concentration (µg/ml)	% Cell Inhibition		
	HeLa	HepG2	MCF-7
62.5	21.25	25.48	12.01
125	36.02	43.10	15.55
250	41.18	56.41	20.18
500	54.41	63.18	36.01

Figure 10. % Cell Inhibition of EEQI in HeLa, Hep G2 and MCF-7 Cell Lines



Cancer is a disease caused by the abnormal proliferation and differentiation of cells and is governed by tumorigenic factors. Cancer is the second most common cause of human death worldwide. Currently, chemotherapy is still one of the best therapeutic methods to treat cancer. With wider application and further understanding, the side effects and acquired drug resistance of synthesized small molecule compounds have caused more and more concerns.[54,55] Therefore, natural

and edible small molecules such as flavones, phenolic compounds which are thought to have remarkable physiological effects, low toxicity and non-mutagenic properties in the human body, have gained more and more interest in anti-cancer agent development.

In the present study, HeLa, Hep G2 and MCF-7 cancer cell lines were used to find the cytotoxicity of the ethanol extract of *Quercus infectoria* due to their well defined characteristics in experimental conditions and their relevance as an *in vitro* system for screening purposes involving natural products and anti-mutagenic effects on this cells.[56,57] The reduction of tetrazolium salts (MTT) is now recognized as a safe, accurate alternative to radiometric testing. Among the applications for the method are drug sensitivity, cytotoxicity, response to growth factors and activation.[58, 59] HepG2 produced more significant *in vitro* anti-cancer activity than other two cell lines. Our findings shown that the antioxidant potentials like phenolic compounds, flavonoids, saponins present in extract might be responsible for the anticancer activities. Spectral datas of EEQI-1 (Gallic acid) also support anti-cancer activity of seeds *of Quercus infectoria.*

CHAPTER 7

SUMMARY AND CONCLUSION

7. SUMMARY AND CONCLUSION

Traditional Indian medicinal herbs have been used in the treatment of different diseases in the country for centuries. There have been claims that some traditional healers can successfully treat cancer using herbal drugs. The seeds of *Quercus infectoria* were selected and authenticated for the present study on the basis of ethanobotanical information.

Literature survey revealed that not much work has done in this plant. So I felt it worthwhile to validate scientifically, the folkclaim for its therapeutic activity. The detailed preliminary phytochemical investigations proved its appropriate identification and rationalized its use as a drug of therapeutic importance. This plant have many phytoconstituents like flavonoids, phenolic compounds, saponins, tannins and so on. The phytoconstituents are found to possess pharmacological activities like antioxidant, antiulcer, antiinflammatory, antimicrobial, wound healing activities. So it was planned to isolate active constituent from ethanol extract of seeds of *Quercus infectoria*. The present study concluded that the phytoconstituent was isolated from the seeds of ethanol extract of *Quercus infectoria* and characterized systematically with IR, H^1 NMR and mass spectroscopy. The spectral datas of the isolated compound suggested that EEQI I showed the structural similarities with **Gallic acid**.

Cancer is a complex multifactorial cell disease characterized by abnormal cellular proliferation. Cancer development is normally caused by oncogene, tumor suppressor gene, and microRNA gene alterations. It imposes a serious burden on the public health system, and its treatment and cure are scientifically challenging. Cancer is expected to claim nine million lives worldwide by 2015. Approximately 60% of anticancer agents are derived from medicinal plants and other natural resources; however, there are still a number of plants that have an anticancer potential but they have not yet been fully investigated. The MTT assay is used in screening the crude extracts as well as in the isolated compounds to assess the toxicity. It could also provide an indication of possible cytotoxic properties of the tested plant extracts. MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. It is frequently used as an *in vitro* model system to measure cytotoxic effects of variety of toxic substances and plant extracts against cancer cell lines. Ethanol extract of seeds of *Quercus infectoria* was screened for *in vitro*

anticancer activitities in three different cell lines such as HeLa, Hep G2 and MCF-7 by MTT assay method. HepG2 produced more significant in *vitro* anticancer activity than other two cell lines. The present study concluded that the antioxidant potentials like phenolic compounds, tannins, flavonoids present in extract might be responsible for the anticancer activities.

In conclusion, it was observed that the plant seeds of *Quercus infectoria* contains a wide variety of secondary metabolites that hold strong antioxidant capacity based on the experiments performed which add scientific evidence to conduct further studies, investigate the lead compounds present in the plant, to evaluate its anticancer potential on *in vivo* animal models and put forward an attempt to carry out trials on human beings.

CHAPTER 8

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8. BIBLIOGRAPHY

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