Studies on *in vitro* antioxidant and *in vitro* anticancer activities of ethanol extract of *Pterocarpus marsupium* ROXB. bark

A Dissertation submitted to

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

CHENNAI- 600 032

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

Branch-IV -- PHARMACOLOGY

Submitted by

Name: SHEMEER.S

REG.No.261425230

Under the Guidance of

Dr. R. SHANMUGA SUNDARAM, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACOLOGY

J.K.K. NATTARAJA COLLEGE OF PHARMACY

KUMARAPALAYAM – 638183

TAMILNADU.

OCTOBER – 2016

Certificates

EVALUATION CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"Studies on *in vitro* antioxidant and *in vitro* anticancer activities of ethanol extract of *Pterocarpus marsupium* ROXB. bark",** submitted to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment to the requirement for the award of Degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide work carried out by **Mr.SHEMEER.S [Reg.No.261425230]**, during the academic year 2015-2016, under my guidance and direct supervision in the Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

Internal Examiner **External Examiner**

CERTIFICATE

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Dr. R. Sambath Kumar, M.Pharm., Ph.D.,
Principal & Professor
Department of Pharmaceutics,
J.K.K. Nattraja College of Pharmacy.

Dr. R. Shanmuga Sundaram, M.Pharm., Ph.D.,
Professor and Head,
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy.

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Place: Komarapalayam
Date:

Dr. R. Shanmuga Sundaram, M.Pharm., Ph.D.,
Vice Principal and Professor,
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy.

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Place: Komarapalayam
Date:

Dr. R. Sambath Kumar, M.Pharm., Ph.D.,
Principal & Professor
Department of Pharmaceutics,
J.K.K. Nattraja College of Pharmacy.

DECLARATION

I hereby declare that the dissertation entitled **"Studies on *in vitro* antioxidant and *in vitro* anticancer activities of ethanol extract of *Pterocarpus marsupium* ROXB. bark",** has been carried out under the guidance and supervision of **Dr. R.SHANMUGA SUNDARAM, M.Pharm., Ph.D.,** Vice Principal, Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam, in partial fulfillment of the requirements for the award of degree of **Master of Pharmacy** in **Pharmacology** during the academic year 2015-2016.

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.

Place: Komarapalayam **Mr.SHEMEER.S,**

Date: **Reg.No.261425230**

Dedicated to
Almighty,
My beloved family,
Teachers and
Friends.

Acknowledgement

ACKNOWLEDGEMENT

I expressmy wholehearted thanks to my guide **Dr. R. Shanmuga Sundaram,** Professor and Vice Principal, for suggesting solution to problems faced by me and providing in dispensable guidance, tremendous encouragement at each and every step of this dissertation work. Without his critical advice and deep-rooted knowledge, this work would not have been a reality.

It is my most pleasant duty to thank our beloved Principal and Professor **Dr. R. SambathKumar,** of J.K.K.Nattraja College of Pharmacy, Komarapalayam for ensuring all the facilities were made available to me for the smooth running of this project.

It is my privilege to express deepest sense of gratitude toward **Mr. S. Venkatesh,** lecturer, department of Pharmacology for their valuable suggestions during my project work.

It is my privilege to express deepest sense of gratitude toward **Dr. M. Senthil Raja,** Prof and head, department of Pharmacognosy, **Dr. M. Vijayabaskaran**, Prof and head, department of Pharmaceutical Chemistry, **Dr. N. Venkateswaramurthy,** head, department of Pharmacy Practice, **Dr. V. Sekar,** Prof and head, department of Analysis, **Mrs. S. Bhama,** Asst Prof, **Mr. T. Thiyagarajan,** Asst. Prof, **Miss. M. Sudha**, Asst. Prof, and **Dr. Prakash,** Associate Professor, all other teachers in all the departments of this institution.

I greatly acknowledge the help rendered by **Mrs. K. Rani,** office superintendent, **Mrs. S. Venkateswari, Mrs. V. Gandhimathi,** librarian, and **Mrs. S. Jayakala,** Asst. librarian for their co-operation.

My special thanks to all the **Technical and Non-Technical Staff Members** of the institute for their precious assistance and help.

I am proud to dedicate my deep sense of gratitude to the founder, (Late) Thiru **J.K.K. Nattraja Chettiar,** for providing us the historical institution to study.

My sincere thanks and respectful regards to our reverent Chairperson **Smt. N. Sendamaraai,** Managing Director **Mr. S. OmmSharravana**, J.K.K. Nattraja Educational Institutions, Komarapalayam,for their blessings, encouragement and support at all times.

Last, but nevertheless, I am thankful to my beloved parents and lovable friends for their co-operation, encouragement and help extended to me throughout my project work.

Mr.SHEMEER.S,
Reg.No.261425230

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ANIMAL ETHICAL COMMITTEE CLEARANCE CERTIFICATE

J.K.K.Nattraja College of Pharmacy, Kumarapalayam, Tamilnadu,

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

Institutional Animal Ethics Committee (IAEC)

REG: NO: 887/PO/Re/S/2005/CPSCEA

CERTIFICATE

Title of the Project	:	Studies on *in vitro* antioxidant and *in vitro* anticancer activities of ethanol extract of *Pterocarpus marsupium* ROXB. Bark.
Department	:	Pharmacology.
Proposal Number	:	JKKNCP/MP/OCT/02/2015-16
Approval date	:	18.01.2016
Animals	:	Swiss Albino Mice(Female)
No of Animals Sancioned	:	15

Dr. R. SAMBATH KUMAR
Chairman IAEC

1. INTRODUCTION

People have been using plants as a source of medicine since the beginning of human Civilization. Perhaps as early as Neanderthal man, plants were believed to have healing power (Anna, 1993). The traditional society across the world have always used herbs to promote healing (Okoli., et al 2007).In today's world herbal medicine is the most predominant means of healthcare in developing countries where about 80% of their total population depends on it for their wellbeing (Akabue., 1982). Plants form the basis of for the development of modern drugs and medicinal plants have been used for many years to treat disease all throughout the world in the daily course of life (W.H.O., 1991, Patil., 2011 and Hamid., 2011). Since a long, time plants have represented the only source of therapeutical agents known to man. Plants have become the primary source of substances for drug development (Zaroni et al., 2004).

This knowledge of plant based drugs developed gradually and was passed on this laying the foundation for many systems of traditional medicine all over the world. Folk medicine does strongly assent its faith in the therapeutic effectiveness of plant preparations (Kumari et al., 2011). Herbs are mines of useful drugs, since ancient past and presently they are becoming popular. Moreover the medicinal plant wealth is our national heritage and it seems to be the first and foremost line of defence for the treatment of various diseases mostly in the tribal and rural communities (Kaul and Dwivedi., 2010).According to the WHO, over 80% of the world's population relies on traditional forms of medicine, largely plant based to meet primary health care needs. The medicinal plants are extensively utilized throughout the world in two distinct areas of health management; traditional system of medicine and modern system of medicine. The medicinal attributes of many plants are found in leaves, used as alterative, tonic diuretic, blood purifier and antiphlogistic. They are used as remedy against chronic eczema, chronic ulcer, chronic rheumatism, chronic nervous diseases, madness, cholera, amenorrhea, piles and fistula. In terms of the number of species individually targeted, the use of the plants as medicines represents by far the biggest human use of the natural world. Plants provide the predominant ingredients of medicines in most medical traditions (Mohd et al., 2009). Recent estimates suggests that over 9000 plants have known

medicinal applications in various cultures and countries, and this is without having conducted comprehensive research amongst several indigenous and other communities (Farnsworth .,1991). Medicinal plants are used at the household level by women taking care of their families at the village level by medicine men or tribal shamns , and by the practitioners of classical traditional systems of medicine such as Ayurveda, Chinese medicine , or the Japanese kampo system.

According to the World Health Organisation, over 80% of the world's population or 4.3 billion people rely upon such traditional plant based systems of medicine to provide them with primary health care (Attiso., 1983). Medicinal plants are used to treat illness and diseases for thousands of years. They have gained economic importance because of their application in pharmaceutical, cosmetic, perfumery and food industries. The interest in herbal systems of medicine is growing day-by-day because nature can cure many diseases. Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security.

Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or other was used for medicinal purposes. It is estimated that world market for plant derived drugs may account for about Rs.2, 00,000 crores. Presently, Indian contribution is less than Rs.2000 crores. Indian export of raw drugs has steadily grown at 26% to Rs.165 crores in 1994-'95 from Rs.130 crores in 1991-'92. The annual production of medicinal and aromatic plant's raw material is worth about Rs.200 crores. This is likely to touch US $5 trillion by the year 2050.

It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Thus, the economic importance of medicinal plants is much more to countries such as India than to rest of the world. These countries provide two third of the plants used in modern system of

medicine and the health care system of rural population depend on indigenous systems of medicine.

Green plants synthesise and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as raw material for various scientific investigations. Many secondary metabolites of plant are commercially important and find use in a number of pharmaceutical compounds. However, a sustained supply of the source material often becomes difficult due to the factors like environmental changes, cultural practices, diverse geographical distribution, labour cost, and selection of the superior plant stock and over exploitation by pharmaceutical industry. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants (Joy et al.,).

Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others. In some cases, the crude extract of medicinal plants may be used as medicaments. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, works in both mixture of traditional medicine and single active compounds are very important. Where the active molecule cannot be synthesised economically, the product must be obtained from the cultivation of plant material (Mukherjee & Mukhhetjee, 2005).

Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value. Interest in herbal medicines has continued to grow. This is shown in several ways, for example, by increased retail sales of herbal medicinal products in Europe and the USA as well as the greater awareness among the public and healthcare professionals about natural health products and complementary therapies. Industrially produced new herbal products, mainly based on single-herb extracts standardized for its specific active ingredient, continue to be developed.

In India knowledge of medicinal plant is very old, and medicinal properties of plants are described in 3500-1500 B.C, from which Ayurveda developed. In Ayurveda the ancient well known treatise are Charak samhita dealing mostly with plants in sashrat samhita in which surgery is also maintained. In Egypt the people were familiar with medicinal properties of plants and animals. Greek scientists contributed much to the knowledge of natural history. Hippocraties (460-370 B.C) is referred to as father of medicine and is remembered for his famous writings on kingdom, which is considered authoritative even in the 20^{th} century. The substances from the plants were isolated, the structure was elucidated and the pharmacologically active constituents were studied. In 1934-1960 simultaneous applications of disciplines developed like organic chemistry, biochemistry, biosynthesis, pharmacology and modern methods and techniques of medicinal chemistry including paper, thin layer chromatography, gas chromatography and spectrophotometry.

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 these substances have been demonstrated to possess interesting biological activities. India is the treasure house of herbs and more than 9000 different herbs with varying medicinal properties are present. Ayurveda an ancient system of Indian medicine has recommended a number of drugs from indigenous plant and animal sources for the treatment of several diseases and disorders. More than 13,000 plants have been studied during the last 5 years (Trease and Evans; Shah & Qadry 1996; Varrote Tyler-iynn et al., 1996).

1.1. Herbal Medicines

Herbal Medicine sometimes referred to as Herbalism or Botanical Medicine, is the use of herbs for their therapeutic or medicinal value. An herb is a plant or plant part valued for its medicinal, aromatic or savory qualities. Herb plants contain a variety of chemical substances that has therapeutic value.

Herbal Medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. The plants provided food, clothing, shelter and medicine. Much of the medicinal use of plants seems to have been developed through

observations of wild animals and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area to its knowledge base. They methodically collected information on herbs and developed well–defined herbal pharmacopoeias. Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs commonly used today are of herbal origin. Indeed, about 25 % of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound.

Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value (WHO, 1993).

1.2. Synthetic Drug: Dominance

Pharmaceutical research took a major leap when alongside natural plants chemistry, pharmacologists, microbiologists and biochemists began to unravel the chemistry of natural products in human beings, animals, plants and microorganisms. Advances in synthetic organic chemistry led to the identification of many chemical molecules that offered more opportunities to develop novel compounds. Many new drugs emerged by this route, particularly those now being used to treat infections, infestations, cancers, ulcers, heart and blood pressure conditions. Many drugs were developed through random screening of thousands of chemicals synthesized as dye-stuffs and the like; many others resulted from serendipity arising from sharp eyed observations of physicians and scientists. Example of such drugs includes sulphonamides, isoniazid, antipsychotics, antihistamines and penicillin. Emergence of the modern pharmaceutical industry is an outcome of all these different activities that developed potent single molecules with highly active for a wide variety of ailments. The drugs produced in many cases improved on nature, a new range of local anesthetics from cocaine avoided its dangerous effects on blood pressure, and chloroquine is much less toxic than quinine. These successes and many more like them resulted in reduced interest in natural products drug discovery and many major drug companies almost neglected such divisions. Work on developing new drugs for the treatment of the world's major diseases such as malaria, trypanosomiasis, filariasis, tuberculosis,

schistosomiasis, leshmaniasis and amoebiasis came almost to a standstill. In addition, although botanical medications continued to be produced in every country, the clinical efficacy of these was usually not evaluated and the composition of these complex mixtures was only crudely analyzed. Thus herbal medicines became the domain of 'old wives tales' and quack medicine, exploitation of the sick, the desperate and the gullible. Sadly, herbal medicines continued to reflect poor quality control both for materials and clinical efficacy (Patwardhan et al.,)

1.3. Development of Phytomedicines for various diseases

Medicinal plants play a key role in the human health care. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant materials (WHO, 1993). The traditional medicine refers to a broad range of ancient natural health care practices including folk tribal practices as well as Ayurveda, Siddha, Amchi and Unani. These medical practices originated from time immemorial and developed gradually, to a large extent, by relying or based on practical experiences without significant references to modern scientific principles. These practices incorporated ancient beliefs and were passed on from one generation to another by oral tradition and guarded literature. Although herbal medicines are effective in the treatment of various ailments very often these drugs are unscientifically exploited and improperly used. Therefore, these plant drugs deserve detailed studies in the light of modern science**.**

1.4. Phytotherapeutic Approach of Drug Development

In phytotherapeutic approach, the emphasis is on the development of a new drug whose extraction and fractionation have emanated on the basis of therapeutic activity. The standard fraction of an active extract or mixture of fractions may prove better therapeutically, less toxic and inexpensive compared to pure isolated compound drugs. However, crude plant preparations require modern standards of safety and efficacy. Modern bioassay methods and physiochemical profile do provide ways and means of developing quality control as well as determining the expiry date of crude preparations or fractions. Standardized herbal preparations may serve as inexpensive and useful drugs to the masses.

Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness. These drugs are invariably single plant extracts or fractions thereof

or mixtures of fractions of extracts from different plants which have been carefully standardized for their safety and efficacy (Suck Dev 1997).

1.5. Traditional Wisdom

Lag phase for botanical medicine is now rapidly changing for a number of reasons. Problems with drug resistant microorganisms, side effects of modern drugs, and emerging diseases where no medicines are available, have stimulated renewed interest in plants as a significant source of new medicines. Pharmaceutical scientists are experiencing difficulty in identifying new lead structures, templates and scaffolds in the finite world of chemical diversity. A number of synthetic drugs have adverse and unacceptable side effects. There have been impressive successes with botanical medicines, most notably quinghaosu, artemisinin from Chinese medicine. Considerable research on Pharmacognosy, Chemistry, Pharmacology and Clinical therapeutics has been carried out on Ayurvedic medicinal plants. Numerous molecules have come out of Ayurvedic experimental base, examples include Rawolfia alkaloids for hypertension, psoriases in vitiligo, hypolipidemic agents, mucana pruriens for Parkinson's disease, piperidines as bioavailability enhancers, baccosides in mental retention, pierosides in hepatic protection, phyllanthins as antivirals, curcumine in inflammation and many other steroidal lactones and glycosides as immunomodulators. A whole range of chronic and difficult to treat diseases such as cancers, cardiovascular diseases, diabetes, rheumatism and AIDS all require new effective drugs. Most developing countries have relied and will continue to rely on traditional natural medicines due to the deterrence of high costs of modern allopathic medicines (Patwardhan et al.,)

Current estimation indicates that about 80% of people in developing countries still rely on traditional medicine based largely on various species of plants and animals for their primary healthcare. Four out of ten Americans used alternative medicine therapies in 1997. Total visits to alternative medicine practitioners increased by almost 50 from 1990 and exceeded the visits to all US primary care physicians (Suck Dev 1997).

1.6. Cancer

Cancer is a general term applied to a series of malignant diseases which may affects many different parts of the body. These diseases are characterized by rapid and

uncontrolled formation of abnormal cells which may mass together to form a growth or tumour, or proliferate throughout the body. Initiating abnormal growth at other sites, if the process is not arrested, it may progress until it causes the death of the organism. Cancer is commonly encountered in all higher animals, and plants also develop growth that resembles cancer. Next to heart disease, cancer is a major killer of mankind. Cancer is basically a disease of cells characterized by the loss of normal cellular growth, maturation and multiplication, and thus homeostasis is disturbed.

Carcinogens

Carcinogens, the agents that cause cancer, have been classified into three broad groups' *viz* physical, chemical and biological.

Physical agents

Ultraviolet and ionizing radiations are mutagenic and carcinogenic, which may damage DNA in several ways. Ultraviolet radiation catalyzes the formation of covalent pyrimidine dimer, thus distorting the A-T, C-G bases pairing sequence in DNA strand. Ionizing radiation (X-rays, G-rays) can break the backbone of DNA molecule either by altering the base sequences structurally or by deletion of the bases from the backbone. Apart from direct effects on DNA, X-rays and G-rays cause free radicals to form in the tissues which can lead to oxidative DNA damage.

Chemical agents

It is estimated that 80% of human cancers are due to environmental factors, principally chemicals. A variety of compounds *viz*., polycyclic aromatic hydrocarbons, nitrosamines, alkylating agents and other inorganic and naturally occurring compounds are carcinogenic. Generally all carcinogens are electrophiles which attack nucleophilic groups in the DNA and RNA and proteins and thus damage the cell.

Many of the alkylating agents can act directly on the target molecules (direct carcinogens) but there are other compounds, which cannot act directly and require prior metabolism to become carcinogens (procarcinogens). Thus chemical carcinogens can be classified as

- Initiating agent – which is capable of initiating cells only.

- Promoting agent – capable of causing the expression of initiated cell clones.
- Progressor agent – which can convert initiated cell or a cell in the stage of promotion to a potentially malignant cell.
- A complete carcinogen has all the properties of initiating, promoting and progressor agents.

Another potential source of exogenous transforming mutations are certain biochemical processes which generate significant quantities of reactive oxygen species (ROS) and free radicals that are estimated to cause alterations in the DNA. Therefore, processes inherent in the cell and not necessarily dependent on exposure to exogenous agents may cause carcinogenesis. But it must be emphasized that environmental carcinogens significantly increase the risk of cancer and carcinogens do accelerate the process of carcinogenesis. The interaction of an "ultimate" carcinogen (i.e. the active electrophile) with DNA targets determines the formation and permanent fixation of transforming lesions.

Biological agents

The oncogenic viruses are well known and form a very diverse group of carcinogenic agents. They include members of all major families of DNA viruses that infect vertebrates except very small Parvovirus's and the very large Poxviruses. On the other hand, only one family of RNA viruses, the retroviruses, can cause tumors. The tumor viruses vary in complexity of their genomes, in the types of neoplasm they induce and in their requirement for co-factors in tumor genesis. RNA tumor viruses are characterized by the presence of RNA-dependant DNA polymerase (reverse transcriptase). Once the virus core enters the cell, the reverse transcriptase transcribes the single stranded RNA viral genome into double stranded DNA copies. The original viral RNA becomes degraded and the viral DNA copy (provirus) is then inserted (integrated) by a covalent linkage into the host cell DNA.

Endogenous DNA damage from normal oxidation is enormous. The steady state of oxidative damage in DNA is about one million oxidative lesions per rat cell. This high background suggests that increasing the cell division rate must be a factor in converting lesions to mutations and thus cancer. Raising the level of either DNA lesions or cell division will increase the probability of cancer. Just as DNA repair protects against lesions, guards the cell cycle and protects against cell division if the

lesion level gets too high. If the lesion level becomes still higher, can initiate programmed cell death (apoptosis).

1.7 Main Features of Cancer

- Excessive cell growth, usually in the form of tumor.
- Invasiveness, i. e. the ability to grow into surrounding tissue.
- Undifferentiated cells or tissue.
- The ability to metastasize or spread to new sites and establish new growth;
- A type of acquired heredity in which the progeny of cancer cells also retain cancerous property.
- A shift of cellular metabolism towards increase in production of macromolecules from nucleosides and amino acids, with an increased catabolism of carbohydrates for cellular energy. Such behavior of cancer cells lead to illness in the host as a result of

- Pressure effect due to local tumor growth;
- Destruction of the organ involved by the primary growth;
- Systemic effect as a result of new growth.

1.8 Causes of Cancer

Many factors are implicated in the causation of cancer. These factors are listed as

- Exposure to the carcinogenic hydrocarbons or to excessive radiation.

- Hereditary factors: A “cancer family syndrome” has been described by the Lynch *et al.,* The hereditary factors involved in the causation of cancer are chromosomal abnormality, enzymes, immune defense system, hormonal imbalance etc.

- Cultural factors: cultural factor play a dominant role by causing about 70 % of all cancers. The important amongst are diet, smoking, drinking and sexual habits.

- Occupational factors: These factors are ionizing radiation, chemicals and other substances for example- coal tar, mustard gas, chromium, hematite, nickel and asbestos can trigger lung cancer in employees working in chemical, insulation and gas factories.

- Viruses: Though it is known that viruses cause cancer in animals, their role in human cancer has not been proved.

1.9 Cell Cycle

Cellular multiplication involves passage of cell through a cell cycle. The various phases of cell cycle are characterized as:

- The interval following cell division to the point where DNA synthesis starts, known as the presynthetic phase G_1.
- After mitosis some of the daughter cells pass into a resting phase or non proliferative phase G_0 and do not re-enter the cell cycle phase G_1 immediately. They may enter the G_1 phase later.
- DNA synthesis phase (S).
- The premitotic or postsynthetic (G2) phase fallows. In this phase RNA and protein synthesis takes place.
- Mitotic phase (M) follows.

1.10. Criteria for Antineoplastic Drug (Anticancer drug)

The term 'anticancer drug' is emotive and can build up false hopes among cancer sufferers. Investigator in the United States National Cancer Institute (NCI) program have used the term cytotoxic, antitumour and anticancer to describe the activity of the compound isolated according to the following definitions. A cytotoxic agent is toxic to tumour cell *in- vitro* and if this toxicity transfers through the tumour cell *in vivo*, the agent is said to have antitumour activity. The term anticancer is reserved for material, which are toxic to tumour cell in clinical trials.

An antineoplastic drug should be:

(1) Cytotoxic: To inhibit the cancer cell metabolism, particularly synthesis of protein and nucleic acid, in order to prevent cell growth, differentiation, vascularization of the new growth etc.

(2) Mitostatic: To disrupt the process of cell division, to prevent the uncontrolled number of cycles of cell division and growth, to retard the proliferation of the cancerous tissue.

(3) Nontoxic: The drug should be nontoxic to the rest of the body of the patient; it should not cause any side effect such as renal or hepatic dysfunction, neurotoxicity, hypersensitivity etc.

(4) **Target oriented:** It should be site-selective targeting in action to the cancerous region and not cause the same effect (or at least not cause them to the same degree as on cancer) on the other part of patient's body.

The drug should be effective in small and few doses, should not be expensive, should have longer half-life, freely available on the market, etc., These criteria for an anticancer drug are a tall order. Hardly there is any drug, synthetic or natural, that meets with all these qualification and so the choice is dictated by the maximum compliance of the criteria and the philosophy of the '*Lesser evil'*. With the presence of a large number of different types of cancer, each a kind of a syndrome, no single drug can be expected to be effective against more that are, at the best a few of related cancers (Barar 2003; Singh & Lippman 1998).

1.11. Plants in the Treatment of Cancer

In the face of failure to find synthetic drugs against cancer, thousands species of plants have been screened since a long time, for antineoplastic activity, in the hope of discovering effective natural products. Compounds have been isolated from hundreds of species and their activities in suppressing tumours induced in laboratory animals 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 3,000 species of plants and animals as useful in dealing with one or the other aspect of cancer management. Basing 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.

Plants in the management of cancer

In addition to the handling of the cancer proper, a host of synthetic or plant based drugs are used in the biomedical system in the management of cancer, which is distinct from treatment aimed at a cure. The better hope of usefulness of plants lies in the areas of detection, prevention, management of symptoms inherent in the disease or incidental to the treatment, post-cure management such as the recovery of the body to full and normal functioning, prevention of remission, and management of symptoms of incurable cancers, to keep the patients in the maximum possible comfort.

Plants in the prevention of cancer

There is a prevailing hope that 'someday people should be able to avoid cancer or delay its onset by taking specially formulated pills or foods. Chemoprevention is the attempt to use natural and synthetic compounds to intervene in the early pre-cancerous stages of carcinogenesis, before the invasive disease begins, as prevention of cancer is immensely better than its uncertain cure.

Food has been identified as one of the most promising sources of chemopreventive agents. These include vitamins A (and its analogues), C and E, which are obtained by us only from plants. Some plant products without any recognised nutritional value such as indoles, isothiocyanates, dithiolthiones and organosulphur compounds have been shown to be chemopreventive. Dithiolthiones and organosulphur compounds are abundant in broccoli, cauliflower and cabbage. Genistein from soyabean, and epigallocatechin, the bulk of solid material in brewed tea have also been found to be chemopreventive, as well as turmeric, ginger and saffron.

Among the plant-based chemopreventives, β-carotenes, the precursors of vitamin A, are rated high. In addition to carrots, they are present in a large number of plants, particularly abundant in the leafy vegetables. These food plants also provide the dietary fibre that is believed to prevent colon cancer. It is not yet very clear how the chemopreventive agents function. Some are believed to prevent the mutations that can lead to cancer, some halt the process of excessive proliferation of altered cells, and some hasten apoptosis (death of cells) of altered cells, while some function as antioxidants and scavenge the free radicals that may trigger cancer (VandeCreek et al., 1999).

1.12. Cancer Chemotherapy

The chemotherapy of neoplastic disease has become increasingly important in recent years. An indication of this importance is establishment of a medical specialty in oncology in which the physician practices various protocol of adjuvant therapy. Most cancer patient now receives some form of chemotherapy, even though it is merely palliative in many cases. The relatively high toxicity of most anticancer drugs has fostered the development of supplementary drugs that may alleviate these toxic effects or stimulate the regrowth of depleted normal cells. There is a cogent reason why cancer is more difficult to cure than bacterial infections. One is that there are

qualitative differences between human and bacterial cells. For example bacterial cell have distinctive cell walls, and their ribosome differ from those of human cells. In contrast, the differences between normal and neoplastic human cells are mostly quantitative. Another difference is that immune mechanism and other host defenses are very important in killing bacteria and other foreign cells, whereas they play a lesser role in killing cancer cells.

1.13. Indian Scenario

The global context sketched above suggest several tremendous opportunities for India, a country with unrivalled terms of diversity of medicinal systems and practices, in addition to being a major storehouse of biological diversity, with 2 of the 4 mega diversity areas of the world located within its borders. In addition several concerns arise in relation to the current consequences of participation in the

market, with regard to the sustainable and equitability of prevailing practices in the

sector. To add to all these aspects, the market in India has been shown to be highly inefficient and imperfect. The need of the hour then is to replant India's participation in the expanding global market. Such an overview could form the basis of a renewed development of India's medicinal plant sector, and a strategic exploitation of other comparative advantage in the global market on a sustainable and equitable basis.

1.14. Drug designing for cancer

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 individual dose and finally, drugs should be used as close as possible to discourage tumor growth and maximize dose intensity (the

dose gives per unit time, a key parameter in the success of chemotherapy). Based on experimental tumor models, it is necessary to eradicate all tumor cells. The fraction of cells killed with each treatment cycle is constant, with regrowth between cycles. Thus, it is desirable to achieve maximal cell kill with each cycle, using the highest drug dose possible, and to repeat dose as frequently as tolerated. Since the tumor 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 treatments in multiple cycles to kill all the tumor cells.

The activity of many of the drugs currently used in cancer chemotherapy can probably be ascribed to inhibition of nucleic acid synthesis, but mechanism of action differs widely. Some compounds are mitotic inhibitors for example colchicine, podophyllotoxin, vincrystine and maytansine, and they act by binding to the protein tubulin in the mitotic spindle, preventing polymerization and assemble into microtubules, and after cell division, the microtubules are transformed back to tubulin.

Although podophyllotoxin is a tubulin binder, it is intriguing that the semisynthetic anticancer drugs etoposide and teniposide derived from it have a different mode of action. These drugs inhibit DNA synthesis and replication via the enzyme topoisomerase II. Camptothecin derivatives, topotecan and irinotecan, exert their cytotoxic action through inhibition of topoisomerase I system. Topoisomerase are fundamental enzyme complex involved in DNA replication by their ability to break and reseal the DNA strands.

With the identification of an increasing number of molecular targets associated with particular cancers, high throughput screening of compounds against a range of such targets now forms the basis of anticancer drug discovery. Examples are the cyclin dependant kinases, which, together with their cyclin parameters, play a key role in the regulation of cell cycle progression, and inhibition of their activity delays or arrest progression at specific stages of cell cycle. These are over 2000 kinases so for identified for genomic studies and all have a common site, the position where the ATP, that is, the source of phosphate that is donated, is bound. The moderately antitumor flavonoids, quercetin, is an early example of natural product compound class that ultimately led to CDK inhibitors. These flavonoids resembles an ATP

mimic where the planar bicyclic chromone ring system is an isostere of adenine. Quercetin exerts its antitumor effect through blocking cell cycle progression at the G0/G1 interface, consistent with Cyclin dependant kinase inhibition.

Taxol is a naturally occurring highly derivatised diterpene belonging to taxane group of compounds present in genus *Taxus* under family Taxaceae. A derivative of taxol-taxofere has been reported to have better bioavailability and pharmacological properties. The bio target of taxol is microtubule responsible for formation of mitotic spindle necessary for cell division which causes detrimental effects leading to blockage of cell cycle (Wilson and Gisvold 2004).

1.15. Antioxidants

Cells in human body use oxygen to breakdown carbohydrates, proteins and fats that give them energy. Metabolically active cells produce by products called free radicals. These are atoms or groups of atoms or groups of atoms that have at least one unpaired electron, which make them highly reactive. They promote beneficial oxidation that produces energy and kill bacterial invaders. If free radicals are at reasonable levels the human body produces enzymes to combat them and useful immune system and antibacterial cell activity.

It is well known that the reactive oxygen species (ROS) are involved in many pathological disorders such as atherosclerosis and related cardiovascular diseases, diabetes, and cancer. Reactive oxygen species, generated *in vivo* mainly by neutrophills, macrophages and xanthenes oxidase system, appears to be responsible in these illnesses by inducing lipid peroxidation via a chain reaction process. Most living species have protective systems against oxidative stress and toxic effects of ROS. Several studies have demonstrated that the antioxidant properties of plant compounds could be correlated with oxidative stress defense. Thus antioxidant compounds can be used to counteract oxidative damage by reacting with free radicals, chelating free catalytic metals, and also by acting as oxygen scavengers.

Antioxidants are compounds which act as inhibitors of the oxidative process. They are quite large in number and diverse in nature, which oppose the process of oxidation largely by neutralizing free radicals. Antioxidants at relatively small concentrations have the potential to inhibit the oxidants chain reactions. Antioxidants are also of

paramount importance in pharmaceutical formulation because there are innumerable medicinal agents possessing diverse chemical functions and are known to undergo oxidative decomposition. The enzymatically potential antioxidants known are superoxide dismutase, glutathione peroxidase, catalase and peroxidases.

In the non enzymatic category, some of the known and documented antioxidants are vitamin C, vitamin E, vitamin A, ß carotenoids, uric acid, ubriquinone and synthetic compounds like melatonin, dihydro epiandrosterone (DHEA) etc. Some of the plant product or extracts viz. Ginkgo biloba extract, spirulina, various spices, like the extract of garlic and onion, turmeric, capsicum, black pepper, amla, tomato, guava, watermelon, tea beverages have been also be reported antioxidants of non-enzymatic category.

1.16. Antioxidant defence

Antioxidant means "against oxidation". Antioxidants work to protect lipids form peroxidation by radicals. Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant it no longer needs to attack the cell and the chain reaction of oxidation is broken[15]. After donating an electron an antioxidant becomes a free radical by definition. Antioxidants in this state are not harmful because they have the ability to accommodate the antioxidant defense system. Antioxidants are manufactured within the body and can also be extracted from the food humans eat such as fruits, vegetables, seeds, nuts, meats, and oil. There are two lines of antioxidant defense within the cell. The first line, found in the fat-soluble cellular membrane consists of vitamin E, β-carotene, and coenzyme (Dekkers et al., 1996).

Antioxidant tends to reduce free radical formation and scavenge free radical. Despite the fact that humans have evolved with antioxidant system to protect against free radicals, which may be endogenous or exogenous, some ROS still escape in quantities sufficient enough to cause damage. Therefore exogenous antioxidants that scavenge free radicals, especially, those from the relatively harmless natural sources play an important role in cardiovascular disease, aging, cancer and inflammatory disorders as well as in ameliorating drug-induced toxicity. This has accelerated the search for potential antioxidants from traditional medicinal plants (Kaczmarski et al., 1999; Veeresham & Asres 2005).

2. Plant Profile

(Wealth of India, 2003 [Anon]; Devasagayam 2007; Wealth of India, CSIR, 1969; Indian Medicinl Plants [Kitikar & Basu] 1975, 1987, 1999; The Flora of Orissa; Saxsena & Brahmam, 1994; Sharma et al., 2005; Sharma 2003; Swain & Das 2007; Nadkarni 1976; Kapoor, 1989).

Botanical Name: *Pterocarpus marsupium* Roxb.
Botanical Source: The plant consists of the bark, leaves, heartwood of *Pterocarpus marsupium P. marsupium*

Order: Fabales

Family: Leguminosaa (Fabaceae)

Subfamily: Faboideae

Tribe: Dalbergieae

Genus: *Pterocarpus*

Species: *marsupium*

Authority: Roxb.

Vernacular names: (Sharma 2003; Swain & Das 2007)

1. Sanskrit: Pitasala, Bijaka, Murga
2. Hindi: Bijasal
3. English: Malabarkino; Indian Kino Tree
4. Bengali: Pitsal
5. Nepalese: Bijasar
6. Sinhalese: Gammalu
7. German: Malabarkino
8. Kannada: Honne
8. French: Pterocarp
9. Unani: Dammul-akhajan
10. Arabian: Dammul Akhwayn
11. Persian: Khoon-e-siyaun-shan
12. Tamil: Vengai
13. Telugu: Yegi
14. Malayalam: Venga

P. marsupium, also known as Malabar kino, (Gamble, 1935) Indian kino tree or vijayasar, is a medium to large, deciduous tree that can grow up to 30 metres tall. It is

native to India, Nepal, and Sri Lanka, where it occurs in parts of the Western Ghats in the Karnataka-Kerala region and also in the forests of Central India. Parts of the Indian kino (heartwood, leaves, and flowers) have long been believed to have medicinal properties in Ayurveda (The Flora of Orissa, Saxsena & Brahmam, 1994). In Karnataka the plant is known as honne or kempu honne. The Kannada people in India make a wooden tumbler from the heartwood of this herb tree (Saldanha, Flora of Karnataka, 1984).

Bark of *Pterocarpus marsupium marsupium*

Leaves of *Pterocarpus*

2.1 Habitat

A moderate to large deciduous tree about 90ft or more high, commonly found in hilly region of central and peninsular India (Andhra Pradesh, Bihar, Gujarat, Kerala, Madhya Pradesh, Maharashtra, Karnataka, Orissa, Tamilnadu, Uttar Pradesh); found at 3000 ft in Gujarat, Madhya Pradesh and Himalayan & sub Himalayan tracts-Nepal (Kapoor, 1989) and Sri Lanka. It grows on a variety of formation provided the drainage is good. It prefers a soil with a fair proportion of sand though it is often found on red loam with a certain amount of clay. The normal rainfall in its natural

habitat ranges from 75 to 200cm but it attains its largest size in parts of Mysore and Kerala, where the rainfall is even higher. It is a moderate light demander and the young seedlings are frost-tender (Wealth of India, CSIR, 1969).

Parts used: Bark, Leaves, Kino (gum)

2.2 Pharmacognostical Characteristics

Morphology

It is a moderate-sized to large deciduous tree. bark grey, longitudinally fissured and scaly. The older trees exude a blood red gum-resin.

Description

Leaves: compound; with 5 to 7 leaflets, 3 to 5 in long, oblong or elliptical with wavy margin or rounded or obtuse or retuse ends, glaucous beneath, secondary nerves close and parallel, over 12 cm each side.

Flowers: yellow, , up to 1.5 cm long, corolla papilionaceous, exserted beyond calyx, Stamen 10, split in 2 bundles , yellow, in very large, dense bunches.

Fruits: 2 to 5 cm long, roundish, winged, with one seed. Legume indehiscent, orbicular, compressed, broadly hardened winged around margin, usually single seeded, seeds subreniform, hilum small.

The Heartwood: is golden to yellowish brown with dark streaks staining yellow when damp and turning darker on exposure, strong and tough.

2.3 Microscopic Characteristics

The wood consists of vessels, tracheids, fibre tracheids and wood parenchyma all the elements being lignified and filled with tannin. Vessels are medium sized drum shaped, scattered, leading to semiring-porous conditions, tyloses present. Tracheids are long, abundant, thick walled, with tapering ends and simple pits on the side walls. Xylem parenchyma is small, thick walled with blunt ends; rectangular simple pitted surrounding the vessel. A few crystal fibers are observed in tangential section of the wood. Tree bark yields a reddish gum known as Kino gum, which becomes brittle on hardening and is very astringent. Sclerenchyma diffused pores Red marks are resin canals 8 Stem hairs overlapping metaxylem and protoxylem.

2.4 Chemical Constituents

Researches in the past have established the genus *Pterocarpus* to be the rich sources of polyphenolic compounds. All active principles of *P. marsupium* are thermostable.

The primary chemical components of P. marsupium are pterosupin, pterostilbene, isoliquiritigenin, liquiritigenin, epicatechin, kinotannic acid, kinoin, kino-red beta-eudesmol,
marsupol, carpusin and marsupinol.

The plant contains pterostilbene 4- 5%, alkaloids 0.4%, tannins 5%, protein, pentosan, pterosupin, pseudobaptigenin, liquiritigenin, isoliquiritigenin, garbanzol, 5- de- oxykaempferol, P- hydroxybenzaldehyde, beudesmol, erythrodirol- 3- monoacetate, l- epicatechin, marsupol, carpusin, propterol, propterol B, marsupinol, irisolidone- 7- O- A- L- rhamnopyranoside, have been obtained mainly from the heartwood and root.
The gum kino from the bark provides non- glucosidal tannins - kinotannic acid, kinonin (C28H24O12), kino- red (C28H22O11), pyrocatechin, pyrocatechin acid & small quantities of resin, pectin and gallic acid.

Aqueous extract of the heartwood of *Pterocarpus marsupium* contains 5 new flavonoids C- glucosides namely 6- hydroxyl- 2- (4- hydroxybenzyl)- benzo- furan- 7- C- â- D- glucopyr anoside, 3- (á - methoxy- 4- hydro xybenzylidene) - 6- hydroxybenzo- 2(3H)- furanone- 7- C- â- D- glucopyranoside, 2-glucosyl, 8- (C- â- D- glucopyranosyl)- 7,3,4- trihydroxyflavone and 1,2- bis (2,4- dihydroxy, 3- C- glucopyranosyl) – ethanedione and two known compounds C- â- D- glucopyranosyl- 2,6- dihydroxyl benzene and sesquiterpene were isolated. Ether extract of the roots of Pterocarpus marsupium consists of a new flavonol glycoside 6- hydroxy- 3,5,7,4- te tramethoxyflavone 6- O- rhamnopyranoside, 8-hydroxy-4’-m ethoxyisoflavone- 7- O- glucopyranoside.

A benzofuranone derivative 2,4’6-trihydroxy-4-methoxy benzofuran-3(2H)-one designated carpus in, 1,3- bis(4- hydroxyphenyl) propan- 2- ol designated propterol, 1- (2,4- di hydroxyphenyl)- 3- (4- hydroxyphenyl) propan- 2- ol designated propterol, 6- hydroxy- 7- O- methyl- 3- (3- hydroxy- 4- O- methyl benzyl) chroman-4-one. Ethyl acetate extract of root contains benzofuranone, marsupin, dihydrochalcone, pterosupin, stilbene, pterostilbene, aliquiritigenin, isoliquiritigenin.

Methanolic extract of heart wood c ontains an isoflavone 7- O- á- L- rham- no pyranosyloxy-4’-methoxy-5-hydroxy-isoflavone. Three new isoflavone glycosides viz retusin 7- glucoside, irisolidone 7- rhamnoside and 5,7- dihydroxy- 6- methoxy

isoflavone 7- rhamnoside have been isolated from the heartwood of Pterocarpus marsupium. 2,6-dihydroxy- 2- (P- hydroxybenzyl)- 3(2H)- benzofuran- 7- C- â- D- glucopyranoside (Maurya et al., 2004; Gairola et a., 2010; Yogesh et al., 2010; Tiwari & Khare 2015).

2.5 Ethnomedicinal Uses (Tiwari et al., 2015)

Useful parts of the herb are heartwood, leaves, flowers, gum. The genus is widely distributed on the Earth and the astringent drug from this genus is known as “Kino”. The phloem of stem contains red astringent fluid present in secretory cell, which exudes after given incision. Kino is odourless but has astringent taste and sticks in the teeth, colouring the saliva red in colour. As astringent it is used in diarrhoea, dysentery etc.

Bruised leaves are applied on fractures, leprosy, leucoderma, skin diseases, sores and boils, Constipation, depurative, rectalgia, opthamology, hemorrhages and Rheumatoid arthritis. Marsupin and Pterostilbene significantly lower the blood glucose levels useful in NIDDM. Bark is used as diuretic in Gabon and fresh leaves are used as food in Nizeria. Also is used in the form of powder or decoction in diarrhoea, and decoction is very useful for diabetic patients.

Stem in the treatment of neurological problems.

Leaves are used in GIT disorders, wood, stem bark, seed and flours are used in African traditional medicine, especially in the Cameroonian pharmacopoeia, for treating various diseases including hypertension, diabetes, intestinal parasitizes, renal and cutaneous diseases. The leaf paste is used as an ointment to treat skin diseases, sores and boils.

Wood: The heartwood is used as an ointment to astringent, bitter, acrid, cooling, anti-inflammatory, union promoter, depurative, urinary astringent, haemostatic, asthelmintic, constipating, anodyne alterant and rejuvenation. It is also useful in elephantiasis, inflammations, fractures bruises, leprosy, skin disease, leucoderma, erysipelas urethrorrhoea, diabetes, rectalgia, rectitis, opthalmopathy, diarrhea, dysentery, cough, asthma, bronchitis and greyness of hair.

Flower: The flower is used as appetizing and febrifuge and also taken to treat anorexia and fever.

Gum-resin: The gum is taken to treat bitter, styptic, vulnerary, antipyretic, anthelmintic and liver tonic. It is useful in spasmodic gastralgia, boils, gleet, urethrorrhoea, odontalgia, diarrhea, psoriasis, wound and ulcers, helminthasis, fevers, hepatopathy and ophthalmia.

Some facts: *P. marsupium* is a plant drug belonging to a group called '*Rasayana*' in Ayurvedic system of medicine. These '*Rasayana*' drugs are immunomodulators and relieve stress in the body. In India, Kannada peoples are used to make a wooden tumbler from the heartwood. Water is left overnight in the wooden tumbler and is consumed in the next morning to cure diabetes. Kol tribes in Odisha pound a paste mixture of the bark of *P. marsupium* with the bark of *Mangifera indica, Shorea robusta & Spondias pinnata* to treat some dysentery illness. The gum resin of this plant is the only herbal product ever found that regenerate beta cells that produce insulin in pancreas.

2.6 Biological activity

Although a large number of compounds have been isolated from various parts of *P. marsupium*, few of them have been studied for biological activity as shown in Table 1. The structure of some of these bioactive compounds has been presented in Figure 1. The bark contains l-epicatechin and a reddish brown colouring matter. The bark is occasionally employed for dyeing. The heartwood yields liquiritigenin, isoliquirtigenin, a neutral unidentified component, alkaloid and resin. The wood also contains a yellow colouring matter and an essential oil and a semi-drying fixed oil.

The tree yields a gum-Kino which exudes when an incision is made through the bark up to the cambium. It is odourless and bitter with astringent taste and colours saliva pink when masticated. Kino contains a non-glucosidal tannin kinotannic acid, kinoin and Kino-red, small quantities of catechol, protocatechuic acid, resin, pectin and gallic acid. The therapeutic value of Kino is due to Kino is due to kinotannic acid. Kino is powerfully astringent and was formerly used widely in the treatment of diarrhea and dysentery. It is locally applied in leucorrhoea and in passive haemorrhages. It is also used for toothache. The bark is used as an astringent and in toothache. The flowers are said to be used in fever. The bruised leaves are considered useful as an external application for boils, sores and skin diseases. The aqueous

infusion of the wood is said to be of use in diabetes and water stored in vessels made of the wood is reputed to have antidiabetic qualities (Anon, Wealth of India, 2003).

2.7 Medicinal use of various parts of *P.marsupium*

Various parts of the *P. marsupium* tree have been used as traditional ayurvedic medicine in India from time immemorial. The medicinal utilities have been described, especially for leaf, fruit and bark. The bark is used for the treatment of stomachache, cholera, dysentery, urinary

complaints, tongue diseases and toothache. The gum exude 'kino', derived from this tree, is used as an astringent (Singh et al., 1965). The gum is bitter with a bad taste. However, it is antipyretic, anthelmintic and tonic to liver, useful in all diseases of body and styptic vulnerant and good for griping and biliousness, opthalmiya, boils and urinary discharges. The flowers are bitter, improve the appetite and cause flatulence (Indian Medicinal Plants 1999). *P. marsupium* has a long history of use in India as a treatment for diabetes. It is a drug that is believed to have some unique features such as beta cell protective and regenerative properties apart from blood glucose reduction (WHO 1980; Chakravarthy et al., 1981). Some of the medicinal attributes of various parts of *P. marsupium* have been summarized (Yogesh et al., 2010) in table 2.

3. Literature Review

3.1. Anti-diabetic and antioxidant activity

P. marsupium demonstrates unique pharmacological properties, which include beta cell protective and regenerative properties as well as blood glucose lowering activity. The animal studies conducted have used various species including rats, dogs, and rabbits with induced diabetes and subsequent treatment with various extracts of *P. marsupium*. In all of these studies, *P. marsupium* was found to reverse the damage to the beta cells and actually repopulate the islets, causing a nearly complete restoration of normal insulin secretion (Chakravarthy et al., 1982; Manickam et al., 1997; Ahmad et al., 1991; Pandey & Sharma 1976; Shah 1967; Chakravarthy et al., 1982).

In one study it was shown that aqueous extract of *P. marsupium* modulates the inflammatory cytokine TNF-alpha in type 2diabetic rats and this has an indirect effect on PPAR-Gamma expression. By decreasing TNF-α, drug can upregulate the PPAR-Gamma and in turn the glucose metabolism (Halagappa et al., 2010).

The bark of *P. marsupium* is traditionally used in the Indian Ayurvedic system of medicine as an anti-diabetic drug. The compound that is responsible for antidiabetic activity is (-) epicatechin, a member of the catechin group of compounds belonging to the class of flavonoids (Zaid et al., 2002).

It has been shown that *P. marsupium* works by the regeneration of the beta cells and increase proinsulin biosynthesis. Marsupin and Pterostilbene significantly lowered the blood glucose level of hyperglycemic rats, and the effect was comparable to that of 1,1-dimethyl biguanide (metformin) (Manickam et al., 1997).

Overnight water stored in water tumblers made out of the heartwood of *P. marsupium* is used as a traditional therapy for patients of Diabetes mellitus especially in the state of Madhya Pradesh (Maheswari et al., 1980).

Isolated compounds from *P. marsupium* have been shown to enhance the conversion of Pro-insulin to insulin and stimulate cAMP content in the islets of Langerhans (Ahmad et al., 1991).

It is proposed that the flavonoid fraction of *P. marsupium* bark effectively reverses the alloxan induced changes in the blood sugar level and the beta cell population in the pancreas (Chakravarthy et al., 1980).

P. marsupium methanol extract has been found to cause normalization of serum protein and albumin levels, possibly through the increase in insulin mediated amino acid uptake, enhancement of protein synthesis and inhibition of protein degradation (Dice et al., 1978).

Administration of the bark extract to diabetic rats restored the levels of serum electrolytes, glycolytic enzymes and hepatic cytochrome p-450 dependent enzyme systems by inhibiting the formation of liver and kidney lipid peroxides (Gayathri and Kannabiran et al., 2010).

3.2. Cardiotonic activity

Cardiotonic activity was reported of the aqueous extract of heartwood of *P. marsupium*. This plant species contains 5,7,2-4 tetrahydroxy isoflavone 6-6 glucoside which are potent antioxidants and are believed to prevent cardiovascular diseases. The cardiotonic effect of the aqueous extract of heartwood of *P. marsupium* was studied by using the isolated frog heart perfusion technique. Calcium free Ringer solution was used as vehicle for administration of aqueous extract of *P.marsupium* as a test extract and digoxin as a standard (Mohire et al., 2007). Liquiritigenin and Pterosupin, the flavonoid constituents of *P. marsupium* are effective against reducing serum cholesterol levels, LDL cholesterol, and atherogenic index. Pterosupin being additionally effective in lowering serum triglycerides (Jahromi and Ray.,1993**).**

3.3. Hepatoprotective activity

Methanol extract of the stem barks of *P. marsupium* possesses significant hepatoprotective activity (Mankani et al., 2005).

3.4. Antioxidant activity

The whole aqueous extract of the stem bark of *P. marsupium* showed high anti-oxidant activity and protects the mitochondria against oxidative damage (Mohammadi et al., 2009).

Heartwood extracts of *P. marsupium* promotes wound healing in both normal and diabetic animals by topical application of the extracts (Singhal et al., 2013).

Ethanol extracts of the heartwood of *P. marsupium* is found to be useful in preventing allergic conditions and diseases such as asthma owing to its ability to decrease the increased eosinophilic , leucocytic count, prevention of mast cell degranulation (Suralkar et al., 2012).

Antidiarrhoeal activity of Ethanol heartwood extract of *Pterocarpus marsupium* was also studied (Dilpesh et al., 2011).

3.5. Antibacterial activity

Hexane, ethyl acetate and methanol extracts were tested against four selected Gram positive and Gram negative bacteria- *S. aureu*, *K. pneuminiae*, and *P. aeruginosa* (Sapha 1956; Gayathri and Kannibaran ., 2010). *In vitro*, it inhibits *Pseudomonas aeruginosa*, *Streptococcus pyrogens* and *Staphylococcus aureus*. Ethyl and methanol extracts were more sensitive to the bacteria than extracts made out of hexane. Both the extracts exhibited concentration dependent variation in their anti-bacterial activity. Similar observations have been reported where it has been showed that ethanol extracts of *P. marsupium* exhibited significant anti-ulcer and antioxidant properties in rats (Nair et al., 2005; Patil & Gaikwad, 2011).

3.6. Anti-inflammatory activity

P. marsupium has also shown strong potential for its antiinflammatory activity. In this study, an extract of *P. marsupium* containing pterostilbene has been evaluated for its PGE2- inhibitory activity in LPS-stimulated PBMC. In addition, the COX-1/2 selective inhibitory activity of *P. marsupium* extract was investigated (Hougee et al., 2005; Salunkhe et al., 2005).

3.7. Central Nervous System

The methanol extracts of *P. marsupium* has potent nootropic activity (Chauhan and Chaudhary, 2012).

3.8. Other Studies

Anti-cataract activity of *P. marsupium* in diabetes was observed (Vats et al., 2004).

Lukewarm aqueous suspension of 2g gum with jaggery is given early in the morning for a week to treat asthma (Patil et al., 2008).

Bark is useful in vitiated condition of kapha, pitta, elephantiasis, erysipelas, urethrorrhea, rectalgia, opthalmopathy, hemorrhages, dysentry, cough, and grayness of hair (Patil and Gaikwad., 2011).

20g of the stem bark boiled with 1 litre of water till 200ml along with 7 black pepper dried seeds of *Piper nigrum*) taken orally cures spermaturia, spermatorrhea, leucorrhoea, amenorrhoea, dysmennorhoea, menorrhagia and impotency (Behera and Mishra ., 2005).

Table 1. Primary chemical components from *Pterocarpus marsupium*

Neem compound	Source Biological	Reference
Liquiritigenin bark	Antidiabetic, Antihyperlipidemic effect	Jahromi & Ray 1993
Isoliquiritigenin bark	Antidiabetic	Jahromi & Ray 1993
Pterosupin	Antihyperlipidemic effect	Jahromi & Ray 1993
Epicatechin bark	Antidiabetic, Anthelmentic properties	
Pterostilbene bark	blood glucose levels, Anti-oxidant and anti tumor effects	Grover et al., 2005
Marsupinol bark	Antihyperlipidemic effect	Jahromi & Ray 1993

Table 2. Some medicinal uses of *Pterocarpus marsupium* as mentioned in Ayurveda

Parts	Medicinal use
Leaf	External application for boils, sores and skin diseases, stomach pain
Bark	Astringent, toothache
Flower	Fever
Gum-Kino	Diarrhea, dysentery, leucorrhoea, passive haemorrhages

3.9. Clinical studies and plausible medicinal applications

Although studies have been carried out on various biological activities of *P. marsupium* extracts and some of the isolated compounds in several animal models, a few reports are available on clinical studies with the extracts or the compounds and their medicinal applications (Anon, Wealth of India, 2003). *Pterocarpus marsupium* (PMS, *Leguminnaceae* family), commonly known as Bija, that has been

recommended as early as 1000 BC, by Sushruta for the treatment of diabetes. Various reports indicate the hypoglycemic activity of PMS both in experimental and clinical studies (Pandey & Sharma 1976; Remsberg et al., 2008; Manlio et al., 2005).

4. Aim and objective of the study

Since pre-historic days attempts are being made to find out suitable drugs from natural sources for treatment of different diseases. The rational approaches, experience of folk medicine provide a valuable approach in the search for the development of new and useful therapeutic agents. Gradually keeping in pace with the scientific interpretations of the drug actions, the causes of the diseases, and the development in the field of chemistry and technology, intensive efforts are being directed towards the design and synthesis of new drugs.

More recently a study by the World Health Organization (WHO) has shown that about 80% of the World's population still relies on traditional medicine. This is of interest to a natural product chemist for many reasons. There is the possibility that the herb used in the traditional medicine is harmful to the patient, in which case the treatment may do more harm the good. Conversely, there is the possibility that the herbs used are not effective at all. That may not be of concern for minor ailments, but in more serious cases an ineffective treatment could result in the death of the patient. Hopefully, however, the herbs used are effective. If that is the case then investigation of that remedy could be of benefit to the remaining 20% of the World's population.

Natural products still play a very important role in the medicine of the remaining 20% of the world's population. Between 1983 and 1994, 41% of new approved drugs have natural products as their source. This percentage becomes even higher when one examines anti-infective and anticancer compounds. For both classes, the percentage of drugs with natural products as their source increases to over 60%.

Because natural products are most important in the areas of anti-infective, antioxidants and anticancer agents, some of the important contributions to these drug classes are worth closer inspection. In particular the anticancer drugs will be examined as that is the area of our research.

Cancer is a multifactorial, multifaceted and multimechanistic disease requiring a multidimensional approach for its treatment, control and prevention. Cancer involves fundamental biological processes concerning disorganised cell replication, cell death and disorganization of organ structure. The annual estimates of cancer for the year 2001 in India is 0.98 million and the annual mortality in 2000 is 0.7 million. The

incidence of cancer is on the rise, with multiple risk factors that involve interplay between genetic and environmental components.

The aim of the research is to find out new anticancer drugs from indigenous plants which are potent and nontoxic agents. These plants are traditional medicinal plants. Their chemical characterization, mode of action and toxicity studies are yet to be established. Present project deals with phytochemical and pharmacological evaluation of *Triumfetta rhomboidea*, *Adiantum venustum*, leaves and stem with special reference to *in-vitro* studies like antioxidant, cytotoxicity and *in-vivo* studies like anticancer, antioxidant, analgesic, antipyretic, anti-inflammatory, and wound healing in animal models. Normally herbal products are free from side effects/adverse effect and they are low cost medicines, which will be beneficial for the people of this country. Keeping this in view, we have selected two plants from the Kolli hills, Namakkal District of Tamil Nadu which are used by the tribes for the treatment of different types of diseases. The main objective of this work is to develop potent anticancer agent having no minimum side effects from indigenous plants for the therapeutic management.

5. Plan of work

Phase I:

5.1. Phytochemical studies:

- Collection and authentication of plants.
- Extraction of plant material by using a suitable solvent system.
- Preliminary phytochemical study for the identification of plant secondary constituents.

Phase II:

5.2. Pharmacological studies:

- Acute oral toxicity and sub-acute toxicity studies
- ***In-vitro*** **antioxidant studies:**

  DPPH assay

  Scavenging of Nitric Oxide radical assay

  Scavenging of ABTS radical cation assay

  Hydroxyl radical scavenging activity

- ***In-vitro*** **Cytotoxicity studies:**

Determination of cell viability by MTT assay

Cytotoxicity tests are being used for the anticancer screening of drugs. A novel anticancer drug should possess cytotoxicity at low concentration against cancerous cell line and should be safe against normal cell lines even at higher concentrations.

Cytotoxicity can also be monitored using the MTT or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. Hence, the ethanol extract of *Pterocarpus marsupium* bark was tested for cytotoxicity study by Micro culture Tetrazolium (MTT) assay method (Sreejayan & Rao 1996; Halliwell & Gutteridge 1998; Scudiero et al., 1998; Ruby et al., 1995; Francis & Rita, 1986).

Phase III:

5.3 Statistical Analysis

The data's were presented as mean ± SEM and were subjected to statistical analysis by Dunnett's test followed by one way ANOVA. P-value less than 0.05 were considered statistically significant.

6. Materials And Methods

6.1. Phytochemical Studies

Collection and Authentication of the Plant

The bark of *P. marsupium* were collected in the month of October 2015, from Kolli Hills, Namakkal district of Tamil Nadu state, India. The collected plants were authenticated by the Dr. G. Murthy, Botanical survey of India, Coimbatore, Tamil Nadu and the voucher specimen has been preserved in our laboratory for future reference (BSI/SC/5/PG/15-16/OCT).

6.1.1 Extraction of *Pterocarpus marsupium* bark using ethanol

The bark of the tree were dried under shade with occasional shifting and made into coarse powder with a mechanical grinder. The powder material were first passed through sieve No.40 and then defatted with petroleum ether. The defatted powder materials thus obtained were further extracted by ethanol for 72 hours in a Soxhlet extraction apparatus. The solvent was removed under reduced pressure and a semisolid mass was vacuum dried so yields a solid residue. After complete drying, the extract material was weighed and the extractive value in percentage was calculated with reference to the air dried sample (Harborne 2005). Thus an ethanol extract of *Pterocarpus marsupium* bark (EEPM) was obtained.

6.1.2. Preliminary phytochemical studies on ethanol extract of EEPM bark

The ethanol extracts were subjected to qualitative tests for the detection of various plant constituents like carbohydrates, glycosides, proteins and amino acids, fixed oils and fats,gums and mucilages, alkaloids, phytosterols, flavanoids, tannins and phenolic compounds, saponins, triterpinoids etc.

Phytochemical Test

The methanol extracts of *P. marsupium was* subjected to various colour reactions to identify the nature of the components (Krishnaswamy, 2003).

i. Test for Carbohydrates

Small quantities of the extracts were dissolved separately in 4ml of distilled water and filtered and the filtrate was subjected to Molisch's test to detect the presence of carbohydrates.

a) Molisch's test: The Molisch's reagent was prepared by dissolving 10 gm of α-napthol in 100ml of 95% alcohol. 1ml of filtrate was mixed with 2 drops of Molisch's reagent. To this solution, was added 1ml of concentrated sulphuric acid from the side of the inclined test tube, so that two acids formed a layer beneath the aqueous solution without mixing with it. If a red brown ring appears at the common surface of the liquids, sugars are present

b) Iodine Test: The iodine solution was prepared by dissolving 2 gm of iodine and 3 gm of potassium iodide in 100ml water. 1ml of above filtrate was mixed with iodine solution, if blue colour appears, sugar is present.

ii. Test for Glycosides

A few mg of residue was dissolved in 4ml of distilled water and filtrated, and collected the filtrate.

a) **Legal Test:** 2ml of filtrate was hydrolysed with dilute hydrochloric acid and heated on a water bath. Then added 1 ml of pyridine and few drops of sodium nitroprusside solution and made alkaline with sodium hydroxide solution. Appearance of pink colour shows the presence of glycosides.

b) **Borntrager's test:** 2ml of filtrate was hydrolyzed with dilute hydrochloric acid and heated on a water bath then treated with chloroform and shaken, after that separated the chloroform layer and added equal quantity of dilute ammonia solution.

iii. Test for Flavonoids

a) 5ml of extract was hydrolyzed with 10% v/v sulphuric acid and cooled. Then it was extracted with diethyl ether. The ether extract was divided into three portions in three separate test tubes. 1ml of dilute ammonia, 1ml of dilute sodium bicarbonate and 1ml of 0.1N sodium hydroxide were added to the 1^{st}, 2^{nd} and 3^{rd} test tubes respectively. In each test tube development of yellow colour indicates the presence of flavonoids.

b) A few mg of extract was mixed with dilute sulphuric acid. Development of yellow to crimson colour shows the presence of flavonoids.

c) Shinoda test: The extracts were dissolved in alcohol. One piece of magnesium followed by concentrated hydrochloric acid was added drop wise

and heated. Appearance of magenta colour demonstrated the presence of flavonoids.

iv. Test for Phytosterols

a) Salkowaski test: 10 mg of extract was dissolved in 2ml of chloroform and 2ml of concentrated sulphuric acid was added from the side of the test tube. The test tube was shaken for few minutes. The development of red colour in chloroform layer indicated the presence of sterols.

b) Liebermann's test: To 10 mg of the extract in a test tube, 1ml of acetic anhydride was added and gently heated. The contents of test tube were cooled. Few drops of concentrated sulphuric acid were added from the side of the test tube. A blue colour gave the evidence of presence of sterols.

c) Liebermann-Burchard Test: 1ml of concentrated sulphuric acid was added to 10 mg of extract in 1ml of chloroform. A reddish-blue colour exhibited by chloroform layer and green fluorescence by the acid layer suggests the presence of sterols.

d) Few mg of extract was dissolved in vanillin solution (100 mg of vanillin dissolved in concentrated sulphuric acid-ethanol in proportion of 4:1) development of Blue to brown colour indicates the presence of sterols.

v. Test for Alkaloids

Few mg of extract was taken in 5ml of 1.5% v/v hydrochloric acid and filtered. These filtrates were then used for testing alkaloids.

a) **Dragendorff's reagent:** The extract was treated with Dragendorff's reagent (Potassium bismuth iodide). Formation of orange-brown precipitate shows the presence of alkaloids.

b) Mayer's reagent: It was prepared by dissolving 1.36 gm of mercuric chloride in 60ml distilled water, added it to a solution of 5 gm of potassium iodide in 20 ml distilled water making volume to 100ml. To a 1ml of test filtrate in a watch glass, a few drops of above reagent were added. The formation of cream coloured precipitate shows the presence of alkaloids.

c) **Wagner's Reagent:** It was prepared by dissolving 1.27 gm of iodine and 2 g of potassium iodide in 5ml of water and made up the volume to 100ml with distilled water.

When few drops of this reagent was added to the filtrate, if it shows reddish brown precipitate indicates the presence of alkaloids.

d) **Hager's reagent:** A saturated aqueous solution of picric acid was employed for this test. When the test filtrate was treated with this reagent, an orange yellow precipitate was formed which indicates the presence of alkaloids.

vi. Test for Tannins

The test extract was taken in water, warmed and filtered. Tests were carried out with the filtrate using following reagents.

a) **Ferric chloride test:** 5ml of filtrate was allowed to react with 1ml of 5% ferric chloride solution. If dark green or deep blue colour is obtained, tannin is present.

b) **Lead acetate test:** 5ml of filtrate was treated with 1ml of 10% lead acetate solution in water. Yellow colour precipitate demonstrated the presence of tannins.

c) **Potassium dichromate test:** 5ml of filtrate was treated with 1ml of 10% aqueous potassium dichromate solution. If yellowish-brown precipitate formed, it suggests the presence of tannins.

d) Bromine water test: Bromine solution was added to the test filtrate. If depolarization of bromine water occurs, tannins are present.

e) **Gelatin Solution test:** 1% w/v solution of gelatin in water, containing 10% sodium chloride was prepared. A little of this solution was added to the filtrate. If white precipitate is obtained, tannins are present.

vii. Test for Saponins

a) Foam test: 1ml solution of extract was diluted with distilled water to 20ml and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

b) 1ml extract was treated with 1% lead acetate solution. Formation of white precipitate indicates the presence of saponins.

viii. Test for Terpenoids

a) **Knoller's test:** 5 mg of extract is treated with 2ml of 0.1% anhydrous stannic chloride in pure thionyl chloride. A deep purple colour that changes to red indicates the presence of terpenoids.

ix. Test for Proteins and Amino Acids

Small quantity of the extracts were dissolved in few ml of water and filtered. Filtrate was treated with following reagent.

a) **Millons reagent:** It was prepared by dissolving 3 gm of mercury in 2ml of fuming nitric acid keeping the mixture well cooled; this solution was then diluted with equal quantity of distilled water. 2ml of residual filtrate was taken and to it, 3ml of millions reagent was added. The white precipitate slowly turns to pink, if proteins are present.
b) **Biuret test:** 1ml of filtrate was taken in water and 1ml of 4% ($CuS0_4$) copper sulphate was added to it. Violet or pink colour is formed, if proteins are present.
c) **Xanthoprotein test:** A little amount of filtrate was taken in 2ml of water and 0.5ml of concentrated nitric acid was added to it. Yellow colour is obtained if proteins are present.
d) **Ninhydrin test:** The ninhydrin reagent is 0.1% w/v solution of ninhydrin in n-butanol. A little of this reagent was added to the test extract. A violet or purple colour is developed, if amino acids are present.

x. Test for Resins

a) Few mg of extract was treated with caustic soda; a red colour develops if resins are present.
b) Few mg of extract was treated with dilute sulphuric acid; a red colour develops, if resins are present. The results were shown in table no.5.

6.2. Toxicological Studies

6.2.1. Acute oral toxicity of *Pterocarpus marsupium* bark

Acute oral toxicity is performed as per OECD guide line 423. Swiss albino mice of female sex weighing 20-25gms were used for the study. The animals were fed with standard mouse diet, had free access to water under well ventilated condition of hours light cycle. They were kept in metal cages with wood shavings as bedding and were adapted to laboratory condition for 7 days prior to the experiments. The study was carried out according to the organization for economic co-operation and Development (OECD) principles (423) (OECD, 2001).

The animals were fasted prior to dosing according to guidelines, food but not water was withheld for 3-4 hours. Following the period of fasting, the animals were weighed to determine the appropriate dose of test drug to be administered to animals.

In all the cases the maximum volume of aliquot portion of the test drug administered did not exceed 1ml/100g of the animal body weight as per guidelines. The animals were randomly assigned to cages for grouping and individual animals were marked with picric acid for identification.

Three animals were used for each step: Since, there is no information on the toxicity of the extracts to be tested, for animal welfare reasons it is decided to use the starting dose of 5mg/kg extract suspended in CMC solution. The test extract is administered orally to first animal and the dosing of second animal is delayed to observe the toxicity of the previously dosed animals. Once no toxic signs were observed in previous dosed animals, third animal is dosed. The animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter for 14 days.

- The animals dosed with 5mg/kg showed no sign of toxicity; the second step is testing the animal with higher dose 50mg/kg. The animals were dosed individually with 50mg/kg and observed for toxic effects.
- The second group animals showed no sign of toxic effects. The third step of testing with 300mg/kg dose. The animals were dosed individually with 300mg/kg and observed.
- The third group animals dosed with 300mg/kg also showed no sign of toxic effects. It is decided to increase the dose to 2000mg/kg in fourth step. The animals were administered orally with 2000mg/kg extract suspended in CMC & observed for toxic effects for 14 days.
- With 2000mg/kg the animals showed no sign of toxic effects; the test extract was found to be nontoxic at 2000mg/kg.

6.2.2. Sub-acute toxicity of *Pterocarpus marsupium* bark

The repeat dose 28 day oral toxicity study was carried out according to OECD guideline, 407 (OECD, 1995). A total of twenty apparently healthy mice of either sex selected randomly into four groups were placed into four different cages (OECD, 1995).

Group I received 10ml/kg of 0.9% saline water and served as control. Group 2, 3 and 4 received test doses of 300, 900 and 2700 mg/kg, respectively of methanol extract of both *P. marsupium.* The animals were dosed daily for a period 28 days and the doses were given at similar time each day. Adjustment was made to maintain a constant dose level in term of animal body weight.

Animals were observed at least twice daily for morbidity and mortality. All the animals were weighed weekly. Animals that survive after 28 days were anaesthetized on the 29th day with chloroform and blood sample for haematological and biochemical analysis were collected and plain vials respectively. The packed cell volume (PCV) was determined by the microhaemocrit method. The haemoglobin concentration (Hb) was evaluated by cyto methaemogloin method using Beckman model of spectrophotometer. White Blood Count (WBC) and Red Blood Count (RBC) were determined using improved Neubauer Haemocytometer. Plasma concentration of glucose, total protein, albumin, globulin and urea were analysed using humalyzer 2000. Necroscopy of all animals was carried out and the organ was then standardized for percentage body weight of each mouse. Subsequently, the animal organs harvested (the brain, liver and kidney) were fixed in 10% formalin saline solution for histopathological examination. Thin crystal sections were stained with haematoxylin and eosin, periodic and Schiff reagent with and without diastase, respectively. The sections were examined under light microscope at high (400x objective) power magnification

6.3. Pharmacological Screening

6.3.1. *In-vitro* anti-oxidant activity of *Pterocarpus marsupium* bark

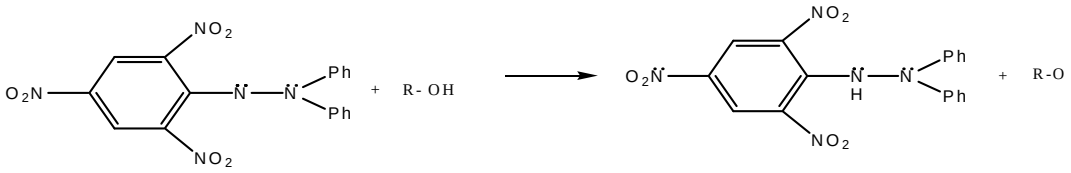
The *in vitro* methods are based on inhibition. Samples are added to a free radical-generating system, inhibition of the free radical action is measured and this inhibition is related to antioxidant activity of the sample. Methods vary greatly as to the generated radical, the reproducibility of the generation process, and the endpoint that is used for the determination. The extract was tested for *in vitro* antioxidant activity using several standard methods. The final concentration of the extract and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.812 μg/ml. The absorbance was measured spectrophotometrically against the corresponding blank solution. The percentage inhibition was calculated by using the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD control - OD sample}}{\text{OD control}} \times 100$$

IC_{50}, which is the concentration of the sample required to scavenge 50 % of free radicals was calculated.

Because of the vast utility of the antioxidants in treatment of various diseases, the following antioxidant activities were investigated on the ethanol extracts of the *P. marsupium* (Sing et al., 2008; Halliwell, 1996; Jayaprakasha et al., 2004).

i) DPPH Assay: DPPH radical scavenging activity was assessed, according to the method of Blois et al (1958). DPPH is a stable free radical with red color which turns yellow color when scavenged. Antioxidants react with DPPH and reduce it to DPPH-H and as a result the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability.



Purple color DPPH Yellow Stable phenoxy radical (2, 2-diphenyl-1-picryl hydrazyl) is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in the absorbance is measured at 490 nm.

Procedure: The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of various concentrations of the extract or the standard solution was added separately in wells of the microtitre plate. The plates were incubated at 37 ºC for 30 min. Absorbance was measured at 517 nm using ELISA reader.

ii) Scavenging of Nitric Oxide radical: The assay was done according to the method of Marcocci *et al* (1994). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modified Griess Ilosvay reaction. In

the present investigation, Griess Ilosvay reagent is modified by using naphthyl ethylenediamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed was measured at 540 nm.

iii) Scavenging of 2, 2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid) diammonium salt) (ABTS) radical cation assay: Total antioxidant potential of extract was determined by scavenging of 2, 2-azinobis (3-ethyl benzoline-6-sulphonic acid) diammonium radical (ABTS) radical cation based on the procedure described by Re *et al.* 1999. ABTS assay is relatively recent one, which involves a more drastic radical, chemically produced and, is often used for screening complex antioxidant mixture such as plant extracts, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS radical for the estimation of the antioxidant activity.

iv) Hydroxyl radical scavenging activity:

There is increasing evidence that hydrogen peroxide, either directly or indirectly via its reduction product hydroxyl radical ($OH^{\bullet}$) causes severe damage to biological systems. Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium based on the method of Halliwell *et al.,* (1987). This assay is based on the quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by Fe3+ ascorbate, EDTA-H2O2 system. The reaction mixture containing FeCl3 (200µM), EDTA [1.04mM], H2O2 (1mM) and 2-deoxy-D-ribose (2.8mM) were mixed with or without extract at various concentration (125-2000µg/ml) in 1mL final reaction volume made with potassium phosphate buffer (20mM, pH 7.4) and incubated for 1 h at 37º C. The mixture was heated at 95ºC in water bath for 15 min followed by addition of 1mL each of TCA (2.8%) and TBA (0.5% TBA in 0.025M NaOH containing 0.02% BHA). Finally, the reaction mixture was cooled on ice and centrifuged at 5000rpm for 15 min. Absorbance of the supernatant liquid was measured at 532nm. The negative control without any antioxidant or extract was considered 100% deoxyribose oxidation. The percentage hydroxyl radical scavenging activity of test sample was

determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive.

6.3.2 *In-vitro* Cytotoxicity activity of *Pterocarpus marsupium* bark

Cytotoxicity tests are being used for the anticancer screening of drugs. A novel anticancer drug should possess cytotoxicity at low concentration against cancerous cell line and should be safe against normal cell lines even at higher concentrations.

Cytotoxicity can also be monitored using the MTT or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. Hence, the ethanol extract of *Pterocarpus marsupium* bark was tested for cytotoxicity study by Micro culture Tetrazolium (MTT) assay method (Sreejayan & Rao 1996; Halliwell & Gutteridge 1998; Scudiero et al., 1998; Ruby et al., 1995; Francis & Rita, 1986).

Cell line and culture medium

HT-29 (Human, colorectal cancer), HeLa (Human, Epithelial cervical cancer) and C_2C_{12} (Mouse, Muscle cell line) cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of HT-29, C_2C_{12} and HeLa were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stockcultures were grown in 25 cm^2 culture flasks and all experiments were carried out in either 96 microtitre plates.

Preparation of test solutions

For cytotoxicity studies, each weighed extracts and essential oils were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not

reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using DMEM medium containing 10% FBS/NBCS. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µl of different test concentrations were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the extract solutions in the wells were discarded and 50µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of drug or test extract needed to inhibit cell growth by 50 % (CTC_{50}) values is generated from the dose-response curves for each cell line (Senthilkumar et al., 2007; Santhoskumar et al., 2007; Vijayan et al., 2003).

$$\%\ Growth\ Inhibition = 100 - \left(\frac{Mean\ OD\ of\ individual\ test\ group}{Mean\ OD\ of\ control\ group} X\ 100 \right)$$

6.4 Statistical Analysis

The experimental results were expressed as the mean ± S.E.M. Data were assessed by the method of analysis of ANOVA, followed by Dunnet's t-test; p value of < 0.05 was considered as statistically significant.

5. RESULTS AND DISCUSSION

5.1. Phytochemical studies

5.1.1. Extraction

Preliminary Phytochemical studies on ethanol extracts of *P. marsupium* bark

Table No.4 Data showing the extractive value of ethanol extract of *Pterocarpus marsupium* bark

Plant Name	Parts used	Method used	Solvent Used	%w/w yield Using
Pterocarpus marsupium	Leaves	Continuous hot percolation process	Ethanol (95 % v/v)	7.06

Table No. 5 Data showing the preliminary phytochemical screening of the ethanol extracts of the *Pterocarpus marsupium* bark

Sl. No	Phytoconstituents	*P. marsupium*
1.	Carbohydrates	+
2.	Glycosides	+
3.	Alkaloids	-
4.	Phytosterol and steroids	+
5.	Flavonoids	+
6.	Proteins&Amino Acids.	+
7.	Tannins	-
8.	Resins	-
9.	Gums and mucilages	-
10.	Triterpenoids	+

+ Present; -Absent

RESULT

The dried powder materials of bark of *P. marsupium* were extracted with ethanol in a Soxhlet apparatus. The yield was 7.06 % w/w. The tested ethanol extracts of *P. marsupium* showed the presence of phytoconstituents such as carbohydrates, sterols,

glycosides, phytosterols and steroids, proteins & amino acids, flavonoids and triterpenoids. The results are shown in Table No.4 and 5.

5.2. Toxicological Studies

5.2.1. Acute oral toxicity of *Pterocarpus marsupium* bark

Acute oral toxicity was performed as per OECD guide line 423

Table No.10 Acute oral toxicity study (423) observations

Respiratory Blockage Innostril	
Dyspnoea	Nil
Apnoea	Nil
Tachypnea	Nil
Nostril discharge	Nil
Motor Activities	
Locomotion	Normal
Somnolence	Nil
Loss of righting reflex	Nil
Anaesthesia	Nil
Catalepsy	Nil
Ataxia	Nil
Toe walking	Nil
Prostration	Nil
Fasciculation	Nil
Tremor	Nil
Convulsion (Involuntary Contraction)	
clonic/tonic/tonic-clonic convulsion	Nil
Asphyxia convulsion	Nil
Opistotones (titanic spasm)	Nil

RESULT AND DISCUSSION

The acute oral toxicity study of methanol extracts of *P. marsupium* was determined as per the OECD guidelines 423 (Acute toxic class method). The animals were observed for 14 days period for any signs of delayed toxicity. Both the extracts have good

margin of safety. The extracts did not show lethal effect on albino mice up to the dose of 2000mg/kg. Hence, submaximal doses, 1/10th (200mg /kg) and 1/20th (100mg/kg) of maximum dose were selected for study.

Table No. 12 Mean body weight of mice after 28 days treatment with EEPM bark

Treatment and Dose mg/kg	Day 1	Day 7	Day 14	Day 21	Day28	Mean wt. gain
Control	25.09 ±1.14	25.85±0.15	26.25±1.21	27.41±0.45	28.51±0.12	3.06
300 EEPM	24.90±025^{ns}	25.12±0.12^{ns}	26.61±0.45^{ns}	27.54±0.13^{ns}	28.52±0.58^{ns}	3.65
900 EEPM	22.59±0.11^{ns}	23.69±0.21^{ns}	24.36±0.65^{ns}	25.16±0.15^{ns}	26.12±0.65^{ns}	3.87
2700 EEPM	23.56±0.26^{ns}	24.21±0.15^{ns}	25.15±0.14^{ns}	26.12±0.26$^{\ns}$	27.36±0.18^{ns}	3.9

Data are mean ± SEM; n=5 A ANOVA; *P < 0.05; **P < 0.01; ***P < 0.001
ns: non-significant (between control and experimental group)

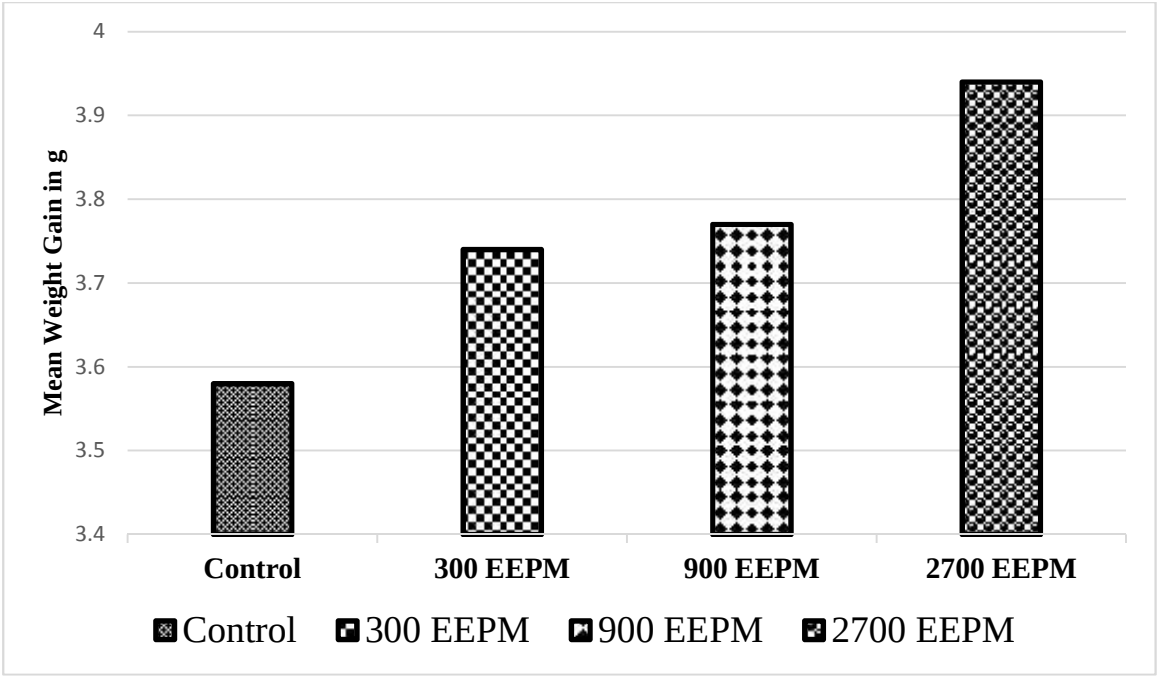


Table No. 13 Haematological parameters of mice after 28 days treatment with EEPM bark

Treatment and Dose mg/kg	RBC(x$10^6 L^{-1}$)	Hb (g/ L^{-1})	PCV (%)	WBC (%)
Control	4.18±0.22	7.19±0.25	21.11±0.52	4.71±1.28
300 EEPM	3.57±0.56 ^{ns}	6.55±1.5 ^{ns}	23.15±0.11 ^{ns}	4.15±0.17 ^{ns}
900 EEPM	4.11±0.12 ^{ns}	6.69±0.21 ^{ns}	20.52±0.62 ^{ns}	3.53±0.61 ^{ns}
2700 EEPM	3.88±0.15 ^{ns}	6.08±0.14 ^{ns}	22.16±0.98 ^{ns}	4.15±0.31 ^{ns}

Data are mean ± SEM; n=5 A ANOVA; *P < 0.05; **P < 0.01; ***P < 0.001
ns: non-significant (between control and experimental group)

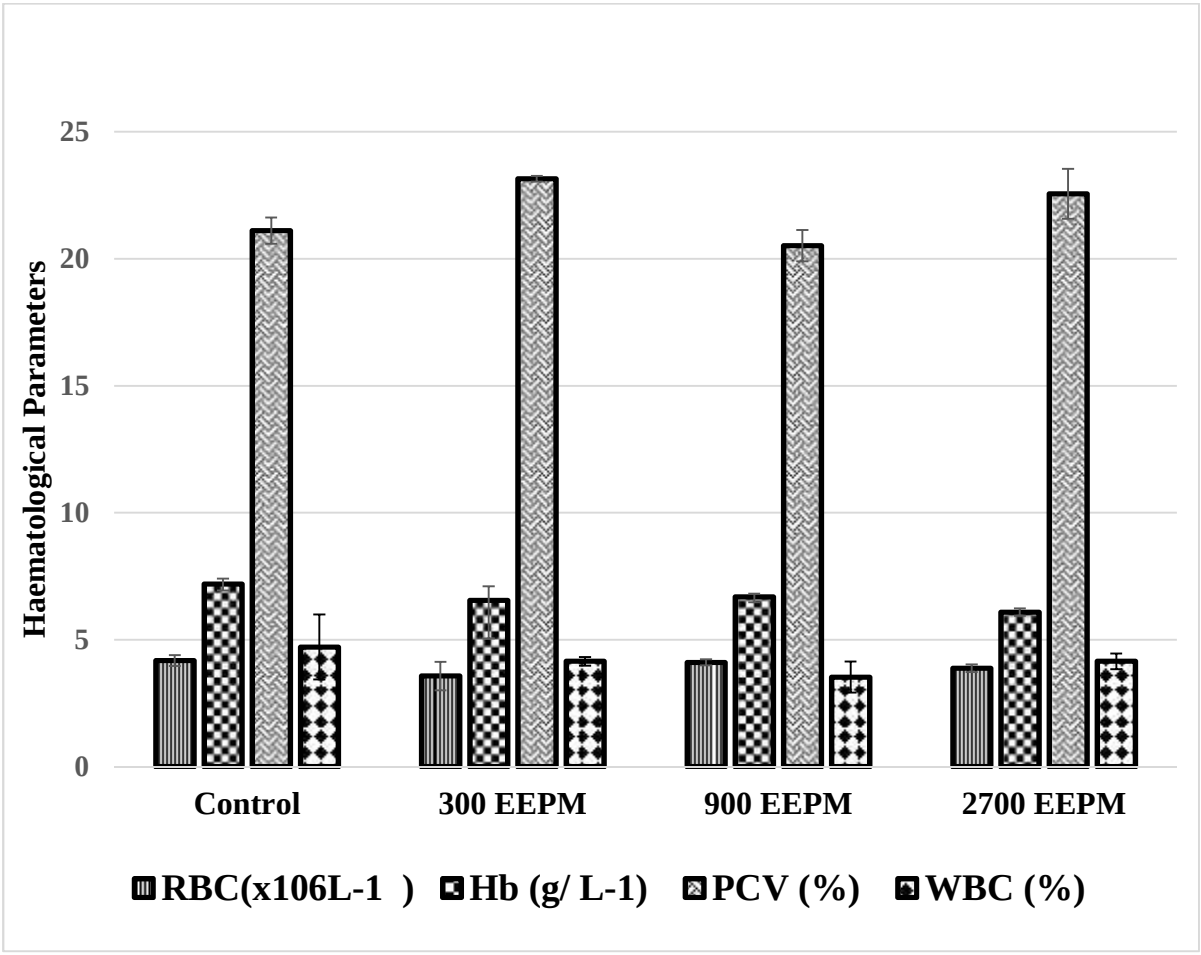
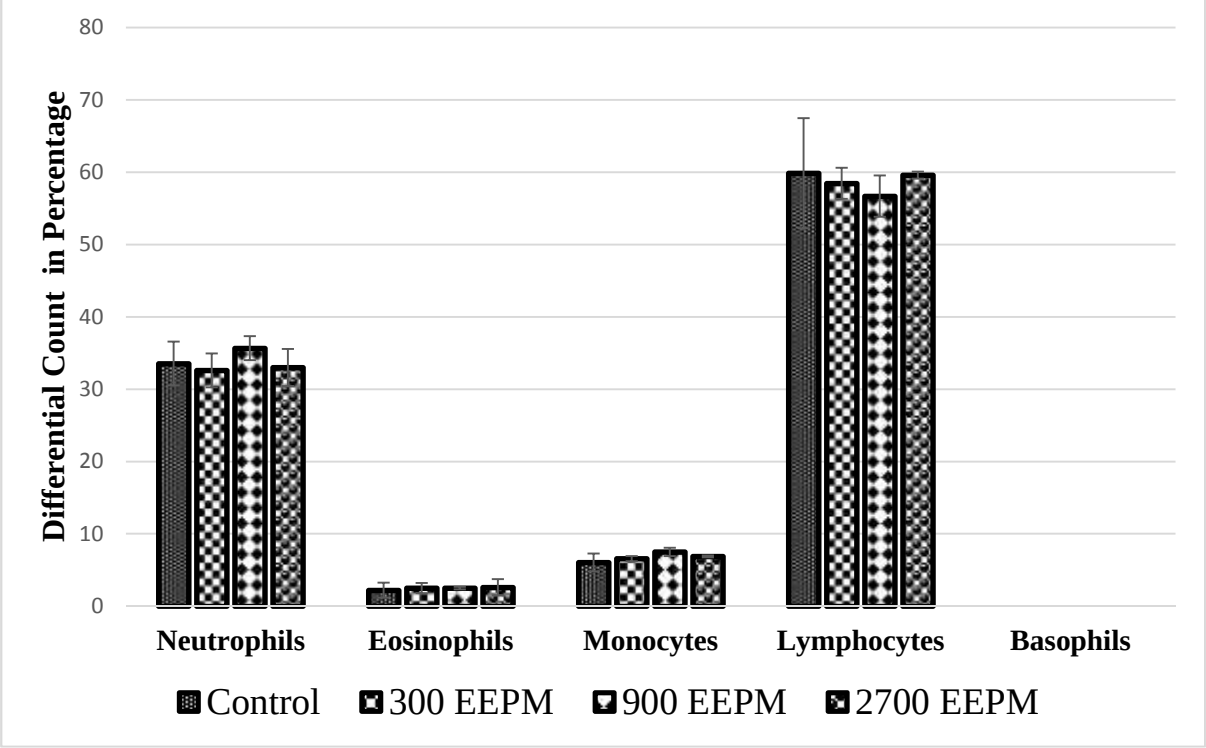


Table No. 14 Leucocytes differential count of mice after 28 days treatment EEPM bark

Treatment and Dose mg/kg	Neutrophils (%)	Eosinophils (%)	Monocytes (%)	Lymphocytes (%)	Basophils (%)
Control	33.54 ±3.05	2.2±1.05	6.05±1.25	59.85±7.6	0.00
300 EEPM	32.59±2.35 ns	2.5±0.71 ns	6.58±0.35 ns	58.45±2.15 ns	0.00
900 EEPM	35.66±1.67 ns	2.5±0.16 ns	7.5±0.57 ns	56.68±2.85 ns	0.00
2700 EEPM	32.98±2.56 ns	2.6±1.12 ns	6.88±0.14 ns	59.58±0.48 ns	0.00

Data are mean ± SEM; n=5 A ANOVA; *P < 0.05; **P < 0.01; ***P < 0.001
ns: non-significant (between control and experimental group)



RESULT AND DISCUSSION

From the sub-acute toxicity studies of EEPM, it was observed that there was no significant difference in the mean organ weight between the control and the animals treated with the EEPM (Table No. 11). Similarly, there was also no significant difference in the mean body weight between control and the animals treated with EEPM (Table No.12).

Haematological analysis showed no significant difference in RBC, Hb, PCV and WBC between control and extract treated groups (Table No.13). The leukocyte differential count between control and extract treated group were also non-significant (Table- 14)

The results of biochemical study also showed no significant difference between the control and the animals treated with the extracts in all the biochemical parameters tested. Observation for gross pathological lesions of the organs examined immediately after dissection showed no visible lesion.

Histopathological examination of liver, kidney and the brain in the control and the animals treated with the extracts showed no lesion that could be attributed to the effect of oral administration of EEPM on mice for 28 days.

The sub-acute toxicity findings therefore indicate that the extracts appeared to have low toxicity and could be as well tolerated for the 28 days study period.

The observations of both acute and sub-acute toxicity could be an indication that EEPM has some high level of safety margin in oral administration.

5.3. Pharmacological Screening

4.3.1 *In vitro* Antioxidant Activity of EEPM bark

Table No.15 *In vitro* DPPH antioxidant activity of EEPM bark

DPPH			
IC_{50} values ± SEM «g/ml* by methods			
Sl:No	Con «g/ml	Percentage inhibition	
		Standard Ascorbic acid	EEPM
1	10	45	40
2	20	48	44
3	40	56	49
4	80	67	55
5	160	88	67
IC_{50} value		**27.43**	**57.03**

*Average of three determinations, values are mean ± SEM

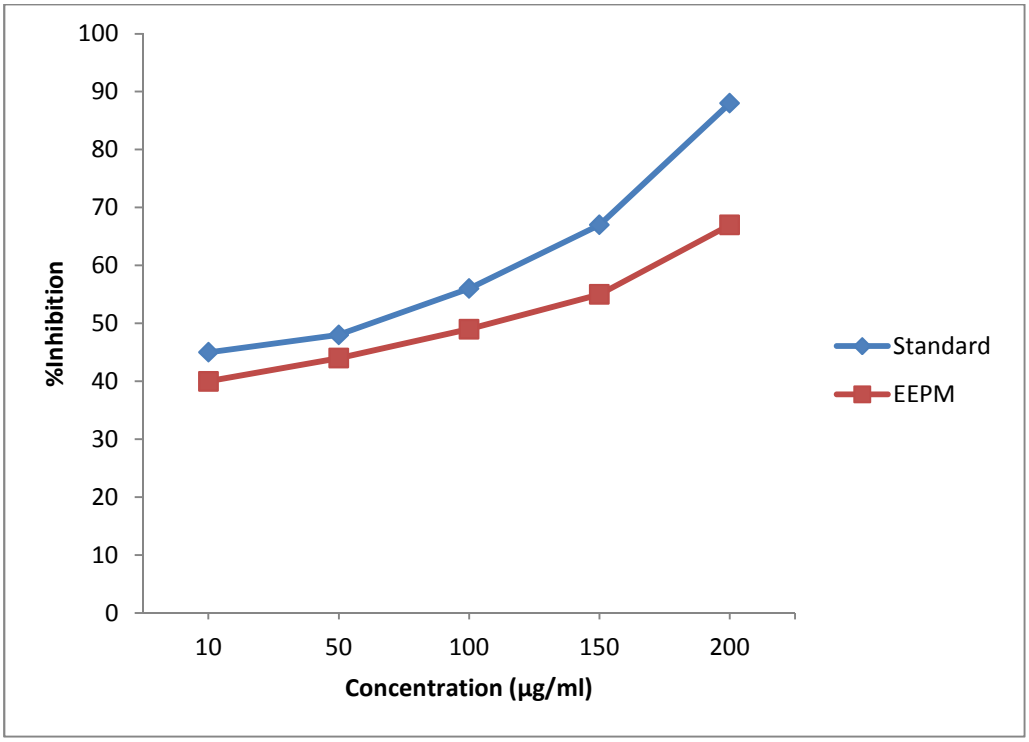


Table No.15 *In vitro* nitric oxide antioxidant activity of EEPM bark

Nitric oxide			
IC_{50} values ± SEM «g/ml* by methods			
Sl:No	**Con «g/ml**	**Percentage inhibition**	
		Standard Rutin	**EEPM**
1	10	46	23
2	20	52	28
3	40	58	40
4	80	67	64
5	160	89	82
IC_{50} value		22.76	59.13

*Average of three determinations, values are mean ± SEM

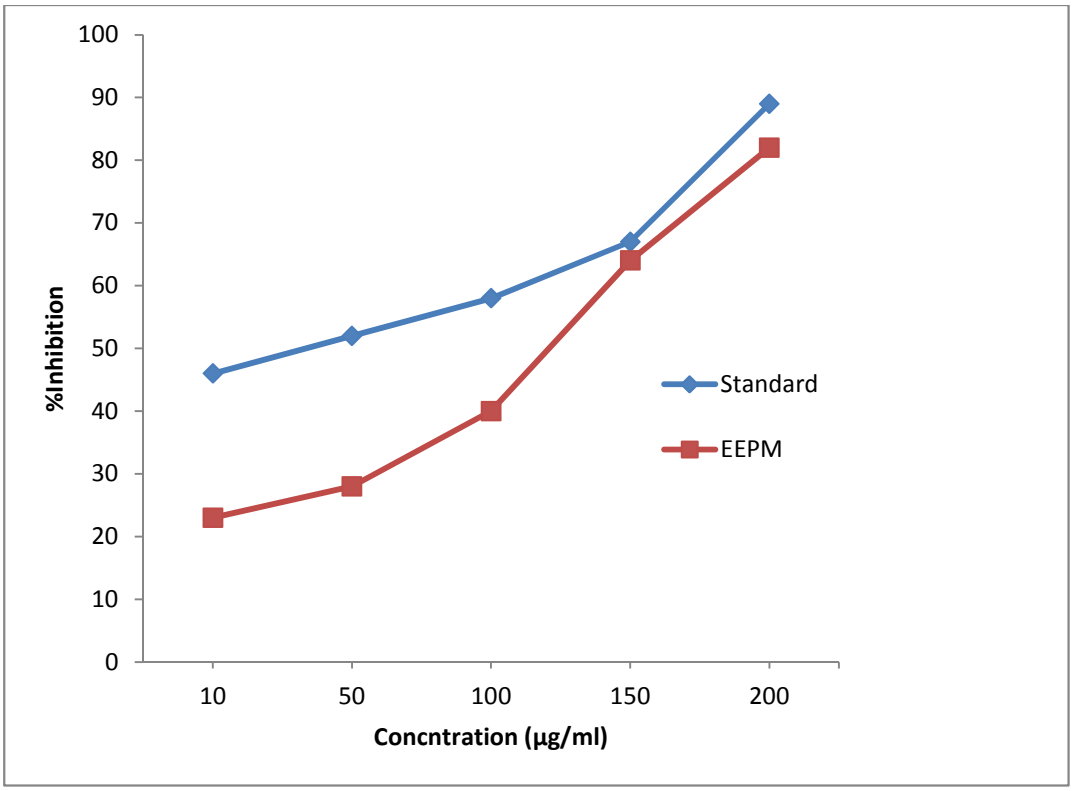


Table No.15 *In vitro* ABTS antioxidant activity of EEPM bark

ABTS			
IC_{50} values ± SEM «g/ml* by methods			
Sl:No	Con «g/ml	Percentage inhibition	
		Standard Rutin	EEPM
1	10	41	12
2	20	54	20
3	40	65	36
4	80	81	63
5	160	92	75
IC_{50} value		**16.23**	**61.62**

*Average of three determinations, values are mean ± SEM

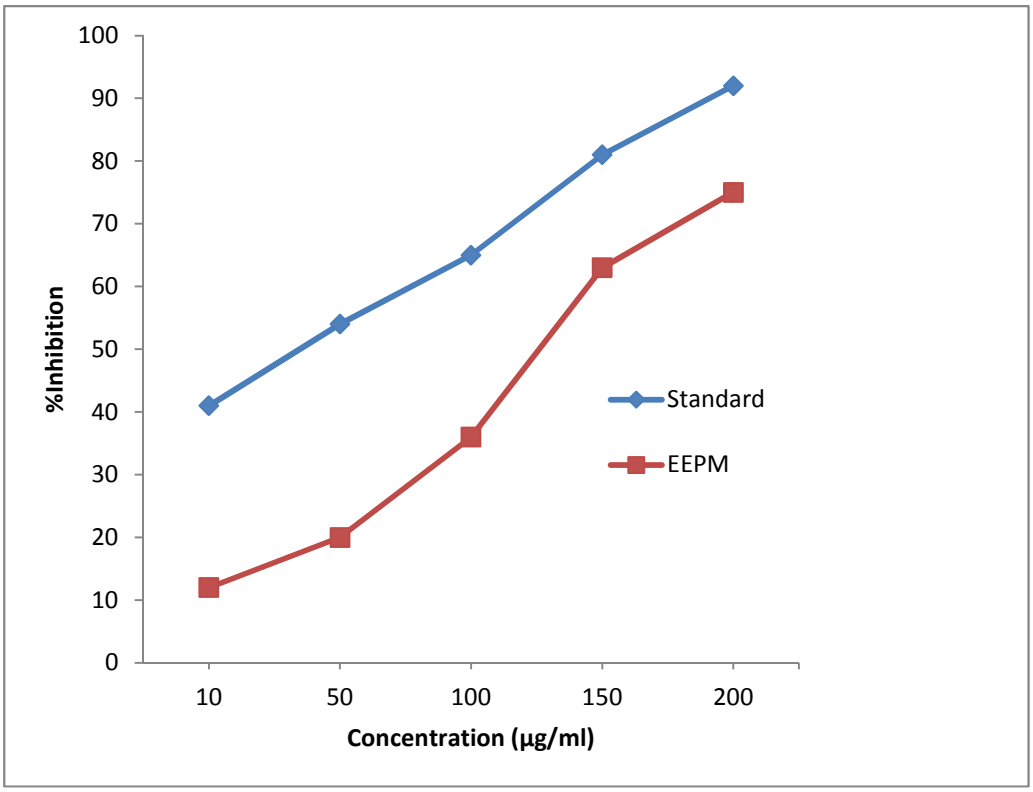
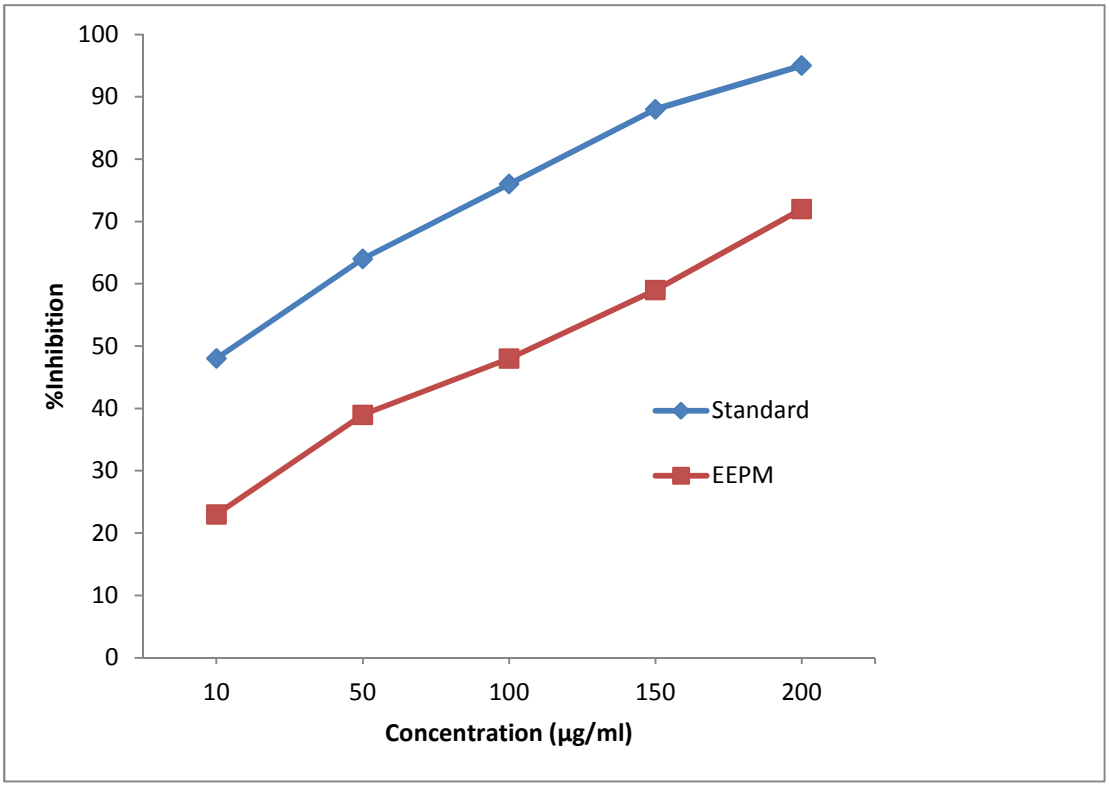


Table No.15 *In vitro* AH2O2 antioxidant activity of EEPM bark

H_2O_2			
IC_{50} values ± SEM µg/ml* by methods			
Sl:No	Con µg/ml	Percentage inhibition	
		Standard Ascorbic acid	EEPM
1	10	48	23
2	20	64	39
3	40	76	48
4	80	88	59
5	100	95	72
IC_{50} value		10.85	44.76



Result

The EEPM exhibited potent DPPH and ABTS radical scavenging activity with IC_{50} values 16.56 ± 0.88 and 39.00 ± 1.31μg/ml, respectively. MEAV showed good antioxidant activity against H_2O_2 radical with IC_{50} values 97.80 ± 4.55 μg/ml which showed better activity than standard Ascorbic acid and moderate antioxidant activity against nitric oxide radical with IC_{50} value 345.50 ± 11.00 μg/ml respectively.

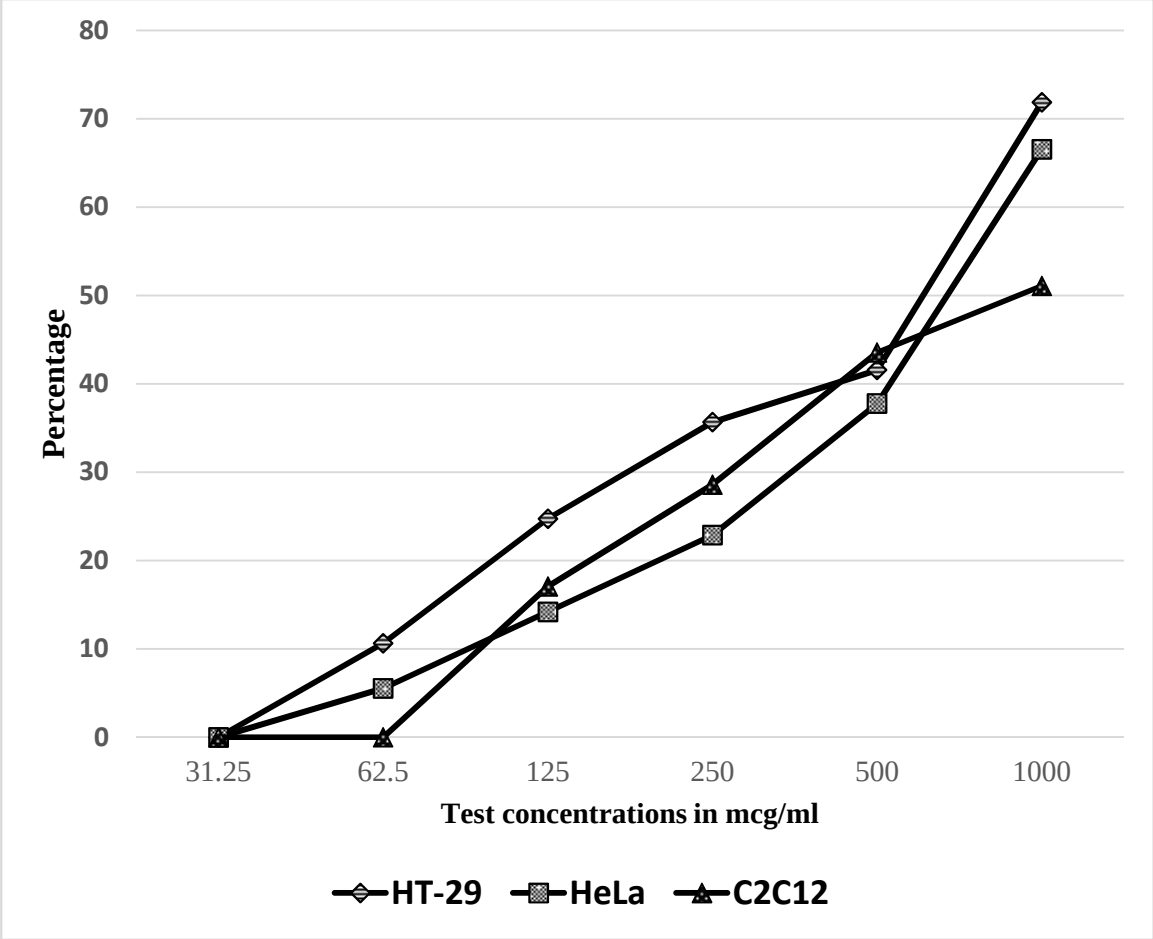
Discussion

The free radical scavenging activity of natural compounds can be evaluated through their ability to quench the synthetic free radicals, in which the absorbance of the reaction mixture is taken in visible range to know whether the compound is having antioxidant property (Halliwell & Gutteridge 1998). The above observations reveal that the drug has considerable antioxidant activity. The EEPM produced significant % inhibition in the levels against ABTS radical scavenging activity, but moderate antioxidant activity against nitric oxide, DPPH and H_2O_2 radicals respectively.

4.3.2 *In Vitro* cytotoxicity activity of ethanol extracts of *Pterocarpus marsupium* bark

Table No.17 Cytotoxicity activity of EEPM bark on different cell lines.

Extract	Cell line	Test Concentrations (μg/ml)	%Cytotoxicity	CTC_{50} (μg/ml)
EEPM	HT-29	1000 500 250 125 62.5 31.25	71.85 41.56 35.65 24.74 10.65 0.00	485.64
EEPM	HeLa	1000 500 250 125 62.5 31.25	66.54 37.75 22.87 14.19 5.54 0.00	625.90
EEPM	C_2C_{12}	1000 500 250 125 62.5 31.25	51.07 43.53 28.57 17.06 0.00 0.00	> 1000



Result

The EEPM exhibited moderate cytotoxic properties towards cancerous cell lines HT-29 and HeLa with CTC_{50} values 701.50 and 785.50µg/ml, respectively. Whereas against C_2C_{12}, test extract failed to exhibit cytotoxicity at test concentrations with only 32% cytotoxicity at 1000µg/ml.

Discussion

Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis).With regard to the possible anticancer mechanisms involved, flavonoids have been reported to exhibit their anticancer activity *via* the modulation of cell cycle arrest at the G1/S phase, down-regulation of anti-apoptotic gene products (Vijayan et al., 2003). On other hand, saponins induce anticancer activity *via* the necrosis cell death, which depends on the types of cancer cells affected (Dymock Indica, 1995).

Furthermore, triterpenoids also induce apoptotic response on cancer cells by inhibiting nuclear factor-kappa B or by causing cell cycle disruption (by decreasing the number of cells in G0/G1 phase with initial increase in S and G2/M) (Sivakumar et al., 2005). In conclusion, EEPM was found to possess moderate cytotoxicity activity against the HT-29 and HeLa cell lines, which could be associated with its antioxidant mechanisms.

6. SUMMARY AND CONCLUSION

The present dissertation work was carried out to evaluate *the in vitro* antioxidant and *in vitro* anticancer activities of ethanol extract of *Pterocarpus marsupium* ROXB. bark.

Literature survey revealed that *P. marsupium* is a versatile medicinal plant and is a unique source of wide range of phytocompounds with diverse chemical structure and pharmacological activity. Very little work has been reported thus far on the biological activity and plausible medicinal applications of these compounds and hence extensive investigation is needed to exploit their therapeutic utility to combat diseases. The plant has been widely studied for its pharmacological activities for antidiabetic activities and regarded as universal panacea in Ayurvedic medicines and is believed to be a versatile plant having a wide spectrum of medicinal activities. With the application of proper scientific and technological advances, a logical interpretation of the codified language of traditional medicine has become a necessity to further promote drug discovery and drug development. Efforts have to be explored and studies need to be undertaken to isolate and develop new molecules especially, with the compounds isolated from *P. marsupium*. Although crude extracts from various parts of *P. marsupium* have been reported to be used as medicinal applications from time immemorial, modern drugs can be further refined and developed after extensive investigation of its bioactivity, mechanism of action, pharmacotherapeutics, and toxicity and after proper standardization and clinical trials.

P. marsupium bark was extracted with ethanol and the yield was 7.06 % w/w. The chemical constituents of the extract were identified by qualitative analysis. Preliminary phytochemical analysis of *P. marsupium* showed the positive test of as carbohydrates, sterols, glycosides, phenols, phytosterols and steroids, proteins& amino acids, flavonoids, triterpenoids.

The acute and sub-acute toxicity study of *P. marsupium* did not show any toxic signs at different increasing dosage levels upto 2000mg for acute and 2700mg for subacute levels. From the results, it was observed that both plant extracts were safe for oral administration on study conducted in mice.

In *in vitro* antioxidant activity, EEPM exhibited potent DPPH and ABTS radical scavenging activity and showed good antioxidant activity against H_2O_2 radical, showed better activity than standard Ascorbic acid and moderate antioxidant activity against nitric oxide radical.

In vitro cytotoxicity activity of EEPM revealed that it exhibited moderate cytotoxic properties towards cancerous cell lines HT-29 and HeLa, whereas against C_2C_{12}, test extract failed to exhibit cytotoxicity at test concentrations with only 32 % cytotoxicity at 1000 µg/ml.

As the global scenario is now changing towards the use of non-toxic plant products having traditional medicinal use, development of modern drugs from *P. marsupium* should be emphasized for the control of various diseases. In fact, time has come to make good use of centuries-old knowledge on *P. marsupium* through modern approaches of drug development. For the last few years, there has been an increasing trend and awareness in *P. marsupium* research. Quite a significant amount of research has already been carried out during the past few decades in exploring the chemistry of different parts of *P. marsupium*. Several therapeutically and industrially useful preparations and compounds have also been marketed, which generates enough encouragement among the scientists in exploring more information about this medicinal plant. An extensive research and development work should be undertaken on *P. marsupium* and its products for their better economic and therapeutic utilization.

In may be concluded that the *in vitro* antioxidant and *in vitro* anticancer activities and other associated biodynamic pharmacological activities of the ethanol extract of *P. marsupium* may be attributed to the presence of triterpenoids and flavonoids and other phytoconsitutents in the extract. However, further studies are needed to isolate the phytoconsitutents and to explore the pharmacological actions of the isolated compounds and the mode of action of these terpenoids and flavonoids and other phytochemicals.

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