IN VITRO PHARMACOLOGICAL INVESTIGATIONS ON AQUEOUS FRACTION OF *PHYLLUNTHUS ACIDUS* LEAF EXTRACT

A Dissertation submitted to the Department of Pharmacy, East West University, in partialfulfillment of the requirements for the degree of Bachelor of Pharmacy.

Submitted To

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DECLARATION BY THE CANDIDATE

I, Sharmin akther hereby declare that this dissertation, entitled "*In Vitro* Pharmacological Investigations on Aqueous Fraction of *Phyllanthus acidus* Leaf Extract" submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic research work carried out by me under the guidance of Abdullah-Al-Faysal, Senior Lecturer, Department of Pharmacy, East West University, Dhaka. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertion, entitled "*In Vitro* Pharmacological Investigations on Aqueous Fraction of *Phyllanthus acidus* Leaf Extract" is a bonafide research work done, under our guidance and supervision by Sharmin Akther (ID: 2013-3-70-034), in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Dedication

This Research paper is dedicated to

My beloved Parents,

Who are my Biggest Inspiration…

ABSTRACT

The purpose of the study was to evaluate the cytotoxic, antioxidant and antimicrobial activity of aqueous fraction of *Phyllunthus acidus* leaf (Family:Phyllunthaceae) extract.The powder of leaf were extracted with methanol and then partitioned with n-hexane, DCM, and ethyl acetate, aqueous fraction was taken for experiment. The aqueous fraction was used to evaluate cytotoxic, antioxidant and antimicrobial activities. The cytotoxic activity was measured by brine shrimp lethality bioassay. LC50 value of aqueous fraction of *Phyllanthus acidus* was 2µg/ml in brine shrimp lethality test. The fraction contained 4.5mg AAE/g of total phenolic content and 4.868 mg AAE/g of total flavonoid content. The results of study clearly indicate the presence of cytotoxic and poor antioxidant properties of aqueous extract. 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 bio-assay, phenolic content, flavonoid content.

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1.1 Introduction to medicinal plants

A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis. When a plant is designated as medicinal‘, it is implied that the said plant is useful as a drug or therapeutic agent or an active ingredient of a medicinal preparation. Medicinal plants may therefore be defined as a group of plants that possess some special properties or virtues that qualify them as articles of drugs and therapeutic agents, and are used for medicinal purposes. There are a huge number of medicinal plants. In the US, almost 1800 medicinal plant species are commercially available. It has been estimated that about 13,000 species of plants have been employed for at least a century as traditional medicines by various cultures around the world. A list of over 20,000 medicinal plants has been published, and very likely a much larger number of the world's flowering plant species have been used medicinally. Sometimes the figure of 70,000 medicinal plant species is cited, but this includes many algae, fungi, and micro-organisms that are not really plants as the word is understood by botanists. In any event, there is no other category of plants useful to man (with the possible exception of ornamental plants) that includes so many species, and the question naturally arises why such a staggering number of plants have useful medicinal properties. The use of medicinal plants is not just a custom of the distant past. Perhaps 90% of the world's population still relies completely on raw herbs and unrefined extracts as medicines. A 1997 survey showed that 23% of Canadians have used herbal medicines. In addition, as much as 25% of modern pharmaceutical drugs contain plant ingredients. (P. P. Joy, J. Thomas,1998)

1.1.1The definition of medicinal plants.

This category includes all plants any or all parts of which are used for therapeutical purposes due to the active ingredients contained in them. They can be wild plants or cultivated ones. Since cultivated plants have numerous beneficial effects too, in a larger sense, any plant can be a medicinal herb, including arable plants, vegetables, fruits, and spices. Presumably many of them had been originally used as medicinal herbs in preserving food or treating gastric disorders, and became spices because of their beneficial effects, pleasant smell and taste. Addictive substances such as caffeine also have curative effects therefore their consumption in

therapeutical doses falls into a different category. The categorization of plants – into arable plants, ornamentals, poisonous plants, weeds, etc. – is always subjective; there is always a human element in it and reflects a certain attitude, economic interests, a purpose or a goal, etc. A plant can belong to several categories, depending on which of its characteristics is emphasized. The above quoted ancient story from the history of Indian therapeutics, in which the studies of Jivaka ended when he could not find a single plant with no beneficial effects after several days of searching, is very relevant and expressive. But our job, besides broadening the selection, is to direct attention to easily obtainable and more effective herbs.

In the history of herbal medicine, there have been extreme views too. For example, it was held that every plant is effective against every disease, which is apparently a wild exaggeration. But it is certainly important to use those plants as medicinal herbs which according to our knowledge and experience have the strongest effects coupled with the least (or no) side effects. We also have to consider that similar to pharmaceuticals, medicinal herbs do not affect everyone in the same way. Depending on the individual's reaction, various herbs can be indicated as the best remedy(Imre Nemeth PhD ,2012)

1.1.2 Example of medicinal plant

1.1.2.1.Anise seed

Uses of anise seed

- The properties of anise make it a natural choice to alleviate flatulence.
- When mixed with other oils, Aniseed oil provides a good antiseptic.
- It is of great help for providing relief when suffering from a dry cough.
- Used as a common cure in cases of infant catarrh.
- Used externally for treating lice infestation and scabies.
- Used commonly in mouthwash

1.1.2.2.Neem

Uses of neem

- Antibacterial Potential.
- Detoxifying Effects.
- Gastric Health.
- Cancer and Chronic Disease
- Fungal Infections
- Diabetes treatment
- Malaria Treatment

1.1.2.3.Basil

Uses of Basil

The health **benefits** of holy basil, also known as **tulsi**, include;

- oral care,
- relief from respiratory disorders,
- as well as treatment of fever, asthma,
- lung disorders,
- heart diseases and stress.

Holy Basil, which has the scientific name *Ocimum sanctum* is undoubtedly one of the best medicinal herb that has been discovered.(Pretz, J.E. 2005)

Fig1.1 Example of medicinal plant(basil,anise seed,neem)

1.2.Role of plants in human history

Plants have also been used in the production of stimulant beverages (e.g. tea, coffee, cocoa, and cola) and inebriants or intoxicants (e.g., wine, beer, and kava) in many cultures since ancient times, and this trend continues till today. Tea (*Thea sinensis*) was first consumed in ancient China (the earliest reference is around CE 350), while coffee *(Coffea arabica*) was

initially cultivated in Yemen for commercial purposes in the 9th century. The Aztec nobility used to consume bitter beverages containing raw cocoa beans (*Theobroma cacao*), red peppers, and various herbs. Nowadays, tea, coffee, and cocoa are important commodities and their consumption has spread worldwide. The active components of these stimulants are methylated xanthine derivatives, namely caffeine, theophylline, and theobromine, which are the main constituents of coffee, tea, and cocoa, respectively. The most popular inebriants in society today are wine, beer, and liquor made from the fermentation of fruits and cereals. Wine was first fermented about 6000–8000 years ago in the Middle East, while the first beer was brewed around 5000–6000 BCE by the Babylonians. The intoxicating ingredient of these drinks is ethanol, a by-product of bacterial fermentation, rather than secondary plant metabolites. Recent studies have shown that a low to moderate consumption of red wine is associated with reduction of mortality due to cardiovascular disease and cancer. (P. P. Joy, J. Thomas,1998)

1.2.1.The value of plants in our lives

Ancient Man is known to have utilized plants as drugs for millennia. Based on current knowledge, at least in the West, we know that extracts of some of these plants are useful in a crude form, i.e. *Atropa belladonna* Tincture as an antispasmodic, *Rauvolfia serpentina* roots for hypertension and as a tranquilizer, *Papaver somniferum* extract or tincture as an analgesic, etc. Further, we know that at least 121 chemical substances of known structure are still extracted from plants that are useful as drugs throughout the world. A large number of plants are used in traditional medical practices, and have been for more than 3000 years, such as in Chinese Traditional Medicine, Indian Traditional Medicine, etc., most of which probably exert therapeutic effects and would be proven as such if they were properly evaluated by Western standards. Still further, plants have been employed for centuries by primitive cultures; most of these are less likely to pass the test of modern experimental verification of efficacy. Finally, there are a large number of so-called herbal remedies, mainly sold in health food stores in developed countries, many of which remain to be verified for their real therapeutic effects. Several years ago the World Health Organization made an attempt to identify all medicinal plants that exist in the world. It was admitted that the compilation of names of medicinal plants undoubtedly contained many replicates since botanical verification was not attempted. Further, the list including more than 20,000 species only provided Latin binomials and the countries where the plants were used, but excluded data indicating what the plants were used for. (P. P. Joy, J. Thomas,1998)

1.2.2.Plants as a basis of some important drugs

Higher plants have been used as a source of drugs by mankind for several thousand years. In fact, ancient man was totally dependent on green plants for his day-to-day needs of medicaments. With the development of modern medicine, synthetic drugs and antibiotics, the importance of plants as raw material for drugs decreased considerably. However, plants were used as a basis of some of the most important drugs, even in the modern system of medicine. With the advancement of synthetic organic chemistry most of the active constituents of plants used in medicine were synthesized. At one time it was thought that ultimately all the plant drugs would be obtained from synthetic sources. However, in spite of phenomenal progress in the development of new drugs from synthetic sources and the appearance of antibiotics as major therapeutic agents, plants continue to provide basic raw materials for some of the most important drugs. Although data are not available for all countries, a study carried out in the United States by Farnsworth and his colleagues between 1958 and 1980 indicated that although the number of prescriptions issued by community pharmacies in the United States increased considerably, the percentage of prescriptions containing one or more plant products remained constant at a figure of 25%. It has been found that in highly developed countries like the United States more than 100 chemical constituents of definite structure derived from 41 species of plants were used in modern medicine. It has also been estimated that in addition to these active constituents, more than 96 crude extracts were also used in the United States. . (P. P. Joy, J. Thomas,1998)

1.2.3.Examples of some modern medicine discovered from plants

- Plants can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced toxicity.
- The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others.
- In some cases, the crude extract of medicinal plants may be used as medicaments. About 121 (45 tropical and 76 subtropical) major plant drugs have been identified for

which no synthetic one is currently available. It has been estimated that more than 400 traditional plants or plantderived products have been used for the management of type 2 diabetes across geographically.

- Galegine, a substance produced by the herb Galega officinalis, provides an excellent example of such a discovery. Experimental and clinical evaluations of galegine provided the pharmacological and chemical basis for the discovery of metformin which is the foundation therapy for type 2 diabetes.
- Plant derived agents are also being used for the treatment of cancer. Several anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world.
- In conclusion, plants have provided humans with many of their essential needs, including life-saving pharmaceutical agents.
- Recently the World Health Organization estimated that 80% people worldwide rely on herbal medicines for some aspect.
- Many developing countries have intensified their efforts in documenting the ethnomedical data and scientific research on medicinal plants.
- Natural products or natural product derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales.
- There are more than 270,000 higher plants existing on this planet. But only a small portion has been explored phytochemically.
- So, it is anticipated that plants can provide potential bioactive compounds for the development of new leads‘ to combat various diseases.

As a vast proportion of the available higher plant species have not yet been screened for biologically active compounds, drug discovery from plants should remain an essential component in the search for new medicines & the scientific study of traditional medicines, concerned medicinal plants are thus of great importance. . (P. P. Joy, J. Thomas,1998)

1.2.4.Ten interesting facts about complementary and alternative medicine

- The World Health Organization estimates that between 65 to 80 percent of the world‘s population (over 4 billion people) rely on alternative medicine as their primary form of health care compared to only 10 to 30 percent of people who use conventional medicine.

- Traditional Chinese medicine has been chosen by the World Health Organization for worldwide propagation to meet the heath care needs of the twenty-first century.
- Medicinal herbs were found amongst the personal effects of the mummified prehistoric ―ice man‖ who was found in the Italian Alps in 1991.
- 19 percent of Fortune 500 companies offer alternative medicine as part of their health care compensation packages.
- One-half of all medical schools now offer courses in alternative medicine.
- Spinal manipulation was used by the Ancient Greeks long before it was incorporated into chiropractic and osteopathic medicine in the 19th Century.
- More than 70% to 90% of physicians consider complementary and alternative medicine therapies, such as diet and exercise, behavioral medicine, counseling and psychotherapy, and hypnotherapy, to be legitimate medical practices.
- Massage therapy dates back thousands of years and has been recorded in ancient writings from the Orient, Asia, Arabia and Greece.
- The National Institutes of Health currently invests about $40 million per year in complementary and alternative medicine related research.
- 2/3 of people who use complementary and alternative medicine do not tell their medical doctor. . (P. P. Joy, J. Thomas,1998)

1.3.Classification of medicinal plant

Classification of medicinal plants is organized in different ways depending on the criteria used. In general, medicinal plants are arranged according to their active principles in their storage organs of plants, particularly roots, leaves, flowers, seeds and other parts of plant. These principles are valuable to mankind in the treatment of diseases. Reports on the classification of many plant species yielding vegetable oils used in cosmetics and body and skin care preparations are sporadic or lacking. Herbs are classified in many ways. Some of them are:

- according to the usage;
- according to the active constituents;
- according to the period of life;
- according to their taxonomy;
- according to their habitats

1.3.1.Classification according to usage

The herbs are classified in four parts: medicinal herbs, culinary herbs, aromatic herbs, ornamental herbs.

- **Medicinal Herbs** have curative powers and are used in making medicines because of their healing properties like marigold, lemon balm, lavender, johnny-jump-up feverfewetc.

- **Culinary Herbs** used as cooking herbs because of flavours like oregano, parsley,
- **Aromatic Herbs** have some common uses because of their pleasant smelling flowers or foliage. Oils from aromatic herbs can be used to produce perfumes, toilet water, and various scents For e.g. mint, rosemary, basil etc.
- **Ornamental Herbs** are used for decoration because they have brightly coloured flowers and foliage like lavender, chives, bee balm, lemongrass etc.(P. P. Joy, J. Thomas,1998)

1.3.2.Classicafication according to according to active constituent

According to the active constituents all herbs are divided into five major categories: Aromatic (volatile oils), Astringents (tannins), Bitter (phenol compounds, saponins, and alkaloids), Mucilaginous (polysaccharides), and Nutritive (food stuffs).

A. **Aromatic Herbs:** The name is a reflection of the pleasant odour that many of these herbs have. They are used extensively both therapeutically and as flavourings and perfumes. Aromatic herbs are divided into two subcategories: stimulants and nervines. Stimulant Herbs increase energy and activities of the body, or its parts or organs, and most often affect the respiratory, digestive, and circulatory systems. E.g. fennel, ginger, garlic, lemongrass. Nerving Herbs are often used to heal and soothe the nervous system, and often affect the respiratory, digestive, and circulatory systems as well. They are often used in teas or in encapsulated form, e.g. ginger, catnip.
B. **Astringent Herbs**: Tannins in Astringent Herbs have the ability to precipitate proteins, and this "tightens," contracts, or tones living tissue, and helps to halt discharges. They affect the digestive, urinary, and circulatory systems, and largedoses are toxic to the liver. They are analgesic, antiseptic, ant abortive, astringent, emmenagogue, hemostatic, and styptic. For e.g. peppermint, red raspberry.
C. **Bitter Herbs:** Bitter Herbs are named because of the presence of phenols and phenol glycosides, alkaloids, or saponins, and are divided into four subcategories:

laxative herbs, diuretic herbs, saponin containing herbs, and alkaloidcontaining herbs.

D. **Laxative Bitter herbs:** include alterative, ant catarrhal, antipyretic, cholagogue, purgative, hypotonic, sialagogue, vermifuge, and blood purifier. For e.g. aloe, cascara, liquorice, pumpkin, senna, yellow dock, yucca, barberry, gentian, safflowers, and golden seal.

E. **Diuretic Herbs:** induce loss of fluid from the body through the urinary system. The fluids released help cleanse the vascular system, kidneys, and liver. They are alterative, antibiotic, ant catarrhal, antipyretic, and antiseptic, lithotripter, and blood purifier in nature. For e.g. asparagus, blessed thistle, burdock, butcher's broom, buchu, chaparral, chickweed, corn silk, dandelion, dog grass, grapevine, and parsley.

F. **Saponin-containing Herbs:** are known for their ability to produce frothing or foaming in solution with water. The name "saponin" comes from the Latin word for soap. They emulsify fat soluble molecules in the digestive tract, and their most important property is to enhance the body's ability to absorb other active compounds. Saponins have the ability to effectively dissolve the cell membranes of red blood cells and disrupt them. They are alterative, ant catarrhal, antispasmodic, and aphrodisiac, emmenagugue, cardiac stimulant, and increased longevity in nature. For e.g. yam root, schizandra, black cohosh, blue cohosh, devil's claw, liquorice, alfalfa, yucca, ginseng, and gotu kola.

G. .**MucilaginousHerbs**: Mucilaginous herbs derive their properties from the polysaccharides they contain, which give these herbs a slippery, mild taste that is sweet in water. All plants produce mucilage in some form to store water and glucide as a food reserve. They eliminate the toxins from the intestinal system, help in regulating it and reduce the bowel transit time. They are antibiotic, antacid, demulcent, emollient, vulnerary, and detoxifier in nature. For e.g. althea, aloe, burdock, comfrey, dandelion, Echinacea, fenugreek, kelp, psylium, slippery elm, dulse, glucomannan from Konjak root, Irish moss, and mullein.

H. **Nutritive Herbs**: These herbs derive both their name and their classification from the nutritive value they provide to the diet. They are true foods and provide some medicinal effects as fibber, mucilage, and diuretic action. But most importantly they provide the nutrition of protein, carbohydrates, and fats, plus the vitamins and minerals that are necessary for adequate nutrition. For e.g. rosehips, acerola, apple, asparagus, banana, barley grass, bee pollen, bilberry, broccoli, cabbage, carrot, cauliflower, grapefruit,

hibiscus, lemon, oat straw, onion, orange, papaya, pineapple, red clover, spirulina, stevia, and wheat germ.

1.3.3.Classification according to the period of life

Herbs also can be classified as annuals, biennials, and perennials. Annuals bloom one season and then die. Biennials live for two seasons, blooming the second season only. Once established, perennials live over winter and bloom each season. They can last for many years with proper care.

i. **Annual** herbs complete their life cycle in one year; start them from seed. The annuals have to be seeded each year unless conditions are favorable enough in the garden to seed themselves. Annual herbs include: Anise, Basil, Borage, Calendula (Pot Marigold), Chamomile, Chervil, Cilantro/ Coriander, Dill Bouquet, Dill Dukat, Fennel smoky, Marjoram, Parsley, Saffron, Summer Savoury.

ii. **Perennial** herbs grow for more than one season and include sweet marjoram, parsley, mint, sage, thyme and chives. Most can be started from young plants. Perennial herbs include: Alfalfa, Allspice, Aloe Vera, Angelica, Bee Balm, Bay leaves, Catnip, Chives Common, Lavender, Lemon Balm, Mints (Spearmint, peppermint, apple mint, orange mint), Mitsuba, Oregano, Rosemary, Sorrel, Salad Burnet, Sage, Tarragon, Thyme, Watercress, Yarrow.

iii. **Biennial** are plants which live two season and bloom in the second season only. They are Caraway seeds, Prime rose, Bai Zhi, Mullein, Teasel, Viper's Bugloss. Like all other plants, herbs can be propagated from seeds, cuttings, divisions, and to a lesser degree, layering. . (P. P. Joy, J. Thomas,1998)

1.3.4.Distribution of medicinal plants by habitats

The Earth has many different environments, varying in temperature, moisture, light, and many other factors. Each of these habitats has distinct life forms living in it, forming complex communities of interdependent organisms. A complex community of plants and animals in aregion and a climate is called a biome.

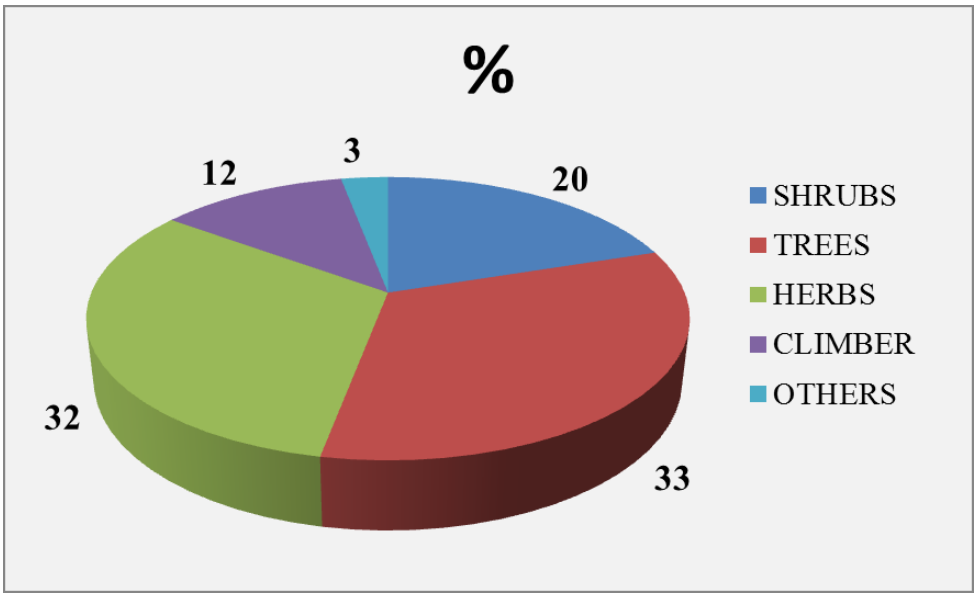


Figure 1.2: Distribution of medicinal plant by habitat. (P. P. Joy, J. Thomas,1998)

1.4.The History of Herbal Medicine

Two thirds of the world's population still use herbs as a first choice to treat diseases. But besides the realm of medicine, several mass-consumed eatables and stimulants also have beneficial sideeffects. For example, coffee and tea are both stimulants and refreshers, but they are also a rich source of fluorine, while ginger, a popular beverage in England, alleviates indigestion. At the beginning of its career, Coca-cola was a beverage for headache. It was invented in the 1880s by a pharmacist in Atlanta, who used his knowledge of the cola nut in its manufacture.

In different parts of the world herbs have been used to treat the same problems. Similarities in herbal medicine are evident despite the fact that American Indian culture was isolated from European, Egyptian, Chinese and Indian influences until the 15th century. Hop and mint species for instance have long been used by every people to treat gastric pain, angelica (Angelica) and liquorice (Glycyrrhizaglabra) were both curative drugs of respiratory diseases, blackberry and raspberry were used against diarrhoea, and bat-willow (Salix alba) was a remedy for inflammation and a natural pain-killer. The name , "aspirin" comes from the old Latin name of meadow sweet, Spirea (today: Filipendula). The medicine was at first extracted from bat-willow and meadow sweet. Both plants contain several active ingredients.

China. Legend has it that around 3400 BCE a mythological emperor called Shennong recognized the curative effect of plants. He carried out his experiments on himself and this became his fate: he died of poisoning. He is held to be the author of the first textbook on the subject, Pen Ts'ao Ching (Great Herbal). The book describes 237 recipes based on several dozens of herbs. From the time of the Shang dinasty (around 1500 BCE), archaeologists unearthed more than a hundred thousand so-called oracle bones with inscriptions of botanic data. The Chinese knew diabetes as early as the 7th century and gave vaccination against smallpox already in the 10th century.

In 1590 Li Shizhen published a monumental book (printing was already invented 800 years before Gutenberg) entitled Compendium of Materia Medica (BencaoGangmu). The book describes 1,094 herbs and 11,000 different prescriptions.

Chinese medicine still considers that disease is caused by the disruption of harmony between the individual and the environment. Its central idea is that nature consists of five elements (wood, fire, earth, metal and water) and every change can be explained by their action. The theory of energy, or life force, is also applied in interpreting the world. Eastern and Western methods have been harmonized since 1949. Nixon's visit to China was a break-through. The public image of acupuncture has since greatly improved, which still in the 1970s was held to be quackery by the official medicine in Hungary for instance.

Egypt. In 1874, a German Egyptologist found a papyrus roll in the Valley of Tombs near Luxor dating from 1500 BCE. The so-called Ebers papyrus is 21 meters long and contains medical descriptions. It lists more than 500 herbs and describes 876 kinds of treatment. The third of current medicinal plants already figures in this document which summarized a thousand years of herbal medicine. Among Egyptians, garlic and onion were the two most popular medicinal plants. Probably that is why the Greek historian Herodotus called the Nilotic people ill-smelling. In the tomb of Pharaoh Tutankhamun (14th century BCE) they found six cloves of garlic. Around 500 BCE, Egyptian herbalists were considered to be the best; court physicians were often Egyptians and it was to Egypt that would-be doctors went to study. Egyptian medicine greatly influenced European medicine.

Europe. In the fifth century BCE, the Greek philosopher Empedocles spoke about four Classical elements (earth, water, air, fire) and associated four bodily fluids to them (black bile, blood, yellow bile, mucus). According to Hippocrates (460-370 BCE), health depends on the

right proportion and balance of these bodily fluids. Therefore diseases are of natural origin and in order to cure them, one has to restore their balance and activate curing forces of the diseased organism. Treatment was always personalised and he observed individual responses to it. He used about three hundred kinds of medicine, among others several common herbs (scented mayweed, blue-bottle, cinnamon, rosemary, garlic, etc.). In the 3rd century BCE, Theophrastus already gave descriptions of 455 herbs. His herbarium was probably the first in Europe in which the preparation and use of medicines were described.

Hippocratic cures were widely applied in the Roman Empire but they were mixed with religion and magic. The Romans achieved good results in preventive medicine too, by purifying their water and building a sewage system.

Dioscorides, who was born in about 40 AD, wrote one of the most extensive herbals of all time. His five-volume book, best known by its Latin title De Materia Medica, gives detailed descriptions of 600 plants with illustrations. Galen, whose authority surpassed even that of Dioscorides, urged the necessity of controlling drugs and he composed complex herbal preparations using multiple ingredients. While his herbal mixtures (based on the so-called Galenic formulation) proved undoubtedly useful, some of his preparations contained up to a hundred ingredients and were used as cure-all panacea. These expensive wonder drugs were very popular among credulous patients and rather hampered the advancement of medicinal practice.

After the fall of the Roman Empire the Classical medicinal tradition was mostly kept alive by the Islamic world. While the Arabs and the Persians substantially enlarged the list of drugs, they did not surpass Galenic principles. The encyclopaedic work entitled Canon Medicinae (The Canon of Medicine) of eleventh-century polymath, Ibn Sīnā, better known as Avicenna, remained a standard medical text for centuries.

In the middle ages, the Church played an important role in this field, Benedictines being the most assiduous herbalists. They copied old books, thus preserving the compiled knowledge of bygone ages. Charlemagne ordained that each monastery should have a garden of medicinal herbs. The abbess Hildegard of Bingen (1098-1179) was also a Benedictine herbalist. Her book Causaeet Curae is a compilation of the knowledge of her age. In England, a Saxon aristocrat called Bald wrote a book in 950 by the command of King Alfred, called the Leech Book of Bald, in which he treats 500 herbs and incorporates Celtic and Druidic wisdom.

Mediaeval witch-hunts may have been the result of professional jealousy of men. Those who were affected by some disease often could only rely on folk medicine, rites and magic, and the wise women experienced in medicinal herbs were often more efficient than "professional" male doctors. The science of medicinal and poisonous herbs was sadly promoted by political and power conflicts that often resorted to the use of poisons and the well-paid help of professional poisoners. But new ideas made their way into mediaeval Europe and one of their most important representative was Aureolus Philippus Theophrastus Bombastus von Hohenheim (1493-1541), who called himself Paracelsus, or "one whose knowledge surpassed that of the ancient physician called Celsus". He held that disease was not caused by the disruption of the balance of bodily fluids but by external factors. He assumed that plants contained medicinal substances.

England Nicholas Culpeper published his Complete Herbal in 1653. It certainly has a historical interest, but the author's view that every plant cures everything is rather disputable.

German Samuel Hahnemann (1755-1843) was the father of homeopathy. He developed his theory as a result of his study of poisonous substances. It is obviously gaining popularity nowadays and provides a completely alternative form of treatment.

America. It seems that Native Americans were rather healthy and resistant to most diseases. Their remedies were fast and effective, but official medicine had neglected them for a long time. George Washington's death in 1799 might have been caused by regular Western medicinal practice, namely the combined effects of bloodletting (two litres of his blood were drained), laxatives and mercury treatment.

Around 1800, Samuel Thompson, who studied from wise women and Native American healers, saved his daughter's life – who was declared incurable – by administering her medicinal herbs and hot baths. He perfected these treatments and started to call himself a "doctor". He successfully practiced for decades, treating millions of people. After his death his method became less popular but his followers carried on his work. The Eclectic Medical Institute, which flourished in the second half of the 19th century, combined the herbalism of European, Asian, Native American and African American traditions. Nowadays, the root extract of Chinese cucumber and St. John's wort are studied by its followers as possible AIDS remedies.

India Indian medicinal plants /Ayurvedic medicinal plants are plants using in Ayurveda mainly as medicinal purpose. They are back bone of Aurveda. Charaka, Susrutha&Vagbatta are main classical text books on Ayurveda where uses, treatment & properties of them describes detailed, but don't think that only usage of medicinal plants have the role. There have most important role to treatment principle. Ayurvedic medicinal plants are gate way miracles, if way of treatment and basic principles of is correct. Vata, pitta &kapha are the three elements s which have definite properties to keep our body, mind and every thing normal .Ayurvedic medicinal plants Classified according to their properties like Rasa, Guna ,Virya , Vipaka (This one of the simple and most important classification I narrate , there have so many classifications.) This classification help us how to manage works medicinal plants to cure diseases. Ayurveda says vitiation/abnormal increase in Vata, pitta &kapha are main cause of disease. Vitiation is due to increase or decrease of similar properties. All the Dravya/ substance has properties (guna). Vata, pitta &kapha have also similar properties. Properties similar to Vata, pitta &kaphahave , do considerably increase in related Doshas, opposite properties decreases the qualities of related Doshas.

So proper identification of Ayurvedic medicinal plants is important in the field of treatment. Medicinal plants using in South India & North India is different, even their Sanskrit names are similar. But they show same action. We classified Ayurvedic medicinal plants according to their synonyms which give exact idea or colourful pictures about medicinal plants. In Ayurveda morphology of medicinal plants planned in the form of synonyms. We designed a data base for Indian medicinal plants which describes 6000 pictures 600 Ayurvedic medicinal plants in India. With coloured photographs, botanical names, Scientific classification morphology, treatment , controversies, adulterations, therapeutic uses, chemical composition, properties, action of the medicinal plants on digestive system, reproductive system, respiratory system, circulatory system, Karma, part used , indications, dosage, Satmikaran, research activities, habitat and distribution.(Imre Nemeth PhD ,2013)

Fig1.3.History of herbal medicine

1.5.The nomenclature of drugs

The drug is that part of the plant, usually preserved by drying, which contains the active ingredient. It is to be noted that most herbs can be used fresh; drying only ensures that we can make a herbal infusion of a given plant any time of the year. There are a few instances when the fresh product has adverse effects. Such is the case for example with black alder, the bark of which contains a substance called rhamnustoxin, an emetic, which breaks up only after a year of storage or following heat treatment. Ricinus seed is also poisonous but the oil is free of poisonous substances after cold pressing, and heat-treatment is also effective.

Herbs are used as raw materials in therapeutics, and this plant material is called drug. In order to avoid misunderstanding, it is better to use the expression “phytogenic drug”, or crude drug, (The word “drug” may be connected to the Germanic verb “droge”, to dry.)

A herbal drug can be:

i. That part of the medicinal plant which contains the most active ingredient(s), and which is preserved by drying. It may be washed, cut and peeled but not otherwise treated.
ii. The essential oil (aetheroleum), resin (resina), balm, fatty acid (oleum), alcoholic extract or tincture (tinctura), etc., produced from the plant material.
iii. A substance produced from the plant material by transformation, e.g., tar (pix, as in juniper tar, Pixjuniperi), active carbon (carbo, as in linden-tree carbon, Carbo tiliae).

The Latin name of the drug consists of two parts. The first name is the genitive of the plant’s Latin name (e. g., Frangulae, Sambuci, Trifolii), the second is the scientific name of the plant’s part, which is in the subjective case (e. g., Sambuciflos – elder flower, Frangulae cortex – black alder bark).

In some cases, when obviously more than one plant species can be taken into account, the full Latin name is given in the drug's name. E. g., Alliisativibulbus, i. e., the clove of *Allium sativum*, or garlic, or *Digitalis lanatae* folium, or the leaf of woolly foxglove, since we use the leaf of another herb of the same family, red foxglove, *Digitalis purpura.*

There are cases when the drug's name is derived from the second part of the herb's Latin name. E. g., drugs extracted from jimsonweed – *Datura stramonium* are called Stramoniisemen and Stramonii folium. The drug of shepherd's purse, Capsella bursa-pastoris is known as *Bursae pastoris* herba, or the essential oil of basil – *Ocimum basilicum* – is called *Aethero leumbasilic*i. Quite a few drugs are known under their traditional name that does not reflect modern taxonomic categories. Thus the flower of forking larkspur, or *Consoli daregalis*, is known as *Calca trippaeflos*, or in the case of rose species, the name of dried rosehip does not come from Rosa sp., but is traditionally Cynosbati pseudofructus cum seminibus. The root drug of liquorice *(Gycyrrhi zaglabra*) is known under the name Liquiriti aerhizomaet radix.

In a few cases the name of the drug may have preserved the older Latin name of the plant, indicating changes in designation or categorization. Such an example is the drug name of the fruit of milk thistle (Silybummarianum),Carduimarianifructus, or the name of the root drug of baby's breath (Gypsophilapaniculata), Saponariaealbae radix.

In some cases the drug has two names. It is often because the name of the plant has changed, like for example, in the case of acacia flower, Robiniaeflos, or Acaciaeflos. In other cases, like for example with bean, the empty pod has several appropriate Latin names, resulting in three names for the drug: Phaseolilegumen, Phaseolipericarpium, Phaseolifructus, sine semine.

The first part of names of products made from plant material is the Latin name of the product itself, e. g., Oleum, Aetheroleum, and the second part is the genitive of the plant's Latin name. E. g., rosemary *(Rosmarinus officinalis*) oil is called *Aetheroleum rosmarini*, peppermint *(Mentha piperita*) oil is called *Aetheroleum menthaepiperitae*, and flaxseed *(Linumus itatissimum*) oil is called *Oleum lini*. The dried opium derived from poppy is called Pulvisopii, and maize starch is called *Amylum maydis.*

Table1.1.The most common morphological expressions used in the names of drugs

amylum, -i	starch	lignum, -i	lignum, woody part
anthodium,-i	inflorescence	nux,	nuces nut

bacca, -ae	berry	oleum, -i	oil
capsula, -ae	capsule	petalum,	a petal
cortex, -icis	bark	pulvis	powder
flos, -ris	flower	Recens fresh	tender
galbulus, -i	Cone	Stigma-ae	stigma
herba, -ae	Grass;herb	tuber, -ecis	Tuber,bulb

(Imre Nemeth PhD,2012)

1.6.Active ingredients

In more recent textbooks, active ingredients (biologically active substances) are classified into a biogenetic system but due to practical reasons, their categorization may vary.

In the biogenetic system, substances are classified into the following five categories according to the five main metabolic pathways: saccharids, phenoloids, polyketides, terpenoids and azotoids. We hereby give a slightly more practical classification, used in most textbooks on medicinal and aromatic plants. In order to give a general overview and directions for practical application, this will suffice, given that the subject of the present textbook is not the chemistry of biologically active substances.

i.Saccharids, or carbohydrates. They are the primary products of photosynthesis. These natural organic compounds consist of carbon, hydrogen and oxygen atoms. They are vitally important for all human and animal organisms. Their anti-inflammatory properties are well-known.This category comprises different sugars, starch, mucilage, inulin, pectine and tree-gum. Sugar alcohols are derived from simple sugars, or monosaccharides (glucose, fructose). Derivatives consisting of two or more component sugars are called oligosaccharides. Derivatives consisting of more than six (or more than ten, according to some sources; classification is rather subjective) component sugars are called polysaccharides. They can be homo-polysaccharides, like starch that consists of glucose units, or inulin that consists of fructose. Products of the partial break-down of starch are called dextrines.

ii.Mucilage is often classified into this category although it may contain uronic acid besides simple sugars. Pectines are hetero-polysaccharides that consist of different kinds of simple sugars and contain some uronic acid. Tree-gum belongs to this category, which is usually produced as a result of pathological processes.

iii.Glycosides may well be classified under the group of carbohydrates because one or more sugar molecules (glucose, galactose, rhamnose, mannose) are bound to a non-carbohydrate moiety (aglycone). They are water-soluble, solid, usually crystalline, organic compounds. They are bitter and have a characteristic aroma, nitrogenous ones are more toxic. Glycosides regulate heart function, are diuretic, laxative and diaphoretic.

The aglycone bound to the sugar molecule can be:

- Alcoholic or phenolic OH group (O-glycosides).
- Organic carbon atom (C-glycosides).
- Thioalcohol (S-glycosides).
- Amin (N-glycosides).
- Their classification according to the aglycone part is as follows:
- Simple phenolic glycosides (arbutin, salycin, populin, primverin, etc.).

iv.Cyanogenic glycosides. Phytogenic hydrogen cyanide (prussic acid) is toxic. Such examples are amigdalin in almond, peach and abricot, durrin in sorghum and Sudan grass.

v.Anthraquinone glycosides. They are special substances with a laxative effect, such as the active ingredients in senna and rhubarb, as well as glucofrangulin.

vi.Steroidal glycosides or cardiac glycosides. These molecules are bound to a steroidal nucleus and contain a 5- or 6-membered lactone ring. These glycosides are found in the plant genera Digitalis, Helleborus, and Adonis.

vii.Thioglycosides (Isothiocyanates). They are often volatile compounds, like the glycoside of mustard oil or sinalbin, sinigrin in crucifers, which break down into allyl izothiocyanate, sulfur, and nitrile.

viii.Indoglycosides. They often have a bitter taste, e.g., the active ingredients of some bitter materials.

- Some classifications mention indoglycosides, such as indigo.
- Some lists mention non-nitrogenous glycosides, among them saponins and plant dyes. They are classified according to another system. They are the following:

ix.Alkaloids. They are natural compounds that contain nitrogen and combine with acids into salts. They have very strong effects. Compounds which contain nitrogen in the heterocycle and originate from amino acids are called true alkaloids. Protoalkaloids are compounds that also

originate from amino acids but contain nitrogen in an aliphatic chain. Pseudoalkaloids are alkaloid-like compounds that do not originate from amino acids but contain nitrogen.

They are strong poisons, usually affecting the nervous system. They are stimulant, excitant, stupefacient and analgesic. Alkaloids are classified into major groups by their structure but we will rather list them by their common natural source, e.g., the given plant families.

- **Solanaceae.**Atropa belladonna, Datura stramonium, Hyoscyamusniger: hyosciamine, atropine, scopolamine, belladonnine; Solanum dulcamara: tomatidenol, solasodine and soladulcidine; Solanum nigrum: solanidine, Nicotianatabacum: nicotine, pyrrolidine.
- **Papaveraceae**. Papaver somniferum: morphine, codeine, narcotine, thebaine, papaverine. Chelidoniummajus: chelidonin, chelerithrine, protopine; Papaver rhoeas: rhoeadine; Glauciumcorniculatum: glaucine.
- **Liliaceae.**Protoveratrine, colchicine and tulipin, which is similar to aconitin.

x.Essential oils. Essential oils are always mixtures and never homogeneous, therefore their classification is purely practical, e. g., ethereal oils, terpenes, camphors. They can be extracted by steam distillation; they are usually lipophilic and not miscible in water; they are nitrogen-free. They are digestant and bactericid. Solid or soft resins are produced from the liquid balm after the essential oil has been volatilised. Substances that are produced from essential oils usually by freeze distillation are called “camphors” in Hungary. . (P. P. Joy, J. Thomas,1998)

1.7.Characteristic ingredients of essential oils:

i..Monoterpenesthat contain ten carbon atoms, most of which originate from geranyl-pirophosphate. Open-chain monoterpenes, e. g., myrcene, ocymene. Their alcohol derivatives are linalool and geraniol, their aldehyde derivative is citral. Cyclic monoterpenes such as menthol and carvone are produced by the cyclisation of the proto-compound.

ii..Sesquiterpenescontain 15 carbon atoms; farnesol is an open-chain and camasulen is a cyclic sesquiterpene.

iii.Non-terpene compounds, terpene intermediates, phenyl propane derivatives such as cinnamic aldehyde, anethole, asarone, methyl chavicole, etc.

iv.Tannins (tannic acid, tannin). Their composition is complex; nowadays the name is used as a collective term. Some of them are derivatives of gallic acid or its derivative ellagic acid

and D-glycose (the glycoside of glycose combined with tannic acids), others are catechin derivatives. Catechin tannic acids are often red, they are called phlobaphenes. The term "tannic acid" can be misleading because most of them do not contain a carboxyl group. Their name comes from their being used for "tanning" by the leather industry. They are chemically heterogeneous phenoloids. They have an acrimonious taste and they are water-soluble. They are astringent, haemostatic and helpful in treating enteritis. The most common plants that contain high levels of tannic acid are oak, birch, heather and horse-chestnut.

v.Bitter materials. Their composition is unclear. They are partly water-soluble, bitter tasting, nitrogen-free substances. They are used for flavouring, preserving and colouring, they are appetitive and digestant.

vi.Organic acids. These compounds can be found in almost all medicinal herbs. The most common organic acids are oxalic acid, citric acid, malic acid, tartaric acid, formic acid, amber acid. Salicylic acid is febrifuge, silica acid strengthens the immune system. Silica acid is contained in horse-tail, lung-wort, knot-grass, hemp-nettle, elm bark, etc.

vii.Fat, fatty acids, waxes. Fats and fatty acids are contained mostly in fruits. Such fruits are cocoa beans, coconut, castor-oil bean, linseed, sunflower seed, etc. They differ from essential oils in that they are not volatile and are easy to dissolve in organic solvents (benzine, ether, chloroform, etc.).

viii.Plant dyes, flavonoids. They are substances of various structures, often bound to sugars, therefore they can also be classified as glycosides. This category comprises flavonoids, isoflavonoids, neoflavonoids, flavones, flavanones, anthocyanidin, proanthocyanidin, apigenin, silybin, chlorophyll and carotenoids. The pharmaceutical and chemical industries use them for their colouring properties. Biologically active flavonoids such as the antispasmodic apigenin and the liver-protecting silybin are often called bioflavonoids.

ix.Milky latex. It is essentially an emulsion of the cell-fluids. It is white or yellow and may contain essential oils, resin and alkaloids. In water it swells into a sticky solution or a sticky mass. Chemically they are not uniform substances. Milky latexes of euphorbia, poppy, composite and asclepiad species are all of differing composition.

x.Vitamins. They are substances of different chemical compositions that are indispensable for the normal functioning of the body. Their deficiency causes diseases.

xi.Antibiotics. According to more recent research, they occur not only in low plant forms but in some high plants as well, among others in garden-cress and other pepper wort species (Lepidiumcrassifolium or cartilegineum), horse-radish and celery. Antibiotics inhibit the growth and reproduction of micro-organisms and sometimes they even kill them. The best

known are phytoncydes, from which allicin is contained in garlic. It has a very strong bactericidal effect, it kills even tubercle bacilli.

1.8.The function of active ingredients in the plant kingdom

i.Growth regulation. E. g., the inhibition of the development of a given tissue structure, the impeding of leaf growth, the reduction of the cross-section of carrier tufts, the inhibition of germination.

ii.Protective function. It only appears in plants attacked by some fungus. Their effect mechanism is not yet clear.

They inhibit intake (antifeddants). E. g., the azotoids of the Solanaceae family inhibit to varying degrees the growth and viability of Colorado beetle larvae. The larvae grow properly and are viable on potato and tomato, while their mortality rate is much higher on related wild species, although the species survives.

iii.Repellents. E. g., essential oils of lavender flowers are repellents, that is why they are often used as moth repellents.

iv.Insecticidal effect. E. g., nicotine in tobacco (Nicotianatabacum), which was in effect used to make insecticides. It has such strong effects that nicotine-based insecticides have been banned for decades (except in the United States). The effect of pyrethrum (Chrysanthemum cinerariaefolium) was already known to the Romans and they used it as a flea powder. Since warm-blooded organisms are unaffected by it, its use is very safe. Unfortunately the active substance is unstable in light, therefore agrochemical producers manufacture pyrethrum-based insecticides with a stabilizing agent.

v.Attracting enemies of pests. In the case of bean and maize, they found compounds that were secreted by the plants after pest damage and sent signals to enemies of the pests. Before pest damage, these active ingredients are untraceable in the undamaged plant. Western corn

rootworms that appeared and proliferated in the last decade in Hungary are trapped by Cucurbitacin traps that are produced from Cucurbitaceae.

vi.Allelopathy. Substances occurring in plants affect the growth and development of other plants and lower organisms. These substances are evaporated by plants, secreted through the roots, washed off by precipitation or they evolve from decayed plant parts. E. g., the foliage of walnut inhibits plant growth and germination, just like goldenrod and couch grass.

vii.Attractants (attractive substances). The repellent lavender is an attractant towards species that pollinate it. Similarly, glycosides of mustard species are repellent to most insect pests but are attractant to cabbage-butterfly.

viii.Reserve nutritives. Especially polysaccharides, like mucilage, which can accumulate up to more than 30% in the roots of e. g., marsh mallow. (P. P. Joy, J. Thomas,1998)

1.9. Antimicrobial screening

Antimicrobial screening is performed to determine the susceptibility of the pathogenic microorganisms to test compound which, in turn is used to selection of the compound as a therapeutic agent. In general, antimicrobial screening in-vitro is undertaken in following two steps:

i) **Primary assay**It is essentially a qualitative or semi qualitative test that indicates the sensitivity or resistance of microorganisms to the compound. However this technique cannot be used to distinguish between bacteriostatic and bactericidal agents (Reiner et al. 1982). The primary assay can be performed in vitro by disk diffusion assay method, which includes

- Plate Diffusion test
- Streak test

The plate diffusion test utilizes different concentrations of a test compound absorbed on sterile filter paper disks on the same plate whereas the streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and is suitable for the estimation of the spectrum of the activity. However, the plate diffusion test is commonly used (Reiner et al. 1982).

ii) **Secondary assay** It quantifies the relative potency such as minimum inhibitory concentration (MIC). The lowest concentration of an antimicrobial agent required to inhibit the growth of the microorganisms in vitro is referred to as minimum inhibitory concentration (MIC). It is done by serial dilution technique (Reiner et al. 1982).

1.10.Antioxidant activity

The main goal of antioxidant activity test is to find the oxidation- reducing power of the plant extract. Oxidation in living organisms is essential for the acquirement of energy in catabolism. However, oxygen-centered free radicals and other reactive oxygen species, which are continuously, produced in vivo result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging, and diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell & Gutteridge 1999).

Free radicals are natural by-products of human metabolism. These are charged molecules which attack cells, breaking cellular membranes and reacting with the nucleic acids, proteins, and enzymes present in the cells. These attacks by free radicals, collectively known as oxidative stress, are capable of causing cells to lose their structure, function and eventually result in cell dysfunction. They are continuously produced by our body's use of oxygen, such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air pollution, pesticides, etc (Li &Trush 1994). Normally, there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems which scavenge these free radicals preventing them from causing deleterious effects in the body (Nose 2000). The antioxidant defense systems in the body can only protect the body when the quantity of free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental conditions or infections, it leads to oxidative stress (Finkel& Holbrook 2000).

When the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system, oxidative stress occurs in cellular system, including the superoxide anion radical, the hydroxyl radical, hydrogen peroxide and the peroxyl are greatly reactive molecules, which consequently generate metabolic products that attack lipids in cel1 membrane or DNA (Halliwell&Gutteridge 1999). Oxidative stress, involves a series of free radical chain reaction processes, is associated with several types of biological damage, DNA damage, diabetes,

respiratory tract disorders, carcinogenesis and cellular degeneration related to aging (Anderson et al. 2000). Continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng et al. 1997). Improved antioxidant status helps to minimize the oxidative damage and thus can delay or decrease the risk for developing many chronic age related, free radical induced diseases (Karuna et al. 2009). The interest in natural antioxidants, especially of plant origin, has greatly increased in recent years as the possibility of toxicity of synthetic antioxidants has been criticized. Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Zheng & Wang 2001). Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Owen et al. 2000).

1.11.Plant Reveiw

- *Phyllanthus acidus i*s commonly known as star gooseberry.
- It is quite a common tree found in the tropics and belongs to the plant family euphorbiaceae.
- *P. acidus* is consumed as herbs by the Indian tribal for remedy of gastro intestinal tract disorders (Supratic Kunder et al.,).
- *Phyllanthus sps* has long been used in folk medicine in many countries as antimicrobial and / or antioxidants (I.M.S Elden et al., 2010).
- *Phyllanthus acidus* leaf extract have antioxidant, analgesic and anti-inflammatory activities (Raja chakraborthy et al.).

The phyto therapeutic can provides many modern drug development can provides many invaluable drugs from traditional medicinal plants. Search for pure phytochemicals as drug is time consuming and expensive. Numerous plants and poly herbal formulations are used for the treatment of liver diseases. World plant biodiversity is the largest source of herbal medicine and still about 60-80% world population rely on plant based medicines which are health care system. India is endorsed with a rich wealth of medicinal plants, which ranked our country in

the list of top producers of herbal medicine. Based on this background the present study was intended to screen the plant *Phyllanthus acidus* ((I.M.S Elden et al., 2010).

Figure 1.4.*Phyllanthus acidus* (Orwa et al.2009)

1.11.1 Taxonomy

Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Order: Malpighiales

Family:Phyllanthaceae

Genus:Phyllanthus

Species*: P.acidus*(I.M.S Elden et al., 2010).

1.11.2.Description

- This is a curious and ornamental shrub or tree,
- 6 1/2 to 30 ft (2-9 m) high, with spreading, dense, bushy crown of thickish, rough, main branches, in general aspect resembling the Bilimbi .
- At the branch tips are clusters of deciduous, greenish or pinkish branchlets 6 to 12 in (15-30 cm) long, bearing alternate, short-petioled, ovate or ovate-lanceolate, pointed

leaves 3/4 to 3 in (2-7.5 cm) long, thin, green and smooth on the upper surface, blue-green with a bloom on the underside; altogether giving the impression of pinnate leaves with numerous leaflets.

- There are 2 tiny, pointed stipules at the base of each leaf. Small, male, female, and some hermaphrodite, 4-parted, rosy flowers, are borne together in little clusters arranged in panicles 2 to 5 in (5-12.5 cm) long,
- Flower hanging directly from leafless lengths of the main branches and the upper trunk, and the fruits develop so densely that they form spectacular masses.
- The fruit is oblate with 6 to 8 ribs; is 3/8 to 1 in (1-2.5 cm) wide; pale-yellow to nearly white when fully ripe; waxy, fleshy, crisp, juicy and highly acid.
- Fruit is tightly embedded in the center is a hard, ribbed stone containing 4 to 6 seeds.
- Gooseberry tree is a deciduous tree with an open, sparingly branched, spreading crown; it can grow about 6 - 9 metres tall.
- The short bole can be 15cm in diameter
- The tree is occasionally cultivated as a garden plant in tropical areas for its fruit and as an ornamental.
- The fruit is sometimes sold in local markets(I.M.S Elden et al., 2010).

1.11.3. Leaves

- 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(I.M.S Elden et al., 2010).

1.11.4. Flower and fruit

- 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 a the center of each fruit.(I.M.S Elden et al., 2010).

Figure1.3. leaves ,fruit,, flower of *Phyllunthus acidus*

1.11.5.Local Name

Burmese (thinbozihpyoo); English (country gooseberry,stargooseberry,plum, Otaheitegooseberry,damsel, Malay gooseberry); Filipino (karmay,bangkiling,iba); French (cerisier de Tahiti); Indonesian (cerme,ceremai,caramele); Lao (Sino-Tibetan) (maknhom,nhombaanz,nhom ban); Malay (kemangul,chermala,chermai); Spanish (grosella); Thai (ma rom); Vietnamese (t[aaf]m ru[ooj]t,ch[uf]m ru[ooj]t)(Eldenshary, E.S. (2003)

1.11.6.Documented Species Distribution

It is documented that the countries where the species neither can be planted in every ecological zone within that country, nor that the species can not be planted in other countries than those depicted. Since some tree species are invasive, one need to follow biosafety procedures that apply to the planting site. Native range Brazil, Colombia India, Indonesia, Laos, Madagascar, Malaysia, Myanmar, Philippines, Thailand, United States of America, Vietnam, Zanzibar. (Orwaet al.2009) .*Phyllanthus acidus* Euphorbiaceae (L.) Skeels.The map above shows countries where the species has been planted. It does neither suggest that the species can be planted in every ecological zone within that country, nor that the species can not be planted in other countries than those depicted. Since some tree species are invasive, you need to follow biosafety procedures that apply to your planting site. This species is believed to have originated in Madagascar and to have been carried to the East Indies. Quisumbing says that it was introduced, into the Philippines in prehistoric times and is cultivated throughout those islands but not extensively. It is more commonly grown in Indonesia, South Vietnam and Laos, and frequently in northern Malaya, and in India in home gardens. The tree is a familiar one in villages and on farms in Guam, where the fruit is favored by children, and occurs in Hawaii and some other Pacific Islands.It was introduced into Jamaica from Timor in 1793 and has been casually spread throughout the Caribbean islands and to the Bahamas and Bermuda. It has long

been naturalized in southern Mexico and the lowlands of Central America, and is occasionally grown in Colombia, Venezuela, Surinam, Peru and Brazil. Formerly an escape from cultivation in South Florida, there are now only scattered specimens remaining here as curiosities.(Eldenshary, E.S. (2003)

1.11.7.Cultivation

Climate

i.Otaheiti gooseberry grows well in the tropics at low and medium altitudes in places with a short or prolonged dry season.

ii.The tree prefers hot, humid tropical lowlands. In north-eastern Brazil, the tree has been found in coastal forest and in Southeast Asia it is cultivated on humid sites, up to 1 000 m altitude.

iii.TheOtaheite gooseberry is subtropical to tropical,

iv. being sufficiently hardy to survive and fruit in Tampa, Florida, where cold spells are more severe than in the southeastern part of the state.

v. In north-eastern Brazil, the tree has been found in coastal forest and in Southeast Asia it is cultivated on humid sites, up to 1 000 m altitude.

vi.TheOtaheite gooseberry is subtropical to tropical,

vii.being sufficiently hardy to survive and fruit in Tampa, Florida, where cold spells are more severe than in the southeastern part of the state.

viii.It thrives up to an elevation of 3,000 ft (914 m) in El Salvador.(Eldenshary, E.S. (2003)

Light requirement: plant grows in part shade/part sun

Soil tolerances: clay; sand; acidic; slightly alkaline; loam

Drought tolerance: high

Soil salt tolerances: poor

Plant spacing: 36 to 60 inches

Light: Bright light. Avoid direct sunlight in summer.

Water: Keep soil lightly moist spring through fall, slightly drier in winter. Do not let soil get waterlogged.

Humidity: Average room humidity

Temperature: Normal room temperatures. 60-75°F/16-24°**C**

Soil The tree grows on a wide range of soils but prefers rather moist sites.Tolerates a variety of soils including very sandy soils.(Eldenshary, E.S. (2003)

Altitude: 0-1 000 m Soil type: It tolerates a variety of soils including very sandy soils.(Eldenshary, E.S. (2003)

Fertilizer: Feed every 2 weeks in spring and summer with a 10-10-10 liquid fertilizer diluted by half.

Propagation The tree is generally grown from seed but may also be multiplied by budding, greenwood cuttings, or air-layers. Seedlings will produce a substantial crop in 4 years.(Eldenshary, E.S. (2003

Figure1.4. propagation of *P.acidus* (Eldenshary, E.S. (2003)

1.12.Flowering and fruiting season

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

explosively dehisce dispersing their seeds. The tree often bears two crops a year in South India, the first in April and May, and the second in August and September. In other areas, the main crop is in January with scattered fruiting 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.(Eldenshary, E.S. ,2003).As the fruit does not soften when ripe, it is harvested when the fruit begins to drop

1.13.Pest

The Otaheite gooseberry is prone to attack by the *phyllanthus caterpiller* in Florida. This pest eats the bark and also the young leaves, causing total defoliation in a few days if not controlled by pesticides.(P., et al. ,2013)

1.14.Human uses of *P.acidus;*

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.It contains 4-hydroxybenzoicacid, caffeicacid. adenosine, kaempferol and hypogallic acid.While the wood is strong and durable if properly treated, the tree is not large and is rarely harvested for wood.The wood is light-brown, fine-grained, attractive, fairly hard, strong, tough, durable if seasoned, but scarce, as the tree is seldom cut down.The root bark has limited use in tanning in India.(Eldenshary, E.S. (2003)

1.15.Medicinal Uses of *P.acidus*

In India, the fruits are taken as liver tonic, to enrich the blood. The sirup is prescribed as a stomachic; and the seeds are cathartic. The leaves, with added pepper, are poulticed on sciatica, lumbago or rheumatism. A decoction of the leaves is given as a sudorific. Because of the mucilaginous nature of the leaves, they are taken as a demulcent in cases of gonorrhea.The

syrup is used to medicate the stomach, and in India the fruit is eaten as a blood-enhancer for the liver.while the seeds are used as a cathartic and the root, if prepared with care, as a purgative .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), The root is drastically purgative and regarded as toxic in Malaya but is boiled and the steam inhaled to relieve coughs and headache.Extract from the plant has shown nematicidal activity against the pine wood nematode. The juice of the root bark is weakly poisonous. The latex is credited with emetic and purgative activity. In Indonesia the bark is heated with coconut oil and spread on eruptions on feet and hands. An infusion of the root is taken to alleviate asthma in Java. In Borneo, roots are used in the treatment of psoriosis of the feet.A leaf decoction is applied to urticaria, a decoction of the bark is used to treat bronchial catarrh in Philippines.The fruit is used as a laxative in Myanmar.The root infusion is taken in very small doses to alleviate asthma. Externally, the root is used to treat psoriasis of the soles of feet.The juice of the root bark, which contains saponin, gallic acid, tannin and a crystalline substance which may be lupeol, has been employed in criminal poisoning. (Eldenshary, E.S. (2003).

1.16.Aim of this experiment

Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi processed plant produce drugs and medicines. Thus huge foreign exchanges can be saved if the manufacturers, to satisfy their needs, utilize the indigenous medicinal plants or their semi processed products. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against the harmful diseases. The increasing failure of chemotherapeutics, severe adverse effects with increase doses and repeated use of drugs ,problems with multiple dosage regimens and antibiotic resistance exhibited by pathogenic microbial infectious agents and emergence of new diseases has led to the screening of medicinal plants throughout the world for their potential activity. The main objective of this study was to discovery of new medicinal compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. *Dracaena spicata* is a medicinal plant used traditionally in Bangladesh. Upon significant literature survey it was found only a little research work has been performed on this plant to evaluate its medicinal value and active constituents those are responsible for its pharmacological activities. Therefore, taking into consideration the traditional uses of the plant

and facilities available for conducting the study, this research work was performed on this plant. The principal aim of the present study was to investigate the scientific basis of the traditional uses of the plant. The methanolic extract of *Dracaena spicata* to evaluate their in- vitro pharmacological activities (like antioxidant, antimicrobial).

1.16.1: Study Area: The research was carried out in the Research Lab, Microbiology Lab and Pharmacognosy Lab of Department of Pharmacy, East West University, Dhaka

1.16.2: Data Collection: All the relevant data has been collected from two types of sources:

i. **Primary sources:** direct personal contact and observations of the experiments carried out in the laboratory.

ii. Secondary sources: various publications like journals, papers, documents and websites

Chapter Two
Literature Review

Literature Review

2.1.Antimicrobial Activity and phytochemical analysis *of Phyllanthus acidus*

Various medicinal plants have been used for years in daily life to treat disease all over the world. In this present study focus the antimicrobial and phytochemical activity *of Phyllanthus acidus* leaf and fruit extracts obtained from different extracts (methanol, ethyl acetate and Diethyl ether) methanol extracts of the *Phyllanthus acidus* showed highest toxicity.

- A qualitative phytochemical analysis was performed for the detection of alkaloids, flavonoids, steroids, terpenoids, anthroquinones, phenols, saponins, tannins, carbohydrates, oils and resin
- *Phyllanthus acidus* is commonly known as star gooseberry. It is quite a common tree found in the tropics and belongs to the plant family euphorbiaceae. *P. acidus* is consumed as herbs by the Indian tribal for remedy of gastro intestinal tract disorders (Supratic,Kunder et al.,).
- *Phyllanthus sps* has long been used in folk medicine in many countries as antimicrobial and / or antioxidants (I.M.S Elden et al., 2010). *Phyllanthus acidus* leaf extract have antioxidant, analgesic and anti-inflammatory activities (Raja chakraborthy et al.).
- The phytotherapeutic can provides many modern drug development can provides many invaluable drugs from traditional medicinal plants.
- Search for pure phytochemicals as drug is time consuming and expensive. Numerous plants and polyherbal formulations are used for the treatment of liver diseases.

World plant biodiversity is the largest source of herbal medicine and still about 60-80% world population rely on plant based medicines which are health care system. India is endorsed with a rich wealth of medicinal plants, which ranked our country in the list of top producers of herbal medicine. Based on this background the present study was intended to screen the plant *Phyllanthus acidus* (leaf and fruit) phytochemical analysis and antimicrobial activity. Screening of antibacterial activity Bacteria tested totally five bacterial strains were used throughout investigation namely *Proteus vulgaris,Shigella boydii,Shigella flexneri, Klebsiella aerogenes* (Gram negative), *Corney bacterium* (Gram positive). All the bacterial cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The young bacterial broth cultures were prepared before the screening procedure. Preparation of inoculums Stock cultures were maintained at 4oC on slopes of

nutrient agar. Active cultures of experiment were prepared by transferring a loopful of cells from the stock cultures to test tube of Muller-Hinton broth (MHB) for bacteria that were incubated without agitation for 24 hrs at 37oC and 25oC respectively. The cultures were diluted with fresh Muller-Hinton broth to achieve optical densities corresponding to 2.0 . 106 colony forming units (CFU/ml) for bacteria.(R.C. Jagessar, A.Mars,2008)

2.2.Antimicrobial susceptibility test

The disc diffusion method (Bauer et al., 1966) was used to screen the antimicrobial activity. Invitro antimicrobial activity was screened by using Muller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes and 0.1% inoculums suspension was swabbed uniformly and the inoculums were allowed to dry for 5 minutes. The concentration of extracts is 40 mg/disc was loaded on 6 mm sterile disc. The loaded disc was placed on the surface of medium and the extract was allowed to diffuse for 5 minutes and the plates were kept for incubation at 37oC for 24 hrs. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter.

Results

The percentage of yield of each extract determined. The methanol extract of leaf and fruit showed the maximum yield. Leaf and fruit of *the P. acidus* methanol extract was found the most potent extract against the bacteria.

Table2.1. Percentage of yield obtained in leaf extracts *Phyllanthus acidus*

EXTRACTS	YIELD OBTAINED (Leaf)	PERCENTAGE OF YIELD (Leaf)
Methanol	9.86	49.3%
Ethyl acetate	3.94	19.7%
Diethyl ether	0.95	4.75%

(A. Jagajothi, G.Manimekalai,2013)

Table 2.2. Antimicrobial activity of *Phyllanthus acidus*

MO	C	Leaf of *P.Acidus*
P. vulgaris	21	9

C. bacterium	10	6
K. aerogenes	29	12
S. boydii	19	16
S. flexneri	19	9

(A. Jagajothi, G.Manimekalai,2013)

Phytochemical screening correlated Alkaloids, flavonoids, steroids and phenols are present in leaf extract. Terpenoids, Anhtroquinone, saponin and tannin are absent in leaf. Leaf extract show better activity when compared to fruit extract. Phytochemical constituents such as alkaloids, flavonoids, phenols and several other aromatic compounds are secondary metabolites of plants that serve a defense mechanism against many microorganisms. In this study P. acidus leaf extract was found more antioxidant than the fruit extract. Leaf extract shows effective result than the fruit extract. Of the three solvents, methanol extract reveals the presence of maximum phytochemical constituents. The antimicrobial assay also proves that the leaf extract obtained high concentration of yield. The presence of alkaloids in the solvent fractions could be well correlated with the antimicrobial activities. Phytochemicals possesses specific physical, chemical and biological activities that make them useful as drugs (Nathiya and Dorcus, 2012).

2.3.Selective Antimicrobial properties of Phyllanthusacidus leaf extract against Candida albicans, Escherichia coli and Staphylococcus aureus using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method

The microbes studied are Eschericia coli, Staphylococcus aureus and Candida albicans. Escherica. coli can cause several intestinal and extra intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia. Staphylococcus aureus can cause furuncles (boils), carbuncles (a collection of furuncles)22. In infants, Staphylococcus aureus can cause a severe disease Staphylococcal scalded skin syndrome (SSSS). Staphylococcal endocarditis (infection of the heart valves) and pneumonia may be fatal. Candida Albicans is a diploid fungus (a form of yeast) and is a casual agent of opportunistic oral and genital infections in humans.

The antibacterial and antifungal activities of *Phyllanthusacidus* was investigated against *S.aureus (*gram+ve*), E.coli* (gram-ve) and*C.albicans* using the Stokes disc diffusion, the Pour plate, Well diffusion and Streak plate methods. The solvent type extracts were obtained by

three extractions with hexane, CH2Cl2, EtOAc and CH3CH2OH respectively. Solvents were removed in vacuo to yield viscous oils and paste which were made up to a concentration of 0.035g in 0.01L(10 mL)of the respective solvents. These were tested in varying volumes of 0.2-0.6 ml/plate. The solvents were used as control whereas ampicillin and nystatin were used as references for bacteria and fungal species respectively. The solvents had no effect on the microorganisms whereas ampicillin and nystatin inhibited microbial growth. *Phyllanthus acidus* showed antimicrobial inhibitory activity at 0.18mg/10mL plate of medium with activity most prominent with the ethanol extracts and negligible with the hexane. This study suggests that the ethanol extracts of *Phyllanthus acidus* ,can be used as herbal medicines in the control of *E.coli* and *S.aureus* following clinical trials.(Nature and Science. 2008)

This paper discusses the antimicro biological (antibacterial and antifungal) activity of leaves of *Phyllanthus acidus* also known as gooseberry from the coastal plain of the Guyana flora and its possible use as an herbal cream/herbal medicine. Its antimicrobial properties were investigated against *S.aureus* (gram+ve*), E.coli* (gram-ve) and *C.albicans* strains using the Stokes disc diffusion sensitivity technique, Well diffusion, Streak plate and a dilution method. An antimicrobial is a compound that kills or inhibits the growth of microbes such as bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity) or parasites (anti parasitic activity). Guyana has a rich bio diversified flora whose crude extracts, both organic and aqueous can be investigated for their antimicrobial activity. Also, the extracts of the specified plants parts of the same species, fractionated for natural products whose antimicrobial activity can also be correlated with that of crude extracts. Following this, clinical trials can lead to the formulation of an herbal plant cream or herbal medicine. A few herbal medicine shops have now been established in Guyana. Plants extracts and fractionated plant extracts have been used for their antimicrobial properties. Besides used as an herbal cream, following clinical trials, crude plant extracts can be chromatographed leading to the isolation and purification of new and known bioactive natural products/phytochemicals

In Guyana, there are many medicinal folklore practises but most are without scientific research. Its our scientific endeavour, to correlate antimicrobial activity of *Phyllanthus acidus* with its folklore practices. In Guyana's traditional medicine, an infusion of the herb is taken for the relief of dysentery and also as a blood purifier (bitter tonic to reduce blood sugar level). Also, an infusion or tea for women who are dieting and wish to remain slim. However, little is known of the antimicrobial properties of *Phyllanthus acidus.*(R.C. Jagessar, A.Mars,2008)

In vitro pharmacological investigation on aqueous fraction of *Phyllanthus acidus* Leaf

Table 2.3. Antimicrobial activity of Plant extract as shown by the inhibition zone diameter.

Area of inhibition. (mm2) using *E.Coli*	Area of inhibition. (mm2) using *S.aureus*	Area of inhibition. (mm2) using *Candida albicans*	Plant Extracts *Phyllanthus acidus*	Reference compound (Ampicillin) (mm2	Control Experiment
<5	<5	<5	Hexane extract	27	No zone of inhibition
<5	<5	<5	CH2Cl2 extract	28	No zone of inhibition
20	15	18	EtOAc extract	28	No zone of inhibition
22	21	20	CH3CH2OH extract	30	No zone of inhibition

(R.C. Jagessar, A.Mars,2008)

2.4.Neuroprotective Effect of *Phyllanthus acidus* L. on Learning and Memory Impairment in Scopolamine-Induced Animal Model of Dementia and Oxidative Stress

Nature is the best source of complementary and alternative medicine.

- The plant *Phyllanthus acidus (PA*) L. has been used traditionally in pain, inflammatory and oxidative stress related disorders.
- Methanolic extract of PA (MEPA) was selected to explore the ability of this plant to enhance cognitive function, brain antioxidant enzymes and anti-acetylcholinesterase

activity which can be used for the treatment of oxidative stress related disorders like Alzheimer's disease (AD).

- The purpose of this study was to investigate the neuroprotective effect of MEPA on learning and memory impairment in scopolamine-induced rats of dementia and oxidative stress. Treatment with MEPA (i.e., 100 and 200 mg/kg b.w.) was investigated in scopolamine-treated Swiss albino male rats for 14 days and its neuroprotective effects were examined using Elevated Plus Maze (EPM) test, Passive Avoidance (PA) test, Novel Object Recognition (NOR) test, Morris Water Maze (MWM) test as well as level of antioxidant enzymes such as catalase (CAT), super oxide dismutase (SOD), glutathione reductase (GSR), glutathione-S-transferase (GST), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), lipid peroxidation (TBARS) contents and acetylcholinesterase (AChE) activity in rat brain tissue homogenates. Administration of MEPA significantly ($P < 0.05$, $P < 0.01$; $P < 0.01$) decreased RTL (retention transfer latency) in rats on 7th and 14th day compared to the disease control and control group in the EPM test. In PA test the doses of MEPA suggestively ($P < 0.05$, $P < 0.001$; $P < 0.05$, $P < 0.01$) increased STL (step-through latency) in rats on 7th and 14th day with respect to disease control and control group. For NOR test administration of MEPA considerably ($P < 0.01$, $P < 0.001$; $P < 0.01$) increased the DI (discrimination index) in rats with respect to that of disease control and control group. The doses of MEPA markedly ($P < 0.05$, $P < 0.01$; $P < 0.01$) decreased EL (escape latency) and significantly ($P < 0.01$, $P < 0.001$; $P < 0.05$, $P < 0.01$) increased TSTQ (time spent in the target quadrant) on successive days as compared to that of disease control and control group in the acquisition trial of MWM test. In case of probe trial of MWM test MEPA administration considerably ($P < 0.01$; $P < 0.05$, $P < 0.01$) increased TSTQ and significantly ($P <0.05$, $P < 0.01$; $P < 0.05$, $P < 0.01$) increased TSA (time spent in the annuli) in rats on successive days as compared to that of disease control and control group. MEPA administration significantly ($P < 0.05$, $P < 0.01$, $P < 0.001$; $P < 0.05$, $P < 0.01$) increased the level of CAT, SOD, GSR, GST GSH, GSH-Px and markedly ($P < 0.01$; $P < 0.01$, $P < 0.001$) decreased TBARS level through inhibiting lipid peroxidation as well as significantly ($P < 0.01$, $P < 0.001$; $P < 0.05$, $P < 0.01$, $P < 0.001$) decreasing AChE activity in rats brain compared to the disease control and control group. The present study demonstrates that MEPA showed the neuroprotective effect by improving cognitive functions and reduces oxidative

stress by increasing the level of brain antioxidant enzymes as well as decreasing lipid peroxidation and acetylcholinesterase activity.

- Therefore, this plant extract can be used for enhancing learning, memory, antioxidant potentiality and anti-acetylcholinesterase activity in neurodegenerative disorders like AD.
- The plant *Phyllanthus acidus (PA)* L. is known in Bengali as Orbori belongs to the family Phyllanthaceae has been explored for cognitive activity .
- This plant has been used traditionally in the treatment of several pain, inflammatory and oxidative stress related disorders such as fever, rheumatism, bronchitis, asthma, respiratory disorder, hepatic disease, diabetes, gonorrhea and gastrointestinal tract disorders .
- Phyllanthus genus is highly enriched in various phyto constituents like alkaloids, phenolics, flavonoids, tannins, terpenes and lignans. Different parts of PA have been reported for excellent medicinal properties.
- The leaves of the plant stated to possess antihypertensive, antimicrobial, hepato protective activity and also used as an antidote to viper venom .
- The root and seed of the plant are useful as cathartic, bark and roots are used to treat fever traditionally.
- The latex of the plant is recognized with purgative and emetic activity . The fruits are used as memory enhancer, blood purifier, appetite stimulant, relief of coughing and preventive action against diabetes .
- Previous studies showed that fruits of this plant showed antioxidant, memory enhancing, anti-cholinesterase, astringent , hepato protective, cytotoxic and antimicrobial activity .

Therefore this study was designed to investigate the neuroprotective effect of MEPA on learning and memory impairment in scopolamine-induced rats by behavioral studies such as Elevated Plus Maze (EPM) test, Passive Avoidance (PA) test, Novel Object Recognition (NOR) test, Morris Water Maze (MWM) test as well as the activity of antioxidant enzymes by biochemical studies such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSR), glutathione-S-transferase (GST), reduced glutathione (GSH), estimation of lipid peroxidation (TBARS) and acetylcholinesterase (AChE) activity in rat brain tissue homogenates.(Md. Sahab Uddin1, Abdullah Al Mamun,2016)

2.5.An extract from the medicinal plant *Phyllanthus acidus* and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease with high frequency among the Caucasian population. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. One mutation alone, F508del-CFTR is present in at least one allele in approximately 90% of CF patients (Bobadilla et al., 2002). CF is characterized by deficient Cl- transport and enhanced airway Na+ absorption, mediated by epithelial Na+ channels (ENaC) along with other abnormalities in ion transport. Pharmacological interventions attempt to correct defective ion transport among other pulmonary phenotypes. Recent strategies make use of natural food components because of their ready accessibility and low toxicity (de Carvalho et al., 2002; Bjarnsholt et al., 2005; Egan et al., 2004). These compounds act in different ways, such as correcting the trafficking defect of mutant CFTR or potentiating residual CFTR activity (Moran & Zegarra-Moran, 2005; Kunzelmann& Mall, 2003; Van Goor et al., 2006).According to previous reports flavonoids and nutraceuticals correct defective electrolyte transport in cystic fibrosis (CF) airways. Traditional medicinal plants from China and Thailand contain phyto-flavonoids and other bioactive compounds. We examined herbal extracts of the common Thai medicinal Euphorbiaceous plant *Phyllanthus acidus (P. acidus*) for their potential effects on epithelial transport. Functional assays by Ussing chamber, patch-clamping, double electrode voltage-clamp and Ca2+ imaging demonstrate activation of Cl- secretion and inhibition of Na+ absorption by *P. acidus*. No cytotoxic effects of *P. acidus* could be detected.

Mucosal application of *P. acidus* to native mouse trachea suggested transient and steady-state activation of Cl- secretion by increasing both intracellular Ca2+ and cAMP. These effects were mimicked by a mix of the isolated components adenosine, kaempferol, and hypogallic acid. Additional experiments in human airway cells and CFTR expressing BHK cells and Xenopusoocytes confirm the results obtained in native tissues. Cl- secretion was also induced in tracheas of CF mice homozygous for F508del-CFTR and in F508del-CFTR homozygous human airway epithelial cells. Taken together, *P. acidus* corrects defective electrolyte transport in CF airways by parallel mechanisms including

- increasing the intracellular levels of second messengers cAMP and Ca2+, thereby activating Ca2+ - dependent Cl- channels and residual CFTR-Cl- conductance;
- stimulating basolateral K+ channels;
- redistributing cellular localization of CFTR;
- directly activating CFTR; and
- inhibiting ENaC
- through activation of CFTR.

These combinatorial effects on epithelial transport may provide a novel complementary nutraceutical treatment for the CF lung disease.The extract of the traditional medicinal plant *Phyllanthus acidus (P. acidus;*) has been shown to be enriched with adenosine (Cohen et al., 1997). Therefore, we have assessed the effects of this extract on the adenosine receptor system in mouse airways and in human airway epithelial cells. In particular, effects on A1 and A2B receptors were examined using pharmacological inhibitors 8-SPT, alloxazine, and DPC-PX. Stimulation of these receptors has been demonstrated to activate both Ca2+ dependent and cAMP (CFTR) regulated Cl- channels and to affect the epithelial Na+ channel ENaC, while other were unable to detect effects of adenosine on Cl- secretion in CF tissues (Clancy et al., 1999). Apart from adenosine, P. acidus also contains other components, which are likely to affect electrolyte transport in the airways, such as the flavonoid kaempferol and 2,3-dihydroxybenzoic acid (DHBA) (Li & Wang, 2004; Illek& Fischer, 1998). We compared the effects of *P. acidus* with the effect of commercially purchased adenosine, kaempferol and DHBA and dissected out the underlying signaling pathways and the conductances affected. The present data indicate that extracts from *P. acidus* activate electrolyte secretion in epithelial tissues by means of intracellular second messengers and by directly increasing membrane expression and activity of ion channels. Thus, medicinal plant extracts from *Phyllanthus acidus* may represent a novel and effective tool to correct defective electrolyte transport in CF(Marisa Sousa, Jiraporn Ousingsawat,2006)

2.6.Anticancer activity

Leaves of *Phyllusthus acidus* have shown anticancer activity. Prepare cermai young leaves as as 1/4 handheld, handheld third starfruit leaves, lote upas halffinger, half finger chinese yam, palm sugar 3 fingers, washed and cut into pieces as needed. The material was then boiled in 3 cups water until 3/4 parts left behind. After chilling filtered, ready to be drunk. 3 times a day.(LaFerla, F.M. (2010)

2.7.Literature review Phytochemicalanalysis antimicrobialscreening andantihelminthic property *Phyllanthus emblica*

In our study, preliminary phytochemical analysis, antimicrobial and antihelminthic investigation on *Phyllunthus emblica* was done. *P.emblica* is a well known and an important medicinally valued plant. Although the efficacy of *P.emblica* fruit is widely proved, use of leaf and bark is less investigated. Present aim of our study is to find the biologically active compound present in this particular plant, check its antimicrobial and antihelminthic property. Different solvent extract of the leaf and bark were used to identify the bioactive compounds present and its antimicrobial activity was checked against different human pathogens(MTCC).Anti helminthic activity was checked against Phertimaposthuma. The study shown it has promising antimicrobial and anti helminthic property. Methanol of the leaf sample showed highest zone of inhibition against Enterobacter aerogens and Enterobacter feacalis. Ethyl acetate of leaf and bark sample showed antifungal activity against Rhizomucor species.The pharmacological property of this medicinally important plant has to be further investigated.(Sukanya, M.K, Shimi Suku,2013)

2.7.1.Anti microbial screening

The crude extract of the plant and drug were tested for antimicrobial activity (antibacterial and antifungal) against strains of pathogenic microbes (MTCC).Drug like ciprofloxacin(3 µg) and kanamycin(15µg)and DMSO were used as controls. Antibacterial activity of samples against human pathogens The crude extract of the plant and drug were tested for their antibacterial activity by disc diffusion method against pathogenic organism like *Pseudomonas aeroginosa, Proteus vulgaris, Klebsiella pneumonia, Enterobacter aerogens, Bacillus megaterium, Pseudomonas putida, Lactococcus lactis, Bacillus substilis, Enterobacter faecalis, Escherichia coli.* Prepared nutrient agar plates were inoculated with pathogenic organism(0.1ml) by spread plate method. Whatmans no.1 filter paper disc were sterilized and inoculated with the sample and DMSO is kept as negative control After incubatione at 30oC for 24 hours zone of inhibition was measured. Antifungal activity of samples against human pathogens The crude extract of the plant and drug were tested for their antifungal activity by disc diffusion method against pathogenic organism like *Aspergillus fumigates, Aspergillus niger, Candida albicans , Candida glabrata, Candida tropicalis , Rhizomu cormiehei*. Prepared Rose Bengal Agar plates were inoculated with pathogenic organism by spread plate method .Whatmans filter

paper were sterilized and inoculated with sample and DMSO were kept as negative controls. After incubation at37oC for two days the zone of inhibition was measured.(Sukanya, M.K, Shimi Suku,2013)

2.7.2.Antihelmintic activity

Anti helminthic activity was conducted using *Pheretima posthuma* (Earth worm) of nearly equal size (±8 cm) due to its anatomical and physiological resemblance with the intestinal round worm parasites of human beings. 2-3 earth worm of nearly equal size were placed in each petridish containing 2 ml (0.4g) sample at room temperature. Observation was made for the time taken for paralysis when there was no movement of any kind except when shaken vigorously and are not revived in normal saline. Time for death were recorded when they lost their mobility even after vigorous movement and also by fading off their body colors(Sukanya, M.K, Shimi Suku,2013)

Result

Phytochemical analysis showed the presence of alkaloids ,caridac glycosides ,saponins, tannins and terpenoids in methanol, butanol and ethyl acetate extract of both bark and leaf samples.Methanol extract of leaf sample showed the highest zone of inhibition against *Enterobacter aerogens* and *Enterobacter feacalis*(18 mm). Ethyl acetate extract also showed high zone of inhibition against E.coli. In Bark sample butanol extract showed an inhibiton zone of 18mm against *Klebsiella pneumoniae*.All other samples obtained almost equal range of inhibition zone(13mm-16mm).

Table2.4.Phytochemical analysis *of Phyllanthus emblica* leaf

	Methanol	Butanol	Ethyle acetate	AquoeuS
Alkaloid	+	+	+	_
Carbohydrate	+	_	+	_
Cardiac glycoside	+	+	+	+
flavanoids	+	_	+	+
saponin	+	+	+	+
tanin	+	+	+	+

terpinoid	+	_	+	+

(Sukanya, M.K, Shimi Suku,2013)

2.7.3.Antibacterial activity Methanol extract of leaf sample showed the highest zone of inhibition against Enterobacter aerogens and Enterobacter feacalis(18 mm). Ethyl acetate extract also showed high zone of inhibition against E.coli. In Bark sample butanol extract showed an inhibiton zone of 18mm against Klebsiella pneumoniae(Sukanya, M.K, Shimi Suku,2013)

Table 2.5.Diameter of Zone of inhibition of *Phyllanthus emblica* Leaf

bacteria	Diameter of zone of inhibition methanol	Diameter of zone of inhibition butanol
Pseudomonas aeroginosa	16	12
Proteus vulgaris	15	12
Klebsiella pneumoniae	11	14
Enterobacter aerogens	18	10
Bacillus megaterium	15	14

(Sukanya M.K, Shimi Suku ,2013)

2.7.4.Anti fungal activity Ethyl acetate extract of both bark and leaf sample showed highest anti fungal activity against Rhizomucor species.(20mm and 19 mm respectively)all other samples showed almost same zone of inhibition(10mm- 16mm)(Sukanya M.K, Shimi Suku ,2013)

Table 2.6. Diameter of Zone of inhibition of *Phyllanthus emblica l*eaf

Fungi	**diameter of zone of inhibition**	
	Methanol	**butanol**
Aspergillus fumigatus	13	15
Aspergillus niger	11	9
Candida albicans	9	9
Candida glabrata	10	14

2.7.5.Anti helminthic activity. Butanol extract of leaf and bark exhibited less time for paralysis and death for Pheretimaposthuma. Aqueous extract of leaf show the maximum time for paralysis and subsequent death(Sukanya, M.K, Shimi Suku,2013)

Table2.7. Antihelminthic activity of *Phyllanthus emblica* leaf

Name of earthworm	**extract**	**paralysis**	**death**
Pheretima posthuma	butanol	14	25
Pheretima posthuma	ethyl acetate	10	21
Pheretima posthuma	aqueous	22	35
Pheretima posthuma	methanol	135	190

(Sukanya M.K, Shimi Suku ,2013)

Here a preliminary phytochemical analysis of various solvent extract of *P. emblica* , its anti microbial and antihelminthic activity were conducted. Phytochemical analysis of the plant emblica showed the presence of many biologically active compounds, such as alkaloids, cardiac glycosides ,saponins, tannins terepenoids, flavinoids and carbohydrates .Different extracts showed promising anti bacterial activity against commonly found human pathogens. Butanol extract showed maximum zone of inhibition (18 mm) for two pathogens,*E.coli* and Enterobacter aerogens and also by ethyl acetate for *E.coli*. Antifungal activity was shown to be highest in ethyl acetate fraction of bark sample. As all the bioactive compounds were present in the butanol and ethyl acetate extract, except falvanoids in case of butanol and methanol bark extract, further studies to identify and isolate the active e compound has to be conducted. Although the fruit of emblica is widely used as an immunity booster ,lower blood cholestrol, enhances memory and intelligence, a natural source of vitamin c and iron,(14,15) the use of leaf and bark is less investigated. Further research has to be conducted to find out the possibility of this medicinally important plant as a potent anti microbial drug and for other pharmacological properties to develop as cost effective formulation (Sukanya M.K, Shimi Suku ,2013)

Chapter Three
Methods and Materials

Methods and Materials

3.1 Collection and preparation of plant material

Plant sample of *Phyllunthus acidus* collected in March, 2017. Then proper identification of plant sample was done by an expert taxonomist. The plant was sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding. The dried plant 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 (5liters) 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 filterpaper 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.

3.4.1. Partition with Pet-ether

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 Pet-ether fraction was then air dried for solid residue.

3.4.2 Partition with Dichloromethane

To the mother solution left after partitioning with Pet-ether, 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 Pet-ether, 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 Pet-ether, 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.

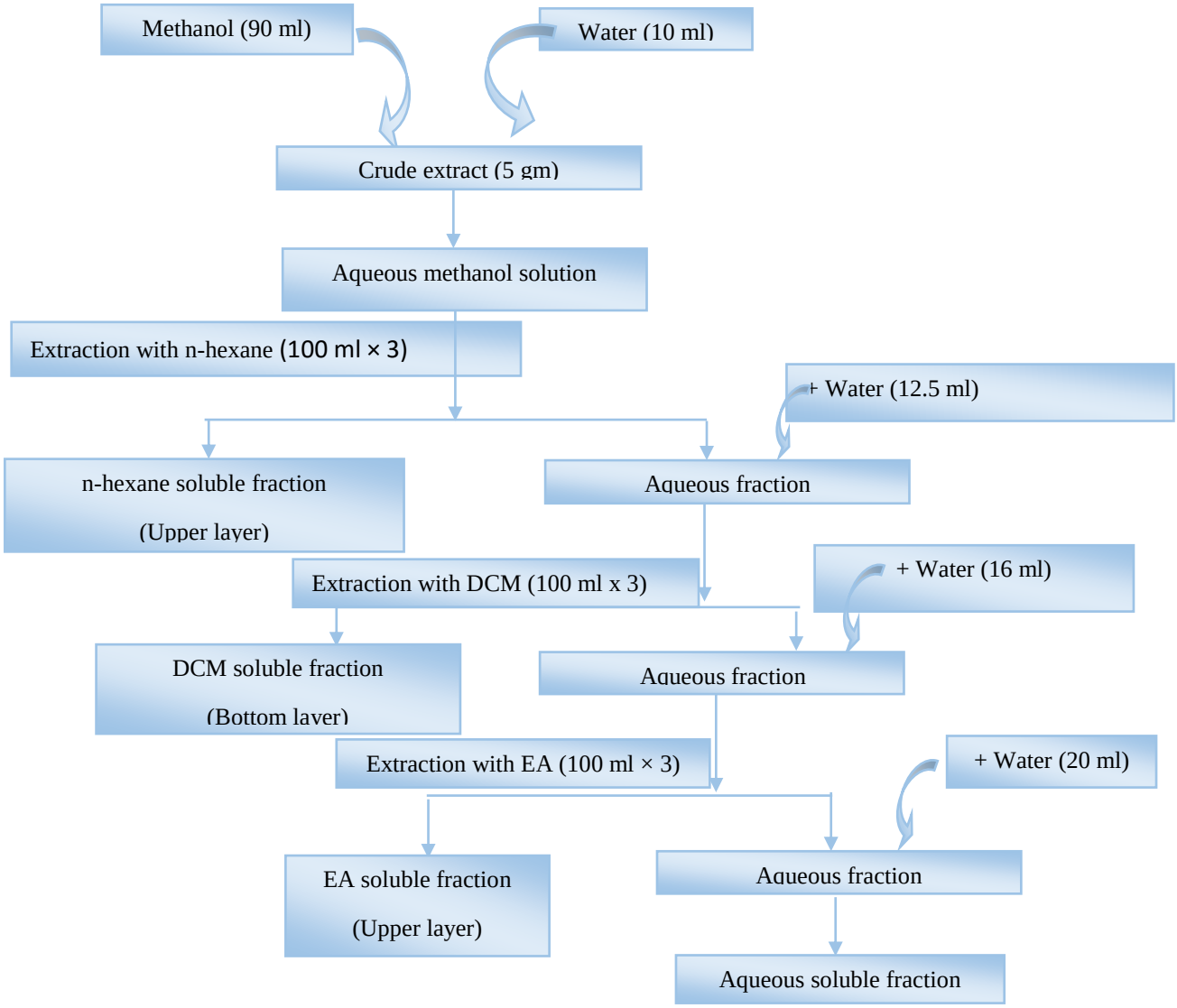


Figure 3.2: Schematic representation of the Partitioning of methanolic crude extract of *P.acidus*

3.4.5 Collection of Aqueous Fraction

After partitioning the mother solution with the four different solvents the Aqueous fraction of them were collected and air dried. This aqueous fraction was further investigated for different

pharmacological properties such as Antioxidant and Cytotoxic (Beckett AH and Stenlake JB, 1986).

3.5 Brine Shrimp Lethality Bioassay

3.5.1 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 or 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 *et al.*, 2013).

3.5.2 Apparatus & Reagents

Table 3.1: 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

3.5.3 Procedure

3.5.3.1 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 38 gm of pure NaCl was dissolved in distilled water and then the volume made up to 1000 ml by distilled water in a 1000 ml beaker for *Artemia salina* hatching. 1-2 drops of 1 N NaOH or 1 N HCl solution was added with a dropper for obtaining the pH 8.4 as sea water.

3.5.3.2 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 18-24 hours. 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 5 ml of seawater. Those freshly hatched free-swimming nauplii were used for the bioassay (Niazi J. *et al*., 2009).

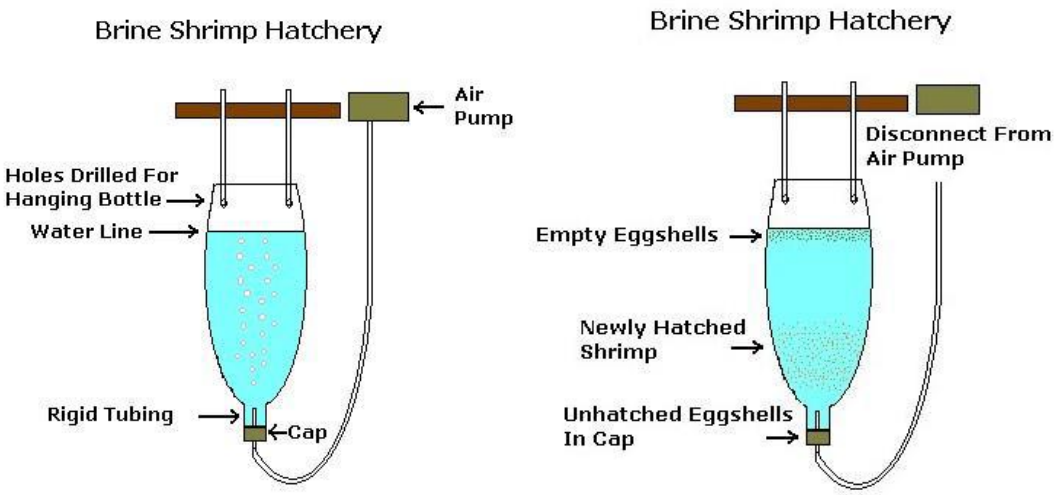


Figure 3.3: Brine shrimp Hatchery.

3.5.3.3 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.

3.5.3.4 Preparation of the Test Samples of Experimental Plant

All the test samples of 4 mg were taken and dissolved in 200 µl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Then 100 µl of solution was taken in test tube each containing 5 ml 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 100 µl sample was added to test tube and fresh 100 µl DMSO was added to vial. Thus the concentrations of the obtained solution in each test tube were 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0.78125 µg/ml for 10 dilutions.

3.5.3.5 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 2000 µg/ml. From that stock solution serial dilutions are made using DMSO to get 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0.78125 µg/ml. Then ten living brine shrimp nauplii in 5 ml simulated seawater are added to the positive control solutions in the pre-marked test-tubes to get the positive control groups.

3.5.3.6 Preparation of the Negative Control Group

100 µl of DMSO was added to the pre-marked test tube containing 5 ml 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).

3.5.3.7 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 (Sleet RB and Brendel K, 1983).

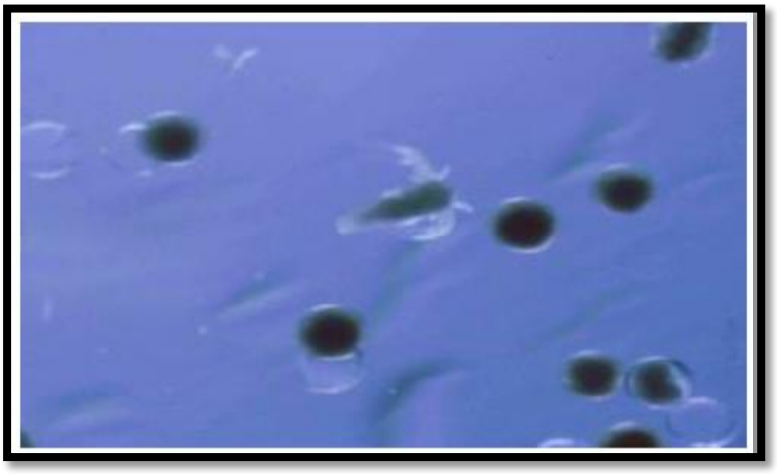


Figure 3.4: Counting of nauplii

3.6 Antioxidant Activity

3.6.1 Total Phenolic Content

The antioxidative 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 antioxidative status occurrences of human diseases. In addition, antioxidant compounds which are responsible for such antioxidant activity could be isolated and then used as antioxidants for the prevention and treatment of free radical-related disorders. Therefore, research to identify antioxidative 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 antioxidant effects have rarely been carried out. The purpose of this study was to evaluate extractives of *P.acidus* new potential sources of natural antioxidants and phenolic compounds. This study also demonstrates a possible relationship between phenolic content and antioxidant activity.

3.6.1.1 Principle

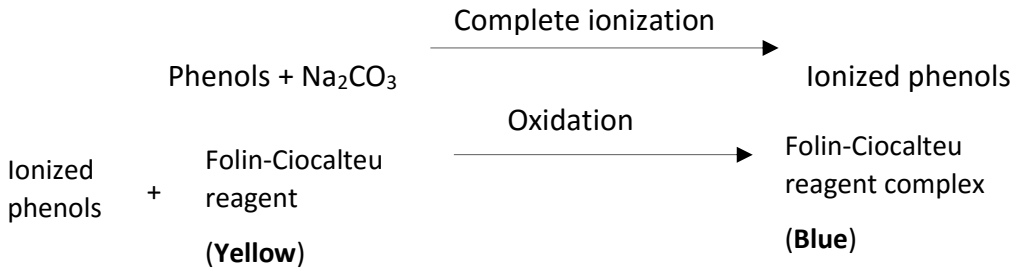
The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. In the alkaline condition phenols ionize completely.

Table 3.2: Composition of 100 mg Folin-Ciocalteu Reagent

Composition of 100 mg Folin-Ciocalteu Reagent	
Water	57.5 ml
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
Lithium Sulfate	15 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-polyphosphotunstates - molybdates. Sequences of reversible one or 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).



3.6.1.2 Apparatus & Reagents

Table 3.3: 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

3.6.1.3 Procedure

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.

Sample preparation

2 mg of the *P.acidus* DCM fraction was taken and dissolved in 1 ml methanol to get a sample concentration of 2 mg/ml.

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.

3.6.2 Total Flavonoid Content

3.6.2.1 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 *et al.*, 2002)

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

3.6.2.2 Apparatus & Reagents

Table 3.4: Apparatus and reagents used for total flavonoid content

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

3.6.2.3 Procedure

Preparation of 10% Aluminium Chloride ($AlCl_3$) Solution: 1gm of $AlCl_3$ was taken into a 10 ml of a volumetric flask and the volume was adjusted by distilled water.

Preparation of 4% NaOH Solution: 4 gm of NaOH was taken into a 100 ml volumetric flask and the volume was adjusted by distilled water.

Preparation of 5% (W/V) NaNO2 Solution: 0.5 gm of $NaNO_2$ was taken into a 10 ml of a volumetric flask and the volume was adjusted by distilled water.

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 200 µg/ml of quercetin. The experimental concentrations were prepared from this stock solution.

Table 3.5: Preparation of standard solution

Concentration (µg/ml)	**Solution taken from stock solution (ml)**	**Solution taken from stock solution (ml)**	**Final volume (ml)**
0	0.0	5.0	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

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.

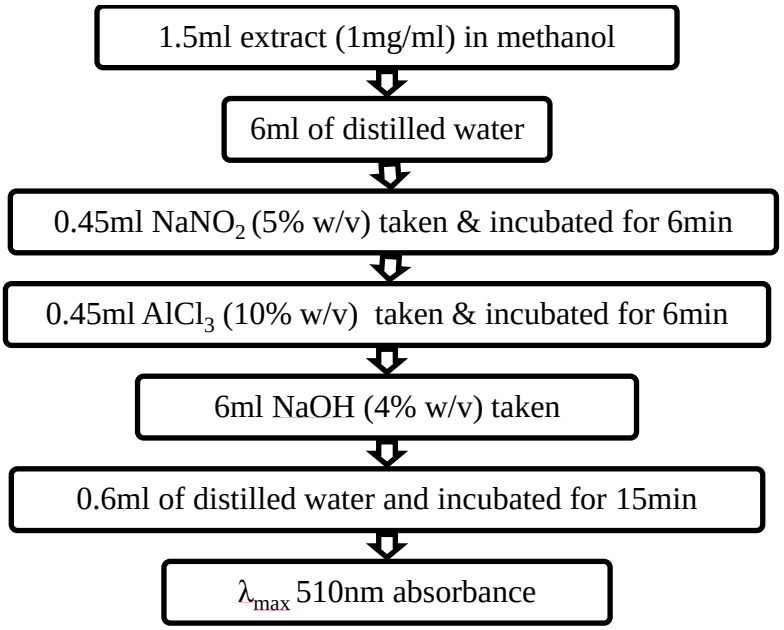


Figure 3.5: Schematic diagram of preparation of extract solution

Preparation of blank solution

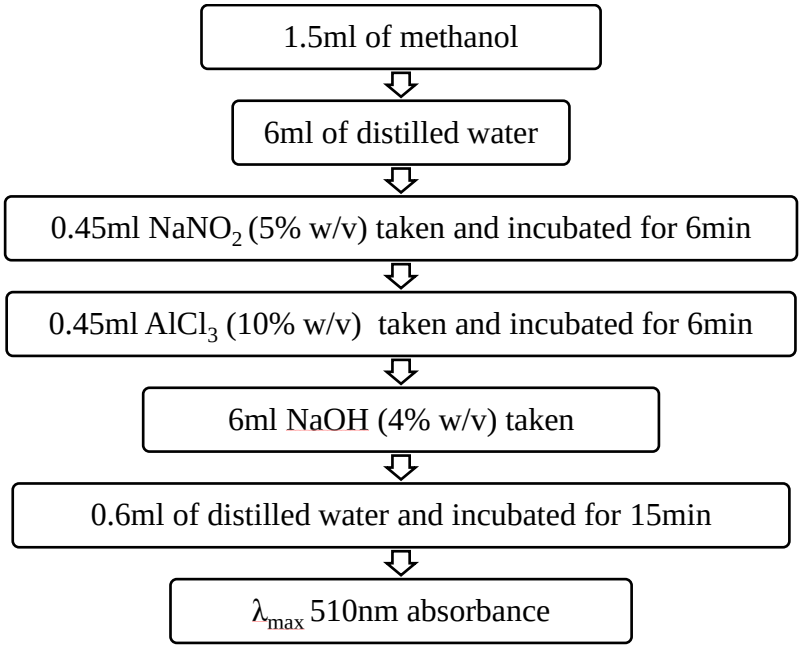


Figure 3.6: Schematic diagram of preparation of blank solution

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 *P.acidus* (leaves)

Aqueous fraction of methanolic extract of *P.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(+ve)	*Bacillus sereus*
	Bacillus subtilis
	Bacillus megaterium
	Staphylococcus aureus
Gram negative (–ve)	*Escherichia coli*
	Salmonella typhi
	Salmonella paratyphi
	Vibrio parahaemolyticus
	Vibrio mimicus
	Shigella dysenteriae

3.7.5 Procedure

3.7.5.1 Preparation of the Medium

To prepare required volume of this medium, 56 gm 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 121º C 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.

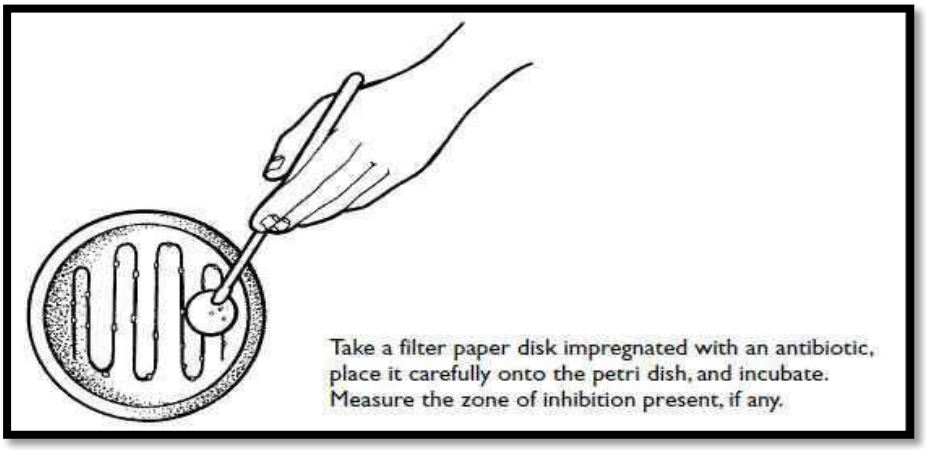


Figure 3.9: Preparation of filter paper discs

- **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, ciprofloxacin (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 ciprofloxain 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 airdrying) 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

3.6.5.8 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.

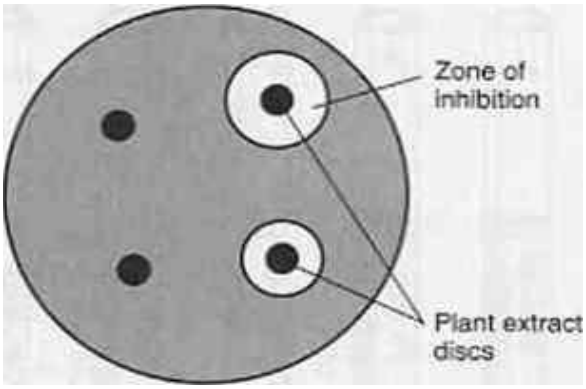


Figure 3.11: Clear zone of inhibition

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 Aqueous 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 aqueous 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.

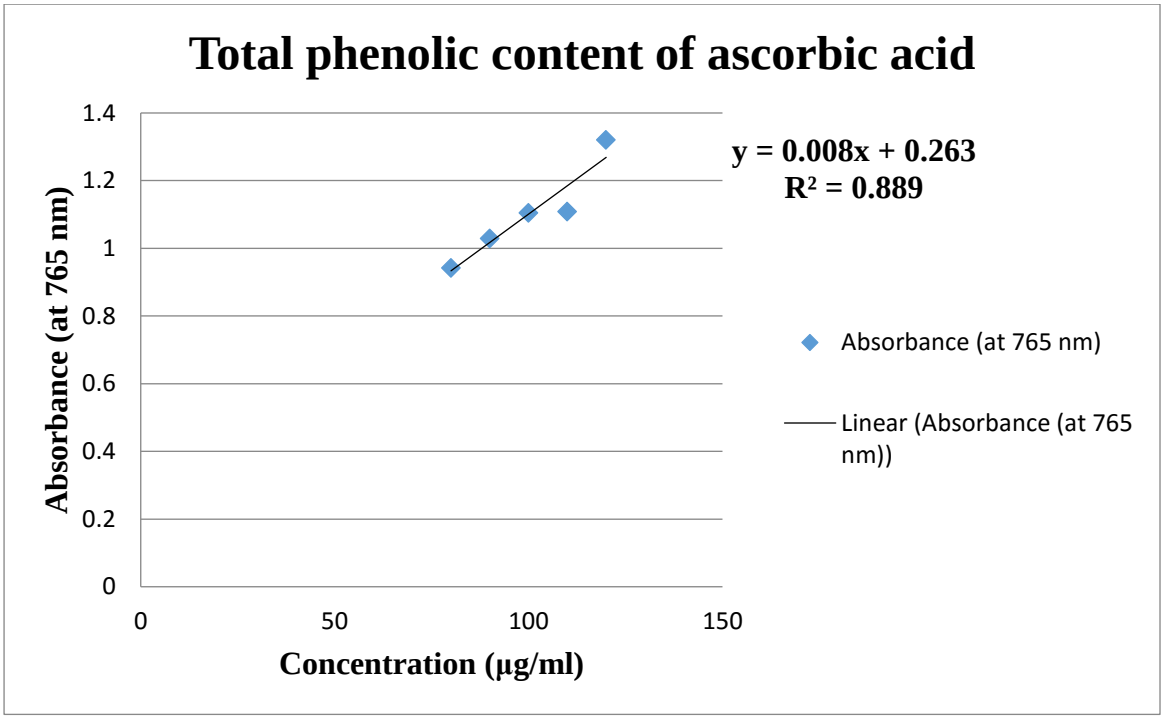


Figure 4.1: Graphical Representation of Assay of Phenolic Content of Ascorbic Acid

4.1.1.2 Total phenol content present in aqueous 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 aquous fraction of *Phyllanthus acidus* (leaves)

Sample	**Concentration (mg/ml)**	**Absorbance (Y value at 765 nm)**	**Total Phenolic (X) value (mg of AAE/gm of dried extract)**
Aqueous fraction of *Phyllanthus 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 aqueous 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 aqueous fraction of *Phyllanthus acidus.*

4.1.2 Result of total flavonoid content

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

4.1.2.1 Preparation of standard curve

Table 4.3: Total flavonoid content of Quercetin

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

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

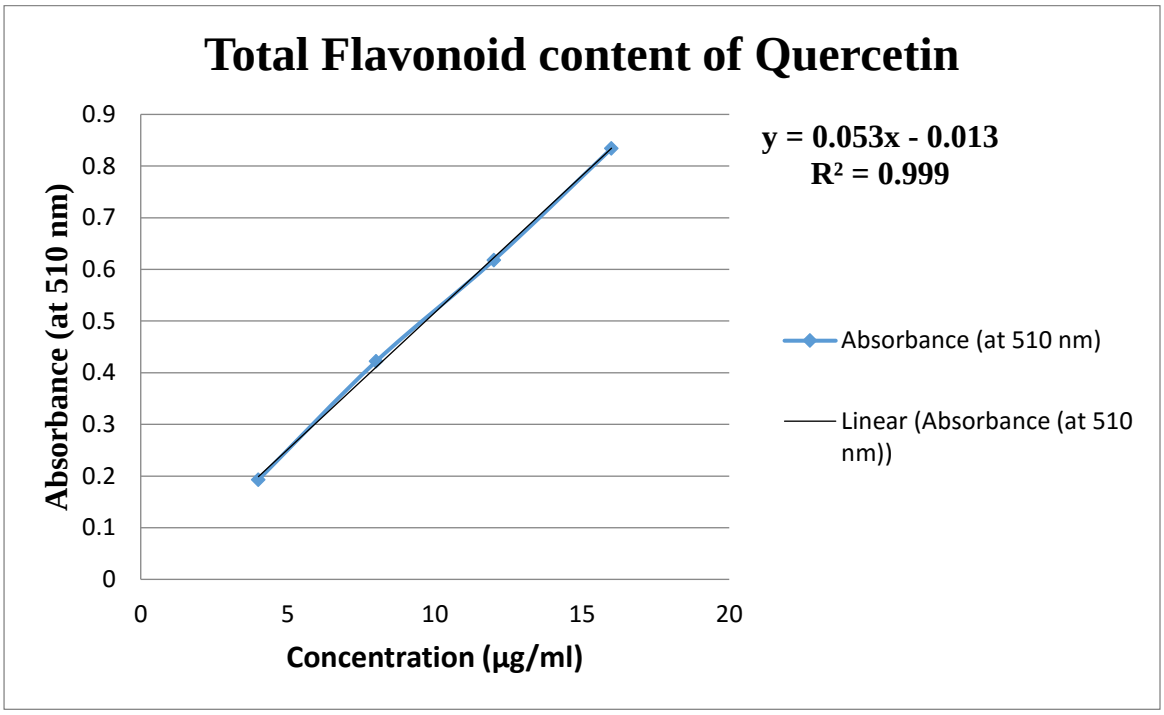


Figure 4.2: Graphical representation of Flavonoid content of quercetin

4.1.1.2 Total flavonoid content present in Aqueous 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 Aqueous 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)**
Aqueous 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 aqueous 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 aqueous fraction of *Phyllanthus acidus* (leaves).

4.2 Result of Antimicrobial Test

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

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

Type of microorganism		**Zone of inhibition (mm)**	
		Standard sample	**aqueous fraction**
Gram positive bacteria	*Bacillus sereus*	31	7
	Bacillus subtilis	31	7
	Bacillus megaterium	31	5
	Staphylococcus aureus	31	8
Gram negative bacteria	*Escherichia coli*	30	7
	Salmonella typhi	30	9
	Salmonella paratyphi	30	8

	Vibrio parahaemolyticus	33	6
	Pseudomonas aureaus	30	7
	Shigella dysenteriae	30	7

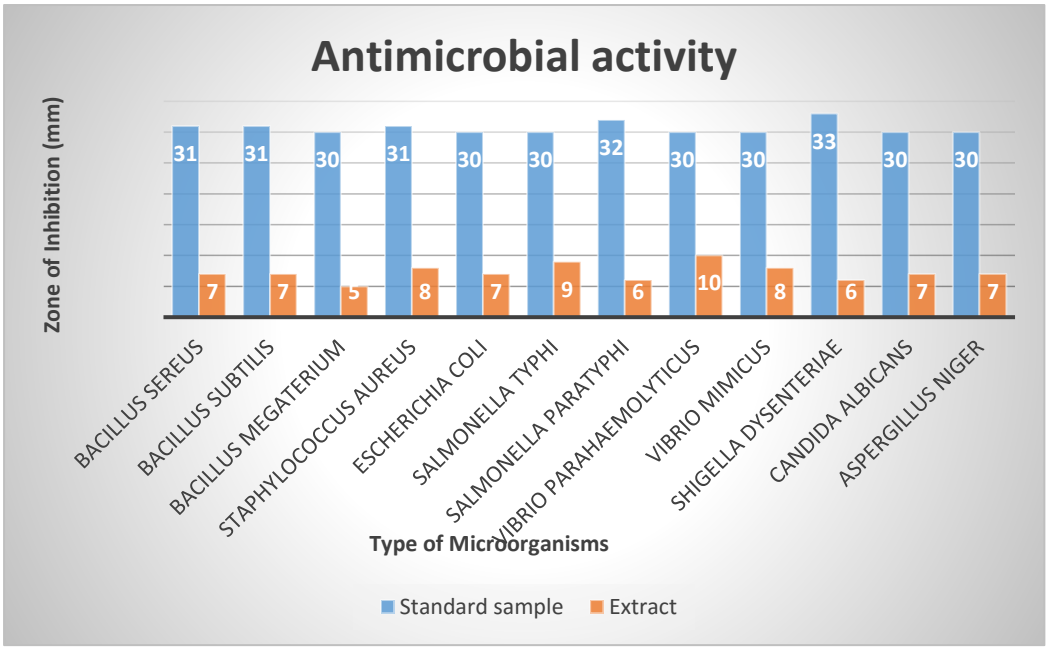


Figure 4.3. Comparison of antimicrobial activity between standard and extract

4.2.2 Discussion

Aqueous 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 aqueous 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 aqueous 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 aqueous 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	2.602	0	10	100	
2	200	2.301	1	9	90	
3	100	2.000	2	8	80	
4	50	1.699	3	7	70	
5	25	1.398	5	5	50	13.38
6	12.5	1.097	5	5	50	
7	6.25	0.796	6	4	40	
8	3.125	0.495	7	3	30	
9	1.5625	0.194	8	2	20	
10	0.78125	-0.107	9	1	10	

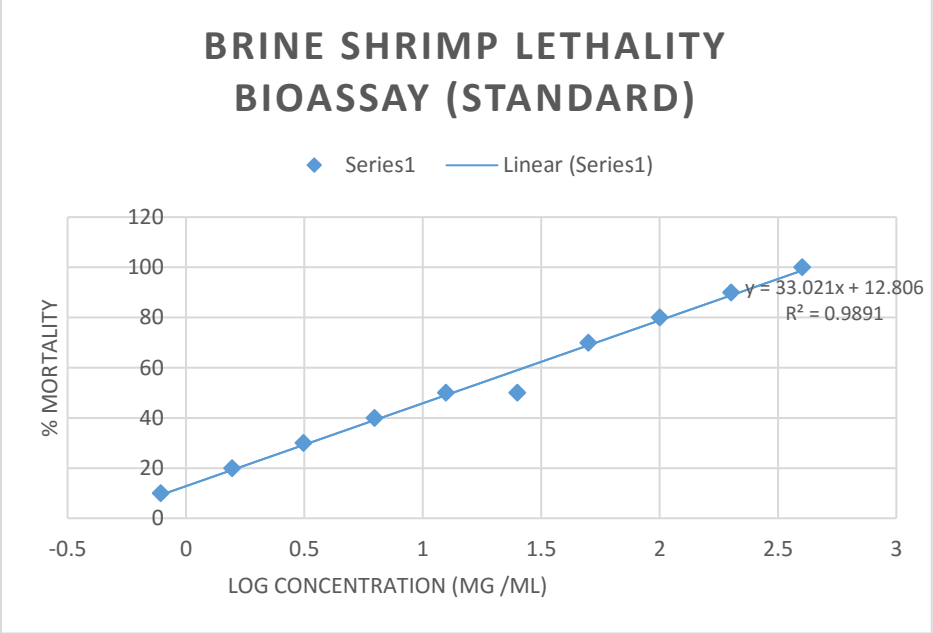


Figure 4.4. % Mortality and Predicted Regression Line of Tamoxifen (standard)

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

Table 4.7: Results of the bioassay in aqueous 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	2.602	0	10	100	
2	200	2.301	0	10	100	
3	100	2.000	1	9	90	
4	50	1.699	1	9	80	
5	25	1.398	2	8	70	2
6	12.5	1.097	2	8	80	
7	6.25	0.796	3	7	70	
8	3.125	0.495	4	6	60	
9	1.5625	0.194	6	4	40	
10	0.78125	-0.107	7	3	30	

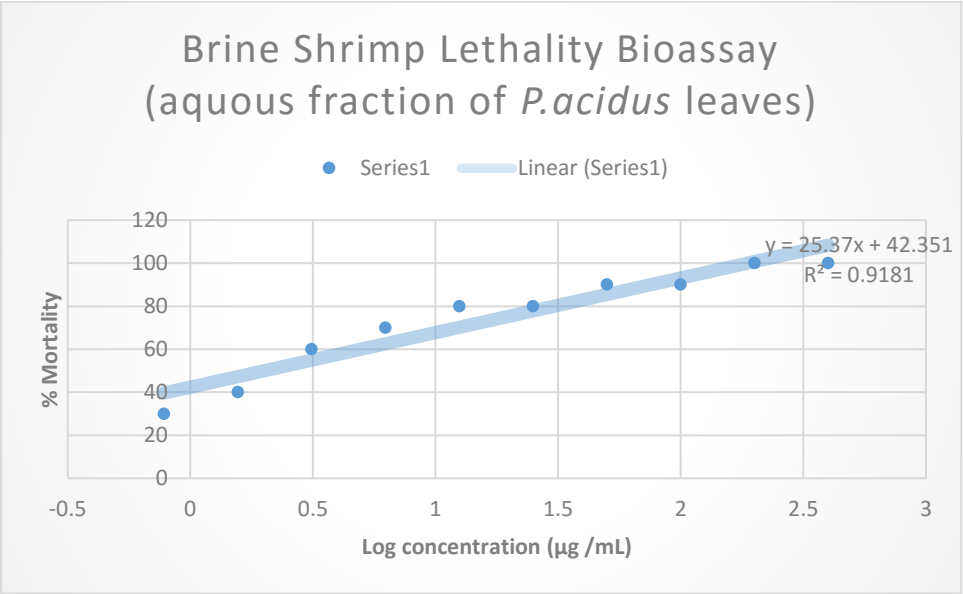


Figure:4.5 Mortality and Predicted Regression Line in aqueous fraction of *P.acidus* (leaves)

4.3.3.Discussion

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

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

Sample	Linear regression equation	R2 value	LC50 (µg/ml)
Standard (Tamoxifen)	y = 33.021x + 12.806	0.9891	13.38
Aqueous fraction	Y=25.37+42.351	0.9181	2

In this investigation, standard and aqueous fraction exhibited cytotoxic activities with the LC50 values at 13.38 µg/ml and 2 µg/ml respectively as shown in Table 4.8. LC50 value of

Phyllanthus acidus (leaves) in aqueous fraction showed more activity of it than Tamoxifen. Further investigation is needed to confirm the activity. Aqueous fraction showed more activity of it than Tamoxifen. Further investigation is needed to confirm the activity.

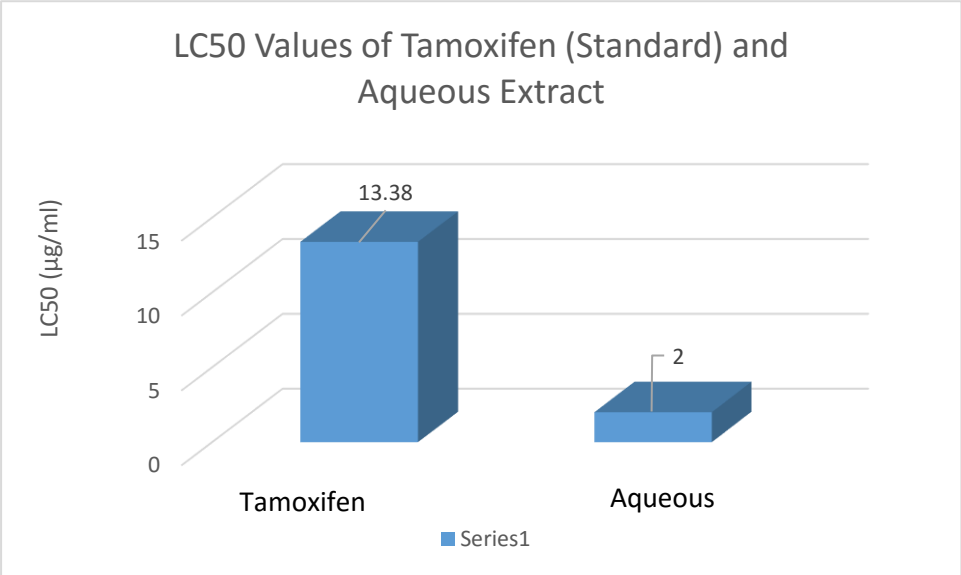


Figure 4.6. Comparison between LC_{50} values of standard and extract

Chapter Five

Conclusion

Conclusion

As the literature review suggests, the presence of several phytochemical compounds in Aqueous fraction of *P.acidus* (leaves), makes the plant pharmacologically active. LC_{50} value of *P.acidus* (leaves) in Aqueous fraction showed more cytotoxic activity than Tamoxifen. Since Aqueous fraction of *P.acidus* (leaves) exhibited potent cytotoxic activity, so it can be investigated for anticancer, pesticidal and antitumor properties in future.

Antioxidant property in Aqueous extract of *P.acidus* (leaves) was determined by Phenolic content assay and Flavonoid content assay. Phenolic content was 4.5mg/gm and Flavonoid content was 4.868 mg/gm in Aqueous extract of *P.acidus (leaves).* So, Aqueous extract of *P.acidus* (leaves) have poor antioxidant property. Mixture of compounds can lower antioxidant property in Aqueous fraction of *P.acidus* (leaves), if any counteracting compounds were present in mixture. So pure compound isolation should be done in future to confirm antioxidant property of Aqueous fraction of *P.acidus*(leaves)

The study also showed that, the extract showed low to moderate antimicrobial activity that could be a better treatment in antimicrobial infections.However, studies are required on higher animal model and subsequently on human subjects to prove efficacy as an antioxidant, cytotoxic and antimicrobial agent. It will help in the development of new novel and safe drugs for the treatment of various diseases.

Chapter Six

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