Evaluation of Antioxidant, Anti-microbial and Cytotoxic activity of Methanolic Extract of *Phyllanthus acidus* leaves

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 Author

I, **Md. Nasib Rahman Arafat**, hereby declare that the dissertation entitled **"Evaluation of Antioxidant, Anti-microbial and Cytotoxic Activity of Methanolic Extract of *Phyllanthus acidus* Leaves"** submitted by me to the Department of Pharmacy, East West University, Dhaka, in the partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy, under the supervision and guidance of **Abdullah-Al-Faysal**, Senior Lecturer, Department of Pharmacy, East West University. The thesis paper has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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Certificate by the Supervisor

This is to certify that the dissertation entitled **"Evaluation of Antioxidant, Anti-microbial and Cytotoxic Activity of Methanolic Extract of *Phyllanthus acidus* Leaves"** submitted to the Department of Pharmacy, East West University, Dhaka, in partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy, was carried out by **Md. Nasib Rahman Arafat** (Student ID: 2013-3-70-010) under my supervision and no part of this dissertation has been or is being submitted elsewhere for the award of any Degree/ Diploma.

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Certificate by the Chairperson

This is to certify that the dissertation entitled **"Evaluation of Antioxidant, Anti-microbial and Cytotoxic Activity of Methanolic Extract of *Phyllanthus acidus* Leaves"** is a genuine research work carried out by **Md. Nasib Rahman Arafat** (Student ID: 2013-3-70-010), under the supervision of **Abdullah-Al-Faisal** (Senior Lecturer, Department of Pharmacy, East West University) in partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy. I further certify that no part of the thesis has been submitted for any other degree and all the resources of the information in this connection are duly acknowledged.

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This Thesis Paper is Dedicated to My Parents and Family Members

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Abbreviations

AChE- Acetylcholinestrase

AD- Alzheimer's Disease

AIDS- Acquired Immune Deficiency Syndrome

ALP-Alkaline Phosphatase

ALT- Alanine Transaminase

APAP- Acetaminophen

AST- Aspartate Transaminase

B- Biological

B.C. - Before Christ

BA- Benzyladenine

BChE- Butyrylcholinestrase

BHK- Baby Hamster Kidney

BUN- Blood Urea Nitrogen

cAMP- Cyclic Adenosine Monophosphate

CAT- Catalase

CEMAT- Central America Centre of Studies on Appropriate Technologies

CF- Cystic Fibrosis

CFTR- Cystic Fibrosis Transmembrane Conductance Regulator

CFTR-Cl^-- Cystic Fibrosis Transmembrane Conductance Regulator-Chloride Ion

CIBA- Chemical Industry Basel

CNS- Central Nervous System

DPPH- 2,2-diphenyl-1-picrylhydrazyl

DMSO- Dimethyl Sulfoxide

ENaC- Epithelial Sodium Channel

FCR- Folin–Ciocalteu reagent

FDA- Food and Drug Administration

GAE- Galic Acid Equivalent

GPx- Glutathione Peroxidase

GSH- Reduced Glutathione

GSH- Reduced Glutathione

GSH-Px- Glutathione Peroxidase

GSR- Glutathione Reductase

GST- Glutathione-S-Transferase

HDL- High Density Liopoprotein

HIV- Human Immunodeficiency Virus

HIV-RT- Human Immunodeficiency Virus- Reverse Transcriptase

IC_{50}- Inhibitory Concentration 50%

IPCB- International Program of Co-operation for Biodiversity

IR- Infrared

LC_{50}- Lethal Concentration 50%

LDL- Low Density Lipoprotein

LPO- Lipid Peroxidation

MEPA- Methanolic Extract of PA

MIC- Minimum Inhibitory Concentration

MS- Murashige and Skoog

NAA- Naphthaleneacetic Acid

NCI- National Cancer Institute

NMR- Nuclear Magnetic Resonance

NP- Natural Product

NPD- Natural Product Derivative

OPS- Organización Panamericana de la Salud

PA- *Phyllanthus acidus*

QE- Quercetine

R&D- Research and Development

RT- Reverse Transcriptase

S- Synthetic

SGOT- Serum Glutamic Oxaloacetic Transaminase

SGPT- Serum Glutamic Pyruvic Transaminase

SNP- Synthetic Derived from NP

SOD- Super Oxide Dismutase

SOD- Super Oxide Dismutase

STL- Step Through Latency

TAA- Thioacetamide

TB- Total Bilirubin

TB- Tuberculosis

TBARS- Lipid Peroxidation

TLC- Thin Layer Chromatography

U.S. – United States

USA- United States of America

UV-Ultra Violet

V- Vaccine

WHO- World Health Organization

Abstract

Medicinal plants are defined as feral and/or cultivated plants that, based on tradition and literature records, can be directly or indirectly used for medical purposes. The basis for this use is that these plants contain so called active ingredients (active principles or biologically active principles) that affect physiological (metabolic) processes of living organisms, including human beings. The plant *Phyllanthus acidus* has been used for the general promotion of health and longevity. It is used as a traditional medicine for the treatment of various diseases. The root is an active purgative. An infusion of the root is taken to alleviate asthma, the leaves are used as one of the ingredients in a Thai remedy to control fevers, an infusion of the leaves is used as a dieting aid for people who are dieting and wish to remain slim, The latex is credited with emetic and purgative activity, the fruit is used as a laxative, they are also taken as a liver tonic to enrich the blood. The aim of the present study is to evaluate the antioxidant, antimicrobial and cytotoxic activity of Ethyl Acetate (EA) extract of *Phyllanthus acidus*. The test sample of *Phyllanthus acidus* exhibited zone of inhibition ranging from 0 to 10.0 mm against the test organisms. The highest (10.0mm) zone of inhibition was demonstrated against *Vibrio parahaemolyticus*. The brine shrimp lethality bioassay was performed to evaluate the cytotoxic activity of EA extract of the *Phyllanthus acidus* by their brine shrimp lethality. From this test, the concentration required for killing 50% of the brine shrimp larva or LC50 of the EA extract of the *Phyllanthus acidus* was calculated approximately as 0.262 µg /mL with a R2 value of 0.225. So, it is evident that the EA extract of *Phyllanthus acidus* is highly cytotoxic as well as biologically active and also has mild to moderate antimicrobial potentiality. The present study showed that it has poor antioxidant activity compared to standard. This is only a preliminary study but the plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of biologically important drug candidates. Still there are plenty of scopes to establish a variety of properties which can be significantly beneficial to mankind.

Keywords: *Phyllanthus* acidus, Ethyl Acetate extract, Antioxidant activity, Anti-microbial activity, Cytotoxic activity, Phytochemistry

CHAPTER ONE: INTRODUCTION

1.1 History and the Earliest Known Medicines to Mankind

For thousands of year's natural products have played a very important role in healthcare and prevention of diseases. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases (Phillipson, 2001).

The earliest known written document is a 4000 year old Sumerian clay tablet that records remedies for various illnesses (Jin-Ming et al., 2003).

For instance, mandrake was prescribed for pain relief, turmeric possesses blood clotting properties, roots of the endive plant were used for treatment of gall bladder disorders, and raw garlic was prescribed for circulatory disorders. These are still being used in several countries as alternative medicines. However, it was not until the nineteenth century that scientists isolated active components from various medicinal plants.

Friedrich Sertürner isolated morphine from *Papaver somniferum* in 1806, and since then natural products have been extensively screened for their medicinal purposes. Atropine obtained from *Atropa belladonna*, strychnine, a CNS stimulant, ziconotide, identified from a cone snail, *Conus magus*, and Taxol® obtained from the bark of the Pacific yew tree are a few examples of active components isolated from natural sources (Newman, Cragg and Snader, 2003).

The use of plants as medicines has a long history in the treatment of various diseases. The earliest known records for the use of plants as drugs are from Mesopotamia in 2600 B.C., and these still are a significant part of traditional medicine and herbal remedies (Koehn and Carter, 2005). To date, 35,000-70,000 plant species have been screened for their medicinal use (Dev, 1999). Their contribution to the world market for herbal remedies is as shown in Figure 1.1 (Farnsworth and Soejarto, 1991).

1: Europe - 33%

2: Asia - 26%

3: North America - 20%

4: Japan - 11%

5: Others - 10%

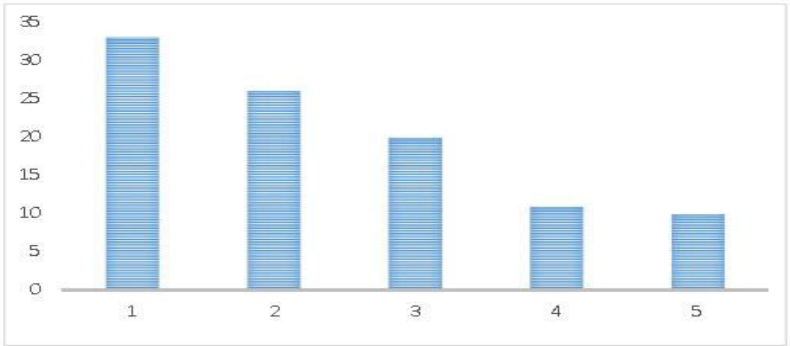


Figure 1.1: World market for drugs from plant sources (Source: Environmental Health Perspectives, 1999).

1.2 Natural Products as Medicines

Collectively, plants produce a remarkably diverse array of over 100,000 low molecular mass natural products, also known as secondary metabolites. Secondary metabolites are distinct from the components of intermediary (primary) metabolism in that they are generally non-essential for the basic metabolic processes of the plant. Most are derived from the isoprenoid, phenyl propanoid, alkaloid or fatty acid/ polyketide pathways. This rich diversity results in part from an evolutionary process driven by selection for acquisition of improved defense against microbial attack or insect/ animal predation. (Pichersky and Gang, 2000)

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, Okpako and Evans, 1996).

1.3 Medicinal Plants

A considerable number of definitions have been proposed for medicinal plants. According to the 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 chemo pharmaceutical semi-synthesis." When a plant is designated as 'medicinal', it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation. Medicinal plants may therefore be defined as a group of plants that possess some special properties or virtues that qualify them as articles of drugs and therapeutic agents, and are used for medicinal purposes (Ghani, 1998).

According to the OPS, (ARIAS, 1999) a medicinal plant is:

- Any plant used in order to relieve, prevent or cure a disease or to alter physiological and pathological process, or
- Any plant employed as a source of drugs or their precursors.

1.4 Herbal Medicines

Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, which contain as active ingredients parts of plants, or other plant materials, or combinations.

1.4.1 Herbs

Crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered.

1.4.2 Herbal materials

In addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir-baking with honey, alcoholic beverages or other materials.

1.4.3 Herbal Preparations

The basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures and fatty oils of herbal materials. They are produced by extraction, fractionation, purification, concentration, or other physical or biological processes. They also include preparations made by steeping or heating herbal materials in alcoholic beverages and/or honey, or in other materials.

1.4.4 Finished Herbal Products

Herbal preparations made from one or more herbs. If more than one herb is used, the term mixture herbal product can also be used. Finished herbal products and mixture herbal products may contain excipients in addition to the active ingredients. However, finished products or mixture products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal.

The proper and judicious use of herbs is often successful in the treatment of illness when other, more conventional medicines and methods fail. Herbs can be used to cleanse the bowels, open congested sinuses, help mend broken bones, stimulate the brain, increase libido, ease pain, aid digestion, and a thousand other purposes. Topically, herbs can repair

damaged skin, soothe a wound, improve complexion, heal bruises and relieve aching muscles. Herbs demonstrate great versatility for the treatment of a broad variety of health needs (Medicinehunter, 2014).

1.5 History

1.5.1 Medicinal Plant in Ancient Time

Plants have been used from ancient times to attempt cures for diseases and to relive physical suffering. Ancient peoples all had acquired some knowledge of medicinal plants. Often times these primitive attempts at medicine were based on superstition and speculation. Evil spirits in the body were thought to be the cause of medical problems. They could be driven out of the body through the use of poisonous or disagreeable plant substances that rendered the body a disagreeable habitat. Medicine men or women of a tribe were usually charged with knowledge of such plants. The progress of medicine has often been guided by the earlier observations and beliefs. Drug plants were always of especial interest. As early as 5,000 B.C. many drugs were in use in China. Sanskrit writings testify to methods of gathering and preparing drugs in these early times. The Babylonians, ancient Hebrews and Assyrians were all familiar with medicinal plants. From Egypt there are records dating to 1,600 B.C. naming many of the medicinal plants used by physicians of that period, among which myrrh, opium, cannabis, aloes, cassia and hemlock are prominent. The Greeks were familiar with many of the drugs of today, evidenced by the works of Hippocrates, Theophrastus, Aristotle and Pythagoras. The supernatural element continued to remain prominent in their culture, however. Only a few individuals were thought able because of some special power to distinguish harmful from valuable plants. This “rhizotomoi” or root diggers were an important caste in ancient Greece. In Rome there was less interest in plants that had healing powers. But by 77 BC Dioscorides wrote in his treatise, “De Materia Medica,” dealing with the nature and properties of all the medicinal substances known at that time. This work was highly esteemed for 15 centuries and to this day is valued in parts of Turkey and North Africa. Pliny and Galen also described the nature of some drug plants.

Following the Dark Ages there began a period of the encyclopedists and herbalists. The monasteries of Northern Europe produced large compendiums of information regarding plants, much of which was false. They stressed the medicinal value and folklore of plants. About the same time there appeared a “Doctrine of Signatures.” This superstitious

doctrine suggested that all plants possessed some sign, given by the Creator, which indicated the use for which they were intended. A plant with heart-shaped leaves was good for heart ailments; the liverleaf with its 3-lobed leaves was good for liver problems, etc. Many of the common names of plants owe their origin to this superstition. Names such as heartease, dogtooth violet, Solomon's seal and liverwort are examples (Hill, 1952).

1.5.2 Medicinal Plant in 21st Century

The development of artemisinin and related antimalarial compounds serves as a modern paradigm for the value of traditional medicines in drug discovery, and we assert the potential exists for additional discoveries of similar importance: of the estimated 250,000 – 500,000 extant plant species, only a fraction have been scientifically investigated for biological activity. Unexplored are untold numbers of species that are likely to be included in traditional medicines. Plants from widely separated regions of the world that are components of traditional medicines used to treat specific conditions such as malaria are phylogenetically clustered ; this principle has been recently described for Pterocarpus, which has significant cross-culture patterns that can inform drug development and supports the value of linking robust ethno botanical and ethno medical studies with 21st century technologies and systems analyses to speed identification of functionally relevant bioactivities .

Inclusion of traditional medicines in development of 21st century treatment paradigms can help assure their convenience, acceptability and accessibility. Furthermore, pharmacological synergism, a principle employed by many traditional medicines lessens the likelihood of development of genetic resistance by the pathogen or disease against drug monotherapies. Synergy research inspired by a "reverse pharmacological approach", could lead to a "new generation of phytopharmaceuticals". The use of powerful technologies facilitates disentangling such complexity, metabolomics analyses enable profiling of major and minor metabolites and bioactive components that contribute to synergism; and computational approaches for analysis of multiple-activity networks have become powerful tools for defining the principal components of mixtures with synergistic modes of action, for prediction of drug metabolism and toxicity, and for high-throughput prioritizing of agent combinations. Data mining approaches to identify active compounds

in mixtures of natural products are being developed and will be essential for the development of effective multiple-agent drugs from traditional medicines.

While U.S. requirements for regulatory approval for health claims made for multi-component medicines present significant challenges for the development of effective multiple-agent drugs from traditional medicines, this is less so for Europe, and especially not for regions of the world highly impacted by TB and Malaria (and in which there are strong traditions of traditional medicine use). As described below, with the establishment of regional research facilities to confirm the safety and efficacy of traditional medicines through the use of tools and robust ethno botanical and ethno medical data, significant improvements in development of improved medicines that are accessible and affordable can be expected (Hill, 1952).

1.5.3 Present Form of Drug Medicinal Plant

Today, approximately 80% of antimicrobial, cardiovascular, immunosuppressive, and anticancer drugs are of plant origin; their sales exceeded US$ 65 billion in 2003. It is widely accepted that more than 80% of drug substances are either directly derived from natural products or developed from a natural compound. And, in fact, around 50% of pharmaceuticals are derived from compounds first identified or isolated from herbs/plants, including organisms, animals, and insects, as active ingredients.

As ancient humans adopted a plant-based (i.e., herbivorous) diet, the body function of humans may have been primed by a large number of secondary metabolites derived from plants.

Considering the extremely high cost and long time of new drug development, as well as the high drug attrition rate, an imminent task for pharmaceutical companies is to explore new ways for drug R&D. Therefore, more and more attention in the field of drug discovery has been focused on the herbal medicine. Herbal medicine as a source of new compounds for drugs is going to become a global trend in the pharmaceutical industry.

An impressive number of chemicals have been isolated either from medicinal plants or synthesized on the basis of natural lead compounds. For instance, schisandrin C present in *Schisandra chinensis* has led to the discovery and development of two potent drug derivatives, bifendate and bicyclol. Artemisinin isolated from *Artemisia annua* has generated at least ten new drugs on the market. Therefore, the use of herbal/plant

medicine has been the single most successful strategy for the development of novel therapeutic agents, and this trend will be continued in the future.

In an era of rapidly advancing science and technology, there is a tendency to ignore traditional values and knowledge, as well as traditional medicines at large. Although the "post genomic" era offers great opportunities for screening active compounds from medicinal plants, one should be aware of traditional knowledge in an attempt to discover drugs derived from herbal medicine. Many medicinal properties of plant species were revealed from experience accumulated from a long history of use in many traditional herbal therapies. Knowledge accumulated in traditional medicine, therefore, plays an important role in enhancing the success rate of drug discovery from herbal medicine. Generally, the success rate of the synthetic route for developing new medicinal agents may be 1/10,000; however, the success rate with search for new therapeutic moieties based on medical plants used in traditional medicinal system can be as high as 1/4 or more. Last but not least, the principle of ecological ethics should be upheld by preserving biodiversity while exploiting natural resources for drug discovery. Man does not have the right to wipe out any species arbitrarily and mess with genes to create transgenic crops for their own benefits.

People are just one of the residents on Earth. As articulated by the ancient Chinese philosopher Lao Zi: "Mother Nature is benevolent to all living things even a stray dog on earth." Modern anthropocentrism with value of philosophical significance for sustainable development should be implemented in the processes of herbal medicine R&D (Sofowora, 1996).

1.6 Traditional Medicine

Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.

It is evident that the non-availability of drugs and commodities, poor access to services by the poor, imposition of unofficial fees, lack of trained providers, a rural-urban imbalance in health providers' distribution, weak referral mechanisms and unfavorable opening hours are contributing to low use of public facilities in Bangladesh. This indicates that

though the health care seeking behavior is partly associated with the socio-economic status of the population, the supply side problems existing within the health system also influence service utilization. (Dr Xiaorui Zhang, 2000)

Table 1.1: Selected Modern Drugs that Came from Traditional Medicine

Drug	What it is for	Derived from	Originally used in
Artemisinin	Antimalarial	Produced from the Chinese herb Qinghao or sweet wormwood	Traditional Chinese medicine for chills and fevers
Cromoglycate	Asthma prevention	Based on the plant Khella, whose active ingredient is khellin	Traditional Middle Eastern remedy for asthma. Also traditionally used in Egypt to treat kidney stones
Etoposide	Anticancer	Synthesized from podophyllotoxin, produced by the American mandrake plant	Various remedies in Chinese, Japanese and Eastern folk medicine
Hirudin	Anticoagulant	Salivary glands in leeches, now produced by genetic engineering	Traditional remedies across the globe, from Shui Zhi medicine in China to eighteenth and nineteenth century medicine in Europe
Lovastatin	Lowers cholesterol	Foods such as oyster, mushrooms and red yeast rice, also used to synthesis other compounds such as Pravastatin	Mushrooms are used to treat a wide range of illnesses in traditional medicine in China, Japan, Europe and Russia

Drug	What it is for	Derived from	Originally used in
Opiates	Painkilling	Unripe poppy seeds	Traditional Arab, Chinese, European, Indian and North African medicines as pain relief and to treat a range of illnesses including diarrhea, coughs and asthma
Quinine	Antimalarial	Bark of the cinchona tree	Traditional remedies to treat fevers and shivers in South America
Vinca alkaloids (vincristine, vinblastine)	Anticancer	Synthesized from indole, alkaloids produced by the rosy periwinkle	Folk remedies across the world use periwinkle plants, including as an antidiabetic in Jamaica.

(Rinaldi and Shetty, 2017)

1.7 Future of Medicinal chemistry

The history of drug research over a period of a century, since Paul Ehrlich introduced the concept of chemotherapeutic agents, is an amazing journey of accomplishments including the serendipitous success of antibiotic. Drug discovery has changed over the years but the goal remains the same- to find safer medicines for the deadliest diseases. Traditionally, the discovery of new drugs has arisen from observations that various plant extracts possess interesting biological effects. However, early users of such plant extracts did not understand or realize which components in the material were responsible for achieving these therapeutic benefits. The main difference between modern and age-old medicine is identifying the composition of matter, or the active form, within the medicine itself. This modern drug discovery and development is mostly a complex, expensive, time consuming and market-driven process with very few novel drug candidates actually making it through the Food and Drug Administration (FDA) for approval. Discovery and development of new drugs does not rely so much on miracles or serendipity anymore, but instead utilizes highly planned processes involving cutting edge technologies. The previous era of modern drug discovery was dominated by chemistry, whereas now a more

rational approach is employed where knowledge about enzymes and receptors has required a unique dialogue between chemists and biologists (Sofowora, 1996).

Finding viable drug targets (the so-called biological approach) has become increasingly used in recent decades with the advancements in the field of genomics and proteomics. A medicinal chemist uses this information to seek out relevant targets capable of being affected by the addition of compounds. These specifically synthesized drug molecules are proposed, synthesized and tested for direct action on these protein targets in order to effectively treat a wide variety of illnesses. In previous times, "classical medicinal chemists" would modify the existing bio-active molecules from natural products. These natural products were the source of most of the active ingredients in most medicine.

In summary, new biological targets, methodologies and advanced computing have improved modern drug discovery and have given medicinal chemists a more profound skill set and toolkit to grasp the nuances of disease pathophysiology. Driven now by target identification and specificity of action, these new molecules and their development are revolutionizing healthcare. Not only are these new techniques and approaches innovative, but they are cost-effective as well. Medicinal chemists are essential players in this process and are relying heavily on new scientific literature to drive this process forward more efficiently. Open access journals such as organic chemistry current research is playing a very important role by providing essential up-to-date research information to scientific community worldwide. The hope here is that these journals will add to the every growing knowledge created by the medicinal chemist to have a great impact in drug discovery process.

1.8 Characteristics of Medicinal Plants

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

1.8.1 Synergic Medicine

The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects.

1.8.2 Support of Official Medicine

In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective.

1.8.3 Preventive Medicine

It has been proven that the component of the plants also characterize 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. (Rasool Hassan, 2012)

1.9 Classification of Medicinal Plants

There are a large number of medicinal and aromatic plants in the nature which are used for medicinal and aromatic purposes. Moreover, medicinal plants are sometimes used for aromatic purposes similarly aromatic plants may also be used for medicinal purpose! Hence, classification of medicinal and aromatic plants is difficult. Since there are a large number of plants in these two groups an attempt has been made in this chapter to facilitate for further study. (My Agricultural Information Bank, 2015)

Medicinal plants are generally classified on the basis of their growth habit. It may be a tree, shrub, herb, annuals, biennial, tubers, rhizomes and climbers.

Table 1.2: Medicinal Trees

Sr. No.	Common Name	Botanical Name	Parts Used
1	Babul	*Acacia nilotice*	Pods, leaves, bark, gum
2	Bael	*Aegle marmelos*	Roots, leaves, fruit
3	Neerh	*Azaflirachta indica*	Bark leaves, flowers, seed, oil
4	Palas	*Butea monossperma*	Bark, leaves, flowers, seed, gum
5	Gugul	*Commiphora mukul*	Resinous gum
6	Olive	*Olea europeae*	Leaves, Oil
7	Arjun	*Terminalia arjuan*	Bark
8	Behela	*Terminalia bellirica Gaertu*	Bark, fruit
9	Hirda Terminalia	*bellirica Gaertu*	Fruits
10	Nagakesar	*Mesua ferrea*	Blowers, oil
11	Markingnut	*Semecarpus & anacardium*	Fruits

Table 1.3: Medicinal Shrubs

Sr. No.	Common Name	Botanical Name	Parts Used
1	Davana	*Artemisia nilagirica*	Leaves, flowering top
2	Safed musli	*Aparagus adscendens*	Tuberous roots
3	Belladonna	*Atropa belladonna*	Leaves and roots
4	Lavender	*Lavandula officinalis*	Flowers
5	Sarpagandha	*Rauvalfia serpentina*	Roots
6	Chitrak	*Plumbage zeylanica*	Leaves, roots

Table 1.4: Medicinal Herbs

Sr. No.	Common Name	Botanical Name	Parts Used
1	Brahmi	*Bacopa monnieri*	Whole plant
2	Am haldi	*Curcuma amada*	Rhizomes
3	Haldi	*Curcuma domestica*	Rhizomes
4	Datura	*Datura metel*	Leaves, flowers
5	Kalazira	*Nigella sativa*	Seed
6	Afim	*Papaver somniferum*	Latex, seed
7	Pipli	*Piper Longum*	Fruits, roots
8	Babchi	*Psoralea corylifolia*	Seed, Fruit

Table 1.5: Medicinal Annuals

Sr. No.	Common Name	Botamical Name	Parts Used
1	Jangali muli	*Blumea lacera*	Whole plant
2	Cockscomb	*Celosia cristala*	Inflorescence
3	Red poppy	*Papaver rhoeas*	Flowers
4	Bhui amla	*Phyllantius niruri*	Whole plant

Table 1.6: Biennial

Sr. No.	Common Name	Botanical Name	Parts Used
1	Bankultthi	*Cassia abus*	Leaves, seeds
2	Caper spurge	*Euphorbia lathyrus*	Seed latex
3	Catchfly	*Melandrium firmum*	Whole plant
4	Chocloate vine	*Akebia quinata*	Deene Stem, fruit
5	Malkunki	*Celustrus paniculatus*	Wild Bark, leaves, seed
6	Hajodi	*Cissus quadrangularis L.*	Whole plant
7	Khira	*Cucumis sativus L.*	Fruit, seed
8	Gudmar	*Gymnema sylvestre Retzx*	Whole plant, leves
9	Kali mirch	*Piper nigrum L.*	Fruit

Table 1.7: Tubers and Rhizomes

Sr. No.	Common Name	Botanical Name	Parts Used
1	Satavar	*Asparagus adscendens*	Tubers
2	Safed musli	*Chlorophytum borivilianum*	Tubers
3	Puskarmul	*Inula racemosa*	Roots
4	Sakarkhand	*Manihot esculenta*	Tubers

(My Agricultural Information Bank, 2015)

1.10 Families of Medicinal Plants

Most of the medicinal plants belong to the following families:

1. Compositae

2. Labiatae

3. Umbelliferae

4. Boraginaceae

5. Cruciferae

1.10.1 Medicinal Plants of the Compositae Family

The Compositae family, also known as the Daisy family, contains the highest number of medicinal plants as compared to other families, all members being sun lovers. They have either a disk flower or a ray flower. Being dry and hard the fruits often have plumes of hairs to aid in wind dispersal. Medicinal plants belonging to this family include chamomile, field and pot marigolds, daisy, wormwood, chicory, thistles, ragwort and artichoke.

1.10.2 Medicinal Plants of the Labiatae Family

A very important medicinal plant family is the Labiatae family, also known as the mint family. Plants in this family are herbs or shrubs often with an aromatic smell. They are often met in the Mediterranean countries for the fact that some of them produce a high amount of essential oil that enables them to survive the hot summer season. The common characteristics are square stems and mostly irregular two-lipped flowers having four stamens. The fruit is small with four (seeds). Some examples from this family include horehound, lavander, balm, micromeria, the mints, thyme and rosemary, basil, sage.

1.10.3 Medicinal Plants of the Umbelliferae Family

The Umbelliferae or parsley members often have hollow stems and flowers in clusters called umbels and a characteristic umbrellaarranged fruit. These plants usually produce an essential oil, an asset to survive during the hot summer days. Bullwort (*Ammi majus*), wild celery (*Apium graveolens*), wild carrot (*Daucus carota*), sea holly (*Eryngium maritima*), fennel (*Foeniculum vulgaris*), anise (*Pimpinella anisum*), wild parsley (*Petroselinium crispum*) are all parsley family members.

1.10.4 Medicinal Plants of the Boraginaceae Family

The Boraginaceae or borage family is made up of herbs or small shrubs with bristly stems and leaves. Members of the Boraginaceae all have tubular flowers mostly in curved racemes, five stamens being attached to the tube. The ovary is superior usually forming a fruit composed of four nutlets. Examples in this family include borage (*Borago officinalis*), common comfrey (*Symphytum officinale*), purple alkanet (*Anchusa asurea*), yellow gromwell (*Neatostema apulum*), viper's bugloss (*Echium vulgare*) and southern hound's tongue (*Cynoglosum creticum*).

1.10.5 Medicinal Plants of the Cruciferae Family

The Cruciferae or mustard (cress) family is characterised by plant that have flowers with cross-like petals. This family groups a large group of medicinal plants that include Wallflower (*Cheiranthus cheiri*), Bitter cress (*Cardamine hirsuta*), Black mustard (*Brassica nigra*), Horseradish (*Armoracia rusticana*), Hedge mustard (*Sisymbrium officinale*), White mustard (*Sinapis alba*), Wild radish (*Raphanus raphanistrum*), Watercress (*Nasturtium officinale*).

There are some other families of plant to which herbs belong such as Rosaceae family, Rutaceae and Solanaceae families, Malvaceae and other families (Bennett, n.d.).

1.11 Benefits of plants

The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs (De Pasquale, 1984). The Industrial Revolution and the development of organic chemistry resulted in a preference for synthetic products for pharmacological treatment. The reasons for this were that pure compounds were easily obtained, structural modifications to produce potentially more active and safer drugs could be easily performed and the economic power of the pharmaceutical companies was increasing. Furthermore, throughout the development of human culture, the use of natural products has had magical-religious significance and different points of view regarding the concepts of health and disease existed within each culture.

Obviously, this approach was against the new modus Vivendi of the industrialized western societies, in which drugs from natural resources were considered either an option for poorly educated or low income people or simply as religious superstition of no pharmacological value. However, 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 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from Digitalis spp., quinine and quinidine from *Cinchona* spp., vincristine and

vinblastine from *Catharanthus roseus*, atropine from *Atropa belladona* 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 (Shu, 1998). The vast majority of these cannot yet be synthesized economically and are still obtained from wild or cultivated plants.

We have so much to benefit from by returning to plants in their most natural state. The famous father of medicine, Hippocrates was quoted as saying: *'Let thy food be thy medicine, and thy medicine shall be thy food'* (Evita Ochel, 2010).

The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. The use of the medicinal herbs for curing disease has been documented in history of all civilizations. Man in the pre-historic era was probably not aware about the health hazards associated with irrational therapy. With the onset of research in medicine, it was concluded that plants contain active principles, which are responsible, for curative action of the herbs.

Before onset of synthetic era, man was completely dependent on medicinal herbs for prevention and treatment of diseases. With introduction of scientific procedures the researchers, were able to understand about toxic principles present in the green flora. The scientists isolated active constituents of the medicinal herbs and after testing some were found to be therapeutically active. Aconitine, Atisine, Lobeline, Nicotine, Strychnine, Digoxin, Atropine, Morphine are some common examples (Wordpress, 2010).

The efficacy of some herbal products is beyond doubt, the most recent examples being *Silybum marianum* (silymarin), *Artemisia annua* (artemesinin) and *Taxus baccata* (taxol). On the other hand, randomized, controlled trials have proved the efficacy of some established remedies, for instance, *Ginkgo biloba* for tinnitus, *Hypericum perforatum* is a reputed remedy for depression. In Hypericum some researchers are of the view that hypericin is the active principle of the herb and some believe that hyperforin is responsible for antidepressant action of the herb.

Recently research has supported biological activities of some medicinal herbs. Cancer is such a segment where researchers are expecting new molecules from herbs that can provide us with tools for fighting this dreaded disease. *Allamanda cathratica* [allamandin], *Elephatopus elatus* [elephantpoin], *Helenium autmnale* [helenalin],

Vernonia hymenlepis, *Heliotropium indicum* [Indicine-N-oxide], *Daphne mezereum* (mezerien) and *Stereospermum suaveolans* [laphacol] are medicinal plants that have shown significant tumor inhibiting effect.

Diabetes mellitus is another area where a lot of research is going on. *Ajuga reptens* (the active principle is said to potentiate effects of insulin), *Galagea officinalis* (galagine), *Bougainvillea spectabilis* (pinitol), *Momordica charantia* (chirantin), *Gymnema sylvestre* (gymnemic acid) are some medicinal herbs that have shown effectiveness in non-insulin dependent diabetes. Recently extract of *Tecoma stans* has shown potent anti diabetic activity. Alkaloid tecomonine is considered to be active principle of the herb.

Arthritis is another potential disease where no satisfactory answer is present in modern medicine. *Commiphora mukul* (guggulsterones), *Boswellia serrata* (boswellic acid), *Withania somnifera* (withanolides), *Ruscus acueleatus* (ruscogenin), *Harpagophytum procumbens* (harpagoside) are prominent plants with anti- arthritic activity. Harpagoside is a precious constituent as it has anti rheumatoid activity. Rest of all natural products has anti-inflammatory activity.

Chrysanthemum parthenium traditionally known as feverfew has shown promising results in migraine, a disease that has eluded the researchers from centuries. The herb contains sesquiterpenes lactones called parthenolides, which are the active principles of the herb.

Hepatoprotective action of certain botanicals deserves attention. *Sedum sarmentosum* (sarmentosin), *Schisandra chinensis* (waweizichun and schisantherin) have shown their ability to lower raised liver enzymes in viral hepatitis.

Croton sublyratus (plaunotol) has potent and wide spectrum anti peptic ulcer action. A number of plant derivatives have shown anti-Aids activity. *Ancistrocladus korupensis* (michellamine-b), *Caulophyllum langigerum* (calanolide-a), *Caulophyllum teymani* (costatolide-a), *Homalanthus nutans* (prostratin), *Conospermum* (concurvone) are the medicinal herbs from African countries that are being employed in research for finding a suitable cure for AIDS.

The concept of antioxidants is firstly catching up and latest research has shown that a number of herbal derivatives have excellent antioxidant action. *Bacopa monnieri* contains bacosides A and B and bacoside A is a strong antioxidant, which reduces several steps of free radical damage. *Coleus forskohlii* (forskolin), *Camellia sinensis* (polyphenols),

Huperzia serrata (huperzine), *Pinus maritima* (Pycnogenol), *Borago officinalis* (gamma linoleic acid) and *Vinca minor* (Vinpocetine) are potential antioxidants.

The plant is a biosynthetic laboratory, not only for chemical compounds, but also a multitude of compounds like glycosides, alkaloids etc. These exert physiological and therapeutic effect. The compounds that are responsible for medicinal property of the drug are usually secondary metabolites. A systematic study of a crude drug embraces through consideration of primary and secondary metabolites derived as a result of plant metabolism. The plant material is subjected to phytochemical screening for the detection of various plant constituents.

With onset of scientific research in herbals, it is becoming clearer that the medicinal herbs have a potential in today's synthetic era, as numbers of medicines are becoming resistant. According to one estimate only 20% of the plant flora has been studied and 60% of synthetic medicines owe their origin to plants. Ancient knowledge coupled with scientific principles can come to the forefront and provide us with powerful remedies to eradicate the diseases. (Pandey and Dilwakar, 2008)

1.12 Uses of Some Medicinal Plants

I. Herbs such as black pepper, cinnamon, myrrh, aloe, sandalwood, ginseng, red clover, burdock, bayberry, and safflower are used to heal wounds, sores and boils.
II. Basil, Fennel, Chives, Cilantro, Apple Mint, Thyme, Golden Oregano, Variegated Lemon Balm, Rosemary, and Variegated Sage are some important medicinal herbs and can be planted in kitchen garden. These herbs are easy to grow, look good, taste and smell amazing and many of them are magnets for bees and butterflies.
III. Many herbs are used as blood purifiers to alter or change a long-standing condition by eliminating the metabolic toxins. These are also known as 'blood cleansers'. Certain herbs improve the immunity of the person, thereby reducing conditions such as fever.
IV. Some herbs are also having antibiotic properties. Turmeric is useful in inhibiting the growth of germs, harmful microbes and bacteria. Turmeric is widely used as a home remedy to heal cut and wounds.

V. To reduce fever and the production of heat caused by the condition, certain antipyretic herbs such as Chirayta, black pepper, sandal wood and safflower are recommended by traditional Indian medicine practitioners.

VI. Sandalwood and Cinnamon are great astringents apart from being aromatic. Sandalwood is especially used in arresting the discharge of blood, mucus etc. Some herbs are used to neutralize the acid produced by the stomach. Herbs such as marshmallow root and leaf, they serve as antacids. The healthy gastric acid needed for proper digestion is retained by such herbs.

VII. Indian sages were known to have remedies from plants which act against poisons from animals and snake bites.

VIII. Herbs like Cardamom and Coriander are renowned for their appetizing qualities. Other aromatic herbs such as peppermint, cloves and turmeric add a pleasant aroma to the food, thereby increasing the taste of the meal.

IX. Some herbs like aloe, sandalwood, turmeric, sheetraj hindi and khare khasak are commonly used as antiseptic and are very high in their medicinal values.

X. Ginger and cloves are used in certain cough syrups. They are known for their expectorant property, which promotes the thinning and ejection of mucus from the lungs, trachea and bronchi. Eucalyptus, Cardamom, Wild cherry and cloves are also expectorants.

XI. Herbs such as Chamomile, Calamus, Ajwain, Basil, Cardamom, Chrysanthemum, Coriander, Fennel, Peppermint and Spearmint, Cinnamon, Ginger and Turmeric are helpful in promoting good blood circulation. Therefore, they are used as cardiac stimulants.

XII. Certain medicinal herbs have disinfectant property, which destroys disease causing germs. They also inhibit the growth of pathogenic microbes that cause communicable diseases.

XIII. Herbal medicine practitioners recommend calmative herbs, which provide a soothing effect to the body. They are often used as sedatives.

XIV. Certain aromatic plants such as Aloe, Golden seal; Barberry and Chirayta are used as mild tonics. The bitter taste of such plants reduces toxins in blood. They are helpful in destroying infection as well.

XV. Certain herbs are used as stimulants to increase the activity of a system or an organ, for example herbs like Cayenne.

XVI. A wide variety of herbs including Giloe, Golden seal, Aloe and Barberry are used as tonics. They can also be nutritive and rejuvenate a healthy as well as diseased individual.

XVII. Honey, turmeric, marshmallow and liquorices can effectively treat a fresh cut and wound. They are termed as vulnerary herbs. (Zahid, 2016)

1.13 Disadvantage of synthetic compound

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank, 1982). This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/ or incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that "natural" products are harmless. However, the use of these substances is not always authorized by legal authorities dealing with efficacy and safety procedures, and many published papers point to the lack of quality in the production, trade and prescription of phytomedicinal products.

1.14 Natural products as medicine in global market

It is estimated that, in 1997, the world market for over-the-counter phytomedicinal products was US$10 billion, with an annual growth of 6.5%. The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries. Eastern countries, such as China and India have a well-established herbal medicines industry and Latin American countries have been investing in research programs in medicinal plants and the standardization and regulation of phytomedicinal products, following the example of European countries, such as France and Germany. In Germany, 50% of phytomedicinal products are sold on medical prescription, the cost being refunded by health insurance. In North America, where phytomedicinal products are sold as "health foods", consumers and professionals have struggled to change this by gathering information about the efficacy and safety of these products, and new guidelines for their registration are now part of FDA policy. In 1997, the North American market for products of plant origin reached US$ 2 billion. According to recent studies conducted by the World Health Organization (WHO), about 80% of the world's population relies on traditional medicine. About 121 drugs prescribed

in USA today come from natural sources, 90 of which come either directly or indirectly from plant sources. Forty seven percent of the anticancer drugs in the market are representation of the contribution of natural products to drug discovery. (Rates, 2001)

V = Vaccine
B = Biological
NP = Natural Product
NPD = Natural Product Derivative
SNP = Synthetic Derived from NP
S = Synthetic

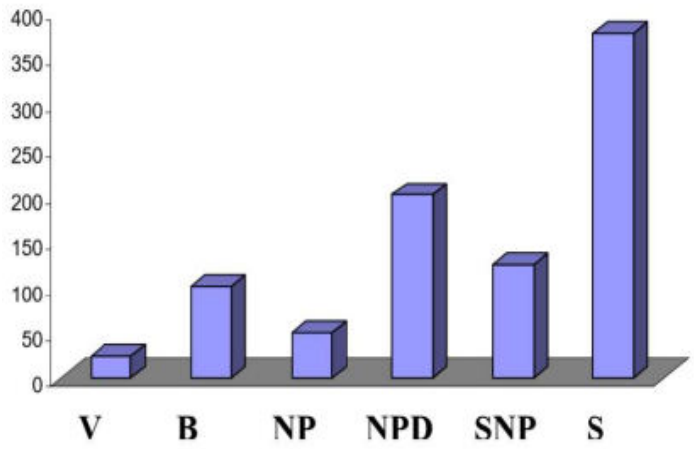


Figure 1.2: Distribution of natural products as drugs

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 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.

The NCI (National Cancer Institute, USA) has tested more than 50,000 plant samples for anti-HIV activity and 33,000 samples for anti-tumour activity. In 1993, the International Program of Co-operation for Biodiversity (IPCB) was launched in order to promote natural products in Latin America and Africa, linking universities, industries and governments in a multidisciplinary program for the sustained development and preservation of the environment (Rouhi, 1997). Large pharmaceutical companies, such as Merck, CIBA, Glaxo, Boehringer and Syntex, now have specific departments dedicated to the study of new drugs from natural sources (Reid et al., 1993). However, the potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250,000–500,000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties. In most cases, only pharmacological screening or preliminary studies have been carried out. It is estimated that 5000 species have been studied for medical use (Payne et al., 1991). Between the years 1957 and 1981, the NCI

screened around 20,000 plant species from Latin America and Asia for anti-tumor activity, but even these were not screened for other pharmacological activities (Hamburger and Hostettman, 1991).

1.15 Challenges of isolating lead compounds from medicinal plants

Research into, and development of therapeutic materials from plant origin is a hard and expensive task (Borris, 1996; Turner, 1996; Williamson, Okpako and Evans, 1996). Each new drug requires an investment of around US$100–360 million and a minimum of 10 years of work, with only 1 in 10,000 tested compounds being considered promising and only 1 in 4 of these being approved as a new drug. Up to 1992, the NCI had only found 3 plant extracts active against HIV out of 50,000 tested, and only 3 out of 33,000 plant extracts tested were found to have anti-tumor activity (Williamson et al., 1996). Quantitative considerations regarding the average yield of active compounds and the amount of starting crude plant material required for the discovery, development and launch of a new drug on the market were presented by McChesney (1995): 50 kg of raw material are necessary to provide 500 mg of pure compound for bioassays, toxicology, and "in vivo" evaluation; full pre-clinical and clinical studies can require 2 kg of pure compounds obtained from 200 ton of raw material. The process is multi-disciplinary (De Pasquale, 1984; Verpoorte, 1989). The basic sciences involved are botany, chemistry and pharmacology, including toxicology. Any research into pharmacological active natural compounds depends on the integration of these sciences. The way they are integrated and the extent of integration depend on the objectives of the study. In any case, a particular discipline should not be seen as secondary to another; quite the opposite, as each step must be carried out considering the theoretical and technical background of each of the sciences involved, otherwise the results may not be robust enough and may lead to break down of the process.

Other fields of challenges may also be involved if the long path from plant to medicine is taken into account. Anthropology, agronomy, biotechnology and organic chemistry can play very important roles. In addition, pharmaceutical technology is fundamental to the development of any drug, including drugs of plant origin (Petrovick, 1999).

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 homemade 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 phyto pharmaceutical preparations or herbal medicines.

1.16 Selecting a plant

The approach for drug development from plant resources depends on the aim. Different strategies will result in a herbal medicine or in an isolated active compound. However, apart from this consideration, the selection of a suitable plant for a pharmacological study is a very important and decisive step. There are several ways in which this can be done, including traditional use, chemical content, toxicity, randomized selection or a combination of several criteria (Soejarto, 1996; Williamson et al., 1996).

The most common strategy is careful observation of the use of natural resources in folk medicine in different cultures; this is known as ethno botany or ethno pharmacology. Information on how the plant is used by an ethnic group is extremely important. The preparation procedure may give an indication of the best extraction method. The formulation used will provide information about pharmacological activity, oral versus non-oral intake and the doses to be tested. However, certain considerations must be taken into account when the ethno pharmacological approach of plant selection is chosen. For instance, each ethnic group has its own concepts of health or illness, as well as different health-care systems. The signs and symptoms should be translated, interpreted and related to western biomedical concepts, thus allowing a focused study of a particular therapeutic property.

Selection based on chemical composition uses phylogenetic or chemotaxonomic information in the search, mainly in certain genera and families, for compounds from a defined chemical class with known pharmacological activity (Souza Brito, 1996).

The search for highly specific potent drugs for therapeutic use and, more precisely, as an investigation tool in biological research has been quite productive in toxic plants. A number of important compounds now used in research came from toxic plants and several examples have been mentioned earlier (Williamson et al., 1996). Observation of the plant's environment has led to the isolation of active compounds, mainly antibacterial and anti-insect drugs (Harmburger and Hostettman, 1991). Another method of selecting a plant is that the investigator decides on a well-defined pharmacological activity and

performs a randomized search, resulting in active species to be considered for further study. The search for anti-tumor drugs is a good example of the use of this strategy.

The search for drugs active against tumors, viruses and cardiovascular and tropical diseases is a priority. The largest research fields, as defined by the number of publications describing bioactive plant-derived compounds in the last few years, are anti-tumor drugs, antibiotics, drugs active against tropical diseases, contraceptive drugs, anti-inflammatory drugs, immune modulators, kidney protectors and drugs for psychiatric use (Hamburger and Hostettman, 1991). Taxol is both an example of the importance of natural products and of the complexity and necessity of finding alternative routes by which it can be obtained. It is the most important natural product derived from diterpene with anti-tumor activity found in recent years. Taxol is isolated from *Taxus* (*T. brevifolia* and *T. bacata*). However, the biggest obstacle to its clinical use is obtaining the material.

In order to produce 2.5 kg of taxol, 27,000 tons of *T. brevifolia* bark are required and 12,000 trees must be cut down. Due to the high demand, this species of *Taxus* will soon be extinct if no alternative source of taxol can be developed. An economically possible and technically realistic alternative is its partial synthesis, in considerable yield, from an analogue found in other species of *Taxus*, as well as the production of other hemi synthetic analogues (Hamburger and Hostettman, 1991).

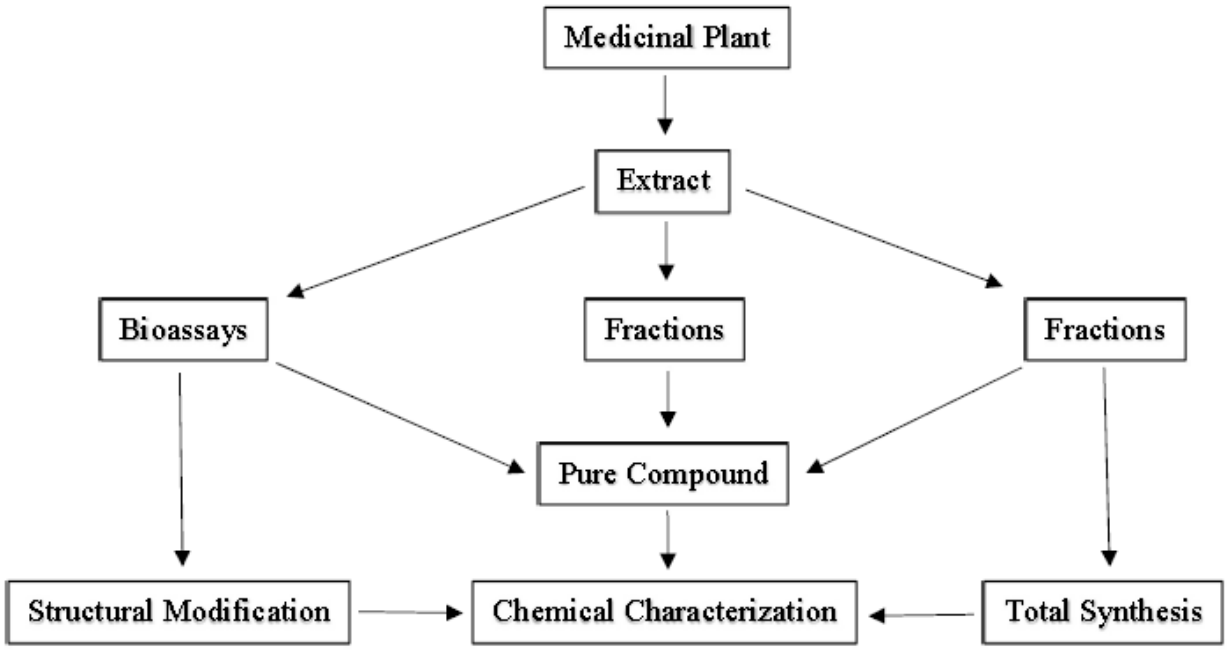


Figure 1.3: Methods for obtaining active substances from plants

1.17 Steps of drug development

Once the plant is chosen, the next step is its collection and botanical identification. Then it should be submitted to a stabilization process. It is important that plant recollection involves a professional botanist who is able to correctly identify the species and prepare part of the material for herbarium preservation in order to have a reference material ("voucher specimen"). Preferably, the place and date of recollection should be recorded and the information retained for further collection, if necessary. Stabilization is usually by drying the material at ambient temperature in a shady place, but can also be carried out in an oven with controlled airflow and temperature. When the stability of the compounds is unknown or if they are known to be unstable, the fresh plant should undergo a stabilization process consisting of freezing, lyophilisation, use of alcohol vapour etc. (Williamson et al., 1996).

The dried or stabilized plant material should then be powdered and subjected to a suitable extraction process. When the chemical nature of the compounds involved is known (once again, chemotaxonomic information and data - bank consultation are crucial), extraction methods should be directed at obtaining these compounds in as high a yield and purity as possible. When the chemical composition is unknown, the extraction procedure can be based on how the plant is used in folk medicine, or several extractions with solvents of increasing polarity can be performed (Williamson et al., 1996). To obtain isolated active compounds, the plant extracts are first qualitatively analysed by thin layer chromatography (TLC) and/or other chromatographic methods and screened to determine the biological activity or to obtain a general evaluation of biological activities. For purification and isolation, the active plant extracts are sequentially fractionated (Verpoorte, 1989), each fraction and/or pure compound being subjected to bioassay and toxicity evaluation in animals. This strategy is called bioactivity-guided fractionation. Bioassays can be performed using micro-organisms, molluscs, insects, cellular systems (enzymes, receptors etc.), cell culture (animal and human), and isolated organs or in vivo (mammals, amphibians, birds etc.) (Hamburger and Hostettman, 1991; Souza Brito, 1996).

After verifying the purity of an isolated active compound, the structure is determined by spectroscopic methods (UV, IR, mass spectrum or NMR) (Verpoorte, 1989). Once the chemical structure is defined, total or partial synthesis and preparation of derivatives

and/or analogues can be considered, and modulation of the biological activity and definition of the structure–activity relationship can be carried out. After completing all these steps, large-scale isolation (it may necessary to collect the plant again) or partial or total synthesis is required for pharmacological evaluation in pre-clinical, clinical and toxicological trials aimed at future therapeutic use (Hamburger and Hostettman, 1991; Borris, 1996). As mentioned above, the final result of this strategy, the drug, is expensive. However, the study of medicinal plants also allows their use "in natura" and/or in pharmaceutical formulations obtained from them, called phytomedicines or herbal remedies. This approach also requires efficacy and toxicity studies, but these are less time-consuming, as the steps of fractionation, purification and bioassay are basically not required or are far less complex.

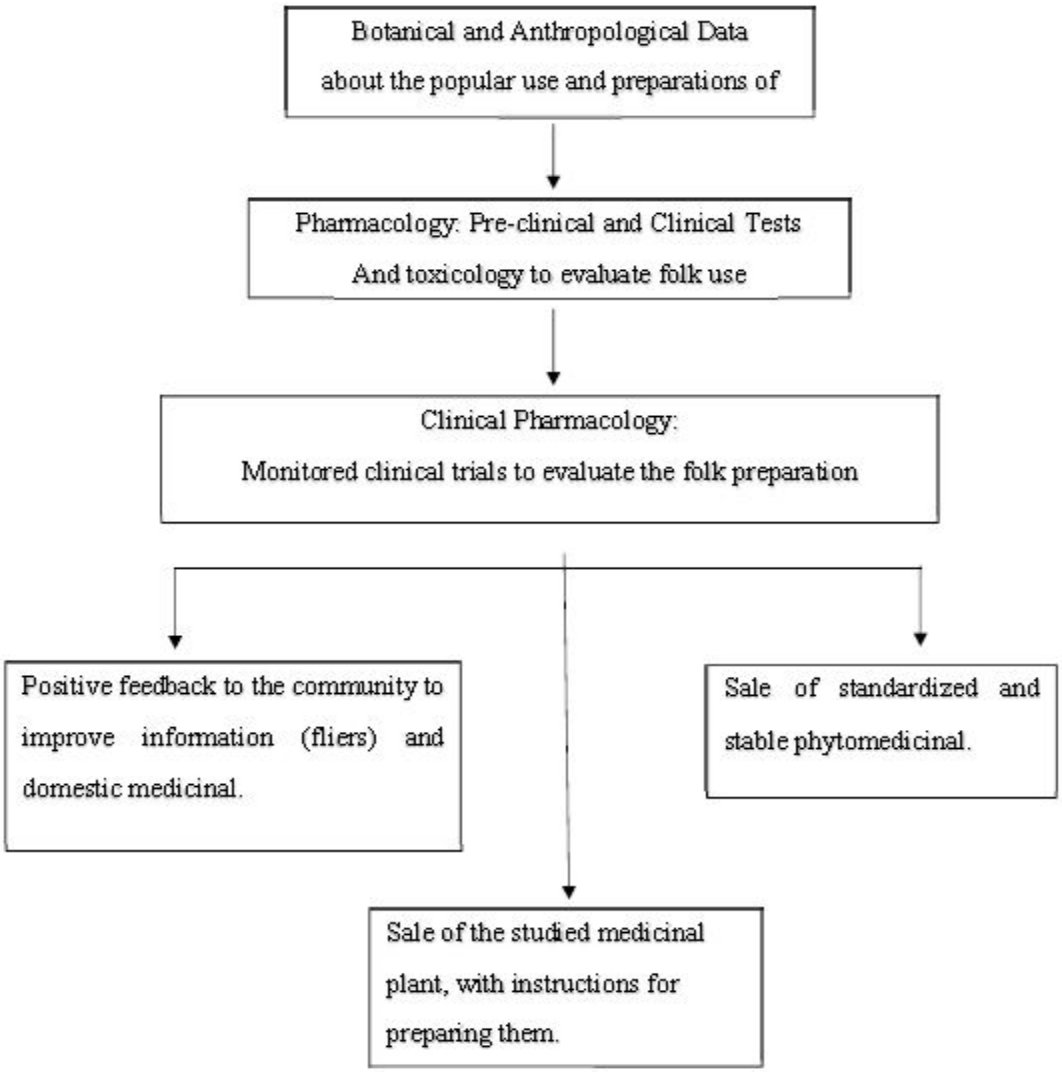


Figure 1.4: Methods for pharmacological validation of the popular use of medicinal plants

1.18 Status of plant research in various countries

Research into medicinal plants and the search or plant-derived drugs require a multidisciplinary approach with integrated projects, financial and technical support, and a very carefully planned strategy. The aims should consider demands in terms of public health, preservation of biodiversity and the technical qualification of each laboratory or research group involved. Advances in technology and knowledge of natural products must be viewed not merely from the perspective of drug development, but also as a special tool for the understanding of biological phenomenon in order to contribute to the well-being of humanity. Various countries around the world have taken steps to do in detail study of medicinal plants. The activities carried out in this field by some of the countries are given below:

Table 1.8: Activities of various countries concerning medicinal plants

Country	Activities	Remark
Armenia	Rich history of use and export of medicinal plants.	Over 3200 species described and conserved for use and export.
Australia	Asian-Australian Centre for the Study of Bioactive Medicinal Plant Constituents was set up in 1992 at La Trobe University for conduction of research in collaboration with Chulalongkorn and Chieng-Mai Universities in Thailand.	Inter-university research work focuses on: - The bioactive constituents of turmeric e.g. curcumin - Islamic medicinal plants - Antifungal proteins and secondary metabolites in crop plants, e.g. in cotton and yellow mustard *Sinapis alba* - Marine toxins - Collaboration on medicinal plants with scientists from Indonesia, Thailand, Bangladesh, Singapore, Kuwait and New-Zealand.
Bangladesh	Research on cultivation and biochemical aspects of medicinal plants.	Bangladesh Council for Scientific and Industrial Laboratory at Chittagong oversees development of appropriate chemical technologies, and pharmacopoeia of plants.

Country	Activities	Remark
Bhutan	Sustainable protection and use of forest resources and development of plant tissue culture.	Capacity building in plant biotechnology for rural markets and development of forest seedlings Indigenous Hospital in Thimphu has recorded 180 species of medicinal and aromatic plants.
Canada	Southern Crop and Food Production Centre of Agriculture and Agrifood Canada mandated to develop novel technologies in production and protection of new crops inclusive of medicinal plants.	Active research on crop production, genetics, germplasm improvement, micro propagation, and protection of medicinal herbs. Attention is given to research in developing base-line agronomic information, elucidating the chemistry of bioactive principles, etc.
China	Chinese root extracts from *Astragalus membranaceus* have been developed for us as a general tonic food and for boosting immunity. Institute of Medicina Plants established in 1983 with branches in provinces of Yunnan, Hainan and Guangxi.	Used in Chinese herbal medicine to strengthen the vital energy Qi in general health and well being. Product widely used in China, and South Asia. Work deals with the development, conservation and utilisation of medicinal plant resources, and the discovery of new potent drugs. The Institute is also recognised as a WHO Collaborating Centre on Traditional Medicine.
Domician Republic	Launched in 1982 as a traditional medicine for the islands (TRAMIL) network with support from IRDC, focus on developing scientific proven medicinal plant remedies as alternatives to patent drugs that are expensive to obtain in rural populations.	Regional node was established in Panama in 1994 to cover area from Belize to Panama. Over 150 medicinal plants evaluated and results disseminated in Caribbean Pharmacopoeia.

Country	Activities	Remark
Estonia	Network of plant genetic resources inclusive of medicinal plants.	Development of computerized gene bank system at Jogeva Plant Breeding Institute linking inputs from the Polli Horticultural Institute and the Estonian Agricultural University and Botanical Garden.
Guatemala	Farmaya Laboratory, following screening of 700 different plants, has developed 15 pharmaceutical products using traditional knowledge of indigenous and rural groups. Farmaya engaged in organic cultivation of medicinal plants, pharmacological research, production of plant-derived pharmaceuticals, and engaged in developing protocols for the safe use of medicinal plants.	Created a National Commission for the Use of Medicinal Plants which serves as a model for other Latin American countries in developing guidelines and standardized protocols for production of plant-based pharmaceuticals. Co-operates in the IDRC project on the Application, Research and Dissemination of the Use of Medicinal Plants in the Caribbean. Collaborates with the Central America Centre of Studies on Appropriate Technologies (CEMAT)
India	Herbal Gene Bank at the Tropical Botanic Garden Research Institute at Thiruvananthapurnam. Germplasm Bank, Point Calimere Wildlife Sanctuary Tamil Nadu.	All-India ethno biological project for the development of drugs from medicinal plants and herbs. Promotion of ethno pharmacological research. More than 40 species of medicinal plants are maintained and protected. Examples are *Manilkara hexandra* to treat jaundice, *Salvadora persicum* to treat ulcers; *Mucuma purata* used for preparation of a health tonic.
Japan	Research Centre of Medicinal Resources Medicinal Plant Gardens, Chiba University.	Chemical, biochemical, and pharmacological studies of plant secondary metabolites, neurotoxic proteins in *Lathyrus sativus.*

Country	Activities	Remark
Malaysia	Network of plant genetic resources.	Screening of marine and terrestrial biochemical diversity for medicinal principles, phyto medicinals and nutraceuticals.
Malta	Medicinal plants are widely used as part of folk medicinal.	Well-known Maltese examples are: fejgel, faqqus il-hmir and hobbeja.
Myanmar	Conservation of plant genetic resources and medicinal plants.	Research programmes at Yangon (formerly Rangoon) University focus on folk medicinal herbs; pharmacognostic studies; and bioassay of plants credited with anti-tumour, antipyretic and anti-diabetic properties.
Nepal	Plant biotechnology, mushroom cultivation, bio-energy production, environmental microbiology and medicinal plants.	University programmes in plant tissue culture and environmental microbial-based technologies; medicinal plants widely cultivated in Shivpuri, Doti, Tistung, Urindavan and Tarakava Herbal farms. Herbal products widely marketed as Ayurvedic therapeutics.
New Zealand	Conservation of medicinal plants used in Maori medicine.	*Coprosma robusta* – a sacred Maori medicinal plant and *Aristotelia serrata* used by early settlers maintained in nurseries.
Nigeria	Preservation of Nigerian genetic patrimony comprised of 5000 acquisitions of edible, fodder, forest, industrial and medicinal plants.	Research supported by National Centre for Genetic Resources and Biotechnology which functions also as affiliate of International Centre for Genetic Engineering and Biotechnology.
Norway	Collaborative project between UNESCO and Governmental agency.	Conduction of research work in the origins, uses, trades and constraints in the cultivation of plants in Mozambique and Madagascar by students.

Country	Activities	Remark
Pacific Islands	Noni, a Tahitian herbal tonic derived from *Morinda citrifolia* is used as a general tonic food and energizer.	Product widely used in China and South Asia and widely marketed throughout the pacific islands.
Russia	Medicinal Plants reared and protected as economic bioresource in Karadag Reserve.	Karadag Reserve serves as a base for studying the bioecological properties for Crimean medicinal plants such as *Rosa canina*.
Sri Lanka	General biotechnologies, medicinal plants.	Possesses rich history of medicinal plants intricately linked with religious and cultural practices. Ayurvedic system of medicine is widespread.
Thailand	Laboratory of Natural Products – research on medicinal plants.	Species been investigated are: Tai Bai (*Phyllanthus amarus*), Chai aim Thai (*Derris escalata* and *Carophyllum inophyllum*). Based at the Chulabhorn Research Institute, research activities deal with the preparation of dietary supplements and therapeutics from traditional medicinal plants.
USA	National Germplasm Resources Laboratory of the US Department of Agriculture hosts Phytochemeco, a phytochemical/geographic database.	Contains unique blend of phytochemicals taxonomic, ecological, geographic and climatic aspects - phytochemical database contains data on over 16,000 chemical compounds present in some 16,000 plants of economic importance, and of some 1500 specific activities of some 4,000 plant-derived chemicals. - taxonomic database contains plant names of over 8,000 taxa - ecological database contains growing locations of some 6,000 taxa - yield database contains crop yields of some 239 taxa

(DaSilva and Hoareau, 1999)

1.19 Medicinal plants in Bangladesh

About 500 medicinal plants grow in Bangladesh, where 80 per cent of the rural population depends on traditional remedies for ailments such as cough, cold, fever, headache and dysentery. Neem, for example, is used to treat skin disease and in beauty care products.

Turmeric is used as an anti-inflammatory, to treat digestive disorders and skin diseases, and in wound healing (Dold & Cocks, 2001). Despite this, and the presence of more than 400 companies producing herbal medicines, more than 90 per cent of the plants and products needed to meet demand are imported by Bangladesh from other countries such as India, Nepal and Pakistan. According to a December 2003 study by the World Bank's South Asia Enterprise Development Facility and a Swiss development organization 'Inter-cooperation', Bangladesh's medicinal plant market is worth US $14 million each year at wholesale for 17,000 tones of final product. The report predicted that the demand for imported raw materials would increase by US $4.9 million within five years. But at present medicinal plants are not commercially farmed in Bangladesh and are only used when gathered from the wild. Plants such as garlic, mint, turmeric and neem could boost Bangladesh's economy if planted on a larger scale, even if it is just in villagers' backyards. Although Bangladesh has no government policy or regulations about growing, conserving and marketing medicinal plants, some universities and non-governmental organizations are collaborating to boost the country's production of the plants (Schippmann et al., 2002).

1.20 Description of Phyllanthus

Trees, shrubs, or herbs, mostly monoecious, less often dioecious; branching often "phyllanthoid": main stems with spiral phyllotaxy, ultimate branchlets sometimes clustered on short shoots, resembling pinnate leaves and often deciduous as a unit, less often stems all similar with spiral or distichous phyllotaxy; hairs simple, often absent, rarely branched.

Leaves alternate, often reduced and scale like on main stems, strongly distichous on leafy stems; stipules small, deciduous or persistent; petiole short; leaf blade simple, margin entire, venation pinnate. Inflorescences axillary, sometimes at leafless nodes, solitary or in fascicles, cymes, glomerules, racemes, or panicles; pedicels delicate.

Male flowers: sepals (2 or)3–6, in 1 or 2 series, free, imbricate, margin entire, eroded, denticulate or fimbriate; petals absent; disk glands 3–6, usually free; stamens 2–6; filaments free or connate; anthers 2- locular, extrorse, thecae 2, connectives obscure, longitudinally or horizontally dehiscent, rarely obliquely so; pistillode absent.

Figure 1.5: *Phyllanthus* Species

Female flowers: sepals as in male or more; disk glands usually small, free or connate into an annulus or urn-shape, surrounding ovary; ovary smooth or less commonly roughened, bullate, or hairy, 3(–12)-locular; ovules 2 per locule; styles 3(–12), apex 2-lobed or 2-branched, rarely entire, erect, spreading, or recurved.

Fruit usually a capsule, globose or depressed globose, smooth or warty, dehiscent into 3 2-valved cocci when mature, less often a fleshy berry or drupe; columella persistent.

Seeds without caruncle or aril, trigonous, surface smooth, sculptured or striate; seed-coat dry crustaceous, endosperm whitish, cartilaginous; embryo straight or slightly curved; cotyledons usually considerably broader than radical. x = 13.

About 750–800 species: primarily in the tropics and subtropics, poorly represented in temperate regions; 32 species (13 endemic, one introduced) in China.

Molecular studies have shown that several long-established genera nest within Phyllanthus, including Breynia, Phyllanthodendron, Glochidion, and Sauropus.

Phyllanthus acidus (Linnaeus) Skeel is recorded from Taiwan and Hong Kong, where it is presumably cultivated for its edible fruits. (zhu shu, Ping-tao and G. Gilbert, 2008)

1.21 Plant Profile

1.21.1 Botanical Name

Phyllanthus acidus (L.) Skeels

Table 1.9: Vernacular Name

Languages	Local Names
Burmese	thinbozihpyoo
English	country gooseberry, star gooseberry, plum, Otaheite gooseberry, damsel, Malay gooseberry
Filipino	karmay, bangkiling, iba
Indonesian	cerme, ceremai, caramele
Lao	Sino-Tibetan, mak nhom, nhom baanz, nhom ban
French	cerisier de Tahiti
Malay	kemangul, chermala, chermai
Spanish	grosella
Thai	ma rom
Vietnamese	t[aaf]m ru[ooj]t, ch[uf]m ru[ooj]t
Bengali	**Orboroi**

(www.worldagroforestry.org, 2017)

1.21.2 Taxonomic Hierarchy of the Plant

Kingdom: Plantae

Angiosperms

Eudicots

Rosids

Order: Malpighiales

Family: Phyllanthaceae

Tribe: Phyllantheae

Subtribe: Flueggeinae

Genus: Phyllanthus

Species: *P. acidus*

(En.wikipedia.org, 2017)

1.21.3 Synonym

Phyllanthus distichus Müll.Arg.
Cicca acida Merr.
Cicca disticha L.
Averrhoa acida L.

1.21.4 Habitat and Distribution

Star gooseberry is an ancient fruit, originating in the tropical climates of Madagascar. Filipino botanist Eduardo Quisumbing explains that although the fruit came to the Philippines in pre-historic times, the star gooseberries did not achieve the same popularity there as it did when it spread to Indonesia, Malaysia, Vietnam and Laos. Nonetheless, countries continue labeling the fruit as their own, hence its other names such as Sri Lankan gooseberry, Malay gooseberry, and Madagascar gooseberry. This pungent, sour fruit bears no relation with the more agreeable, reddish European gooseberry (Ribes uva-crispa).

Today, the fruit grows throughout Asia, parts of Central America, the Caribbean and parts of South America. Garden hobbyists in Hawaii and Florida dabble with star gooseberries as well.

Pinpointing the fruit's arrival in India is difficult because of its ancient history. Star gooseberry's close relative—the amla—is distinctly native to parts of India. How and when these two fruits crossed paths is a mystery. (Reddy, 2017)

1.21.5 Description

Phyllanthus acidus is a small, glabrous tree up to 10 m tall with phyllanthoid branching, bark rough, grey, with prominent lenticels; cataphylls not persistent, blackish-brown, their stipules triangular-ovate; deciduous branchlets ascending, (20-)25-52 cm long, with 25-40 leaves. Leaves pinnate, 20-40 cm long. Leaflets alternate, simple, entire, shortly petiolate, broadly ovate to ovate-lanceolate, (4-)5-9 cm x (2-)2.5-4.5 cm, base obtuse to rounded, apex acute, petiole 2.5-4 mm long, stipules triangular-acuminate.

Flowers small, pink, in dense, cushion-shaped cymules at the nodes of leafless branches on older wood, and usually also on proximal branchlets of current year's growth, pale green to reddish; male flowers 4-merous, filaments and anthers free, dehiscing vertically;

female flowers on a stout pedicel, 4-merous, disk deeply lobed or split, styles connate, deeply bifid, staminodes present, ovary superior.

Fruit drupaceous, oblate, 1-1.5 cm x (1.2-)1.5-2(-2.5) cm when fresh, shallowly 6- or 8-lobed, greenish yellow to creamy-white; flesh firm, sour with a hard, bony, grooved stone containing 6-8 smooth seeds.

Phyllanthus, the generic name is derived from the Greek 'phullon'-leaf and 'anthos'-flowers from the fact that members of this genus have flowers in dense clusters in leaf axils. (www.worldagroforestry.org, 2017)

1.21.6 Biology

Otaheiti gooseberry is monoecious. Flowering and fruiting is mostly in January-May in the Caribbean and throughout the year in Java. The tree flowers between February-April in Florida. Fruits mature in 90-100 days. *P. acidus* trees start producing a substantial crop at the age of 4 years. The peak fruiting season in the Philippines is in April to June. The fruits often explosively dehisce dispersing their seeds. (www.worldagroforestry.org, 2017)

1.21.7 Ecology

Otaheiti gooseberry grows well in the tropics at low and medium altitudes in places with a short or prolonged dry season. The tree prefers hot, humid tropical lowlands. In north-eastern Brazil, the tree has been found in coastal forest and in Southeast Asia it is cultivated on humid sites, up to 1000 m altitude. (www.worldagroforestry.org, 2017)

1.21.8 Biophysical Limits

Altitude: 0-1 000 m

Soil type: It tolerates a variety of soils including very sandy soils. (www.worldagroforestry.org, 2017)

1.21.9 Documented Species Distribution

Native: Brazil, Colombia

Exotic: India, Indonesia, Laos, Madagascar, Malaysia, Myanmar, Philippines, Thailand, United States of America, Vietnam, Zanzibar

Figure 1.5: Documented Species Distribution

The map above shows countries where the species has been planted. It does neither suggest that the species can be planted in every ecological zone within that country, nor that the species cannot be planted in other countries than those depicted. Since some tree species are invasive, you need to follow bio-safety procedures that apply to your planting site. (www.worldagroforestry.org, 2017)

1.21.10 Traditional Uses of *Phyllanthus acidus*

Food: The mature sour fruits may be eaten fresh but usually they are sprinkled with salt to neutralize the acidity. Used in cooking to flavor dishes, the fruits are excellent raw materials for processing into pickle and sweetened dried fruits; fruit juice is used in cold drinks and fruit to make vinegar. In Malaysia, ripe and unripe fruit are served as a relish, syrup or sweet preserve. The fruits, combined with other fruits are used in chutney or jam, because of their setting properties. Young leaves are cooked as a vegetable in Indonesia, Thailand and India.

Fuel: The tree is used as fuel wood.

Timber: The wood is fairly hard, strong, tough and durable if seasoned. It is used for utensils and other small objects.

Tannin or dyestuff: The bark is used in India as a tanning agent.

Poison: Extract from the plant has shown nematicidal activity against the pine wood nematode, Bursaphelenchus xylophilus. The juice of the root bark is weakly poisonous.

Medicine: The latex is credited with emetic and purgative activity. In Indonesia the bark is heated with coconut oil and spread on eruptions on feet and hands. An infusion of the root is taken to alleviate asthma in Java. In Borneo, roots are used in the treatment of psoriosis of the feet. A leaf decoction is applied to urticaria, a decoction of the bark is used to treat bronchial catarrh in Philippines. The fruit is used as a laxative in Myanmar. In India, the fruits are taken as a liver tonic to enrich the blood.

Other products: Triterpenoids (phyllanthol and ?-amyrin) have been isolated from the Otaheiti gooseberry. The root bark contains saponins, gallic acid and tannins. (www.worldagroforestry.org, 2017)

1.21.11 Tree Management

It is grown at a spacing of 8 m x 8 m in Indonesia. (www.worldagroforestry.org, 2017)

1.21.13 Pests and Diseases

Caterpillars of *Parallelia absentimacula* and *P. joviana* feed on the cerme in Indonesia. The only serious pest is the oriental fruitfly (*Dacus dorsalis*) which infests maturing fruits. (www.worldagroforestry.org, 2017)

1.22 Study

1.22.1 Study Design

Modern medicines are intimately related to chemistry and detailed examinations of active principles of plants and other products from an essential part of it. The healing properties of the plants are due to the presence of physiologically active chemical compounds inside the plant materials. The phytochemical investigation or screening is an evaluatory process for the detection of plant constituents through chemical analysis; phytochemical screening is co-related with phytochemical study. The compounds isolated through phytochemical study are applied on treated animal to find out the pharmacological effect either beneficial or toxic and thus toxic plants are separated. In this research work methanol extract of *Phyllanthas acidus* leaves were evaluated for antioxidant activity, anti-inflammatory activity, cytotoxic activity and anti-microbial activity. It is found that chemical

constituent such as lupeol, b-amyrin, phyllanthol, phyllanthoside is present in the leaves of *Phyllanthus acidus* which can be extracted out by methanol extract.

1.22.2 Study Protocol

Present study was designed so that the medicinal effect of methanol extract of *Phyllanthas acidus* plant can be observed. The study protocol consists of following steps:

1. Preparation of methanol extract of *Phyllanthas acidus* leaves.
2. Determination of antioxidant activity of methanol extract of *Phyllanthas acidus* leaves.
3. Determination of anti-inflammatory activity of methanol extract of *Phyllanthas acidus* leaves.
4. Investigation of cytotoxic activity of methanol extract on *Artemia salina* leaches (brine shrimp).
5. Investigation of anti-microbial activity of methanol extract on different strains of gram positive and gram negative bacteria.

1.22.3 Study Objective

The objective of this study was to biologically evaluate the effect of methanol extract of *Phyllanthas acidus* on antioxidant activity, anti-inflammatory activity, cytotoxic activity and anti-microbial activity. There is medical flow about this plant and that is possesses various medicinal properties due to which it was extensively used as traditional medicine. Therefore, the objective of this work is to explore the possibility of developing new drug candidates from this plant for the treatment of various diseases.

CHAPTER TWO: LITERATURE REVIEWS

2.1 In Vitro Antibacterial, Antioxidant, Total Phenolic Contents and Anti-HIV-1 Reverse Transcriptase Activities of Extracts of Seven *Phyllanthus* Sp

In this study eighty percent methanol extracts obtained from seven *Phyllanthus* sp. were evaluated for antibacterial activity using the broth micro-dilution assay, anti-HIV-1 reverse transcriptase (RT) activity using the HIV-RT assay, antiradical scavenging effects and phenolic contents using the DPPH assay and Folin–Ciocalteau colorimetric method, respectively. Best antibacterial activity as indicated by the minimum inhibitory concentration (MIC) values was obtained by *Phyllanthus amarus* against *Staphylococcus aureus* (Gram-positive) with a MIC value of 17.7 µg/ml. *Phyllanthus myrtifolius* and *Phyllanthus urinaria* inhibited growth of *Pseudomonas stutzeri* (Gram-negative) with MIC values of 78 µg/ml and 117 µg/ml, respectively. A strong inhibition of HIV-RT was obtained by *Phyllanthus pulcher* (IC_{50} 5.9 µg/ml) followed by *P. urinaria* and *P. myrtifolius* (IC_{50} of 10.4 and 12.7 µg/ml, respectively). A remarkable DPPH scavenging effect was observed with *P. myrtifolius*, *Phyllanthus reticulatus* and *P. urinaria* (IC_{50} of 10.2, 10.8 and 17.4 µg/ml, respectively). Highest total phenolic contents were recorded for *P. myrtifolius* and *P. urinaria* (207 and 205 mg/GAE/g respectively). With the exception of *P. amarus*, *Phyllantus debilis* and *P. pulcher*, total phenolic contents correlated with DPPH radical scavenging activity (Eldeen et al., 2011).

2.2 Six Weeks Oral-gavage of a *Phyllanthus acidus* Leaf Water Extract Decreased Visceral Fat, the Serum Lipid Profile and Liver Lipid Accumulation in Middle-Aged Male Rats

In the study the researchers aimed to investigate the effects of a chronic oral administration of PA extracts to middle-aged (12–14months) rats on their body weight, food intake, body fats, liver and kidney functions, fasting blood glucose and lipid profiles, liver lipid accumulation and on blood pressure. Three different kinds of PA extracts were used: (1) a PA water extract, (2) a heated PA water extract, and (3) an n-butanol fraction of the PA water extract, prepared from fresh leaves of *Phyllanthus acidus*. The rats were orally gavaged with the three PA extracts at 1.0g/kg bodyweight or, as a control, with distilled water once a day for 6 weeks. Fasting blood sugar, lipid profile and ALP, SGOT, SGPT, BUN and creatinine levels were measured by enzymatic methods. Liver lipid accumulation was measured using oil red O staining on fresh thin cryostat liver tissue sections. The animal basal blood pressure and heart rate were measured in anesthetized

rats via a common carotid artery using a polygraph. Results showed that after 6 weeks of treatment using gavaged heated PA extract and PA n-butanol extract there were no changes in any of the parameters studied. However, the initial PA water extract caused a slight decrease in the animal body weight with no change in food intake. No changes were observed in the liver and kidney functions (serum ALP, SGOT, SGPT, BUN and creatinine did not change), nor did the fasting blood sugar or triglyceride levels differ significantly. Serum cholesterol, HDL and LDL levels, as well as visceral and subcutaneous adipose tissue and liver lipid accumulation were significantly decreased compared to that of the control group. There were no differences found in the basal systolic and diastolic blood pressure and the basal heart rate between the PA water extract treatment and the control group. These results indicated that the PA water extract had an effect on lipid metabolisms that resulted in a decrease of the serum lipid profile, visceral and subcutaneous fat, as well as on liver lipid accumulation in middle-aged rats. The active component that is responsible for these effects is likely to be a water soluble substance(s) and is heat labile (Chongsa, Radenahmad and Jansakul, 2014).

2.3 Protective Effects of *Phyllanthus acidus* (L.) Skeels Leaf Extract on Acetaminophen and Thioacetamide Induced Hepatic Injuries in Wistar Rats

In the study two different sets of experiments, the *P. acidus* extracts (200 and 400 mg/kg, body weight) and silymarin (100 mg/kg, body weight) were given orally for 7 days and a single dose of APAP (2 g/kg, per oral) or TAA (100 mg/kg, subcutaneous) were given to rats. The level of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and total protein were monitored to assess hepatotoxicity and hepatoprotection. APAP or TAA administration caused severe hepatic damage in rats as evident from significant rise in serum AST, ALT, ALP, total bilirubin and concurrent depletion in total serum protein. The *P. acidus* extracts and silymarin prevented the toxic effects of APAP or TAA on the above serum parameters indicating the hepatoprotective action. The aqueous extract was found to be more potent than the corresponding ethanolic extract against both toxicants. The phenolic and flavonoid content (175.02±4.35 and 74.68±1.28, respectively) and 2, 2-diphenyl-1-picrylhydrazil (DPPH) [IC_{50} = (33.2±0.31)µg/mL] scavenging potential was found maximum with aqueous extract as compared to ethanolic extract. The results of present study suggests that the aqueous extract of *P. acidus* leaves has significant hepatoprotective activity on

APAP and TAA induced hepatotoxicity, which might be associate with its high phenolic and flavonoid content and antioxidant properties (Jain and Singhai, 2011).

2.4 Anti-inflammatory, Anti-nociceptive and Antioxidant Activities of *Phyllanthus acidus* L. Extracts

In this study anti-inflammatory activity was evaluated using carrageenan induced paw oedema, cotton pellet induced granuloma, membrane stabilizing activity method. Analgesic activity of the extracts was estimated against acetic acid induced writhing, tail immersion method, formalin test. Free radical scavenging and antioxidant potential of the extracts of *Phyllanthus acidus* leaves was performed using several in vitro and ex vivo assay models. Total phenolic and total flavonoid contents of the extracts were determined using standard chemical methods. The extracts exhibited significant anti-inflammatory and analgesic activities at dose dependent manner. Methanol extract at a dose of 500 mg/kg showed superior activity which was comparable with the standard drugs. Ethyl acetate extract showed moderate activity while petroleum ether extract showed least activity. Total phenolic and total flavonoid content in methanol extract were 73.08±0.682 mg GAE/g and 61.28±0.062 mg QE/g respectively. The extracts possess significant antioxidant activity, methanol extract showed highest IC_{50} value. The contents of flavonoid and phenolic compounds could be correlated with the antioxidant, analgesic and anti-inflammatory activities observed for *Phyllanthus acidus* leaves. Our findings suggest that *Phyllanthus acidus* contains potential antioxidant, analgesic and anti-inflammatory compounds which could be tested as drug candidates against oxidative stress, pain and inflammation related pathological diseases (Chakraborty et al., 2012).

2.5 An Extract from the Medicinal Plant *Phyllanthus acidus* and Its Isolated Compounds Induce Airway Chloride Secretion: A Potential Treatment for Cystic Fibrosis

According to previous reports, flavonoids and nutraceuticals correct defective electrolyte transport in cystic fibrosis (CF) airways. Traditional medicinal plants from China and Thailand contain phytoflavonoids and other bioactive compounds. We examined herbal extracts of the common Thai medicinal euphorbiaceous plant Phyllanthus acidus for their potential effects on epithelial transport. Functional assays by Using chamber, patch-clamping, double-electrode voltage-clamp and Ca^{2+} imaging demonstrate activation of Cl^-

secretion and inhibition of Na^+ absorption by *P. acidus*. No cytotoxic effects of *P. acidus* could be detected. Mucosal application of *P. acidus* to native mouse trachea suggested transient and steady-state activation of Cl^- secretion by increasing both intracellular Ca^{2+} and cAMP. These effects were mimicked by a mix of the isolated components adenosine, kaempferol, and hypogallic acid. Additional experiments in human airway cells and CF transmembrane conductance regulator (CFTR)-expressing BHK cells and Xenopus laevis oocytes confirm the results obtained in native tissues. Cl^- secretion was also induced in tracheas of CF mice homozygous for Phe508del-CFTR and in Phe508del-CFTR homozygous human airway epithelial cells. Taken together, *P. acidus* corrects defective electrolyte transport in CF airways by parallel mechanisms including 1) increasing the intracellular levels of second messengers cAMP and Ca^{2+}, thereby activating Ca^{2+}-dependent Cl^- channels and residual CFTR-Cl^- conductance; 2) stimulating basolateral K^+ channels; 3) redistributing cellular localization of CFTR; 4) directly activating CFTR; and 5) inhibiting ENaC through activation of CFTR. These combinatorial effects on epithelial transport may provide a novel complementary nutraceutical treatment for the CF lung disease (Sousa et al., 2006).

2.6 Neuroprotective Effect of *Phyllanthus acidus* L. on Learning and Memory Impairment in Scopolamine-Induced Animal Model of Dementia and Oxidative Stress: Natural Wonder for Regulating the Development and Progression of Alzheimer's Disease

In this consequence, methanolic extract of PA (MEPA) was selected to explore the ability of this plant to enhance cognitive function, brain antioxidant enzymes and anti-acetyl cholinesterase activity which can be used for the treatment of oxidative stress related disorders like Alzheimer's disease (AD). The purpose of this study was to investigate the neuroprotective effect of MEPA on learning and memory impairment in scopolamine-induced rats of dementia and oxidative stress. Treatment with MEPA (*i.e.*, 100 and 200 mg/kg b.w.) was investigated in scopolamine-treated Swiss albino male rats for 14 days and its neuroprotective effects were examined using Elevated Plus Maze (EPM) test, Passive Avoidance (PA) test, Novel Object Recognition (NOR) test, Morris Water Maze (MWM) test as well as level of antioxidant enzymes such as catalase (CAT), super oxide dismutase (SOD), glutathione reductase (GSR), glutathione-S-transferase (GST), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), lipid peroxidation (TBARS) contents and acetylcholinesterase (AChE) activity in rat brain tissue homogenates.

Administration of MEPA significantly ($P < 0.05$, $P < 0.01$; $P < 0.01$) decreased RTL (retention transfer latency) in rats on 7^{th} and 14^{th} day compared to the disease control and control group in the EPM test. In PA test the doses of MEPA suggestively ($P < 0.05$, $P < 0.001$; $P < 0.05$, $P < 0.01$) increased STL (step-through latency) in rats on 7^{th} and 14^{th}day with respect to disease control and control group. For NOR test administration of MEPA considerably ($P < 0.01$, $P < 0.001$; $P < 0.01$) increased the DI (discrimination index) in rats with respect to that of disease control and control group. The doses of MEPA markedly ($P < 0.05$, $P < 0.01$; $P < 0.01$) decreased EL (escape latency) and significantly ($P < 0.01$, $P < 0.001$; $P < 0.05$, $P < 0.01$) increased TSTQ (time spent in the target quadrant) on successive days as compared to that of disease control and control group in the acquisition trial of MWM test. In case of probe trial of MWM test MEPA administration considerably ($P < 0.01$; $P < 0.05$, $P < 0.01$) increased TSTQ and significantly ($P < 0.05$, $P < 0.01$; $P < 0.05$, $P < 0.01$) increased TSA (time spent in the annuli) in rats on successive days as compared to that of disease control and control group. MEPA administration significantly ($P < 0.05$, $P < 0.01$, $P < 0.001$; $P < 0.05$, $P < 0.01$) increased the level of CAT, SOD, GSR, GST GSH, GSH-Px and markedly ($P < 0.01$; $P < 0.01$, $P < 0.001$) decreased TBARS level through inhibiting lipid peroxidation as well as significantly ($P < 0.01$, $P < 0.001$; $P < 0.05$, $P < 0.01$, $P < 0.001$) decreasing AChE activity in rats brain compared to the disease control and control group. The present study demonstrates that MEPA showed the neuroprotective effect by improving cognitive functions and reduces oxidative stress by increasing the level of brain antioxidant enzymes as well as decreasing lipid peroxidation and acetylcholinesterase activity. Therefore, this plant extract can be used for enhancing learning, memory, antioxidant potentiality and anti-acetylcholinesterase activity in neurodegenerative disorders like AD (Uddin et al., 2016).

2.7 Evidence of Trimonoecy in Phyllanthaceae: *Phyllanthus acidus*

Pollen from staminate flowers and pistillate flowers with ‘‘staminodes’’ of *Phyllanthus acidus* Skeels were analyzed under scanning electron microscopy, and tests of pollen viability and in vitro germination were carried out to verify possible similarities between the three types of flowers. The results show that pistillate flowers with ‘‘staminodes’’ are

bisexual, indicating the occurrence of trimonoecy in this species. (Cardoso-Gustavson, Demarco and Carmello-Guerreiro, 2011)

2.8 In Vitro Antioxidant and Cholinesterase Inhibitory Activities of Methanolic Fruit Extract Of *Phyllanthus Acidus*

In this study, *P. acidus* was evaluated for its cholinesterase inhibitory and antioxidant activities. Methods In this study, we evaluated the antioxidant potential and neuroprotective activity of *P. acidus* by assessing total phenol content (FCR assay), total flavonoid content, total antioxidant capacity, Fe^{3+} reducing power capacity, DPPH (2, 2-diphenyl-1-picrylhydrazyl) and hydroxyl radical scavenging capacity, lipid peroxidation inhibition activity & metal chelating activity. In addition acetyl cholinestrase (AChE) and butyrylcholinestrase (BChE) inhibitory activities were performed using Ellman's method. Results Total phenolic content and total flavonoid content of the extract were 116.98 mg of Gallic acid equivalent and 168.24 mg of quercetin equivalent per gm of dried extract. The methanolic extract of *P. acidus* (MEPA) showed considerable total antioxidant activity and reducing capacity. In DPPH scavenging assay and hydroxyl radical scavenging assay, the MEPA showed 84.33 % and 77.21 % scavenging having IC_{50} of 15.62 and 59.74 µg/ml respectively. In lipid peroxidation inhibition activity MEPA showed moderate inhibition of peroxidation at all concentrations with IC_{50} value of 471.63 µg/ml and exhibited metal chelating activity with IC_{50} value 308.67µg/ml. The MEPA exhibited inhibition of rat brain acetyl cholinesterase and human blood butyryl cholinesterase in a dose dependent manner and the IC_{50} value was found to be 1009.87µg/ml and 449.51µg/ml respectively. Conclusion These results of the present study reveal that MEPA has considerable amount of antioxidant activity as well as anti-acetyl cholinesterase and anti-butyryl cholinesterase activity which suggest its effectiveness against Alzheimer's disease and other neurodegenerative disorders. (Moniruzzaman et al., 2015)

2.9 Protective Effects of *Phyllanthus acidus* (L.) Skeels Extract on Acetaminophen Mediated Hepatic Injury and Oxidative Stress in Wistar Rats

The present study was undertaken with a view to validate the traditional use of *Phyllanthus acidus* (L.) Skeels fruit as a hepatoprotective agent. The 70% ethanolic extract of *P. acidus* fruit (100, 200 and 400 mg/kg, p.o.), and reference drug silymarin (100 mg/kg, p.o.) were given to rats of different groups respectively once a day for 5 d

and the carbon tetrachloride (CCl_4) (2 mL/kg, subcutaneously) was given on days 2 and 3. Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (TB) and total protein (TP) were assessed along with liver histopathological examination. The effects on oxidative stress markers such as lipid peroxidation (LPO), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were also assessed in liver tissue homogenate to evaluate in vivo antioxidant activity. In addition, the effects on hexobarbitone-induced sleeping time were observed and the free radical-scavenging potential was determined by using 2, 2-diphenyl-1-picrylhydrazil (DPPH) in mice. *P. acidus* extracts and silymarin exhibited a significant hepatoprotective effect as evident from the decreases of serum AST, ALT and ALP levels and LPO and increases in the levels of TP, GSH, SOD, CAT, and GPx compared with control group ($P<0.01$ or $P<0.05$). The biochemical results were supplemented with results of histopathological sections of the liver tissues. *P. acidus* extracts considerably shortened the duration of hexobarbitone-induced sleeping time in mice compared with control group ($P<0.01$) and showed remarkable DPPH-scavenging activity. The present findings suggest that the hepatoprotective effect of *P. acidus* against CCl_4-induced oxidative damage may be related to its antioxidant and free radical-scavenging potentials (Jain and Singhai, 2011).

2.10 Effect of Auxin and Cytokinin on Phyllanthusol A Production by Callus Cultures of *Phyllanthus acidus* Skeels

Callus cultures of *Phyllanthus acidus* Skeels were established to verify whether they produce Phyllanthusol A as the intact plant does. Different growth regulator combinations were applied to MS medium to influence the level of production of Phyllanthusol A. The effects of various combinations of auxin and cytokinin on the growth and accumulation of Phyllanthusol A were investigated. MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) 1 mg lG1 and 6-furfurylaminopurine (kinetin) 1 mg lG1 was used to support the growth of callus cultures and the maximum amount of dry biomass (613 mg) was produced after 42 days of culture. High Performance Liquid Chromatographic analysis of methanol extracts from callus cultures of *P. acidus* revealed that the cultures produced Phyllanthusol A. The concentrations of the growth regulators á-naphthaleneacetic acid (NAA) and benzyladenine (BA) played a critical role in the production of Phyllanthusol A. The callus cultures accumulated 20 mg/g.dry weight of

Phyllanthusol A in MS medium supplemented with NAA (2 mglG1) and BA (0.5 mglG1). (Duangporn and Siripong, 2009)

2.11 Proximate Analysis on Biochemical Study of *Phyllanthus acidus*, *Phyllanthus emblica* and *Citrus limon*

The present study was under taken to investigate the biochemical characterization of *Phyllanthus acidus, Phyllanthus emblica and Citrus limon*. The results suggest that among the three varieties the level of storage polysaccharides (Total Carbohydrate & Starch) and total protein seems to be higher in *Phyllanthus emblica* than *phyllanthus acidus* and *citrus limon*. The amount of cholesterol in *Phyllanthus acidus* showed better level than the rest of the two varieties. When compared to *Phyllanthus acidus* and *Phyllanthus emblica*, the ascorbic acid content was found to be higher in *Citrus limon*. Phyllanthus emblica contain high amount of calcium than the other two varieties. The amount of phosphorous and iron in *Citrus limon* posses better level than *phyllanthus acidus* and *emblica*. Thereby to conclude the fruits selected *Phyllanthus acidus, Phyllanthus emblica and Citrus limon* varieties showed very impressive biochemical components which is of therapeutic and economic importance. (Suriyavathana and Subha, 2011)

2.12 Selective Antimicrobial Properties of *Phyllanthus acidus leaf* Extract Against *Candida albicans, Escherichia coli* and *Staphylococcus aureus* Using Stokes Disc Diffusion, Well Diffusion, Streak Plate and a Dilution Method

The antibacterial and antifungal activities of *Phyllanthus acidus* was investigated against *S.aureus* (gram+ve), *E.coli* (gram-ve) and *C.albicans* using the Stokes disc diffusion, the Pour plate, Well diffusion and Streak plate methods. The solvent type extracts were obtained by three extractions with hexane, CH_2Cl_2, EtOAc and CH_3CH_2OH respectively. Solvents were removed in vacuo to yield viscous oils and paste which were made up to a concentration of 0.035g in 0.01L (10mL) of the respective solvents. These were tested in varying volumes of 0.2-0.6 ml/plate. The solvents were used as control whereas Ampicillin and Nystatin were used as references for bacterial and fungal species respectively. The solvents had no effect on the microorganisms whereas Ampicillin and Nystatin inhibited microbial growth. Phyllanthus acidus showed antimicrobial inhibitory activity at 0.18mg/10mL plate of medium with activity most prominent with the ethanol extracts and negligible with the hexane. This study suggests that the ethanol extracts of

Phyllanthus acidus , can be used as herbal medicines in the control of *E.coli* and *S.aureus* following clinical trials. (Jagessar, Mars and Gomes, 2008)

2.13 In Vitro Evaluation Of Cytotoxic, Antibacterial, Antioxidant And Phytochemical Screening Of Petroleum Ether Extract Of *Phyllanthus Acidus*

The *in vitro* activity of the petroleum ether extract of fruit part of *Phyllanthus acidus* was tested for cytotoxic antibacterial and antioxidant activities as well as for phytochemical screening. The plant was collected from Savar, Dhaka. Phytochemical screening of petroleum extract of *phyllanthus acidus* revealed the presence of carbohydrate, glycoside and steroid. The extract exhibited antibacterial activity was determined by the disc diffusion method against thirteen pathogenic bacteria and the cytotoxic activity was performed by brine shrimp lethality bio-assay method. The higher concentrations showed antimicrobial activity against a number of bacteria including *Salmonella typhi, Staphylococcus aureus, Bacillus cereus, Escherichia coli, Bacillus megaterium.* In brine shrimp lethality bio-assay, the LC_{50} value was 3.12. The phenolics contents was 159.601 mg/g GAE and the amount of flavonoid was 24.183 mg/g of quercetin equivalent. The DPPH radical scavenging activity of *Phyllanthus acidus* was found to slight increase with increasing concentration of the extract and IC_{50} value showed 1192.263 µg mL−1 for plant extract compared to 13.37 µg mL−1 which was the IC_{50} value for the reference ascorbic acid (Razibul Habib et al., 2011).

CHAPTER THREE: METHODS AND MATERIALS

3.1 Collection & Preparation of Plant Material

Plant sample (Leaves) of *Phyllanthus acidus* was collected from Mirpur Cantonment, Dhaka and Rupgonj, Narayangonj in November 2016. Then proper identification of plant sample was done by an expert taxonomist. The leaves of the plant were sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding. The dried leaves was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Department of Pharmacy, East West University.

3.2 Extraction of the plant material

About 650 gm of the powdered material was taken in separate clean, round bottomed flask (5 liters) and soaked in 3.5 liter of methanol. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture was then filtered through cotton followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 390°C with a Heidolph rotary evaporation.

Figure 3.1: Drying of extract using rotary evaporator

The concentrated extract was then air dried to solid residue. The weight of the crude methanol extract obtained from the powdered whole plant was 25.18 gm respectively.

3.3 Preparation of Mother Solution

5 gm of methanol extract was triturated with 90 ml of methanol containing 10 ml of distilled water. The crude extract was dissolved completely. This is the mother solution.

3.4 Partition of Mother Solution

The mother solution was then partitioned off successively by four solvents of different polarity.

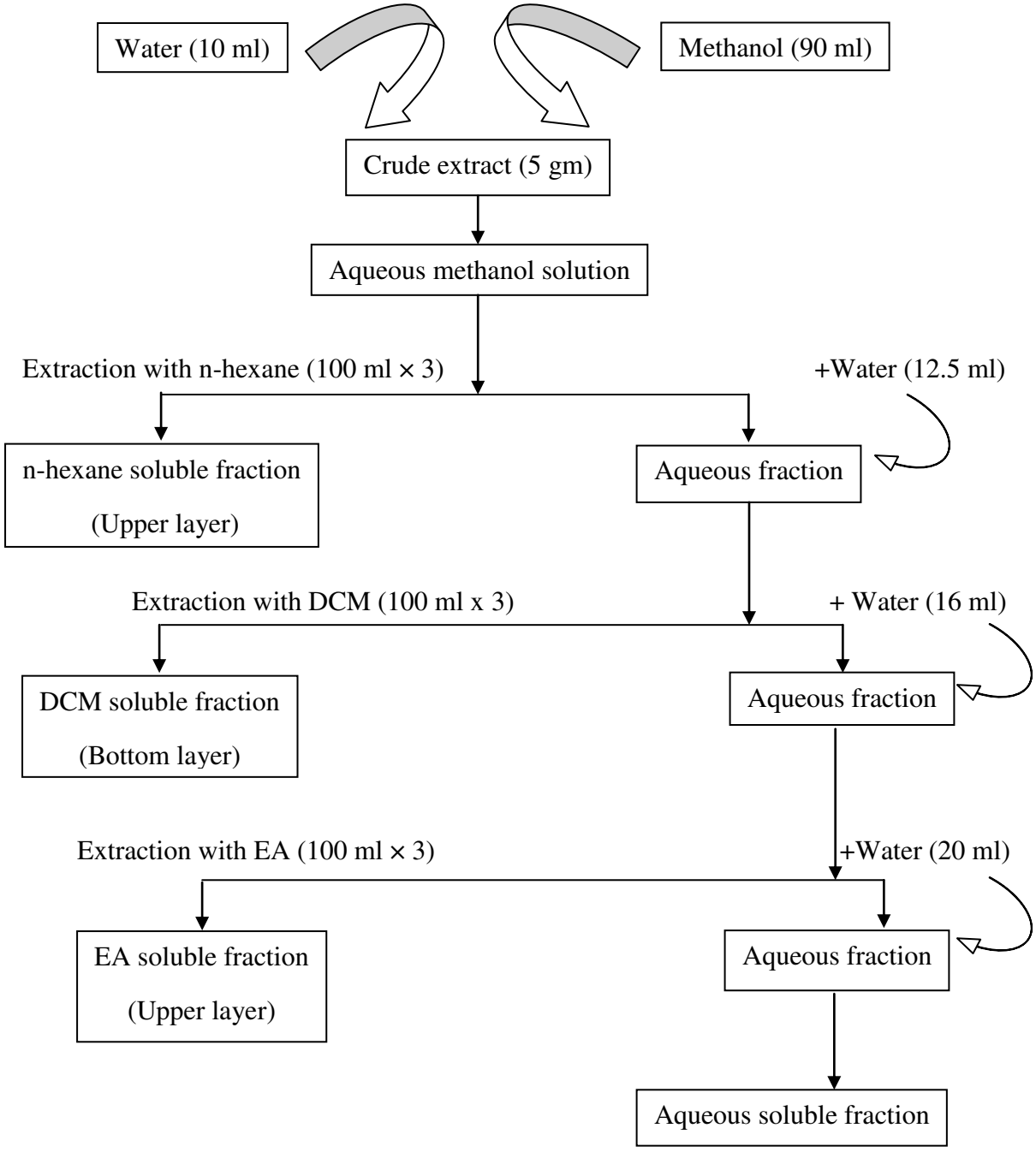


Figure 3.2: Schematic representation of the Partitioning of methanolic crude extract of *Phyllanthus acidus* leaves.

3.4.1 Partition with n-Hexane

The mother solution was taken in a separating funnel. 100 ml of the Pet-ether was added to it and the funnel was shaken and then kept undisturbed. The organic portion was collected. The process was repeated thrice (100 ml X 3). The n-hexane fraction was then air dried for solid residue.

3.4.2 Partition with Dichloromethane

To the mother solution left after partitioning with n-hexane, 12.5 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with Dichloromethane (DCM). The process was repeated thrice (100 ml X 3). The DCM fraction was then air dried for solid residue.

3.4.3 Partition with Ethyl acetate

To the mother solution that left after washing with n-hexane, and Dichloromethane, 16 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with ethyl acetate. The process was repeated thrice (100 ml X 3). The ethyl acetate fraction was then air dried for solid residue.

3.4.4 Partition with Aqueous Fraction

After partitioning the mother solution with n-hexane Dichloromethane and Ethyl acetate, 20 ml of distilled water was added and mixed. The mother solution was then taken in a separating funnel and extracted with aqueous fraction. The process was repeated thrice (100 ml X 3). The aqueous fraction was then air dried for solid residue.

3.4.5 Collection of n-hexane Fraction

After partitioning the mother solution with the four different solvents the n-hexane fraction was collected and air dried. This n-hexane was further investigated for different pharmacological properties (antioxidant, cytotoxic and antimicrobial) (Beckett and Stenlake, 1988).

3.5 Antioxidant Activity

3.5.1 Total Phenolic Content

The anti-oxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes. The antioxidant activity of phenolic compounds is mainly due to their redox properties, it has been reported that there is an inverse relationship between the anti-oxidative status occurrences of human diseases. In addition, antioxidant compounds which are responsible For Such antioxidants activity could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders. Therefore, research to identify anti-oxidative compounds is an important issue. Although it remains unclear which of the compounds, of medical plants are the active ones, polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as free radical scavenging and inhibition of lipid per oxidation, are the most crucial. Even though a variety of herbs are known to be sources of phenolic compounds, studies isolating polyphenols and evaluating their anti-oxidative effects have rarely been carried out. The purpose of this study was to evaluate extractives of Opuntiaelatior as new potential sources of natural antioxidants and phenolic compounds. This study also demonstrates a possible relationship between phenolic content and antioxidant activity. 50 Cytotoxic and Antioxidant activity in aqueous fraction of Opuntiaelatior extract.

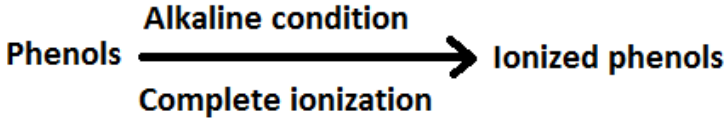
3.5.1.1 Principle

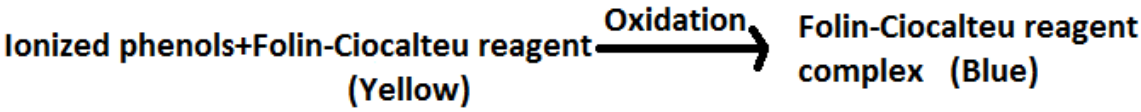
The content of total phenolic compounds in plant methanolic extracts was determined by Folin– Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. In the alkaline condition phenols ionize completely.

Table 3.1: Composition of 100 mg Folin-Ciocalteu Reagent:

Water	57.5 ml
Lithium Sulfate	15.0 mg
Sodium Tungstate Dihydrate	10.0 mg
Hydrochloric Acid (25%)	10.0 mg
Phosphoric Acid 85% solution in water	5.0 mg
Molybdic Acid Sodium Dihydrate	2.5 mg

When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution become blue. The exact chemical nature of the FC reagent is not known, but it is believed to contain hetero poly phosphotunstates - molybdates. Sequences of reversible oneor two-electron reduction reactions lead to blue species, possibly $(PMoW11O40)^{4-}$. The intensity of the color change is measured in a spectrophotometer at 765 nm. The absorbance value will reflect the total phenolic content of the compound (Singleton, Orthofer and Lamuela-Raventós, 1999).





3.5.1.2 Apparatus & Reagents

Table 3.2: Apparatus and reagents used for total phenolic content:

Folin-Ciocalteu reagent (10 fold diluted)	UV-spectrophotometer
Ascorbic acid	Beaker (100 & 200 ml)
Na_2CO_3 solution (7.5%)	Test tube
Methanol	Micropipette (50-200µl)
Distilled water	Cuvette

3.5.1.3 Procedure

3.5.1.3.1 Standard curve preparation

Ascorbic acid was used here as standard. Different ascorbic acid solutions were prepared having a concentration ranging from 120 µg/ml to 80 µg/ml. 5 ml of FCR (diluted 10 times with water) and 4 ml of Na_2CO_3 (7.5% w/v) solution was added to ascorbic acid

solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 765 nm. After plotting the absorbance in ordinate against the concentration in abscissa a linear relationship was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples.

3.5.1.3.2 Sample preparation

2 mg of the Opuntiaelatior aqueous fraction was taken and dissolved in 1 ml methanol to get a sample concentration of 2 mg/ml.

3.5.1.3.3 Determination of total phenol content

- ✓ ml plant extract of different concentrations (120 µg/ml, 110 µg/ml, 100 µg/ml, 90 µg/ml and 80 µg/ml) was taken in test tubes.
- ✓ 5 ml of Folin–ciocalteu (Diluted 10 fold) reagent solution was added into the test tube.
- ✓ 4 ml of Sodium carbonate solution was added into the test tube.
- ✓ The test tubes containing the samples were incubated for 1 hour at the room temperature to complete the reaction.
- ✓ Absorbance of solution was measured at 765 nm using a spectrophotometer against blank.
- ✓ A typical blank solution containing methanol was taken.

3.5.2 Total Flavonoid Content

3.5.2.1 Principle

Aluminium chloride ($AlCl_3$) colorimetric method is incorporated to determine the total flavonoid contents of the crude plant extract. The basic principle of the assay method is that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols of the crude extract. In addition aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A or B-ring of flavonoids. The formed flavonoid-aluminium complex between flavonoid of the crude extract and aluminium chloride has an absorbance maximum at 510 nm. Therefore, the amount of flavonoid in the crude extract can be quantified by measuring the absorbance of reaction mixture at 510 nm using a UV-visible

spectrophotometer against a blank containing all reagents except the extracts. Quercetin at various concentrations was used as standard (Chang C et al., 2002).

Flavonoid (Extract) + $AlCl_3$ (reagent) = Formation of flavonoid-aluminium complex (λ_{max} = 510 nm)

3.5.2.2 Apparatus & Reagents

Table 3.3: Apparatus and reagents used for total flavonoid content:

Aluminium chloride	Spatula
Methanol	Analytical balance
Ascorbic acid	Pipette and pumper
Sodium hydroxide	Aqueous fraction
Sodium nitrite	Test tubes and beaker

3.5.2.3 Procedure

3.5.2.3.1 Preparation of 10% Aluminium Chloride ($AlCl_3$) Solution

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

3.5.2.3.2 Preparation of 4% NaOH Solution

4 mg of NaOH was taken into a 100 ml volumetric flask and the volume was adjusted by distilled water.

3.5.2.3.3 Preparation of 5% (W/V) $NaNO_2$ Solution

5 mg of $NaNO_2$ was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water.

3.5.2.3.4 Preparation of Standard Solution

The stock solution was prepared by taking 10 mg of quercetin and dissolved into 50 ml of methanol. Concentration of this solution was 200µg/ml. The experimental concentrations were prepared from this stock solution.

Table 3.4: Preparation of standard solution:

Concentration (µg/ml)	Solution taken from stock solution (ml)	Volume adjusted by methanol (ml)	Final volume (ml)
0	0.0	5	5
4	0.1	4.9	5
8	0.2	4.8	5
12	0.3	4.7	5
16	0.4	4.6	5

3.5.2.3.5 Preparation of Extract Solution

5 mg of each plant extracts were taken and dissolved into 5 ml of methanol. The concentration of the solution was 1 mg/ml of plant extracts. Then the following steps were carried out. 1.5 ml extract was taken in a test tube and then 6 ml of distilled water was added. Then 5% of $NaNO_2$was added and incubated for 6 minutes. 10% $AlCl_3$ was added and incubated for 6 minutes. 4% NaOH and 0.6 ml distilled water was added. Then it was incubated for 15 minutes. For blank solution 1.5 ml methanol was taken and same procedure was repeated. Then the absorbance of the solution was measured at 510 nm using a spectrophotometer against blank.

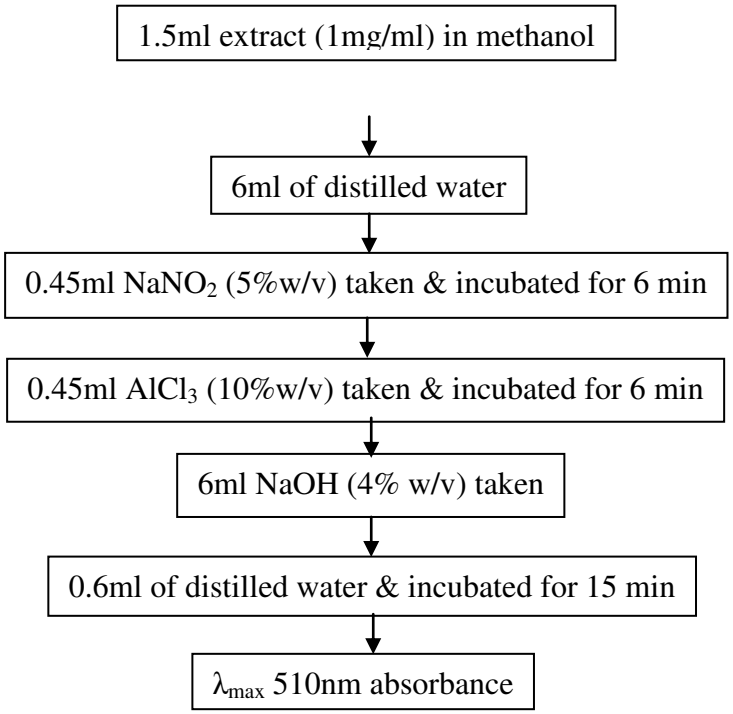


Figure 3.3: Schematic diagram of preparation of extract solution

3.5.2.3.6 Preparation of blank solution

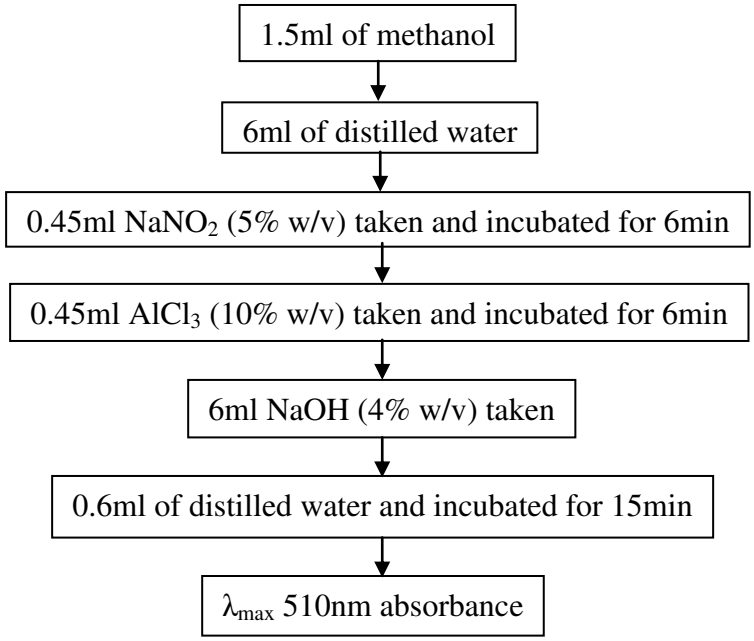


Figure 3.4: Schematic diagram of preparation of blank solution

3.6 Brine Shrimp Lethality Bioassay

3.6.1 Principle

Brine shrimp lethality bioassay is a recent development in the assay procedure for the bioactive compounds and natural product extracts, which indicates cytotoxicity as well as a wide range of pharmacological activities e.g. anticancer, antiviral, and pharmacological activities of natural products etc. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Thus (in-vivo) lethality, a simple zoological organism, (Brine shrimp napulii- *Artemia salina*) can be used as a convenient monitoring for screening and fractionation in the discovery of new bioactive natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. This bioassay is indicative of cytotoxicity and a wide range of pharmacological activity of natural products. Brine shrimp is the English name of the genus Artemia of aquatic crustaceans. Artemia is the only genus in the family Artemiidae (Olowa and Nuñeza, 2017).

3.6.2 Apparatus & Reagents

Table 3.5: Apparatus and reagents for Brine shrimp lethality bioassay:

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

3.6.3 Procedure

3.6.3.1 Preparation of Sea Water

To hatch the brine shrimp nauplii for the assay, sea water representing brine should be prepared at first. To prepare sea water 38gm of pure NaCl was dissolved in distilled water and then the

volume made up to 1000ml by distilled water in a 1000ml beaker for *Artemia salina* hatching. 1-2 drops of NaOH solution of 1N was added with a dropper to obtain the pH 8.4 as sea water.

3.6.3.2 Hatching of Brine Shrimp

A rectangular tank was divided in to two unequal compartments by a porous separator. The larger compartment was darkened while the smaller one was kept illuminated. Then a dry preserved egg of *Artemia salina* Leach was added in the artificial sea water. Oxygen was supplied through an air pump and a table lamp was placed near the beaker. The eggs of *Artemia salina* were hatched at room temperature (25-30ºC) for 18-24hr. The larvae (nauplii) were attracted by the light and moved to the smaller compartment through the holes. 10 living shrimps were then collected by a pipette and then added to each of the test tubes containing 5ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay (Niazi et al., 2009).

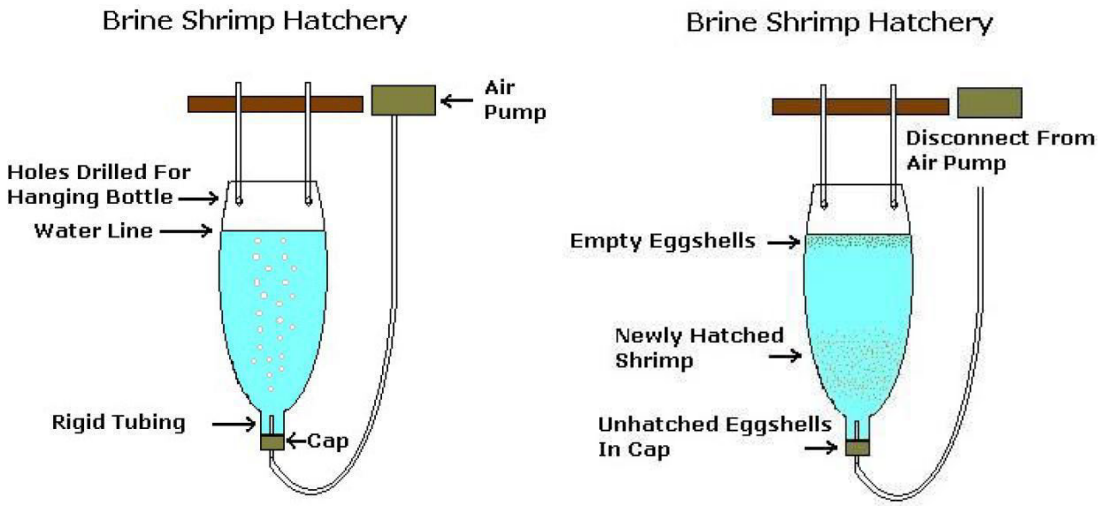


Figure 3.5: Brine shrimp Hatchery

3.6.3.3 Preparation of Test Solutions

Clean test tubes were taken. These test tubes were used for ten different concentrations (one test tube for each concentration) of test samples and ten test tubes were taken for standard drug tamoxifen for ten concentrations of it and another one test tube for control test.

3.6.3.4 Preparation of The Test Samples of Experimental Plant

All the test samples of 4mg were taken and dissolved in 200µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100µl of solution was taken in test tube each containing 5ml of simulated seawater and 10 shrimp nauplii. Thus, final concentration of the prepared solution in the first test tube was 400µg/ml. Then a series of solutions of varying concentrations were prepared from the stock solution by serial dilution method. In each case 100µl sample was added to test tube and fresh 100µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml for 10 dilutions.

3.6.3.5 Preparation of the Positive Control Group

In the present study tamoxifen is used as the positive control. Measured amount of the tamoxifen is dissolved in DMSO to get an initial concentration of 20µg/ml. From that stock solution serial dilutions are made using DMSO to get 400µg/ml, 200µg/ml,

100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.125µg/ml, 1.5625µg/ml and 0.78125µg/ml. Then ten living brine shrimp nauplii in 5ml simulated seawater are added to the positive control solutions in the pre-marked test-tubes to get the positive control groups.

3.6.3.6 Preparation of the Negative Control Group

100µl of DMSO was added to the pre-marked test tube containing 5ml of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds (Goldstein, Aronow and Kalman, 1974).

3.6.3.7 Counting Of Nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

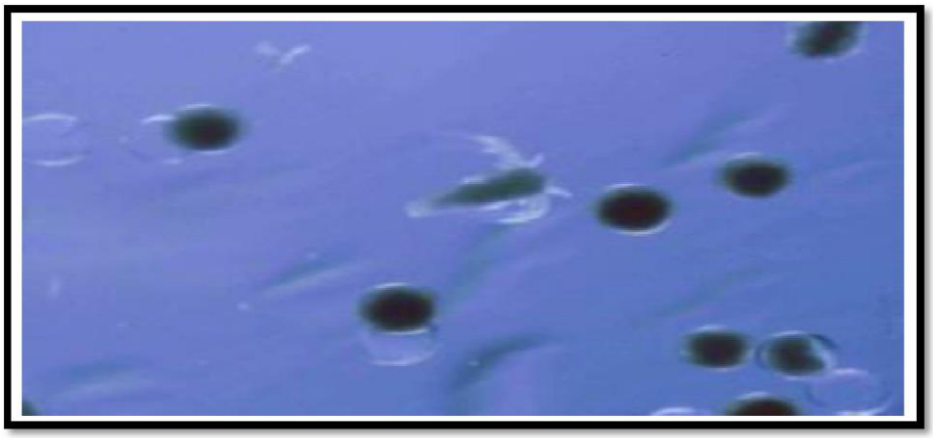


Figure 3.6: Counting of nauplii

3.7 Antimicrobial Activity by Disc Diffusion Method

3.7.1 Principle

The disk diffusion susceptibility method is simple and well-standardized. Bacterial inoculums are applied to the surface of a large agar plate. Antibiotic discs and disc of test materials are placed on the inoculated agar surface. Plates are incubated for 16–24hr at 35°C prior to determination of results. The test materials having antimicrobial property inhibit microbial growth in the media surrounding the discs and thereby yield a clear, distinct area defined as zone of inhibition. The zones of growth inhibition are measured to the nearest millimeter around each of the antibiotic disks. 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 (Barry, 1976).

3.7.2 Apparatus &Reagents

Table 3.6: Apparatus and reagents for antimicrobial test:

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

3.7.3 Test Sample of *Phyllanthus acidus*

n-hexane fraction of methanolic extract of *Phyllanthus acidus* leaves were taken as test sample.

3.7.4 Test Organisms

The bacterial strains used for the experiment were collected as pure cultures from the East West University microbiology laboratory. Both gram positive and gram-negative organisms were taken for the test and they are listed in the following table.

Table 3.7: List of micro-organisms:

Type of Bacteria	Name of Bacteria
Gram positive Bacteria	***Bacillus megaterium***
	Bacillus subtilis
	Bacillus sereus
	Staphylococcus aureus
Gram Negative Bacteria	***Escherichia coli***
	Salmonella paratyphi
	Salmonella typhi
	Vibrio parahaemolyticus
	Shigella dysenteriae
	Pseudomonas aureaus

3.7.5 Procedure

3.7.5.1 Preparation of the Medium

To prepare required volume of this medium, 5.6gm of agar medium was taken in a bottle with a cap and distilled water was added to it to make 200ml volume. The contents were then autoclaved to make a clear solution.

Figure 3.7: Autoclave machine

3.7.5.2 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 glassware were sterilized by autoclaving at a temperature of 121^0C and a pressure of 15-lbs/sq. inch for 20 minutes. Micropipette tips, cotton, forceps, blank discs etc. were also sterilized.

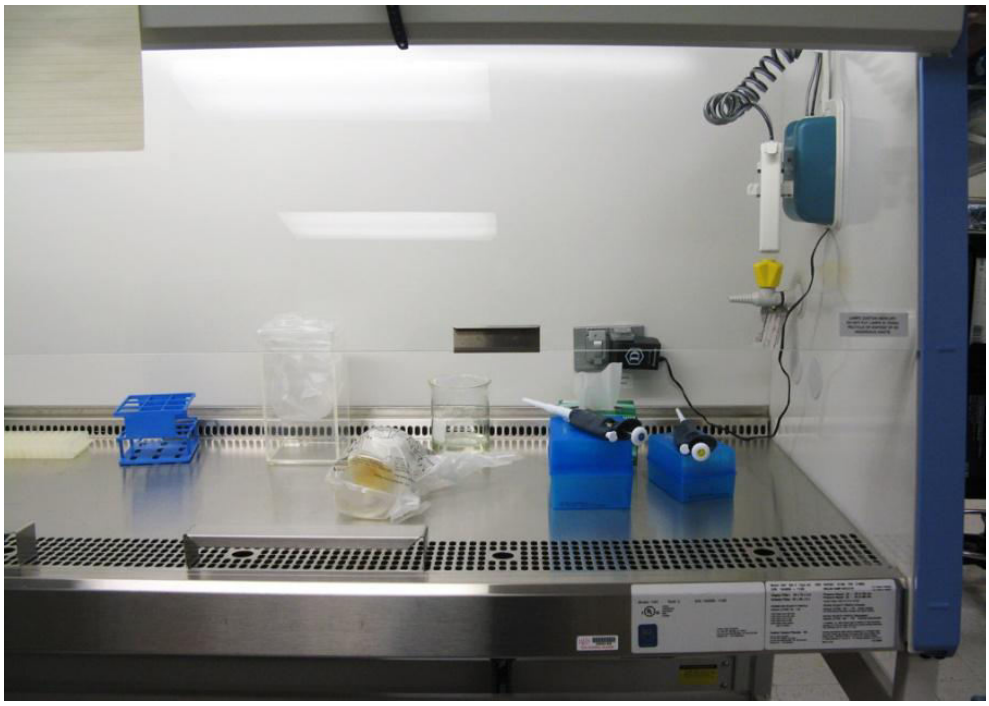


Figure 3.8: Laminar hood

3.7.5.3 Preparation of the Test Plate

The test organisms were transferred from the subculture to petridish containing about 10 ml of melted and sterilized agar medium. The bacterial and fungal suspension was taken by a loop mixed with normal saline with the help of vortex machine. Then a sterilized cotton bud was taken and dipped into the bacterial suspension. Then the bacterial sample is applied to the petridish with the help of this cotton bud.

3.7.5.4 Preparation of Discs

Three types of discs were used for antimicrobial screening.

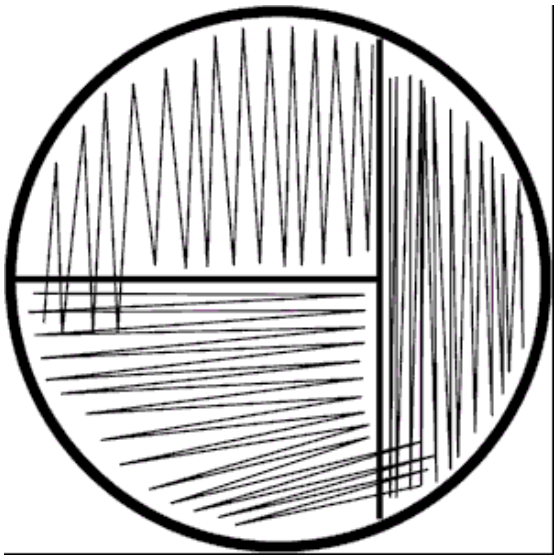


Figure 3.9: Preparation of filter paper discs.

- ✓ 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, azithromycin (30µg/disc) disc was used as the reference.
- ✓ 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.
- ✓ Sample Discs: These discs were soaked with solutions of test samples of known concentration, dried and used to determine the anti-activity of the samples.

3.7.5.5 Preparation of Test Sample

Measured amount of test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. Sterilized metrical filter paper discs were taken in a blank petridish under the laminar hood. Then discs were soaked with solutions of test samples and dried.

3.7.5.6 Application of Test Samples

Standard azithromycin discs 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 produced by the test sample. Methanol discs were used as negative controls which ensure that the residual solvents (left over the discs even after air drying) and the filter paper were not active themselves.

3.7.5.7 Diffusion & 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°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°C for 24 hours.

Figure 3.10: Incubator

3.7.5.8 Determination of Antimicrobial Activity by Measuring the Zone Of Inhibition

The antimicrobial potency of the test agents are 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.

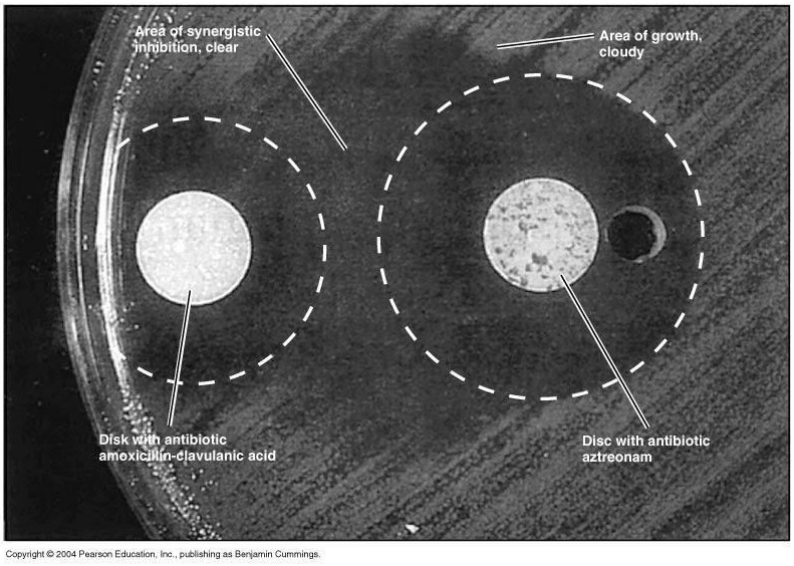


Figure 3.11: Clear zone of inhibition

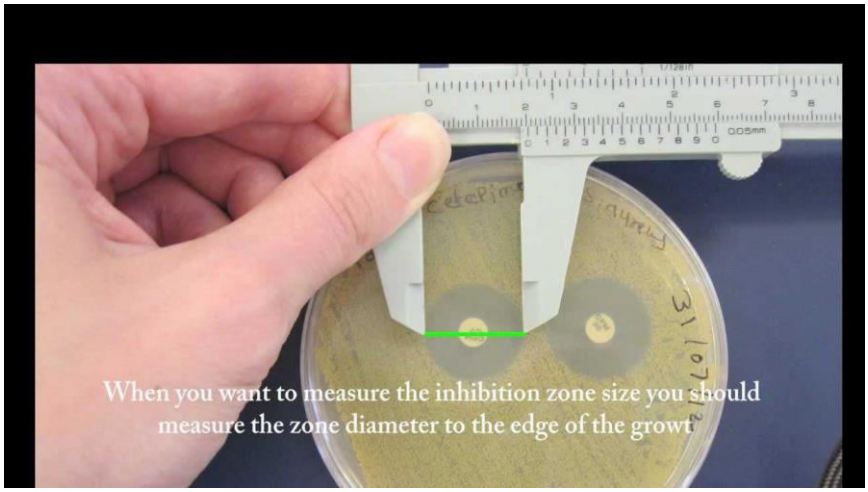


Figure 3.12: Determination of clear zone of inhibition

CHAPTER FOUR:
RESULTS
AND DISCUSSIONS

4.1 Antioxidant test results

Antioxidant tests are classified by various methods. Samples were subjected to various standard methods to determine various scavenging capacity and amount that is equivalent to the standard like ascorbic acids. Antioxidant property of the Ethyl Acetate (EA) fraction of methanolic extract of *Phyllanthus acidus* (leaves) was determined by following methods-

- ✓ Determination of total phenolic content
- ✓ Determination of total flavonoid content

4.1.1. Result of Total Phenolic Content

The Ethyl Acetate extract of leaves of *Phyllanthus acidus* were subjected to determine total phenolic content. Ascorbic acid was used as reference standard (Singleton, Orthofer and Lamuela-Raventós, 1999).

4.1.1.1. Preparation of Standard Curve

Table 4.1: Total phenolic content of ascorbic acid

Concentration (µg/ml)	Absorbance (at 765 nm)	Regression line	R2 value
80	0.942	y = 0.008x + 0.263	0.889
90	1.029		
100	1.105		
110	1.109		
120	1.321		

A linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.1. This linear curve was considered as a standard curve.

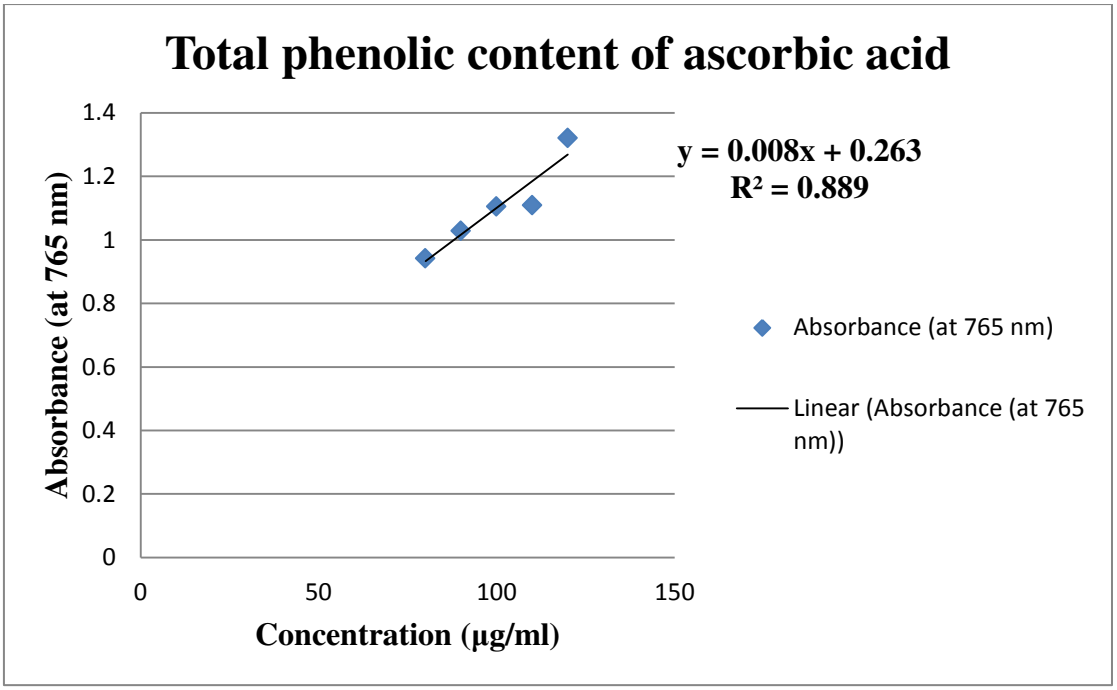


Figure 4.1: Graphical Representation of assay of phenolic content of Ascorbic Acid

4.1.1.2. Total Phenol Content Present in EA Extract of *Phyllanthus acidus*

Based on the absorbance values of the extract solution, reacted with Folin-Ciocalteu reagent and compared with the standard solutions of ascorbic acid equivalents (AAE), the total phenolic content present in the extract is calculated and given in the table below.

Table 4.2: Total Phenolic content in EA fraction of *Phyllanthus acidus* (leaves)

Sample	Concentration (mg/ml)	Absorbance (Y value at 765 nm)	Total Phenolic (X) value (mg of AAE/gm of dried extract)
EA fraction of *Phyllanthus acidus*	2	0.977	89.25

4.1.1.3. Discussion

The absorbance was found to be directly proportional to the concentration. Absorbance increased with the increase in concentration indicating increase in phenolic content. Absorbance of the EA fraction is between the absorbances of standard of 80 µg/ml and 90 µg/ml. Based on the absorbance values of extract solution and using the regression line equation of the standard curve, 89.25 mg of AAE/gm of dried extract of phenolic content was found in the EA fraction of *Phyllanthus acidus* (leaves).

4.1.2. Result of Total Flavonoid Content

The EA fractions of *Phyllanthus acidus* (leaves) were subjected to determine total flavonoid content. Quercetin was used as reference standard.

4.1.2.1. Preparation of Standard Curve

Table 4.3: Total Flavonoid content of Quercetin

Concentration (µg/ml)	Absorbance (at 510 nm)	Regression line	R2 value
4	0.193	y = 0.053x - 0.013	0.999
8	0.422		
12	0.618		
16	0.834		

After absorbances were taken of different solution of Quercetin of concentrations ranging from 4 µg/ml to 16µg/ml, a linear relationship was observed when the absorbance were plotted against concentrations, as shown in Figure 4.2. This linear curve was considered as a standard curve.

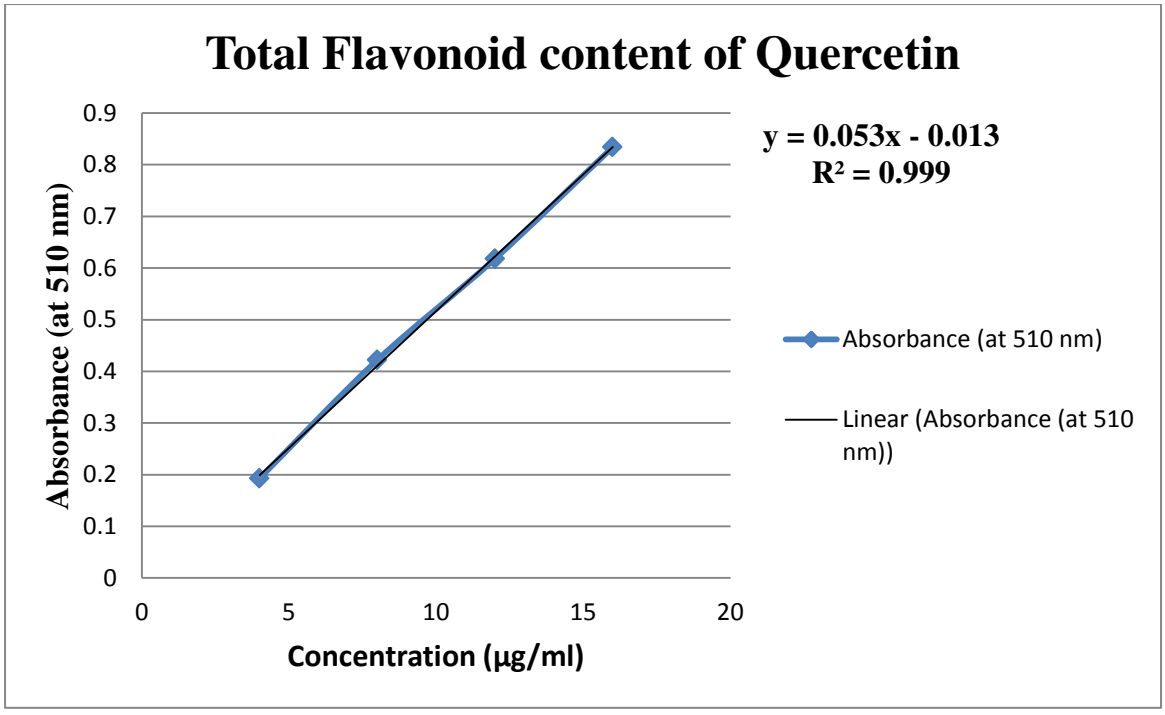


Figure 4.2: Graphical representation of Flavonoid content of quercetin.

4.1.2.2. Total Flavonoid Content Present in EA fraction of *Phyllanthus acidus* (leaves)

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract is calculated and is given in Table 4.4.

Table 4.4: Total Flavonoid content in EA fraction of *Phyllanthus acidus* (leaves)

Sample	Concentration (mg/ml)	Absorbance (Y value at 510 nm)	Total Flavonoid (X) value (mg of quercetin/gm of dried extract)
EA fraction of *Phyllanthus acidus*	1	0.157	3.208

4.1.2.3. Discussion

The absorbance was found to be directly proportional to the concentration. Absorbance increased with the increase in concentration indicating increase in flavonoid content. Absorbance of the EA fraction is less than the absorbance of standard. Based on the absorbance values of extract solution and using the regression line equation of the standard curve, 3.208 mg of Quercetin/gm of dried extract of flavonoid content was found in the EA fraction of Phyllanthus acidus (leaves).

4.2. Result of Antimicrobial Test

The antimicrobial activities of EA fraction of *Phyllanthus acidus* leaves extract were subjected in the study against various Gram positive bacteria, Gram negative bacteria and fungi. The EA fraction was subjected to the various bacterial and fungal cultures and from that zones of inhibition were measured. Ciprofloxacin was used as standard reference.

4.2.1. Zone of Inhibition of Standard and EA Fraction

Table 4.5: Antimicrobial activity of standard sample (Ciprofloxacin) and EA fraction

Type of microorganism		Zone of inhibition (mm)	
		Standard sample	EA fraction
Gram positive bacteria	*Bacillus megaterium*	30	6
	Bacillus subtilis	31	7
	Bacillus sereus	31	5
	Staphylococcus aureus	31	8.5
Gram negative bacteria	*Escherichia coli*	30	7
	Salmonella paratyphi	32	7
	Salmonella typhi	30	0
	Vibrio parahaemolyticus	30	10
	Shigella dysenteriae	33	0
	Pseudomonas aureaus	30	7

4.2.2. Discussion

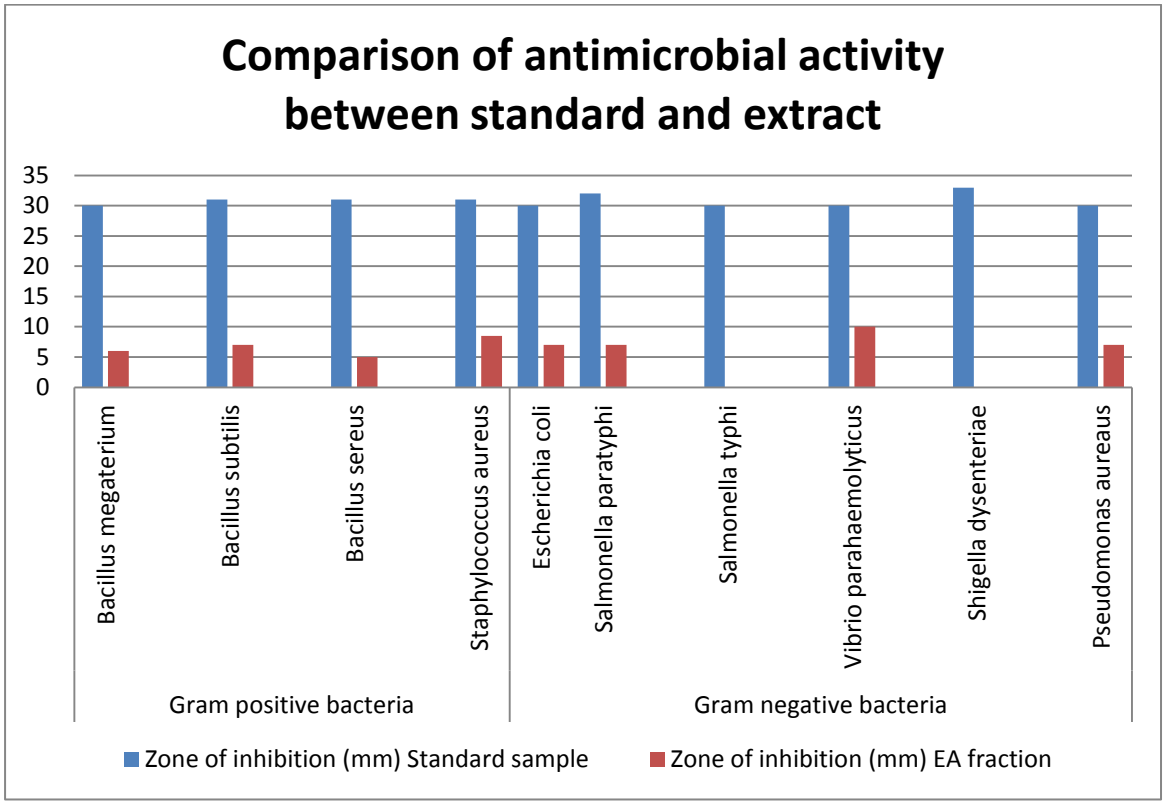


Figure 4.3: Comparison of antimicrobial activity between standard and extract

EA fraction of *Phyllanthus acidus* leaves extract showed low to moderate antimicrobial activity when compared to reference standard drug Ciprofloxacin. None of the zone of inhibition of EA fraction is equal to Ciprofloxacin against any bacteria as shown in the Figure: 4.3. Among all the microbiological cultures, the fraction showed the best antimicrobial activity against *Vibrio parahaemolyticus* (10 mm) comparable to the standard (30 mm).

4.3. Result of Brine Shrimp Lethality Bio-Assay

The EA fraction of the *Phyllanthus acidus* (leaves) extract was subjected to brine shrimp lethality bioassay. After 24 hours, the test tubes were inspected using a magnifying glass and the number of survivors counted. The effectiveness of the concentration and % mortality relationship of plant product was expressed as a Median Lethal Concentration (LC_{50}) value. LC_{50} represents the concentration of the standard and EA 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.

$$\%\ Mortality = \frac{\text{Number of dead nauplii } \times 100}{\text{Total number of nauplii}}$$

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.

4.3.1. Preparation of Curve for Standard

Here, Tamoxifen was used as reference standard.

Table 4.6: Results of the bioassay of Tamoxifen (standard)

Test tube number	Concentration (C) (µg */ml*)	Log C	Number of alive nauplii	Number of dead nauplii	% Mortality	LC_{50} (µg */ml*)
1	400	2.60205999	8	2	20	16.437
2	200	2.30103	9	1	10	
3	100	2	10	0	0	
4	50	1.69897	10	0	0	
5	25	1.39794001	0	10	100	
6	12.5	1.09691001	0	10	100	
7	6.25	0.79588002	10	0	0	
8	3,125	0.49485002	2	8	80	
9	1.5625	0.19382003	0	10	100	
10	0.78125	-0.10720997	2	8	80	

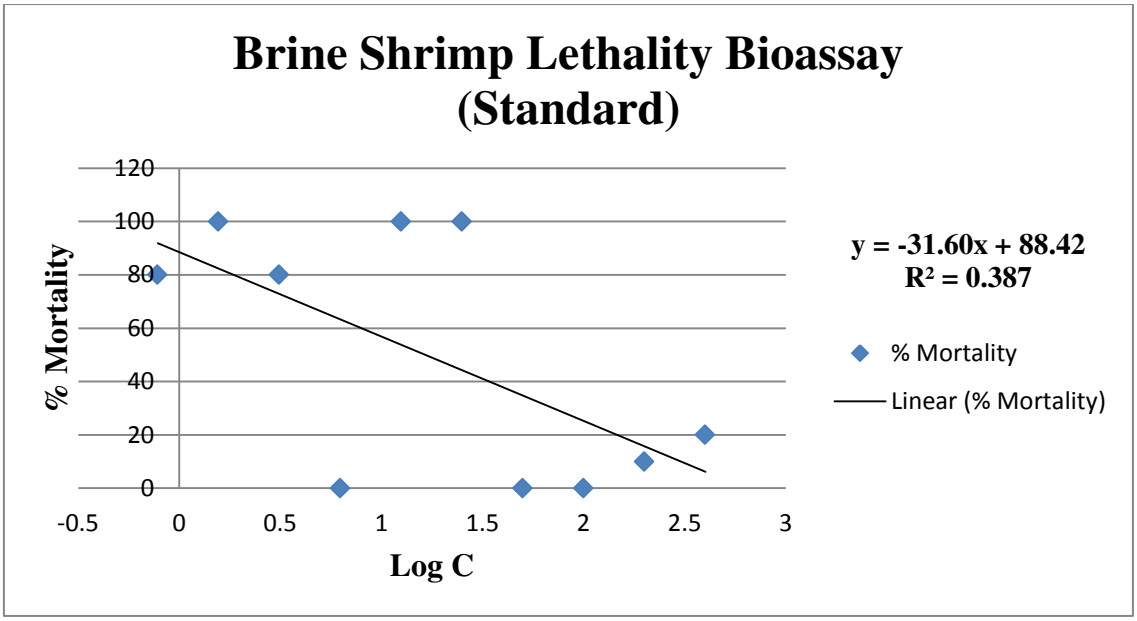


Figure 4.4: % Mortality and Predicted Regression Line of Tamoxifen (standard)

4.3.2. Preparation of EA Fraction Curve of *Phyllanthus acidus* (leaves)

Table 4.7: Results of the bioassay in EA fraction of *Phyllanthus acidus* (leaves)

Test tube number	Concentration (C) (µg */ml*)	Log C	Number of alive nauplii	Number of dead nauplii	% Mortality	LC_{50} (µg */ml*)
1	400	2.60205999	2	8	80	0.262
2	200	2.30103	1	9	90	
3	100	2	0	10	100	
4	50	1.69897	1	9	90	
5	25	1.39794001	1	9	90	
6	12.5	1.09691001	3	7	70	
7	6.25	0.79588002	0	10	100	
8	3,125	0.49485002	1	9	90	
9	1.5625	0.19382003	10	0	0	
10	0.78125	-0.10720997	3	7	70	

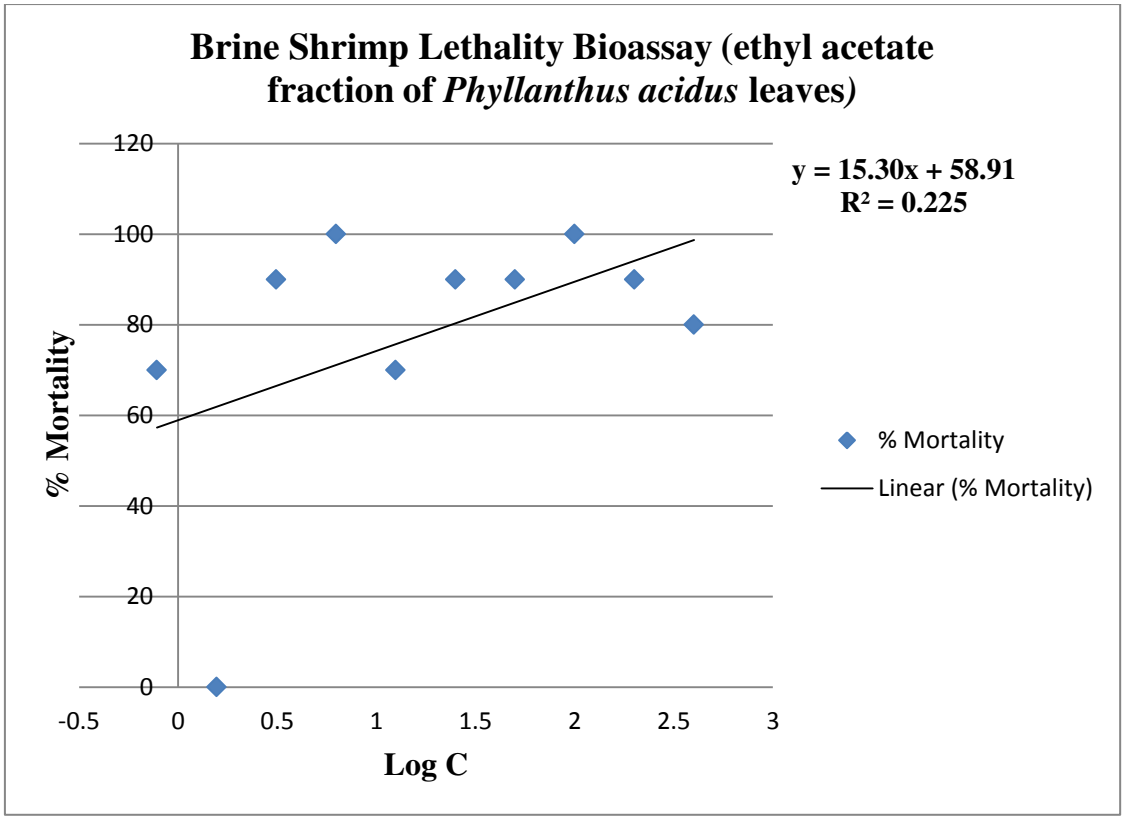


Figure 4.5: % Mortality and Predicted Regression Line in EA fraction of *Phyllanthus acidus* (leaves)

4.3.3 Discussion

In Brine Shrimp Lethality bioassay, varying degree of lethality was observed with exposure to different concentrations of the test samples. The degree of lethality was found to be directly proportional to the concentration. Maximum mortalities took place at the concentration of 400 and 200 µg/ml, whereas the least mortalities at the concentration of 0.78125µg/ml as shown in Table 4.7.

Table 4.8: Cytotoxic activity of Tamoxifen and EA fraction of *Phyllanthus acidus* (leaves).

Sample	Linear regression equation	R2 value	LC50 (µg/ml)
Standard (Tamoxifen)	y = -31.60x + 88.42	0.387	16.437
EA fraction	y = 15.30x + 58.91	0.225	0.262

In this investigation, standard and EA fraction exhibited cytotoxic activities with the LC50 values at 16.437 µg/ml and 0.262 µg/ml respectively as shown in Table 4.8. LC50 value of *Phyllanthus acidus* (leaves) in EA fraction showed more activity of it than Tamoxifen. Further investigation is needed to confirm the activity.

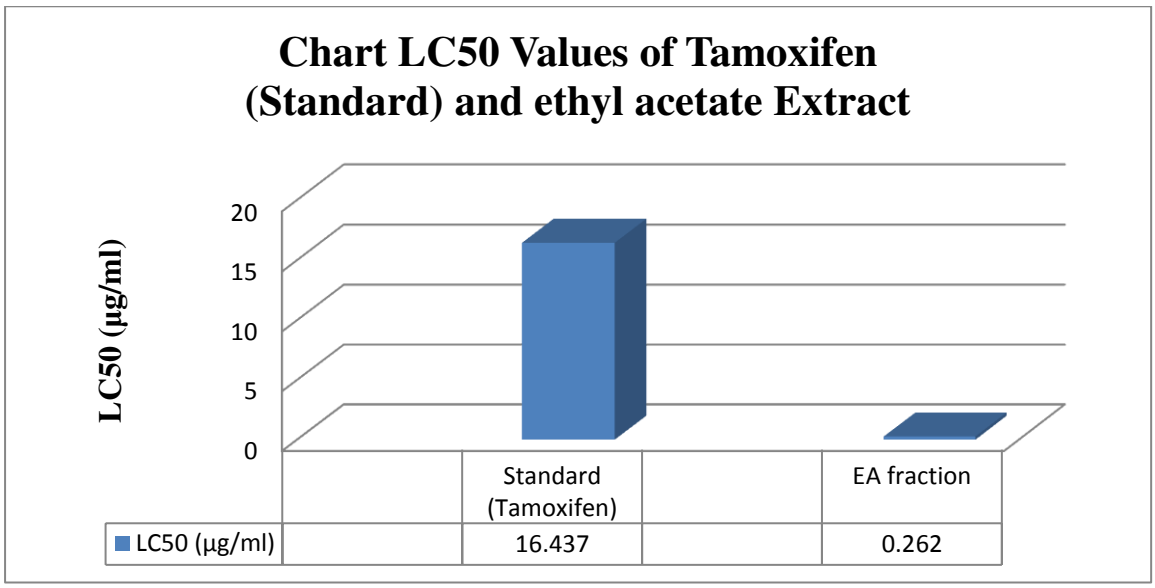


Figure 4.6: Comparison between LC50 values of standard and extract

CHAPTER FIVE: CONCLUSION

5.1 Conclusion

Traditional medicines are mostly utilized by means of the natural products isolated from natural resources such as plant extracts. Pharmacological studies always reveal the potential medicinal properties of plants of our surroundings. Ethno botanical data on the traditional uses of plants encourage the isolation of secondary metabolites leading to new lead compounds. With the increasing demands of inventing new drugs the pharmacological assay of natural plant resources play a non-parallel role in the traditional drug discovery. Day by day the study of traditional medicinal plants is increasing in significant rate with the view to invention and establishment of new therapy line.

As the literature review suggests, the presence of several phytochemical compounds in *Phyllanthus acidus* makes the plant pharmacologically active. The present study showed that it has poor antioxidant activity.

The ethyl acetate extract possesses cytotoxic activity that could be a better treatment in tumor as well as cancer.

The study also showed that, the extract showed low to moderate antimicrobial activity that could be a better treatment in antimicrobial infections. However, studies are required on higher animal model and subsequently on human subjects to prove efficacy as an antioxidant, cytotoxic and antimicrobial agent.

The medicinal values of the leaves of this plant may be related to their phytochemical constituent. So, further investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be tested. It will help in the development of new novel and safe drugs for the treatment of various diseases.

CHAPTER SIX:

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