Phytochemical and Pharmacological Investigation on *Phyllanthus acidus* Leaf

A Dissertation submitted to the Department of Pharmacy, East West University, Bangladesh, in partial fulfillment of the requirements for the Degree of Bachelor of Pharmacy.

Submitted by

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Declaration by the Candidate

I, Maliha Binta Saleh, hereby declare that the dissertation entitled **"Phytochemical and Pharmacological Investigation on *Phyllanthus acidus* Leaf"** submitted by me to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the award of the degree 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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Certification by the Supervisor

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This is to certify that the thesis entitled **"Phytochemical and Pharmacological Investigation on *Phyllanthus acidus* Leaf"** submitted to the Department of Pharmacy, East West University for the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy, was carried out by Maliha Binta Saleh, ID: 2013-3-70-049.

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Dedication

This research paper is dedicated to
my beloved Parents
and my family members

Abstract:

The purpose of the study was to evaluate the cytotoxic, antimicrobial and antioxidant activity of n-hexane fraction of *Phyllanthus acidus* leaves extract.

The powder of *Phyllanthus acidus* were extracted with methanol and then partitioned with petroleum ether, DMSO, ethyl acetate and crude fraction was taken for experiment. The n-hexane fraction was used to evaluate cytotoxic, antimicrobial and antioxidant activities. The cytotoxic activity was measured by brine shrimp lethality bioassay. LC50 value of n-hexane fraction of *Phyllanthus acidus* leaves is 158951614.2 µg/ml in brine shrimp lethality test. The fraction contained 4.5 mg AAE/gm of total phenolic content, 4.868 mg of Quercetin/gm of total flavonoid content. The results of study clearly indicate the presence of very poor cytotoxic and poor antioxidant properties of n-hexane extract. The study also showed low to moderate antimicrobial activity. The obtained results provide a support for the use of this plant in traditional medicine and its further investigation.

Key words: *Phyllanthus acidus*, Brine shrimp lethality bioassay, phenolic content, flavonoid content.

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Abbreviated Form	Meaning
DMSO	Dimethyl Sulfoxide
gm	Gram
Hr	Hour
LC50	Lethal concentration required to kill 50% of the sample population
µg	Micro gram
Ml	Milliliter
Mg	Milligram
µl	Microliter
UV	Ultraviolet
WHO	World Health Organization

Chapter One
INTRODUCTION

1.1 Overview

A plant is any one of the vast number of organisms within the biological kingdom Plantae; in general, these species are considered of limited motility and generally manufacture their own food. They include a host of familiar organisms including trees, forbs, shrubs, grasses, vines, ferns, and mosses. Conventionally the term plant implies a taxon with characteristics of multicellularity, cell structure with walls containing cellulose, and organisms capable of photosynthesis. Modern classification schemes are driven by somewhat rigid categorizations inherent in DNA and common ancestry. (Hogan, 2017)

Figure 1.1: Dusky Rock Moss (*Andreaea rothii*)

1.2 Taxonomy and Terminology

Throughout most of the history of science from Aristotle to Linnaeus and into the 20th century, species were divided into two kingdoms: animals and plants. Driven by DNA characterizations and other modern analysis, fungi and bacteria have now been removed to separate kingdoms; in particular, fungi have cell walls that contain chitin rather than cellulose. Lichens, which are a symbiotic association of a fungal and photosynthetic organism, are generally not considered plants in the purest sense of taxonomy, although earlier classification schemes viewed them as plants. Viruses are also not considered to be plants, since they do not have a cell of their own, but inhabit

a host cell of another organism; moreover, in many classifications they are not considered a living organism at all. Myxomycetes, or slime molds, are also not considered plants, but rather are heterotrophs that can ingest bacteria, fungal spores, and other items.

The scientific study of plants, known as botany, has identified about 350,000 extant taxa of plants, defined as seed plants, bryophytes, ferns and fern allies. As of 2008, approximately 400,000 plant species have been described, of which roughly ninety percent are flowering plants.

Vascular plants have lignified tissue and specialized structures termed xylem and phloem, which transport water, minerals, and nutrients upward from the roots and return sugars and other photosynthetic products. Vascular plants include ferns, club mosses, flowering plants, conifers and other gymnosperms. A scientific name for this vascular group is Tracheophyta. (Hogan, 2017)

1.3 The Major Divisions of Plantae

- Anthocerotophyta (hornworts: nonvascular plants with one chloroplast per thallus cell)
- Bryophyta (mosses: nonvascular plants with wiry stems that reproduce by spores)
- Cycadophyta (cycads: nonflowering vascular plants with large pinnately compound leaves)
- Ginkgophyta (gymnosperm with one extant tree species, Ginkgo biloba)
- Gnetophyta (woody plants having some angiosperm and some gymnosperm features)
- Lycopodiophyta (vascular fern allies without seeds or flowers, having single microphyll leaf veins)
- Magnoliophyta (flowering plants that have vascular systems and are seed producing)
- Marchantiophyta (liverworts: nonvascular plants with one-celled rhizoids)
- Pinophyta (gymnosperm conifers that have vascular systems and cones, but no flowers)
- Pteridophyta (ferns: vascular plants lacking flowers and seeds, reproducing by spores)

Several groups of algae are under debate as to whether they should be included in Plantae; however, we will follow a definition of plants that excludes algae. Green plants, often termed Viridiplantae, derive the majority of their energy from sunlight via photosynthesis and are a subset of Plantae. (Hogan, 2017)

1.4 Morphology

Plant morphology involves the study of organism structures, including reproductive structures, and also addresses the pattern of development of these structures as the plant matures. For vascular plants the principal structures involved are roots, stems, and leaves; for flowering plants the development of floral structures and seeds is of great importance in plant identification. When structures in different species are thought to result from common, inherited genetic pathways, those structures are termed homologous. For example, cacti spines share the same fundamental structure and development as leaves of other vascular plants, thus cactus spines are homologous to leaves. Plant morphology observes both the vegetative structures of plants and reproductive structures. The vegetative structures of vascular plants includes the study of the shoot system, composed of stems and leaves, as well as the subsurface or root system. The reproductive structures are more varied, and are usually specific to a particular group of plants, such as flowers and seeds for flowering plants, sori for ferns, and capsules for mosses. Analysis of plant reproductive structures has led to the discovery of the alternation of generations present in most plants (as well as algae). This area of plant morphology overlaps with the study of biodiversity and plant systematics.

Plant structure manifests at a range of geometric scales. For the genetic level, intricate microbiology analysis of DNA and RNA structure is required. At the cellular level, optical microscopy must be used. At the macroscopic scale, the visually observable architecture of a plant's structure is under scrutiny. Plant morphology also addresses the pattern of development: the process by which structures originate and mature as a plant grows. While animals produce all the body parts they will ever have from early in their life, plants periodically produce new tissues and structures throughout their life cycles. A living plant continues to have embryonic tissues even in advanced stages of development. The way in which new structures mature as they are produced may be affected by the point in time when the plant begins to develop, as well as by the habitat. (Hogan, 2017)

1.5 Metabolism and Growth

Plant growth is governed by environmental and ecological factors. Chief environmental factors include meteorological parameters such as temperature, precipitation, wind velocity, and available sunlight, and edaphic factors such as soil nutrients, soil moisture, soil granularity and compaction, as well as topographic factors. Ecological factors include competition for water, nutrient, and light resources from other members of the plant community, as well as herbivory and trampling factors. In addition, the presence of plant diseases plays a role in the successful growth and propagation of plant species. In the last millennium, the role of humans has become a major factor in habitat destruction and fragmentation, and there is evidence of the imprint of humans on selective cultivation of species.

The majority of biomass created by a plant is typically derived from the atmosphere. Through a process known as photosynthesis, most plants use the energy in sunlight to convert carbon dioxide from the atmosphere, plus water, into simple sugars, which are used as building blocks and form the main structural components. Chlorophyll, a molecule that lends a green appearance, is typically present in plant leaves as well as and often in other plant parts to absorb sunlight to power the photosynthetic process. Parasitic plants, conversely, derive nutrient resources from a host. Carnivorous plants actually capture small animal prey to gain many essential nutrients. Plants typically depend on soil for architectural support and water uptake, but also obtain nutrients such as nitrogen and phosphorus from soil. Epiphytic and lithophytic plants often depend on rainwater or other sources for nutrients. Some specialized vascular plants, such as mangroves, can grow with their roots in anoxic conditions. (Hogan, 2017)

1.6 Ecology

Plants constitute most of the primary production of the Earth's ecosystems; that is, they produce the bulk of the biomass from light, carbon dioxide, and basic nutrients. The cornerstone of this primary productivity is photosynthesis, which has radically altered the composition of early Earth's atmosphere, resulting in air that is 21% oxygen. Animals rely on oxygen as well as food sources for herbivores; plants also provide shelter and nesting locations for many species.

Land plants are key components of the water cycle and several other biogeochemical cycles. Some plants have coevolved with nitrogen- fixing bacteria, making plants an important part of the nitrogen cycle. Plant roots play an essential role in soil development and prevention of soil erosion.

The majority of plants have fungi associated with their root systems in a kind of mutualistic symbiosis known as mycorrhiza; an important function of this type of symbiosis is the enhancement of phosphorus uptake. The fungi help the plants gain water and mineral nutrients from the soil, while the plant gives the fungi carbohydrates manufactured in photosynthesis. Some plants serve as homes for endophytic fungi that protect the plant from herbivores by producing toxins. In fact, most plants contain a variety of endophytic micro-organisms, each of which produces a unique set of chemicals that can be useful to the host plant.

Various forms of parasitism are also fairly common among plants, from the semi-parasitic mistletoe that merely extracts nutrients from its host, but also has photosynthetic capability, to the fully parasitic toothwort that acquire all their nutrients through conduits to the roots of other plants. (Hogan, 2017)

1.7 Plant Associations

In a given ecosystem there is typically a well-defined plant association, which commonly is characterized by a canopy layer, an intermediate (or shrub) layer, and an understory or forest floor layer. In the case of grasslands, tundras, and certain other treeless habitats, the upper one or two layers may be absent, although in those cases there are often material differences in the grassland plant height layering. A given plant association will, of course, be dependent on certain soil types, meteorology, and mixture of fauna; moreover, the plant association may manifest marked seasonal differences in temperate and boreal settings, although this appearance will simply conceal certain plants that are dormant or leafless in a given season. Characterization of a plant association is helpful to botanists as a guide to plant identification and other ecological research; furthermore, understanding of a plant association is critical to studies of plant succession, where environmental changes in a given landscape lead to a series of plant communities, before a stable equilibrium is attained. (Hogan, 2017)

1.8 Interactions with Humans

Most of the human diet is plant derived; in addition, a large fraction of raw materials for shelter, clothing and other life necessities of Homo sapiens is obtained from plant products. Furthermore, countless medicinal extracts have been produced from plants. Tree rings are a method of dating in archaeology and serve as a record of past climates. Basic biological research has often been done with plants, such as use of pollen core records to study the distant past or the pea plants used to derive Mendel's laws of genetics. The field of ethnobotany studies plant use by indigenous cultures, which helps to conserve endangered species as well as discover new medicinal herbs. Gardening is the top leisure activity in many world regions.

Plants have served as a source of interest to humans for millennia beyond their use as food. Gardening for ornamental purposes and use of cut flowers for decoration have been noted at least as early as the Bronze Age by Egyptian, Cretan, and Celtic cultures, for example. Early scientists such as the Greeks spent considerable effort engaging in describing and characterizing morphology of various species. Plants have been an important element of human art, with elements of plant architecture appearing as ornamentation for ceramics and other decoration in Neolithic and Bronze ages in China, Crete, Southern Africa, British Isles, Egypt, and in the Mayan civilizations. As an example, glyphs found in Middle Minoan pottery as early as 1850 BC contain designs of olive sprig, saffron, wheat, and silphium. In the history of art, plants played an important role as subjects in classical still-life paintings, and may have reached a crescendo with obsessions by 18th- and 19th-century European printmakers in creating myriads of botanical prints.

Specific to gardening, plants have been used throughout history not only as adornment for indoor and outdoor spaces of human habitation, but also to modify microclimates for more comfortable habitation. For example, treelines and shrub borders have been used, particularly in the last millennium in Europe to provide windscreens for livestock and separation of pastures to secure livestock ownership. Landscaping has also been used for centuries as a method of microclimate amelioration for human habitation, including wind protection, thermal buffering, and atmospheric humidity modification. (Hogan, 2017)

1.9 The Value of Plants in Our Lives

Plants are fundamental to life. For millennia the plants, animals, rocks, and trees were the only pharmaceutical giants we had. Like all living things on Earth, every one of us is still a shareholder in Nature – the greatest pharmacy on Earth.

Plants are the most formidable chemists. They are constantly producing an arsenal of chemical compounds, in order to respond to different challenges and threats in their environment. They materialise chemical compounds that make them impervious to particular climatic conditions, certain microorganisms, bacteria, viruses, insects, numerous animals, including us.

We humans are still learning and re-learning how to harness the self healing ability of plants, in order to enhance or rebalance the health of our own body, mind and spirit.

It is this ever expanding and evolving field of knowledge that inspires research and re-education. Throughout human history we have learned a lot from plants and we have continually endeavoured to pass this knowledge on to the next generation. (Vilinac, 2017)

1.9.1 A wide range of uses

The art of truly relating to the plants is to choose the ones that you feel an affinity with. If you have the ability to grow them for yourself – in a garden or in a pot – this is of enormous value. Growing your own plants and medicinal herbs, endeavouring to learn more about them, puts you in touch with Nature in a very deep way. We have never in the history of human kind had so much information about medicinal nature of plants on our fingertips.

The mystery of their magic still stands, but what was magic and mystery to our ancestors is a science to us today. The more we learn about plants the more we find ways to use them to support health.

At a very basic level we can use them as a condiment or seasoning in food, enhance all those otherwise ‘dull’ dishes by the fragrance and flavour only herbs can provide.

The vibrant natural toiletries and cosmetics industry thrives on the power of plants to impart their healing, nourishing, soothing, invigorating, relaxing and other effects onto our skin and hair. (Vilinac, 2017)

1.9.2 Traditional use

All cultures have a history of herbal medicine use, usually making use of the plants found closest to home. Even today in the times of advanced technology and medical science still depend on plants for their healing.

Western culture, however, is predominantly excited by the new and upcoming and the novel – and perhaps most importantly the patentable. This means that the good, tried and tested tools of survival become relegated to historical anecdote.

But herbal medicines – the original human health care products – are still fully present and available to our lives if you look out for them.

Common herbs and spices – including ginger, turmeric and garlic, and cinnamon and rosemary as well as fenugreek seeds and leaves, artichoke leaf extract, yarrow, and holy basil all may help lower cholesterol. For lowering blood pressure, herbs and spices including cloves, ground Jamaican allspice, cinnamon, sage, marjoram, tarragon, and rosemary are beneficial. Thyme tincture can outperform conventional acne treatments.

One has to observe that the western culture is predominantly excited by the new and upcoming and often relegates the good, tried and tested tools of survival to a historical anecdotes.

Until the beginning of 1900s medicinal plants from all over the world were fully monographed in all pharmacopoeias as legitimate medicinal ingredients. They are now presented in relatively small numbers but that is slowly changing as we rediscover the true medicinal value of plants. European laws continue to restrict not only what can be sold, but what can be said about traditional herbal remedies insisting on the randomized trial being the only source of legitimate information.

It is good, then, to see some scientists acknowledging that ancient research is research and that traditional use, or 'herbal lore' – as often passed down orally as in written form – can also help us understand the uses and relevance of herbs in our lives. (Vilinac, 2017)

1.9.3 Plants are Food

Either indirectly or directly, human nutrition is dependent on plants. Throughout the history of human beings, about seven thousand various species of plants have been used as food for humans. To a large extent, human nutrition depends on corn or maize, rice, wheat and other cereals.

Other crop staples include legumes, cassava and potato. Human food also includes edible flowers, herbs, nuts, certain fruits, spices and vegetables. From plants, beverages produced include alcohol, beer, wine, tea and coffee.

We obtain sugar from sugar beet and sugar cane and from flowers comes honey. From olives, sunflowers, safflowers, rapeseed and soybean comes margarine and cooking oils. Additives in food include pectin, starch, locust bean gum, guar gum and gum Arabic.

Animals of livestock which are all herbivores include camels, goats, sheep, pigs and cows and most manly feed on grasses and cereal plants. Brewer's yeast and other fungi provide human beings with numerous beverages and foods including staples like beer and bread.

Brewer's yeast is not only important to producing great food but it is especially rich in vitamin B12 and is quite nutritious. In the maturation of cheese, some molds are important such as the kind you see in blue cheese. A great source of dietary fiber is found in edible mushrooms, which also happen to be a complete protein. Plus, some mushrooms used as food are medicinal, and provide a smattering of benefits to health. Mushrooms also make great ingredients in nutritious dishes that heal. (Jenniferc, 2014)

1.9.4 Plants Provide Air and Regulate the Water Cycle

Plants brings us oxygen which is a by-product of photosynthesis. Carbon is also stored by plants and they help in keeping a lot of the produced carbon dioxide from burning fossil fuels out into

the earth's atmosphere. Also, the water cycle is regulated by plants. The help in purifying and distributing the water of the planet. They also help in moving water from the soil to the atmosphere through a method called 'transpiration.' (Jenniferc, 2014)

1.9.5 Plants Are Important to Science

Plants and fungi in particular have industrial applications and many model organisms enable comprehension of fundamental biology such as development and genetics. Entrepreneurs apply plants such as fungi to provide biodegradable and sustainable products that are structural such as vehicle bumpers, packing materials and building materials. Enzymes produced by plants and fungi are valuable in the industry of paper pulp, for fashion and even bioremediation. Denim jeans are softened by enzymes from plants.

In the world of science, brewer's yeast and the mold Neurospora crassa are organism models used globally in applied and basic science laboratories. In the year 1996, the first eukaryole to have a sequenced genome was saccharomyces cerevisiae. Industrial synthesis basic chemicals include a fast array of organic chemicals that you can get from plants. These chemicals are utilized in vast varieties of experiments and studies.

The rings of trees serve as a record of climates from the past and are an important dating method in archaeology. Often, basic biological research has been done with plants such as the peas used for deriving the laws of genetics by Gregor Mendel. Space colonies or space stations may one day depend on plants for the support of life.

The ethnobotany field studies the use of plants by indigenous cultures which help in discovering new medicinal plants and in conserving species that are endangered. In the US, gardening is the most popular activity of leisure. Horticulture therapy or working with plants is beneficial for rehabilitating persons with disabilities.

Certain plants contain chemicals psychotropics which are ingested after extraction including opium, cannabis and tobacco. It is also from plants that we get pesticides such as pyrethrin, strychnine, rotenone and nicotine. Poisonous substances are also derived from plants including curare, hemlock and ricin. (Jenniferc, 2014)

1.9.6 Aesthetic Uses

For aesthetic purposes, thousands of species of plants are cultivated. In addition, plants can help prevent soil erosion, provide privacy, abate noise, reduce wind, modify temperatures and provide shades. Often, people cut dried flowers to frame, and display house plants in greenhouses or indoors.

In gardens, bedding plants, herbaceous perennials, vines, shrubs, ornamental trees, shade trees, lawn grasses and outdoor gardens are planted. Often, in textiles, photography, language, humor, architecture and art, images of plants are used often.

These are also used on coats of arms, flags, stamps and money. There are art forms made of living plants as well including espalier, ikebana, bonsai and topiary. The course of history has sometimes been changed by plants such as the tulipomania.

Each year, plants are the reason for the existence of a multi-billion dollar per year industry of tourism which includes traveling to forests, rainforests, tulip festivals, national parks, historic gardens, botanical gardens and arboretums. In the National Cherry Blossom Festival, there are forests filled with colorful leaves of autumn. Plants that are sold as novelties include the resurrection plant, sensitive plant and the Venus flytrap. (Jenniferc, 2014)

1.9.7 Plants Are Used for Natural Products

It is from plants that you get natural products that include cork, amber, alkaloids, resins, gums, latex, tannins, waxes, pigments, natural dyes, essential oils and fibers. Other products also include hemp rope, chewing gum, inks, plastics, linoleum, lubricants, varnish, rubber, turpentine, cosmetics, perfumes, shampoos, paints and soaps.

Cotton is made from cellulose-derived synthetic fibers like acetate and rayon as well as rame, flax and cotton. From plants, renewable fuel comes as well including biofuels like peat and firewood. From plants, you can derive fossil fuel like petroleum and coal.

Aside from its other myriad uses, the backbone of all habitats is also made up from plants. Other species of wildlife and fish also depend on pants for shelter and food. Wood is used for sports equipment, musical instruments, cardboard, paper, furniture and buildings. (Jenniferc, 2014)

1.9.8 Medicine

One fourth of the drugs that are prescribed is derivatives of or come directly from plants. In addition, 4 out of 5 people around the globe at the moment rely on plants for primary healthcare. Medicines derived from plants include vincristine, digitalis, colchicine, reserpine, quinine, morphine, taxol and aspirin. There are also herbal supplements by the hundreds such as Saint John's wort, feverfew, Echinacea and ginkgo.

Plants are also important in the search for cancer drugs. Current therapeutics of cancer include paclitaxel, isolated from the Chinese happy tree camptothecin. It is also derived from the South African willow and the Himalayas and eastern US etoposide. There has been a long history for the search of anti-cancer drugs from plants and other natural sources.

Promising sources of drugs are found in algae, sea squirts and sea sponges, which are all drug sources undergoing studies at the moment. Plants are the anchor structures of these organism's ecosystems. When there is rapid loss of plant life, the consequences are far-reaching and the losses will affect future cancer discovery of drugs adversely.

Also, extraordinarily powerful medicines are provided by fungi which have revolutionized massive economic worth (like cholesterol medicine, immuno-suppressants and antibiotics) and human health.

The cephalosporins, statins, cyclosporines and penicillin drugs are all based on fungi-produced natural chemicals. In TCM or Traditional Chinese Medicine, mushrooms are also important ingredients and myriad activities that are therapeutic such as anti-tumor, anti-viral and anti-inflammatory effects have been attributed to them. Plants can be a part of natural approaches to chronic conditions like obesity, heart disease and cancer. (Jenniferc, 2014)

1.9.9 Plants Relieve Stress

To our living spaces, plants bring natural beauty. By creating balance and texture, a patio or room can be transformed instantly into a welcoming, comfortable environment. Just like beauty pageant queens, however, plants can do so much more than just look beautiful. They clean the air we breathe by acting as oxygen factories and absorbing toxins.

They are also proven for reducing stress. Generally, plants simply make people feel better. By improving air quality and easing mental fatigue, plants manage to find themselves an indispensable part of life at home. Plants reduce stress-related muscle tension, lower blood pressure and calm the heart rate.

Plants also help us focus and relax, leading to increased capabilities of solving problems, idea generation, creativity and increased productivity. Plants also help surgery-recovering patients and ease Alzheimer's symptoms. As a matter of fact, the existence of plants is so significant that they have even been shown to relieve the experienced symptoms of children with ADD.

At this point it is clear that our ancestors were on to something and that plants are so much more than just ornamental. We are deeply connected to plants on some level that just living with them in our home brings us repose and health. (Jenniferc, 2014)

1.10 History of Plants

Over 3,000 million years ago, the first living-organism which resembled a plant appeared. It was a blue-green algae which lived in the sea and can still be found in the water today. When the plants made their first appearance on Planet Earth the atmosphere was unlivable for all oxygen breathing creatures. The air was made out of carbon dioxide, a gas which to us is deadly. Then photosynthetic plants came along and slowly over several million years, cleaned the atmosphere and filled it with oxygen. (Odec, 2017)

1.10.1 Discovery of Photosynthesis

In 1649, Jan Baptista Van Helmont did the first biological experiment in which the ingredients were measured accurately and all changes noted precisely. Van Helmont began by transplanting the shoot of a young willow tree into a large bucket of soil. He weighed the willow and then the soil separately. If the willow tree formed its tissues by absorbing the nutrients from the soil then the soil should lose weight as the plant grew. Van Helmont carefully kept the soil covered so that absolutely nothing could interfere with his experiment.

Naturally, Van Helmont had to water the willow tree or else it wouldn't grow. He concluded that the water he was adding helped carry the nutrients to the tree and then simply evaporated into the air.

For five years, Van Helmont waited patiently, watching the tree grow until finally he removed it from the pot, shook off all the soil and and weighed the plant. In five years the willow tree had added 164 pounds to its original weight. Then, for the second part of the experiment, Van Helmont dried and weighed the soil.

From this, Van Helmont concluded that the willow tree drew its nutrients, not from the soil but from water. Accidentally, he made a mistake and said that the material that made up the bark, wood, roots and leaves came from the water he had added over the five years.

The next big important step in the understanding of photosynthesis came in the early 1770's. Joseph Preistly, the British man who received the recognition of discovering oxygen, found that a piece from a mint plants could restore the air in a container with a burning candle, so that it could be used again. Accidentally, one day, Joseph Preistly placed the candle in a dark corner of his laboratory. Since the mint plant could not photosynthesize, the candle's flame extinguished. Unfortunately, Mr. Preistly never did really understand that great role which light played in his experiment.

Several years later, in 1979, a Dutch physician, Jan Ingenhousz, wanted to find out whether flowers really did help cure illnesses. After many different tests, he finally concluded that only the green

parts of plants cleaned the air and only when placed in strong light. Flowers and other non-green parts of plants used up oxygen just like animals.

In 1796, Ingenhousz suggested that this process of photosynthesis causes carbon dioxide to split into carbon and oxygen. Then the oxygen is released as a gas.

Later, other scientists discovered that sugars contain carbon, hydrogen and oxygen atoms in a ratio of one carbon molecule per molecule of water (CH2O). This is where the word carbohydrate comes from, *carbo*- for "carbon" and *hydrate* for "water". Carbohydrates are a family of chemical compounds including sugars and starches, which are made up of large numbers of sugar units linked together.

In 1804, the Swiss scientists, Nicholas Theodore de Saussure repeated Van Helmont's experiment but carefully measured the amounts of carbon dioxide and water that were given to the plant. He showed that the carbon in the plants came from carbon dioxide and the hydrogen from water. Then, forty years later, a Garman scientist, Julius Mayer, showed that the energy of sunlight is captured in photosynthesis. (Odec, 2017)

1.11 Medicinal Plants

A medicinal plant is a plant that has similar properties as conventional pharmaceutical drugs. Humans have used them throughout history to either cure or lessen symptoms from an illness. A pharmaceutical drug is a drug that is produced in a laboratory to cure or help an illness. Typically, pharmaceutical drugs are modeled after compounds found in medicinal plants. (Peterson, 2017)

1.11.1 Historical review of medicinal plants usage

Healing with medicinal plants is as old as mankind itself. The connection between man and his search for drugs in nature dates from the far past, of which there is ample evidence from various sources: written documents, preserved monuments, and even original plant medicines. Awareness of medicinal plants usage is a result of the many years of struggles against illnesses due to which man learned to pursue drugs in barks, seeds, fruit bodies, and other parts of the plants. Contemporary science has acknowledged their active action, and it has included in modern

pharmacotherapy a range of drugs of plant origin, known by ancient civilizations and used throughout the millennia. The knowledge of the development of ideas related to the usage of medicinal plants as well as the evolution of awareness has increased the ability of pharmacists and physicians to respond to the challenges that have emerged with the spreading of professional services in facilitation of man's life.

Ever since ancient times, in search for rescue for their disease, the people looked for drugs in nature. The beginnings of the medicinal plants' use were instinctive, as is the case with animals. In view of the fact that at the time there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. In time, the reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered; thus, the medicinal plants' usage gradually abandoned the empiric framework and became founded on explicatory facts. Until the advent of iatrochemistry in 16th century, plants had been the source of treatment and prophylaxis. Nonetheless, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again. (Petrovska, 2012)

1.11.2 Historical Sources Relevant for Study of Medicinal Plants Use

The oldest written evidence of medicinal plants' usage for preparation of drugs has been found on a Sumerian clay slab from Nagpur, approximately 5000 years old. It comprised 12 recipes for drug preparation referring to over 250 various plants, some of them alkaloid such as poppy, henbane, and mandrake.

In 2500 BC

The Chinese book on roots and grasses "Pen T'Sao," written by Emperor Shen Nung, treats 365 drugs (dried parts of medicinal plants), many of which are used even nowadays such as the following: *Rhei rhisoma*, camphor, *Theae folium, Podophyllum*, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra.

The Indian holy books Vedas mention treatment with plants, which are abundant in that country. Numerous spice plants used even today originate from India: nutmeg, pepper, clove, etc.

In 1550 BC

The Ebers Papyrus, written circa 1550 BC, represents a collection of 800 proscriptions referring to 700 plant species and drugs used for therapy such as pomegranate, castor oil plant, aloe, senna, garlic, onion, fig, willow, coriander, juniper, common centaury, etc.

According to data from the Bible and the holy Jewish book the Talmud, during various rituals accompanying a treatment, aromatic plants were utilized such as myrtle and incense.

In 800 BC

In Homer's epics The Iliad and The Odysseys, created circa 800 BC, 63 plant species from the Minoan, Mycenaean, and Egyptian Assyrian pharmacotherapy were referred to. Some of them were given the names after mythological characters from these epics; for instance, Elecampane (*Inula helenium* L. Asteraceae) was named in honor of Elena, who was the centre of the Trojan War. As regards the plants from the genus *Artemisia*, which were believed to restore strength and protect health, their name was derived from the Greek word *artemis*, meaning “healthy.”

In 500 BC

Herodotus referred to castor oil plant, Orpheus to the fragrant hellebore and garlic, and Pythagoras to the sea onion (*Scilla maritima*), mustard, and cabbage.

In 459-370 BC

The works of Hippocrates contain 300 medicinal plants classified by physiological action: Wormwood and common centaury (*Centaurium umbellatum Gilib*) were applied against fever; garlic against intestine parasites; opium, henbane, deadly nightshade, and mandrake were used as narcotics; fragrant hellebore and haselwort as emetics; sea onion, celery, parsley, asparagus, and garlic as diuretics; oak and pomegranate as adstringents.

In 371-287 BC

Theophrast founded botanical science with his books “De Causis Plantarium”— Plant Etiology and “De Historia Plantarium”—Plant History. In the books, he generated a classification of more than 500 medicinal plants known at the time. Among others, he referred to cinnamon, iris rhizome, false hellebore, mint, pomegranate, cardamom, fragrant hellebore, monkshood, and so forth. In the description of the plant toxic action, Theophrast underscored the important feature for humans to become accustomed to them by a gradual increase of the doses. Owing to his consideration of the said topics, he gained the epithet of “the father of botany,” given that he has great merits for the classification and description of medicinal plants.

In 25 BC-50 AD

In his work “*De re medica*” the renowned medical writer Celsus quoted approximately 250 medicinal plants such as aloe, henbane, flax, poppy, pepper, cinnamon, the star gentian, cardamom, false hellebore, etc.

In 77 AD

In ancient history, the most prominent writer on plant drugs was Dioscorides, “the father of pharmacognosy,” who, as a military physician and pharmacognosist of Nero's Army, studied medicinal plants wherever he travelled with the Roman Army. Circa 77 AD he wrote the work “De Materia Medica.” This classical work of ancient history, translated many times, offers plenty of data on the medicinal plants constituting the basic *materia medica* until the late Middle Ages and the Renaissance. Of the total of 944 drugs described, 657 are of plant origin, with descriptions of the outward appearance, locality, mode of collection, making of the medicinal preparations, and their therapeutic effect. In addition to the plant description, the names in other languages coupled with the localities where they occur or are grown are provided. The plants having mild effect are dominant, but there are also references to those containing alkaloid or other matter with strong effect (fragrant hellebore, false hellebore, poppy, buttercup, jimson weed, henbane, deadly nightshade). Dioscorides’ most appreciated domestic plants are as follows: willow, camomile, garlic, onion, marsh mallow, ivy, nettle, sage, common centaury, coriander, parsley, sea onion,

and false hellebore). Camomile (*Matricaria recucita* L.), known under the name Chamaemelon, is used as an antiphlogistic to cure wounds, stings, burns, and ulcers, then for cleansing and rinsing the eyes, ears, nose, and mouth. Owing to its mild carminative action, it is particularly appropriate for usage with children. Dioscorides deemed that it had abortive action, on which he wrote, "The flower, root, and the entire plant accelerate menstruation, the release of the embryo, and the discharge of urine and stone, provided that they are used in the form of an infusion and baths." This untrue belief was later embraced by both the Romans and the Arabs; hence the Latin name *Matricaria*, derived from two words: *mater* denoting "mother," i.e. matrix, denoting 'uterus'. Dioscorides differentiated between a number of species from the genus *Mentha*, which were grown and used to relieve headache and stomach ache. The bulbs of sea onion and parsley were utilized as diuretics, oak bark was used for gynaecological purposes, while white willow was used as an antipyretic. As maintained by Dioscorides, *Scillae bulbus* was also applied as an expectorant, cardiac stimulant, and antihydrotic. It is worth underscoring that Dioscorides pointed to the possibility of forgery of drugs, both the domestic ones such as opium forged by a yellow poppy (*Glaucium flavum*) milk sap and poppy, and the more expensive oriental drugs, transported by the Arab merchants from the Far East, such as iris, calamus, caradmomum, incense, etc.

In 23 AD-79

Pliny the Elder, a contemporary of Dioscorides, who travelled throughout Germany and Spain, wrote about approximately 1000 medicinal plants in his book "Historia naturalis." Pliny's and Dioscorides' works incorporated all knowledge of medicinal plants at the time.

In 131 AD-200

The most distinguished Roman physician (concurrently a pharmacist), Galen, compiled the first list of drugs with similar or identical action (parallel drugs), which are interchangeable—"De succedanus." From today's point of view, some of the proposed substitutes do not correspond in a pharmacological context and are absolutely unacceptable. Galen also introduced several new plant drugs in therapy that Dioscorides had not described, for instance, *Uvae ursi folium*, used as an uroantiseptic and a mild diuretic even in this day and age.

In the seventh century AD

The Slavic people used *Rosmarinus officinalis, Ocimum basilicum, Iris germanica*, and *Mentha viridis* in cosmetics, *Alium sativum* as a remedy and *Veratrum album, Cucumis sativus, Urtica dioica, Achilea millefolium, Artemisia maritime* L., *Lavandula officinalis, Sambuci flos* against several injurios insects, i.e. louses, fleas, moths, mosquitos, and spiders and *Aconitum napellus* as a poison in hunting.

In the Middle Ages

The skills of healing, cultivation of medicinal plants, and preparation of drugs moved to monasteries. Therapy was based on 16 medicinal plants, which the physicians-monks commonly grew within the monasteries as follows: sage, anise, mint, Greek seed, savory, tansy, etc.

In 742 AD-814

Charles the Great, the founder of the reputed medical school in Salerno, in his "Capitularies" ordered which medicinal plants were to be grown on the state-owned lands. Around 100 different plants were quoted, which have been used till present days such as sage, sea onion, iris, mint, common centaury, poppy, marsh mallow, etc. The great emperor especially appreciated the sage (*Salvia officinalis* L.). The Latin name of sage originates from the old Latins, who called it a salvation plant (*salvare* meaning "save, cure"). Even today sage is a mandatory plant in all Catholic monasteries.

The Arabs introduced numerous new plants in pharmacotherapy, mostly from India, a country they used to have trade relations with, whereas the majority of the plants were with real medicinal value, and they have persisted in all pharmacopoeias in the world till today. The Arabs used aloe, deadly nightshade, henbane, coffee, ginger, strychnos, saffron, curcuma, pepper, cinnamon, rheum, senna, and so forth. Certain drugs with strong action were replaced by drugs with mild action, for instance, *Sennae folium* was used as a mild laxative, compared to the purgatives *Heleborus odorus* and *Euphorbium* used until then.

Throughout the Middle Ages European physicians consulted the Arab works “De Re Medica” by John Mesue (850 AD), “Canon Medicinae” by Avicenna (980-1037), and “Liber Magnae Collectionis Simplicum Alimentorum Et Medicamentorum” by Ibn Baitar (1197-1248), in which over 1000 medicinal plants were described.

For Macedonia, St Clement and St Naum of Ohrid's work are of particular significance. They referred to the Nikeian pharmacological codex dating from year 850, and transferred his extensive knowledge on medicinal plants to his disciples and via them to the masses.

Marco Polo's journeys (1254-1324) in tropical Asia, China, and Persia, the discovery of America (1492), and Vasco De Gama's journeys to India (1498), resulted in many medicinal plants being brought into Europe. Botanical gardens emerged all over Europe, and attempts were made for cultivation of domestic medicinal plants and of the ones imported from the old and the new world. With the discovery of America, materia medica was enriched with a large number of new medicinal plants: *Cinchona, Ipecacuanha, Cacao, Ratanhia, Lobelia, Jalapa, Podophylum, Senega, Vanilla, Mate,* tobacco, red pepper, etc. In 17th century, *Cortex Chinae*, yielded from quinine bark *Cinchona succirubra* Pavon, under the name countess’ powder, since the Countess of Chinchon was the first one who used it, was introduced to European medicine. Quinine bark rapidly overwhelmed England, France, and Germany despite the fact that there was many an opponent to its use among distinguished physicians—members of a range of academies.

Paracelsus (1493-1541) was one of the proponents of chemically prepared drugs out of raw plants and mineral substances; nonetheless, he was a firm believer that the collection of those substances ought to be astrologically determined. He continuously emphasized his belief in observation, and simultaneously supported the “Signatura doctrinae”—the signature doctrine. According to this belief, God designated his own sign on the healing substances, which indicated their application for certain diseases. For example, the haselwort is reminiscent of the liver; thus, it must be beneficial for liver diseases; St John's wort *Hypericum perforatum* L. would be beneficial for treatment of wounds and stings given that the plant leaves appear as if they had been stung.

While the old peoples used medicinal plants primarily as simple pharmaceutical forms—infusions, decoctions and macerations—in the Middle Ages, and in particular between 16th and 18th

centuries, the demand for compound drugs was increasing. The compound drugs comprised medicinal plants along with drugs of animal and plant origin. If the drug the theriac was produced from a number of medicinal plants, rare animals, and minerals, it was highly valued and sold expensively.

In 18th century

In his work *Species Plantarium* (1753), Linnaeus (1707-1788) provided a brief description and classification of the species described until then. The species were described and named without taking into consideration whether some of them had previously been described somewhere. For the naming, a polynomial system was employed where the first word denoted the genus while the remaining polynomial phrase explained other features of the plant (e.g. the willow Clusius was named *Salix pumila angustifolia antera*). Linnaeus altered the naming system into a binominal one. The name of each species consisted of the genus name, with an initial capital letter, and the species name, with an initial small letter.

Early 19th century

It was a turning point in the knowledge and use of medicinal plants. The discovery, substantiation, and isolation of alkaloids from poppy (1806), ipecacuanha (1817), strychnos (1817), quinine (1820), pomegranate (1878), and other plants, then the isolation of glycosides, marked the beginning of scientific pharmacy. With the upgrading of the chemical methods, other active substances from medicinal plants were also discovered such as tannins, saponosides, etheric oils, vitamins, hormones, etc.

In late 19th and early 20th centuries

There was a great danger of elimination of medicinal plants from therapy. Many authors wrote that drugs obtained from them had many shortcomings due to the destructive action of enzymes, which cause fundamental changes during the process of medicinal plants drying, i.e. medicinal plants' healing action depends on the mode of drying. In 19th century, therapeutics, alkaloids, and glycosides isolated in pure form were increasingly supplanting the drugs from which they had been isolated. Nevertheless, it was soon ascertained that although the action of pure alkaloids was faster,

the action of alkaloid drugs was full and long-lasting. In early 20th century, stabilization methods for fresh medicinal plants were proposed, especially the ones with labile medicinal components. Besides, much effort was invested in study of the conditions of manufacturing and cultivation of medicinal plants.

On account of chemical, physiological, and clinical studies, numerous forgotten plants and drugs obtained thereof were restored to pharmacy: *Aconitum, Punica granatum, Hyosciamus, Stramonium, Secale cornutum, Filix mas, Opium, Styrax, Colchicum, Ricinus,* and so forth. The active components of medicinal plants are a product of the natural, most seamless laboratory. The human organism accepts the drug obtained from them best in view of the fact that man is an integral part of nature. There are scores of examples of this kind; perhaps they will instigate serious research into the old manuscripts on medicinal plants, which would not be observed out of curiosity about history but as potential sources of contemporary pharmacotherapy.

In present days

Almost all pharmacopoeias in the world—Ph Eur 6, USP XXXI, BP 2007-proscribe plant drugs of real medicinal value. There are countries (the United Kingdom, Russia, Germany that have separate herbal pharmacopoeias. Yet, in practice, a much higher number of unofficial drugs are always used. Their application is grounded on the experiences of popular medicine (traditional or popular medicine) or on the new scientific research and experimental results (conventional medicine). Many medicinal plants are applied through self-medication or at the recommendation of a physician or pharmacist. They are used independently or in combination with synthetic drugs (complementary medicine). For the sake of adequate and successfully applied therapy, knowledge of the precise diagnosis of the illness as well as of medicinal plants, i.e. the pharmacological effect of their components is essential. Plant drugs and phytopreparations, most commonly with defined active components, verified action and, sometimes, therapeutic efficiency, are applied as therapeutic means. In the major European producer and consumer of herbal preparations—Germany, rational phytotherapy is employed, based on applications of preparations whose efficiency depends on the applied dose and identified active components, and their efficiency has been corroborated by experimental and clinical tests. Those preparations have been manufactured

from standardized plant drug extracts, and they adhere to all requirements for pharmaceutical quality of drugs.

With the new Law on Drugs and Medical Devices dated September 2007 and enacted in the Republic of Macedonia, dry or sometimes fresh parts of medicinal plants (herbal substances) may be used for preparation of herbal drugs, herbal processed products, and traditional herbal drugs. Herbal substances may also be utilized for manufacture of homeopathic drugs, which are stipulated in the current law, too. In the Republic of Macedonia herbal preparations are dispensed without a medical prescription, as "over the counter" (OTC) preparations. (Petrovska, 2012)

1.12 Herbal Medicine

Herbal medicine, also called botanical medicine or phytomedicine, refers to using a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. Herbalism has a long tradition of use outside conventional medicine. It is becoming more mainstream as improvements in analysis and quality control, along with advances in clinical research, show the value of herbal medicine in treating and preventing disease. (Ehrlich, 2015)

1.12.1 History of Herbal Medicine

Plants have been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) used herbs in their healing rituals, while others developed traditional medical systems (such as Ayurveda and Traditional Chinese Medicine) in which herbal therapies were used. Researchers found that people in different parts of the world tended to use the same or similar plants for the same purposes.

In the early 19th century, when chemical analysis first became available, scientists began to extract and modify the active ingredients from plants. Later, chemists began making their own version of plant compounds and, over time, the use of herbal medicines declined in favor of drugs. Almost one fourth of pharmaceutical drugs are derived from botanicals.

Recently, the World Health Organization estimated that 80% of people worldwide rely on herbal medicines for some part of their primary health care. In Germany, about 600 to 700 plant based medicines are available and are prescribed by some 70% of German physicians. In the past 20 years in the United States, public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has led to an increase in herbal medicine use. (Ehrlich, 2015)

1.12.2 Functions of Herbs

In many cases, scientists are not sure what specific ingredient in a particular herb works to treat a condition or illness. Whole herbs contain many ingredients, and they may work together to produce a beneficial effect. Many factors determine how effective an herb will be. For example, the type of environment (climate, bugs, and soil quality) in which a plant grew will affect it, as will how and when it was harvested and processed. (Ehrlich, 2015)

1.12.3 Uses of Herbs

The use of herbal supplements has increased dramatically over the past 30 years. Herbal supplements are classified as dietary supplements by the U.S. Dietary Supplement Health and Education Act (DSHEA) of 1994. That means herbal supplements, unlike prescription drugs, can be sold without being tested to prove they are safe and effective. However, herbal supplements must be made according to good manufacturing practices.

The most commonly used herbal supplements in the U.S. include:

- Echinacea (*Echinacea purpurea* and related species)
- St. John's wort (*Hypericum perforatum*)
- Ginkgo (*Ginkgo biloba*)
- Garlic (*Allium sativum*)
- Saw palmetto (*Serenoa repens*)
- Ginseng (*Panax ginseng* or Asian ginseng) and *Panax quinquefolius* or American ginseng)
- Goldenseal (*Hydrastis canadensis*)
- Valerian (*Valeriana officinalis*)

- Chamomile (*Matricaria recutita*)
- Feverfew (*Tanacetum parthenium*)
- Ginger (*Zingiber officinale*)
- Evening primrose (*Oenothera biennis*)
- Milk thistle (*Silybum marianum*)

Practitioners often use herbs together because the combination is more effective. Health care providers must take many factors into account when recommending herbs, including the species and variety of the plant, the plant's habitat, how it was stored and processed, and whether or not there are contaminants (including heavy metals and pesticides). (Ehrlich, 2015)

1.12.4 Benefits of Herbal Medicine

Herbal medicine is used to treat many conditions, such as allergies, asthma, eczema, premenstrual syndrome, rheumatoid arthritis, fibromyalgia, migraine, menopausal symptoms, chronic fatigue, irritable bowel syndrome, and cancer, among others. It is best to take herbal supplements under the guidance of a trained provider. For example, one study found that 90% of people with arthritic use alternative therapies, such as herbal medicine. Since herbal medicines can potentially interact with prescription medications, and may worsen certain medical conditions, be sure to consult with your doctor or pharmacist before taking any herbs. Some common herbs and their uses are discussed below.

- **Ginkgo** (*Ginkgo biloba*) has been used in traditional medicine to treat circulatory disorders and enhance memory. Although not all studies agree, ginkgo may be especially effective in treating dementia (including Alzheimer disease) and intermittent claudication (poor circulation in the legs). It also shows promise for enhancing memory in older adults. Laboratory studies have shown that ginkgo improves blood circulation by dilating blood vessels and reducing the stickiness of blood platelets. By the same token, this means ginkgo may also increase the effect of some blood-thinning medications, including aspirin. People taking blood-thinning medications should ask their doctor before using ginkgo. People with a history of seizures and people with fertility issues should also use concern; Speak with your physician.

- **Kava kava** (*Piper methysticum*) is said to elevate mood, enhance wellbeing and contentment, and produce a feeling of relaxation. Several studies show that kava may help treat anxiety, insomnia, and related nervous disorders. However, there is serious concern that kava may cause liver damage. It is not clear whether the kava itself caused liver damage in a few people, or whether it was taking kava in combination with other drugs or herbs. It is also not clear whether kava is dangerous at previously recommended doses, or only at higher doses. Some countries have taken kava off the market. It remains available in the United States, but the Food and Drug Administration (FDA) issued a consumer advisory in March of 2002 regarding the "rare" but potential risk of liver failure associated with kava-containing products.
- **Saw palmetto** (*Serenoa repens*) is used by more than 2 million men in the United States for the treatment of benign prostatic hyperplasia (BPH), a noncancerous enlargement of the prostate gland. Several studies suggest that the herb is effective for treating symptoms, including frequent urination, having trouble starting or maintaining urination, and needing to urinate during the night. But not all studies agree. At least one well-conducted study found that saw palmetto was no better than placebo in relieving the signs and symptoms of BPH.
- **St. John's wort** (*Hypericum perforatum*) is well known for its antidepressant effects. In general, most studies have shown that St. John's wort may be an effective treatment for mild-to-moderate depression, and has fewer side effects than most other prescription antidepressants. But the herb interacts with a wide variety of medications, including birth control pills, and can potentially cause unwanted side effects, so it is important to take it only under the guidance of a health care provider.
- **Valerian** (*Valeriana officinalis*) is a popular alternative to commonly prescribed medications for sleep problems because it is considered to be both safe and gentle. Some studies bear this out, although not all have found valerian to be effective. Unlike many prescription sleeping pills, valerian may have fewer side effects, such as morning drowsiness. However, Valerian does interact with some medications, particularly psychiatric medications, so you should speak to your doctor to see if Valerian is right for you.

- **Echinacea preparations** (from *Echinacea purpurea* and other *Echinacea* species) may improve the body's natural immunity. Echinacea is one of the most commonly used herbal products, but studies are mixed as to whether it can help prevent or treat colds. A review of 14 clinical studies examining the effect of echinacea on the incidence and duration of the common cold found that echinacea supplements decreased the odds of getting a cold by 58%. It also shortened the duration of a cold by 1.4 days. Echinacea can interact with certain medications and may not be right for people with certain conditions, for example people with autoimmune disorders or certain allergies. Speak with your physician. (Ehrlich, 2015)

1.12.5 Storage of Herbal Medicine

The herbs available in most stores come in several different forms: teas, syrups, oils, liquid extracts, tinctures, and dry extracts (pills or capsules). Teas can made from dried herbs left to soak for a few minutes in hot water, or by boiling herbs in water and then straining the liquid. Syrups, made from concentrated extracts and added to sweet-tasting preparations, are often used for sore throats and coughs. Oils are extracted from plants and often used as rubs for massage, either by themselves or as part of an ointment or cream. Tinctures and liquid extracts are made of active herbal ingredients dissolved in a liquid (usually water, alcohol, or glycerol). Tinctures are typically a 1:5 or 1:10 concentration, meaning that one part of the herb is prepared with 5 to 10 parts (by weight) of the liquid. Liquid extracts are more concentrated than tinctures and are typically a 1:1 concentration. A dry extract form is the most concentrated form of an herbal product (typically 2:1 to 8:1) and is sold as a tablet, capsule, or lozenge.

No organization or agency regulates the manufacture or certifies the labeling of herbal preparations. This means you cannot be sure that the amount of the herb contained in the bottle, or even from dose to dose, is the same as what is stated on the label. Some herbal preparations are standardized, meaning that the preparation is guaranteed to contain a specific amount of the active ingredients of the herb. However, it is still important to ask companies making standardized herbal products about their product's guarantee. It is important to talk to your doctor or an expert in herbal medicine about the recommended doses of any herbal products. (Ehrlich, 2015)

1.12.6 Experts in Herbal Medicine

Herbalists, chiropractors, naturopathic physicians, pharmacists, medical doctors, and practitioners of Traditional Chinese Medicine all may use herbs to treat illness. Naturopathic physicians believe that the body is continually striving for balance and that natural therapies can support this process. They are trained in 4-year, postgraduate institutions that combine courses in conventional medical science (such as pathology, microbiology, pharmacology, and surgery) with clinical training in herbal medicine, homeopathy, nutrition, and lifestyle counseling. (Ehrlich, 2015)

1.13 Plant Based Drugs and Medicines

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world. Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. For example, many years ago a plant chemical was discovered in a tropical plant, Cephaelis ipecacuanha, and the chemical was named *emetine*. A drug was developed from this plant chemical called *Ipecac* which was used for many years to induce vomiting mostly if someone accidently swallowed a poisonous or harmful substance. Ipecac can still be found in pharmacies in many third world countries but has been mostly replaced by other drugs in the United States. Another example of this is the plant chemical named *taxol* shown in the drug column below. The name taxol is the name of the plant chemical orginally discovered in the plant. A pharmaceutical company copied this chemical and patented a drug named *Paclitaxel*™ which is used in various types of tumors today in the U.S. and many other countries.

The 120 substances are sold as drugs worldwide but not in all countries. Some European countries regulate herbal sustances and products differently than in the United States. Many European countries, including Germany, regulate herbal products as drugs and pharmaceutical companies prepare plant based drugs simply by extracting out the active chemicals from the plants. A good example is the plant substance/drug, cynarin. Cynarin is a plant chemical found in the common artichoke (*Cynara scolymus*). In Germany, a cynarin drug is sold for liver problems and hypertension which is simply this one chemical extracted from the artichoke plant or a plant extract which has been standardized to contain a specific milligram amount of this one chemical. These

products are manufactured by pharmaceutical companies, sold in pharmacies in Germany and a doctor's prescription is required to purchase them. In the United States artichoke extracts are available as natural products and sold in health food stores. Some products are even standardized to contain a specific amount of the cynarin chemical. You can purchase these natural and standardized extracts over the counter without a prescription and you could not go to a pharmacy in the U.S. and obtain a cynarin drug with a prescription. Another similar example is the plant chemical, silymarin. Silymarin is a chemical found in the milk thistle plant and natural milk thistle extracts standarized to contain specific amounts of silymarin are found in just about every health food store in the United States. However in Germany, silymarin drugs and milk thistle standardized extracts are sold only in pharmacies and require a doctor's prescription for liver problems.

Some of the drug/chemicals are still sold as plant based drugs requiring the processing of the actual plant material. Others have been chemically copied or synthesized by laboratories and no plant materials are used in the manufacture of the drug. A good example of this is the plant chemical quinine, which was discovered in a rainforest tree (*Cinchona ledgeriana*) over 100 years ago. For many years the quinine chemical was extracted from the bark of this tree and processed into pills to treat malaria. Then a scientist was able to synthesize or copy this plant alkaloid into a chemical drug without using the original tree bark for manufacturing the drug. Today, all quinine drugs sold are manufactured chemically without the use of any tree bark. However, another chemical in the tree called *quinidine* which was found to be useful for various heart conditions couldn't be completely copied in the laboratory and the tree bark is still harvested and used to extract this plant chemical from it. Quinidine extracted from the bark is still used today to produce quinidine-based drugs. In the U.S. there are four patented brand-name heart drugs sold in pharmacies containing bark-extracted quinidine: Cardioquin™, Quinaglute Dura-tabs™, Quinidex Extentabs™ and Quin-Release™. (Beingfit, 2017)

1.14 *Phyllanthus acidus*

Phyllanthus acidus, known as the Otaheite gooseberry, Malay gooseberry, Tahitian gooseberry, country gooseberry, star gooseberry, starberry, West India gooseberry, (in Bangladesh) Orboroi, grosella (in Puerto Rico), jimbilin (in Jamaica), damsel (in St Vincent and the Grenadines and

Grenada), karamay (in the Northern Philippines),layuan (in the Bicol region of the Philippines), (Rata nelli in Sri lanka) bangkiling (in the Southern Philippines), cermai (in Brunei Darussalam, Indonesia and Malaysia), Goanbili (in Maldives), Sapra (in Belize) or simply gooseberry tree, is one of the trees with edible small yellow berries fruit in the Phyllanthaceae family. Despite its name, the plant does not resemble the gooseberry, except for the acidity of its fruits. It is mostly cultivated for ornamentation. (Beingfit, 2017)

1.14.1 Scientific Classification

Kingdom:	Plantae
Order:	Malpighiales
Family:	Phyllanthaceae
Tribe:	Phyllantheae
Subtribe:	Flueggeinae
Genus:	*Phyllanthus*
Species:	***P. acidus***

1.14.2 Description

The plant is an intermediary between shrubs and tree, reaching 2 to 9 m (6½ to 30 ft) high. The tree's dense and bushy crown is composed of thickish, tough main branches, at the end of which are clusters of deciduous, greenish, 15-to-30-cm long branchlets. The branchlets bear alternate leaves that are ovate or lanceolate in form, with short petioles and pointed ends. The leaves are 2-7.5 cm long and thin, they are green and smooth on the upperside and blue-green on the underside. In general, the Otaheite gooseberry tree very much looks like the bilimbi tree. (Beingfit, 2017)

Figure 1.2: Leaves of *Phyllanthus acidus*

The flowers can be male, female or hermaphrodite. They are small and pinkish and appear in clusters in 5-to-12.5-cm long panicles. Flowers are formed at leafless parts of the main branches, at the upper part of the tree. The fruits are numerous, oblate, with 6 to 8 ribs, and densely clustered. They are pale yellow or white, waxy, crisp and juicy, and very sour. 4 to 6 seeds are contained in a stone at the center of each fruit.

1.14.3 Cultivation and human use

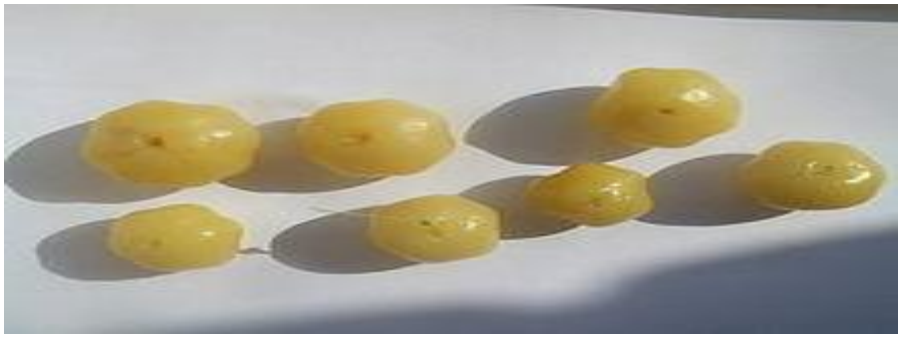


Figure 1.3: Fruits of *Phyllanthus acidus*

Figure 1.4: *Phyllanthus acidus* in India

The Otaheite gooseberry prefers moist soil. It can be cultivated in a variety of ways—budding, cutting and air-layering—in addition to the usual seed growth. The tree is cultivated for its ornamental value, but also for food and medicinal purposes. While it produces some fruit throughout the year, it is mainly harvested in January except in South India, where it bears crops in April–May and again in August–September. As the fruit does not soften when ripe, it is harvested when the fruit begins to drop.

Various parts of the plant are used for food. In India and Indonesia, the cooked leaves are eaten. While the fruit is eaten fresh, and is sometimes used as flavoring for other dishes in Indonesia, it is generally regarded as too tart to eat by itself in its natural form and is processed further. It is candied in sugar or pickled in salt, used in chutney, relish or preserves. In the Philippines, it is used to make vinegar as well as eaten raw, soaked in salt or vinegar-salt solution and sold along the roadside. It is candied as well, usually stored in jars with syrup. They make these into a syrup in Malaysia. Liberally sugared, it is also used to make fruit juice. In Thailand it is used as an ingredient to make Som tam.

The plant is also used medicinally. The peppered leaves are used to make a poultice to treat sciatica, lumbago and rheumatism (but have been observed to cause low blood pressure when combined with nitrates), while the seeds are used as a cathartic and the root, if prepared with care, as a purgative. The syrup is used to medicate the stomach, and in India the fruit is eaten as a blood-enhancer for the liver. *P. acidus* contains 4-hydroxybenzoic acid, caffeic acid, adenosine, kaempferol and hypogallic acid. (Beingfit, 2017)

While the wood is strong and durable if properly treated, the tree is not large and is rarely harvested for wood. In India, the root bark is sometimes used for tanning.

1.14.4 Origin of Star Gooseberry (*Phyllanthus acidus*)

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. (Beingfit, 2017)

1.14.5 Checking for Ripeness in Star Gooseberry

When unripe, gooseberries appear whitish green and are hard to the touch. As they ripen on the vine, they turn pale gold. The fruits do not ripen further once plucked from the tree, and are therefore harvested once they begin to drop.

1.14.6 Taste of Star Gooseberry

Few eat raw star gooseberries due to its pungent, overwhelmingly sour and astringent taste. The flesh is juicy, watery, crisp and densely compact, not unlike the texture of amla. Use caution when eating, as the stone waiting in the middle of the fruit is rock-hard. (Beingfit, 2017)

1.14.7 Nutritional Value of Star Gooseberry

According to Thailand's nutritive department in the Ministry of Public Health, 100g of edible star gooseberry contains the following values:

- 91.7% and 91.9g* Water
- 28kcal
- .7% and .155g* Protein
- .52% and .52g* Fat
- 6.4% Carb
- .6% and .8g* Fiber
- .51% Ash
- 5mg Calcium
- .4mg Iron
- 23mg Phosphorous
- 8mg Ascorbic acid
- .01mg Thiamine
- .05mg Riboflavin

*Values taken from a separate study conducted in El Salvador
**Interestingly, star gooseberries have a pitiful amount of vitamin C despite being closely related to amla, the latter of which is packed with the nutrient. (Beingfit, 2017)

1.14.8 Health Benefits of Star Gooseberry

Though star gooseberries do not receive as much attention as amla—the star of the *Phyllanthus* genus—these fruits may still be considered superfruits for their incredible health benefits.

Traditionally, star gooseberries are used in India to treat a number of illnesses. According to the book, "Biodiversity in India," these fruits are used as a blood purifier and appetite stimulant. They are also used to remedy bronchitis, biliousness, and treat digestive disorders such as urinary

concretions, diarrhea, and piles. As is the case with amla, star gooseberry concoctions also act as a liver tonic and blood enrichment remedy. Another concoction includes making a leaf poultice with added pepper to treat sciatica and rheumatism. (Beingfit, 2017)

1.14.9 Scientific studies also prove the efficacy of star gooseberry as a health remedy

- According to a 2011 study published in the *Journal of Chinese Integrative Medicine*, the antioxidants in star gooseberry fruit have a **hepatoprotective** effect on the liver
- A 2007 study published in *Molecular Pharmacology* indicates that star gooseberry plant extracts may provide treatment against **cystic fibrosis** of the lungs.
- A 2010 article published in the *European Journal of Pharmacology* found that leaf extracts **reduced blood pressure**, thereby suggesting potent **hypotensive** properties.
- A 2012 study published in the *Asian Pacific Journal of Tropical Biomedicine* indicates that leaf extracts exhibit strong **anti-inflammatory, analgesic** and **antioxidant** properties. The study suggests that gooseberry leaves may remedy oxidative stress, pain and inflammation.
- A 2008 study published in *Nature and Science* indicates that the leaves have **antimicrobial** activities that inhibited growth of **E.coli.** A 2006 study published in *Phytomedicine* confirms its antibacterial activity when tested for inhibition against E.coli and staph. (Reddy, 2017)

Chapter Two
Literature Review

2.1 Literature Review on *Phillanthus acidus*

An attempt was made to explore the ethnobotanical, economical and biological importance of *Cicca acida* or *Phyllanthus acidus*. The plant is usedfor 28 types of remedies like cathartic, emetic, coughs, hypertension, asthma, skin diseases etc and as a food in the raw form. In India, it isfound in different states including Maharastra, Assam, Manipur, Tamilnadu and South Indian states as home garden ornaments. The paper reviews the data related to scientific works carried out with the plant and listed the bioactive compounds isolated from the plant till date. Based on the review made, present paper highlights the need of future research with *Cicca acida* or *Phyllanthus acidus* so that more active principle for treating new ailments can be isolated and made available from the plant. (Devi & Paul, 2011)

2.1.1 Phytochemical composition of methanolic extract of Phyllanthus acidus L (Skeels) fresh leaves by GC/MS Analysis

Secondary metabolites and different phytochemicals are present in the medicinal plant *Phyllanthus acidus* belonging to family Phyllanthaceae. Scientific evidence data related GC-MS analysis of methanolic extract of *Phyllanthus acidus* fresh leaves has not been reported till date during literature review. Objective: The study was aimed to investigate phytochemicals present in the methanolic extract of *Phyllanthus acidus* fresh leaves. Material and Methods: Methanolic extraction of fresh leaves *Phyllanthus acidus* was prepared by simple maceration and GC-MS analysis data was provided by IICPT, Thanjavur. Phytochemical investigation was carried out to identify the possible components from *Phyllanthus acidus* fresh leaves by evaluating retention time in the GC-MS chromatogram. Results: Eleven peaks have revealed in the GC-MS spectrum of the methanolic extract of *Phyllanthus acidus.* From the results, squalene is present as major bioactive compounds in methanolic extract of *Phyllanthus acidus* fresh leaves. Conclusion: The present data provides to find the molecular formula and weight of 11 biomolecules. Further investigation should be done to isolate bioactive compounds and their structural elucidation and screening of pharmacological activity in the drug development. (Phatak & Hendre, 2017)

2.2 Literature Review on Other *Phyllanthus* Species

2.2.1 Pharmacognostic evaluation of leaves of certain *Phyllanthus* species used as a botanical source of *Bhumyamalaki* in *Ayurveda*

Today, World over, there is a great deal of interest in Ayurvedic system of medicine and thus the demand for various medicinal plants in the production of Ayurvedic medicines is ever increasing. Due to varied geographical locations where these plants grow, a great deal of adulteration or substitution is encountered in the commercial markets. Histological studies of the plant drugs are not only to study the adulterants but also are indispensable in accurate identification. Microscopic observations of the *Phyllanthus* species revealed the occurrence of anisocytic and paracytic type of stomata in *Phyllanthus amarus*, while only anisocytic type of stomata is present in *P. fraternus* and *P. maderaspatensis*. Epidermal cell walls of *P. amarus* and *P. fraternus* are wavy and straight walled epidermal walls are observed in *P. maderaspatensis*. In India all the above-mentioned species of *Phyllanthus* are called "*Bhumyamalaki*" and they are being used in the treatment of various liver disorders. However, all the species of *Phyllanthus* doesn't have the active constituents responsible for the treatment of liver disorders. In the present investigation by using simple micro techniques accurate identification of different species of *Phyllanthus* has been established. (Sharma & Sheela, 2011)

2.2.2 Antiinflammatory activity of *Phyllanthus emblica*, *Plumbago zeylanica* and *Cyperus rotundus* in acute models of inflammation

Experimental studies conducted earlier have proved that *Phyllanthus emblica* (*Pe*), *Plumbago zeylanica* (*Pz*) and *Cyperus rotundus* (*Cr*), plants from the *medohara* group of Ayurveda possess antiatherosclerotic activity. As inflammation is also one of the pathophysiological factors, it was of interest to evaluate whether these drugs exhibit any antiinflammatory activity. Two models of acute inflammation, namely carrageenan induced rat paw edema and acetic acid induced peritonitis in mice were used. In the model of carrageenan induced paw edema *Pe*, *Pz* and *Cr* showed a trend to reduce the edema while the combination of *Pe* + *Pz* (PI: 20.64%) showed results comparable to aspirin (23.74%). Whereas in a model of acetic acid induced peritonitis, all the plant drugs i.e. *Pe*, *Pz*, *Cr* and a combination of *Pe* + *Pz* showed a significant decrease in the protein content of the

peritoneal exudates compared with the disease control group ($p < 0.05$), however, only *Pe* + *Pz* exhibited activity comparable to aspirin. (Dang & Parekar, 2010)

2.2.3 Antibacterial activities of *Emblica officinalis* and *Coriandrum sativum* against Gram negative urinary pathogens

Present investigation is focused on antibacterial potential of aqueous infusions and aqueous decoctions of *Emblica officinalis* (amla) and *Coriandrum sativum* (coriander) against 345 bacterial isolates belonging to 6 different genera of Gram negative bacterial population isolated from urine specimens by employing well diffusion technique. Aqueous infusion and decoction of *Emblica officinalis* exhibited potent antibacterial activity against *Escherichia coli* (270), *Klebsiella pneumoniae* (51), *K. ozaenae* (3), *Proteus mirabilis* (5), *Pseudomonas aeruginosa* (10), *Salmonella typhi* (1), *S. paratyphi* A (2), *S. paratyphi B* (1) and *Serratia marcescens* (2) but did not show any antibacterial activity against Gram negative urinary pathogens. (Saeed & Tariq, 2007)

2.2.4 Ethnopharmacology of *Phyllanthus emblica* L.

This papper peals with the ethnopharmacology of *P. emblica*, a traditional herbal medicine used by many peoples in the world. The paper introduces the biological characters,geographical distribution-patterns, chemical constitution and pharmacology of the plant. By cross-cultural comparative study, this paper indicates that there are 17 countries and nations of the world using various parts of *P.emblica* in their medical treatment. The medicinal plant is good for anti-hepatitis, anti-cancer, anti-tumor and regulation of stomachal function. The plant is also regarded as a trditional immunomodulator anda natural adaptogen. The result of the study reveals that *P.emblica* is an important traditional medicine with broad prospects. (Quan & Peigen, 1997)

2.2.5 A Chemical and Ethnopharmalogical Study on *Phyllanthus emblica* (Euphorbiaceae)

The use of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) in the separation of phenolic antioxidants was investigated. A simple and fast MEKC method provided sufficient selectivity for the satisfactory resolution of gallates, butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT). Application of the marker technique

improved the repeatability of the analysis and the reliability of identification. The standard deviations for the migration indices were less than 1 % and the technique can therefore be used for rapid purity testing of real samples extracted from plant material, i.e. *Phyllanthus emblica* L. (Euphorbiaceae) leaf extracts.

The Automated Multiple Development (AMD) technique is suitable for the separation of multicomponent mixtures in thin layer chromatography (TLC). The main challenge in optimization AMD is the handling of the numerous instrumental parameters. The wide variation in the polarity of phenolic compounds, e.g.in medicinal plants, can cause problems in identification and separation. Systematic experiments were therefore performed to evaluate gradient elution in AMD for finding a suitable set of operating parameters for the separation of a phenolic reference mixture. Careful optimization leads to an efficient and reliable separation that can be repeated automatically. AMD TLC is a useful tool that provides more powerful screening than conventional, non-instrumental TLC methods.

The effect of experimental parameters on the separation of phenolic compounds was studied by densitometry and video TLC-documentation system. Both the video and densitometer methods are suitable for any analyst lacking the special skills needed to make documents on TLC developments. The video storage system is easy to use when most of the parameters are kept constant. The strong points of the video documentation are the independence of the mode of separation and the structure of the chromatoplate used, the speed of evaluation, and the archiving the captured image for further reporting purposes, e.g. to satisfy the demands of Good Laboratory Practice (GLP). The limitation of both reflectance densitometry and video documentation is the detection of compounds distributed vertically inside thedepth of the chromatoplate.

A computer program for the mobile phase optimization of TLC was employed to enhance the quality of TLC separations. The desirability function technique was combined with the “PRISMA” model. The study showed that the dependence between the eluent composition and retardation for the phenolic test mixtures can be expressed to a high degree of accuracy using quadratic regression models. The optimum eluent mixtures for the separation of compounds were read from the contour plot inside the horizontal plane of the “PRISMA” , and a good separation was achieved using the optimized solvent combination.

P. emblica L. (Euphorbiaceae), a tree growing in subtropical and tropical areas of Far-Eastern countries has been reported to contain constituents with variable biological effects. The activities

of crude leaf extracts were evaluated in human polymorphonuclear leukosytes (PMNs) and platelets. The study showed that the plant leaves have antineutrophil and antiplatelet properties in vitro. This agrees with the anti-inflammatory and antipyretic usage of this tree in traditional medicine by rural populations in Asia. Calcium (Ca2+) is a key mediator of various intracellular processes. Excitable cells contain voltage dependent, receptor operated and stretch operated channels at the plasmalemma. These channels enable the cells to increase cytosolic Ca2+ levels. Calcium channels are highly interesting because they are targets for the drugs used in cardiovascular therapy. The cells of rat pituitary gland (GH4C1) have been found to possess voltage operated Ca2+ channels (VOCCs), and can therefore be used in models studying compounds that interact with Ca2+ channels. The calcium transport activity of 9 phenylpropanes and methanes, and 20 flavonoids was studied in cultured rat pituitary cells (GH4C1) in order to determine their possible interaction with VOCCs. Flavones (flavone and isoflavone genistein) and phenylmetane derivative octyl gallate displayed clear inhibition of Ca2+ entry. The action of the octyl gallate and quercetin on VOCCs was further studied by the means of whole-cell patch-clamp technique. Quercetin markedly enhanced both transient and delayed Ca2+ currents, indicating that quercetin may affect both T- and L-type VOCCs. Onset of action of octyl gallate was clearly slower than that of quercetin. (Summanen, 1999)

2.2.6 Anticancer activity of *Phyllanthus Amarus*

The aqueous extract of *Phyllanthus amarus* demonstrates potent anticancer activity against 20 metylcholanthrene (20-MC) induced sarcoma development. The aqueous extract inhibits DNA topoisomerase II of mutant cell cultures and inhibited cell cycle regulatory enzyme cdc 25 tyrosine phosphatase of *Saccharomyces cerevisiae*. The anticarcinogenic and anti-tumour activity of *Phyllanthus amarus* proposed to be inhibition of metabolic activation of carcinogen as well as the inhibition of cell cycle regulators responsible for cancerous growth and DNA repair. (Verma & Sharma, 2014)

2.2.7 Antiamnesic Activity of *Phyllanthus Amarus*

Antiamnesic activity of aqueous extract of leaves and stems of *Phyllanthus amarus* were evaluated for nootropic effects and brain cholinesterase activity in male Swiss albino mice. Scopolamine and diazepam were used as standard drugs to produce amnesia and elevated plus maze and passive avoidance paradigm as models for evaluation of cognitive functions. The result reveals a dose dependent attenuation of diazepam and scopolamine induced amnesic deficits and reduction in brain cholinesterase activity. Since the reduction in cholinesterase in linked with increase acetylcholine concentration in brain which further is responsible for improving memory, provide a rationale to use this therapeutic potential in the management of patients with cognitive disorders. (Verma & Sharma, 2014)

2.2.8 Antioxidative Activity of *Phyllanthus Amarus*

The DPPH assay is used to determine antioxidant potential, which is based on the reduction of stable radical DPPH to yellow coloured diphenyl picryl hydrazine. Thus, the ability of the test samples to quench this radical is a measure of its antioxidative ability.
Phyllanthus amarus have powerful antioxidant property which is evident from the present study in which phyllanthin and *Phyllanthus amarus* extract were evaluated. In the experiment, it was observed that the DPPH free radical scavenging activity was concentration dependent and reaches maximum at a concentration of 20 mol/ml for phyllanthin and 300 g/ml for *Phyllanthus amarus* extract. Further, since phyllanthin possess very high antioxidative property as evident by its low IC value of 7.4 mol/ml as compared to *Phyllanthus amarus* extracts suggest its contribution in antioxidative effects. In another study, it has been found that boiled water extract of the fresh and dried *Phyllanthus amarus* plant had comparatively greater antioxidant activity than microwave assisted extraction method employed for the extraction. (Verma & Sharma, 2014)

2.2.9 Antinociceptive Activity of *Phyllanthus Amarus*

The hydroalcoholic extract of four *Phyllanthus* species namely *Phyllanthus amarus, Phyllanthus orbiculatus, Phyllanthus fraternus* and *Phyllanthus stipulatuswere* given intraperitoneally and

evaluated in acetic acid induced writhing and formalin and Capsaicin induced licking effects. In acetic acid induced writhing test it was found that all produced significant inhibition of acetic acid induced abdominal constrictions, with mean ID values of 0.3, 1.8, 7.4 and 26.5 mg/kg for *Phyllanthus amarus, Phyllanthus Orbiculatus, Phyllanthus fraternus* and *Phyllanthus stipulates*, respectively. Similarly, in the formalin test, it was observed that the hydroalcoholic extract of four species produced graded inhibition against both phases of formalin induced licking, inhibition in licking being more active in the late phase. Apart from the above models,hydroalcoholic extract of the species also elicited significant reduction in the capsaicin-induced neurogenic pain. It was also observed that hydroalcoholic extract of the *Phyllanthus* species was less potent and efficacious when given orally compared to intraperitoneal route. (Verma & Sharma, 2014)

2.2.10 Antimicrobial activity of *Phyllanthus Amarus*

Antimicrobial activity of ethanol and water extracts of *Phyllanthus amarus* were evaluated against the test organisms *Salmonella typhi*. Ethanolic, cold water extract and hot water extract of *Phyllanthus amarus* were employed for antimicrobial evaluation by agar cup diffusion method which were compared against standard antibiotics that were evaluated by disk diffusion method. The result demonstrates ethanolic extract to be most potent against the test bacteria with diameter of 8.0 mm as growth inhibition zone. This study establishes one of the traditional uses of *Phyllanthus amarus* against typhoid fever. In another study, hexane, petroleum ether, chloroform, acetone and methanol extract of *Phyllanthus* leaves were tested for antibacterial activity against *Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Streptococcus faecalis, Enterobacter species, Serratia marcescens, Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method. The results demonstrated methanol extract of *Phyllanthus amarus* for highest inhibitory activity against above bacterial species. Similarly, in another study antimicrobial potential of *Phyllanthus amarus* were investigated using agar well diffusion method for activity against several drug resistant pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella* Species. The results revealed minimum inhibitory concentration (MIC) of the ethanolic plant extracts on *Escherichia coli, Staphylococcus aureus, Pseudomona aeruginosa* and *Klebsiella* Species were at 10 mg/ml, 50 mg/ml, 150 mg/ml

and 100 mg/ml while the minimum bactericidal concentration were at 50 mg/ml, 100 mg/ml, 150 mg/ml and 150 mg/ml respectively. Further studies on hexane, chloroform, ethyl acetate, acetone and methanol extract of stem bark extracts of *Phyllanthus amarus* demonstrated the antimicrobial activity for all these extracts with a diameter that ranges between 11 mm 24 mm against *Streptococcus pyogenes, Staphylococcus aureus, Salmonella typhi, Escherichia coli, Candidas albican, Aspergillus flavus*. The antimicrobial activity of the methanolic extract of *Phyllanthus amarus* as studied by agar dilution method and disc diffusion showed significant concentration dependent antibacterial activity specifically for gram-negative microbes. It was also observed that antibacterial action was mainly due to the isolated phyllanthin. These studies signify the antimicrobial potential of *Phyllanthus amarus* and need of isolation of some potential phytoconstituents from this species. (Verma & Sharma, 2014)

2.2.11 Antileptospiral Activity of *Phyllanthus Amarus*

Leptospirosis is globally important disease found mainly wherever human come in contact with the urine of infected animals or urine contaminated environment. *Phyllanthus amarus* have been investigated for the antileptospiral activity by micro dilution tests and tube dilution technique. The results revealed the inhibitory action of methanolic and aqueous extract of whole plant of *Phyllanthus amarus* against leptospira. (Verma & Sharma, 2014)

2.2.12 Anticonvulsant Activity of *Phyllanthus Amarus*

Epilepsy is a major neurological disorder characterized by the occurrence of recurrent seizures. The two widely proposed mechanisms involve alterations in the voltage-dependent ion channels such as reduction in inhibitory GABA-mediated drive or increase in excitatory glutamate mediated inputs. This chronic progressive CNS disorder affects a large population of the world. In search of herbal treatment, aqueous and ethanolic extract of *Phyllanthus amarus* were evaluated for anticonvulsant effect using pentylenetetrazole (PTZ) and maximal electroshock-induced seizures (MES) in swiss albino rats. The result showed ethanolic and aqueous extract of leaves and stem

of *Phyllanthus amarus* significantly effective in abolishing hind limb extension induced by MES as well as PTZ induced seizures. (Verma & Sharma, 2014)

2.2.13 Antidiabetic Activity of *Phyllanthus Amarus*

Diabetes is a metabolic disorder of carbohydrate, fat and protein and is considered as the world's largest endocrine disease. The antidiabetic potential of *Phyllanthus amarus* investigated in an experiment model where fasted rats were made diabetic by single intraperitoneal injection of 120 mg/kg of alloxan monohydrates and then two doses of the aqueous and hydroalcoholic extract of *Phyllanthus amarus* administered orally which were then compared with the normal control group that received distilled water only. After 15 days treatment the result demonstrates aqueous and hydroalcoholic extract of *Phyllanthus amarus* decrease the blood glucose level significantly. Serum analysis of the treated experimental animals showed an increase in insulin and reduction in the malondialdehyde concentration, therefore demonstrated the potential antidiabetic property of aqueous and hydroalcoholic extract of *Phyllanthus amarus*. In an other study the methanolic extract of *Phyllanthus amarus* was found to inhibit lipid perxidation & scavenge hydroxyl and superoxide radicals. Since free radicals are linked with diabetes, therefore quenching of free radical could be one mechanism of action. However, there is a need of further experimental studies in order to isolate chemical constituents and their mechanism of action. (Verma & Sharma, 2014)

2.2.14 Anti-Inflammatory Activity of *Phyllanthus Amarus*

The anti-inflammatory potential of *Phyllanthus amarus* was evaluated using different models such as rat Kupffer cells, macrophages RAW264.7, human whole blood and in mice. Two different extracts of *Phyllanthus amarus* (hexane and ethanol/water extracts) and their anti-inflammatory effect was evaluated against the lipopolysaccharide stimulated above mentioned test cells. In addition, anti-inflammatory effect was evaluated in mice that were treated with galactosamine/lipopolysaccharide for inducing acute toxic hepatitis. The evaluation parameters were production of nitrite, prostaglandin E and cytokines that were measured by Griess assay, prostaglandin E by radioimmunoassay and latter by enzyme-linked immunosorbent assay. The

other inflammatory markers such as endotoxin-induced nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) were determined by Western blot and activation of NF-κB and activator protein 1 (AP-1) by electrophoretic mobility shift assay (EMSA). The results revealed ethanol/water extracts and hexane extracts effective in inhibition of liopolysaccharide induced production of nitric oxide (NO) and prostaglandin E2 (PGE) in Kupffer cells and in macrophages RAW264.7. The extracts also attenuated the lipopolysaccharide induced secretion of tumor necrosis factor (TNF-α) in macrophages RAW264.7 as well as in human whole blood. Hexane and ethanol/water extracts of *Phyllanthus amarus* reduced expression of endotoxin-induced nitric oxide synthase iNOS and cyclooxygenase COX-2 and inhibited activation of nuclear factor NF-κB. *Phyllanthus amarus* also inhibited induction of interferon-γ (IFN-γ), interleukin (IL) 1β and interleukin (IL)-10 in human whole blood and reduced tumor necrosis factor (TNF-α) production in-vivo. Further, experimental studies have been done to determine the chemical compounds responsible for the activity. In an attempt to test phytoconstituents and extracts of *Phyllanthus amarus* for anti-inflammatory effect, the purified lignans of *Phyllanthus amarus* and different extracts obtained from this plant were evaluated in carrageenan induced paw edema and neutrophil influx model of inflammation. The result showed that hexane extract and the lignin-rich fraction, or lignans phyltetralin, nirtetralin and niranthin inhibited carrageenan-induced rat paw edema, lower the increase of interleukin (IL)-1β tissue levels induced by carrageenan and inhibited neutrophil influx, bradykinin activating factor, platelet activating factor and endothelin-1-induced paw oedema. These results show that the hexane extract, the lignin rich fraction and the lignans niranthin, phyltetralin and nirtetralin exhibited marked anti-inflammatory properties. Another interesting study where anti-inflammatory effect of soft drink prepared from the leaf extract of *Phyllanthus amarus* was evaluated for its anti-inflammatory effect and the result revealed anti-inflammatory activity of soft drink similar to reference compound Ibuprofen. All these studies acknowledge *Phyllanthus amarus* as potent anti-inflammatory plant and lignins as potent phyto-compounds. (Verma & Sharma, 2014)

2.2.15 Antifertility Activity of *Phyllanthus Amarus*

Phyllanthus amarus possess antifertility activity. This activity was shown in the experimental study where alcoholic extract of *Phyllanthus amarus* brought changes in 3-beta and 17-beta hydroxyl steroid dehydrogenase (HSDs) levels, thereby effecting hormonal conversions in the female mice that confirmed by observation of no pregnancy in cohabited normal females and male mice. (Verma & Sharma, 2014)

2.2.16 Nephroprotective and cardioprotective activity of *Phyllanthus Amarus*

Nephroprotective and cardioprotective effect of *Phyllanthus amarus* is evident from the study in which methanol extract of *Phyllanthus amarus* leaves caused a significant dose dependent decrease in the levels of total cholesterol, urea, total protein, uric acid, and prostatic, alkaline and acid phosphatases, aspartate transaminase (AST) and alanine transaminase (ALT). Since increase in these enzymes is related to hepatic and heart disorders therefore their reduction shows that the leaves of *Phyllanthus amarus* have hepato protective, nephroprotective and cardioprotective properties. (Verma & Sharma, 2014)

2.2.17 Hepatoprotective effect of *Phyllanthus Amarus*

Hepatoprotective effects of aqueous extract from *Phyllanthus amarus* on ethanol-induced rat hepatic injury were studied in in vitro study where *Phyllanthus amarus* increases the percentage 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reduction assay and decreased the release of aspartate transaminase (AST) and alanine transaminase (ALT) in rat primary cultured hepatocytes treated with ethanol. The results reveal that treatment of rats with *Phyllanthus amarus* extract orally brought cell recovery in ethanol-induced liver injury by bringing the levels of aspartate transaminase (AST), alanine transaminase (ALT), high-sensitivity human thyroglobulin (HTG) and Tumor necrosis factor (TNF-α) to normal. Histopathological study confirmed the beneficial effect of *Phyllanthus amarus* with its potential antioxidant activity. (Verma & Sharma, 2014)

2.2.18 Antiviral activity of *Phyllanthus Amarus*

Phyllanthus amarus possess antifungal, antiviral and anticancerous properties. Further, evaluation of antiviral activity of *Phyllanthus* species were evident from experiment study where aqueous extract of *Phyllanthus amarus* along with other species of *Phyllanthus* genus were evaluated against Herpes Simplex Virus type-1 and Herpes Simplex Virus type-2 in vero cells by quantitative polymerase chain reaction. Western blot and 2D-gel electrophoresis were used to study protein expressions of treated and untreated infected vero cells. *Phyllanthus amarus* along with *Phyllanthus urinaria* demonstrate the strongest antiviral activity against Herpes Simplex Virus type-1 and Herpes Simplex Virus type-2 which is proposed to its action in the early stage of infection and replication. (Verma & Sharma, 2014)

2.2.19 Haematological Properties of *Phyllanthus Amarus*

Phyllanthus amarus has been found to produce some haematological changes in experimental studies. When albino rats were treated with the *Phyllanthus amarus* aqueous extract prepared from the whole plant, dose dependent decrease in erythrocyte sedimentation rate (ESR) and packed cell volume (PCV) was observed. Circulating leucocytes and neutrophils count were significantly increased in rats treated with 100 mg/kg of aqueous extract of *Phyllanthus amarus* as evident by total and different count studies of blood of experimental animals. In addition quantitative analysis of alanine aminotransferases (ALT) and aspartate aminotransferases (AST) gave significantly higher values of alanine aminotransferases (ALT) in treated rats. Author has suggested immunostimulant potential of plant. (Verma & Sharma, 2014)

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.

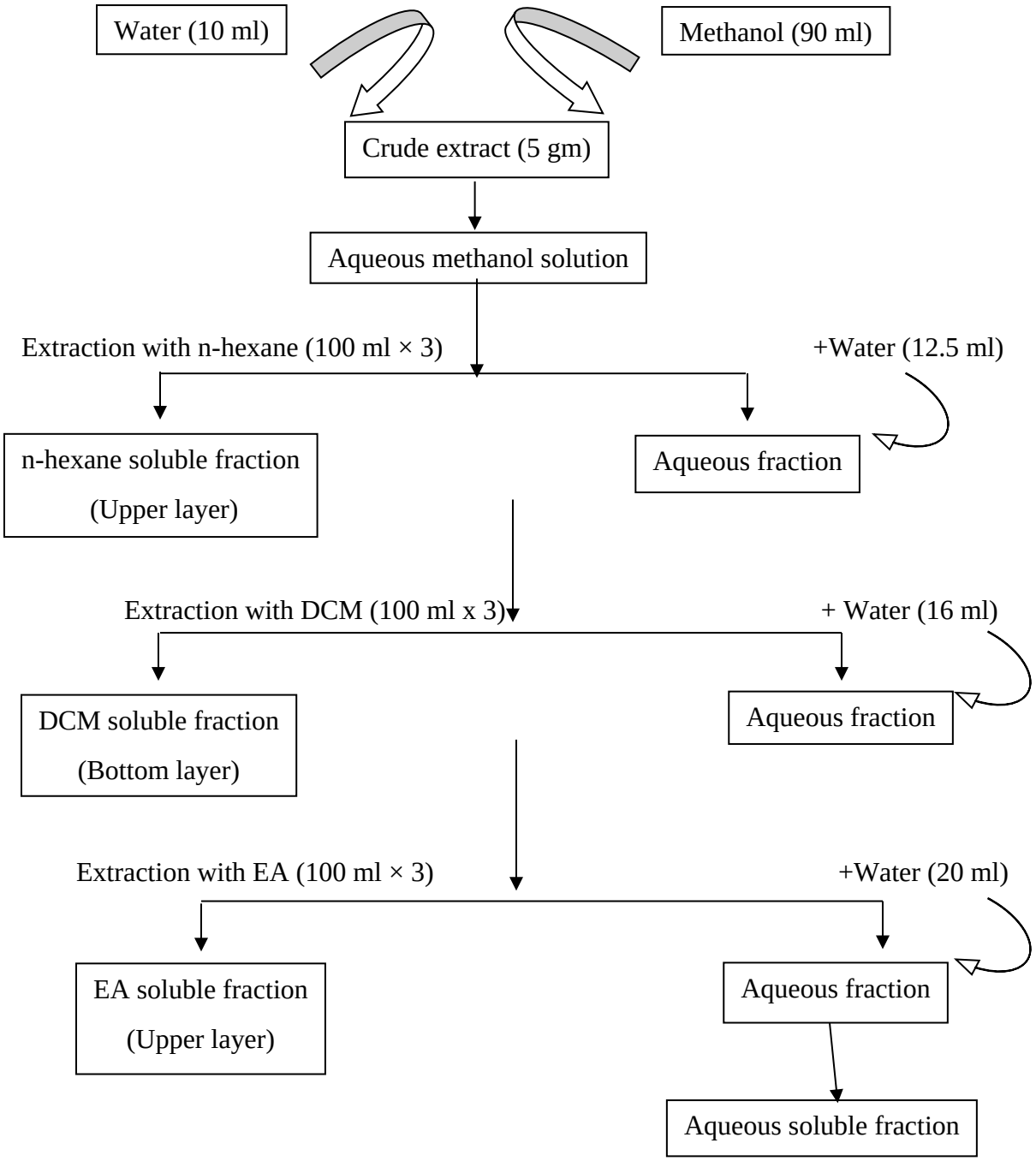


Figure 3.2: Schematic representation of the Partitioning of methanolic crude extract of *Phyllanthus acidus* leaves.

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 AH and Stenlake JB, 1986)

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.

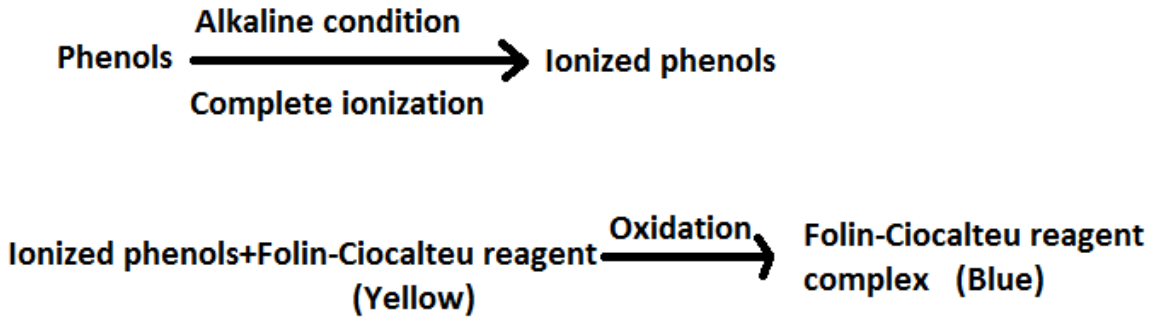
3.5.1.1 Principle

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 et al., 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)
Na2CO3 solution (7.5%)	Test tube
Methanol	Micropipette (50-200 µl)
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

- 1.0 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.

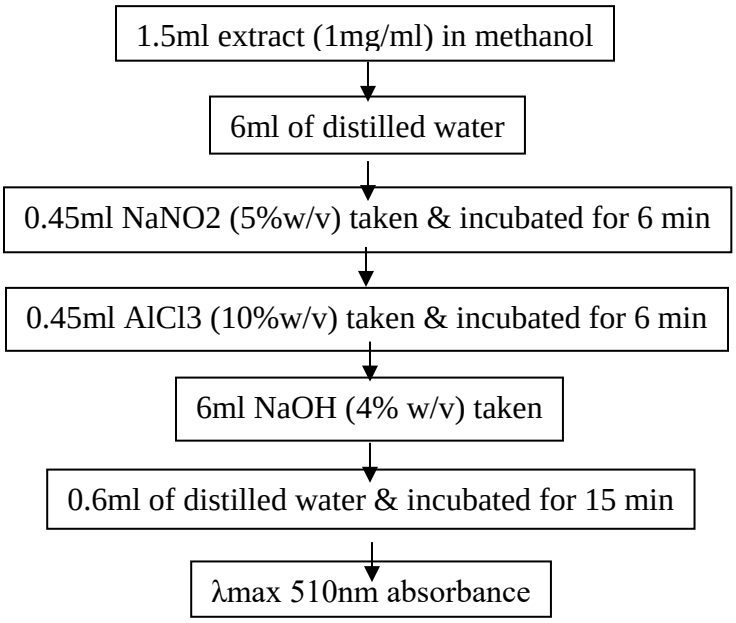


Figure 3.3: Schematic diagram of preparation of extract solution

3.5.2.3.6 Preparation of blank solution

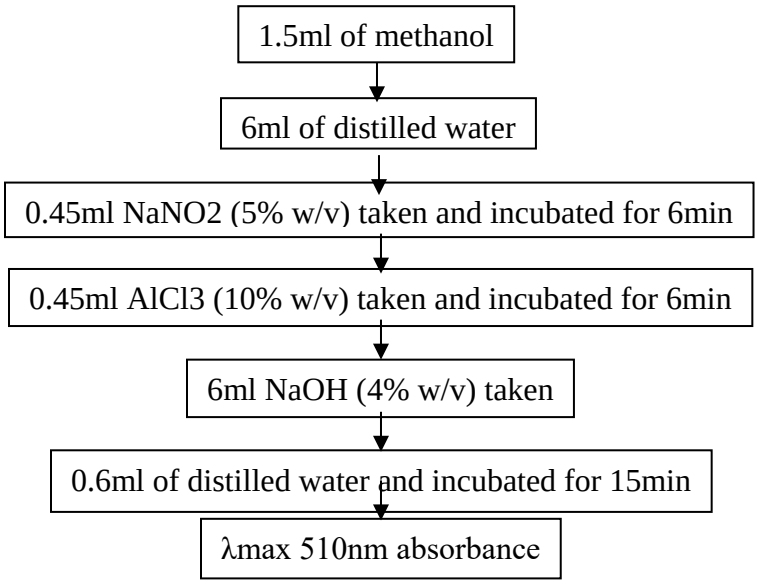


Figure 3.4: Schematic diagram of preparation of blank solution

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 Nuneza, 2013).

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 J. *et al*., 2009).

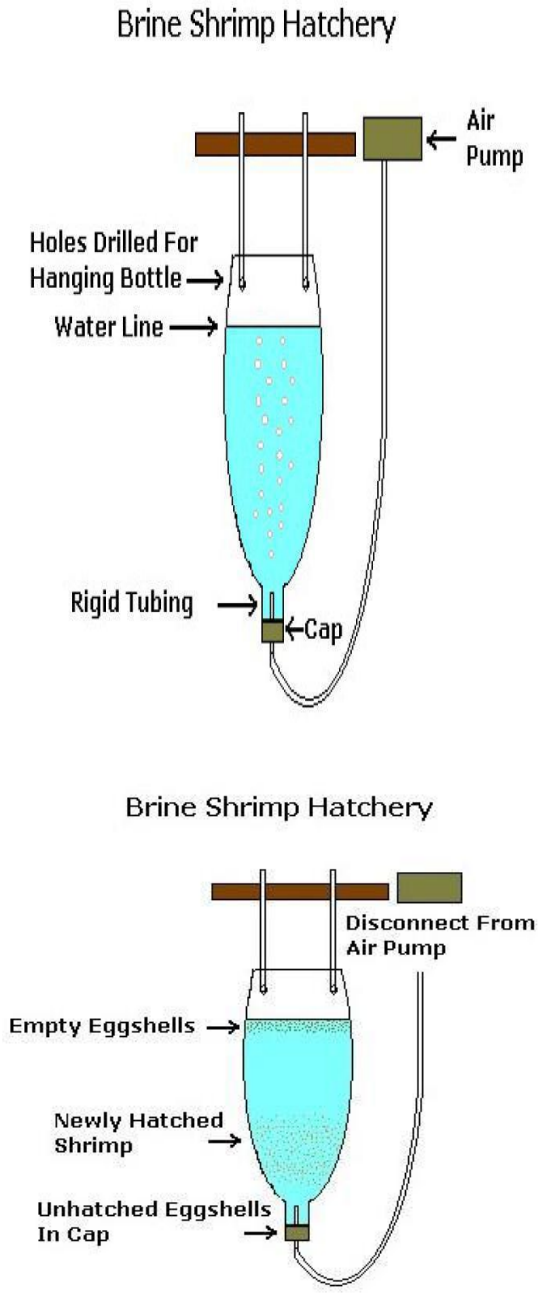


Figure 3.5: Brine shrimp Hatchery

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 et al., 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.

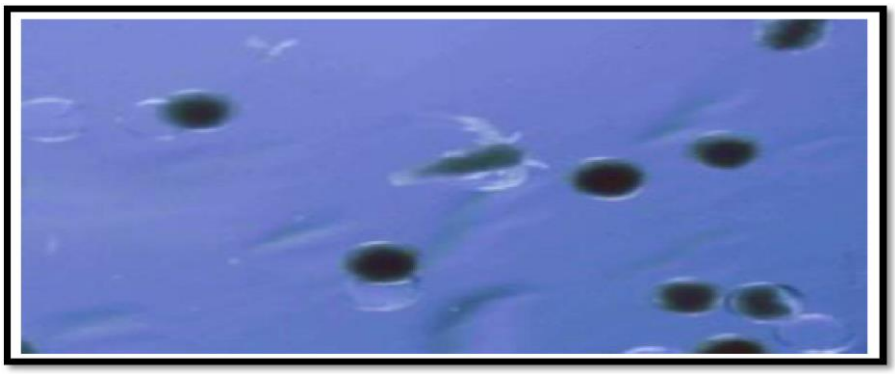


Figure 3.6: Counting of nauplii

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.

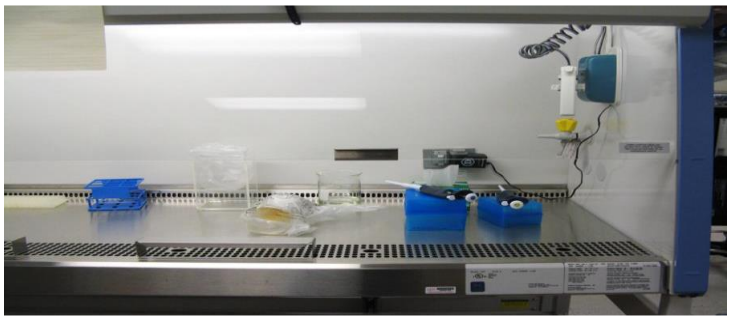


Figure 3.8: Laminar hood

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.

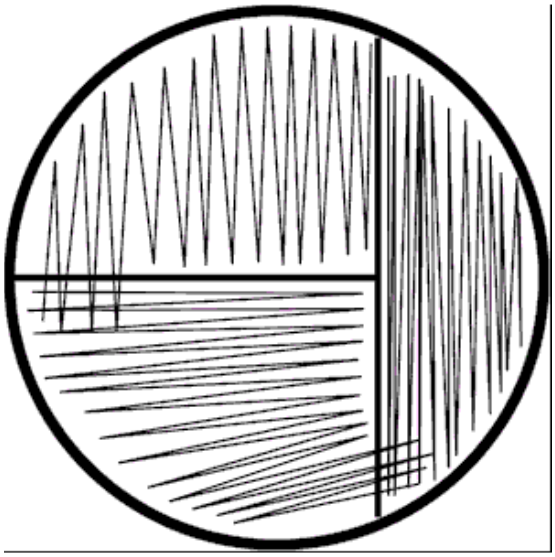


Figure 3.9: Preparation of filter paper discs

- 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.

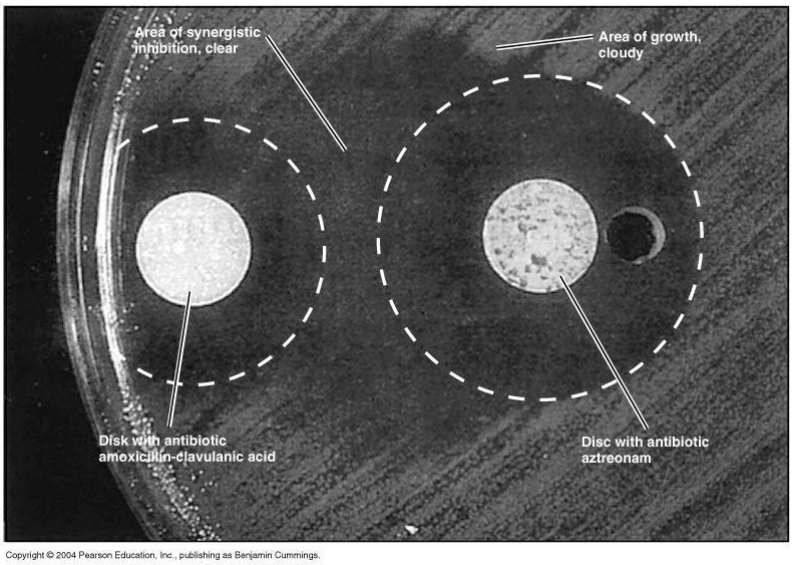


Figure 3.11: Clear zone of inhibition

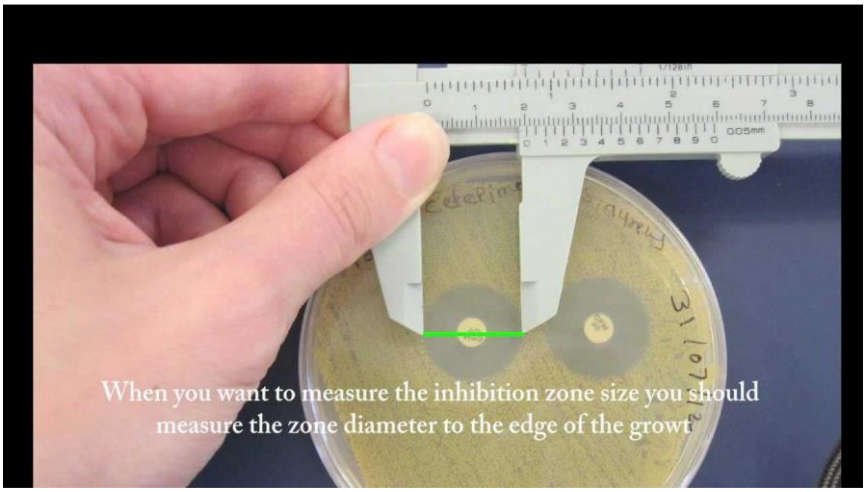


Figure 3.12: **Determination of clear zone of inhibition**

Chapter Four

Results and Discussion

Result and Discussion

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 n-hexane 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 n-hexane extract of leaves of *Phyllanthus acidus* were subjected to determine total phenolic content. Ascorbic acid was used as reference standard. (Singleton et al., 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.

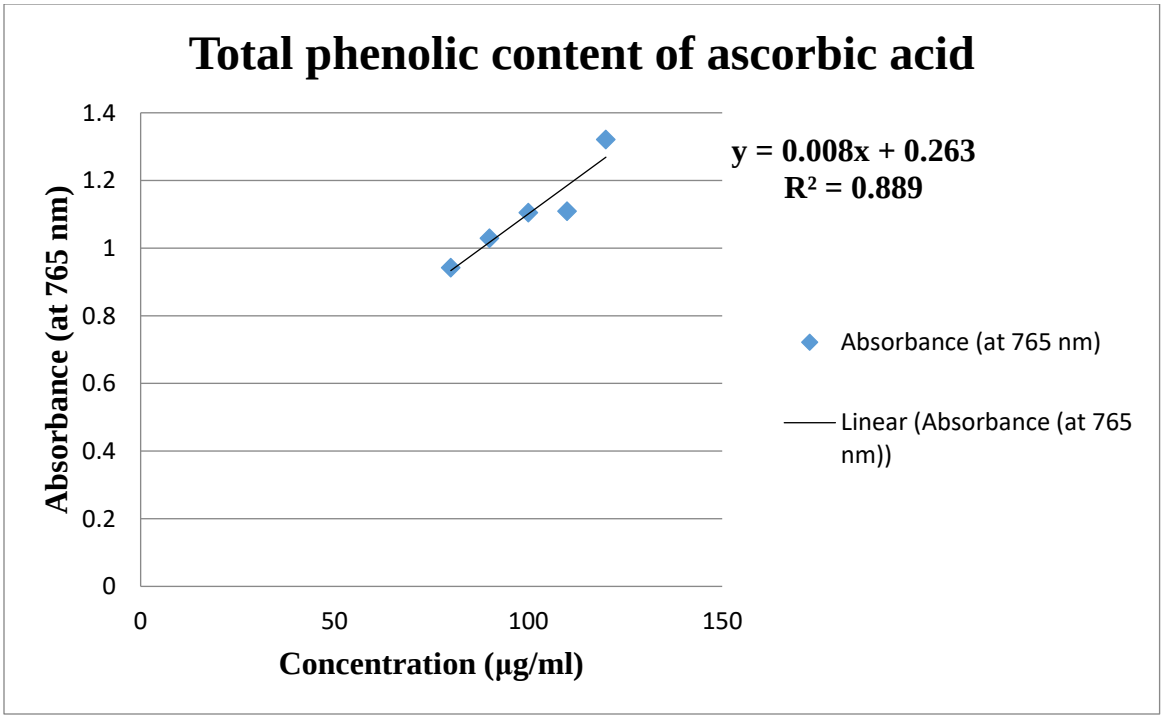


Figure 4.1: Graphical Representation of Assay of Phenolic Content of Ascorbic Acid

4.1.1.2 Total phenol content present in n-hexane extract of *Phyllantus 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 n-hexane fraction of *Phillanthus acidus* (leaves)

Sample	Concentration (mg/ml)	Absorbance (Y value at 765 nm)	Total Phenolic (X) value (mg of AAE/gm of dried extract)
n-hexane fraction of *Phillanthus acidus*	2	0.299	4.5

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 n-hexane fraction is lower than the absorbance of standard. Based on the absorbance values of extract solution and using the regression line equation of the standard curve, 4.5 mg of AAE/gm of dried extract of phenol content was found in the n-hexane fraction of *Phyllanthus acidus.*

4.1.2 Result of total flavonoid content

The n-hexane fractions of *Phillanthus 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.

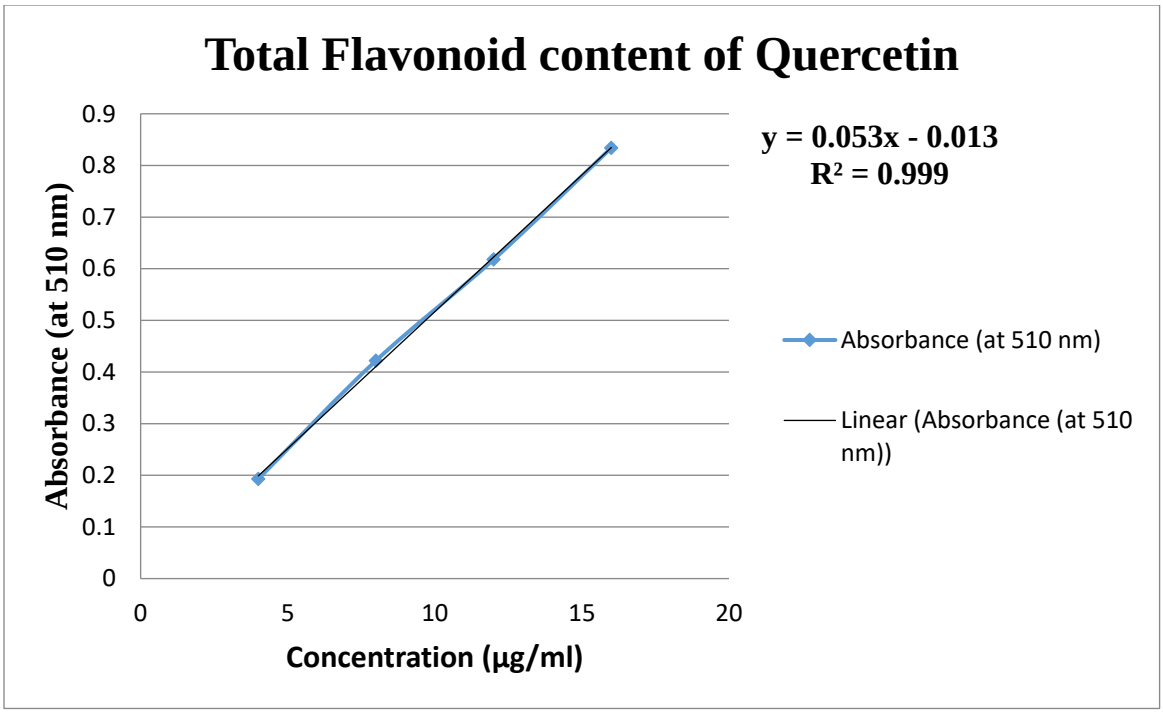


Figure 4.2: Graphical representation of Flavonoid content of quercetin

4.1.2.2 Total flavonoid content present in n-hexane fraction of *Phyllanthus acidus* (leaves)

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

Table 4.4: Total Flavonoid Content in n-hexane 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)
n-hexane fraction of Phyllanthus acidus	1	0.245	4.868

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 n-hexane 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, 4.868 mg of Quercetin/gm of dried extract of flavonoid content was found in the n-hexane fraction of *Phyllanthus acidus* (leaves).

4.2 Result of Antimicrobial Test

The antimicrobial activities of n-hexane fraction of *Phyllanthus acidus* leaves extract were subjected in the study against various Gram positive bacteria and Gram negative bacteria. The n-hexane 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 n-hexane Fraction

Table 4.5 Antimicrobial activity of standard sample (Ciprofloxacin) and n-hexane fraction

Type of microorganism		**Zone of inhibition (mm)**	
		Standard sample	**n-hexane fraction**
Gram positive bacteria	*Bacillus sereus*	31	7
	Bacillus subtilis	31	7
	Bacillus megaterium	30	1
	Staphylococcus aureus	31	7
Gram negative bacteria	*Escherichia coli*	30	6
	Salmonella typhi	30	9
	Salmonella paratyphi	32	6
	Vibrio parahemolytics	30	0
	Pseudomonas aureaus	30	6
	Shigella dysenteriae	33	0

4.2.2 Discussion

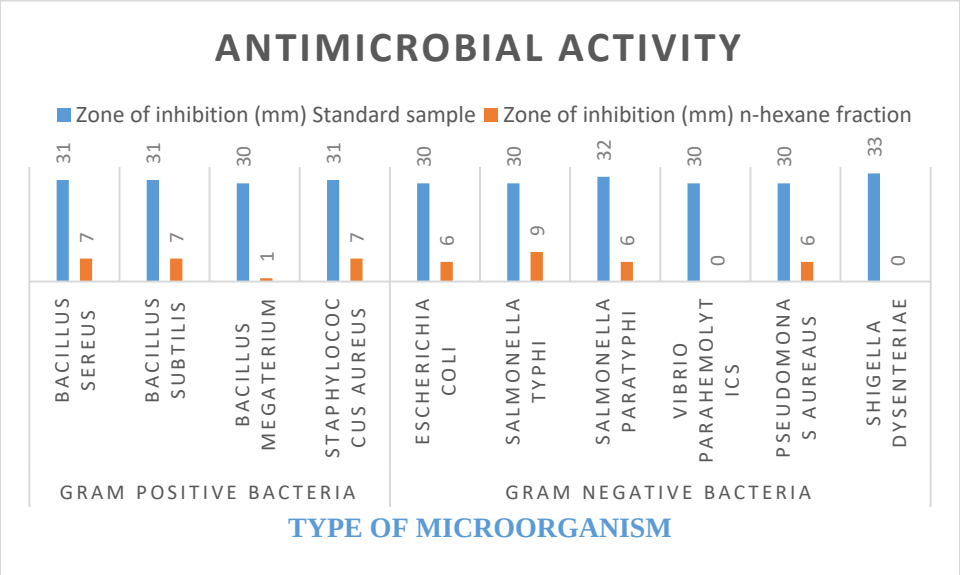


Figure 4.3: Comparison of antimicrobial activity between standard and extract

n-hexane 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 n-hexane fraction is equal to Ciprofloxacin against any bacteria as shown in the figure 4.3. among all the microbial cultures, the fraction showed the best antimicrobial activity against Salmonella typhi (9mm) comparable to the standard (30mm).

4.3 Result of Brine Shrimp Lethality Bio-Assay

The n-hexane 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 n-hexane 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	16.405
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	

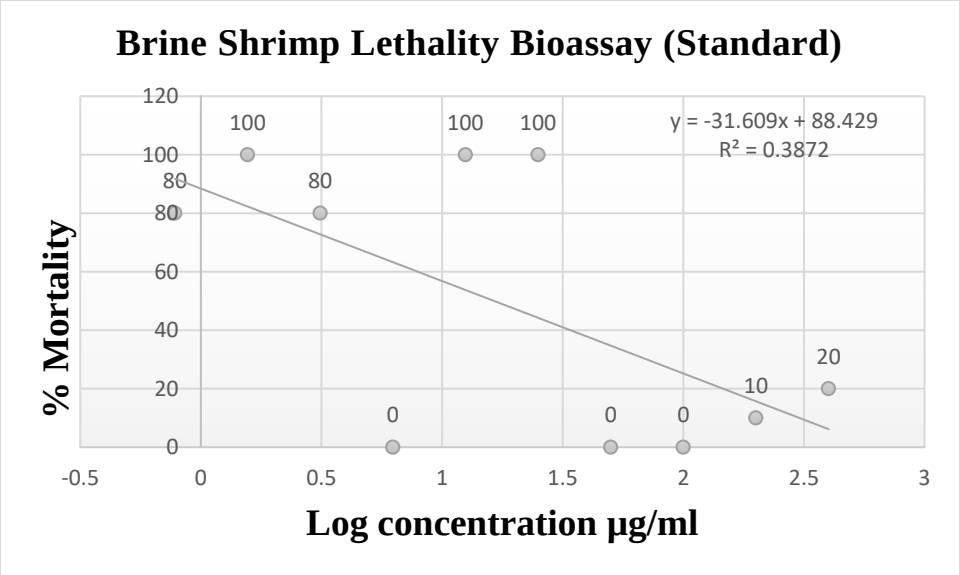


Figure 4.4: % Mortality and Predicted Regression Line of Tamoxifen (standard)

4.3.2. Preparation of n-hexane Fraction Curve of *Phyllanthus acidus* (leaves)

Table 4.7: Results of the bioassay in n-hexane 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	1589516
2	200	2.30103	4	6	60	14.2
3	100	2	4	6	60	
4	50	1.69897	3	7	70	
5	25	1.39794001	4	6	60	
6	12.5	1.09691001	3	7	70	
7	6.25	0.79588002	1	9	90	
8	3,125	0.49485002	2	8	80	
9	1.5625	0.19382003	3	7	70	
10	0.78125	-0.10720997	3	7	70	

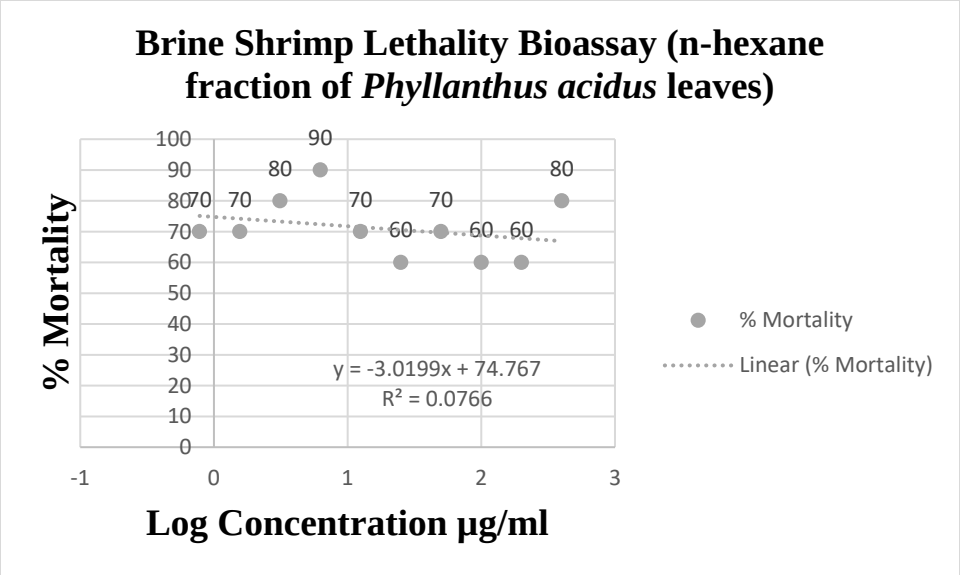


Figure 4.5: % Mortality and Predicted Regression Line in n-hexane fraction of *Phyllanthus acidus* (leaves)

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 6.25 µg/ml, whereas the less mortalities at the concentration of 200 and 100 µg/ml as shown in Table 4.7.

Table 4.8: Cytotoxic activity of Tamoxifen and n-hexane fraction of *Phyllanthus acidus* (leaves)

Sample	Linear regression equation	R2 value	LC50 (µg/ml)
Standard (Tamoxifen)	y = -39.609x + 12.806	88.429	16.405
n-hexane fraction	y = -3.0199x + 74.767	0.0766	158951614.2

In this investigation, standard and n-hexane fraction exhibited cytotoxic activities with the LC50 values at 16.405 µg/ml and 158951614.2 µg/ml respectively as shown in Table 4.8. LC50 value of *Phyllanthus acidus* (leaves) in n-hexane fraction showed very less activity of it than Tamoxifen. Further investigation is needed to confirm the activity.

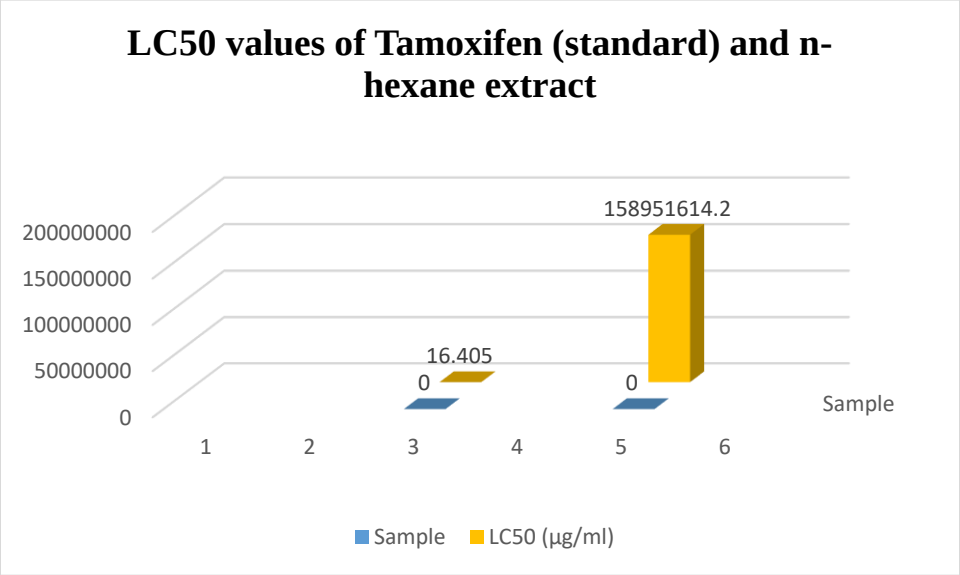


Figure 4.6. Comparison between LC50 values of standard and extract

Chapter Five

Conclusion

Conclusion

As the literature review suggests, the presence of several phytochemical compounds in n-hexane fraction of *Phyllanthus acidus*, makes the plant pharmacologically active.

LC50 value of *Phyllanthus acidus* in n-hexane fraction showed very less cytotoxic activity than tamoxifen. Antioxidant property in n-hexane extract of *Phyllanthus acidus* was determined by Phenolic content assay and flavonoid content assay. Phenolic content was 4.5 mg of AAE/gm, Flavonoid content was 4.868 mg of quercetin/gm in n-hexane extract of *Phyllanthus acidus*. So, n-hexane extract of *Phyllanthus acidus* have poor antioxidant property. Mixture of compounds can lower antioxidant property in n-hexane fraction of *Phyllanthus acidus*, if any counteracting compounds were present in mixture. So pure compound isolation should be done in future to confirm antioxidant property of n-hexane fraction of *Phyllanthus acidus*.

n-hexane 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 n-hexane fraction is equal to Ciprofloxacin against any bacteria.

Further investigations can be carried out to isolate and identify the active compounds present in the plant that are responsible for pharmacological activity in the development of novel and safe drugs. Other tests can be performed to evaluate some other pharmacological activities.

Chapter Six

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