Phytochemical Screening and Investigation of *in-vitro* Antioxidant and Antibacterial Activity of *Leea aequata* leaf extract

A project submitted

by

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in partial fulfillment of the requirements for the degree of

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This experiment is dedicated to my parents and siblings for their love and constant support.

Certification Statement

This is to certify that this project titled "**Phytochemical Screening and Investigation of *in-vitro* Antioxidant and Antibacterial Activity of *Leea aequata* leaf extract**" submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Dr. Raushanara Akter, Assistant Professor, Department of Pharmacy, BRAC University. This project is the result of the author's original research and has not previously been submitted for a degree or diploma in any university. This project contains no material previously published or written by another person and the appropriate reference is given where I have used the others language or ideas.

Signed,

_____

Countersigned by the supervisor,

_____

Acknowledgement

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Abstract

Leea aequata (*L. aequata*) is a traditional medicinal plant in Bangladesh belonging from leeaceae family. The roots, stems and tubers of this plant are used as astringent. It is used as antiseptic, anesthetics, bronchitis, fever, itching and in tuberculosis. The objective of this study was to determine the phytochemical constituents and investigate *in-vitro* antioxidant and antibacterial activity of methanolic extract of *L. aequata* leaves. The phytochemical screening of methanolic extract of *L. aequata* leaves showed the presence of alkaloids, flavonoids, phenolic compounds, glycosides, tannins, carbohydrates and phytosterols. The antioxidant study was performed by using DPPH free radical scavenging activity, total phenolic content, total flavonoid content and total antioxidant capacity. The DPPH free radical scavenging activity study had exhibited that with increasing of concentration of the plant extract ranging from 50 to 1200 µg/mL, the free radical scavenging activity increased from 84.87% to 89.92%. The highest free radical scavenging activity (89.92%) found at 1200 µg/mL and the lowest free radical scavenging activity (84.87%) found at 50 µg/mL**.** The total phenolic content study revealed that with the increase in concentration from 200 to 1200 µg/mL, the total phenolic content of plant sample increases from 41.08 to 132.4 mg/g of gallic acid. Moreover, the total flavonoid content increased from 4.86 to 13.09 mg/g of quercetin at 200–1200 µg/mL and the total antioxidant capacity was found to be 44 to 60.96mg/g of ascorbic acid. In addition, the antibacterial activity of *L. aequata* leaf extract was determined by disc diffusion method against four bacterial strains such as, *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Vibrio cholerae*. Kanamycin and ampicillin were used as standard antibacterial drugs. The study revealed that this plant did not show any antibacterial effects on any of the four bacterial strains whilst kanamycin and amoxicillin had effects against the bacterial strains. This study has founded the possibility of using *L. aequata* leaf extract as an antioxidant and also encourages performing different pre-clinical and clinical studies for assuring the potential antioxidant effect in other methods along with different bioactivity studies.

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INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Phytotherapy of medical plant and National health care systems

Medicinal plants are used worldwide including Bangladesh from the prehistoric period for the medicinal purpose. The medicinal plants are useful in treating disease due to the presence of its constituents, more precisely phytochemical constituents which have the protective and defense mechanism against disease. It is also known as phytotherapy. It is more traditional approaches but proven by modern science. Phytotherapy concept was first originated by French physician Henri Leclerc, who published different editions of the Précis de phytotherapy ('Handbook of Phytotherapy'). Though numerous countries were introduced herbal preparations in the pharmacopoeias by realizing the benefits of medicinal plants and plant extracts, the practice of phytotherapy differs throughout the world. For example, South Korea and Japan have integrated phytotherapy products into health insurance coverage. On the other hand, China, India, and Nepal, consider herbal medicines as a wide health care coverage, which belongs to traditional medicine services (Heinrich, 2013).

1.2 History of medicinal plant

Before the drug development, ancient people have been used many plants or species for several centuries to cure different diseases. At the beginning, medicinal plants were used as nutrition derived by naturally which was later replaced by cultivated flora. After that, different herbs and plants such as- lavender, thym, neem, cintella, mint leaves were introduced for their oil and extract. Castor oil and coriander have thousands of recipes including treating disease, application of cosmetics and preservatives. Around 2000 herbs which have medicinal properties is described by Chinese manuscripts. The Greek and Roman people use medicinal plant since antiquity. Herodotus (5th century B.C.) describes in his writing that how ancient Europe and China people used 'Mother wort' which is an extract from *Leonurus cardiaca* for increasing blood circulation, diuretic agent and regulating menstruation. "Hungarian water", the first alcoholic extract, known since 1380 was derived from rosemary, and since then, it was broadly used in Europe for five centuries (Vinatoru, 2001).

From the Ancient Egypt and Babylon to till now herbal bath or 'hot bath' is being practiced to remove physical- mental tiredness and also useful for the skin. It is proven by the Modern science that aromatic bathing containing medicinal plant can dilate blood vessels, relax muscle tension and so on. For instance, Cleopatra, the queen of Egypt (69—30 b.c.e.), bathed with rose petals (Rosa spp., Rosaceae) petals and after bathing she would apply loitions and perfumes from cinnamon (*Cinnamomum verum*), white lily (*Lilium candidum*) and oils from almond (*Prunus dulcis*) (Alakbarov, 2003). There are many evidence showing that Unani Hakims and European cultures used herbs for about 4000 years in order to cure diseases. In ancient time different concoctions, plant extract extracts or brews were prepared by the herbalists for healing wounds. Those primitive people had learned that which plants or specific plant parts may help to reduce certain diseases such as induce labor, toothaches etc.

Thus, knowledge and personal experience have been passing over from generations to generations. Over the time, population rising, disease condition, insufficient supply of medicine with vast side effects of synthetic drug have emphasised on using different plant part as a source of medicine (Balunasa & Kinghorn, 2005).

1.3 Medicinal plants in Bangladesh

Nature has innumerable number of important plants that have indispensable therapeutic value in the treatment of different diseases. There are about 2000 plants were enlisted as medicinal plant for having medicinal properties within the Asian subcontinent and from them more than around 722 medicinal plants grow in Bangladesh. According to (Ghani, 2003a), about 500 of herbal or medicinal plants name was enlisted so far as found in Bangladesh. Medicinal plants are widely used as nutrient, antibiotics (penicillin, streptomycin) and in Unani, Herbal, Homeopath medicine.

Table 1.1: List of some medicinal plants in Bangladesh with their medicinal uses (Sadi, 2012).

Scientific name	Using part	Medicinal uses
Terminalia arjuna	Bark	Heart disease, tuberculosis
Centella asiatica	Whole plant	Metabolic problem, ulcer, dysentery
Adhatoda vasica	Leaf, root	Asthma, cold, tuberculosis
Mentha viridis	Whole plant	Metabolic disorder, Gastritis
Azadirachta indica	Leaf, root, bark	Skin diseases, worm killer, arthritis
Hemidesmus indicus	Root, whole plant	Arthritis, diabetes
Aloe indica	Leaf	Headache, sexual disease

1.4 Phytochemical constituents of crude plant extracts

The extract of medicinal plants contains different compounds which are responsible for treating diseases. The compounds can be:

1.4.1 Alkaloids: It is a naturally occurring cyclic organic nitrogenous bases. Chemical classification of alkaloids:

- Pyrrolidine group e.g. Hygrine
- Piperidine e.g. Lobeline
- Pyrrolizidine e.g. Senecionine
- Tropane e.g. Atropine
- Quinoline e.g. Quinine
- Isoquinoline e.g. Morphine

- Aporphine e.g. Boldine
- Indole e.g. Ergometrine
- Imidazole e.g. Pilocarpine
- Diazocin e.g. Lupanine
- Purine e.g. Caffeine
- Steroidal groupe.g. Solanidine
- Amino group e.g. Ephedrine
- Diterpene e.g. Aconitine.

Taxonomic classification of alkaloids

- Cannabinaceous Alkaloids: *Cannabis sativa* Linn.
- Rubiaceous Alkaloids: Cinchona Sp. (Quinine)
- Solanaceous Alkaloids: *Atropa belladonna* (Hegnauer, 1963)

1.4.2 Flavonoids

Flavonoid consists of two phenyl rings (A and B) as well as a heterocyclic ring. Contents of flavonoid mainly determines the anti-oxidant activity of a plant. Moreover, antibacterial, anti-inflammatory and hepatoprotective activity may also possess.

1.4.3 Phenols

Phenolic compounds show antioxidant activity which particularly depends on the structure, positions, number of hydroxyl groups and nature of substitutions attached to the aromatic rings.

1.4.4 Tannins

Tannins are astringent and polyphenolic biomolecule which binds and precipitates different organic compounds such as, alkaloids, amino acids and proteins. Tannins can

inhibit the growth of bacteria, viruses, yeast and fungi. Presence of tannins in the medical plant indicates its antioxidant and anticancer properties.

1.4.5 Glycosides

Glycosides can be of different types-

- By glycone or presence of sugar
- By the type of glycosidic bond
- By aglycone- Anthraquinone glycosides, alcoholic glycosides, coumarin glycosides, cyanogenic glycoside, flavonoid glycosides, phenolic glycosides, steroidal glycosides and thioglycosides. Different glycosides have analgesic, anti-inflammatory, antipyretic and cardiovascular effects on human.

1.4.6 Saponins

Saponins are widely used as adjuvants in vaccines and also have anti-inflammatory and expectorant effect.

1.5 Significance of medicinal Plant

Medicinal plants derived from naturally have significant importance on human health.

Table 1.2: Some medicinal plant with their significance (Sadi 2012).

Scientific name	Part used	Medicinal use
Aloe barbadensis	Leaf gel	Skin diseases- psoriasis, eczema, abrasion
Asparagus recemosus	Leaf, root	Fever, dysentery, diabetes
Rauwolfia serpentine	Stem, leaves	high blood pressure, insanity and insomnia

Characteristics of medicinal plants when using as a treatment:

- Synergic medicine- The components of medicinal plant interact simultaneously to complement with each other or neutralize the possible side effects.

- Supporting the official medicine- In case of cancer or other complex cases the ingredients of the plants are proved to be effective.

- Preventive medicine- Medicinal plants are used to prevent the presence of different diseases (Hassan, 2012).

US pharmacies and in Middle European drugstores have approximately 40% of medicines of biogenic stock such as antibiotics from bacteria, sera extracted from animals and other compounds from higher plants. These medicinal plant can be used as nutrition as well as alternative and complementary medicines in drug development and synthesis. Different Medicinal plants are identified with anti-microbial, analgesic, anti-oxidant, anti-depressant, anti-cancer, cytotoxic activity etc. Thus, along with the other diseases, National Cooperative Drug Discovery Group (NCDDG) which is a research project, have been identified some important compounds from the tropical rainforest plant species having anticancer activity. Thus, modern drug therapy is largely depending on such products (Balunasa & Kinghorn, 2005).

1.6 Marketed products derived from medicinal plants

Modern science has proved the role of medicinal plants on human body. Liu *et al*., (2000) said that about 50% of all marketed drugs were derived from natural products as well as from their derivatives.

Table 1.3: Some marketed drug derived from medicinal plants.

Drug	Chemical class	Source	Medicinal use	Mechanism of action
Penicillin	Penicillin	Fungus, *Penicillium niger*	Antibiotic (Bédoyère, G. 2005)	Inhibition of peptidoglycan synthesis
Salicylic acid	Beta-hydroxy acid	Barks of willow tree	Analgesic, anti-inflammatory (NSAID)	Inhibition of COX Enzymes
Digoxin	Cardiac glycoside	*Digitalis purpurea*	Congestive heart failure, atrial fibrilation	Inhibition of the Na+/K+-ATPase membrane pump
Morphine	Alkaloid	Opium poppy, *Papaver somniferum*	Analgesic, (Sertürner, 1805)	Opioid agonist by binding to opiate receptors (mu,delta, kappa)
Paclitaxel (Taxol^{TM})	Terpenoid	*Taxus brevifolia*	Tubulin polymerization stabilizer (Sneader, 2005)	Tubulin polymerization Stabilizer
Quinine	Alkaloid	Cinchona bark, *Cinchona officinalis*	Anti-malarial agent (Meshnick & Dobson, 2001).	Protein synthesis Inhibitor
Mevastatin[2]	Polyketide	Fungus, *Penicillum citrinum*	Cholesterol lowering drug, (Li, 2009).	Inhibition of the HMG-coA

1.7 Antioxidants

Antioxidants can be derived from both naturally and synthetically for different human diseases. From all of the phytochemicals, flavonoids are considered as potent antioxidants for their potential effect on human health. The chemical structure, position of hydroxyl groups and free radical scavenging property are important for having antioxidant property. Quercetin which contains flavonol in abundant has potent antioxidant property because of the right molecular structure for showing potent free radical scavenging activity (Sannigrahi, Mazumder & Pal, 2009).

1.7.1 Classification of antioxidants

Antioxidants are categorized based on the chemical structure, mechanism, solubility and kinetics.

Table 1.4: Alphabetical classification of antioxidants
(Source: Flora, 2009)

Name of antioxidant	Categories of antioxidants	Examples
Antioxidant C	Carotenoids	β-carotene, Lycopene, Lutein
Antioxidant E	Enzymes	SOD, Catalase, GPx
Antioxidant G	Glutathione	Glutathione
Antioxidant H	Hormones	Melatonin, oestrogen
Antioxidant L	Lipid associated chemicals	Ubiquinol-10, M-acetyl cysteine, lipoic acid
Antioxidant M	Minerals	Zinc, Selenium, Copper
Antioxidant P	Phenolics	Quercetin, catechin
Antioxidant S	Saponins, Steroids	Cortison, estradiol, estriol
Antioxidant V	Vitamins	α-tocopherol, ascorbic Acid

1.7.2 Principle of antioxidant study

Antioxidant study of medicinal plant can be determined by DPPH free radical scavenging assay, total phenolic content, total flavonoid content and total antioxidant content of the plant extract.

DPPH free radical scavenging assay

The DPPH (1, 1-diphenyl-2-picryl-hydrazyl) free radical scavenging assay for determining antioxidant activity of methanolic leaf extract *L. aequata*, is described by Braca *et al*. (2001).

DPPH determines the free radical scavenging activity of the antioxidant present in the plant extract. Generally, DPPH produces stable free radicals in aqueous solution or methanol by delocalizing of free electrons which generates a deep purple color when absorption is taken at 517nm. If antioxidants/hydrogen present in the sample then DPPH is reduced into hydrazine that produces purple to yellow colored solution (Brand & Willams, 1995). Thus, a decrease in the absorbance of DPPH with the increase in the concentration of sample at 517nm shows scavenging activity by the sample where DPPH changes its color for the conformation.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the plant extract of *L. aequata* was determined by the modified folin-ciocalteu method as mentioned by Wolfe, Wu & Liu (2003).

Folin-ciocalteu reagent (FCR) is used to measure the reducing ability of the sample. This compound undergoes reduction reactions that involves transfer of electrons and forms blue colored complex. In the complex, molybdenum is reduced where electron-transfer occur between the reducing agent and Mo (VI) as follows:

$$Mo\ (VI) + e^- \Longrightarrow Mo\ (V)$$

The absorbance of the complex is measured at 765nm against blank (methanol and reagents) by using the UV-Visible Spectrophotometer and the total phenolic content of the fractions were expressed as gallic acid equivalents (GAE) using the following equation:

$C = (c \times V) \div m$

Where, C = Total content of phenolic compounds, mg of gallic acid per gram of dried plant extract, expressed as gallic acid equivalent (GAE)

c = concentration of gallic acid obtained from calibration curve (mg/mL)

V= Volume of sample solution (mL)

m = weight of the sample (g)

Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) of the *L. aequata* extracts was determined according to the method described by Kumaran & Karunakaran (2007) with slight modification. Here, an acidic complex is produced between 3 substrates –a C-4 keto-group with either C-3 or C-5 hydroxyl group of the flavanols and flavones present in the extract sample with aluminium chloride (Pal *et al*., 2009). Moreover, aluminium chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of the flavonoids and the absorbance is taken at 415nm using UV-Visible Spectrophotometer.

The calibration curve was made using quercetin which is the standard with different concentrations and absorbance was measured spectrophotometrically at 415nm. The total phenolic content of each of the fractions were then, calculated and expressed as quercetin equivalents (QE) using the following equation:

$C = (c \times V) \div m$

Where, C = Total content of flavonoid compounds, mg of quercetin per gram of dried plant extract, expressed as quercetin equivalent (QE)

c = concentration of quercetin obtained from calibration curve (mg/mL)

V= Volume of sample solution (mL)

m = weight of the sample (g)

Determination of total antioxidant capacity (TAC)

The total antioxidant capacity of plant extract of *L. aequata* was determined using the method described by Prieto *et al*. (1999) which is also known as the phosphomolybdenum method.

This assay is based on the reduction reaction between Mo(VI) to Mo(V) by the sample extract which forms a green-colored phosphate-molybdenum(V) complex at an acidic pH.

The calibration curve was made by using Ascorbic acid as the standard in different concentrations and absorbance was taken at 695nm spectrophotometrically. The total antioxidant capacity of each of the fractions was expressed as ascorbic acid equivalent (AAE) using the following equation:

$A = (c \times V) \div m$

Where, A = Total antioxidant capacity, mg of ascorbic acid per gram of dried plant extract, expressed as ascorbic acid equivalent (GAE)

c = concentration of ascorbic acid obtained from calibration curve (mg/mL)

V= Volume of sample solution (mL)

m = weight of the sample (g)

1.7.3 Mechanism of antioxidant activity

- Chain breaking reaction- alpha tocopherol.
- Reducing the connection of Reactive Oxidative Substances- glutathione.
- Scavenging initiating radicals- superoxide dismutase.
- Chelating the transition metal catalysts- transferrin.

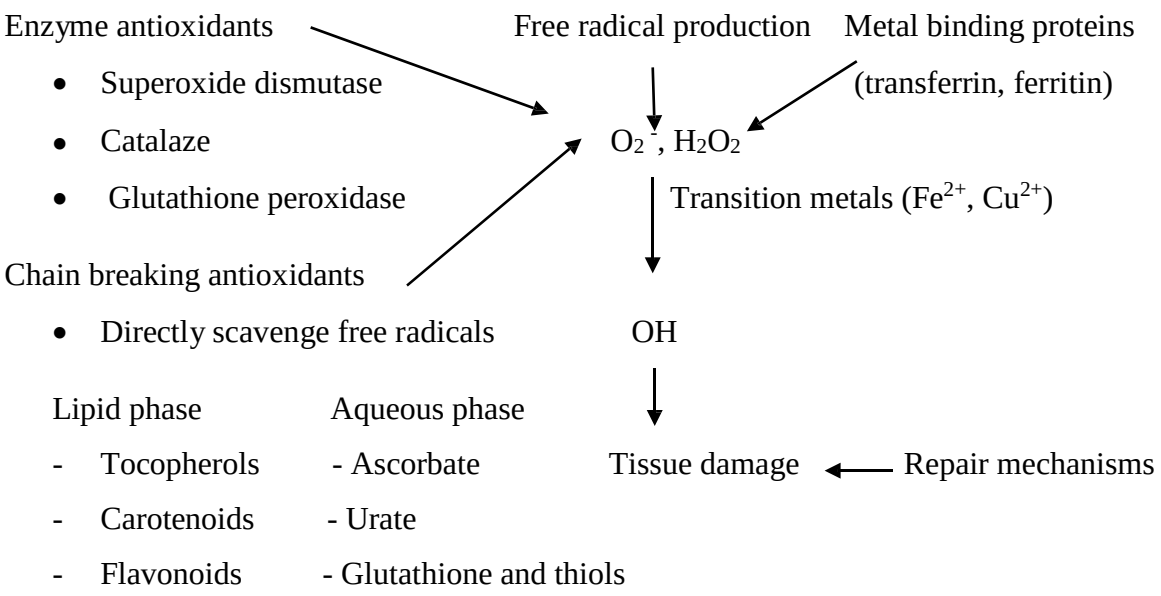

Figure 1.1: Flow chart showing mechanism of antioxidants.

1.8 Antibacterial agents

Antibacterial agents refer to the naturally, semi-synthetically or synthetically derived compound that inhibit or kill the growth of microorganism or bacteria without causing no harm to the host. Bacteria contains only one cell which is different in nature. Hence, Microorganisms become easily susceptible to antibacterial drugs. But inventing new antibacterial drugs chemically with the increasing demand is not possible. Therefore, medicinal plants act as a good source which can give antibacterial effect as well as other therapeutic effects (Prasad, Sudha, Khadri, & Riazunnisa, 2015).

1.8.1 Principle of antibacterial study

Disc diffusion method

The most popular disc diffusion method is used to determine antimicrobial study of medicinal plant. Through the presence of zone of inhibition on the disc determine the antibacterial effect. Firstly, Muller Hinton Agar and Nutrient Agar are used as solid and liquid medium respectively for culturing the bacterial strains. After incubation for about 24

hours, the absorbance of bacterial mixer is taken. Secondly, sample of different concentration is prepared where small filter paper is soaked. Then petri dish is streaked with the previous bacterial culture and placed the soaked filter paper and standard filter paper (kanamycin, amoxicillin) on the disc following by incubation for 24 hours. Lastly, zone of inhibition is measured to determine the antibacterial effect of the plant extract.

1.8.2 Mechanism of antibacterial activity

Medicinal plants having antimicrobial property show their action through different ways.

- Inhibition of cell wall synthesis
- Inhibition of protein synthesis
- Inhibition of synthesis of essential metabolites
- Injury to plasma membrane

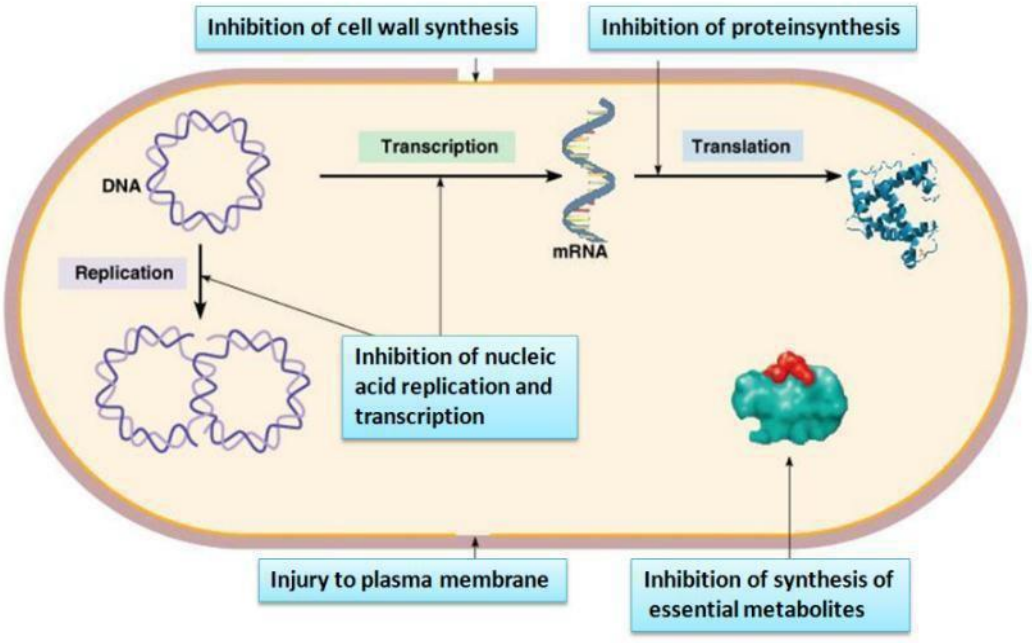

Figure 1.2: Mechanism of antibacterial drug

1.9 Phytochemical constituents showing antibacterial activity

Different studies have showed that phenols, polyphenolic compounds flavonoids, tannins, alkaloids and coumarins show the antibacterial activity.

Table 1.5: Phytochemical constituents with antibacterial activity (Nandagopal, Sankar, Ramamurthy, Sathish, & Sridharan, 2011).

Class of compounds	Mechanism of action	Activity
Phenols and phenolic Acids	oxidized compounds inhibit enzyme with sulfhydryl groups.	Bacteriostatic
Flavanoids, flavonols and flavones	Through complex formation with extracellular or other soluble proteins or interacting with bacterial cell walls and lipophilic flavonoid which disrupt microbial membranes. Other	Inhibits bacterial growth such as- *Vibrio cholerae, Streptococcus mutans* and *Shigella*
Tannins	By interaction with DNA	Anti-viral
Alkaloids	Intercalate with DNA	HIV infection
Polypeptides and Lectin	By Forming ion channels into the microbial membrane.	Antibacterial

Table 1.6: Some examples of medicinal plant showing antibacterial activity (Sharma, Chandraker, Patel & Ramteke, 2009).

Medicinal plant	Bacteria
Cinnamomum verum	*Helicobacter pylori*
Myristica fragrans	*S. mutans, Porphyromonas gingivalis*
Cinnamomum cassia	*Pseudomonas aeruginosa*
Zingiber officinale	*Escherichia coli*

Allium cepa	*Streptococcus mutans, Porphyromonas gingivalis*, and *Prevotella intermedia*
Terminalia chebula	*Klebsiella pneumonia*

1.10 Selection of *L. aequata* leaf for the present study

After doing extensive literature review of medicinal plant in Bangladesh showing their different bioactivity studies, it was found that neither *in vivo* nor *in-vitro* antioxidant potential and antimicrobial activity against selected bacterial strains was performed on the *L. aequata* leaf extract. Thus it was taken as initiative to determine its antioxidant and antimicrobial activity.

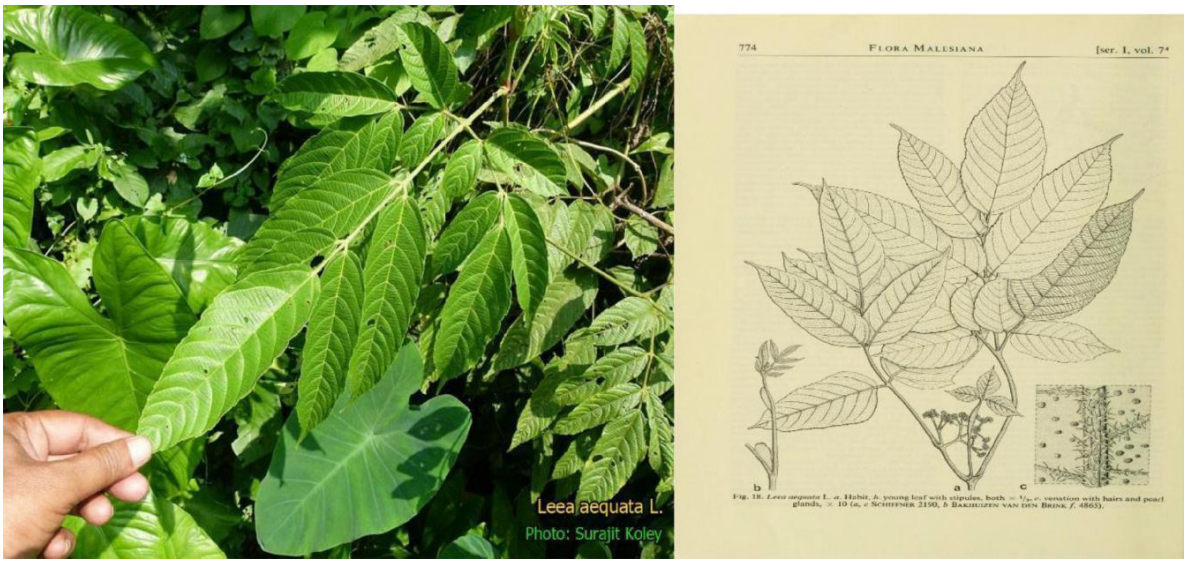

Figure 1.3: *L. aequata* plant

1.11 Previous study of *L. aequata* plant

The previous study of *L. aequata* plant revealed that the stems, tubers and roots of *L. aequata* plant are astringent and mucilaginous. The root has also anesthetics, anthelmintic, vulnerary, alexiteric, analgesic and antipyretic effects on human body. The roots are useful in dyspepsia, leprosy, tuberculous ulcers, itching, bronchitis (Yusuf *et al.*, 2009).

Young shoots from the *L. aequata* produces sap which is mashed with ashes and used as cleansing wounds. On steam distillation, this plant produces 0.15% essential oil which has been shown to inhibit the growth of *Mycobacterium tuberculosis* at 10 µg/mL of concentration, *Micrococcus pyogenes* at 100 µg/mL, and *Pasteurella pestis* at 50 µg/mL concentration *in-vitro* (Fern, 2014).

1.12 Plant Description

Name: L. aequata

Family: Leeaceae

Synonyms:

Leea ancolona

Leea hirsuta Blume ex Spreng

Leea hirta Roxb. ex Hornem.

Leea hispida Gagnep

Leea kurzii C.B. Clarke

Leea sambucina M. Laws.

Leea scabra Roxb. ex Roem. & Schult

Bengali/Vernacular Name: Kakjangha

Tribal Name: Sine Sa Apang (Marma)

Classification:

IBP taxonomy Hierarchy

Kingdom- Plantae

Phylum- Tracheophyta

Class- Magnoliopsida

Order- Vitales

Family- Leeaceae

Genus- Leea

Species- *L. aequata*

A shrub which is 1.2-3meter high, young shoots, more or less pubescent. Leaves are bipinnate, leaflets are 7.5-18 * 2.5-4.5 cm long. Rachis is angled, terete, hirsute, hardly winged and pubescent. Petioles are not dilated at their base, ovate-oblong or oblong and also acuminate. Both of the surface are more or less hairy, membranous. Beneath is covered with flat circular disks, main nerves 7-12 pairs, ascending and curved. Flowers are white, in short compact, pubescent, subterminal or leaf opposed cymes. Moreover, bracts large, broadly ovate. Calyx pubescent and outside is sparsely covered with glandular disks where cleft is about one-fourth. Lobes are ovate or subacute. Lobes of the staminal tube are oblong, bifid and anthers united in bud. Berry is 6 mm in diameter, depressed-globular about 2 to 6 lobed and form black color when ripe. Chromosome number is unknown. Time of flowering and fruiting: June- December.

Distribution: Myanmar, India, Malay, Indonesia and Peninsula. In Bangladesh, it is available in the forest of Chittagong and Sylhet.

Traditional use:

- The roots, stems exhibit astringent property.
- Tubers, stems, roots act as anthelmintic, analgesic, anesthetics, vulnerary, alexiteric and antipyretic.
- Also useful in dyspepsia, leprosy, tuberculous ulcers, itching, bronchitis.
- Plant oil acts as antibacterial drug by inhibiting some bacterial growth.

1.13 Rationale of this study

Previous study showed some bioactivity properties such as analgesic, anthelmintic, alexiteric, antipyretic and anesthetics effects of the stems, tubers and roots of *L. aequata* plant. Moreover, *in-vitro* antibacterial study which was done before from the essential oil of this plant that inhibited the growth of *Mycobacterium tuberculosis, Micrococcus pyogenes* and *Pasteurella pestis* in certain concentration.

However, it was found that the antioxidant and antibacterial activity study against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Vibrio cholerae* was not performed previously. By analyzing the phytochemical screening of this plant it was found that leaf extract contains flavonoids, alkaloids, phenolic compounds, tannins, glycosides, carbohydrates and phytosterol. Thus, this study will focus mainly on determining the antioxidant and antibacterial activity of methanolic leaf extract of *L. aequata.*

1.14 Aim of the project

The aim of the study is to determine phytochemical constituents and to investigate *in-vitro* antioxidant and antibacterial activity of *L. aequata* leaf extract.

1.15 Objectives of the project:

The objectives of the project are:

a) To carry out phytochemical screening of leaf extracts of *L. aequata* in order to qualitatively determine the presence of phytochemical constituents.

b) To determine its antioxidant activity.

c) To investigate the antibacterial activity of leaf extracts of *L. aequata* comparable to the standard ampicillin and kanamycin.

CHAPTER TWO

METHODOLOGY

CHAPTER 2: METHODOLOGY

2.1 Collection of plant material

L. aequata plant was selected for this investigation. As no previous study was conducted on the antioxidant potential and antimicrobial activity of this plant, so *L. aequata* leaves were chosen for the investigation.

The whole *L. aequata* plant was collected in the month of January 2017 from Sylhet, Bangladesh. Then it was submitted to the National Herbarium of Bangladesh (NHB), Mirpur, Dhaka for its authentication. After a week, its voucher specimen was collected and the plant was identified (ACCESSION NO.: DACB-44008) and authentication is done by the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka.

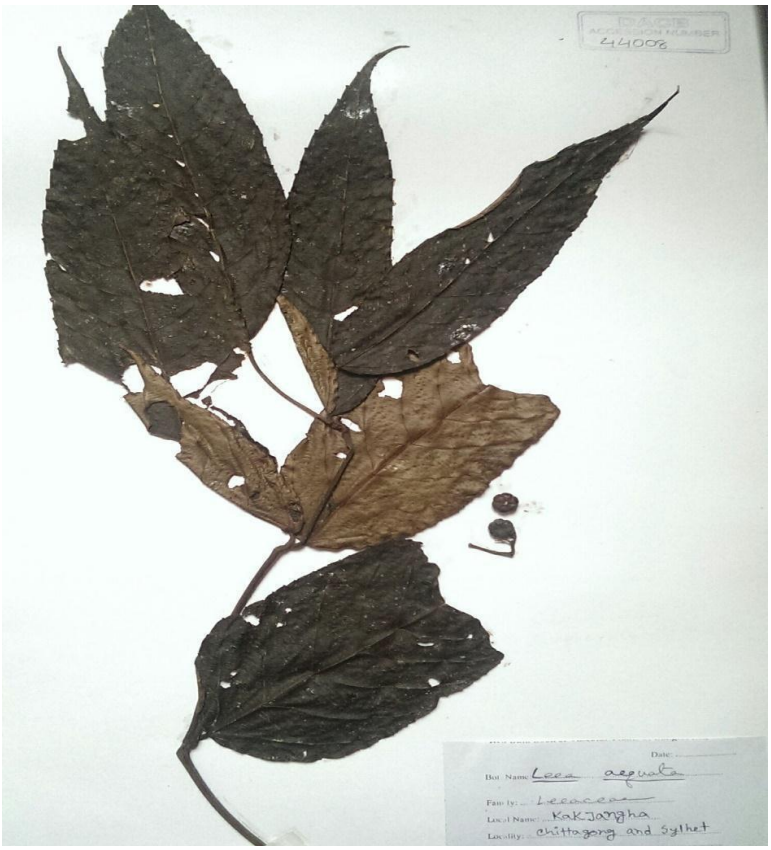

Figure 2.1: *L. aequata* leaves obtained from the National Herbarium of Bangladesh, Mirpur, Dhaka

2.2 Extraction procedure

The total extraction procedure can be divided into 2 parts:

a. Plant material preparation and drying (2 steps-from washing to oven drying)

b. Extraction process (5 steps- from size reduction to drying of plant concentrate)

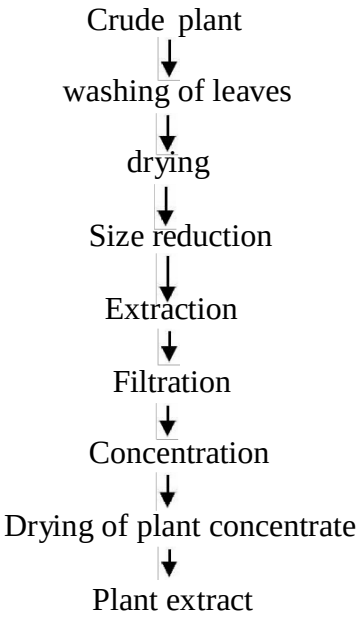

Figure 2.2: Flow chart showing leaf extraction procedure.

a. Plant material preparation and drying

The leaves were separated from the stem and washed with clean water to remove dust particles and debris. The clean leaves were then allowed to shade drying for about 10 days.

b. Extraction process

Size reduction and weighing

High capacity grinding machine was used to grind the dried and crispy leaves into coarse powder. Then leaves powder was packaged into air-tight plastic container which was stored in a dry, cool, and dark place until further investigation. The final weight of the powdered plant material was 178gm.

Extraction

To get the leaves extract different aqueous and organic solvent can be used. Here methanol was used as a solvent. *L. aequata* leaves powder were soaked into 900mL volume of methanol for t 2 days at room temperature (22-25°C) with prior agitation.

Filtration

After two days, the beaker containing the leaves powder and methanol was filtered using Whatman filter which pore size: 110mm.

Concentration

By using rotary evaporator (Heidolph) at 30°C, and at 100rpm the collected filtrate was concentrated into concentrated methanolic extract. Then, the extract was transferred onto petri-dishes for drying under laminar air flow.

Drying

Laminar air flow (LAF) was used to evaporate the solvent from the extract on petri dish leaving with a semi-solid and dried extract. LAF also prevent any microbial growth on the extract while drying. Finally, aluminum foil was used to cover petri-dishes and refrigerated.

Figure 2.3: The petri-dishes containing the methanolic extract of *L. aequata* leaves placed under laminar air flow for drying.

2.3 Phytochemical screening

In order to identify the qualitative chemical compositions such as- carbohydrates, alkaloids, flavonoids, glycosides and tannin etc. phytochemical screening was performed on the *L. aequata* crude extracts.

The following qualitative tests were performed:

Detection of alkaloid

For the detection of alkaloid, three tests were done. 0.5g of *L. aequata* leaf extract was dissolved in 5mL of 1% hydrochloric acid which was boiled in a water bath and then filtrated. Then the filtrate was used to do the tests for alkaloid.

Hager's test

A few drops of Hager's reagent (1% picric acid solution) was added in to 2mL of the filtrate and yellow precipitation confirms the presence of alkaloids (Waldi, 1965).

Mayer's test

According to Evans & Trease (1997), 10mL Mayer's reagent is made by dissolving 0.1358g of Mercuric (II) Chloride with 0.5g of Potassium Iodide in 10mL distilled water.

A few drops of Mayer's reagent was added to a 2mL of the filtrate and creamy or white precipitation formation indicates the presence of alkaloid.

Wagner's test

According to Wagner (1993), a 10mL Wagner's reagent is prepared by dissolving 0.2g of Iodine crystals with 0.6g of Potassium Iodide in 10mL distilled water.

A few drops of Wagner's reagent was added into 2mL of the filtrate and the presence of alkaloid was confirmed by the formation of a brownish black precipitation.

Detection of carbohydrates

According to Ramkrishnan, Prasannan & Rajan (1994), carbohydrate can be detected by dissolving 0.5g of methanolic extract of *L. aequata* in 5mL of distilled water and filtering the raw mixture. Then the filtrate was used for the presence of carbohydrate.

Molisch's test

2 drops of Molisch's reagent and 2mL of concentrated sulfuric acid were added to the 2mL of the filtrate which was allowed to stand for a while. The presence of carbohydrate is confirmed by the formation of a violet ring.

Fehling's test

1mL of each of the Fehling's solution A and B were added into 2mL of the filtrate in 1:1 ratio which was boiled for few minutes. The presence of carbohydrate is detected by the formation of a brick-red precipitation.

Detection of flavonoids

Lead acetate test

A few drops of lead acetate was treated with the methanolic extract and the presence of flavonoid identifies by the formation of yellow colored precipitation.

Zinc ribbon test

0.5mL of alcoholic extract was treated with 6-10 drops of conc. Hydrochloric acid (HCl) and a small piece of zinc. Then the solution was boiled and left to stand. The presence of red to crimson color formation in solution signifies the presence of flavonoid.

Detection of phenols or phenolic compounds

Ferric Chloride test

According to Soni & Sosa (2013), this test is done by treating 2mL of extract with 3-4drops of 15% (w/v) ferric chloride solution. The presence of phenol is confirmed by the formation of a bluish-black precipitation.

Detection of phytosterols

Libermann Burchard's test

1mL of chloroform was added to a small amount of extract and filtered. 2mL of acetic anhydride was added to the filtrate, boiled and then cooled. After that, 1mL conc. sulfuric acid was added to this solution. The existence of phytosterols is indicated by the presence of a brown ring at the junction (Soni & Sheetal, 2013).

Detection of steroids

Salkowski test

According to Ghani (2003), 1mL of extract was treated with 2mL of chloroform and 1mL of sulfuric acid. The presence of red color signifies the detection of steroids in the extract.

Detection of tannin

Lead acetate test

A few drops of 1% lead acetate was added to 1mL of the leaf extract and yellow-colored precipitation confirms the presence of tannin (Tiwari & Bimlesh, 2011).

Potassium dichromate test

10% potassium dichromate solution is made by dissolving 1g of potassium dichromate in 10mL distill water. Then 1mL of 5% potassium dichromate was added to 5mL aqueous crude extract solution and the presence of tannin identifies by the formation of yellow precipitation (Ghani, 2003).

Ferric chloride test

5% ferric chloride solution is made by dissolving 0.5g of ferric chloride in 10mL distilled water. 1mL of 5% ferric chloride was added to 5mL aqueous crude extract and greenish black precipitation identifies the presence of tannin (Ghani, 2003).

Detection of resin

2mL of the crude extract was treated with 5-10 drops of acetic anhydride following with gently heating the solution. 0.5mL of sulfuric acid was added to the solution. The presence of bright purple color identifies the presence of resin (Soni & Sosa, 2013).

Detection of glycosides

The crude extract was hydrolyzed with dilute HCl prior to the Borntrager's Test (Mariappansenthilkumar, 2013).

Borntrager's test (modified)

5mL of 5% Ferric (III) chloride and 5mL of dilute hydrochloric acid were added to 5mL of filtrate with gently heating the mixture for 5minutes in a water-bath and cooled. 5mL of benzene was added to this mixture and shaken thoroughly. The organic layer was made separated by using a separating funnel and then diluting it an equivalent volume of ammonia solution. The formation of a pinkish-red color ammonical layer signifies the detection of glycosides in the extract (Kamalakar *et al*., 2014).

Detection of saponins

Froth test

In a graduated cylinder the crude extract was mixed with 20mL volume of distilled water and the cylinder was shaken for about 15min. The formation of a foam layer of about 2cm in height indicates the existence of saponins (Kokate, 1999).

2.4 *In-vitro* antioxidant activity of *L. aequata* leaf extract

Among different methods of determining antioxidant activity of a medicinal plant, here 4 methods were chosen to determine the antioxidant activity in leaves extract of *L. aequata*.

2.4.1 *DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging assay*

The DPPH free radical scavenging assay of plant extract *L. aequata* was determined using the method described by Braca *et al*. (2001).

Reagents and chemicals

- DPPH – supplied from Sigma Aldrich, U.S.A.
- Methanol – supplied from Active Fine Chemicals Ltd., Bangladesh
- L-ascorbic acid – supplied from Merck, Germany.

Reagent preparation

Preparation of 0.004% (w/v) DPPH solution: 2mg of DPPH was dissolved in 50mL distilled water and then stored in refrigerator at -4°C till before use.

Sample and standard preparation

Preparation of sample stock solution: 120mg of crude extract was dissolved in 10mL of methanol to make a concentration of 12 mg/mL.

From the stock solution 6 diluted concentrations- 1200, 800, 400, 200, 100 and 50 µg/mL were prepared by serial dilution.

Preparation of standard solution: L-ascorbic acid was used as standard and it was prepared in the same procedure as the sample where 6 concentrations are prepared ranging from 1200 to 50 µg/mL from the standard through serial dilution.

Preparation of blank solution

The blank contained only 3mL methanol.

Experimental procedure

I. 1mL of each of 6 concentrations from sample and standard (L-ascorbic acid) were taken in test tubes.
II. 2mL of 0.004% (w/v) DPPH was added to each of the test tube.
III. The test tubes were incubated for about 30minutes at room temperature and measured the absorbance of sample, standard against the blank solution at 517nm using spectrophotometer (U-2910 UV-Vis Spectrophotometer).
IV. Finally, the percentage of free radical scavenging activity was calculated from the equation as follows:

% Free Radical Scavenging (%FRS) activity = $(A_0 – A_1) \div A_0 * 100$

Where, A_0 = The absorbance of the control

A_1 = The absorbance of the sample/standard

V. Finally, the % Free Radical Scavenging activity was plotted against concentration.

2.4.2 *Determination of total phenolic content* (TPC)

The total phenolic content of the *L. aequata* was determined by using modified Folin- Ciocalteu method which is mentioned by Wolfe, Wu & Liu (2003).

Reagents and chemicals

The following reagents and chemicals and reagents were required for determining total phenolic content.

- Folin-Ciocalteu Reagent (FCR)- supplied from LOBA Chemie Pvt. Ltd., India
- Gallic acid monohydrate (Standard)- supplied from Sigma Aldrich, USA
- Sodium carbonate- supplied from Merck Specialities Pvt. Ltd., Mumbai
- Methanol- supplied from Active Fine Chemicals Ltd., Bangladesh

Reagent preparation

Preparation of 250mL of 10% FCR solution: It was made by dissolving 25mL of FCR in distilled water up to 250mL mark.

Preparation of 100mL of 7.5% (w/v) Sodium carbonate: It was made by dissolving 7.5g of Sodium carbonate in distilled water up to 100mL mark.

Preparation of sample and standard

Preparation of sample stock solution: 120mg of *L. aequata* extract was dissolved in 10mL of methanol to make a concentration of 12 mg/mL which is known as sample stock solution.

Then from the sample stock concentration, 4 other diluted concentrations- 1200, 800, 400 and 200 µg/mL were derived through serial dilution.

Preparation of standard solution: Gallic acid was used as the standard and the standard was prepared in the same way as the stock resulting in the 4 serially diluted concentrations, ranging from 1200, 800, 400 and 200 µg/mL.

Preparation of the blank

The blank contained 5mL of FCR solution, 4mL of Sodium carbonate and 1mL of methanol and making the volume up to 10mL.

Experimental procedure

I. 1mL of each of 5 concentrations from sample and standard (gallic acid) solutions were taken in test tubes.
II. 25mL of FCR and 4mL of Sodium Carbonate solution were added.
III. All the test tubes containing mixture was vortexed for 15s and allowed to stand for 30minutes at 40°C in water bath.
IV. Then, the absorbance of all standard and sample solutions was measured at 765nm against blank using spectrophotometer (U-2910 UV-Vis Spectrophotometer).
V. Finally, the total phenolic content of each of the fractions were expressed as Gallic Acid Equivalents using the following equation:

$C = (c \times V) \div m$ Where,

C = Total content of phenolic compounds, mg of gallic acid per gram of dried plant extract, expressed as gallic acid equivalent (GAE)

c = concentration of gallic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

2.4.3 ***Determination of total flavonoid content (TFC)***

The total flavonoid content of the crude extracts of *L. aequata* was determined according to the method which is described by Kumaran & Karunakaran (2007).

Reagents and chemicals

Following reagents and chemicals were required for determining the TFC.

- Quercetin (Standard)- supplied from Sigma Aldrich Co., Germany

- Methanol- supplied from Active Fine Chemicals Ltd., Bangladesh
- Potassium Acetate- supplied from Merck KGaA, Germany
- Aluminium Chloride-supplied from Merck Specialities Pvt. Ltd.

Reagent preparation

Preparation of aluminium chloride solution: 100mL of 10% aluminium chloride solution was made by dissolving 10g of aluminium chloride in 100mL distilled water.

Preparation of potassium acetate solution: 100mL of 1M potassium acetate solution was made by dissolving 9.815g of potassium acetate in 100mL distilled water.

Preparation of sample and standard

Preparation of sample stock solution: 120mg of *L. aequata* leaves extract was dissolved in 10mL of methanol to have a concentration of 12 mg/mL.

Then 4 sample concentrations- 1200, 800, 400 and 200 µg/mL were prepared by serial dilution from the stock solution.

Preparation of standard: The standard was quercetin and the other 4 concentrations ranging from 1200, 800, 400 and 200 µg/mL were prepared in the same manner as the sample.

Preparation of the blank

The blank contained 200uL of 10% aluminium chloride solution, 200µL of 1M Potassium acetate solution, 4mL of methanol and 5.6mL of distilled water to make the volume of 10mL.

Experimental procedure

I. 1mL of each of 5 concentrations were taken from sample and standard (quercetin) in each test tubes.
II. Adding 3mL methanol to each test tubes.
III. Then, 200uL of 10% aluminium chloride and 200µL of 1M Potassium acetate were added to the mixture using 1000µL micropipette.

IV. Finally, to make the volume of 10mL, 5.6mL of distilled water was added to each test tubes.

V. The test tubes were incubated at room temperature for 30minutes.

VI. Lastly, the absorbance of each concentration from the sample and standard solutions were taken against blank using spectrophometer at 415nm.

VII. So, the total flavonoid content of each of the fractions were expressed as Quercetin Equivalents using the following equation:

$C = (c \times V) \div m$ Where,

C = Total content of flavonoid compounds, mg of quercetin per gram of dried plant extract, expressed as quercetin equivalent (QE)

c = concentration of quercetin obtained from calibration curve (mg/mL)

V= Volume of sample solution(mL)

m = weight of the sample (g)

2.4.4 *Determination of total antioxidant capacity (TAC)*

The TAC of leaves extract of *L. aequata* was determined by using a method described by Prieto *et al*. (1999).

Reagents and chemicals

Following reagents and chemicals were required for determining the TAC.

- Ammonium Molybdate- supplied from Active Fine Chemicals Ltd., Bangladesh
- L-Ascorbic Acid (Standard)- supplied from Merck, Germany
- Sodium Triphosphate (Na3PO4.12H2O)- supplied from Merck KGaA, Germany
- Concentrated sulfuric acid (98%)- supplied from Merck, Germany
- Methanol- supplied from Active Fine Chemicals Ltd., Bangladesh

Reagent preparation

3.28mL of 98% concentrated sulfuric acid was dissolved in 100mL distilled water to make 0.6M 100mL of Sulfuric acid.

4.494g of Ammonium molybdate was dissolved in 100mL distilled water to prepare 100mL 0.004M Ammonium molybdate.

1.0645g of Sodium phosphate was dissolved in 100mL distilled water to make 100mL of 0.028M Sodium phosphate

Sample and standard preparation

Preparation of sample stock solution: 120mg of *L. aequata* extract was dissolved in 10mL of methanol to prepare 12 mg/mL of concentration.
The 4 sample concentrations ranging from 1200, 800, 400 and 200 µg/mL were prepared from the stock through serial dilution.

Preparation of standard solution: Ascorbic acid was used as standard and the other 4 concentrations ranging from 1200, 800, 400 and 200 µg/mL were prepared in the same manner as the sample concentration through serial dilution.

Preparation of the blank

The blank contained 3mL of reagent solution and 300µL of methanol

Experimental procedure

I. 300uL of each of the concentrations from sample and standard (L-ascorbic acid) were taken in test tubes.
II. 3mL of each reagent solution (0.6M sulfuric acid, 0.004M Ammonium molybdate and 0.028M Sodium phosphate) were added into the solutions.
III. The test tubes containing sample, standard and blank were incubated in water bath at 95°C for 90min.
IV. Then, the absorbance of each standard and sample solutions were taken against blank at 695nm using spectrophotometer.

V. The TAC of each of the concentrations were expressed as Ascorbic Acid Equivalents (AAE) using the equation as follows:

$A = (c \times V) \div m$ Where,

A = Total antioxidant capacity, mg of ascorbic acid per gram of dried plant extract, expressed as ascorbic acid equivalent (GAE)

c = concentration of ascorbic acid obtained from calibration curve (mg/mL)

V = Volume of sample solution (mL)

m = weight of the sample (g)

2.5 Antibacterial activity of *L. aequata* leaf extract

The antibacterial activity was studied by Disc Diffusion method.

Table 2.1: List of bacteria used in the test

Type of Bacteria	Name of Bacterial Stains
Gram (+)ve Bacteria	*Bacillus subtilis*
Gram (-)ve Bacteria	*Escherichia coli*
Gram (+)ve Bacteria	*Staphylococcus aureus*
Gram (-)ve Bacteria	*Vibrio cholerae*

Preparation of liquid medium by nutrient agar

Firstly, 0.25gm of nutrient agar was dissolved in 10mL distilled water in four conical flasks to make volume 10mL and 1.25gm of nutrient agar was dissolved in 50mL distilled water in another conical flask to make volume 50mL. Then the five test tubes were autoclaved at 121ºC for 1hour.

Pre-culturing the bacterial strains

The Strains were collected previously and preserved from long term STGG medium in an ultra-low temperature (ULT) freezer at -80°C temperature and the medium contains trypsin, glucose, skim milk and glycerin to preserve the strains.

Introduction of bacterial strains into liquid medium

The four bacterial strains - *Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Vibrio cholerae* were taken by a loop with sterilization and dipped into freshly prepared four nutrient agar medium which had volume of 10mL and 50mL agar medium was remained as blank. All the conical flasks were incubated for 24 hours to grow the bacterial colony. Thereafter, 24 hours of incubation, some new bacterial colony was formed and the absorbance of each solution was taken. The strains were then preserved in refrigerator for further use.

Preparation of solid medium

3.8gm of Molar Hington agar was measured and dissolved in 100mL distilled water and autoclaved at 121ºC for 1hour.After autoclave, the solution was stand for sometimes for cooling.

Preparation of crude extract sample

The sample was prepared by dissolving 7mg methanolic extract of *L. aequata* leaves 20mL of methanol which concentration was 350 μg/mL. Then it was serial diluted to get 300 μg/mL, 250 μg/mL, 200 μg/mL respectively. Afterwards, some sterilized small Whitman filter paper (6mm in diameter) was allowed to soak into four concentration of sample for 15-20 mints and then the filter paper was allowed to dry.

Standard

Here kanamycin and ampicillin were used as the standard.

Preparation of disc

The Muller Hington agar solid medium was poured into four petri dish and four different cotton swab was dipped into four bacterial mixers and streaked to the dish. Then all the petri

dish were allowed to dry for 5 min. Afterwards, all the soaked filter paper and kanamycin, amoxicillin filter paper (standard) were individually placed on the surface of the petri dish by using forceps and pressed gently on the disc. To avoid bacterial cross contamination, every time the forceps were sterilized by flame. Finally, the petri dishes were incubated for 24 hours at about 37°C to observe the action of crude extract and the standard on bacterial culture. This each of the work was done under the laminar air flow to avoid bacterial contamination.

CHAPTER THREE

RESULTS

CHAPTER 3: RESULTS

3.1 Phytochemical screening of *L. aequata*

Phytochemical screening was done by different methods and the result is presented in the following table.

Table: 3.1: Phytochemical screening of *L. aequata* leaf extract

Class of compound	Result
Alkaloids	+++
Flavonoids	++
Phenol/ Phenolic compound	+
Glycosides	+
Tannins	+++
Carbohydrates	++
Phytosterols	+
Resins	-
Steroids	-
Saponins	-

(+) means present in a single experiment test

(++) means present in two methods of experiment

(+++) means present in three methods experiment

(-) means absent

Interpretation: The phytochemical screening of *L. aequata* showed the presence of carbohydrates, flavonoids, alkaloids, phenolic compounds, tannins, glycosides, and phytosterol. However, resins, steroids and saponins were absent.

3.2 Antioxidant activity of *L. aequata* leaf extract

The presence of flavonoids, phenols, phenolic compounds and tannins in the phytochemical screening of *L. aequata* leaf extract contribute to the moderate antioxidant activity found in the study.

3.2.1 *DPPH free radical scavenging activity of L. aequata leaf extract*

DPPH free radical scavenging assay of methanolic leaf extract *L. aequata* was done by method which is described by Braca *et al*. (2001). The calibration curve of L-ascorbic acid (Standard) is showed in figure 3.1 and table 3.2 contains the absorbance and % inhibition by the standard. In addition, free radical scavenging activity of *L. aequata* leaf extract is showed in table 3.3.

Calibration curve of L-ascorbic acid (Standard)

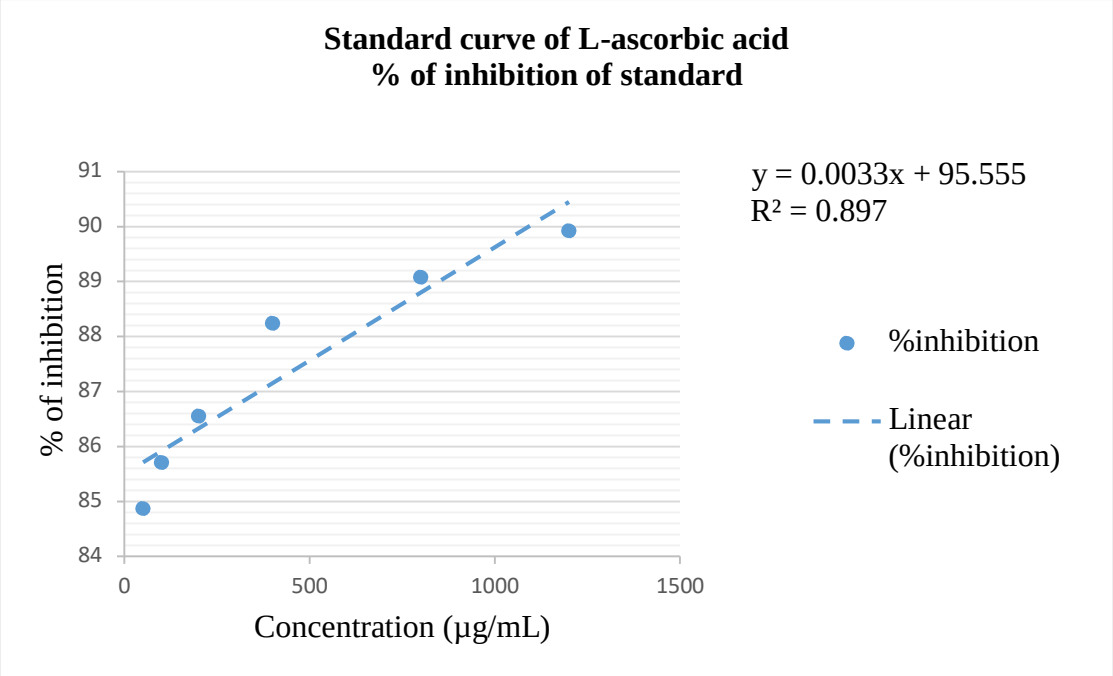

Figure 3.1: Graphical presentation of % free radical scavenging activity of standard (L- ascorbic acid).

Table 3.2: DPPH free radical scavenging activity of standard (L-ascorbic acid)

Where A_0= 0.119 (absorbance of control)

Conc. of Standard (µg/mL)	Absorbance of Standard (L-ascorbic acid)	% free radical scavenging activity
50	0.006	94.96%
100	0.005	95.79%
200	0.004	96.64%
400	0.003	97.48%
800	0.002	98.32%
1200	0.001	99.16%

Interpretation: It was observed that as the concentration of standard (L-ascorbic acid) was increased from 50 to 1200 µg/mL, the absorbance decreased slowly from 0.006 to 0.001 with the increased in the free radical scavenging activity from 94.96% to 99.16%.

Table 3.3: Free radical scavenging activity of *L. aequata* leaf extract

Conc. of Standard (µg/mL)	Absorbance of Sample	% free radical scavenging activity
50	0.019	84.87
100	0.018	85.71
200	0.017	86.55
400	0.016	88.24
800	0.015	89.08
1200	0.014	89.92

Interpretation: Findings showed that the % free radical scavenging activity was concentration dependent. The % free radical scavenging activity of methanolic extract of *L. aequata* was observed to be 84.87% to 89.92% when concentration ranging from 50 to 1200

µg/mL. The highest % free radical scavenging activity was found at 1200 µg/mL concentration.

Curve of % free radical scavenging activity of *L. aequata* leaf extract.

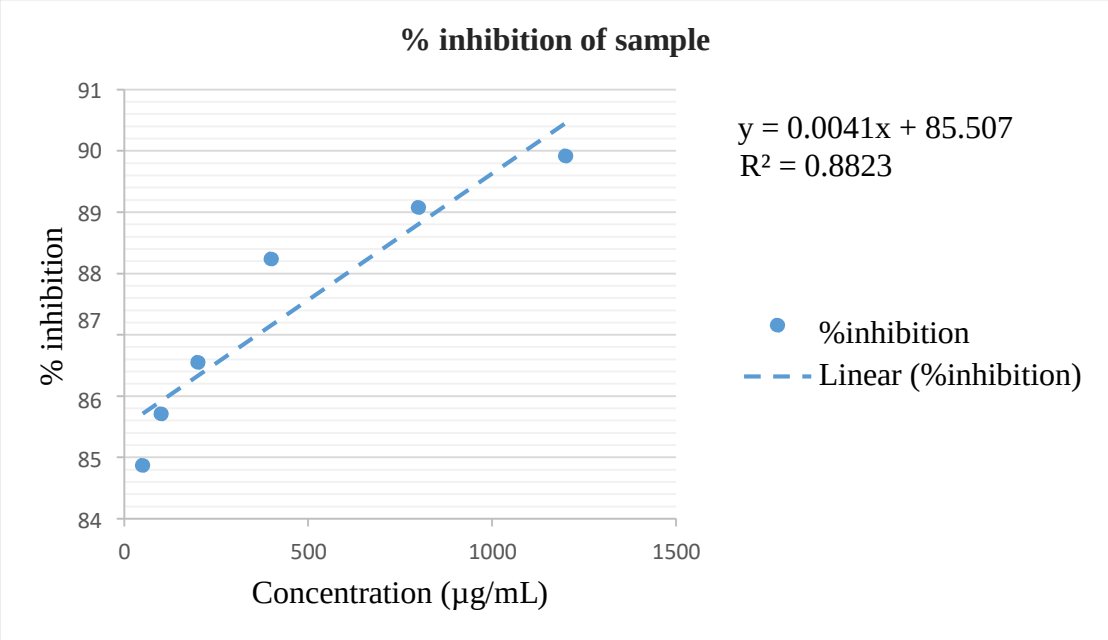

Figure 3.2: Graphical presentation of % free radical scavenging activity of *L. aequata* leaf extract.

3.2.2 *Determination of total phenolic content (TPC)*

The total phenolic content of the selected plant was determined by using modified Folin-Ciocalteu method which is mentioned by Wolfe, Wu & Liu (2003). Figure 3.3 represents the graphical presentation of standard gallic acid and table 3.4 includes the total phenolic content of *L. aequata* leaf extract.

Calibration curve of gallic acid (standard)

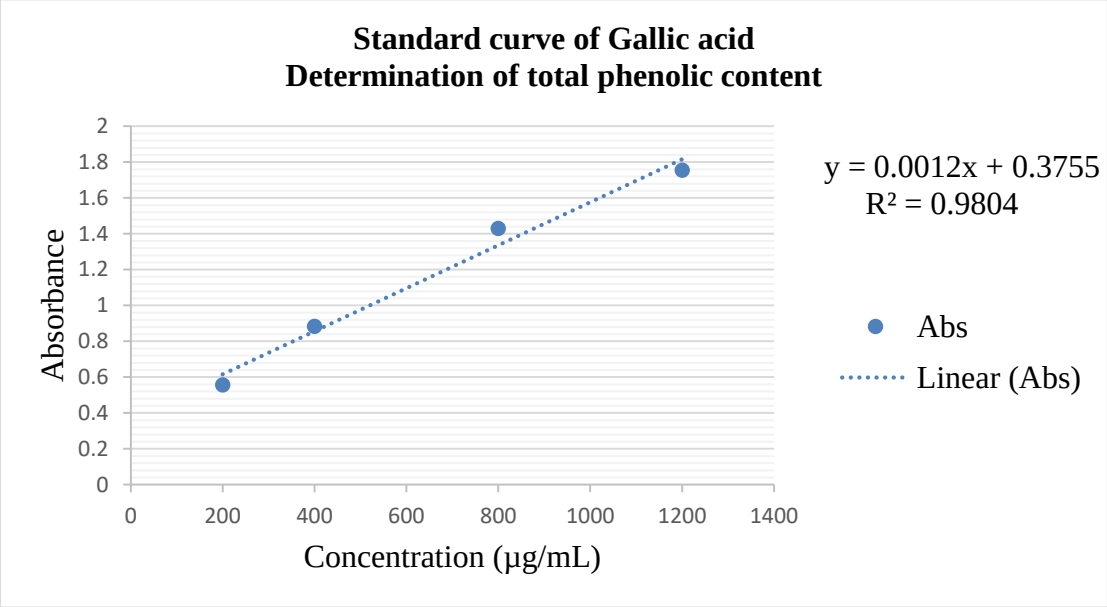

Figure 3.3: Graphical presentation of standard gallic acid (GA) at 765nm for determining total phenolic content.

Interpretation: The equation of the calibration curve of gallic acid was found to be y=0.0012x+0.3755 which was used to determine the total phenolic content of *L. aequata*. A regression coefficient of 0.9804 was also obtained indicating a very strong relationship between the concentration and the absorbance.

Table 3.4: Total phenolic content of *L. aequata* leaf extract.

Concentration (µg/mL)	**Absorbance of Standard (GA)**	**Absorbance of *L. aequata* leaf extract**	**TPC present in sample (mg) of gallic acid per gram of dried extract**
200	0.556	0.967	41.08
400	0.883	0.999	43.3
800	1.43	1.010	44.06
1200	1.754	2.282	132.4

Interpretation: Study showed that as the concentration of *L. aequata* was increased from 200 to 1200 µg/mL, the total phenolic content also increased from 41.08 to 132.4mg of gallic acid per gram of dried extract. The highest total phenolic content of this plant extract was 132.4gm found at 1200 µg/mL concentration.

3.2.3 *Determination of total flavonoid content (TFC)*

The total flavonoid content (TFC) was determined according to the method Kumaran & Karunakaran (2007) with slight modification. Here, graphical presentation of standard quercetin is showed in figure 3.4 and Table 3.5 includes total flavonoid content of *L. aequata* leaf extract.

Calibration curve of quercetin (standard)

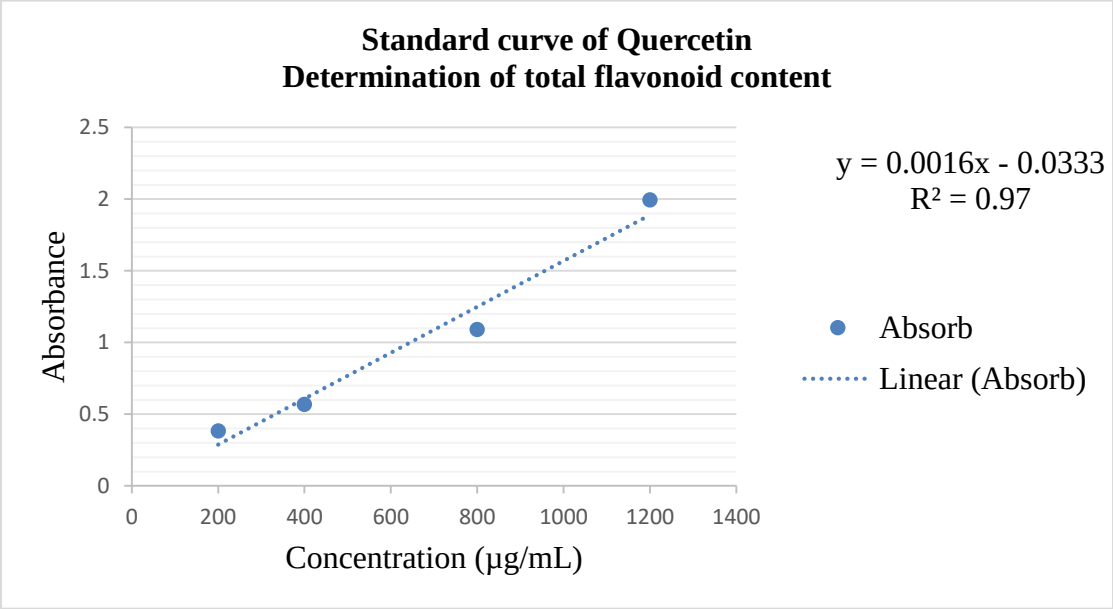

Figure 3.4: Graphical presentation of standard quercetin (QE) at 415nm for determining total flavonoid content.

Interpretation: The equation of the calibration curve of quercetin was found to be y= 0.0016x-0.0333 which was used to determine the total flavonoid content of *L. aequata*. A regression coefficient of 0.97 was also obtained indicating a very strong relationship between the concentration and the absorbance.

Table 3.5: Total flavonoid content of *L. aequata* leaf extract.

Concentration (µg/mL)	**Absorbance of Standard (QE)**	**Absorbance of *L. aequata* leaf extract**	**TFC present in sample (mg) of quercetin per gram of dried extract**
200	0.383	0.060	4.86
400	0.568	0.075	5.64
800	1.09	0.136	8.82
1200	1.994	0.218	13.09

Interpretation: Result of total flavonoid content showed that at 200 to 1200 µg/mL concentration of *L. aequata* leaf extract contained only 4.86 to 13.09mg of quercetin per gram of dried extract which was very low content. The highest total flavonoid content was found 13.09gm at 1200 µg/mL concentration.

3.2.4 *Determination of total antioxidant capacity (TAC)*

The TAC of *L. aequata* leaf extract was determined according to the method described by Prieto *et al*. (1999). Calibration curve of ascorbic acid (standard) is showed in figure 3.5 and Table 3.6 represents total antioxidant capacity of *L. aequata* leaf extract.

Calibration curve of ascorbic acid (standard)

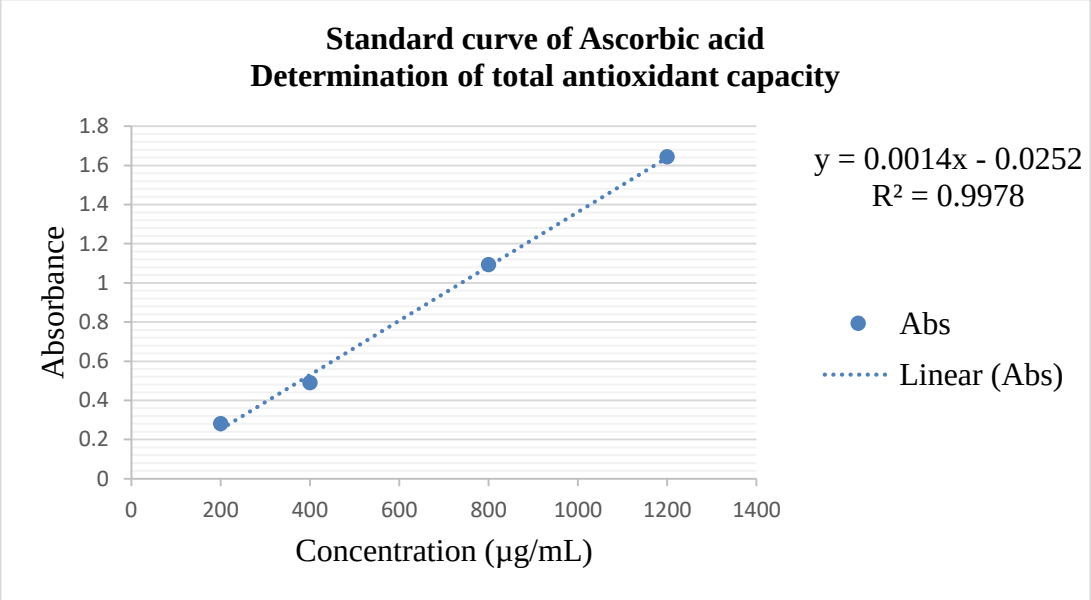

Figure 3.5: Graphical presentation of standard ascorbic acid (AA) at 695nm for determining total antioxidant capacity.

Interpretation: The equation of the standard curve of ascorbic acid was found y=0.0014x-0.0252 which would be used to determine the total antioxidant capacity of *L. aequata*. A regression coefficient of 0.9978 was also obtained indicating a good relationship between the concentration and the absorbance.

Table 3.6: Total antioxidant capacity of *L. aequata* leaf extract

Concentration (µg/mL)	**Absorbance of standard (AA)**	**Absorbance of *L. aequata* leaf extract**	**TAC present in sample (mg) of ascorbic acid per gram of dried extract**
200	0.281	0.715	44
400	0.49	0.730	44.95
800	1.092	0.810	49.71
1200	1.643	0.999	60.96

Interpretation: The experiment revealed that flavonoid content of *L. aequata* leaf extract was increased with the increase in concentration. When concentration of *L. aequata* was increased from 200 µg/mL to 1200 µg/mL, the total flavonoid content subsequently increased from 44 to 60.96mg of ascorbic acid per gram of dried extract. The highest total antioxidant capacity was 60.96gm found at 1200 µg/mL concentration.

3.3. Antibacterial activity of *L. aequata* leaves extract

The disc diffusion method was used to do the antibacterial test on *L. aequata* leaf extract. The disc was contained 350 µg/mL, 300 µg/mL, 250 µg/mL and 200 µg/mL of sample solution, four different bacterial culture and the standard (Kanamycin, ampicillin) was used as positive control. The experiment had found that the leaves extract of *L. aequata* have no antibacterial activity against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Vibrio cholerae.* There was no zone of inhibition against those four bacteria. Both ampicillin and kanamycin have antibacterial effect on *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Vibrio cholerae* by showing their zone of inhibition in the experiment. The zone of inhibition of kanamycin against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Vibrio cholerae* was 15mm, 13mm, 17mm and 12mm, respectively and the zone of inhibition of ampicillin against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Vibrio cholerae* was 3mm, 7mm, 2mm and 10mm, respectively. Methanol was used as solvent for diluting the plant extract and no antibacterial activity was seen for methanol.

Table 3.7: Antibacterial activity of methanolic leaf extract of *L. aequata*.

Different conc. of sample (µg/mL)	**Bacterial strains and zone of inhibition**			
	Bacillus subtilis mm	*Escherichia coli* mm	*Staphylococcus aureus* mm	*Vibrio cholerae* mm
350	-	-	-	-
300	-	-	-	-
250	-	-	-	-
200	-	-	-	-
Kanamycin	15	13	17	12
Ampicillin	3	7	2	10
Methanol	-	-	-	-

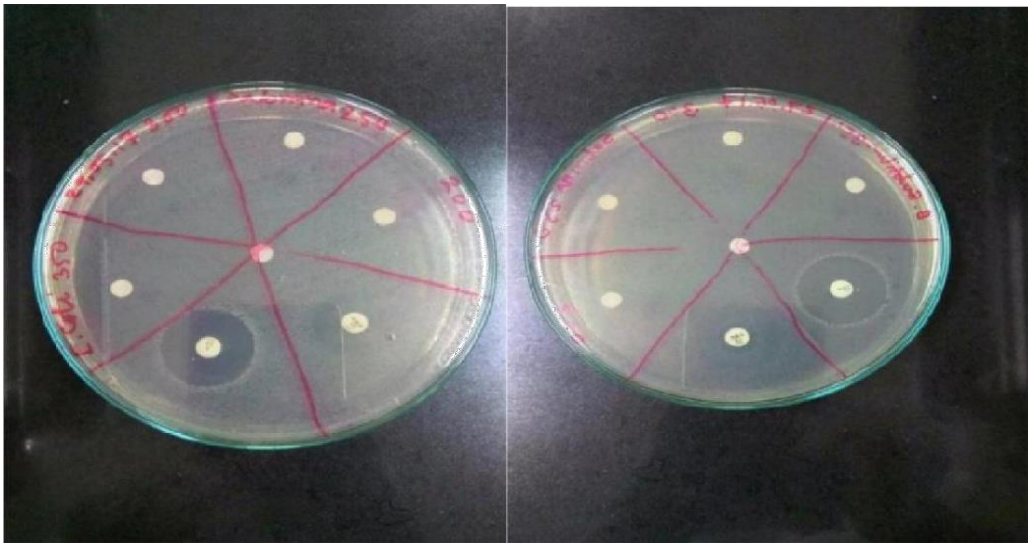

Figure 3.6: Zone of inhibition shown by standard (kanamycin and ampicillin) on *E. coli*, *B. subtilis*, respectively.

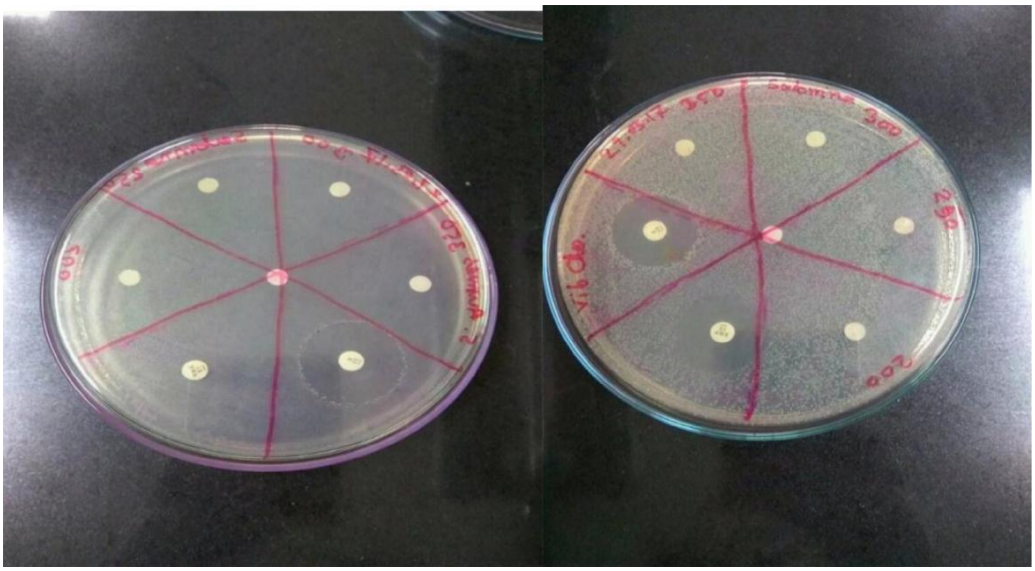

Figure 3.7: Zone of inhibition shown by standard (kanamycin and ampicillin) on *S. aureus*, *V. cholerae*, respectively.

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CHAPTER FOUR

DISCUSSION

CHAPTER 4: DISCUSSION

L. aequata is a traditional medicinal plant which is native to Myanmar, India, Malay Indonesia, Peninsula and also in the forest of Chittagong and Sylhet in Bangladesh. From the history, we have seen that people are using medicinal plants for curing different types of diseases. Modern science has proven the benefit of different medicinal plant along with the synthetic drug. Medicinal plants have analgesic, anti- inflammatory, antibacterial, antioxidant, cytotoxic, anticancer and others effects on human body. By realizing the therapeutic value of herbal medicine, scientists are trying to discover new drug from nature derived medicinal plant. Thus, medicinal plant has achieved its own place in the drug development.

Literature review had showed that, the roots, stems and tubers of *L. aequata* are astringent with different medicinal value but there is no antioxidant study found on this leaf extract before. In addition, the antibacterial study of various parts of extracts *L. aequata* had been performed on *Mycobacterium tuberculosis, Micrococcus pyogenes, and Pasteurella pestis* previously. So, this study was focused on phytochemical constituents, antioxidant and antibacterial study using other bacterial strains (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Vibrio cholerae*) of *L. aequata* leaf extract to find out its bioactivity property which may help in the drug development in the future.

Phytochemical screening of methanolic extract of *L. aequata* leaves showed the presence of alkaloids, tannins, flavonoids, phenols, carbohydrates, phytosterols and glycosides. It does not show the presence of resins, steroids and saponins in this plant.

The antioxidant test showed that the leaves extract of *L. aequata* has moderate antioxidant effect which was confirmed by DPPH free radical scavenging activity, total phenolic content, total flavonoid content and total antioxidant capacity. Possible mechanisms such as- chain breaking reaction or reducing the ROS or Scavenging free radicals and chelating the metal by the leaf extract may contribute to the antioxidant activity.

Firstly, in the DPPH method, % free radical scavenging activity (% FRS) of standard L-ascorbic acid was increased from 94.96%, 95.79%, 96.64%, 97.48%, 98.32% and 99.16% at 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL and 1200 µg/mL concentration,

respectively (table 3.2). Whereas, the % free radical scavenging activity of *L. aequata* extract increased from 84.87%, 85.71%, 86.55%, 88.24%, 89.08% and 89.92% at the same concentration which is close to the standard (table 3.3). Thus, DPPH free radical scavenging method had showed significant antioxidant activity of the plant extract. The % FRS of standard was always found to be higher than that of crude extract of *L. aequata*.

Secondly, total phenolic content in the dried extract of *L. aequata* was determined by using the folin-ciocalteu method where gallic acid is the standard. The results showed that at 200, 400, 800 and 1200 µg/mL of dried extract of *L. aequata*, the total phenolic content (TPC) obtained were respectively 41.08, 43.3, 44.06 and 132.4mg of gallic acid per gram of dried extract which shows a positive correlation with the standard (table 3.4). So, *L. aequata* possesses sufficient content of phenolic content to exhibit moderate antioxidant activity.

Moreover, total flavonoid content of *L. aequata* leaves extract was evaluated by using quercetin as standard. According to the result, at 200, 400, 800 and 1200 µg/mL of dried extract of *L. aequata*, the total flavonoid content obtained were respectively, 4.86, 5.64, 8.82, 13.09mg of quercetin per gram of dried sample (table 3.5). This shows very little antioxidant activity of the selected plant.

Finally, the total antioxidant capacity of the extract of *L. aequata* was determined using the phosphomolybdenum method, where the results were expressed as ascorbic acid equivalent (AAE) as ascorbic acid was the standard. The result showed that the total antioxidant capacity (TAC) of *L. aequata* was 44, 44.95, 49.71, and 60.96mg of ascorbic acid at 200, 400, 800 and 1200 µg/mL concentration, respectively (table 3.6).

In addition, this study was focused on the determination of the antibacterial activity of *L. aequata* leaf extract using 4 different bacterial strains: *Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Vibrio cholera*e. Generally antibacterial agents inhibit bacterial growth by Inhibition of cell wall synthesis or protein synthesis or other essential metabolites. The test had shown that *L. aequata* leaf extract have no antibacterial effect on this four (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Vibrio cholerae*) bacterial stains. On the other hand, kanamycin and ampicillin were used as standard which have antibacterial effects by showing their zone of inhibition against the same four bacterial

strains on the same disc. Findings showed that the largest zone of inhibition was found in standard kanamycin against *Staphylococcus aureus* and it was 17 mm (table 3.7).

Lastly, the presence of components such as- phenols or phenolic compounds, flavonoids, tannins etc in the phytochemical screening of methanolic leaf extract of *L. aequata* may justify its use in traditional or folkloric remedies in treating different diseases.

CHAPTER FIVE

CONCLUSION & FUTURE DIRECTION

CHAPTER 5: CONCLUSION

"Alternative Medicine" this term has become very familiar in our culture. Medicinal plants are widely used as raw ingredients for the extraction from the active ingredients as well as in the synthesis of t important drugs. Therefore, the objective of this study was to determine the antioxidant and antibacterial study of *L. aequata* leaf extract which has never been done.

L. aequata leaf contains flavonoids, alkaloids, glycosides, phenolic compounds, tannins, phytosterol, carbohydrates in the phytochemical screening. The antioxidant study was done by using DPPH free radical scavenging activity, total phenolic content, total flavonoid content and total antioxidant capacity. In DPPH free radical scavenging method, the % FRS increased from 84.87% to 89.92% at 50 to 1200 µg/mL concentration, respectively. Then, the total phenolic content of plant sample was increased from 41.08 to 132.4mg/g of gallic acid at concentration 200 to 1200 µg/mL, respectively. The total flavonoid content increases from 4.86 to 13.09mg/g of quercetin at 200 to 1200 µg/mL concentration, respectively. Lastly, the total antioxidant capacity of selected plant was 44 to 60.96mg/g of ascorbic acid at 200 to 1200 µg/mL, respectively. Moreover, the antibacterial study performed on *L. aequata* leaf extract was determined by disc diffusion method against four bacterial strains: *Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Vibrio cholerae.* The study showed that *L. aequata* leaf extract cannot be used as antibacterial agent against those four bacterial strains as there was no zone of inhibition shown by the extract.

To conclude, this current study of *L. aequata* plant has established that it has moderate antioxidant effect but has no antibacterial activity on *Escherichia coli, Bacillus subtilis, Staphylococcus aureus and Vibrio cholerae.*

FUTURE DIRECTIONS

The result of the present study has established that methanolic extract of *L. aequata* possesses moderate antioxidant effect with no antibacterial activity against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Vibrio cholerae* which showing different scopes for further studies:

Different antioxidant test method such as: nitric oxide, hydrogen per-oxide, lipid per oxidation method may carry out to find its significant antioxidant activity.

No pharmacological studies have yet been performed on cytotoxic, anti-cancer and antihyperlipidemic, and anti-atherosclerotic activities of this plant. Further different bioactivity studies of this plant should carried out that have not yet been done. Such initiatives into these research activities may lead to the new drug isolation and development of novel drug discovery.

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