A STUDY ON CYTOTOXIC, ANTIMICROBIAL & ANTIOXIDANT INVESTIGATIONS OF METHANOL EXTRACT OF ROOT OF *Bombax ceiba*

A Dissertation Submitted to the Department of Pharmacy, East West University in the Partial Fulfillment of the Requirements for The Degree of Bachelor of Pharmacy

Submitted by

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DECLARATION BY THE RESEARCH CANDIDATE

I, Sumiya Ferdous, ID: 2012-1-70-055, hereby declare that the dissertation entitled **"A Study on Cytotoxic, Antimicrobial & Antioxidant Investigations of Methanol Extract of Root of *Bombax ceiba*"** submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic research work carried out by me. The contents of this dissertation, in full or in parts, have not been submitted to any other institute or University for the award of any degree or Diploma of Fellowship.

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CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertation, entitled **"A Study on Cytotoxic, Antimicrobial & Antioxidant Investigations of Methanol Extract of Root of *Bombax ceiba*"** is a bona fide research work done by Sumiya Ferdous (ID: 2012-1-70-055), in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy under my supervision.

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ENDORSEMENT BY THE CHAIRPERSON

This is to certify that the dissertation, entitled **"A Study on Cytotoxic, Antimicrobial & Antioxidant Investigations of Methanol Extract of Root of *Bombax ceiba*"** is a bona fide research work done by Sumiya Ferdous (ID: 2012-1-70-055), under the guidance of **Nazia Hoque**, Senior Lecturer, in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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DEDICATION

This research paper is dedicated to my beloved parents for their unconditional support.

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ABSTRACT

Bombax ceiba has risen the interest of scientists and researchers since some of the active constituents and phytochemicals found on this plant are known to exert many beneficial effects. It is a rich source of phytochemicals, as for example apigenin**,** a well-known anti-cancer agent, or luteol, triterpene with many health benefits among which can mention its anti-inflammatory, anti-arthritic, anti-mutagenic, antimalarial and anti-plasmodial properties.

The aim of the present study was to evaluate the cytotoxic activity, antimicrobial activity and antioxidant activity of methanol extract of *Bombax ceiba.*

In vitro antioxidant activity test performed by aluminum chloride colorimetric method in which flavonoid concentration was found 13.816±1.06 mg/g. In flavoinoid antioxidant test, we measured the absorbanace of methanol extract of our sample at 695 nm.

The antimicrobial activities of methanol solvent extract of plant were tested against the Gram-negative bacterial strains and 2 yeast strains by observing the zone of inhibition. The antimicrobial test was performed by disc diffusion method. The crude methanol extract of *Bombax ceiba* plant showed poor antimicrobial activities against Gram-negative bacteria at concentrations of 300 μg/disc and 600 μg/disc.

The cytotoxic activity of the plant was done by using *Artemia saline* Leach. The LC_{50} was observed approximately as 20.165 μg/ml with a R^2 value of 0.9766. Cytotoxicity of methanol extract of *Bombax ceiba* was good because LC_{50} value was found more than 5 μg/ml which indicates excellent cytotoxic effect. So, further studies are needed to evaluate the cytotoxicity of isolated pure compounds.

In conclusion, further investigations are needed to identify the active constituents and the exact mechanism(s) of action responsible for the reported antimicrobial and cytotoxic and antioxidant properties of *Bombax ceiba.*

Key Words: *Extract, Methanol, Bombax ceiba, Cytotoxic, Antimicrobial, Antioxidant*

CHAPTER ONE

INTRODUCTION

1.1. General Introduction

Plants and man are inseparable. Plants existed on the earth in the geological past form the early history of the earth. The use of plants to alleviate human suffering is as old as the evolution of human civilization itself. From the early stages of human civilization, plants, especially medicinal plants have played a pioneering role for the welfare of human beings. Recently, dramatic changes have taken place in the primary health care system of world population through the development of science, technology and medical science, but till to day 400 cores of people of the world are totally dependent on herbal medicine. It is revealed that even in the developed countries 25%, of the prescribed drugs come from plant sources and herbal medicines are used by about 75-80% of the world's population for primary health care because of their better cultural acceptability, better compatibility with human body and lesser side effects. WHO consultative body of medicinal plants has formulated a definition of medicinal plants in the following way "A medicinal plant is any plant which in one or more of its organs, contains substances that can be used for therapeutic purposes or which is a precursor for synthesis of useful drugs" (Sofowora, 1982).

Ever since ancient times, in search for rescue for their disease, the people looked for drugs in nature. The beginnings of the medicinal plants use were instinctive, as is the case with animals. In view of the fact that at the time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. In time, the reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants' usage gradually abandoned the empiric framework and became founded on explicatory facts. Until the advent of iatrochemistry in 16th century, plants had been the source of treatment and prophylaxis. Nonetheless, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again. (Khan *et al*., 2005).

Healing with medicinal plants is as old as mankind itself. The connection between man and his search for drugs in nature dates from the far past, of which there is ample evidence from various sources like written documents, preserved monuments, and even original plant

medicines. Awareness of medicinal plants usage is a result of the many years of struggles against illnesses due to which man learned to pursue drugs in barks, seeds, fruit bodies, and other parts of the plants. Contemporary science has acknowledged their active action, and it has included in modern pharmacotherapy a range of drugs of plant origin, known by ancient civilizations and used throughout the millennia. The knowledge of the development of ideas related to the usage of medicinal plants as well as the evolution of awareness has increased the ability of pharmacists and physicians to respond to the challenges that have emerged with the spreading of professional services in facilitation of man's life. (Rates, 2001).

In Bangladesh there are about 297 Unani, 204 Ayurvedic and 77 Homeopatheic drug manufacturing industries where the medicinal plants are extensively used in both raw and semi– processed forms of medicine in various pharmaceutical dose formulations. These plants also serve as important raw materials for many modern medicinal preparations. The market value of drugs produced by these industries from medicinal plants is about Tk. 300 cores. (The Daily Jugantor, 2003).

1.1.1. Medicinal Plants as Drugs

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal and human body are generally designated as medicinal plants (Ghani, 1998).

Or, according to the World Health Organization (WHO),

> "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemopharmaceutical semi-synthesis" (Sofowara, 1982).

The history of the use of medicinal plants for alleviating diseases had its origin in the activities of the most primitive man of the remote past (Ghani, 1998). Our ancestors were forced to use any natural substances that they could find to ease their sufferings caused by acute and chronic illness, physical discomforts, wounds and injuries and even terminal illness. Since that ancient time, plants with therapeutic properties have occupied an important place in the disease treatment practices (Khan *et al*., 2005).

Concerning drugs of plant origin, it is important to bear in mind certain conceptual distinctions. Plants can be used as therapeutic resources in several ways. They can be used as herbal teas or other home-made remedies, when they are considered as medicinal plants. They can be used as crude extracts or ―standard enriched fractions‖ in pharmaceutical preparations, such as tinctures, fluid extracts, powder, pills and capsules, when they are considered as phytopharmaceutical preparations or herbal medicines.

Finally, plants can be subjected to successive extraction and purification procedures to isolate the compounds of interest, which can themselves be active and used directly as a drug, examples being quinine, digoxin and ergotamine, or they can be used as precursors (e.g. diosgenin) in semi-synthetic processes or as models for total synthesis, with well-defined pharmacological activity or structure–activity relationship studies determining a prototype drug (e.g. morphine) (Rates, 2001).

1.1.2. Medicinal Plants from Ancient Times

Archaeological evidence indicates that the use of medicinal plants dates at least to the Paleolithic, approximately 60,000 years ago. Written evidence of herbal remedies dates back over 5,000 years, to the Sumerians, who created lists of plants. A number of ancient cultures wrote on plants and their medical uses. In ancient Egypt, herbs are mentioned in Egyptian medical papyri, depicted in tomb illustrations, or on rare occasions found in medical jars containing trace amounts of herbs. The earliest known Greek herbals were those of Diocles of Carystus, written during the 3rd century B.C, and one by Krateuas from the 1st century B.C. Only a few fragments of these works have survived intact, but from what remains scholars have noted that there is a large amount of overlap with the Egyptian herbals. Seeds likely used for herbalism have been found in the archaeological sites of Bronze Age China dating from the Shang Dynasty. Over a hundred of the 224 drugs mentioned in the Huangdi Neijing, an early Chinese medical text, are herbs. Herbs were also common in the medicine of ancient India, where the principal treatment for diseases was diet. *De Materia Medica* by Pedanius Dioscorides, a Roman physician, is a particularly important example of such writings. The documentation of herbs and their uses was a central part of both Western and Eastern medical scholarship through to the 1600s, and

these works played an important role in the development of the science of botany (Nunn, 2002; Robson et. al., 2009, Hong, 2004; Ackerknecht, 1982).

Human beings have used plants for the treatment of diverse ailments for thousands of years. According to the World Health Organization, most populations still rely on traditional medicines for their psychological and physical health requirements, since they cannot afford the products of Western pharmaceutical industries, together with their side effects and lack of healthcare facilities. Rural areas of many developing countries still rely on traditional medicine for their primary health care needs and have found a place in day-to-day life. These medicines are relatively safer and cheaper than synthetic or modern medicine. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses (Ernst, 2007).

Herbal medicines are in great demand in both developed and developing countries as a source of primary health care owing to their attributes having wide biological and medicinal activities, high safety margins and lesser costs. Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell (Ernst, 2007). Even with the advent of modern or allopathic medicine, Balick and Cox (1996) have noted that a number of important modern drugs have been derived from plants used by indigenous people.

Traditional use of medicine is recognized as a way to learn about potential future medicines. Researchers have identified number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources. Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Fabricant and Farnsworth, 2001).

1.1.3. Traditional Medicine

Traditional medicines have existed in Bangladesh as an important basis of health care since olden times. Because of their potentialities and close association with the culture and

tradition of the people, traditional systems of medicine have assumed a unique position in the health care of the people living in even the remotest areas of the country. Although the use of traditional medicine is so deeply rooted in the cultural heritage of Bangladesh the concept, practice, type and method of application of traditional medicine vary widely among the different ethnic groups. Traditional medical practice among the tribal people is guided by their culture and life style and is mainly based on the use of plant and animal parts (Samy *et al*., 2008). Among the largest ethnic group, the bangles on the main land, there are two distinct forms of Traditional medicine practice (Ghani, 1998):

1. One is the old and original form based on old knowledge, experience and belief of the older generations. This includes:

- *Folk medicine*, which uses mainly plant and animal parts and their products as medicines for treating different diseases and also includes treatments like bloodletting, bones setting, hot and cold baths, therapeutic fasting and cauterization.
- *Religious medicine*, which includes use of verses from religious books written on papers and given as amulets, religious verses recited and blown on the face or on water to drink or on food to eat, sacrifices and offerings in the name of God and gods, etc. and
- *Spiritual medicine*, which utilizes methods like communicating with the supernatural beings, spirits or ancestors through human media, torturous treatment of the patient along with incantations to drive away the imaginary evil spirits and other similar methods.

2. The other is the improved and modified form based on the following two main traditional systems:

- Unani-Tibb or Graeco-Arab system, which has been developed by the Arab and Muslim scholars from the ancient Greek system, and
- Ayurvedic system, which is the old Indian system, based on the Vedas the oldest scriptures of the Hindu saints of the Aryan age.

Both the Unani and Ayurvedic systems of traditional medicine have firm roots in Bangladesh and are widely practiced all over the country. Apparently the recipients of these systems of medicine appear to be the rural people, but practically a good proportion of the urban population still continues to use these traditional medicines, although organized modern health care facilities are available to them (Ghani, 1998).

As only a certain percentage of plants are used in traditional medicines, it is roughly estimated that of the discovered 17,000 species, nearly 3,000 species are used in medicinal field (Samy *et al*, 2008). All plants produce chemical compounds as part of their normal metabolic activities. These phytochemicals are divided into (Bernhoft, 2010)-

(a) Primary metabolites such as sugars and fats, which are found in all plants; and

(b) Secondary metabolites—compounds which are found in a smaller range of plants, serving a more specific function. For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination.

It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs—examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove. Toxic plants even have use in pharmaceutical development (Bernhoft, 2010).

Plants synthesize a bewildering variety of phytochemicals but most are derivatives of a few biochemical motifs (Bernhoft, 2010).

Some medicinal uses of common plants in Bangladesh are reported in following table.

Table 1.1: **Name & Medicinal Uses of Some Common Plants in Bangladesh** (Samy *et al*, 2008)

Common name	Botanical name	Parts Used	Uses
Pudina	*Menthe arvensis*	Whole plant	Indigestion, stomach disorder, stimulant.
Kalmegh/ Bhui neem	*Andrographis paniculata*	Whole plant	Fever, Weakness, Release of gas.
Kalmishak	*Smilax zeylanica*	Roots, steams	blood dysentery, rheumatisms, abscess
Dhutara	*Datura metel*	Roots, leaves, seeds	Anesthesia, pain, asthma, epilepsy, rheumatic fever, hypertension.
Tulsi	*Ocimum sanclum*	Leaves, flower, seeds	Cough, Cold, Bronchitis, Expectorant.
Henna/Mehdi	*Lawsennia iermis*	Leaves, flower	Burning, Steam, Anti-inflammatory
Gritkumari	*Aloe verra*	Leaves	Laxative, Wound healing, Skin burns & care, Ulcer.
Anantamul/sariva	*Hemidesmus indicus*	Root, leaves	Appetizer, Carminative, Aphrodisiac, Astringent
Sharisa	*Brassica napus*	Leaves, seeds.	Fever, common cold, stomachache, itching, headache.
Vringraj	*Eclipta alba*	Whole plant	Anti-inflammatory, Digestive, Hair tonic
Neem	*Azardirchata indica*	Leaves	Sedative, Analgesic, Epilepsy,Hypertensive

1.1.4. Significances of Medicinal Plants to Mankind

Even if we only consider the impact of the discovery of the penicillin, obtained from micro-organisms, on the development of anti-infection therapy, the importance of natural products is clearly enormous. About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 examples of important drugs obtained from plants are digoxin from Digitalis spp., quinine and quinidine from Cinchona spp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin (Yue-Zhong Shu, 1998). The vast majority of these cannot yet be

synthesized economically and are still obtained from wild or cultivated plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds (Hamburger and Hostettmann, 1991). In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies (Williamson *et al*., 1996).

Medicinal plants have many characteristics when used as a treatment, as follow:

- Synergic medicine- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.
- Support of official medicine- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.
- Preventive medicine- It has been proven that the component of the plants also characterizes by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment.

1.1.5 Advantages of Drug Discovery from Natural Resources

Usage of botanical sources as starting point in the drug development program is associated with few specific advantages:

- Mostly, the selection of a candidate species for investigations can be done on the basis of long-term use by humans (ethnomedicine). This approach is based on an assumption that the active com-pounds isolated from such plants are likely to be safer than those derived from plant species with no history of human use. At certain time point afterward, one may attempt upon synthesis of active molecule and reduce pressure on the resource. Drug development from *Rauwolfia serpentina*, *Digitalis purpurea*, etc. in the past fall under this category of approach.
- Sometimes, such approaches lead to development of novel molecules derived from the source due to inherent limitations of the original molecule. For instance, podophyllin derived from *Podophyllum hexandrum* was faced with dose-limiting

toxicities. Such limitations could be overcome to a great extent by semi-synthesis of etoposide, which continues to be used in cancer therapy today. Similar was the case with camptothecin (originally isolated from *Camptotheca* sp. And subsequently from *Mappia* sp.), which led to development of novel anticancer molecules like topotecan and irinotecan.

- Natural resources as starting point has a bilateral promise of delivering the original isolate as a candidate or a semi-synthetic molecule development to overcome any inherent limitations of original molecule.

1.1.6 Value of Medicinal Plants

Plants are valuable for modern medicine in four basic ways:

1. They are used as sources of direct therapeutic agents
2. The chemical structures derived from plant sources can be used as models for new synthetic compounds.
3. Finally, plants can be used as taxonomic markers for the discovery of new compounds. (Rahman *et. al*., 2007)

1.1.7 Global Scenario of Medicinal Plants

According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Herbal medicine is a common element in Ayurvedic, homeopathic, naturopathic, traditional, oriental, Native American & Indian medicine. Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. The present global herbal market is worth about US$ 62 billion per annum. The annual growth of herbal market is about 15percent and the global herbal market by 2050 is expected to be about US$ 5 trillion (Payyappallimana, 2009).Thus, the modern social context and economic view of health services, the needs of the pharmaceutical market and the recognition that research on medicinal plants used in folk medicine represents a suitable approach for the development of new drugs (Calixto,2000) have led to an increase in the number of publications in this field, and

private and governmental institutions are now financially supporting research programs worldwide (Rates, 2001).

1.1.8 Medicinal Plants in Bangladesh

In an estimate, the international market of medicinal plants related to trade stood at 60 billion US Dollar per year. The demand for medicinal plants based raw materials are growing at an approximate rate of 10-15% per year internationally. Medicinal plant sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives of Bangladesh. In recent years, the growing demand for herbal product has led to a quantum jumping in volume of plants material trade within and across the country. Bangladesh there is no systematic cultivation processor conservation strategies about medicinal plants. The local people conserve traditional knowledge through their experience and practice, which is handed down orally without any documentation. This knowledge is now under threat to extinction. This is a very alarming situation with regard to natural growth of medicinal plants in the wilderness in this country. In this scenario, the survey on ―Traditional and industrial use and market Scenario of Medicinal plants in Bangladesh" which has been conducted by the DEBTEC researchers at Chakbazar, Dhaka, Bangladesh. It is found that there is worth of 11million US dollars medicinal plant market in Bangladesh, which have been imported but not in the name of medicinal plants rather in the name of spices and other products. This research aimed at documenting the 'Present Status and Market Scenario of Medicinal Plants' in Bangladesh. Our research finding shows that 84.1% of the respondent use medicinal plants in health care. 18.3% of the villagers use Kabirazi in the disease in medium category.55.0% of our respondent's source of knowledge of using medicinal plant is family where 34.7% gained knowledge from neighbor. Only 14.3% of the respondents are involved with trading of medicinal plant. About 10.4% of the villagers are involved in cultivation, collection or business of medicinal plant. From the survey report it has been found that 46.6% industries are using above 60% of imported medicinal plants as their raw materials and 53.3% of the industries are using below 40%. The study revealed that 86.7% industries are importing Indian raw materials, 53.3% are importing the Pakistani one and very few of them are importing the raw materials from Nepal, Iran and Korea. According to the response of shop owners, the local raw materials of their products are mostly coming from 5 different areas

of the country. Among those 90% are coming from Chittagong and again 76.6% from Tangail, 30% from Gazipur and another 30% from Khulna. In this scenario, appropriate steps must therefore be taken immediately in order to save this situation with regard to growth, conservation and supply of medicinal plants in the country. The best possible way of doing this is to bringing this more and more of these plants under planned cultivation. The cultivation of medicinal plants in Bangladesh will lead to the conservation and also protect the biodiversity. Ecological and biotic factors are suitable in Bangladesh for the cultivation of medicinal plants. We have been successful to sensitize the policy makers. In Bangladesh there is no develop processing unit and to train the garden owner for skilled manpower to value addition of MP, which will create the income generating women in rural areas. In Bangladesh, about 500 plant species have been identified as medicinal plants because of their therapeutic properties, Approximately, 400 herbal factories have been established in this country for producing Ayurvedic and Unani medicines. It has been estimated that Bangladesh has a market of about 100-core taka worth herbal products annually. The total size of the medicinal plant market at wholesale prices was estimated at some US$ 14 million per annual which corresponds to 17000 tons of products. It has been estimated that 12,500 tons of dried medicinal plant products are sold in Bangladesh that have a worth of Tk 255 million to rural economy. At the factory level, 5000 tons of medicinal plants are imported annually that cost around 480 million taka (Alam *et al*., 1996). Although modern medicinal science has been developed to a great extent, many rural people of Bangladesh still depend on plant products and herbal remedies for treating their ailments (Bregum, 2004).

1.2 Approaches for Isolation of Active Compounds from Natural Origin

1.2.1 Random Approach

Generally, two approaches have been followed for screening of the plants selected randomly for the purpose of new drug discovery (Katiyar *et al*., 2012)-

a) Screening for selected class of compounds like alkaloids, flavonoids, etc.: While this route is simple to perform, however, it is flawed in the sense that it provides no idea of the biological efficacy. However, chances of getting novel structures cannot be denied following this approach.

b) Screening of randomly selected plants for selected bioassays: Central Drug Research Institute, a premier R and D organization of Council of Scientific and Industrial Research of India, followed this approach about three decades ago. They screened almost 2000 plants for biological efficacy. However, the screening did not yield any new drug. National Cancer Institute (NCI) of National Institute of Health, USA, studied about 35,000 plant species for anticancer activity, spending over two decades from 1960 to 1980. It resulted in proving two success stories, which were those of paclitaxel and camptothecin. This route, therefore, has been applied for both focused screening as well as general screening, showing some success in focused screening. If target-based bioassays are used, e.g. screening against PTP1B, chances of success would probably be more. This approach, however, needs a huge library of extracts, which very few organizations in the world are having.

1.2.2 Ethnopharmacology Approach

The approach of ethnopharmacology essentially depends on empirical experiences related to the use of botanical drugs for the discovery of biologically active New Chemical Entities (NCEs). This process involves the observation, description, and experimental investigation of indigenous drugs, and is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines like anthropology, archaeology, history, and linguistics. This approach based on ethnomedicinal usage history has seen some success, e.g. *Andrographis paniculata* was used for dysentery in ethnomedicine and the compounds responsible for the activity were isolated as andrographolide. Morphine from *Papave rsomniferum*, Berberine from *Berberis aristata*, and Picroside from *Picrorrhiza kurroa* are some examples of this approach. Some of the plants which are not selected on the basis of ethnomedical use also had some success stories, like L-Dopa from *Mucuna prurita* and paclitaxel from *Taxus brevifolia* (Katiyar *et al*., 2012).

1.2.3 Traditional System of Medicine Approach

Countries like India and China have a rich heritage of well-documented traditional system of medicine in vogue. Though these codified systems of medicine use largely botanical

sources as medicines, however, these stand apart from ethnomedicine specifically on three accounts (Katiyar *et al*., 2012):

- The ethnomedicinal practice is based on empirical experiences. On the other hand, these codified systems built up the empirical practices on strong conceptual foundations of human physiology as well as of pharmacology (though the tools of their investigations in those times were far different from the existing ones).
- The pharmaceutical processes have been more advanced as against the use of crudely extracted juices and decoctions in ethnomedicinal practices. Due to this phenomenon, the concept of standardization was known to the system.
- They are well documented and widely institutionalized. On the other hand, the ethnomedicinal practices are localized and may be largely controlled by few families in each of the community.

However, in terms of historicity, ethnomedicinal practices might be older than codified systems of medicine (Katiyar *et al*., 2012).

The first time discovery of artemisinin from *Artemesia alba* for malaria, guggulsterones which is found from *Commiphora mukul* (for hyperlipidemia), boswellic acids which are coming from *Boswellia serrata* (anti-inflammatory), and bacosides from *Bacopa monnieri* (nootropic and memory enhancement) was based on the leads from these codified systems of medicine prevailing in China and India. However, it can be stated that such approach for selecting candidates in drug discovery programs has not been adopted much so far. Nonetheless, the approach has a distinct promise in terms of hit rates. But the distinct example for this approach has been the discovery of reserpine from *Rauwolfia serpentine*, which was based on the practices of Unani medicine (Katiyar *et al*., 2012).

1.3 Procedure for Development

Since drug development is an expensive practice, careful phytochemical analysis and pharmacological screening and if promising clinical tests are required. The way of developing drugs from plants involves several stages (Ghani, 1998), which include:

1. Selection and correct identification of the proper medicinal plant.
2. Extraction with suitable solvent(s).
3. Detection of biological activity of crude extract and establishment of a bioassay system to permit the identification of the active fractions and rejection of the inactive ones.
4. Fractionations of crude extract using the most appropriate chromatographic procedures, biological evaluation of all fractions and separation of the active fractions.
5. Repeated fractionation of active fractions to isolate pure compound(s).
6. Elucidation of chemical structure of pure compound(s) using spectroscopic methods.
7. Evaluation of biological activity of pure compound(s)
8. Toxicological tests with pure compound(s).
9. Production of drug in appropriate dosage forms.

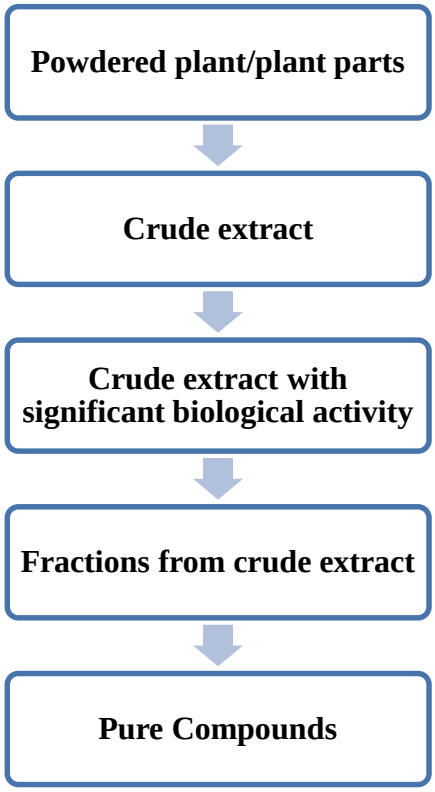


Fig 1.1: Schematic Diagram of Development of Procedure

The way we have worked on

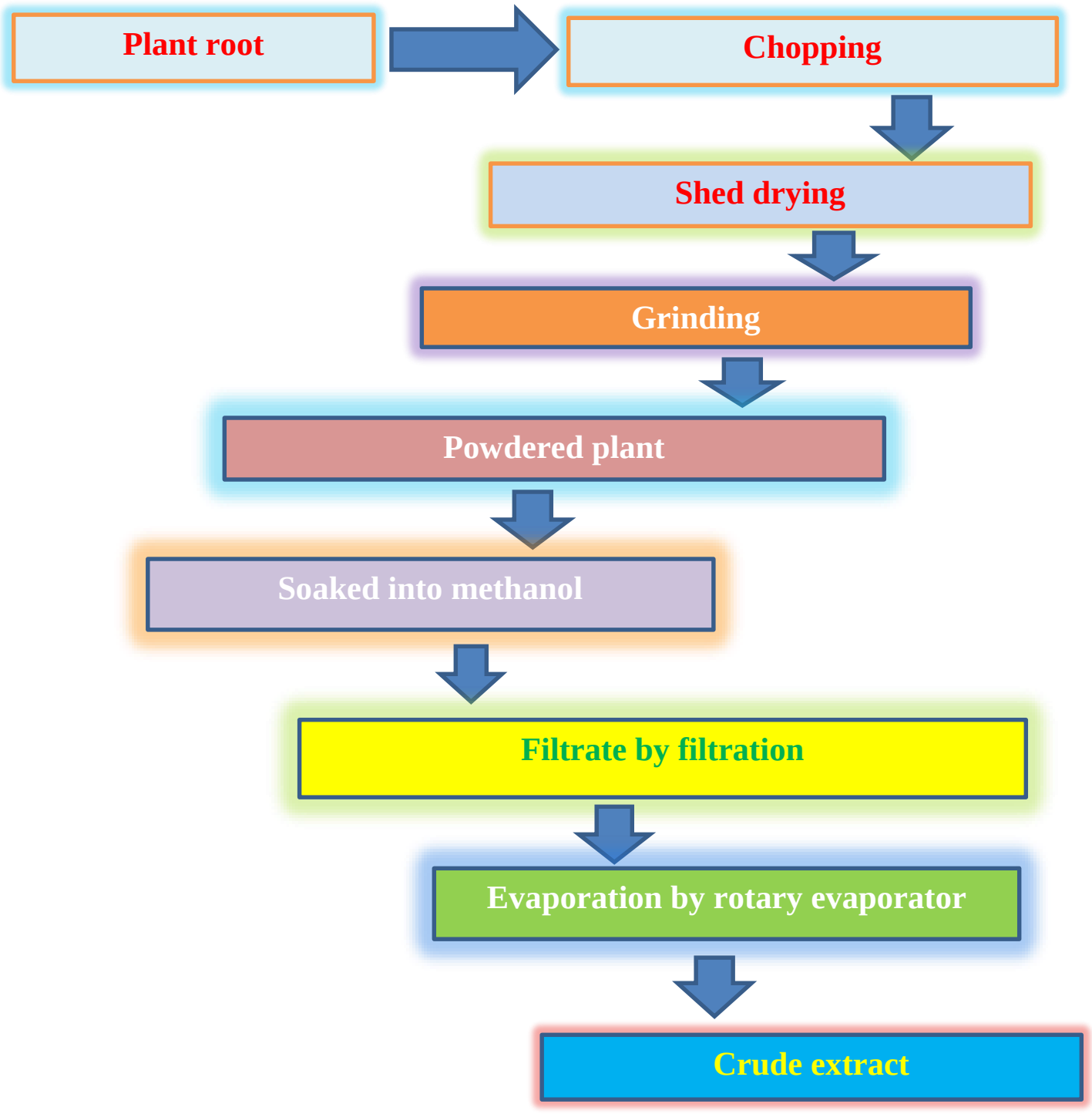


Figure-1.2: Schematic Presentation of the Crude Preparation from The Plant

1.3.1 Selection and Identification of Plant

As per WHO guidelines (WHO 2003), the plant selected for collection should be taxonomically same as recommended by the national pharmacopoeia or other related documents. If a new plant is being selected for collection then it should be properly identified and documented. The botanical identity, scientific name including genus, species, subspecies or variety and family of the plant should be recorded. If available, the local name should also be verified. Complete taxonomical identification is an important factor during selection as taxonomy of the plant species can play an important role in their biological activity. In general, the search for the medicinal plants can follow three main routes: random, ethno (including ethnobotanical, ethnomedical and ethnopharmacological) and ecological search. Random search is extremely laborious and the success rate could be very low. Nevertheless, important drugs such as taxol, derivatives of camptothecin have been discovered by the National Cancer Institute (NCI) in collaborations with the United States Department of Agriculture (USDA) using this method (Rates, 2001).

1.3.2 Collection of Plant

Medicinal plant materials should be collected in the proper season so as to ensure the best possible quality of both the starting material as well as the finished product. Seasonal variations can affect the chemical composition of the plants and thus its biological activity. In most cases, maximum accumulation of chemical constituents occurs at the time of flowering which then declines at the beginning of the fruiting stage. The time of harvest should also depend on the plant part to be used since it is well known that depending on the plant species the level of biologically active constituents can vary in different parts at different stages of the plant growth and development (Rates, 2001).

1.3.3 Drying and Grinding

Plants can be dried in a number of ways: in the open air (shaded from direct sunlight); placed in thin layers on drying frames, wire-screened rooms, or in buildings; by direct sunlight, if appropriate; in drying ovens/rooms and solar dryers; by indirect fire; baking; lyophilization; microwave; or infrared devices. In the situation where possible, temperature and humidity should be controlled to avoid damage to the active chemical constituents.

The method and temperature used for drying may have a considerable impact on the quality of the resulting medicinal plant materials. For example, shade drying is preferred to maintain or minimize loss of color of leaves and flowers; and lower temperatures should be employed in the case of medicinal plant materials containing volatile substances. The drying conditions should be recorded. In the case of natural drying in the open air, medicinal plant materials should be spread out in thin layers on drying frames and stirred or turned frequently. Drying medicinal plant material directly on bare ground should be avoided. Insects, rodents, birds and other pests, and livestock and domestic animals should be kept away from drying sites. For indoor drying, the duration of drying, drying temperature, humidity and other conditions should be determined on the basis of the plant part concerned (root, leaf, stem, bark, flower, etc.) and any volatile natural constituents, such as essential oils. If possible, the source of heat for directs drying (fire) should be limited to butane, propane or natural gas, and temperatures should be kept below 30 °C. If other sources of fire are used, contact between those materials, smoke, and the medicinal plant material should be avoided. Grinding improves efficiency of extraction by increasing the surface area of plant material. This decreases the amount of solvent needed for extraction as it allows the plant material to pack more densely. Therefore, it is essential to grind samples into finer size for better extraction results (Ghani, 1998).

1.4 Cytotoxic Screening

Cytotoxicity is the quality of being toxic to cells. Treating cells with the 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). To measure the cytotoxicity of a compound derived from plant, a bioassay can be employed in order to provide an estimation of concentration or potency of a substance (drugs, hormones, vitamins, toxins, and antitoxin) by measurement of the biological response that it produces.

Some of the traditional medicine involves the use of crude plant extracts which may contain an extensive diversity of molecules, often with indefinite biological effects. However, most of the available information regarding the medicinal potential of these plants is not

provided with credible scientific data. For this reason, several researches have been conducted to determine the toxicity of medicinal plants. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the Brine Shrimp (*Artemia* sp.) Lethality Assay (BSLA). BSLA is used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds. The low cost and ease of performing the assay and the commercial availability of inexpensive brine shrimp eggs makes BSLA a very useful bench top method. This assay has been noted as a useful tool for the isolation of bioactive compounds from plant extracts (Olowa and Nuneza, 2013).

In this present study, methanol extracts of the selected medicinal plant were tested *in vivo* for their cytotoxic effect against the brine shrimp nauplii and relate toxicity results with their known ethno-pharmacological activities. (Rates, 2001).

1.5 Antimicrobial Test:

An important task of the clinical microbiology laboratory is the performance of antimicrobial susceptibility testing of significant bacterial isolates. The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections. The most widely used testing methods include broth microdilution or rapid automated instrument methods that use commercially marketed materials and devices. Manual methods that provide flexibility and possible cost savings include the disk diffusion and gradient diffusion methods. Each method has strengths and weaknesses, including organisms that may be accurately tested by the method. Some methods provide quantitative results (e.g., minimum inhibitory concentration), and all provide qualitative assessments using the categories susceptible, intermediate, or resistant. In general, current testing methods provide accurate detection of common antimicrobial resistance mechanisms. However, newer or emerging mechanisms of resistance require constant vigilance regarding the ability of each test method to accurately detect resistance.

1.5.1 Emergence of Antimicrobial Resistance and the Rationale for Performing Susceptibility Testing

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates. Empirical therapy continues to be effective for some bacterial pathogens because resistance mechanisms have not been observed e.g., continued penicillin susceptibility of *Streptococcus pyogenes*. Susceptibility testing of individual isolates is important with species that may possess acquired resistance mechanisms (e.g., members of the Enterobacteriaceae, Pseudomonas species, Staphylococcus species, *Enterococcus* species, and *Streptococcus pneumoniae*).

1.5.2 Overview of Commonly Used Susceptibility Testing Methods

1.5.2.1 Broth Dilution Tests

One of the earliest antimicrobial susceptibility testing methods was the macro broth or tube-dilution method. This procedure involved preparing two-fold dilutions of antibiotics in a liquid growth medium dispensed in test tubes. The antibiotic-containing tubes were inoculated with a standardized bacterial suspension. Following overnight incubation at 35°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The advantage of this technique was the generation of a quantitative result (i.e., the MIC). The principal disadvantages of the macro dilution method were the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test. The test by use of small, disposable, plastic "microdilution" tray. has made broth dilution testing practical and popular. Standard trays contain 96 wells, each containing a volume of 0.1 mL that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray. A broth microdilution susceptibility panel containing 98 reagent wells and a disposable tray inoculator (Jorgensen, J. and Ferraro, M. 2009).

1.5.2.2. The Advantages of the Microdilution

This procedure includes the generation of MICs, the reproducibility and convenience of having prepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. There is also assistance in generating computerized reports if an automated panel reader is used. The main disadvantage of the microdilution method is some inflexibility of drug selections available in standard commercial panels. (Jorgensen, J. and Ferraro, M. 2009).

1.5.2.3. Antimicrobial Gradient Method.

The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. It employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. The gradient diffusion method has intrinsic flexibility by being able to test the drugs the laboratory chooses but it is an expensive approach if more than a few drugs are tested. This method is best suited to situations in which an MIC for only 1 or 2 drugs is needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is to be tested. Example, penicillin and ceftriaxone with pneumococci. (Jorgensen, J. and Ferraro, M. 2009).

1.5.2.4. Disk Diffusion Test.

The disk diffusion susceptibility method is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum to the surface of a large (150 mm diameter) agar plate. At a fixed concentration, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35°C prior to

determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks. The results of the disk diffusion test are "qualitative," in that a category of susceptibility (ie, susceptible, intermediate, or resistant) is derived from the test rather than an MIC (Jorgensen and Ferraro, 2009).

1.5.3 Interpretation of Susceptibility Test Results

The results of a susceptibility test must be interpreted by the laboratory prior to communicating a report to a patient's physician. Optimal interpretation of MICs requires knowledge of the pharmacokinetics of the drug in humans, and information on the likely success of a particular drug in eradicating bacteria at various body sites. This is best accomplished by referring to an expert source such as the CLSI, which publishes interpretive criteria for MICs of all relevant antibiotics for most bacterial genera. Indeed, both MIC values and disk diffusion zone diameters must be interpreted using a table of values that relate to proven clinical efficacy of each antibiotic and for various bacterial species. The CLSI zone size and MIC interpretive criteria are established by analysis of 3 kinds of data: (1.) microbiologic data, including a comparison of MICs and zone sizes on a large number of bacterial strains, including those with known mechanisms of resistance that have been defined either phenotypically or genotypically; (2) pharmacokinetic and pharmacodynamic data; and (3) clinical studies results (including comparisons of MIC and zone diameter with microbiological eradication and clinical efficacy) obtained during studies prior to FDA approval and marketing of an antibiotic.

A "susceptible" result indicates that the patient's organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species. Conversely, an organism with a MIC or zone size interpreted as "resistant" should not be inhibited by the concentrations of the antibiotic achieved with the dosages normally

used with that drug. An "intermediate" result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. Exceptions can occur if the antibiotic is highly concentrated in a body fluid such as urine, or if higher than normal dosages of the antibiotic can be safely administered (e.g., some penicillins and cephalosporins). At times, the "intermediate" result can also mean that certain variables in the susceptibility test may not have been properly controlled, and that the values have fallen into a "buffer zone" separating susceptible from resistant strains. Generally, reporting of a category result of susceptible, intermediate, or resistant provides the clinician with the information necessary to select appropriate therapy. Reporting of MICs could aid a physician is selecting from among a group of similar drugs for therapy of infective endocarditis or osteomyelitis, in which therapy is likely to be protracted.

It is important that the tables used for susceptibility test interpretations represent the most current criteria. Indeed, the CLSI documents are reviewed and updated frequently, usually once per year. Use of old or outdated information from the original editions of FDA-approved drug labels or older CLSI tables could represent a serious shortcoming in the reporting of patients' results (Jorgensen and Ferraro,2009).

1.5.4 The Acceptable Accuracy of a Susceptibility Test Method

When assessing the accuracy of various susceptibility testing methods as compared to standard reference methods, the terms very major and major errors have been used to describe false-susceptible or false-resistant results, respectively. In evaluations of new susceptibility testing methods it is important to examine a representative number of strains that are resistant to various drugs to verify the ability of the new test to detect resistance and to test a number of susceptible strains to determine the rate of major errors that might be expected in a typical clinical laboratory setting. The emergence of new antimicrobial resistance mechanisms, including some that may be difficult to detect (e.g., vancomycin intermediate susceptibility in *S. aureus* and carbapenemase production in some gram-negative organisms) requires that the performance of susceptibility devices be constantly

reassessed and updated when needed. In some cases, it has been necessary to employ special ancillary testing methods (e.g., single concentration screening agars, modified Hodge test for carbapenemase production) to supplement routine testing by a commercial instrument system (Jorgensen and Ferraro,2009).

1.5.5 Current Test Methods and Future Directions

The antimicrobial susceptibility testing methods described in this article provide reliable results when used according to the procedures defined by the CLSI or by the manufacturers of the commercial products. However, there is considerable opportunity for improvement in the area of rapid and accurate recognition of bacterial resistance to antibiotics. There is a need for development of new automated instruments that could provide faster results and also save money by virtue of lower reagent costs and reduced labor requirements. To accomplish this, it will likely be necessary to explore different methodologic approaches for detection of bacterial growth (Jorgensen and Ferraro, 2009).

1.6 Antioxidant Potential of Medicinal Plants

Antioxidants are substances that may protect human cells against the effects of free radicals. Dietary plants contain variable chemical families and amounts of antioxidants. It has been hypothesized that plant antioxidants may contribute to the beneficial health effects of dietary plants. Studies suggest that a diet high in antioxidants from fruits and vegetables is associated with a lower risk of cancer, cardiovascular disease, Parkinson's disease and Alzheimer's disease. Such diseases have been found to be the result of damage of cells due to free radical generation (Singh et.al., 2013).

1.6.1 Free Radicals and Oxidative Stress

Free radicals are natural by-products of human metabolism. These are charged molecules which attack cells, breaking cellular membranes and reacting with the nucleic acids, proteins, and enzymes present in the cells. These attacks by free radicals, collectively known as oxidative stress, are capable of causing cells to lose their structure, function and eventually result in cell dysfunction. They are continuously produced by our body's use of oxygen, such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust,

radiation, air pollution, pesticides, etc. (Li & Trush, 1994). Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems which scavenge these free radicals preventing them from causing deleterious effects in the body (Nose, 2000). The antioxidant defense systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental conditions or infections, it leads to oxidative stress (Finkel & Holbrook, 2000).

When the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system, oxidative stress occurs in cellular system, including the superoxide anion radical, the hydroxyl radical, hydrogen peroxide and the peroxyl are greatly reactive molecules, which consequently generate metabolic products that attack lipids in cel1 membrane or DNA (Halliwell & Gutteridge, 1995). Oxidative stress, involves a series of free radical chain reaction processes, is associated with several types of biological damage, DNA damage, diabetes, respiratory tract disorders, carcinogenesis and cellular degeneration related to aging (Anderson *et al*., 2000). Continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng *et al*., 1997). Improved antioxidant status helps to minimize the oxidative damage and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases (Karuna *et al*., 2009). The interest in natural antioxidants, especially of plant origin, has greatly increased in recent years as the possibility of toxicity of synthetic antioxidants has been criticized (Jayaprakash and Rao, 2000).

Several herbs and herbal formulations are available for the scavenging activity. In addition to this there is a global trend to revive the traditional systems of medicines and renewed interest in the natural remedies for treating human ailments. Antioxidants have important preventive roles, not only on undesirable changes in the flavor and nutritional quality of food, but also on tissue damage in various human diseases. Almost all organisms are well protected against free radical damage by either enzymes or compounds, such as ascorbic acid, α- tocopherol and gluthione (Singh et.al., 2013).

Phenolic compounds from medicinal plants, such as flavonoids, phenolic acids, stilbenes, tannins,coumarins, lignans and lignins, possess strong antioxidant activity and may help toprotect the cells against the oxidative damage causedby free-radicals. They are well known as radical scavengers, metal chelators, reducing agents, hydrogendonors, and singlet oxygen quenchers. (Kähkönen *et al*., 1999; Proestos *et al*., 2006) There is a growing interest all over the world for discovering the untapped reservoir rof medicinal plants. Hence, the present study was aimed at determining the antioxidant capacities of the plant chosen.

1.6.2 Classification of anti-oxidants

It is of two types (Gupta *et al*., 2006):

1. Based on solubility:

(a) Hydrophilic antioxidants- They are soluble in water. Water soluble antioxidants react with oxidants react with oxidants in the cell cytoplasm and blood plasma.

(b) Hydrophobic antioxidants- They are soluble in lipids. Lipid soluble antioxidants protect cell membranes from lipid peroxidation.

2. Based on line of defense:

(a) First line defense (preventive antioxidant)-

These are enzymes like superoxide dismutase (SOD), catalases (CAT), glutathione peroxidase (GTX), glutathione reductase and some minerals like Se,Mn,Cu etc. SOD mainly acts by quenching of superoxide (O_2), catalase by catalyzing the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. GTX catalyzes the reduction of H_2O_2 and lipid peroxide generated during lipid peroxidation to water using reduced glutathione as substrate.

(b) Second line defense (Radical scavenging antioxidant)-

These are glutathione, Vit C, uric acid, albumin, bilirubin, Vit E, carotenoids, flavonoid etc. β-carotene is an excellent scavenger of singlet oxygen. Vit C interacts directly with

radicals like O_2, OH. GSH is a good scavenger of many free radicals like O_2, OH and various lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone.

(c) Third line defense (Repair and de-novo enzymes)-

These are a complex group of enzymes for repair of damaged DNA, protein, oxidized lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. These enzymes repair the damage to biomolecules and reconstitute the damaged cell membrane.

1.7 Review on *Bombax ceiba*

Plants have been an important source of medicines since the beginning of cultivation. There is a growing demand for plant-based medicines, health products, pharmaceuticals, food supplements, cosmetics etc. *Bombax ceiba* Linn. (Bombacaceae) is a tall tree buttressed at the base that is widely distributed throughout India, Ceylon and Malaya, upto 1500 m of altitude. Many parts of the plant (root, stem bark, gum, leaf, prickles, flower, fruit, seed and heartwood) are used by various tribal communities and forest dwellers for the treatment of a variety of ailments. The plant literature survey shows the plant possesses astringent, cooling, stimulant, diuretic, aphrodisiac, demulcent, and tonic effects and also helps in dysentery. It also possesses important pharmacological activity such as aphrodisiac, anti-inflammatory and hepatoprotective activity in addition to anticancer and anti-HIV activity, anti-*Helicobacter pylori*, antiangiogenic, analgesic and antioxidant activity and hypotensive, hypoglycemic and antimicrobial activity. It is reported to contain important phytoconstituents such as naphthol, naphthoquinones, polysaccharides, anthocyanins, shamimin and lupeol. (Chaudhary and Khadabadi,2012)

1.7.1 Vernacular names of *Bombax ceiba*

Local name:	Semal
Sanskrit name:	Salmali
Common Name:	Silk cotton tree, Red silk cottontree, Semal
Trade Name:	Semal

1.7.2 Taxonomical Classification

Kingdom:	Plantae
Division:	Magnioliophyta
Class:	Magniolipsida
Order:	Malvales
Family:	Malvaceae (Bombacaceae)
Genus:	Bombax
Species:	ceiba
Binomial name:	*Bombax ceiba* L., *Bombax malabaricum* D.C.

1.7.3 The Plant Family of Bombacaceae

The family of Bombacaceae consists of about 22 tropical genera and 150 species. The largest genera include bombax (60 species), ceiba (15 species). *Bombax ceiba* produces large sized timber of light weight which is used for variety of purposes that is from manufacture of match box and match splint to veneer and plywood. This is very important for the conservation of Asian vultures which is near to extinction. Other commercial products derived from this family include Floss (Kapok) from *Ceiba pentendra* and Silk cotton from *Bombax ceiba* (Semal). (Rajendra, K.,2007)

1.7.3.1 Salient Features of Family Bombacaceae

Semal is a very large, conspicuous and attractive tree, growing to 40-meter-tall on occasions. It is deciduous, but even when leafless, the tree can be easily recognized by the arrangements of branches which grow out from the trunk in regular whorls (whorl branching) (Rajendra, K.,2007).

This family has mostly the large trees with buttressed trunks. This family has the Leaves Simple or Digitate, often curved with scurfy scales, alternate.

Fig 1.3: Whole Plant and Flower

In most of the species it has the palmate type of compound leaves but in many species we can find also the simple leaves. It has the complete flowers which are bisexual, generally large and showy, arranged in crowded fascicles with the Calyx mostly five toothed, persistent and Petal often elongated. It bears medium to large size fruits (capsules) which dry in most cases but also has the fleshy, loculicidally dehiscent or indehiscent in nature. It has the valves and because of large size and weight, it could not fall away from the tree base. The seeds are found generally embedded in hairs from the wall of the fruit, with little or no albumen (Rajendra, K.,2007).

5 species of trees belonging 3 genera occur in India. They are *Bombaxceiba, Bombax insigne, Bombax scopuloram, Ceiba pentendra and Cullenia rosayroana.* But in Nepal, only 3 species *Bombax ceiba, Ceiba pentendra and Bombax insigne*are found. *Bombax ceiba* is found in tropical areas (in south) whereas *Bombax insigne* is found in low hills i.e subtropical areas of Nepal. Besides, *Ceiba pentendra* is the exotic species for Nepal which is mostly found in plantations. This *Ceiba pentendra* has been introduced since roughly 2 decades earlier. This tree, which was previously known as *Salmalia malabarica,* is a species of the moist tropics and occurs in India, Burma, and the sub Himalayan region.

1.7.4. Growth Habit

Semal is called Kings of the Forest due to their massive size and showy flowers. It is a large deciduous tree with a straight cylindrical stem and horizontally spreading

branches in whorls. This horizontally branching system in whorls, large size and the buttress at the base are the first seen characteristics to distinguish the species in the forest. The tree reaches up to 40 meter in height and 2 meter in diameter with the clear bole of 24-30 meter. Large trees are invariably buttressed at the base. Stem buttresses at the base and go up to 5-6 meter in height. 5-6 m in height (Rajendra, K.,2007).

1.7.5. Morphology

The young stem and branches are covered with sharp, straight, stout prickles up to 1.2 cm long with woody conical bases.

1.7.5.1. Bark

Bark of Semal looks pale ashy to silver grey, 1.8 -2.5 cm thick, smooth up to middle age, becoming rough with irregular vertical cracks on older trees. (Rajendra, K.,2007)

Fig 1.4: Bark of *Bombax ceiba*

1.7.5.2. Leaves

Semal tree has the compound leaves which is palmate in appearance. It is exactly appearing as the palms appear in man. It is digitate, large, spreading, glabrous which has common petiole, and the size of leaf is 15-30 cm long. One leaf is composed of several leaflets. Five leaflets are common in one leaf but sometimes upto the seven leaflets could be found. The

size of leaflets varies from10 to 20 cm. generally the leaflets found in the center are longer as in the fingers in palm. The leaflets are lanceolate, acuminate, more or less coriaceous and entirely glabrous (Rajendra, K.,2007).

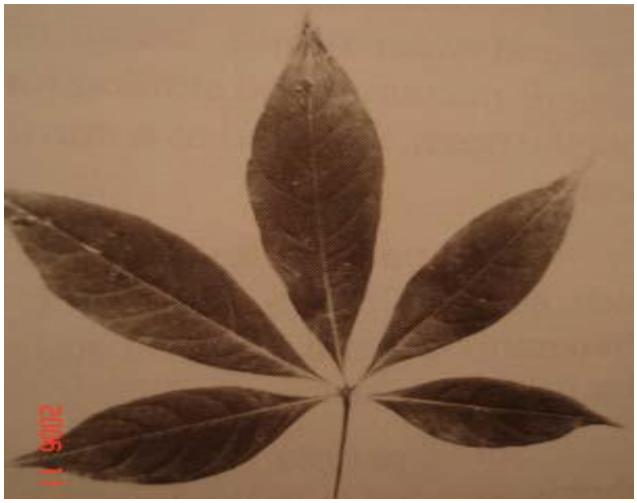


Fig 1.5: Leaves of Bombax ceiba

1.7.5.3. Flowers

The bright red flowers, which appear in January to March, are large and conspicuous on the leafless trees. It presents a strikingly remarkable sight in winter and spring when the usually bare branches are covered with large, fleshy, red flowers. Birds are attracted to them and are probably responsible for their pollination. These flowers form a scarlet carpet on the ground for few weeks (2-3weeks) after dropping. The flowers of semal are very showy, attractive and visible from long distances also. Because of its beautiful and attractive flowers, people like to plant it as the ornamental plant in the botanical garden, garden or as the avenue species. Flowers are numerous, large, 10-12.5 cm across. It clustered towards the ends of branches at the time of flowering. It has the thick, fleshy and cup shaped Sepals. It bears generally 5 petals in one flower which are 7.5-15 cm long oblong, recurved above, and fleshy, of bright crimson (rarely yellow or orange) color (Rajendra, K.,2007).

Fig 1.6: Flower of *Bombax ceiba*

1.7.5.4 Capsule:

The pods are about 10-18 cm in length, oblong-oval in shape, locucidally 5 valved; valves woody, downy outside, lined with silky hairs within.

1.7.5.5 Seeds

Within the capsule it has many seeds which are obovoid, smooth, 6-9 mm long in size. These seeds are oily and surrounded by a thick mass of long silky hairs or floss, hence easily blown about by wind.

Fig 1.7: Seeds of *Bombax ceiba*

1.7.6. Habitat and Distribution

Bombax ceiba Linnaeus belongs to the family Bombacaceae which contains about 26 genera and nearly 140 pantropical species. It is commonly known as simbal, simul, Indian kapok, katsavar, Indian bombax or red silk cotton tree. It is widely found in temperate Asia, tropical Asia, Africa and Australia. In India, it can be found at altitudes up to 1500 m. In peninsular India, the tree is very common in the dry as well as moist deciduous forests and near rivers. The tree is a strong light-demander and fast growing. It grows best on deep sandy loams or other well-drained soils, particularly in valleys, in regions receiving 50 to 460 cm annual rainfall well distributed throughout the year (Chaudhary and Khadabadi, 2012).

Semal is widely distributed in Indian subcontinent except extremely arid regions ascending up to 1200 meters and occasionally up to1500 meters. In Nepal, it is found from Terai (70 m) up to about 1300 meters. It seeks moist, protected valleys preferably flat ground near stream banks where it is often gregarious. Though typical of the alluvial Savanna type of forests, it also grows sporadically in mixed deciduous forests in the lower valleys and in the Sal (*Shorea robusta*) forest. Though it is generally scarce in the hills. It is very common in the Bhabar and Terai tracts (tropics) of Nepal and India especially in the open grazing grounds in miscellaneous forests. It is often found growing in association with Sal (*Shorea robusta*). It is often only tree species left in villages in the Terai. Although it has a very wide range of distribution, it is nowhere very common, usually occurring scattered in mixed deciduous forests. Occasionally, it tends to be gregarious on alluvial soils near river banks and grassy savanna lands. It also occurs in India, Sri Lanka, Pakistan, Bangladesh, Myanmar, Java, Sumatra and Northern Australia. (Rajendra k., 2007)

1.7.7 Ethanobotony

According to (Chaudhary and Khadabadi, 2012)

1.7.7.1 Abortifacient

Tribal people throughout India are well-acquainted with the knowledge of the plant's usage. Preparation of about 30g of seed powder of *B. ceiba* and about 10g hing *(Ferula foeitida)* are used as an abortifacient by the Oraon tribe in West Bengal.

1.7.7.2 Aphrodisiac, Birth Control, Sexual Diseases & Tonic

An ethnobotanical survey of the tribal area of southern Rajasthan was carried out during the year 2001–2002 for ethnosexicological herbal medicines. *B. ceiba* was used as described: half a cup of ethanol extract of bark and flower was given for 3 days to both men and women with sexual diseases like hydrocele, leucorrhoea, gonorrhea and was also used to check menstrual disorders in women. Studies on the ethnomedicobotany of the Kandha tribe of Orissa showed that one teaspoon juice of fresh stem bark of *B. ceiba*, one teaspoon juice of fresh root of *Asparagus racemosus*, powder of seven black peppers (dried seed of *Piper nigrum*L., Piperaceae) and one teaspoon of processed sugar or gum taken orally on an empty stomach two times daily for 21 days to cure gonorrhoea, impotency, spermatorrhea, sterility, nocturnal emission and leucorrhoea. It is also prescribed for increasing sperm in semen and to act as aphrodisiac (Manu Vhokta). Another study was carried out in Sitamata Wildlife Sanctuary of Chittorgarh and Udaipur district located in the southwest region of Rajasthan. This study showed that bark, flower and powdered root barks of *B. ceiba* are used in hydrocele, leucorrhoea, gonorrhoea and to regularize menstruation, urinary problems and as a tonic. An ethnobotanical study has very often resulted in the discovery of important drug plants. An infusion of the bark of *B. ceiba* is used as a tonic.

1.7.7.3 Anti-Inflammatory Activity*:* An ethnobotanical study of traditional anti-inflammatory plants used by the Lohit community of Arunachal Pradesh showed that fresh paste prepared from the bark of *B. ceiba* mixed with cow dung was applied over back muscle of leg at night to treat hotness and inflammation.

1.7.7.4 Impotency, Asthma & Small-Pox Boils*:* An ethnobotanical study examined the folk medicinal uses of certain plants by tribes of the Sonbhadra district in Uttar Pradesh. Root powder of *B. ceiba* was used as a tonic to treat impotency, 10 g of root powder was advised daily with a glass of milk. A powder of stem prickles was used to treat asthma; about 10 g (one spoonful) powders was taken with a glass of cow's milk/fresh water in the morning for 3-4 months. Seed paste prepared in water was applied on small-pox boils.

1.7.7.5 Muscular Injury*:* An ethnobotanical study on medicinal plants around Mtyinggeling, Hainan, China showed that *B. ceiba* barks androots were used to treat muscular injury.

1.7.7.6 Wounds: Ethnomedicinal uses of useful plants from Mysore and Coorg districts, Karnataka included using the paste of *B. ceiba* bark externally for cattle wounds.

1.7.7.7 Anti-Diarrheal*:* The native people of state Mizoram used traditional methods of treatment based on herbal drugs. Decoction of theleaves of *B. ceiba* and the bark of *Mangifera indica* was taken (5 ml, 2-3 times daily) orally to treat diarrhea. An ethnobotanical survey of medicinal plants used by traditional practitioners and religious healers of Bangladesh has shown that seeds and roots of *B. ceiba* were used in the treatment of leprosy, Pimples and skin disease. The ethnopharmacology of medicinal plants among the tribal communities of North-West Frontier Province, Pakistan showed applications of *B. ceiba* in the treatment of skin diseases and in folk cosmetics. Fresh bark of *B. ceiba* was crushed and applied topically on pimples, carbuncles and boils.

1.7.7.8 Anthelmintics

A survey was conducted in southern Punjab, Pakistan, to document existing ethnobotanical knowledge by the herdsmen respondents about anthelmintics in ruminants. Flowers of *B. ceiba* (25–50g as feedstuff) were fed to the animal as anthelmintics.

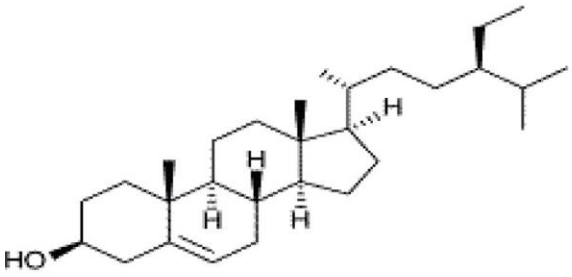
1.7.7.9 Miscellaneous Uses

An ethnomedicinal and ethnopharmaco-statistical studies in Eastern Rajasthan shows multiple uses of *B. ceiba*. The tender twig was used as a toothbrush to cure mumps. Powdered flowers mixed with honey were given in menorrhagia. The thorn was rubbed on stone with unboiled milk, made into paste and applied for 5–6 days as ointment on the face to get rid of acne. The thorn was crushed and chewed with stem bark of *Cordia gharaf* to cure mouth sores. The roots powdered with those of *Chlorophytum, Capparis sepiaria* and fruits of *Pedalium murex* were taken with water as a tonic for 7–8 days to calm body heat.

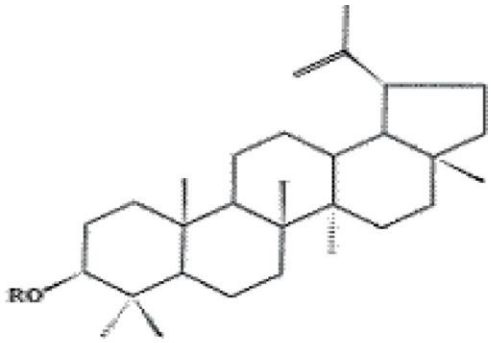
Root bark extract was given as a tonic in case of sexual debility and also as nervine tonic. Root powder mixed with sugar candy and milk was taken to avoid impotency.

1.7.8 Phytochemistry

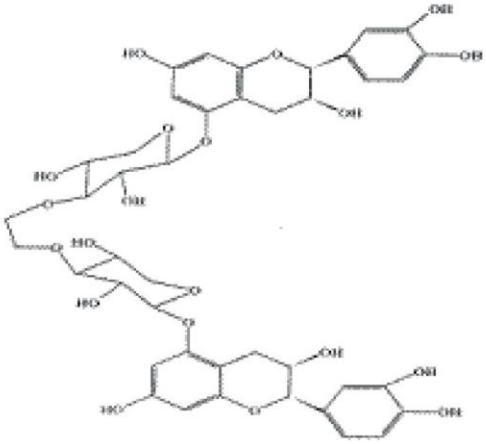
B. ceiba flowers have been shown to contain the β-Dglucoside of β-sitosterol, free β-sitosterol, hentriacontane, hentriacontanol, traces of an essential oil, kaempferol, and quercetin. Shamimin, a newly discovered flavonol C-glycoside has been isolated as a pale yellow powder from the ethanolic extract of fresh, undried leaves of *B. ceiba*. Its structure has been elucidated as 2-(2, 4, 5-trihydroxyphenyl)-3, 5, 7-trihydroxy-6-C-glucopyranosyloxy-4H-1-benzopyran-4-one through extensive spectroscopic methods (IR, mass, 1H- and 13C-NMR), and 2D-NMR experiments. The Ph.D work presented by Muhammad Ali Versianire viewed the phytochemical studies of *B. ceiba.* Dried leaf extracts of the plant were subjected to chemical investigation, which led to the isolation of three new compounds [4-C-β-D Glucopyronosyl-1, 3, 6, 8-tetrahydroxy- 7-O-(4”-hydroxybenzoyl)-9H-xanthen-9-One (I), 2-C-β-D Glucopyronosyl-1, 6, 7-trihydroxy-3-O-(4”-hydroxybenzoyl)- 9H-xanthen-9-One (II), 4-C-β-D Glucopyronosyl-1, 6, 8-trihydroxy-3, 7-di-O-(4”-hydroxybenzoyl)-9H-xanthen- 9-One (III)] and one known compound mangiferin. A sesquiterpene lactone isolated from the roots of a plant species identified as *Salmalia malbaricum* (*Bombaxceiba*) was previously identified as hemigossylic acid lactone-7-methyl ether. 2D NMR experiments have shown that this was a new compound, isohemigossylic acid lactone-2-methyl ether (Chaudhary and Khadabadi, 2012).



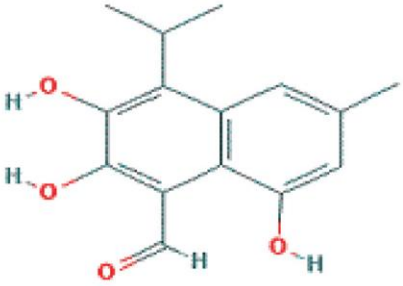
b-sitosterol



Lupeol



Shamimin



Hemigossipol

1.7.9 Pharmacology of *Bombax ceiba*

1.7.9.1 Hypotensive Activity

Shamim in along with lupeol [lup-20 (29) en-3b-ol], which possesses potent hypotensive activity, have been isolated from *B. ceiba* stem bark. BCBMM [filtrate from BCBM (Methanol extract of defatted stem bark)] One of the most active fractions has revealed its adverse effects on heart, liver and kidneys of mice at the dose of 1000 mg/ kg/d.

1.7.9.2 Antioxidant Activity

The antioxidant activity of a methanol extract of *B. ceiba* was evaluated using several antioxidant assays, in terms of its: (i) ability to scavenge DPPH (1, 1-diphenyl-2-picrylhydrazyl) and hydroxyl free radicals; (ii) action against lipidperoxidation (in rat liver

microsomes and soy bean phosphatidylcholine liposomes), induced by ascorbyl radicals and peroxynitrite; and (iii) effect on myeloperoxidase activity. The cytotoxicity was monitored through the mitochondrial activity in the Vero cell line. The extract showed antioxidant activity in all assays. The EC (50) for DPPH was 87 μg/ml; lipid peroxidation of microsomes and soy bean liposomes induced by ascorbyl radicals were

141 μg/ml and 105 μg/ml, respectively, and by peroxynitrite were 115 μg/ml and 77 μg/ml, respectively. The K (0.5) value for myeloperoxidase activity inhibition by the extract was 264 μg/ml. The extract showed very low toxicity toward Vero cells. The total phenolic content present in water extracts of *B. ceiba* (elaimbul; gum), was determined by Folin-Ciocalteu method. Caffeine and gallic acid were quantified by high performance liquids chromatography (HPLC). Total free radical scavenging activity of each ingredient was investigated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and the values were compared with phenolic and gallic acid present in each plant. The polyphenol content of *B. ceiba* were 32.57 ± 5.04% of total extractable. Detectable levels of gallic acid were present only in *B. ceiba* 1.46 mg/g of total extractable). The EC50 values for DPPH radicalscavenging activity for *B. ceiba* were 15.47 ± 1.80 μg cm-3, The mean values of EC50 (y) for DPPH radical scavenging activity were correlated with total phenolics (x) present in plant extracts (Dar *et al*.,2005).

1.7.9.3 Analgesic Activity

Mangiferin, 2-Beta-D-glucopyranosyl-1,3,6,7-tetrahydroxy- 9H-xanthen-9-one, obtained directly from methanol extracts of *B. ceiba* leaves demonstrated strong antioxidant activity (EC50 (5.8 +/- 0.96 μg/ml) using DPPH assay. The acetyl and cinnamoyl derivatives were found to be less active than mangiferin whereas methyl and 3, 6, 7-trimethylether tetraacetate derivatives were inactive implying that for antioxidant activity, free hydroxyl groups and catechol moiety are essential. Moreover, mangiferin showed hepatoprotective activity against carbon tetrachloride induced liver injury further supporting the free radical scavenging property in the *in vivo* system. Additionally, crude plant extracts and purified mangiferin failed to exhibit acute anti-inflammatory activity whereas, extracts displayed significant analgesic effect in acetic acid-induced writhing and hot plate tests in mice.

Using naloxone, it was revealed that plant extract induced analgesia was independent of the opioid receptor; whereas, mangiferin demonstrated significant interaction with the receptor at a peripheral site, with a slight contribution at the neuronal level not affect the growth of tumor cell lines such as SK-MEL-2, A549 and B16-F10 melanoma.

1.7.9.4 Hypotensive and Hypoglycemic activity

Shamimin, a *C*-flavonolglucoside from *B. ceiba* leaves showed significant potency as a hypotensive agent at the doses of 15 mg/kg, 3 mg/kg, 1mg/kg and significant hypoglycaemic activity at 500 mg/kg in sprague dawley rats. (Saleem et al,1999)

1.7.9.5 Antimicrobial and Antibacterial activity

Plant extracts (methanol and aqueous) were assayed for their activity against multi-drug resistant *Salmonella typhii*. Strong antibacterial activity was shown by the methanol extracts of *Salmalia malabarica.*Or plant parts were collected, dried, homogenized and extracted in two organic solvents viz. methanol and acetone. The antibacterial activity against *Klebsiella pneumonia* was done agar disc diffusion method. The activity was compared with standard antimicrobials Amikacin and Piperacillin.

1.7.9.6 3

A methanol extract of the stem barks of *B. ceiba* was found to exhibit a significant antiangiogenic activity on *in vitro* tube formation of human umbilical venous endothelial cells (HUVEC). Bioactivity-guided fractionation and isolation carried out on this extract identified lupeol as an active principle. At 50 and 30 μg/ml, lupeol showed a marked inhibitory activity on HUVEC tube formation while it did not affect the growth of tumor cell lines such as SK-MEL-2, A549 and B16-F10 melanoma.

1.7.10.1 Cytotoxicity

Aqueous extracts of the plants were screened for their cytotoxicity using the brine shrimp lethality test. The present study supports that brine shrimp bioassay is simple reliable and convenient method for assessment of bioactivity of medicinal plants and lends support for their use in traditional medicine (Alluri and Gottumukkala, 2005).

1.7.10.2 Hepatoprotective activity

The hepatoprotective activity of a methanol extract of flowers of *B. ceiba* (MEBC) was investigated against hepatotoxicity produced by administering a combination of two anti-tubercular drugs isoniazid (INH) and rifampicin (RIF) for 10 and 21 days by intraperitoneal route in rats. MEBC were administered at three graded dose i.e. 150, 300and 450 mg/kg i.p. 45 min prior to anti-tubercular challenge for 10 and 21 days. MEBC was evident in all doses as there was a significant decrease in alkaline phosphatase (ALP), alanine transaminases (ALT), aspartate transaminases (AST) and total bilirubin levels, but increase in the level of total protein in comparison to control. MEBC significantly decreased the level of TBARS (thiobarbituric acid reactive substances) and elevated the level of GSH (reduced glutathione) at all doses as compared to control. The results obtained from the analysis of biochemical parameters and histopathological studies, resulted in the conclusion that the MEBC were not able to completely revert the hepatic injury induced by INH and RIF, but it could limit the effect of INH and RIF to the extent of necrosis (Saleem *et al*, 2003).

1.7.10.3 Inhibitory Effects on Fatty Acid Syntheses

Fatty acid syntheses (FAS) had been found to be over express and hyperactive in most cancers. Pharmacological inhibitors of FAS activity preferentially repress cancer cell proliferation and induce cancer cell apoptosis without affecting nonmalignant fibroblasts. These made FAS an excellent drug target for cancer therapy. The FAS activity is the lowest in gastric cancer cell N87 (15.91 ± 3.61 U/ mg protein) and the highest in lung cancer cell A549 (127.36 ±.14 U/mg protein). The cancer cell A549 was used as a cell model to test the inhibitory effort of flavonoid extracts on FAS. The minimum inhibitory concentration of *B. ceiba* Linn was 247.98 μg/ml.

1.7.10.4. Antipyretic

The methanol extract of *Bombax malabaricum* (syn *Bombax ceiba*) leaves (MEBM) was investigated for the antipyreticactivity in rats. MEBM possessed significant antipyretic

activity in Baker's yeast-induced pyrexia. Phytochemical tests showed the presence of steroids, carbohydrates, tannins, triterpenoids, deoxy-sugars, flavonoids and coumarin glycosides.

1.7.10.5. Aphrodisiac

The aphrodisiac activity of *B. ceiba* root extract was investigated. The extract (400 mg/kg body wt./day) was administered orally by gavage for 28 days. Mount latency (ML), intromission latency (IL), ejaculation latency (EL), mounting frequency (MF), intromission frequency (IF), ejaculation frequency (EF) and post-ejaculatory interval (PEI) were the parameters observed before and during the sexual behavior study at day 0, 7, 14, 21, and 28 days. The extract reduced significantly ML, IL, EL and PEI ($p < 0.05$). The extract also increased significantly MF, IF and EF ($p < 0.05$). These effects were observed in sexually active and inactive male mice. (Singh and Singh, 2002)

1.8. Medicinal uses of different parts of *Bombax ceiba.*

Root: Diarrhoea, dysentery, boils & burns, diabetes, impotence& as aphrodisiac, night pollution, scorpion sting &snakebite, sex tonic, urinary troubles, brain tonic, gonorrhoea, syphilis, bedwetting, leucorrhoea, & spermatorrhoea.

Stem & Bark: Acterial, viral, protozoal infection &digestive disturbances, Boil, heartburn, heart tonic, kidney stone, spermatorrhoea & weakness, headache, dislocated bones, easy delivery, snakebite, scorpion, centipede & spider stings.

Gum: Asthma, giardiasis, bleeding piles, diarrhea &dysentery, dental caries, aphrodisiac & in scabies.

Leaf: Glandular swellings, rheumatism, antidysenteric, haematinic, menorrhagia, leucorrhoea, Anaemi a& infertility.

Flower: Haematuria, anaemia, lecucorrhoea, haemorrhoids, hydrocoele, gonorrhoea, menstrual disorders & leucorrhoea, boils & sores, splenomegaly, internal bleeding & cancer, colitis, premature ejaculation, snakebite, permanent sterilization, diuretic & laxative.

Fruit: Antifertility agent, uterus protrusion, leucorrhoea.

Fruit & heartwood: Antidiabetic, antidiarrhoeal, snakebite.

Seed: Chicken pox & small pox (Jain, Verma and Katewa, 2007).

1.9 Different Works Done on Genus Bombax:

Name of the plant	Used Part	Activity
Bombax buonopozense (Akuodor *et al*.,2011)	Leaf and Root	The root extract demonstrated antibacterial activity against all the organisms tested, while the leaf extract had activity on *S. aureus and B. subtilis* only.
Bombax pentadrum (Sciencedirect.com, 2015)	Different Parts of These Plants	Antisickling activity
Bombax insigne (Sint *et al*., 2012)	Wood Tisue	Physico- mechanical property, anti-fungal and hydrophobicity by N- methylol melamine compound
Bombax munguba (Fehling *et al*., 1998)	Seed Oil	Preparation of malvalic and sterculic acid
Bombax buonopozense (Chris, 2012)	Aqueous Stem Bark Extract	Antimalarial potential

Table 1.2 Different Works Done on Genus Bombax

CHAPTER TWO

LITERATURE REVIEW

Literature Review

Though we have worked on root of this plant, we have studied on various part of this plant. Here is some literature review on different part of this plant.

2.1 *In Vitro* Antioxidant Activity of *Bombax Ceiba*

A study was undertaken to evaluate the antioxidant potential of bark of *Bombax ceiba* (Bombacaceae). Aqueous and ethanolic extracts of the bark were subjected to *in vitro* antioxidant activity screening models such as DPPH, ABTS, nitric oxide and superoxide radical scavenging activity, inhibition of lipid peroxidation, reduction of ferric ions and total antioxidant capacity. Ascorbic acid was used as the standard. In all the models studied, the extracts showed potent antioxidant activity, thereby augmenting it into the present day system of medicine (Gandhare, Soni and Dhongade, 2011).

2.2. Potential Anti-Diabetic Property of *Bombax ceiba*

Bombax ceiba bark extract was evaluated for its hypoglycemic and hypolipidemic potential through normal and streptozotocin-induced diabetic rats administered with graded oral doses(200,400,600mg/kg/day) for 21 days. The result showed that a dose of 600mg/kg of *B. ceiba* extract is the most effective to cause significant ($p<0.001$) hypoglycemic and/or hypolipidemic effect on strepzotocin-induced diabetic rats. This dose also significant hypoglycemic activity. The present study thus provides a scientific rationale for the traditional use of this plant in the management diabetics (Bhavasar and Tatele, 2013).

2.1.3. Free Radical Scavenging Property of *Bombax ceiba*

Silk cotton tree (*Bombax ceiba Linn*.) is a well-known ethnomedical plant. Root of this plant was investigated for its antioxidant potential for the first time. Assesment of antioxidant was done using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and reducing power assay. Preliminary phytochemical screening of the roots showed activity presence of phenolic, tannins, flavins, flavonoids, steroids, saponins and cardiac glycosides. Methanol extract of the root showed high amount of phenolics (30.95% w/w) and tannins (15.45% w/w) and a very good DPPH radical scavenging activity in a dose-dependent reduction ability with a maximum absorbance of 1.11 at a concentration of 500 micro gram of the extract. Acute study in healthy volunteers showed a significant rise in

antioxidant status at the ends of 4 h after administration of 3 gm root powder. This strong *in vitro* and in vivo antioxidant potential of *B.ceiba* dry root powder validates its uses in diabetes mellitus and heart disease as described in the traditional medicine. (Jain *et al*., 2011)

2.4. Anabolic Effect of *Bombax ceiba linn.* Root in Idiopathic Involuntary Weight Loss – A Case Study

Bombax ceiba Linn.is a popular plant among native communities for its medicinal properties. The root is specially used for debility and impotence. We report a case study of a patient of involuntary weight loss without any detectable cause who was administered 1.5 g of *B. ceiba* root powder with milk for 24 weeks. He regained his weight and achieved normal body mass index (19.9 Kg/m2) with 147% rise in fibrinolytic activity and marked improvement in his total antioxidant status without any undesirable side effects with its administration or withdrawal symptoms after its discontinuation. This case study, first time scientifically documents anabolic potential of B. ceiba root powder, which the indigenous communities have been utilizing since ages (Verma *et al*., 2010).

2.5 Analgesic & Antioxidant Activity of Mangiferin & Its Derivatives: The Structure Activity Relationship

Mangiferin, 2-b-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthen-9-one, obtained directly from methanol extracts of *Bombax ceiba* leaves in substantial amounts demonstrated strong antioxidant activity (EC50 5.80.96m g/ml or 13.74mM) using DPPH assay comparable to rutin, commonly used as antioxidant for medical purposes. The acetyl and cinnamoyl derivatives were found to be less active than mangiferin whereas, methyl and 3,6,7-trimethylether tetraacetate derivatives were inactive implying that for antioxidant activity, free hydroxyl groups and catechol moiety are essential. Moreover, mangiferin showed hepatoprotective activity against carbon tetrachloride induced liver injury further supporting the free radical scavenging property in the in vivo system. Additionally, plant extracts and mangiferin failed to exhibit acute anti-inflammatory activity whereas, it displayed significant analgesic effect in acetic acid-induced writhing and hot plate tests in mice. Using naloxone, it was revealed that plant extracts induced analgesia was

independent of opioid receptor, whereas, mangiferin demonstrated significant interaction with it at peripheral site with a slight contribution at the neuronal level (Verma *et al*., 2010).

2.6 *Bombax ceiba* Linn.: Pharmacognosy, Ethnobotany & Phyto-Pharmacology

Plants have been an important source of medicines since the beginning of cultivation. There is a growing demand for plant-based medicines, health products, pharmaceuticals, food supplements, cosmetics etc. *Bombax ceiba* Linn. (Bombacaceae) is a tall tree buttressed at the base that is widely distributed throughout India, Ceylon and Malaya, upto 1500 m of altitude. Many parts of the plant (root, stem bark, gum, leaf, prickles, flower, fruit, seed and heartwood) are used by various tribal communities and forest dwellers for the treatment of a variety of ailments. The plant literature survey shows the plant possesses astringent, cooling, stimulant, diuretic, aphrodisiac, demulcent, and tonic effects and also helps in dysentery. It also possesses important pharmacological activity such as aphrodisiac, anti-inflammatory and hepatoprotective activity in addition to anticancer and anti-HIV activity, anti-Helicobacter pylori, antiangiogenic, analgesic and antioxidant activity and hypotensive, hypoglycemic and antimicrobial activity. It is reported to contain important phytoconstituents such as naphthol, naphthoquinones, polysaccharides, anthocyanins, shamimin and lupeol (Chaudhary and khadabadi,2012).

2.7 A New Lignan with Anti-HBV Activity from the Roots of *Bombax ceiba*

A new lignin bombasinol A (**1**), together with three known compounds was obtained from the ethanol (95%) extract of roots of *Bombax ceiba* L. through its being subjected to silica gel and Sephadex LH-20 chromatography. Their structures were elucidated as 4-(4-(3,5-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxy-phenol (**1**),5,6-dihydroxymatairesinol (**2**), (+)-pinoresinol (**3**) and matairesinol (**4**) on the basis of spectroscopic methods, including 1-D and 2-D NMR (HSQC and HMBC) experiments and by comparison of the data with those previously reported literatures. All these compounds were the first reported from Bombacaceae. The anti-Hepatitis B Virus (HBV) activity of all compounds isolated from *B. ceiba* in the research was evaluated. From the results of the HBV assay, these tested compounds showed inhibitory activity against HepG2 2.2.15 cell lines (Wang *et al*.,2012)

2.8 Cardioprotective Effect of *Bombax ceiba* Flowers against Acute Adriamycin-Induced Myocardial Infarction in Rats

The present study was designed to evaluate the cardioprotective potential of aqueous flower extract of *Bombax ceiba* L., Malvaceae (BC), on the basis of biochemical and histopathological parameters in Adriamycin (Adr) induced myocardial infarction in rats and to compare with vitamin E, a known cardioprotective antioxidant. Male Wister rats were used as in vivo model for the study. BC was administered orally to Wister rats at different doses (150 mg/kg, 300 mg/kg and 450 mg/kg, b.w.) for six days/week for four weeks. Thereafter, all the groups except saline were administered (20 mg/kg, *i.p.*). There was a significant decrease in myocardial superoxide dismutase, catalase and reduced glutathione in animals treated with Adr. Concurrently marked increase in extent of lipid peroxidation was reported. Co-treatment of BC/vitamin E and Adr resulted in an increase in the cardiac antioxidant enzymes and reduction in lipid peroxidation as compared to Adr-treated animals. Adr showed significant decrease ($p<0.001$) in the level of cardiac marker enzymes [Lactate dehydrogenase (LDH) and Serum glutamic oxaloacetic transaminase (SGOT)] in heart homogenate with corresponding increase in their level in serum. In BC/vitamin E treated groups significant increase ($p<0.001$) of LDH in heart homogenate and decrease of SGOT and LDH in serum were observed. Microscopic studies in Adr-treated animals revealed mitochondrial swelling, leukocyte infiltration, lipid inclusions and myofibrillar loss whereas the pre-treatment with BC/vitamin E led to a lesser degree of Adr-induced histological alterations. These findings suggest that aqueous flower extract of BC has protective effect against Adr-induced cardiotoxicity and may have potential as a cardioprotective agent (Patel *et al*., 2011).

2.9 Curative Treatment with Extracts of *Bombax ceiba* Fruit Reduces Risk of Calcium Oxalate Urolithiasis in Rats

Oral administration of ethylene glycol resulted in hyperoxaluria and increased renal excretion of calcium and phosphate. However, supplementation with aqueous and ethanol extracts of *B. ceiba* fruit significantly ($p < 0.05$) reduced the elevated urinary oxalate, showing a regulatory action on endogenous oxalate synthesis. The increased deposition of

stone forming constituents in kidneys of calculogenic rats was also significantly lowered with curative treatment of aqueous and ethanol extract (Gadge and Jalalpure, 2012).

2.10 Ethanobotanical, Pharmacognostical & Physico-Chemical Studies of Stem Bark of *Bombax ceiba* L., Commonly Growing in Eastern Uttar Pradesh Region of India

To evaluate the pharmacognostic, physico-chemical characters and ethanobotany of an important medicinal plant, *Bombax ceiba* L. The pharmacognostic studies out in terms of various investigations like organoleptic or morphological characters, microscopic or anatomical studies, physico-chemical evaluations (loss on drying, ash values, extractive values), preliminary phytochemical screening, TLC finger print profiling and fluorescence analysis of powdered crude drug as per WHO recommended guidelines for standardizations. The detail microscopy revealed the presence of collapsed phloem, non-collapsed phloem, sieve elements, sieve tubes, companion cells and starch grains. Physiochemical parameters such as percentage of foreign matters, ash values, loss on drying, swelling index extractive values were determined. Preliminary phytochemical screening showed the presence of carbohydrates, terpenoids, glycosides, Flavonoids, tannins and phenolic compounds. These studies provided referential information for correct identification and standardization of this plant material. This information will also be helpful to differentiate *Bombax ceiba* from the closely related other species (Wahab *et al*., 2012).

2.11 Effect of *Bombax ceiba* L. on Spermatogenesis, Sexual Behavior & Erectile Function in Male Rats

A number of herbal drugs are advocated in the traditional Ayurvedic literature for the improvement of overall sexual function. Young roots of *Bombax ceiba* Linn. (Fam. Bombacaceae) also known as Semal. Musliare used traditionally in Indian subcontinent as sexual stimulant. Its juice is considered nutritive and restorative tonic. Lyophilised aqueous extract of roots was studied for effect on sexual behavior and spermatogenesis in male albino rats. Administration of 100 mg Kg^{-1} body weight of aqueous extract influenced the five parameters evaluated *in vivo. S*exual behavior analysis in the presence of a female rate,

serum testosterone level, anabolic effects, epididymal sperm count and seminal fructose level were the parameters evaluated. In *B. ceiba* extract-treated animals, a gain in body and sexual organ weights was observed. Mount, intromission and ejaculation frequencies were significantly improved ($P < 0.05$). An increase in serum testosterone levels was also observed, but it was not statistically significant ($P > 0.05$). Seminal fructose content and epididymal sperm count were significantly improved as well. Penile erection index was also higher compared to control group animals. Hesitation time was significantly reduced ($P < 0.01$), and copulatory rate was doubled in treated animals compared with control group animals (Bhargava, Thakur and Yadav, 2011).

2.12 Possible Modulation of FAS and PTP-1B Signaling in Ameliorative Potential of *Bombax Ceiba* Against High Fat Diet Induced Obesity

Bombax ceiba Linn., commonly called as Semal, is used in various gastro-intestinal disturbances. It contains Lupeol which inhibits PTP-1B, adipogenesis, TG synthesis and accumulation of lipids in adipocytes and adipokines whereas the flavonoids isolated from *B. ceiba* has FAS inhibitory activity. The present study was aimed to investigate ameliorative potential of *Bombax ceiba* to experimental obesity in Wistar rats, and its possible mechanism of action.MaleWistar albino rats weighing 180-220 g were employed in present study. Experimental obesity was induced by feeding high fat diet for 10 weeks. Methanol extract of *B. ceiba* extract 100, 200 and 400 mg/kg and Gemfibrozil 50 mg/kg as standard drug were given orally from 7^{th} to 10^{th} week. Induction with HFD for 10 weeks caused significant ($p < 0.05$) increase in % body wt, BMI, LEE indices; serum glucose, triglyceride, LDL, VLDL, cholesterol, free fatty acid, ALT, AST; tissue TBARS, nitrate/nitrite levels; different fat pads and relative liver weight; and significant decrease in food intake (g and kcal), serum HDL and tissue glutathione levels in HFD control rats. Treatment with *B. ceiba* extract and gemfibrozil significantly attenuated these HFD induced changes, as compared to HFD control. The effect of *B. ceiba* 200 and 400 mg/kg was more pronounced in comparison to gemfibrozil. On the basis of results obtained, it may be concluded that the methanol extract of stem bark of *Bombax ceiba* has significant ameliorative potential against HFD induced obesity in rats, possibly through modulation

of FAS and PTP-1B signaling due to the presence of flavonoids and lupeol (Gupta *et al*., 2013).

2.13 Hepatoprotective Activity of Bombax ceiba Linn against Isoniazid and Rifampicin-Induced Toxicity in Experimental Rats

Hepatoprotective activity of methanol extract of flowers of Bombax ceiba L. (MEBC) was investigated against hepatotoxicity produced by administering a combination of two anti-tubercular drugs Isoniazid and Rifampicin for 10 and 21 days by intraperitoneal route in rats. MEBC were administered at three graded dose i.e. 150, 300 and 450 mg/kg i.p. 45 min prior to anti-tubercular challenge for 10 and 21 days. MEBC was evident in the all doses as there was a significant decrease in AST, ALT, ALP, and Total Bilirubin levels, but increased the level of total protein in comparison to control. MEBC significantly decreased the level of TBARS and elevated the level of GSH at all doses as compared to control. Histology of the liver section of the animals treated with MEBC improved the hepatotoxicity caused by antitubercular drugs. The results obtained from the analysis of biochemical parameters and histopathological studies, enabled to conclude that the MEBC were not able to revert completely the hepatic injury induced by INH + RIF, but it could limit the effect of INH + RIF to the extent of necrosis (Ravi *et al*., 2010).

2.14 Hypotensive Activity and Toxicology of Constituents from Bombax ceiba Stem Bark

A novel constituent, shamimicin, 1‴, 1⁗⁗-bis-2-(3,4-dihydroxyphenyl)-3,4-dihydro-3,7-dihydroxy-5-O-xylopyranosyloxy-2H-1-benzopyran alongwith lupeol, which possesses potent hypotensive activity has been isolated from Bombax ceiba stem bark. BCBMM—one of the most active hypotensive fractions has revealed its adverse effects on heart, liver and kidneys of mice at the dose of 1000 mg/kg/d (Saleem et al., 2003).

2.15 Antioxidant activity of methanol extract of *Bombax ceiba*

The antioxidant activity of a methanol extract of Bombax ceiba was evaluated using several antioxidant assays, in terms of its: (i) ability to scavenge DPPH (1,1-diphenyl-2-picryl-hydrazyl) and hydroxyl free radicals; (ii) action against lipid peroxidation (in rat liver microsomes and soybean phosphatidylcholine liposomes), induced by ascorbyl radicals

and peroxynitrite; and (iii) effect on myeloperoxidase activity. The cytotoxicity was monitored through the mitochondrial activity in the Vero cell line. The extract showed antioxidant activity in all assays, the EC50 (μg/ml) for DPPH was 87 and for lipid peroxidation of microsomes and soy bean liposomes induced by ascorbyl radicals were 141 and 105, respectively, and by peroxynitrite were 115 and 77, respectively. The K0.5 value for myeloperoxidase activity inhibition by the extract was 264 μg/ml. The extract showed very low toxicity toward Vero cells (Vieira *et al*., 2009).

2.16 Antiangiogenic Activity of Lupeol from *Bombax ceiba*

In the search for antiangiogenic agents from medicinal plants used in Vietnam, a methanol extract of the stem barks of *Bombax ceiba* was found to exhibit a significant antiangiogenic activity on *in vitro* tube formation of human umbilical venous endothelial cells (HUVEC). Bioactivity-guided fractionation and isolation carried out on this extract afforded lupeol as an active principle. At 50 and 30 μg/mL lupeol showed a marked inhibitory activity on HUVEC tube formation while it did not affect the growth of tumor cell lines such as SK-MEL-2, A549, and B16-F10 melanoma (Patel *et al*., 2011).

2.17 Impregnation of *Bombax ceiba* and *Bombax insigne* Wood with a N-Methylol Melamine Compound

Methylated N-methylol melamine (NMM) is known for its ability to enhance physico-mechanical properties, anti-fungal ability, and hydrophobicity and was therefore used to impregnate two less used and non-durable wood species from Myanmar, Bombax ceiba and Bombax insigne. Solution uptake, weight percent gain and nitrogen content were increased by increasing melamine concentrations with B. ceiba always achieving higher values compared with B. insigne. According to the leaching results, a higher degree of condensation after curing as well as a better crosslinking of NMM could be obtained at higher temperatures. However, both curing temperatures used (90 and 120 °C) resulted in almost the same amount of nitrogen fixed in the cell wall. UV microspectrophotometry confirmed the penetration of the NMM into different morphological regions of wood tissues, which was again supported by the analysis of point measurement spectra of treated and untreated specimens (Sint *et al*., 2012).

CHAPTER THREE

METHODOLOGY

23. Materials and Methods

3.1. Preparation of the Plant Sample

3.1.1 Collection & Proper Identification of the Plant Sample

At first with the help of a comprehensive literature review *Bombax ceiba* was selected for this investigation. The whole plants were collected from a Rampua in Dhaka, Bangladesh and identified by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. The voucher specimens of the plants have been deposited in the herbarium for further reference.

3.1.2 Plant Material Preparation

The leaves of the plants were collected in fresh condition. It was sun-dried and then, dried in an oven at reduced temperature (not more than 50 ^{0}C) to make suitable for grinding purpose. The coarse powders were then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

3.1.3 Extraction Procedure

The powdered plant materials were submerged into methanol in an air-tight flat bottomed container for seven days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant materials were dissolved in the solvent. After that, by using rotary evaporator. pure solvent is separated.

Fig 3.1: Rotary Evaporator

3.2. Brine Shrimp Lethality Bioassay

3.2.1. Preamble

Bioactive compounds are always toxic to living body at some higher doses and it justifies the statement that 'Pharmacology is simply toxicology at higher doses and toxicology is simply pharmacology at lower doses. Brine shrimp lethality bioassay is a rapid and comprehensive bioassay for the bioactive compound of the natural and synthetic origin. By this method, natural product extracts, fractions as well as the pure compounds can be tested for their bioactivity. In this method, *in vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products.

This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal & anti-tumor etc. of the compounds.

Brine shrimp lethality bioassay technique stands superior to other cytotoxicity testing procedures because it is rapid in process, inexpensive and requires no special equipment or

aseptic technique. It utilizes a large number of organisms for statistical validation and a relatively small amount of sample. Furthermore, unlike other methods, it does not require animal serum.

3.2 .1. Materials

- *Artemia salina* leach (brine shrimp eggs)
- Sea salt (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipettes
- Micropipette
- Glass vials
- Magnifying glass
- Test samples of experimental plants.

Test samples that used in this experiment were crude methanol extract of *Bombax ceiba*

3.2.2. Experimental Procedure

Preparation of seawater

38 g sea salt (pure NaCl) was weighed, dissolved in 1 litre of distilled water adjusted to pH 8.5 using 1N NaOH and was filtered off to get clear solution.

Hatching of Brine Shrimps

*Artemia salina*Leach (Brine Shrimp eggs) collected from pet shops was used as the test organism. Artificial seawater was taken in the small tank and Shrimp eggs were addedto one side of the tank and then that side was covered. The tank was kept underconstant aeration for 48 hrs to hatch the Shrimp and to be matured as nauplii. Thehatched Shrimps were attracted to the lamp through the perforated dam and with thehelp of a Pasteur pipette 10 living shrimps were added to each of the test tubes containing 5 ml of Brine solution.

Preparation of Test Solutions

2mg of each sample is measured sample was dissolved in 60µl of DMSO. A series of solutions of lower concentrations were prepared by serial dilution with DMSO. From each of these test solutions 30µg were added to pre-marked glass vials/test tubes containing 5 ml of seawater and 10 Shrimp nauplii. So, the final concentration of samples in the vials/test tubes were 320µg/ml, 160 µg/ml, 80µg/ml,40 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1.25 µg/ml for 9 dilutions.

Preparation of Controls

Vincristine sulphate served as the positive control. 0.2 mg of vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 µg/ml from which serial dilutions were made using DMSO to get 10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1.25 µg/ml,0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml, 0.0390 µg/ml. The control groups containing 10 living Brine Shrimp nauplii in 5 ml simulated seawater received the positive control solutions. As for negative control, 30 µg of DMSO was added to each of the pre-marked test tubes containing 5 ml of simulated seawater and 10 Shrimp nauplii. The test was considered invalid if the negative control showed a rapid mortality rate and therefore has to conduct again. The test tubes (containing nauplii) were then maintained at room temperature for 24 hrs under the light for observing the survival rate.

Counting of nauplii and analysis of data

After 24 hours, the test tubes were inspected using a magnifying glass and the number of survivors was counted. The percent (%) mortality was calculated for each dilution.The concentration-mortality data were analyzed by using Microsoft Excel. The effectiveness or the concentration-mortality relationship of plant product is usually expressed as a median lethal concentration (LC50) value. This represents the concentration of the chemical that produces death in half of the test subjects after acertain exposure period. However, LC90 values were also calculated in the similar way for all fractions and the reference cytotoxic drug vincristine sulphate.

3.3. Preparation & Procedure for Antioxidant Property

3.3.1. Preamble

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. The most common reactive oxygen species (ROS) include superoxide (0_2.) anion, hydrogenperoxide (H_20_2), peroxyl (ROO-) radicals, and reactive hydroxyl (OH.) radicals. The nitrogen derived free radicals are nitric oxide (NO.) and peroxynitriteanion(ONOO.). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome. In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc. They were also suggested to be a potential iron chelator. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties.

3.3.2. Methods

The antioxidant activity of the mehanolic extracts of *Bombax ceiba* were determined by using determination of Flavonoids content.

3.3.2.2 Determination of Flavonoids Content

3.3.2.2.1 Principle

Aluminum chloride colorimetric method was used for flavonoids determination.1 ml of sample was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 510 nm with UV/Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at various concentrations in methanol. The concentration of flavonoids was expressed in terms of mg/100ml of sample.

3.3.2.2.2. Reagent

- Aluminum Chloride ($AlCl_3$)
- Potassium Acetate
- Ethanol or Methanol
- Quercetin (Analytical or Reagent grade)

3.3.2.2.3.

10 gm of $AlCl_3$ was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water.

3.3.2.2.4. Preparation of 1M Potassium Acetate Solution

9.815 gm of potassium was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water.

3.3.2.2.5. Preparation of Standard Solution

Quercetin stock solution of concentration 5µg/µl was prepared by dissolving 0.025 gm of quercetin into 5 ml of methanol. The experimental concentrations from the stock solution were prepared by the following manner:

Concentration (µg/ml)	Solution Taken from Stock Solution	Solution Taken from Others	Adjust The Volume by Distilled Methanol	Final Volume
100	100 µl	-	4.90 ml	5 ml
50	-	2 ml (100µg/ml)	2 ml	4 ml
25	-	2 ml (50µg/ml)	2 ml	4 ml
12.5	-	2 ml (25µg/ml)	2 ml	4 ml

Table 3.1 Preparation of Standard Solution

3.3.2.2.6 Preparation of Extract Solution

0.050 gm of each plant extracts were taken and dissolved into 5 ml of methanol. The concentration of the solution was 10µg/µl of plant extracts.

3.3.2.2.7 Experimental Procedure

- 1.5 ml of each plant extracts (1mg/ml) or standard of different concentration solutions were taken in different test tubes and 3 ml of methanol were added into the test tubes.
- 6 ml of distilled water were added into the test tubes with 0.45 ml solution Sodium Nitrate (5% w/v)
- The test tubes were incubated for 6 minutes at room temperature to complete the reaction. Then 0.45 ml Aluminum chloride (10%) taken and again incubated for 6 minutes.
- Then 6 ml distilled water added.
- The absorbances of the reaction mixtures were measured at 510 nm using a spectrophotometer against blank.
- The calibration curve was prepared by preparing quercetin solution at various concentration in methanol. The concentration of flavonoids was expressed in terms of mg/100 ml of sample which is calculated by the following formula equation.

3.4 *In vitro* Antibacterial Screening

3.4.1 Preamble

Bacteria is responsible for many infectious diseases. The increasing clinical implications of drug resistant fungal and bacterial pathogens have lent additional urgency to antimicrobial drug research. The antimicrobial screening which is the first stage of antimicrobial drug research is performed to ascertain the susceptibility of various fungi and bacteria to any agent. This test measures the ability of each test sample to inhibit the *in vitro* bacterial growth. This ability was estimated by disc diffusion method.

We have used disc diffusion method for our sample. But there is no standardized method for expressing the results of antimicrobial screening. Some investigators use the diameter of zone of inhibition and/or the minimum weight of extract to inhibit the growth of microorganisms. However, a great number of factors viz., the extraction methods, inoculums volume, culture medium composition, P^H and incubation temperature can influence the results.

Among the above mentioned techniques the disc diffusion is a widely accepted *in vitro* investigation for preliminary screening of test agents which may possess antimicrobial activity. It is essentially a quantitative or qualitative test indicating the sensitivity or resistance of the microorganisms to the test materials. However, no distinction between bacteriostatic and bactericidal activity can be made by this method.

3.4.2 Principle of Disc Diffusion Method

Solutions of known concentration (μg/ml) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4 ^{0}C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test

materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel. As a result, there is a gradual change of test materials concentration in the media surrounding the discs. The whole process is done under laminar air flow. The plates are then incubated at 37 ^{0}C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter.

The experiment is carried out more than once and the mean of the readings is required.

In the present study the crude methanol extract of *Bombax ceiba* was tested for antibacterial activity by disc diffusion method.

3.4.3 Experimental

3.4.3.1 Apparatus and Reagents

- Filter paper discs
- Petri dishes
- Inoculating loop
- Sterile cotton
- Sterile forceps
- Spirit burner
- Micropipette
- Screw cap test tubes
- Nose mask and Hand gloves
- Laminar air flow hood
- Autoclave
- Incubator
- Refrigerator
- Nutrient Agar Medium
- Ethanol

- Chloroform

3.4.3.2 Test materials

3.4.3.2.1 Test samples

Crude methanol extract of *Bombax ceiba*

3.4.3.2.2 Test Organisms

- The bacterial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both Gram positive and Gram-negative organisms were taken for the test

List of Test Bacteria

1. *Shigella dysentriae*
2. *Pseudomonas aureus*
3. *Saccharromyces cerevaceae*

3.4.4. Culture Medium & Their Composition

The following media is used normally to demonstrate the antimicrobial activity and to make subculture of the test organisms.

Nutrient Agar Medium

Ingredients	Amounts
Bacto peptone	0.5 gm
Sodium chloride	0.5 gm
Bacto yeast extract	1.0 gm
Bacto agar	2.0 gm
Distilled water q.s. to	100 ml
P^H	7.2 ± 0.1 at 25^0C

Nutrient agar medium (DIFCO) used most frequently for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

3.4.4.1 Preparation of Culture Medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The P^H (at 25 ^{0}C) was adjusted at 7.2 – 7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/ sq. inch at 121 ^{0}C for 20 minutes. The slants were used for making fresh culture of bacteria that were in turn used for sensitivity study.

3.4.5 Sterilization Procedure

In order to avoid any type of contamination and cross contamination by the test organisms the antimicrobial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on one hour before working in the Laminar Hood. Petri dishes and other glasswares were sterilized by autoclaving at a temperature of 121 ^{0}C and a pressure of 15-lbs./sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

Fig 3.2: Laminar Air-Flow

3.4.6 Preparation of Subculture

In an aseptic condition under laminar air hood cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 ^{0}C.

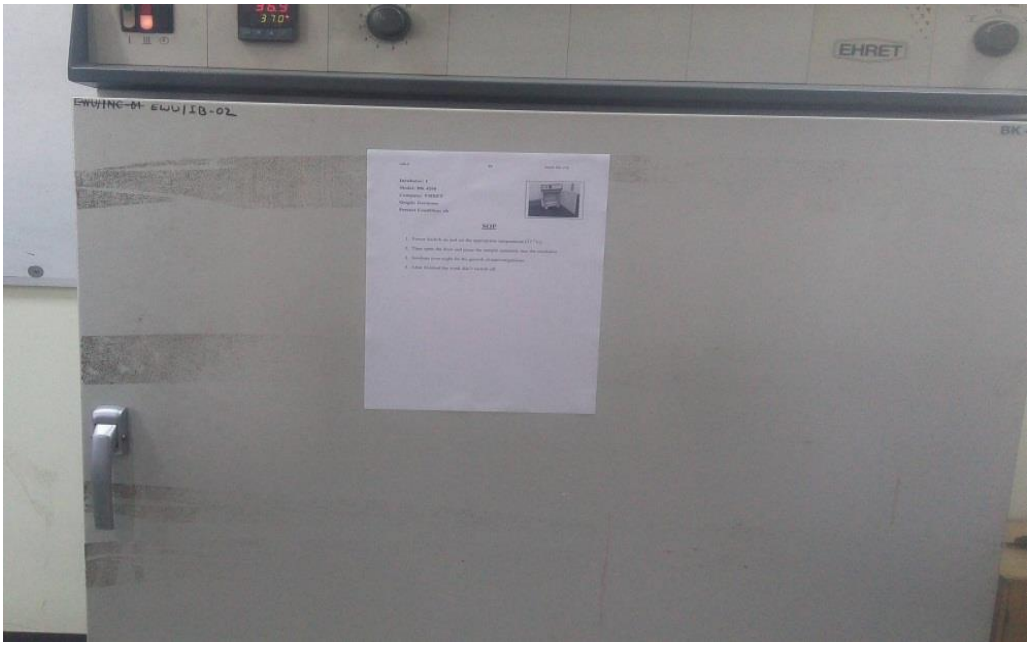


Fig 3.3: Auto-Clave

3.4.7 Preparation of the Test Plates

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

3.4.8 Preparation of Discs

3.4.8.1. Standard Discs

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, Amoxicillin (10µg/disc) standard disc was used as the reference.

3.4.8.2 Blank Discs

These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

3.4.8.3 Preparation of Sample Discs with Test Samples

50 mg of each test samples were dissolved in 2 ml of ethanol to obtain the concentration 25 mg/ml in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

3.4.8.3.1 Preparation of Sample Discs

Methanol extracts of *Bombax ceiba*were tested for antimicrobial activity against a number of both grams positive and gram negative bacteria The amount of sample per disc was 100 µg and 1 mg.

3.4.9 Preparation and Application of the Test Samples

Sample discs were prepared by adding 20µl of the test solutions to the sterile filter paper discs. The discs were then allowed to dry for sufficient period of time until complete evaporation of the solvent. The test samples were applied to previously sterilized discs using adjustable micropipette under aseptic conditions.

3.4.9.1. Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator at 4 ^{0}C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^{0}C for 24 hours.

3.4.10 Determination of Antimicrobial Activity by the Zone of Inhibition

The antimicrobial potency of the test agents were measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

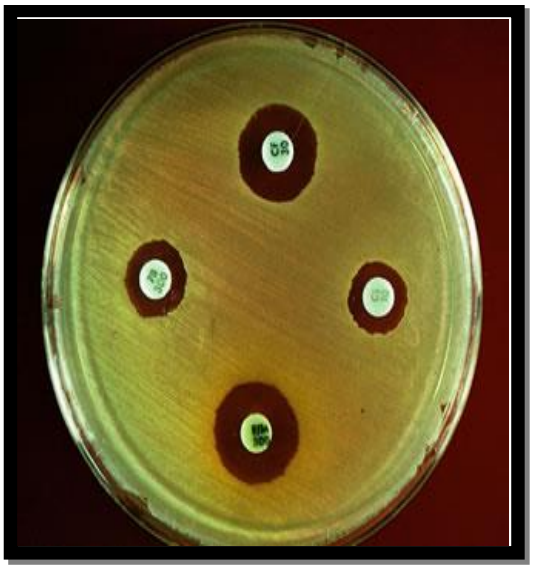


Figure: 3.4 Measuring Zone of Inhibition

CHAPTER FOUR

RESULT & DISCUSSION

4.1 Results and Discussions

4.1.1 Results of Cytotoxicity Assay of *Bombax ceiba*

After 24hrs, the test tubes were inspected using a magnifying glass and the number of survivors counted. The results of brine shrimp lethality bioassay are provided in the table. Test samples showed different mortality rate at different concentration. The effectiveness of the concentration and % mortality relationship of plant product was expressed as a median Lethal Concentration (LC_{50}) value. This represents the concentration of the methanol extract that produces death in half of the test subjects after a certain period. The percentage mortality at each concentration was determined using the following formula. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

$$\text{Percentage (\%) Mortality} = \frac{\text{No of Shrimp Died}}{\text{Total No of Shrimp in Each Test}} \times 100$$

The LC_{50} of the test samples was obtained by a plot of percentage of the shrimps died (%Mortality) against the logarithm of the sample concentration (Log C) and the best-fit line was obtained from the curve data by means of regression analysis.

Table 4.1: Results for Cytotoxic Assay of *Bombax ceiba* (Methanol Extract) on Shrimp Nauplii.

Concentration(C) (µg/ml)	Log C	No. of Nauplii Taken	No. of Nauplii Dead	% mortality	Value of x (log LC_{50})	LC_{50} (µg/ml)
400	2.60206	10	8	80		
200	2.30103	10	7	70		
100	2.00000	10	7	70		
50	1.69897	10	6	60		

25	1.39794	10	5	50	1.3046	20.165
12.5	1.09691	10	5	50		
6.25	0.79588	10	4	40		
3.125	0.49485	10	3	30		
1.5625	0.19382	10	2	20		

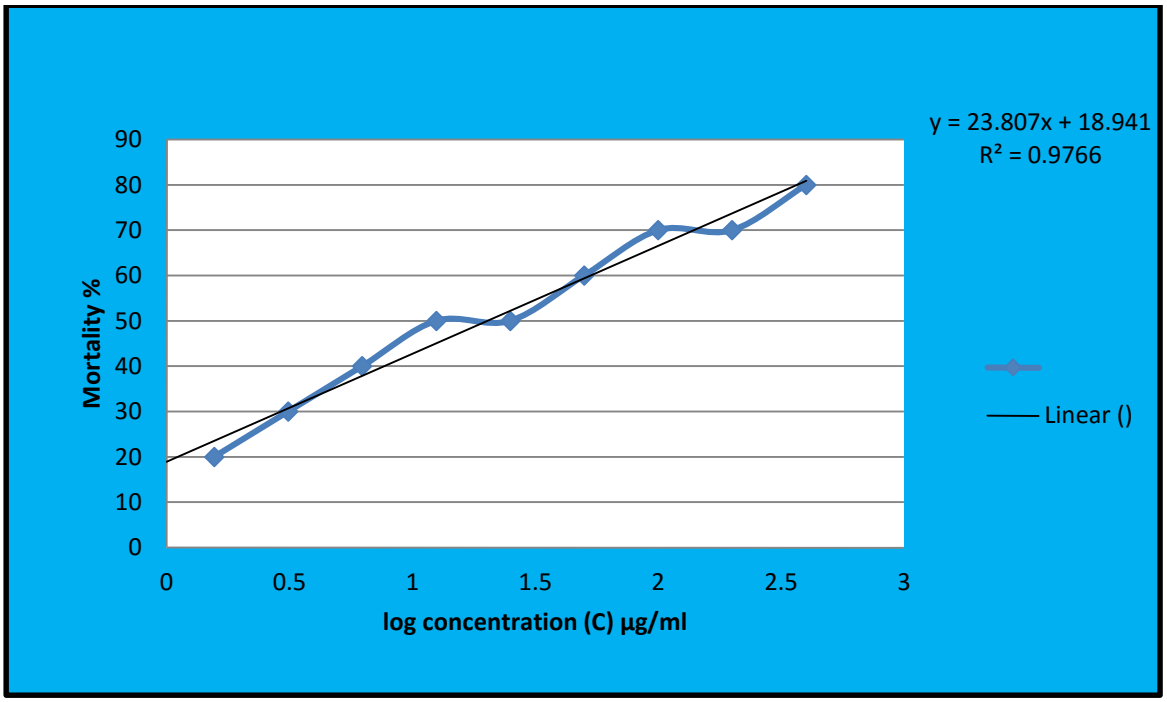


Figure 4.1: Effects of Various Concentrations of Methanol Extract of *Bombax ceiba* on the Viability of Brine Shrimp Nauplii after 24 Hours of Incubation.

From the investigation of in vivo lethality of brine shrimp, it was observed that when the shrimps were exposed to different concentrations of the samples, there was varying degree of lethality. The regression analysis produced a linear correlation between the mortality rate and the concentration of samples in both case of Tamoxifen and methanol extract. It is evident from the data, the percent mortality of brine shrimp nauplii was found to increase gradually with the increase in the concentration of the test samples. Maximum mortalities

occurred at the highest concentration of 400µg/ml, whereas the least mortalities occurred at concentration 1.5625 µg/ml as shown in Table 4.1.

Table 4.2 Linear Regression, R^2 value & LC_{50} Value

Sample	Linear Regression Equation	R^2 Value	LC_{50} (µg/ml) after 24hr
Methanol extract of *Bombax ceiba*	Y=23.407x+18.941	0.9766	20.165

From this experiment, it is observed that methanol extract of *Bombax ceiba* possess cytotoxic activities having LC_{50} values of 20.165µg/ml**.**

4.2.1 Results of antioxidant assay of *Bombax ceiba*

In flavoinoid antioxidant test,we measured the absorbanace of methanol extract of sample at 695 nm.

The total flavonoid content is measured with the aluminium chloride colorimetric assay using quercetin as standard. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition, it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids.

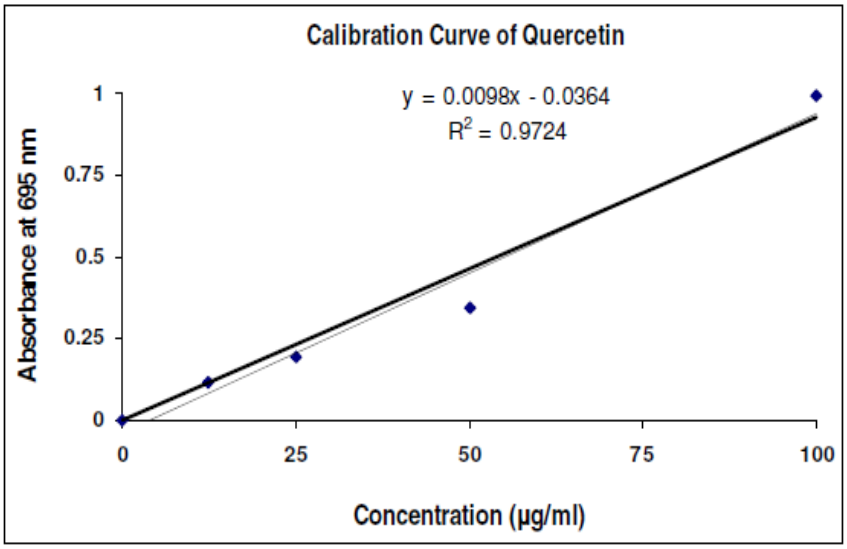


Fig 4.2: Calibration Curve of Quercetin

Table 4.3: Determination of Total Flavonoid Concentration

Sample Containing Methanol Extract of *B. Ceiba*	**Concentration of Sample**	**Equation**	**Value of Y**	**Total Flavonoid Concentration in mg/g** **Which Is the Value of X**	**Average Mean Concentration ± std Deviation**
Sample 1	1mg/ml	Y=0.0098x-0.0364 Where The Value of Y (Absorbance))	0.102	14.122	13.816 ± 1.06
Sample 2	1mg/ml		0.096	13.51	

4.2.2 Results of Antimicrobial test of *Bombax ceiba*

Table 4.4: Observation of Zone of Inhibition

Name of Bacteria	Methanol Concentration of 300µg/disc	Methanol Concentration of 600µg/disc	Zone of Inhibition of Kanamycin µg/disc
Shigella dysenteriae **(Gram Negative)**	10 mm	**7**mm	24mm
Saccharomyces cerevaceae **(Yeast)**	10 mm	8 mm	12mm
Pseudomonas aureus **(All Gram Negative)**	7 mm	7 mm	23mm

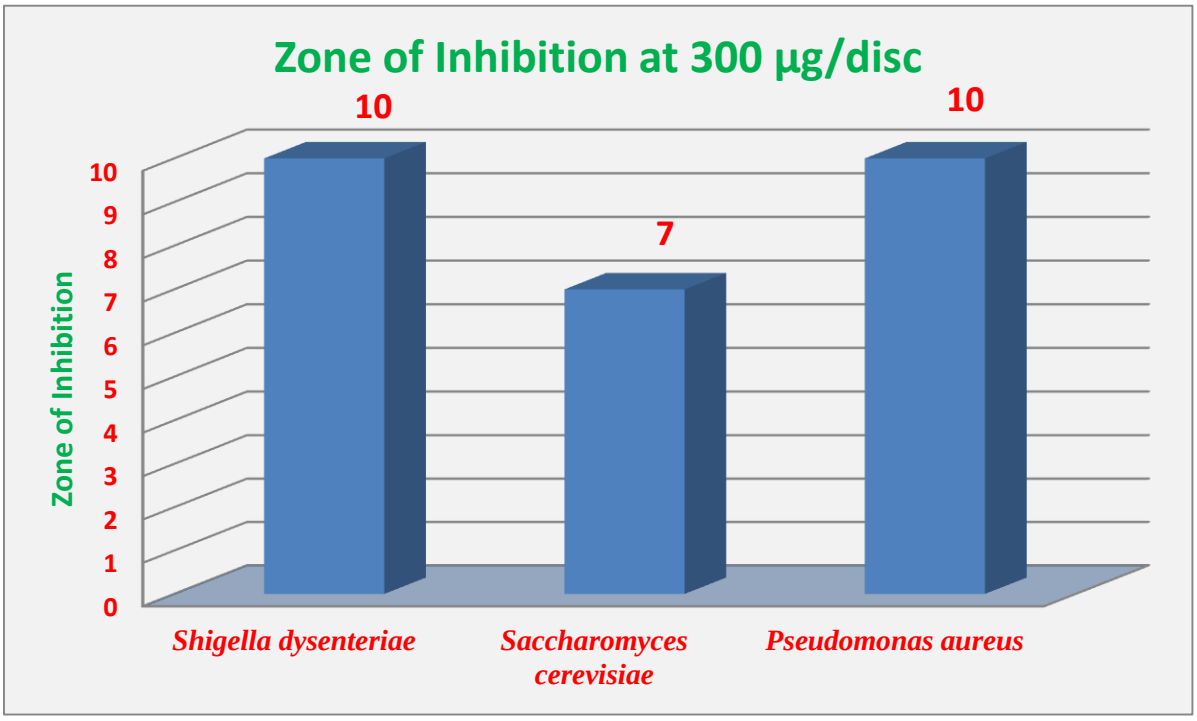


Fig 4.3: Zone of Inhibition at 300 µg/disc of *Shigella dysenteriae, Saccharomyces cerevisiae and Pseudomonas aureus*

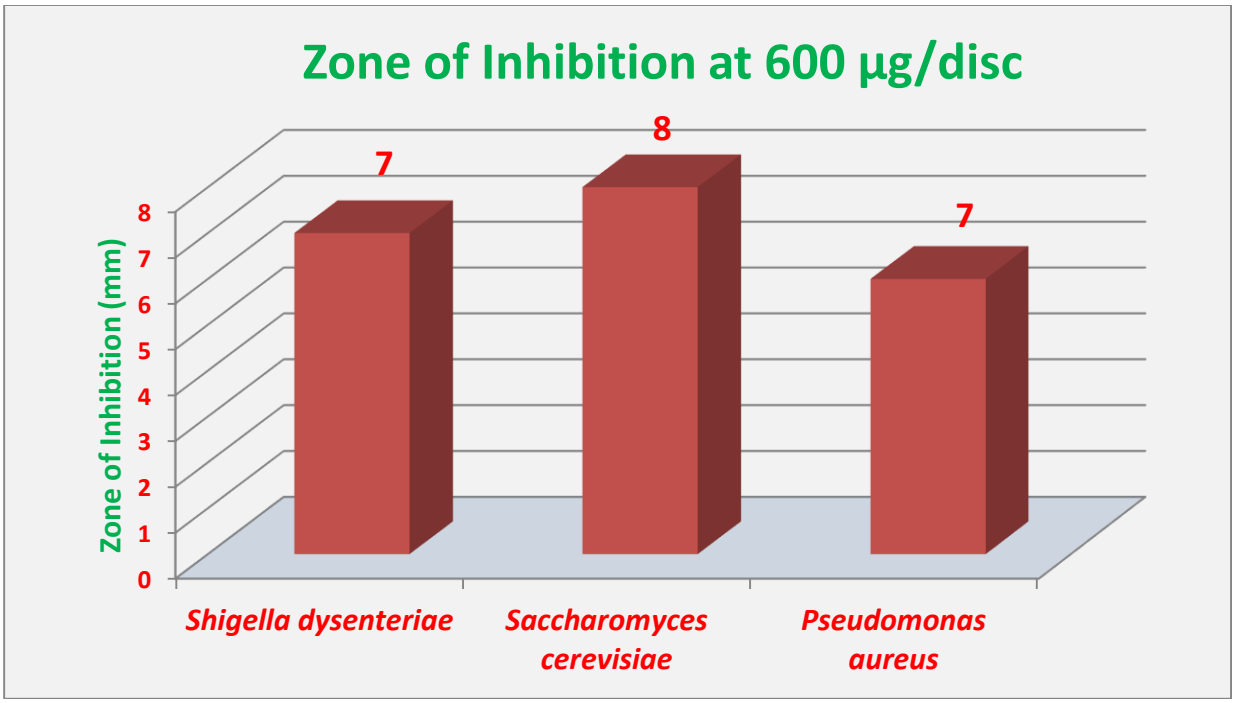


Fig 4.4: Zone of Inhibition at 600 µg/disc of *Shigella dysenteriae, Saccharomyces cerevisiae and Pseudomonas aureus*

Acording to zone of inhihibition of kanamycin, the methanol extract (300 & 600 µg/disc) of *Bombax ceiba* showed antibacterial activity against Gram negative bacteria but the antibacterial activity is quite weaker than kanamycin.

4.3 Discussion

Bombax ceiba is a medicinal plant enriched with various chemical constituents having different medicinal activities. The study has shown the cytotoxic, antioxidant and antimicrobial activities.

The cytotoxic activity of the methanol root extract of *Bombax ceiba* was tested by using brine shrimp in proper condition. The brine shrimp was hatched in the laboratory. The standard conditions were to check the result in 8 hours but due to some problems the result was checked after 16 hours. But the results were sufficient and in acceptable level. From this experiment, it is observed that methanol extract of *Bombax ceiba* possess cytotoxic activities having LC_{50} values of 20.16 5 µg/ml. we can conclude that the methanol root extract of *Bombax ceiba* gives lethal effect.

In the antimicrobial test, the zone of inhibition was tested against Shigella dysenteriae, Saccharomyces cerevaceae & pseudomonas aureus. the zone of inhibition was produced by the methanol root extract of *Bombax ceiba* ranged from 7-10 mm at 300 µg/disc and 7-8 at 600 µg/disc. There resultant zone of inhibition was not good enough. The test result may vary due to some experimental error. The zone of inhibition of the methanol root extract of Bombax ceiba. It is clear that it has activity against gram negative bacteria as zone of inhibition is noticed against *Shigella dysenteriae* & *Pseudomonas aureus*. Not also gram negative but also it has shown its activity against *Saccharomyces cerevaceae* which is a yeast. So we can conclude that it has mild to moderate antimicrobial activity.

The methanol root extract of *Bombax ceiba* has also antioxidant activity. The presence of antioxidants such as phenolics, flavonoids, tannins and proanthocyanidins in plants may provide protection against a number of diseases; for example, ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders. Total flavonoid contents can be determined in the sample extracts/fractions by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminum complex formation using aluminum chloride in alkaline condition which can be monitored spectrophotometrically at wavelength of 695nm. Several studies reported that flavonoids present in herbs significantly contributed to their antioxidant properties. It has been shown

to be highly effective scavengers of most oxidizing molecules, including single oxygen and various free radicals.

Further investigations are required & researches should focus and explore the specific cellular and molecular targets of various constituents which can develop an unknown medicine for a known fatal disease.

CHAPTER FIVE

CONCLUSION

5.1. Conclusion

The results of my study clearly establish the fact that the methanol extract of possesses both cytotoxic and antioxidant activity and antimicrobial activity of *Bombax ceiba.*

The crude methanol extract of *Bombax ceiba* was evaluated for the screening of antioxidant activity by determination of flavonoid content method. Total flavonoid content was measured with the aluminum chloride colorimetric assay. Finally, volume was making up to 10 ml with distilled water and mix well. Orange yellowish color was developed. The absorbance was measured at 695 nm spectrophotometer using UV-visible instrument. The blank was performed using distilled water. Quercetin was used as standard. The samples were performed in triplicates. The calibration curve was plotted using standard quercetin. The data of total flavonoids of polyherbal formulation were expressed as mg of quercetin equivalents/ 100g of dry mass.

The extract can be evaluated using different solvents to obtain further understanding of the antioxidant property because the antioxidant activity of plant origin is dependent on the type and polarity of the extracting solvent as well as on the test system and the substrate to be protected by the antioxidant. Solvent extraction is frequently used for isolation of the antioxidants and both extraction yield and antioxidant activity of the extracts are strongly dependent on the solvent, due to the different antioxidant potentials of compounds with different polarity. For these reasons, comparative studies for selecting the optimal solvent providing maximum antioxidant activity are required for each substrate (Sarikurkcu, 2011).

However, the components responsible for antimicrobial activity of the extracts are clear. Future studies will be aimed at investigating the effects of different parts upon isolating and identifying the substances responsible for the antimicrobial effects of the solvent extracts.

The extract also displayed significant cytotoxic activity as observed in the brine shrimp lethality test, which has been successfully used as a simple biological test to guide the fractionation process of plant extracts in order to detect antitumor compounds. The LC_{50} value was <1000 μg/ml so the extract can be regarded as a promising candidate for a plant-derived antitumor compound. This bioassay has good correlation with the human solid

tumors cell lines. However, further and more specific bioassays are necessary in order to confirm these conclusions.

CHAPTER SIX

REFERENCE

6. REFERENCES

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