Phytochemical and Pharmacological Investigation on *Syzygium samarangense* Leaf

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

Submitted By

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DECLARATION BY THE CANDIDATE

I, Kaniz Sadia Tabassum, hereby declare that this dissertation, entitled "Phytochemical and Pharmacological Investigation on *Syzygium samarangense* Leaf" submitted to the Department of Pharmacy, East West University, in the partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Honors) is a genuine & authentic research work carried out by me. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any Degree or Diploma of Fellowship.

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CERTIFICATION BY THE SUPERVISOR

This is to certify that the dissertation, entitled "Phytochemical and Pharmacological Investigation on *Syzygium samarangense* Leaf" is a research work done, under our guidance and supervision by Kaniz Sadia Tabassum (ID: 2013-1-70-052), 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, they are my biggest inspirations.

ABSTRACT

The purpose of the study was to evaluate the cytotoxic, antioxidant and antimicrobial activity of aqueous fraction of *Syzygium samarangense* leaf (Family: Myrataceae) extract.

The powder of *Syzygium samarangense* leaf were extracted with methanol and then partitioned with n-hexane, DCM, and ethyl acetate 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. LC_{50} value of aqueous fraction of *Syzygium samarangense* was 1.64 µg/ml in brine shrimp lethality test. The fraction contained 117.8125 mg AAE/g of total phenolic content and 2.339623 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: *Syzygium samarangense*, Brine shrimp lethality bio-assay, phenolic content, flavonoid content.

List of Contents

List of Figures

List of Tables

Abbreviations

Meaning of abbreviated form	**Abbreviated form**
Ascorbic Acid Equivalent	AAE
Dichloromethane	DCM
Gram	g or gm
Hour	hr
Lethal concentration required to kill 50% of the sample population	LC_{50}
Microgram	µg
Micro liter	µl
Milligram	mg
Milliliter	ml
Ultraviolet	UV
World Health Organization	WHO
Syzygium	S.
Folin– Ciocalteu Reagent	FCR

-CHAPTER ONE-

INTRODUCTION

1.1 Overview

Humans have evolved with herbs and plants for hundreds of thousands of years. Using herbal medicine brings harmony and balance back to the body, because it allows the body to be just as responsible for the healing as the plant. Using harsh, synthetic chemical compounds, which have only been around for a hundred years or so (and have not usually been properly tested for long term safety), comes with the mentality that the body is a broken machine and needs to be fixed. The number of plant species discovered by humans since they began exploring the planet is some 270,000 species, although it is entirely possible that the total number of species on earth is closer to 400,000. Providing the major source of food for people the world over plants, however, are responsible for very much more than just nourishment (Plotkin, 2014).

1.2 Role of Plants as a Source of Drug in Human History

- The oldest written evidence of medicinal plants usage for preparation of drugs has been found on a Sumerian clay slab from Nagpur, approximately 5000 years old. It comprised 12 recipes for drug preparation referring to over 250 various plants, some of them alkaloid such as poppy, henbane, and mandrake.
- The Chinese book on roots and grasses Pen T'Sao, written by Emperor Shen Nung Circa 2500 BC, treats 365 drugs (dried parts of medicinal plants), many of which are used even nowadays such as the following: Rhei rhisoma, camphor, Podophyllum, ginseng, cinnamon bark, and ephedra.
- The Indian holy books Vedas mention treatment with plants, which are abundant in that country. Numerous spice plants used even today originate from India: nutmeg, pepper, clove, etc.
- The works of Hippocrates (459–370 BC) contain 300 medicinal plants classified by physiological action: Wormwood was applied against fever; garlic against intestine parasites; opium, henbane, deadly nightshade, and mandrake were used as narcotics; fragrant hellebore and haselwort as emetics; sea onion, celery, parsley, asparagus, and garlic as diuretics; oak and pomegranate as astringents.

- Theophrastus (371-287 BC) founded botanical science with his books ―De Causis Plantarum― Plant Etiology and ―De Historia Plantarium―Plant History. In the books, he generated a classification of more than 500 medicinal plants known at the time. Among others, he referred to cinnamon, iris rhizome, false hellebore, mint, pomegranate, cardamom, fragrant hellebore and monkshood. He describe about plant's toxic action.
- Dioscorides, ―the father of pharmacognosy, wrote ―De Materia Medica. 944 drugs became described in book, 657 are of plant origin, with descriptions of the outward appearance, locality, mode of collection, making of the medicinal preparations, and their therapeutic effect. Camomile, garlic, onion, marsh mallow, ivy, nettle, sage, common centaury, coriander, parsley, sea onion were described by Dioscorides.
- Galen (131 AD–200) had described Uvae ursi folium and a mild diuretic.
- Charles the Great (742 AD–814) had described sage, sea onion, iris, mint, common centaury, poppy, marsh mallow, etc.
- The Arabs used aloe, deadly nightshade, henbane, coffee, ginger, strychnos, saffron, curcuma, pepper, cinnamon, rheum, senna and so forth. Certain drugs with strong action were replaced by drugs with mild action, for instance, Sennae folium was used as a mild laxative, compared to the purgatives Hellebores odorous and Euphorbium used until then.
- Paracelsus (1493-1541) was one of the proponents of chemically prepared drugs out of raw plants and mineral substances. For example, the haselwort is beneficial for liver diseases.
- St John's wort (*Hypericum perforatum* L.) would be beneficial for treatment of wounds and stings.
- The discovery, substantiation, and isolation of alkaloids from poppy (1806), ipecacuanha (1817), strychnos (1817), quinine (1820), pomegranate (1878), and other plants, then the isolation of glycosides, marked the beginning of scientific pharmacy. With the upgrading of the chemical methods, other active substances from medicinal plants were also discovered such as tannins, saponosides, etheric oils, vitamins, hormones, etc.
- In 19th century, therapeutics, alkaloids, and glycosides isolated in pure form were increasingly supplanting the drugs from which they had been isolated. It was soon

ascertained that although the action of pure alkaloids was faster, the action of alkaloid drugs was full and long-lasting.

- In early 20th century, stabilization methods for fresh medicinal plants were proposed, especially the ones with labile medicinal components. Besides, much effort was invested in study of the conditions of manufacturing and cultivation of medicinal plants (Petrovska B, 2012).
- 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 (Plotkin, 2014).

1.3 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 (Akerele, Heywood and Synge, 1991).

1.3.1 A Wide Range of Plant Uses

The art of truly relating to the plants is to choose the ones that you feel an affinity with. If you have the ability to grow them for yourself – in a garden or in a pot – this is of enormous value. Growing your own plants and medicinal herbs, endeavoring to learn more about them put you in touch with Nature in a very deep way.

In the history of human kind we have never had so much information about medicinal nature of plants on our fingertips. The mystery of their magic still stands, but what was magic and mystery

to our ancestors is a science to us today. The more we learn about plants the more we find ways to use them to support health. At a very basic level we can use them as a condiment or seasoning in food, enhance all those otherwise dull dishes by the fragrance and flavor only herbs can provide. The vibrant natural toiletries and cosmetics industry thrives on the power of plants to impart their healing, nourishing, soothing, invigorating, relaxing and other effects onto our skin and hair (Bgci.org, 2016).

1.3.2 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 (Akerele, Heywood and Synge, 1991).

According to recent studies conducted by the World Health Organization (WHO), about 80% of the world's population relies on traditional medicine.

- Mandrake was prescribed for pain relief
- Turmeric possesses blood clotting properties
- Roots of the endive plant were used for treatment of gall bladder disorders (Nahain *et al*., 2002).
- Paclitaxel from *Taxus brevifolia* used for the treatment of lung, ovarian and breast cancer.
- The alkaloid, forskolin from *Coleus forskohlii* and phytochemicals from *Stephania glabra*, are now being rediscovered as adenylate cyclase and nitric oxide activators, which may help in preventing conditions including obesity and atherosclerosis.

- Apomorphine is a semi synthetic compound derived from morphine (*Papaver somniferum*) used in Parkinson's disease.
- Cannabidiol obtained from cannabis plant (*Cannabis sativa*) and Capsaicin active compound from *Capsicum annuum* are used as pain relievers (Veeresham C, 2012).

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 11 been estimated that in addition to these active constituents, more than 96 crude extracts were also used in the United States (Akerele, Heywood and Synge, 1991).

1.3.3 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 plant derived 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 12 researches 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 (Ahmed, 2016).

1.3.4 Necessity of Drug Development from Plant Sources

The traditional medicinal preparations are generally supplied as crude extract of a medicinal plant. Since plant extracts possess a number of chemical constituents, each of them may expert some effect on the living body. On the contrary, a plant extract may have a chemical component in such a low concentration that it may not elicit the therapeutic action of interest.

Besides, the crude extract may contain a number of ingredients performing the same therapeutic role. Ingestion of such an extract may cause serious side-effects due to synergistic action of the constituents. So the application of herbal drug in crude form may be ineffective or may cause a toxic reaction. Vincristine, a prominent anticancer drug, was developed from periwinkle plant (*Vinca rosea*) which was formerly prescribed for treating diabetes.

The efficient hypotensive drug, reserpine, was developed from *Rauwolfia serpentine* which was previously provided as an antidote to snake-bites and in the treatment of lunatic patients (Chopra RN et al., 1982).

Khelin, a coronary vasodilator drug prescribed as an effective remedy for angina pectoris, was developed from Ammi visnaga which was formerly used as a diuretic and antispasmodic in renal colic. Thus drug development from medicinal plants gives effective result (Ghani, 1998).

1.3.5 Procedure for Development

Since drug development is an expensive practice, careful phytochemical analysis and pharmacological screening and if promising clinical tests are required. Pharmacology is the study of the therapeutic value and/or potential toxicity of chemical agents on biological systems. It targets every aspect of the mechanisms for the chemical actions of both traditional and novel therapeutic agents. In its entirety, pharmacology embraces knowledge of the sources, chemical properties, biological effects and therapeutic uses of drugs.

Phytochemical analysis refers to the extraction, analysis and identification of the medicinally active substances found in plants. Some of the bioactive substances that can be derived from plants are flavonoids, alkaloids, carotenoids, tannin, antioxidants and phenolic compounds.

Pharmacological studies range from those that examine the effects of chemical agents on subcellular mechanisms, to those that deal with the potential hazards of pesticides and herbicides, to those that focus on the treatment and prevention of major diseases with drug therapy. Several medicinal plants can be employed to produce extracts exhibiting biological effects. It is estimated that only 500 medicinal plant species had been recorded in Bangladesh out of approximately 1900 species regarded as having medicinal value (Yusuf *et al.*, 1994).

The way of developing drugs from plants involves several stages (Ghani, 1998), which include:

- Selection and correct identification of the proper medicinal plant.
- Extraction with suitable solvent(s).
- Detection of biological activity of crude extract and establishment of a bioassay system to permit the identification of the active fractions and rejection of the inactive ones.

- Fractionations of crude extract using the most appropriate chromatographic procedures, biological evaluation of all fractions and separation of the active fractions.
- Repeated fractionation of active fractions to isolate pure compound(s).
- Elucidation of chemical structure of pure compound(s) using spectroscopic methods.
- Evaluation of biological activity of pure compound(s)
- Toxicological tests with pure compound(s).
- Production of drug in appropriate dosage forms.

1.4 Medicinal Plants and Their Importance as Alternative Medicine

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. World Health Organization (WHO) has provided a definition of medicinal plants, which is

"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".

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. (canada.ca, 2016)

This scenario is similar to the one occurring in Bangladesh. Bangladesh is an Asian country where only 20 % of the people can be provided with modern healthcare services while the rest 80 % are dependent on traditional plant-based systems. In the Plant Kingdom, Medicinal plants form the largest single grouping of plants. It is estimated that 30,000 species worldwide fall in this group, of which around 33% are trees (UNDP, 1999).

Medicinal plants are various plants thought by some to have medicinal properties, but few plants or their phytochemical constituents have been proven by rigorous science or approved by regulatory agencies such as the United States Food and Drug administration or European Food Safety Authority to have medicinal effects (Newman *et al.,* 2003).

1.4.1 Value of Medicinal Plants

Many of the plants could be used as stimulants, poisons, hallucinogens or as medicine because of the presence of unique or rich biological-active plant chemicals (i.e. Chemical compounds that have a biological effect on another organism). Chemicals that make a plant valuable as medicinal plant are:

- Alkaloids (compounds has addictive or pain killing or poisonous effect and sometime help in important cures).
- Glycosides (use as heart stimulant or drastic purgative or better sexual health).
- Tannins (used for gastrointestinal problems like diarrhea, dysentery, ulcer and for wounds and skin diseases).
- Volatile/essential oils (enhance appetite and facilitate digestion or use as antiseptic and insect repellent properties).
- Fixed oils (present in seeds and fruits could diminish acidity).

- Gum-resins and mucilage (possess analgesic property that suppress inflammation and protect affected tissues against further injury and cause mild purgative).
- Vitamins and minerals (Fruits and vegetables are the sources of vitamins and minerals and these are used popularly in herbals) (Ghani A, 1998).

1.4.2 The Importance of Medicinal Plant in Drug Discovery

Development of new drug is a complex, time-consuming, and expensive process. The time taken from discovery of a new drug to its reaching the clinic is approximately 12 years, involving more than 1 billion US$ of investments in today's context. Essentially, the new drug discovery involves the identification of new chemical entities (NCEs), having the required characteristic of drug ability and medicinal chemistry. These NCEs can be isolated from natural products. More than 80% of drug substances were purely natural products or were inspired by the molecules derived from natural sources (including semi-synthetic analogs).

- Morphine was isolated from opium produced from cut seed pods of the poppy plant (*Papaver somniferum*) approximately 200 years ago.
- Few drugs developed from natural sources have undoubtedly revolutionized medicine, like:
    - ✓ Antibiotics (e.g. penicillin, tetracycline, erythromycin), antiparasitics (e.g. avermectin).
    - ✓ Antimalarials (e.g. quinine, artemisinin), lipid control agents (e.g. lovastatin).
    - ✓ Immunosuppressant for organ transplants (e.g. cyclosporine, rapamycins).
    - ✓ Anticancer drugs (e.g. paclitaxel, irinotecan).

Clinical trials are ongoing on more than 100 natural product derived drugs and at least 100 molecules/compounds are in preclinical development stage. Cancer and infections are the two predominant therapeutic areas for which the drug discovery program is based on natural products, but many other therapeutic areas also get covered, such as cardiovascular, gastrointestinal, inflammation etc.

The botanical sources are known to provide the following classes of NCEs for drug discovery processes.

- Bioactive compounds for direct use as drug, e.g. digoxin.
- Bioactive compounds with structures which themselves may act as lead compounds for more potent compounds, e.g. paclitaxel from *Taxus* species.
- The novel chemophore which may be converted into druggable compounds with/without chemical analoging.
- Pure photochemical for use as marker compounds for standardization of crude plant material or extract.
- Pure photochemical which can be used as pharmacological tools.
- Herbal extracts as botanical drugs, e.g. green tea extract (Katiyar *et al.*, 2012).

1.5 Traditional Use of Medicinal Plants

- Numerous clinical and animal studies document the efficacy of hawthorn as a cardio tonic. Cardio tonics help to improve blood supply to the heart, increase the tone of the heart muscle, stimulate cardiac output, dilate coronary arteries, stabilize blood pressure, prevent atherosclerosis (the accumulation of arterial plaque), and prevent or help improve congestive heart failure. Many herbs used for cardiovascular health, such as hawthorn and gingko, have antioxidant properties, which may help prevent hardening of the arteries or other circulatory insufficiencies.
- Some herbs used for cardiovascular health are commonly taken to lower cholesterol. Garlic is one notable example, and a number of clinical studies have shown that garlic is effective in moderately reducing serum cholesterol (Frishman WH et al., 2005).
- Clinical research indicates that ginger is a very effective herb for nausea, indigestion, and minor gastric upsets. Ginger is also effective for morning sickness in the early stages of pregnancy and for motion sickness.
- Peppermint oil has demonstrated clinical efficacy for irritable bowel syndrome.
- Many herbs are liver protective and restorative-they can help to protect a healthy liver and restore function to a liver that has suffered impaired functions due to disease or

injury, such as cirrhosis, hepatitis, or exposure to hepatotoxic agents. (Langmead L and Rampton DS, 2001).

- Adaptogenic herbs, such as ginseng, owe much of their activity to stimulation of pituitary and adrenal activity (Gaffney BT et al., 2001).
- Many plants are diuretics. They can help eliminate disease-carrying microorganisms from the urinary tract, and they can help prevent kidney stone formation and bladder inflammation resulting from bladder irritation-whether or not it's due to microbial infection. Others are effective urinary tract disinfectants. One that has been studied clinically and found effective for both prevention and treatment of urinary tract infections is cranberry, which may be taken as cranberry juice or in the form of concentrated cranberry juice solids (Jepson RG and Craig JC, 2007)
- Some nursing women use herbs to induce milk production during lactation, or conversely, to reduce milk production during weaning. For example, Fenugreek is an herb that has been successfully used to induce lactation.
- Black cohosh and red clover are effective for treating menopausal symptoms (Gabay MP, 2002).
- Anti-inflammatory botanicals, of which there are many (examples include ginkgo, ginger, hawthorn, and St. John's wort) are useful in suppressing various immune functions involved in the inflammatory response (Geller SE and Studee L, 2005).
- Extracts of immune-stimulating medicinal mushrooms, such as reishi or turkey tail can be used as adjunct therapies to help maintain immune functions during radiation and chemotherapy (Park EJ and Pezzuto JM, 2002).
- Fruit and vegetables rich in antioxidants are best as antioxidant supplements.
- Green tea, in its natural form or as a concentrated supplement.
- Dark chocolate contains many of the same beneficial compounds, known as catechins (Engler M and Chen C, 2004).
- One application of botanical medicines in this area is to lower blood sugar in individuals who may be diabetic or pre-diabetic. Popular botanical medicines thought to have this effect include: Ginseng, Ayurvedic medicine, Green tea (Chantre P and Lairon D, 2002).

All cultures have a history of herbal medicine use, usually making use of the plants found closest to home. Even today in the times of advanced technology and medical science still depend on plants for their healing. Western culture, however, is predominantly excited by the new and upcoming and the novel and perhaps most importantly the patentable. This means that the good, tried and tested tools of survival become relegated to historical anecdote.

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

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

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

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

1.6 Research on Herbal Drug

Herbal drug may be defined as the plants, plant parts and plant products of all description, particularly those with medicinal properties. Herbal drugs are generally manufactured by the combination of two or more natural substances. The utility of these combinations are:

- To increase efficacy of the drug.
- To remove toxic effects.
- To reduce side-effects.
- To maintain stability.
- To keep pleasant taste, color and odor.

1.6.1 Scientific Basis of Herbal Drug

Herbal drug is often criticized as non-scientific, inactive and erroneous medicine. But phytochemical and biological investigation proves its medicinal value and therapeutic utility. Traditional medicines that are used topically to treat skin disease contain tannin. Tannin is chemical having antiseptic and astringent property. When it is used topically it reacts with the proteins on infected area to produce a thin but strong barrier. This layer protects the infected area from micro-organism. Besides, tannin has antibiotic property. So it is said that there is no basic difference between herbal drug and allopathic medicine.

1.6.2 Necessity of Herbal Drug Research in Bangladesh

Most of the people of our country have no or little access to allopathic medicine due to their uncompromisable low income in respect of high cost of allopathic medicine. A survey conducted in 1990 in different villages of Bangladesh shows that on average of 14% if people suffering illness approach qualified allopathic doctors, 29% contact unqualified village doctors, 10% contact mollahs, 29% contact quack and 19% contact homeopaths.

The survey indicates an extensive use of medicinal plants, most of which are served in a crude and substandard form, by our people. The use of such crude and substandard herbal drug is dangerous and may threaten public health. Thus the analysis of plants for exploring the bounty of

chemical entities and their biological screening is the current need for standardization of herbal medication (Ghani, 1998). Since Bangladesh is a country of low economic growth, a proper health care system can be established by supplying low cost medicines to its population. This may be only possible by utilizing our natural resources of medicinal plants and their constituents. So, scientific exploration and standardization of these potential crude drugs is an urgent need to revolutionize our drug sector.

Besides, Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi-processed plant products to produce drugs and medicines. During the last five years Bangladesh has spent more than 1500 crore Taka for importing chemicals, raw materials and semi-processed drugs of plant origin from neighboring and other countries and this trend is growing upwards day by day. This huge foreign exchange can be saved if the indigenous medicinal plants or its semi processed products are utilized by the manufacturer to satisfy their need (Ghani, 1998).

1.6.3 Interesting Truths and Facts about Herbal Remedies

Herbal remedies are the use of plants or plant extracts to medicate certain illnesses, minor or serious illnesses even cancer treatment. It has been used by our ancestors historically - the Chinese, Arabs, Africans for centuries. According to the World Health Organization, traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses.

Fact Number 1

The effectiveness of herbal medicine is not corroborated by scientific evidences but it is continually used by almost 80 percent of people in other parts of the world like Asia and Africa and Arab nations.

Fact Number 2

In some parts of the world specifically the United States herbal remedies are classified as dietary supplement because it cannot either be classified as food or drugs. Laws pertaining to dietary supplements are relatively lax comparing to drugs or foods.

Fact number 3

The World Health Organizations stated that ―Not many countries have national policies for traditional medicine. Regulating traditional medicine products, practices and practitioners is difficult due to variations in definitions and categorizations of traditional medicine therapies. A single herbal product could be defined as either dietary supplement food or an herbal medicine, depending on the country. This disparity in regulations at the national level has implications for international access and distribution of products.

Fact Number 4

The availability of herbs and herbal products in some countries is a problem that is why it is difficult to standardize it.

Fact Number 5

Culture plays an important part whether to use herbal remedies or not. Availability of immediate herbal remedies is also a factor in using them.

Fact Number 6

In some countries, there are well defined rules and laws pertaining to herbal medicines as well as professionals who are certified to practice "the scientific study of herbals", case in point is China where there are medical doctor who are giving prescriptions pertaining to herbals.

Fact Number 7

The people in the oldest civilization used herbal remedies to treat diseases specially the Chinese, Arabs and Africans as evidence into eh different writings. It has been fact that herbal medicine is prevent o be effective even in history.

Fact Number 8

About the safety and effectiveness plus quality: The World health Organization also stated that: the ―Scientific evidence from tests done to evaluate the safety and effectiveness of traditional medicine products and practices is limited. For example, it can be difficult to assess the quality of finished herbal products. The safety, effectiveness and quality of finished herbal medicine products depend on the quality of their source materials (which can include hundreds of natural constituents), and how elements are handled through production processes.

Putting all this information together it is up to the individual whether to medicate with herbal remedies or not. As they say health is wealth and since herbal remedies have been proven historically not scientifically, it is a nice alternative as herbal is natural and it comes from nature as well. In the same manner as there should always be precaution, we should talk to a physician first if we have doubts about herbal remedies especially if we are taking medicines for other sickness. Like in scientific medicine, there is always risk in taking medicines whether it is herbal or scientific medicines which are artificial. Scientific medicines are artificial and taken in by our body in the same manner as herbal remedies are provided by nature (HubPages, 2014).

1.7 *Syzygium samarangense:* A Brief Outlook

Syzygium samarangense (Family: Myrtaceae), is a tropical tree growing to 16 to 50 ft (5- 15 m) tall. The tree has a short trunk with thick and open, wide spreading crown and pinkish-gray, flaking bark. It is also called as Wax apple, Rose apple and Java apple. This species presumably originated in Malaysia. It is widely under cultivated and grown throughout Malaysia and in neighboring countries such Thailand, Indonesia and Taiwan. From South America to Southeast Asia, these fruits have been used for a wide variety of ailments, including cough, diabetes, dysentery, inflammation and ringworm. It is also under cultivation in different parts of India for their edible fruits. Different varieties of fruits vary in skin color, ranging from white, green to deep red with white flesh. The pulp is crisp and watery, with a scented flavor. It needs an extra-tropical climate growing at the lower altitudes up to 4,000 ft (1,220 mt) in India. It does best in parts of the Philippines that have a long dry season.

1.7.1 Plant Family: Myrtaceae

Myrtaceae, a family of mainly tropical and subtropical plants in which the leaves are simple, commonly opposite, finely dotted with oil glands, and without stipules. The flowers are regular, with 4 or 5 petals, numerous exerted stamens, and the ovary more or less inferior. The fruit is a woody capsule or a berry. There is one to many seeds. Some are ornamentals with showy flowers. Many produce valuable timbers. There are 121 genera, comprising about 3850 species, occurring in tropical and warm regions, and abundant in Australia (Encyclopedia.com, 1998).

Four important groups (genera) in the family Myrtaceae are:

- *Eucalyptus*
- *Callistemon*
- *Melaleuca*
- *Leptospermum* (Australian National Botanic Gardens, 2013).

1.7.2 Plant Profile

1.7.2.1 Scientific Name: *Syzygium samarangense* (Blume) Merr. & Perry.

1.7.2.2 Common/English Names: Java Apple, Java Roseapple, Mountain Apple (Pacific Islands), Samarang Rose Apple, Wax Apple, Wax Jambu.

1.7.2.3 Botanical Names: Synonyms

Table 1.1: Other scientific names of *S. samarangense*

Eugenia javanica Lam.
Eugenia samarangensis(Blume) O.Berg
Jambosa javanica (Lam.) K.Schum. & Lauterb.
Jambosa samarangensis(Blume) DC.
Myrtus javanica (Lam.) Blume

Myrtus samarangensis Blume

Different scientists in different part of the world may think they discovered the plant first, so each of them gave different names. But the first given name takes precedence, the other names are rejected, and thereafter referred to as synonyms.

1.7.2.4 Taxonomic Tree

Table 1.2: Scientific classification of *S. samarangense*

Domain: Eukaryota
Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Dicotyledonae
Order: Myrtales
Family: Myrtaceae
Genus: *Syzygium*
Species: *Syzygium samarangense*

(Cabi.org, 2016)

1.7.2.5 Traditional Names

Classical or traditional names of plant include:

Table 1.3: Vernacular/ Local names of *S. samarangense*

Location	Names
Filipino	Java apple, makopa
Indonesian	Jambu klampok (java)
Malay	Jambu air mawar
Hindi	Jamrul, amrool

Telugu	Gulaabijaamichettu
Thai	Chomphu-khieo
Vietnamese	Roi
Malayalam	Chambekka
Jamaica	Otaheti apple
Bengali	Jamrul
Sri Lanka	Jumbo
French	Jamalac

(*Syzygium Samarangense:* A Review on Morphology, Phytochemistry & Pharmacological Aspects, 2011)

1.7.2.6 Origin/Distribution

Java apple is indigenous in Bangladesh to the Solomon Islands. It has naturalized in the Philippines since prehistoric times. It is commonly and widely cultivated in Malaysia, Indonesia, Thailand, Cambodia, Laos, Vietnam and Taiwan. It is frequently cultivated in India and in Africa (Zanzibar and Pemba) and also in the Antilles, Suriname and northern Australia.

1.7.2.7 Agroecology

This species thrives in the warm fairly moist tropical lowlands up to 1,200 m altitude. It is more adaptable than *S. malaccense* to drier conditions and can withstand long dry season with a reliable water supply from streams and ponds. This species prefers heavy, fertile soils.

1.7.2.8 Edible Plant Parts and Uses

The ripe fruit is eaten fresh out of hand or they are sliced and eaten in fruit cocktails, or dipped in salt, in a sweetened dark soya sauce, in sambal or in a fruit salad “rujak” mixed with a spicy peanut sauce. In Indonesia the fruit are also preserved in pickles, “asinan”.

Java apple can also be stewed like apples. The fruit is frequently used in salads, and used in light sautéed dishes in Indian ocean island cuisine. The ripened fruit varies in hue and can be from yellowish-greenish white to light pink to red to a dark, maroon purple (Lim, 2012).

1.7.2.9 Morphological Description

- **Tree:** The tree is 16 to 50 ft (5-15 mt) tall, has a short trunk 10 to 12 in (25-30 cm) thick, and open, wide spreading crown, and pinkish-gray, flaking bark.

- **Leaves:** rounded or slightly cordate at 5 cm) long and 5-12 cm wide;

Figure 1.2: *S. samarangense* leaves

- **Flowers:** Flowers, borne in drooping panicles of 3 to 30 at the branch tips or in smaller clusters in the axils of fallen leaves, are fragrant, yellowish-white, 3/4 to 1 1/2 in (2-4 cm) broad, 4-petalled, with numerous stamens 3/5 to 1 in (1.5-2.5 cm) long.

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Figure 1.4: *S. samarangense* fruits

- **Seeds:** Each fruit contains a single large, subglobose seed or a pair of subglobose to emispherical seeds 1.6–2 cm (0.6–0.8 in) in diameter, lightbrown externally, green internally, and somewhat meaty in texture. The fruits of some trees are entirely seedless.

Figure 1.5: *S. samarangense* seeds

1.7.2.10 Traditional Claims

- **Leaves:** It is used as astringent, to treat fever and halt diarrhoea. Powdered leaves are used for cracked tongues. Juice of leaves is used in baths and lotion. It is also used in diabetes, cough and headaches.
- **Fruits:** It is used in diabetes, stomatitis aphtosa, diuretic, emmenagogue, abortifacient and febrifuge. Decoction of fruits is used in fever.
- **Root-bark:** The root bark decoction is used in dysentery and amenorrhea and also used as abortifacient.
- **Root:** It is used as diuretic and is given to alleviate edema. Malayans use powdered dried root preparations for itching.
- **Bark:** Juice of bark is used to treat wounds and the bark is used as astringent in mouthwash preparations for the treatment of thrush.
- **Stem:** Decoction of stem is used to treat diarrhea (*Syzygium Samarangense:* A Review on Morphology, Phytochemistry & Pharmacological Aspects, 2011).

1.7.2.11 Nutritive/Medicinal Properties

The nutrient composition of *S. samarangense* fruit per 100 g edible portion (Leung et al. 1972) was reported as: water 91.5 g, energy 30 kcal, protein 0.4 g, fat 0.1 g, carbohydrate 7.8 g, fibre 0.8 g, ash 0.2 g, Ca 17 mg, P 9 mg, Fe 0.3 mg, Na 2 mg, K 105 mg, b-carotene 0 m g, thiamin 0.03 mg, riboflavin 0.01 mg, niacin 0.3 mg, ascorbic acid 13 mg. Another analysis conducted in Australia reported that wax jambu had the following food value per 100 g edible portion (Wills 198): water 90.3%, protein 0.7 g, fat 0.2 g, glucose 2.1 g, fructose 2.4 g, dietary fibre 1.9 g, malic acid 0.10 g, citric acid 0.12 g, oxalic acid 0.02 g, energy 94 kJ, vitamin C 8 mg, thiamin 0.02 mg, riboflavin 0.04 mg, niacin 0.5 mg, K 38 mg, Na 1 mg, Ca 13 mg, Mg 5 mg, Fe 0.8 mg and Zn 0.1 mg. A total of 39 volatile constituents were identified in wax jambu *(S. samarangense*) (Wong and Lai 1996). The volatiles of wax jambu were characterized by the presence of a large number of C9 aldehydes and alcohols. A triterpene, methyl 3-epi-betulinate in its native form and 4,6-dihydroxy-2-methoxy- 3, 5 -dimethyl chalcone along with ursolic acid, jacoumaric acid and arjunolic acid were isolated from the aerial parts of *Syzygium samarangense* (Srivastava et al. 1995). Various parts of the plant have been reported to have bioactive compounds and to exhibit antioxidant, anticancer, antiviral, antimicrobial, spasmolytic, antihyperglycaemic, protease inhibition, anti-amnesiac and immunomodulatory activities.

1.7.2.12 Other Uses

- Java apple is also cultivated as ornamental or wind break.
- The red hard wood is used for house construction in the Nicobar and Andaman Islands (Lim, 2012).

1.8 Aims of the Present Study

Attempts should be continued for the evaluation of the cytotoxic, antimicrobial, and antioxidant activity of the aqueous fraction of *S. samarangense* leaves extract. To conduct cytotoxic investigation of aqueous extract by brine shrimp lethality bioassay. To investigate in vitro antioxidant property of aqueous extract by total phenolic content and total flavonoid

content. *S. samarangense* is a very common plant which is used in our country as well as in world by a lot of people for several purposes. All the parts of this plant are used for medicinal activity. To achieve this objective, the whole work was designed in the following way:

1. Cytotoxic study with aqueous fraction.

2. Antioxidant study with aqueous fraction.

3. Observation of antimicrobial action with aqueous fraction.

-CHAPTER TWO-

LITERATURE REVIEW

2.1 Phytoconstituents of *S. samarangense*

Investigators have found their principal constituent to be tannins

- **Leaves contains:** (1) lupeol (triterpenoid); (2) betulin (triterpenoid); (3) epi-betulinicacid (triterpenoid); (4) 2, 4-dihydroxy-6-methoxy-3-methylchalcone; (5) 2-hydroxy-4, 6-dimethoxy-3-methylchalcone; (6) 2, 4-dihydroxy-6-methoxy-3, 5-dimethylchalcone; (7) 2, 4-dihydroxy-6-methoxy-3-methyldihydrochalcone; (8) 7-hydroxy-5-methoxy-6, 8-dimethylflavanone; (9) 2-hydroxy-4, 6-dimethoxy-3-methyldihydrochalcone; (10) 2, 4-dihydroxy-6-methoxy-3, 5-dimethyldihydrochalcone; (11) sitosterol; (12) alpha-carotene and Beta-carotene.
- **Leaf oil** is largely composed of monoterpenes (30% sesquiterpenes, 9 % caryophyllene).
- **Aerial parts** contains Ursolic acid, Jacoumaric acid and Arjunolic acid, Mearnsitrin, 2 - C-Methyl-5- O-Galloylmyricetin-3-O-α-l-Rhamnopyranoside, desmethoxymatteucinol, 4, 6 Dihydroxy-2-Methoxy- 3, 5-Dimethylchalcone, Methyl 3-epi-betulinate, Oleanolic acid. They also contain Desmethoxymatteucinol, 5-O-Methyl-4-desmethoxymatteucinol, Oleanic acid.
- **Quercetin glycosides** are also present in this plant which includes Reynoutrin, Hyperin, Myricitrin, Quercitrin, Quercetin, Guaijaverin. It also contains Flavanone - (S)-pinocembrin, and Phenolic acids- Gallic acid and Ellagic acid.

2.2 Pharmacological Activities of *S. samarangense*

WHO recognized several *Syzygium* species were reported to possess antibacterial, antifungal and anti inflammatory activities. The flavonoids, isolated from *S. samarangense*, were reported to possess antihyperglycemic activity, spasmolytic and immunomodulatory activities.

2.2.1 Antidiarrhoeal Activity

The hexane extract of *S. samarangense* was found to dosedependently (10-3000 microg/mL) relax spontaneously contracting isolated rabbit jejunum. When tested for a possible calcium channel blocking (CCB) activity, the extract (10-1000 microg/mL) relaxed the high K+-induced contractions and also decreased the Ca++ dose-response curves in a dosedependent manner (30-100 microg/mL), confirming the CCB activity. The flavonoids isolated from the hexane extract were tested for a possible spasmolytic activity. All flavonoids, showed dosedependent (10-1000 microg/mL) spasmolytic activity. These indicate that the presence of compounds with spasmolytic and calcium antagonist activity may be responsible for the medicinal use of the plant in diarrhea.

2.2.2 Anticholinesterase Activity

The actual inhibitory assay involves the addition of 30 µL of test sample solution and 30 µL of enzyme stock solution to 2.81 µL of phosphate buffer. The mixture was incubated for 5 – 10 min at 25°C. A 100 µL of DTNB stock solution and 30 µL of substrate stock solution were then added and absorbance at 412 nm was recorded. The control used was physostigmine. The percent inhibition was calculated. When tested against butyrylcholinesterase, it exhibited 68.0% inhibitory activity at 0.20 mM concentration and its IC50 was determined to be 127 µM. The IC50 of physostigmine, the positive control, was 0.041 µM and 0.857 µM against acetylcholinesterase and butyrylcholinesterase respectively.

2.2.3 Immunopharmacological Activity

The flavonoids isolated from *S. samarangense* were evaluated for immunopharmacological activity. Human peripheral blood mononuclear cells (PBMC) were used as target cells, and cell proliferation was determined by 3H-thymidine uptake. Among the flavanoids, (-)-strobopinin, myricetin 3-O-(2"-O-galloyl)-alpha-rhamnopyranoside, (-)- epigallocatechin 3-O-gallate and myricetin 3-O-alpha-rhamnopyranoside showed inhibitory potency on PBMC proliferation activated by phytohemagglutinin (PHA). The IC50 values of compounds 1, 2, 3,

and 4 on activated PBMC proliferation were 36.3, 11.9, 28.9, and 75.6 µM, respectively. The inhibitory mechanisms may involve the blocking of interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) production, since compounds 1, 2, 3 and 4 reduced IL-2 and IFN gamma production in PBMC in a dose-dependent manner**.**

2.2.4 Cytotoxic Activity

The flavonoids 2'-hydroxy-4',6'-dimethoxy-3'-methylchalcone , 2',4'- dihydroxy-6'-methoxy 3',5'-dimethylchalcone , 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone , 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyldihydrochalcone, isolated from *S. samarangense*, were subjected to cytotoxicity testing using the dimethylthiazoldiphenyl tetrazolium (MTT) assay. The cell lines used were the Chinese hamster ovarian (CHO-AA8) and the human mammary adenocarcinoma, (MCF-7 and SKBR- 3). Among the test compounds, 2 exhibited significant differential cytotoxicity against the MCF-7 cell line with an IC50 of 0.0015 ± 0.0001 nM. It was also cytotoxic against the SKBR-3 cell line with an IC_{50} of 0.0128 ± 0.0006 nM. Doxorubicin, the positive control, had an IC50 of 2.60 ± 0.28 × 10-4 nM against the MCF-7 cell line and an IC_{50} of 2.76 ± 0.52 × 10-5 nM against the SKBR-3 cell line. When tested in a mechanism-based yeast bioassay for detecting DNA-damaging agents using genetically engineered *Saccharomyces cerevisiae* RS322Y (RAD52) mutant strain and (LF15/11) (RAD+) wild type strain, 2 showed significant selective cytotoxicity against the RAD52 yeast mutant strain. It had an IC12 of 0.1482 nM, as compared with the positive control, streptonigrin, which had an IC12 of 0.0134 nM. Hence, 2 is a cytotoxic natural product with potential anticancer application.

2.2.5 Antibactirial Activity

According to Asian Journal of Biochemical and Pharmaceutical Research the methanolic and the petroleum ether extracts of *S. samarangense* exhibited significant antimicrobial activity on certain pathogens. The minimum inhibition and minimum bacterial/fungal concentrations were determined by microdilution method using 96-well microtitre plate method. As the disc

dosage level increases the inhibitory effect is also increased. The extracts were proved as strong inhibitors against gram negative bacteria than gram positive bacteria.

2.2.6 Analgesic and Anti-Inflammatory Activity

Cycloartenyl stearate, lupenyl stearate, sitosteryl stearate, and 24-methylenecycloartanyl stearate (sample 1) from the air-dried leaves of *Syzygium samarangense* exhibited potent analgesic and anti-inflammatory activities at effective doses of 6.25 mg/kg body weight and 12.5 mg/kg body weight, respectively. Sample 1 also exhibited negligible toxicity on zebra fish embryonic tissues. There were incidences of mortality upon direct exposure of sample 1 to dechorionated embryos, but higher mortality and aberration were observed during intact chorion treatment.

2.2.7 Antioxidant Activities

Antioxidant activity of *S. samarangense* was investigated in fruits. For this, at first matured fruits of them were sliced into small pieces and dried in the sun and finally crushed in a grinder to make powder. Ethanolic extracts of fruit powder were prepared using 99.99% ethanol. The antioxidative activities of the extracts were determined according to their abilities of scavenging 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. It was demonstrated that the ethanolic extracts of *S. samarangense* showed antioxidant activity. The IC_{50} of the ethanolic extract *S. samarangense* was 200 µ/mL. This indicates the fruit is beneficial to human health (Syzygium Samarangense: A Review On Morphology, Phyt ochemistry & Pharmacological Aspects, 2011).

2.2.8 Anticancer Activity

Ethanolic extracts of the fruit powder showed antioxidant activities indicating java apple fruit to be beneficial to human health. Studies reported the methanolic extracts of the pulp and seeds of the fruits of *Syzygium samarangense* yielded four cytotoxic flavanoid compounds and eight antioxidant compounds (Simirgiotis et al. 2008). Three C-methylated chalcones, 2′ ,4′ -dihydroxy-3′ ,5′ - dimethyl-6′ -methoxychalcone, 2′ ,4′ -dihydroxy- ′3 -methyl-6′ -

methoxychalcone (stercurensin), and 2′ ,4 ′-dihydroxy-6′ -methoxychalcone (cardamonin,) were isolated and displayed cytotoxic activity (IC 50 = 10, 35, and 35 mM, respectively) against the SW-480 human colon cancer cell line.

Among the flavonoids isolated from *Syzygium samarangense* , namely 2-hydroxy-4,6-dimethoxy-3-methylchalcone (1), 2,4-dihydroxy-6- methoxy-3,5-dimethyl chalcone (2), 2,4′ -dihydroxy-6-′methoxy-3-′methylchalcone (3), 2′,4′- dihydroxy-6′-methoxy-3′-methyldihydrochalcone (4) and 2′, 4′ -dihydroxy-6′-methoxy-3′,5 ′-dimethyldihydrochalcone (5), compound 2 exhibited cytotoxicity testing using the dimethylthiazoldiphenyl tetrazolium (MTT) assay (Amor et al. 2007) . Compound 2 exhibited significant differential cytotoxicity against the human mammary adenocarcinoma MCF-7 cell line with an IC 50 of 0.0015 nM. It was also cytotoxic against the human mammary adenocarcinoma SKBR-3 cell line with an IC 50 of 0.0128 nM. Doxorubicin, the positive control, had an IC 50 of 2.60 × 10 −4 nM against the MCF-7 cell line and an IC 50 of 2.76 × 10 −5 nM against the SKBR-3 cell line. Compound 2 showed significant selective cytotoxicity against the RAD52 yeast mutant strain. It had an IC 12 of 0.1482 nM, as compared with the positive control, streptonigrin, which had an IC 12 of 0.0134 nM. Hence, compound 2 was deemed a cytotoxic natural product with potential anticancer application.

Results of studies suggested that dimethylcardamonin (2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone; DMC), a naturally occurring chalcone, and major compound isolated from *Syzygium samarangense* leaves, possessed antiproliferative activity (Ko et al. 2011) . DMC suppressed colorectal carcinoma HCT116 and LOVO cell proliferation through a G (2) /M phase cell-cycle delay, and induced autophagy, the hallmark of Type II programmed cell death.

2.2.9 Antiviral Activity

Oleanolic acid, an anti-HIV compound and ursolic acid have also been isolated from leaves. Both oleanolic acid and ursolic acid were found effective in protecting against chemically induced liver injury in laboratory animals (Liu 1995). Oleanolic acid has been marketed in

China as an oral drug for human liver disorders. Oleanolic acid and ursolic acid have also been long recognized to have anti-inflammatory and antihyperlipidemic properties in laboratory animals. Oleanolic acid and ursolic acid are relatively nontoxic, and have been used in cosmetics and health products (Liu 1995). Oleanolic acid also possessed anti-HIV activity. Studies showed that oleanolic acid inhibited the human immunodeficiency virus-1 (HIV-1) replication in all the cellular systems (cultures of human peripheral mononuclear cells (PBMC) and of monocyte/ macrophages) (Mengoni et al. 2002).

2.2.10 Protease Inhibitory/Antiamnesiac Activity

Compounds isolated from the hexane extract of the leaves of *Syzygium samarangense* exhibited inhibitory activity against the following serine proteases: trypsin, thrombin and prolylendopeptidase (Amor et al. 2004). The compounds were identified as a mixture of a -carotene and b -carotene (1), lupeol (2), betulin (3), epi-betulinic acid (4), 2,4′ -dihydroxy-6′ -methoxy-3′ - methylchalcone (5), 2′ -hydroxy-4′ ,6′ -dimeth oxy-3′ -methylchalcone (6), 2 ′ ,4′ -dihy droxy-6′ - methoxy-3 ′,5 ′ -dimethylchalcone (7), 2′ ,4′ -dihydroxy-6′ -methoxy-3′ -methyldihy drochalcone (8) and 7-hydroxy -5-methoxy-6,8-dimethylfl avanone (9). Hydro genation of compounds 5, 6 and 7 yielded compound 8, 2 ′ -hydroxy-4′ ,6 ′ -dimethoxy -3′ -methyldihydrochalcone (10) and 2′ ,4′ -dihydroxy-6′ -methoxy-3′,5′-dimethy l dihydrochalcone (11), respectively. In addition, b-sitosterol (12) and b -D-sitosterylglucoside (13) were also isolated. Compounds 3–8 and 10 exhibited significant and selective inhibition against prolylendopeptidase among three serine proteases. Inhibitors of prolyl endopeptidase may improve memory by blocking the metabolism of endogenous neuropeptides and may have possible potential as anti-amnesiac drugs (Yoshimoto et al. 1987).

Currently, new drugs are required that can improve memory and learning or delay the neurodegenerative process in conditions such as Alzheimer's disease. Prolylendopeptidase (PEP) an enzyme with a role in metabolism of prolinecontaining neuropeptides, such as vasopressin, substance P and thyrotropin-releasing hormone (TRH), were suggested to be involved with learning and memory process (Tezuka et al. 1999).

2.2.11 Antihyperglycaemic Activity

Research reported that the flavonoid, 2′ ,4′-dihydroxy-3′,5′ -dimethyl-′6-methoxychalcone 1, its isomeric flavanone 5-O-methyl- 4′ -desmethoxymatte-ucinol 2 and 2′ 4′ dihydroxy- 6′ methoxy-3′ -methylchalcone 3 isolated from the leaves significantly lowered the blood glucose levels (BGLs) in glucose-hyperglycaemic mice when administered 15 minutes after a glucose load, indicating their antihyperglycaemic property (Resurreccion-Magno et al. 2005).

-CHAPTER THREE-

METHODS and MATERIALS

3.1 Collection and Preparation of Plant Material

Plant sample of *Syzygium samarangense* was collected from Narayangonj in March, 2016. 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.

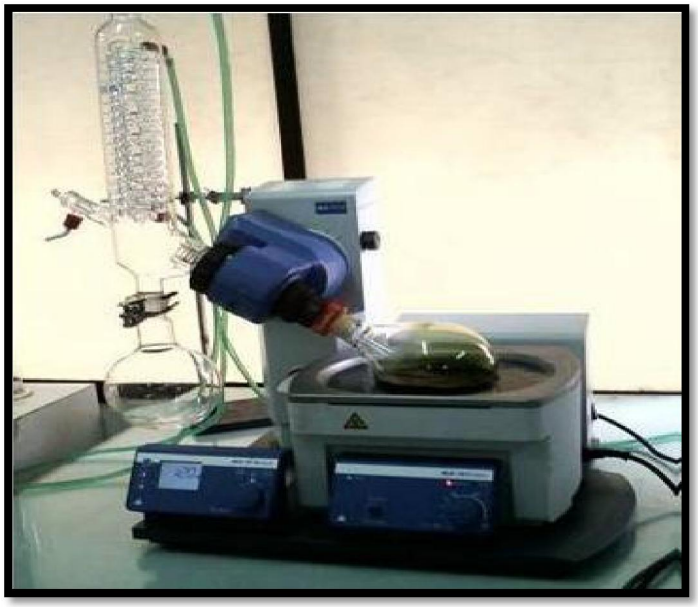


Figure 3.1: Drying of extract using rotary evaporator

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 n-hexane

The mother solution was taken in a separating funnel. 100 ml of the n-hexane 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×3). The n-hexane fraction was then air dried for solid residue.

3.4.2 Partition with Dichloromethane

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

3.4.3 Partition with Ethyl Acetate

To the mother solution that left after washing with n-hexane, and Dichloromethane, 16 ml of distilled water was added and mixed. The mother solution was then taken in a separating

funnel and extracted with ethyl acetate. The process was repeated thrice (100 ml ×3). The ethyl acetate fraction was then air dried for solid residue.

3.4.4 Partition with Aqueous Fraction

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

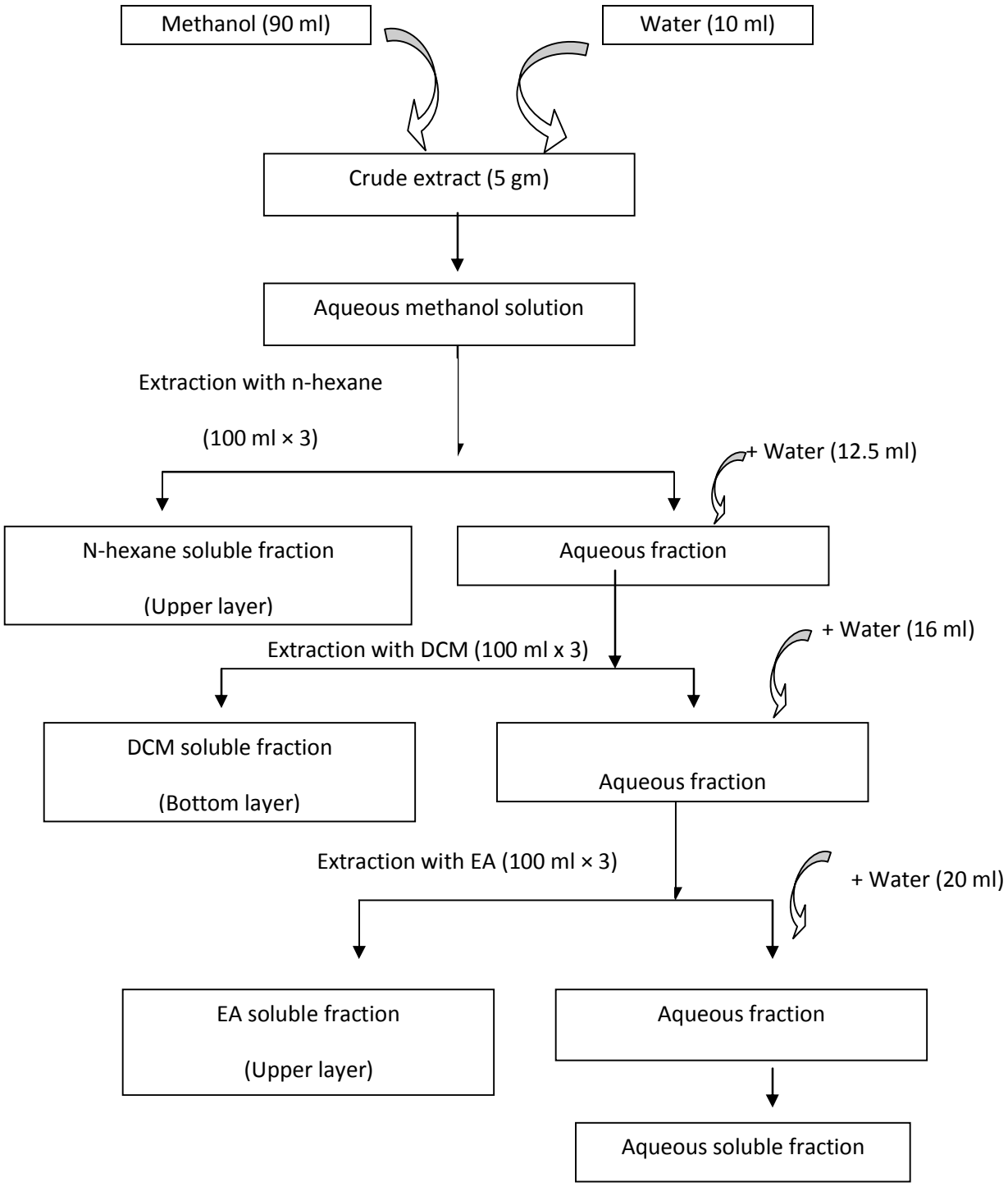


Figure 3.2: Schematic representation of the partitioning of methanolic crude extract of *S. samarangense.*

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 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 and 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).

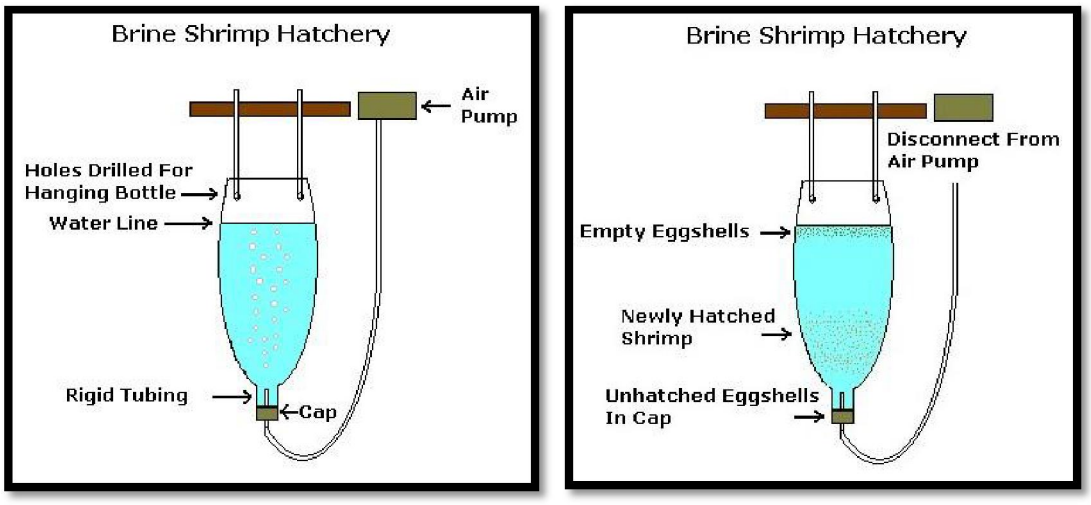


Figure 3.3: Brine shrimp Hatchery

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).

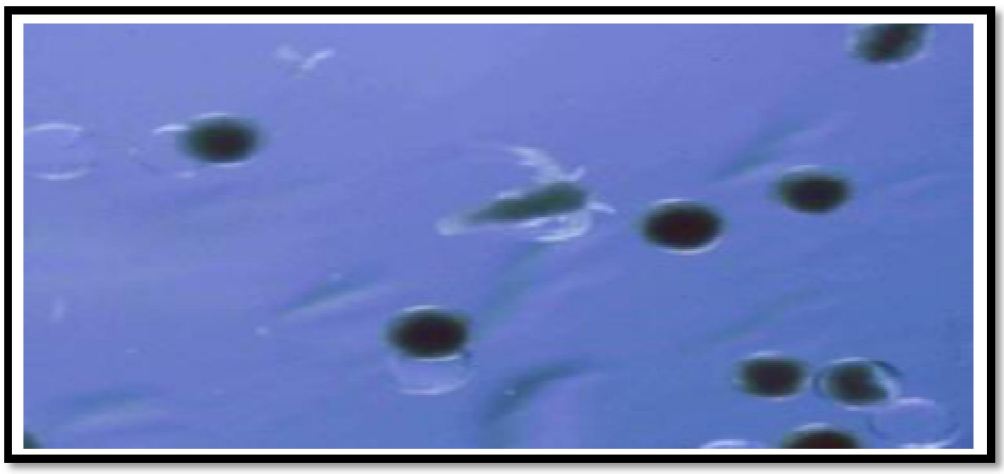


Figure 3.4: Counting of nauplii

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 antioxidants 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 antioxidative effects have rarely been carried out. The purpose of this study was to evaluate extractives of *S. samarangense* 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

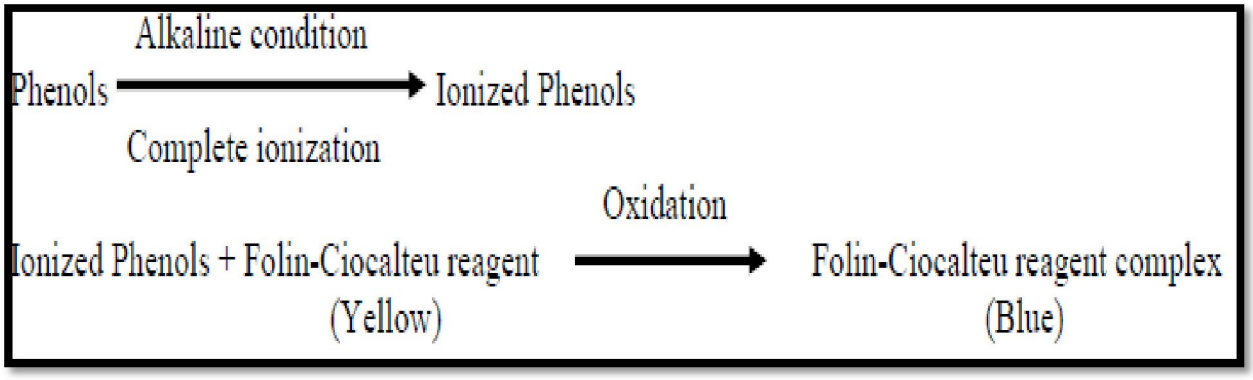
The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. In the alkaline condition phenols ionize completely.

Table 3.2: Composition of 100 mg Folin-Ciocalteu Reagent

i.	Water	57.5 ml
ii.	Lithium Sulfate	15.0 mg
iii.	Sodium Tungstate Dihydrate	10.0 mg
iv.	Hydrochloric Acid (25%)	10.0 mg
v.	Phosphoric Acid 85% solution in water	5.0 mg
vi.	Molybdic Acid Sodium Dihydrate	2.5 mg

When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution become blue. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$.

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 and 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)
Na_2CO_3 solution (7.5%)	Test tube
Methanol	Micropipette (50-200 µl)
Distilled water	Cuvette

3.6.1.3 Procedure

3.6.1.3.1 Standard Curve Preparation

Ascorbic acid was used here as standard. Different ascorbic acid solutions were prepared having a concentration ranging from 120 µg/ml to 80 µg/ml. 5 ml of FCR (diluted 10 times with water) and 4 ml of Na_2CO_3 (7.5% w/v) solution was added to ascorbic acid solution. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 765 nm. After plotting the absorbance in ordinate against the concentration in abscissa a linear relationship was obtained which was used as a standard curve for the determination of the total phenolic content of the test samples.

3.6.1.3.2 Sample Preparation

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

3.6.1.3.3 Determination of Total Phenol Content

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

3.6.2 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 C et al., 2002).

Flavonoid (Extract) + $AlCl_3$ (reagent) = Formation of flavonoid-aluminium complex (λmax = 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

3.6.2.3.1 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.

3.6.2.3.2 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.

3.6.2.3.3 Preparation of 5% (W/V) $NaNO_2$ 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.

3.6.2.3.4 Preparation of Standard Solution: The stock solution was prepared by taking 10 mg of quercetin and dissolved into 50 ml of methanol. Concentration of this solution was 200 µg/ml of quercetin. The experimental concentrations (0, 4, 8, 12, and 16 µg/ml) were prepared from this stock solution.

Table 3.5: Preparation of standard solution

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

3.6.2.3.5 Preparation of Extract Solution: 5 mg of each plant extracts were taken and dissolved into 5 ml of methanol. The concentration of the solution was 1 mg/ml of plant extracts. Then the following steps were carried out.

1.5 ml extract was taken in a test tube and then 6 ml of distilled water was added. Then 5% of $NaNO_2$was added and incubated for 6 minutes. 10% $AlCl_3$ was added and incubated for 6 minutes. 4% NaOH and 0.6 ml distilled water was added. Then it was incubated for 15 minutes. For blank solution 1.5 ml methanol was taken and same procedure was repeated.

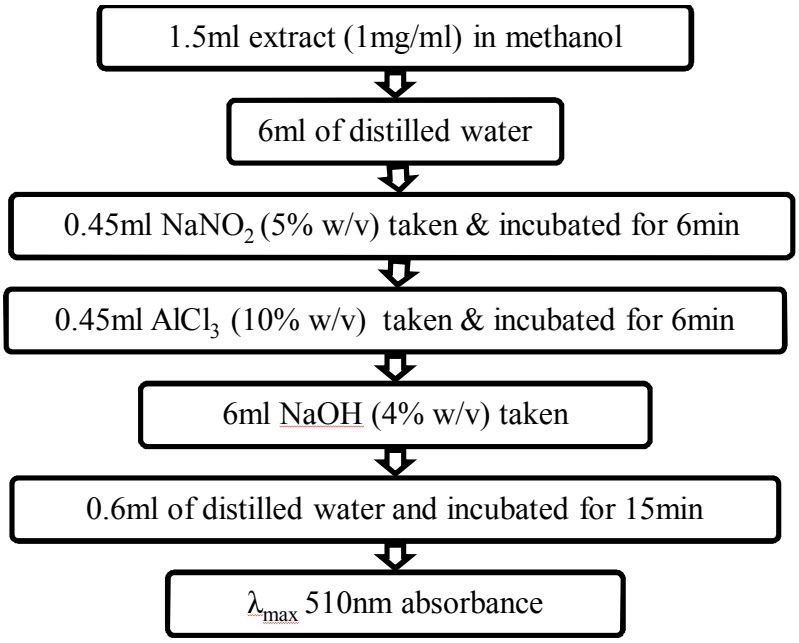


Figure 3.5: Schematic diagram of preparation of extract solution

Preparation of blank solution

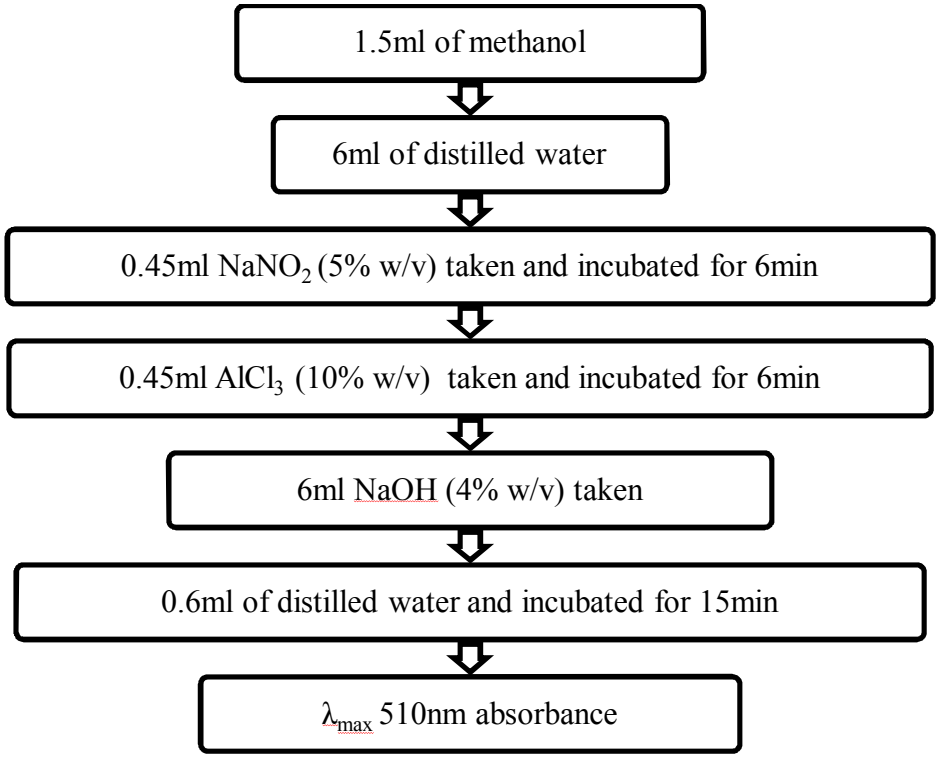


Figure 3.6: Schematic diagram of preparation of blank solution

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 and 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 *Syzygium samarangense*

Aqueous fraction of methanolic extract of *Syzygium samarangense* 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 +ve	*Bacillus subtilis* *Bacillus cereus* *Bacillus megaterium* *Staphylococcus aureus*
Gram –ve	*Escherichia coli* *Salmonella typhi* *Salmonella paratyphi* *Vibrio parahaemolyticus* *Vibrio mimicus* *Shigella dysenteriae*
Fungi	*Candida albicans* *Aspergillus niger*

3.7.5 Procedure

3.7.5.1 Preparation of the Medium

To prepare required volume of this medium, 4.6 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.

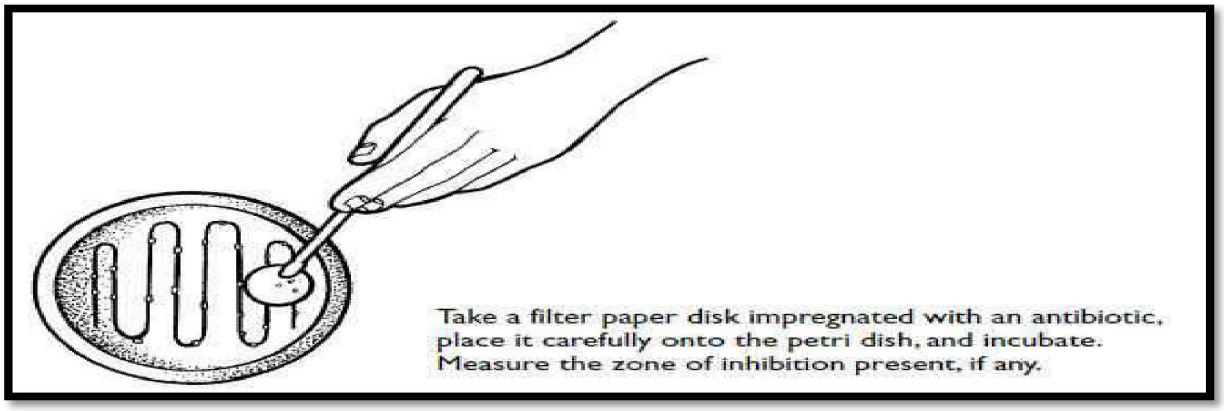


Figure 3.9: Preparation of filter paper discs

- **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 air drying) and the filter paper were not active themselves.

3.7.5.7 Diffusion and Incubation

The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator at 4°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.

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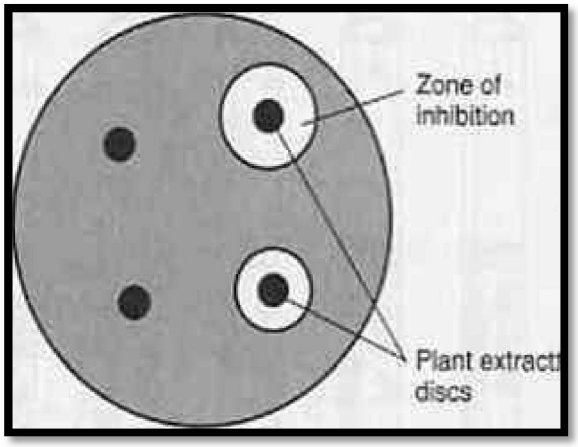

Figure 3.11: Clear zone of inhibition

Figure 3.12: Determination of clear zone of inhibition

-CHAPTER FOUR-

RESULT and DISCUSSION

4.1 Result of Brine Shrimp Lethality Bio-Assay

The aqueous fraction of the *Syzygium samarangense* 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:

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

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.

4.1.1 Preparation of Curve for Standard

Here, Tamoxifen was used as reference standard.

Table 4.1: Results of the bioassay of Tamoxifen (standard)

Test tube no.	**Concentration (C) (µg/ml)**	**Log C**	**Number of Nauplii alive**	**Number of Nauplii dead**	**% Mortality**	**LC_{50} (µg/ml)**
1	400	2.602	0	10	100	12.51
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	4	6	60	
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	.078125	-0.107	9	1	10	

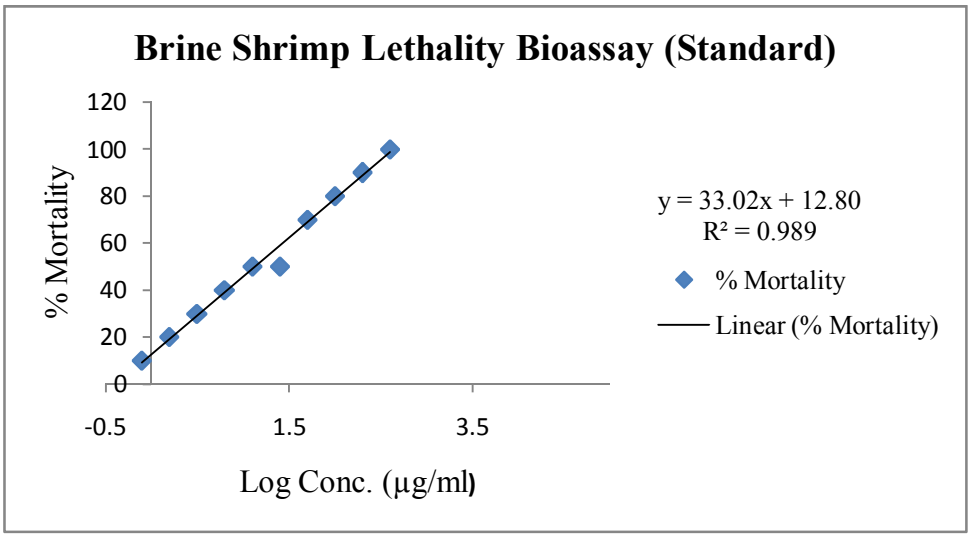

Figure 4.1: % Mortality and predicted regression line of Tamoxifen (standard)

4.1.2 Preparation of Aqueous Fraction Curve

Table 4.2: Results of the bioassay of aqueous fraction (extract)

Test tube no.	**Concentration (C) (µg/ml)**	**Log C**	**Number of nauplii alive**	**Number of naupliidead**	**% Mortality**	**LC_{50} (µg/ml)**
1	400	2.602	0	10	100	1.64
2	200	2.301	1	9	90	
3	100	2.000	1	9	90	
4	50	1.699	2	8	80	
5	25	1.398	3	7	70	
6	12.5	1.097	4	6	60	

7	6.25	0.796	3	7	70	
8	3.125	0.495	2	8	60	
9	1.5625	0.194	3	7	50	
10	.078125	-0.107	4	6	40	

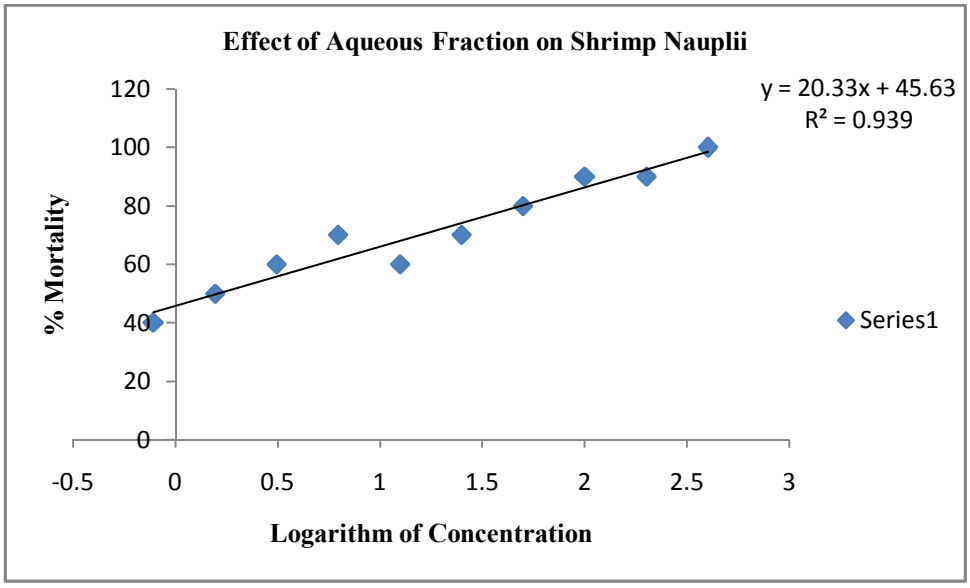

Figure 4.2: % Mortality and predicted regression line of aqueous fraction (extract)

4.1.3 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 ranging from the lowest concentration to the highest concentration in both standard and aqueous fraction samples. Mortality increased gradually with an increase in concentration of the test samples. Maximum mortalities took place at the highest concentration of 400µg/ml, whereas the least mortalities at lowest concentration 0.78125µg/ml as shown in Table 4.1 and Table 4.2.

Table 4.3: Cytotoxic activity of Tamoxifen and aqueous fraction of *S. samarangense* leaves

Sample	Linear regression equation	R^2 value	LC_{50} (µg/ml, 24hr)
Standard (Tamoxifen)	y = 33.021x + 12.806	0.989	12.51
Extract (Aqueous fraction)	y = 20.33x + 45.63	0.939	1.64

In this investigation, standard and aqueous fraction exhibited cytotoxic activities with the LC_{50} values 12.51µg/ml and 1.64µg/ml respectively as shown in Table 4.3. For aqueous fraction, LC_{50} value is less than the standard which indicates that the extract has more potent activity than standard against brine shrimp nauplii.

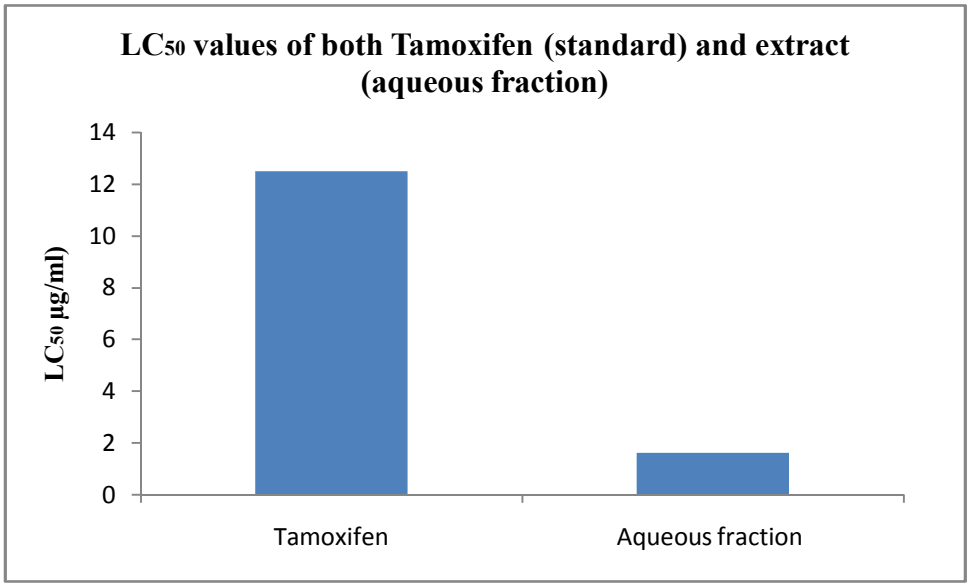

Figure 4.3: Comparison between LC_{50} values of standard and extract

From the above figure it can be concluded that for aqueous fraction the lethal concentration required to kill 50% of the sample population is lower than the standard. So the extract is more potent than Tamoxifen (Standard) at lower concentration.

4.2 Result of Antioxidant Tests

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 *Syzygium samarangense* extract was determined by following methods:

- Determination of total phenolic content.
- Determination of total flavonoids content.

4.2.1 Result of Total Phenolic Content

The aqueous extract of *S. samarangense* and the aqueous fractions of the methanol extract of *S. samarangense* were subjected to determine total phenolic content. Ascorbic acid was used as reference standard (Singleton et al., 1999).

4.2.1.1 Preparation of Standard Curve

Table 4.4: Total Phenolic content of ascorbic acid

Concentration (µg/ml)	**Absorbance (at 765 nm)**	**Regression line**	**R^2value**
80	1.306	y = 0.016x-0.19	0.654
90	1.009		
100	1.529		
110	1.794		
120	1.747		

A linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.4. This linear curve was considered as a standard curve.

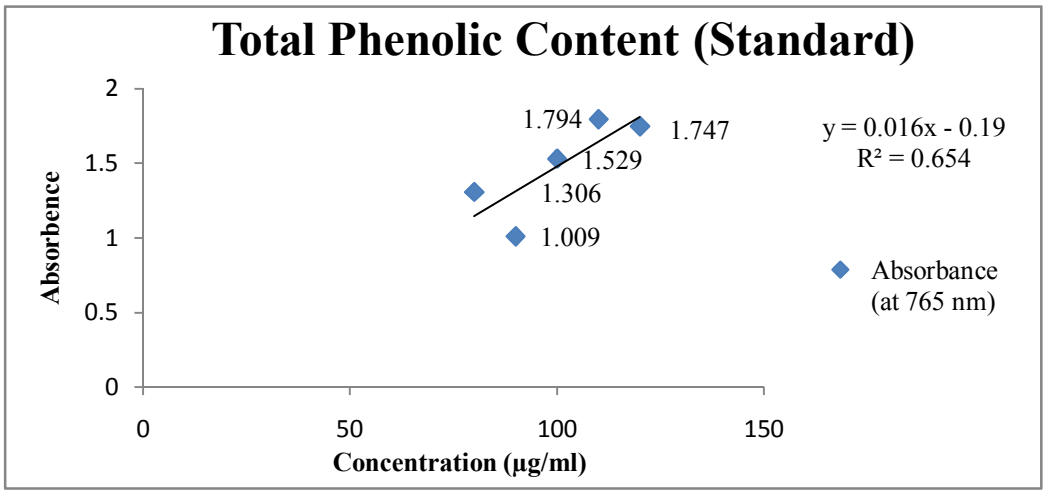

Figure 4.4: Graphical representation of Phenolic content of ascorbic acid

4.2.1.2 Total Phenolic content present in aqueous extract of *S. samarangense*

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 4.5: Total Phenolic content in aqueous fraction of *S. samarangense*

Concentration (mg/ml)	Absorbance	mg AAE/g
2	1.695	117.8125

4.2.1.3 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 less than the absorbance of standard. Based on the absorbance values of extract solution and using the regression line equation of the standard curve, 117.8125 mg of AAE/gm of dried extract of phenol content was found in the aqueous fraction of *S. samarangense.*

4.2.2 Result of Total Flavonoid Content

The aqueous fractions of *S. samarangense* leaves were subjected to determine total flavonoid content. Quercetin was used as reference standard.

4.2.2.1 Preparation of Standard Curve

Table 4.6: Total flavonoid content of Quercetin.

Concentration (µg/ml)	Absorbance (At 420 nm)	Regression line	R^2 value
0	0	y = 0.053x-0.013	0.999
4	0.193		
8	0.422		
12	0.618		
16	0.834		

After absorbances were taken of different concentrations of quercetin ranging from 0µg/ml to 16µg/ml, a linear relationship was observed when the absorbances were plotted against concentrations, as shown in Figure 4.5. This linear curve was considered as a standard curve.

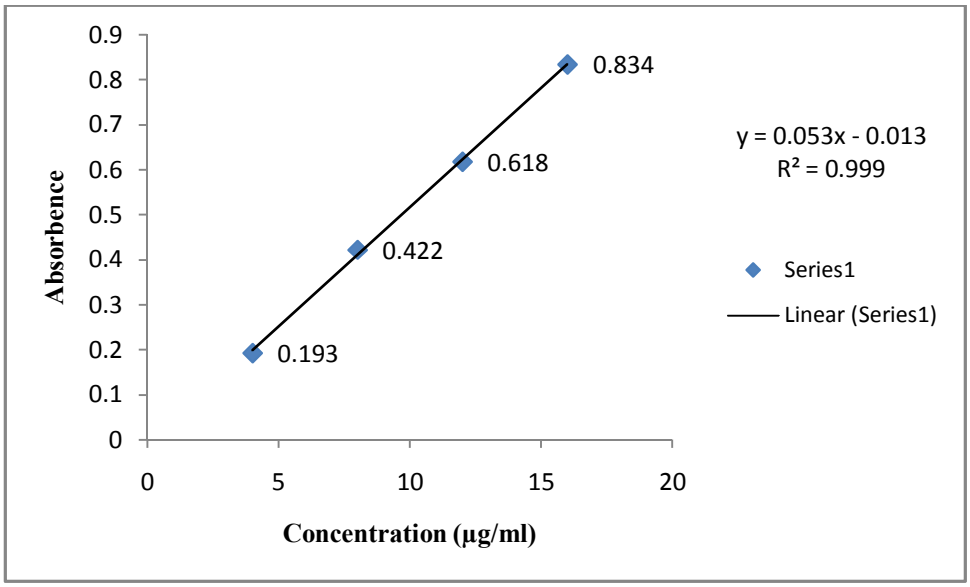

Figure 4.5: Graphical representation of assay of flavonoid content of quercetin

4.2.2.2 Total Flavonoid Content Present in Aqueous Extract

Based on the absorbance value of extract solution and using the regression line equation of the standard curve, the total flavonoid present in the extract is calculated and is given in Table 4.8.

Table 4.7: Total flavonoid content of aqueous fraction of *S. samarangense* leaves extract.

Sample	Concentration (mg/ml)	Absorbance	Total flavonoid content (mg of AAE/g of dried extract)
Aqueous fraction of *S. samarangense*	1	0.111	2.339623

4.2.2.3 Discussion

To determine the total flavonoid content of the test samples the standard curve was used. For 1mg/ml concentration of aqueous fraction of *S. samarangense* (leaves), 2.339623 mg of AAE/gm of dried extract of flavonoid content was found. So it can be said that, the extract contains very low antioxidative compounds.

4.3 Result of Antimicrobial Screening Test

4.3.1 The result of Antimicrobial Test

The antimicrobial activities of aqueous fraction of *S. samarangense* leaves extract were subjected in the study against various Gram positive bacteria, Gram negative bacteria and fungi. 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.

4.3.2 Zone of Inhibition of Standard and Aqueous Fraction

Table 4.8: Antimicrobial activity of standard (Ciprofloxacin) and aqueous fraction

Types of microorganism		**Zone of inhibition(mm)**	
		Standard sample (mm)	**Aqueous Fraction (mm)**
Gram positive bacteria	*Staphylococcus aureus*	31	7
	Bacillus subtilis	31	6
	Bacillus ceresus	31	9
	Bacillus megaterium	30	6

Gram negative bacteria	*Escherichia coli*	30	7
	Vibrio parahaemolyticus	30	9
	Vibrio mimicus	30	8
	Shigella dysenteriae	33	8
	Salmonella typhi	30	7
	Salmonella paratyphi	32	8
Fungi	*Candida albicans*	30	8
	Aspergillus niger	30	7

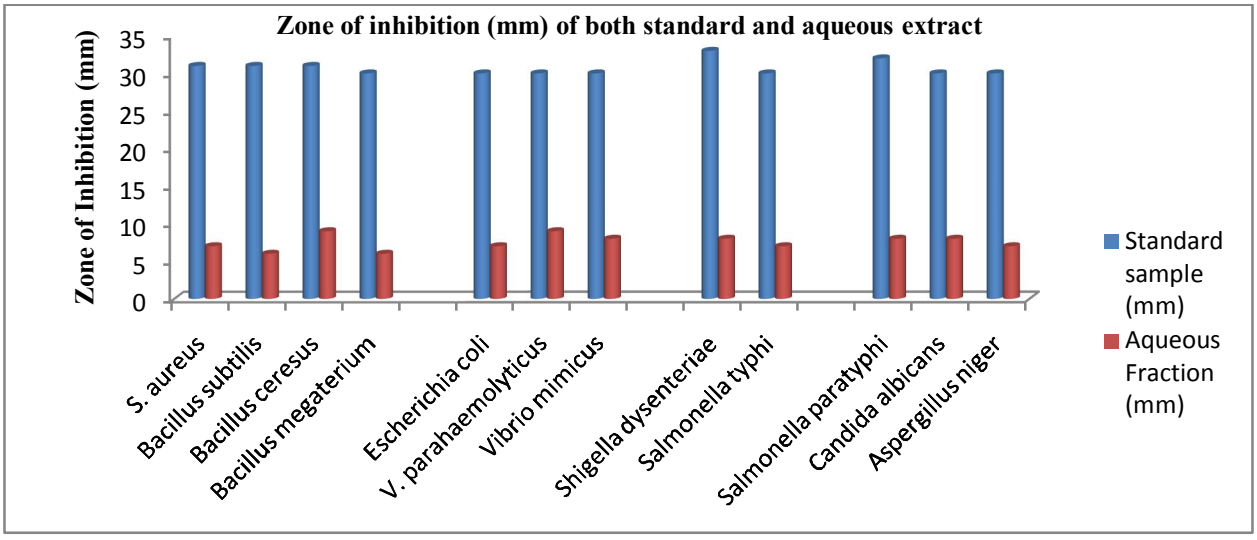

Figure 4.6: Comparison between zone of inhibition of standard and aqueous extract

4.3.3 Discussion

Aqueous fraction of *S. samarangense* leaves extract showed low to moderate antimicrobial activity when compared to ciprofloxacin reference standard drug. None of the zone of inhibition of aqueous fraction is equal to ciprofloxacin against any bacteria or fungi as shown in the Figure: 4.6. Among all the microbiological cultures, the fraction showed the best antimicrobial activity against *Bacillus ceresus* (9 mm) and *Vibrio parahaemolyticus* (9 mm) comparable to the standard (30-31 mm).

-CHAPTER FIVE-

CONCLUSION

5.1 Conclusion

As the literature review suggests, the presence of several phytochemical compounds in aqueous fraction of *Syzygium samarangense*, makes the plant pharmacologically active.

LC_{50} value of *Syzygium samarangense* in aqueous fraction showed more cytotoxic activity than Tamoxifen. Since aqueous fraction of *Syzygium samarangense* exhibited potent cytotoxic activity, so it can be investigated for anticancer, pesticidal and antitumor properties in future.

Antioxidant property in aqueous extract of *S. samarangense* was determined by Phenolic content assay and Flavonoid content. Phenolic content was 117.8125 mg/gm and Flavonoid content was 2.339623 mg/gm in aqueous extract of *S. samarangense.* So aqueous extract of *S. samarangense* have poor antioxidant property. Mixture of compounds can lower antioxidant property in aqueous fraction of *S. samarangense*, 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 *S. samarangense.*

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

-CHAPTER SIX-

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