In-vitro Antioxidant and Cytotoxicity Screening of Ethanolic Extract of *Lagerstroemia thorelli* (Lythraceae) Leaves

By

Amir Hamja Tipu

ID: 19146022

A Project Submitted to The School of Pharmacy in Partial Fulfillment of The Requirements for The Degree of Bachelor of Pharmacy (Hons.)

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February, 2023

Declaration

I, **Amir Hamja Tipu**, do hereby declare that

1. This project titled **"In-vitro Antioxidant and Cytotoxicity Screening of Ethanolic Extract of *Lagerstroemia thorelli* (Lythraceae) Leaves"**, constitutes my own work while completing my degree at BRAC University.
2. This project does not contain material previously published or written by a third party, except where this is clearly cited and referenced.
3. This project has not been submitted for any other degree or diploma at a university or other educational institution.
4. I have acknowledged all main sources of information.

Signature,

Amir Hamja Tipu

19146022

Date

Approval

The project titled **"In-vitro Antioxidant and Cytotoxicity Screening of Ethanolic Extract of *Lagerstroemia thorelli* (Lythraceae) Leaves"** submitted by Amir Hamja Tipu (ID: 19146022) of Summer, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on March, 2023.

Supervised By:

Dr. Shahana Sharmin
Assistant Professor
School of Pharmacy
BRAC University

Approved By:

Program Director:

Professor Dr. Hasina Yasmin
Program Director and Assistant Dean
School of Pharmacy
BRAC University

Dean:

Professor Dr. Eva Rahman Kabir
Dean
School of Pharmacy
BRAC University

Ethics Statement

This project comprises in-vitro analytical methods only. No animal or human subjects were involved in this study.

Abstract

Substantial antioxidant activity of *Lagerstroemia thorelli* leaves extract was revealed in DPPH free radical scavenging assay with an IC_{50} value of 83.05085 µg/mL compared to that of 47.55245 µg/mL demonstrated by ascorbic acid. Besides, the total phenolic content test exhibited concentration of phenolic compounds in the extract to be 122.6309 mg/g GAE of dry extract. Afterwards, cytotoxicity of the extract was assessed in terms of LC_{50} value. During the brine shrimp lethality bioassay, cytotoxicity of the leaf extract was evaluated to be impotent based on the LC_{50} at 28861.40 µg/mL against that of 0.000145 µg/mL for vincristine sulfate. However, more advanced researches must be performed to discover other pharmacological properties of *L. thorelli*. Conclusively, on the basis of the present preliminary study, it can be claimed that the profound antioxidant property can be utilized to treat many oxidative stress-associated diseases.

Keywords: *Lagerstroemia thorelli*; Antioxidant; Ascorbic acid; Phenolic compounds; Vincristine sulfate.

Dedication

This project is dedicated to my beloved mother and the soul of my father for being my source of support and inspiration. Also, their affection, efforts, sacrifices and struggles have given me the master key to unlock many invaluable opportunities and to dream higher. Moreover, they have contributed to develop focus, concentration and self-control in me. May Allah (SWT) bless them and grant them Jannah. A special feeling of gratitude to my sister, Dr. Taslima Rahman, for encouraging me throughout my whole academic life. Truly I love you all to the moon and back.

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Words cannot express how profound and sincere my gratitude is to my honorable supervisor, Dr. Shahana Sharmin, Assistant Professor, School of Pharmacy, BRAC University, for her continuous guidance, support and motivation. I could not have imagined having the heartiest administrator for my project work and I feel so fortunate about that. Her prudence, dynamism, enthusiasm, professionalism, immense knowledge and patience have driven me to complete this project successfully.

I am eager to extend my sincere thanks to Dr. Eva Rahman Kabir, Professor and Dean, School of Pharmacy, BRAC University and also to other honorable faculty members of the School of Pharmacy.

I am also thankful to the laboratory officers, the authority of Ayesha Abed library and all the other staff of university facilities for giving me the golden opportunity to carry out my project work without any hindrance.

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Amir Hamja Tipu

February, 2023

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List of Abbreviations

- gm = Gram
- mg = Milligram
- µg = Microgram
- m = Meter
- cm = Centimeter
- mL = Milliliter
- IC_{50} = Median Inhibitory Concentration
- LC_{50} = Median Lethal Concentration
- DPPH = 2,2-Diphenyl-1-Picrylhydrazyl
- TPC = Total Phenolic Content
- GAE = Gallic Acid Equivalent
- DMSO = Dimethyl Sulfoxide
- R^2 = Regression Coefficient
- Conc.= Concentration
- *L. thorelli* = *Lagerstroemia thorelli*
- UV-vis = Ultraviolet Visible
- BNH = Bangladesh National Herbarium
- NaCl = Sodium Chloride

Chapter One

Introduction

1. Introduction

Since the beginning of creation, mankind has been dependent on natural resources for fulfilling their daily needs. Nature has been arranged by the Creator with hundreds and thousands of plant and animal species, the majority of which have provided humanity with food, shelter, cloth and remedies for various maladies. Unani, Siddha, Ayurveda, Homeopathy and other traditional systems of medicine have been treating human beings with plant based medicines. Traditional practitioners and ancient scholars, most notably Charaka, Sushrutha, Hippocrates, Aristotle, Rhazes and Avicenna, have gathered knowledge from nature in a long and tedious process to formulate their own medicines of phytochemicals and secondary metabolites from the crude extracts of plant materials and passed it down from generation to generation.

Ahmad & Ahamad reported that in recent days traditional medicines are still being preferred over synthetic combinatorial chemicals to treat cancer, diabetes, obesity, cardiovascular complications and many more chronic diseases and lifestyle disorders (2020). According to Newman and Cragg, in the time frame of 1981-2014, 1562 drugs were approved, of which 25% were directly made from natural compounds or their derivatives and 21% were made by combining synthetic compounds with pharmacophores from natural origin or their derivatives (2016). Plants, as natural resources, provide significant contributions to modern drug discovery and development processes. These plants contain a variety of biologically active chemicals and are regarded as medicinal plants.

1.2. Availability and Usage of Medicinal Plants in Bangladesh

Bangladesh, although being a subtropical country, has seven distinct climatic zones due to variations in rainfall, temperature and humidity (Sarker et al., 2017). It serves as a great source of innumerable medicinal plants, especially the hilly areas and the mangrove forests. These plants play an important role as natural reservoirs of many pharmacologically active compounds such as alkaloids, terpenes, flavonoids, sterols, phenolics, resins, lactones, volatile oil, tannins etc. Not too long ago, the majority of the population used to be dependent on folk medicinal healers, also known as Kabiraj. Those kabiraj used to treat patients with their expertise gained through constant practice, utilizing natural remedies gathered from the wild. Among 5000 plant species found in Bangladesh, around 20% are said to possess medicinal values (Kadir, 1990).

Table 1.1: Medicinal Plants of Bangladesh and Their Usage as Traditional Medicine (Hossan et al., 2010)

Taxonomic Name	Local Name	Segment(s) Used	Therapeutic Use
Plumbago indica L.	Agni-chita	Leaves and stems	Syphilis, Rheumatoid arthritis, Dysmenorrhea
Grewia asiatica L.	Chandani shewra	Leaves	Gonorrhea, diarrhea, anorexia
Urena lobata L.	Bulbuli gach	Leaves	Presence of pus or semen in urine, UTIs, Dysmenorrhea
Clitoria ternatea L.	Umaio	Leaves and roots	Edema, Indigestion, Frequent urination
Emblica officinalis Gaertn.	Amla	Leaves and fruits	Leucorrhea, Diabetes, Jaundice
Streblus asper Lour.	Aurga	Leaves and stems	Cholera, Cancer, Leucoderma
Ipomoea paniculata Burm.f.	Bhui kurma	Whole plant	Menstrual problems, Liver cirrhosis, Stimulant
Costus speciosus (Koen.) Sm.	Tiatui	Roots	Leucorrhea, Smallpox, Eczema
Glycosmis pentaphylla (Retz.) DC.	Ash-sheora	Whole plant	Migraine, Rheumatoid arthritis, Gastritis

According to Islam et al., 250 plant species are serving as an integral part of traditional medicine in Bangladesh (2018). These medicinal plants are now applied through self-medication or recommended by physicians or pharmacists. Nevertheless, only a few of them are investigated scientifically to validate their therapeutic value and mechanism of action.

1.3. Selection of *Lagerstroemia thorelli*

Despite being recognized as an ornamental tree for decades, the medicinal value of *L. thorelli* is yet to be discovered. There is no adequate information about *L. thorelli* available in any journal or publication. So, the goal of this project is to reveal different properties of *L. thorelli* such as antioxidant and cytotoxic activity.

1.3.1. Introduction to the Selected Plant - *Lagerstroemia thorelli*

Lagerstroemia thorelli falls under the family Lythraceae, also known as loosestrife and pomegranate family. Lythraceae plants are distributed worldwide, mainly in tropical and subtropical areas. This family comprises about 32 genera and approximately 620 species. Most of them are annual or perennial herbs, along with some shrubs and trees (Xu & Deng, 2017). In Bangladesh, *L. thorelli* is mostly found in the Sylhet division, but also available in the locality of Savar. It can easily be propagated from seeds and so it is often seen in the side-ways of roads and parks all over the country. *L. thorelli* is basically a dwarf bushy tree with both white and mauve mottled flowers and the flowering season is from the month of July to September (Randhawa & Mukhopadhyay, 1986).

1.3.2. Morphology of *Lagerstroemia thorelli*

Lagerstroemia thorelli is a medium sized deciduous tree having a height of 8-10 m, but it can reach up to 15 m. Bole is fairly straight at the bottom, sometimes crooked, branchless up to 5 m and 1-1.5 m in diameter. Its bark surface is smooth, thornless and free of latex, gray to fawn-brown mottled. On the contrary, the inner bark is fibrous, gray-fawn to yellow in color, turning purple upon exposure to air. The bark easily comes off the trunk, but from the cambium to the pith of the trunk is woody. The crown is bushy and spreading and exudes leaves in the dry season. Leaves are simple, opposite, distichous and have an average size of 10 × 4 cm. Matured leaves are bright green in color and have a decussate arrangement. Inflorescence is irregular, branched, multi-flowered, large, axillary or terminal panicle. Both mauve and white flowers have wide-open petals, often 6, inserted near the mouth of the calyx tube, having many stamens in several rows. Fruits are large woody capsules on the persistent calyx and brown in color. Seeds are with apical wings and brown to black in color. Root system is dense underneath and can extend a considerable distance, but is not aggressive to nearby foundations (Orwa et al., 2009).

It has been estimated that the origin of *L. thorelli* is in the tropical regions of Asia, most probably in the Indian subcontinent (Randhawa & Mukhopadhyay, 1986). These deciduous and evergreen flowering plants are also recognised as native to South-East Asia, Northern Australia and parts of Oceania. *L. thorelli* is known as ‘Choto Jarul’ locally in Bangladesh (Uddin & Hassan, 2016).

1.3.3. Taxonomic Classification of *Lagerstroemia thorelli*

Table 1.2: Taxonomic Hierarchy of *L. thorelli*

Rank	**Scientific Name (Common Name)**
#Kingdom	Plantae (Plants)
#Phylum	Magnoliophyta (Flowering Plants)
#Class	Magnoliopsida (Dicotyledons)
#Order	Mytrales
#Family	Lythraceae
#Genus	*Lagerstroemia* (Crapemyrtle)
#Species	*Lagerstroemia thorelli* Gagnep.

(*BOLD Systems: Taxonomy Browser - Lagerstroemia thorelli {species}*, n.d.)

1.3.4. Related Publication on *Lagerstroemia thorelli*

No research studies have yet been conducted on *L. thorelli* to reveal its chemical constituents, pharmacological properties or medicinal uses. Potential properties of the unrevealed plant are yet to be discovered in future research studies.

1.4. Project Justification/Rationale

Literature review of available academic studies revealed inadequate information about *Lagerstroemia thorelli* Gagnep., since no significant studies have been conducted on the selected plant. Earlier research studies in several other species of this genus have reported to exert powerful antidiabetic, anti-inflammatory, antioxidant, diuretic, anti-obesity and antimicrobial activities (Swamy, 2012). So, this project is designed and conducted to find out the potential pharmacological properties of the leaf extract of the plant.

1.5. Aim of the Project

This project aims to investigate and disclose unknown biological potentials of the leaves extract of *Lagerstroemia thorelli*.

1.6. Objectives of the Project

i. To evaluate the antioxidant activity of the leaves extract of *Lagerstroemia thorelli* by in-vitro DPPH free radical scavenging method and the total phenolic content test,
ii. To assess and collect data for cytotoxic potentials of the ethanolic extract of the *L. thorelli* leaves.

Chapter Two

Methodology

2. Methodology

2.1. Leaves Collection and Verification

Healthy green leaves of *L. thorelli* were collected by the second week of September 2022, from Nabiganj upazila in Habiganj district, Sylhet division, Bangladesh. A herbarium sheet of *L. thorelli* leaves was prepared according to the instructions of the Bangladesh National Herbarium (BNH), Mirpur, Dhaka and was sent to the herbarium for verification. DACB-87494 is the accession number for the given specimen.

Figure 2.1: *Lagerstroemia thorelli* Leaves Collection

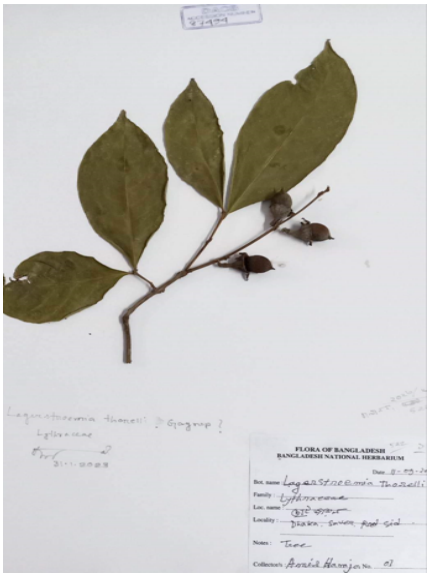

Figure 2.2: *L. thorelli* Leaves Herbarium Sheet

2.2. Preparation and Extraction of Leaves

Preparation and extraction of *Lagerstroemia thorelli* leaves involve several steps. According to Clemen-Pascual et al., the entire process can be segmented into two main parts: "Plant Material Preparation" and "Extraction" (2022).

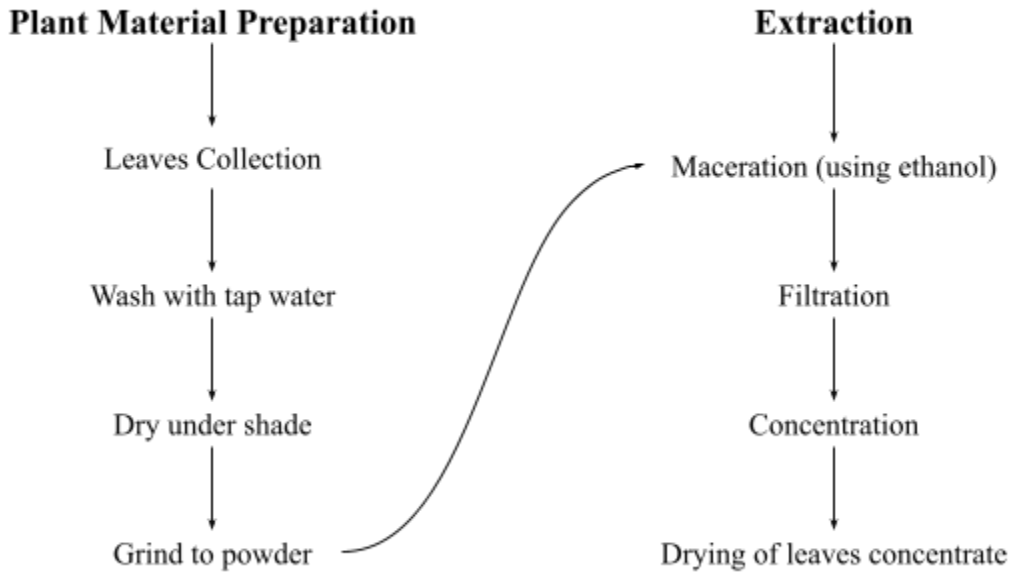

Figure 2.3: Flowchart of Steps of Extraction of Ingredients from *L. thorelli* Leaves

2.2.1. Plant Material Preparation

Plant material preparation is the preparatory stage and one of the most important steps. This is because success and quality of the research/project findings are exclusively dependent on this step (Abubakar & Haque, 2020). The roadmap of this step begins with collecting the whole plant or part of the plant.

For this project, after collecting the leaves, they were separated from the stalks and washed in clean tap water to get rid of dust particles and other foreign matters. The leaves were then allowed to dry under shade at room temperature for fourteen days (air-drying method). Essential precautions were taken to avoid cross-contamination. Completely dried and brittle leaves were pulverized in a high capacity mixer grinder to obtain approximately 300 g of powder. Stored in air-tight glass containers with necessary labeling, the powder was preserved in a cool, dry and dark place for further processing.

Figure 2.4: *L. thorelli* Leaves Powder

2.2.2. Extraction

For extraction of phytochemicals from the leaf powder, maceration technique was used. Maceration was originally used in winemaking, but was later adopted and widely used in the extraction of medicinal plants. Azwanida detailed in his paper that this technique involves placing the powdered material in a stoppered container with a suitable solvent and soaking it at room temperature for at least three days with occasional agitation (2015). Ethanol was used as the solvent in this study and the powder was immersed in 1 L of ethanol for sixteen days ensuring normal ambient temperature (22-25 °C) as well as frequent stirring.

Figure 2.5: Ethanol 2.5 L - Solvent Used for Extraction

Figure 2.6: Maceration of *L. thorelli* Leaves

Filtration is a critical step and occurs in two sequential stages (Odey et al., 2012). First, a white cloth, autoclaved for 45 minutes, was used to remove large particles, then Whatman filter papers (pore size: 110 mm) were used to remove smaller particles, resulting in a clear solution.

The filtrate was collected in a beaker and for three days, the beaker was placed in a laboratory water bath maintaining a temperature of 53±1 °C. Thereafter, the thick concentrated mixture was transferred to a petri-dish and again placed in the water bath at the same temperature to vaporize the residual solvent from the extract. After taking all possible safety measures to avoid contamination, the petri-dish was kept under laminar air flow for several hours to obtain a completely dried extract. Finally, the petri-dish was wrapped with aluminium foil, labeled and stored in the refrigerator for further investigation.

Table 2.1: Weight of Obtained Ethanolic Extract of *L. thorelli* Leaves

Initial Weight of the Petri-dish (g)	146.05
Weight of Petri-dish With the Extract (g)	157.26
Weight of the Extract (g)	11.21

So, about 11.21 g of leaf extract was obtained after ethanol extraction.

Figure 2.7: Evaporating Solvent from the Extract Using Laboratory Water Bath

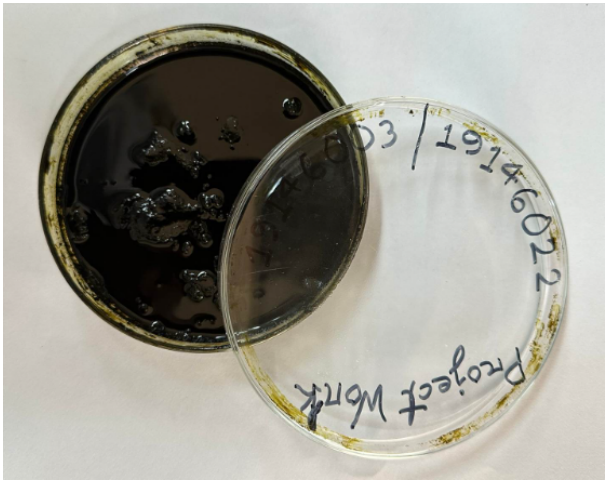

Figure 2.8: Ethanolic Extract of *L. thorelli* Leaves

2.3. In-vitro Antioxidant Property Analysis

2.3.1. Evaluation of DPPH Free Radical Scavenging Assay

In research from Rahman et al., DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging method is extensively accepted for screening antioxidant activity of plant extracts due to its ease of use, low cost and relatively fast analysis time (2015). In this method, antioxidant properties are assessed by decolorization of the DPPH solution by addition of the extract solution in a concentration-dependent manner.

2.3.1.1. Mechanism of DPPH Assay

DPPH, a primarily stable free radical, possesses an unpaired electron which is delocalized over the entire atom during the DPPH assay. Giving a violet/purple color in ethanol, DPPH offers maximum absorbance at a wavelength of 517 nm. Scavenging of DPPH free radicals by the plant extractives results in decrease in absorbance value with time. There lies a proportional relation between the rate of decrease in DPPH absorption and the concentration of radicals that are being scavenged. Employing a UV-visible spectrophotometer, absorption readings were taken and the scavenging activity is represented as the inhibition percentage of DPPH radicals. This assay is based on both electron transfer and hydrogen atom transfer reactions. This assay considers concentration of the test sample and temperature and time of reaction as well, which are to be controlled carefully. Presence of ethanol, contributing to a background antioxidant activity, is another critical factor that needs to be taken into consideration whilst constructing the standard (Liang & Kitts, 2014).

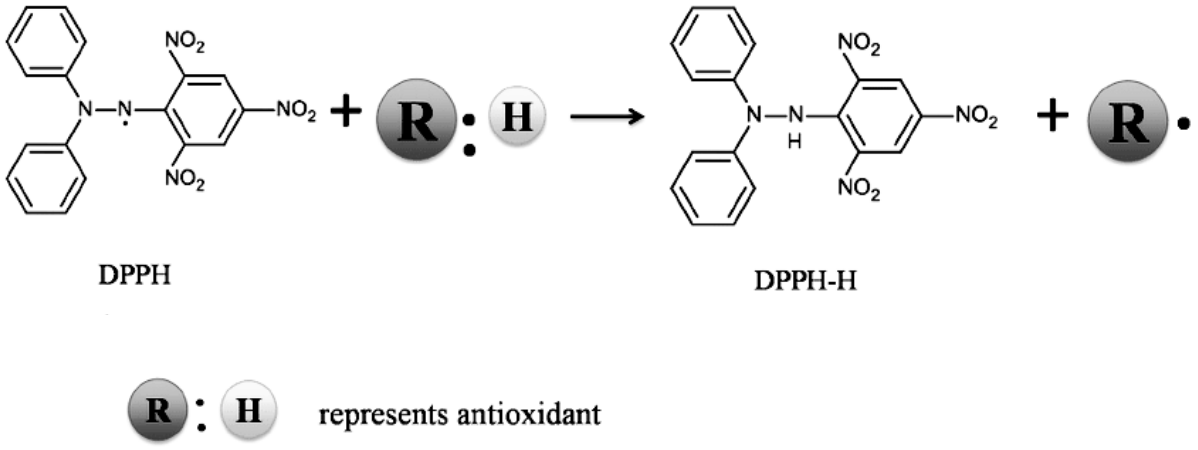

Figure 2.9: Reaction Mechanism of DPPH with Antioxidant (Liang & Kitts, 2014)

2.3.1.2. Materials and Reagents

List of materials and reagents used in the DPPH test is as follows:

Table 2.2: Materials and Reagents

Materials	**Reagents**
UV-visible Spectrophotometer	2,2-diphenyl-1-picrylhydrazyl (DPPH)

Test tubes	Ascorbic acid
Volumetric flasks	Ethanol
Pipette	Leaves extract
Weighing machine	Distilled water

2.3.1.3. Procedure for DPPH Assay

Control Preparation

2 mg of ascorbic acid was dissolved in 4 mL of ethanol to obtain a mother liquor with a concentration of 500 µg/mL. The mother liquor was serially diluted to obtain solutions with concentrations varying from 500 to 0.977 µg/mL. As a standard, ascorbic acid is used here to check the antioxidant activity of the extract.

Sample Preparation

Test sample with a concentration of 500 µg/mL was prepared using 2 mg leaf extract and 4 mL ethanol. Desired concentrations varying from 500 to 0.977 µg /mL for different solutions were achieved by serial dilution of the mother test sample. Afterwards, they were preserved in a dry and dark place with proper labeling.

DPPH Solution Preparation

1 mg of DPPH powder was accurately measured and dissolved in 50 mL of ethanol for preparing a DPPH solution of concentration 20 µg/mL. Thereupon, immediately the solution was covered with aluminium foil and stored in a dark place.

Assay of Free Radical Scavenging Activity

For the assay, each test tube, possessing 1 mL of sample solution or standard solution of different concentrations ranging from 500 to 0.977 µg/mL, was filled with 3.0 mL of DPPH ethanol solution. For thirty minutes, these test tubes were kept in a dark place. Thenceforth, absorbance was recorded at 517 nm by UV-visible spectrophotometer against ethanol as blank.

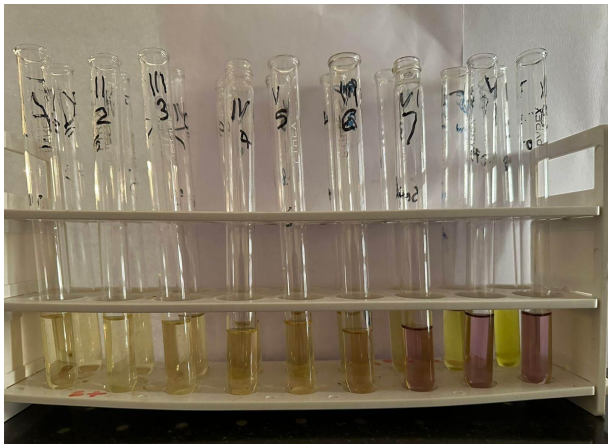

Figure 2.10: Change in Color Due to Reaction Between DPPH and Antioxidant Agent(s)

Calculation

Percent inhibition (% I) of DPPH free radical was calculated as:

$$\% \text{ Inhibition} = (1 - A/A_0) \times 100$$

Where, absorbance of the blank (1 mL ethanol + 3 mL DPPH solution) is indicated as A_0 and absorbance of the sample or the standard is indicated as A (Salar et al., 2015).

Subsequently, IC_{50} values were obtained by graphing the percentage of inhibition (% I) against the concentration (µg/mL) of the extract.

2.3.2. Evaluation of Total Phenolic Content (TPC)

Total phenolic content test is performed involving Folin-Ciocalteu's reagent as oxidative agent and solution of gallic acid as the standard (Sharmin et al., 2018). This test quantifies concentration of phenolic compounds in the plant extract in a simple and reproducible manner.

2.3.2.1. Mechanism of TPC Test

This test, performed in alkaline medium, is based on the electron transfer from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes. As a result, the yellow color of the Folin-Ciocalteu's reagent changes to dark blue in proportion to the concentration of phenolic compounds. The exact chemical mechanism of the Folin-Ciocalteu's reagent is yet to be known. However, reversible one- or two-electron reduction reactions have been postulated to cause the blue color (Ainsworth & Gillespie, 2007).

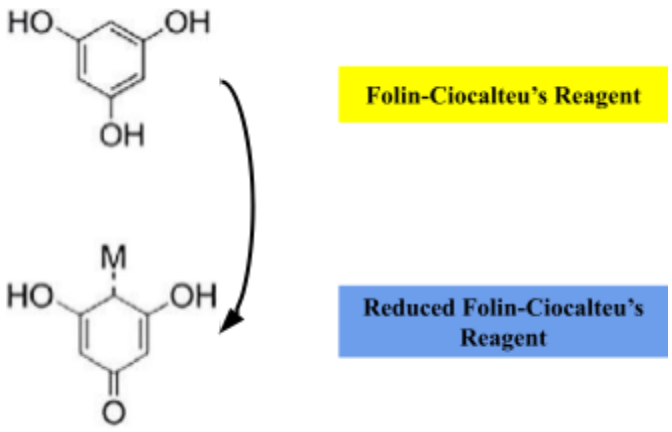

Figure 2.11: Reduction of Folin-Ciocalteu's Reagent in Presence of Phenolics (Ford et al., 2019)

2.3.2.2. Materials and Reagents

List of materials and reagents used in the TPC test is as follows:

Table 2.3: Materials and Reagents

Materials	**Reagents**
Test Tubes	Folin-Ciocalteu's Reagent
Erlenmeyer Flasks	Na_2CO_3
UV-visible Spectrophotometer	Gallic Acid
Micropipette	Distilled Water

2.3.2.3. Procedure for TPC Test

Sample Preparation

10 mg of the *L. thorelli* leaf extract and 5 mL of distilled water were taken into a test tube and shaken until dissolved to get a concentration of 2 mg/mL.

Na_2CO_3 Solution Preparation

A digital balance was used to accurately measure 7.5 g of Na_2CO_3 into a clean Erlenmeyer flask. 92.5 mL of distilled water was carefully pipetted into the flask. Thereupon, the Erlenmeyer flask was shaken vigorously to obtain a 7.5% w/v solution of Na_2CO_3.

Standard Preparation

Using 1 g of gallic and 10 mL of distilled water, a standard solution was prepared having a concentration of 100 µg/mL. It was used as a stock/mother solution for serial dilutions. Subsequently, solutions of concentration 75, 50, and 25 µg/mL were achieved through diluting the stock with a measured amount of distilled water.

Analysis of Total Phenolic Content

A slightly modified method from the research of Ainsworth and Gillespie was used for performing the total phenolic content test (2007). Market preparation of Folin-Ciocalteu's reagent was tenfold diluted with distilled water to maintain a 1:9 ratio. Clean test tubes were arranged in a holder with required labeling and 2 mL of Na_2CO_3 solution was precisely pipetted into each of them. Then, 0.5 mL standard solutions of gallic acid of various concentrations ranging from 100 µg/mL to 25 µg/mL were taken in pre-labeled test tubes and 0.5 mL of sample solution was taken in a separate test tube containing Na_2CO_3 solution. A blank was also prepared to nullify the effects of the solvent. Later on, 2.5 mL of Folin-Ciocalteu's reagent was added in each of the test tubes. For incubation, the mixture was preserved at room temperature for about twenty minutes. Thereafter, absorbance was read with a UV-visible spectrophotometer at a wavelength of 765 nm. The total phenolic content in the test sample was estimated using a standard curve of gallic acid. Sample's phenolic content is revealed as units of mg/g gallic acid equivalent (GAE) of dry extract (Alhakmani et al., 2013).

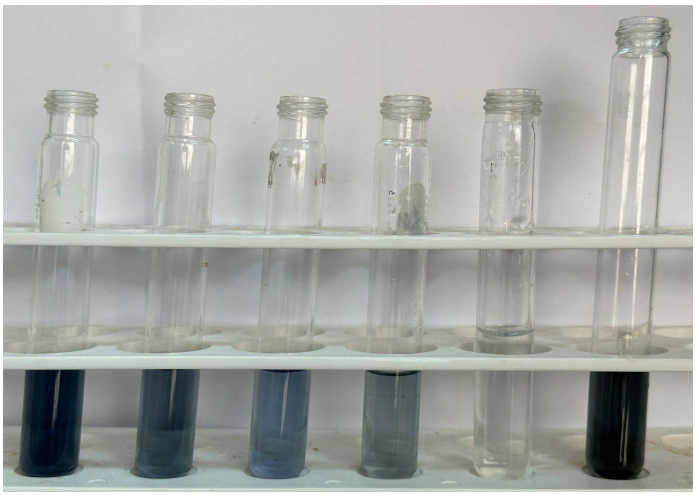

Figure 2.12: Dark Blue Color Indicates Presence of Phenolic Content

2.4. In-vitro Cytotoxicity Property Analysis

2.4.1. Evaluation of Brine Shrimp Lethality Bioassay

Artemia salina, commonly known as brine shrimp, is a simple zoological organism that is highly sensitive to many synthetic as well as natural compounds. Michael et al. first proposed using them to assess the cytotoxic effects or antitumor properties of plant extracts (1956). Later on, Meyer et al. emphasized this bioassay as a simple, economical, rapid, reliable and in-house tool (1982). LC_{50} values of active compounds or extracts are determined through this technique.

2.4.1.1. Materials and Reagents

List of materials and reagents used in the brine shrimp lethality bioassay is as follows:

Table 2.4: Materials and Reagents

Materials	**Reagents/Others**
Small Tank to Hatch Eggs	*Artemia salina* Leach (Brine Shrimp Eggs)
Pipette and Micropipette	Dimethyl Sulfoxide (DMSO)
Lamp (Light Source)	Vincristine Sulfate
Magnifying Glass	Oxygen Supply
Pasteur Pipette	Sea Salt (Pure NaCl)

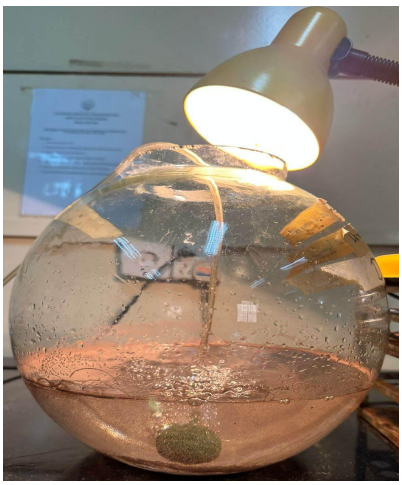

Figure 2.13: Hatching of Brine Shrimp Eggs

2.4.1.2. Procedure for Brine Shrimp Lethality Bioassay

Sea Water Preparation

At first, 38 g of sea salt (non-iodized salt) was accurately weighed into a beaker and dissolved in 1 L of distilled water to mimic the salinity of seawater. To obtain a clear solution, the mixture was then carefully filtered off.

Brine Shrimp Hatching

Brine shrimp eggs were collected from a pet shop for this bioassay. Seawater was filled in a small glass tank. One teaspoon of brine shrimp (*Artemia salina*) eggs was then added in the tank. Oxygen was supplied continuously during hatching. Lamp was used as a light source to draw the nauplii on one-side of the tank. After two days, adequately motile nauplii were obtained to apply for this procedure. Using a pasteur pipette 15±2 live shrimps were collected and placed into test tubes containing 2.5 mL of seawater.

Test Solution Preparation

32 mg of leaf extract was properly mixed with 200 µL of pure DMSO. After that, seawater was used to bring the volume to 20 mL. The final solution concentration was 1600 µg/mL. The solution was then serially diluted with seawater to 800, 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL. Next, 2.5 mL of leaves extract solution was poured in 2.5 mL of seawater containing 15 nauplii (Asaduzzaman et al., 2015).

Table 2.5: Preparation of Test Solutions with Sample for Brine Shrimp Lethality Bioassay

Extract Solution (Preparatory Concentration; µg/mL)	Seawater Containing Nauplii (mL)	Final Volume (mL)	Final Concentration (µg/mL)
2.5 mL (1600)	2.5	5	800
2.5 mL (800)	2.5	5	400
2.5 mL (400)	2.5	5	200
2.5 mL (200)	2.5	5	100
2.5 mL (100)	2.5	5	50

2.5 mL (50)	2.5	5	25
2.5 mL (25)	2.5	5	12.5
2.5 mL (12.5)	2.5	5	6.25
2.5 mL (6.25)	2.5	5	3.125
2.5 mL (3.125)	2.5	5	1.5625

Control Group Preparation

According to Asaduzzaman et al., control groups are vital aspects of true experimental designs as they provide comparative analysis of the cytotoxic effect of test sample (leaf extract) and confirm that the study results are due to equivalent performance of the test sample and not because of any extraneous effects (2015).

For this bioassay, two sorts of reference control groups were utilized:

1) Positive Control and
2) Negative Control.

Positive Control Preparation

Vincristine sulfate was employed in this current study as the positive control. 100 µL from the market formulation of vincristine sulfate solution (2mg/2mL) was mixed with 200 µL of pure DMSO and 4.70 mL of seawater to get a concentration of 20 µg/mL. Afterwards, the stock solution was serial diluted to obtain various concentrations ranging from 10 µg/mL to 0.039 µg/mL. 2.5 mL of each concentration of positive control was poured in 2.5 mL of seawater containing 15 nauplii.

Table 2.6: Preparation of Positive Control for Brine Shrimp Lethality Bioassay

Positive Control (Preparatory Concentration; µg/mL)	Seawater Containing Nauplii (mL)	Final Volume (mL)	Final Concentration (µg/mL)
2.5 mL (20)	2.5	5	10
2.5 mL (10)	2.5	5	5

2.5 mL (5)	2.5	5	2.5
2.5 mL (2.5)	2.5	5	1.25
2.5 mL (1.25)	2.5	5	0.625
2.5 mL (0.625)	2.5	5	0.3125
2.5 mL (0.3125)	2.5	5	0.15625
2.5 mL (0.15625)	2.5	5	0.078125
2.5 mL (0.078125)	2.5	5	0.0390625
2.5 mL (0.0390625)	2.5	5	0.01953125

Negative Control Preparation

To prepare negative control, 50 μL of dimethyl sulfoxide analytical reagent was added into three test tubes with proper labeling, containing 4.95 mL of seawater along with 15±2 nauplii. If brine shrimp nauplii has a high mortality rate in the negative control, then the experiment is thought to be invalid. Because the nauplii may have died due to some unwanted causes, such as lack of oxygen or food, change in pH etc., other than the cytotoxic effect of the test compound.

Nauplii Counting

A magnifying glass was used and the number of survivors in each tube were counted after 24 hours. Based on the inspection, the following equation was applied to compute the mortality percentage (%):

$$\% \text{ mortality} = (\text{No. of dead nauplii} / \text{Initial count of nauplii in test tube}) \times 100$$

Where, Number of dead nauplii = (Initial count of nauplii in test tube − No. of survivors) (Waghulde et al., 2019).

Efficacy of the test sample is assessed by the LC_{50} value which indicates the concentration of any substance at which half of the test subject dies after a certain time. This value is calculated from the regression equation obtained by graphing the mortality percentage (%) against the logarithm of the corresponding concentration.

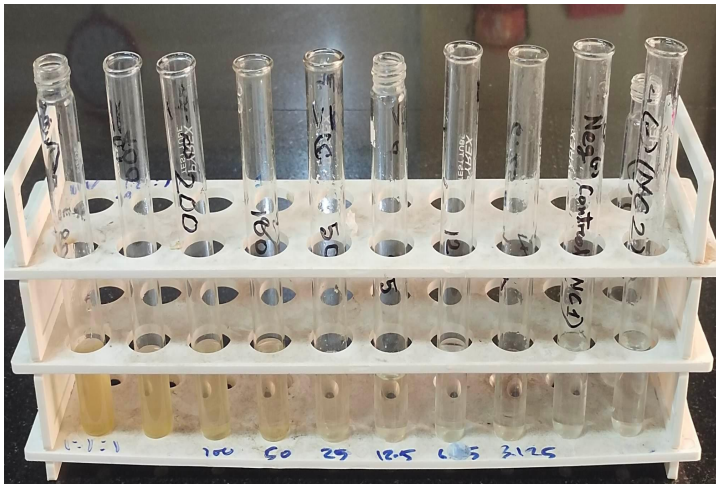

Figure 2.14: Serial Dilution of Test solution for Determination of Cytotoxic Effect

Chapter Three

Observation and Results

3. Observation and Results of All Experiments

3.1. Antioxidant Property Analysis

3.1.1. Evaluation of DPPH Free Radical Scavenging Assay of *L. thorelli*

Change in color of the test mixtures from violet to yellow indicated strong presence of one or multiple scavengers in the ethanolic extract of leaves which contributed in free radical neutralization and therefore exerted antioxidant potentials.

Absorbance was taken using a Shimadzu UV-1280 UV-Vis spectrophotometer. % of inhibition was calculated using the aforementioned equation in section 2.3.1.3. The blank solution exhibited an absorbance (A_0) of 0.623 at 517 nm wavelength. IC_{50} value was determined by the formula "y = mx + c" obtained from the slope of the graph plotted using conc. against percentage (%) of inhibition.

Table 3.1: Estimation of IC_{50} Value (µg/mL) of Ascorbic Acid

Conc. (µg/mL)	Absorbance of Ascorbic Acid (Standard)	% of Inhibition	IC_{50} (µg/mL)
500	0.021	96.62921348	47.55245
250	0.038	93.90048154	
125	0.061	90.20866774	
62.5	0.154	75.28089888	
31.25	0.262	57.94542536	
15.625	0.356	42.85714286	
7.8125	0.398	36.11556982	
3.90625	0.425	31.78170144	
1.953125	0.452	27.44783307	
0.9765625	0.483	22.47191011	

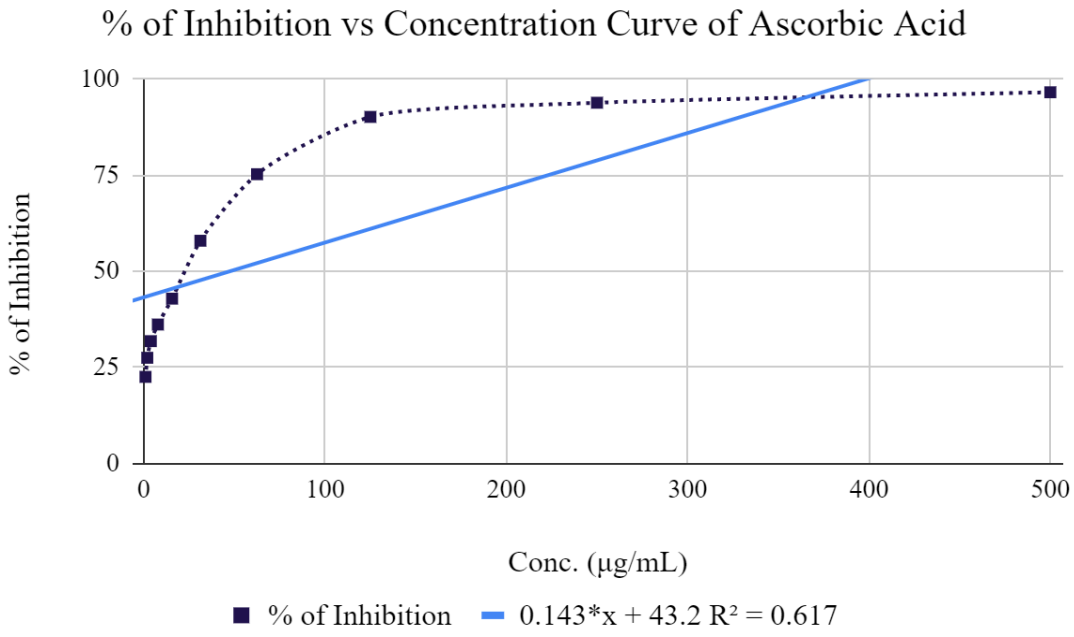

Figure 3.1: % Inhibition vs. Concentration Curve of Ascorbic Acid (Standard)

Table 3.2: Estimation of IC_{50} Value (µg/mL) of *L. thorelli* Leaves Extract

Conc. (µg/mL)	Absorbance of *L. thorelli* Leaves Extract	% of Inhibition	IC_{50} (µg/mL)
500	0.072	88.44301766	83.05085
250	0.134	78.49117175	
125	0.198	68.21829856	
62.5	0.210	66.29213483	
31.25	0.255	59.06902087	
15.625	0.303	51.36436597	
7.8125	0.393	36.91813804	
3.90625	0.438	29.69502408	
1.953125	0.479	23.11396469	
0.9765625	0.507	18.61958266	

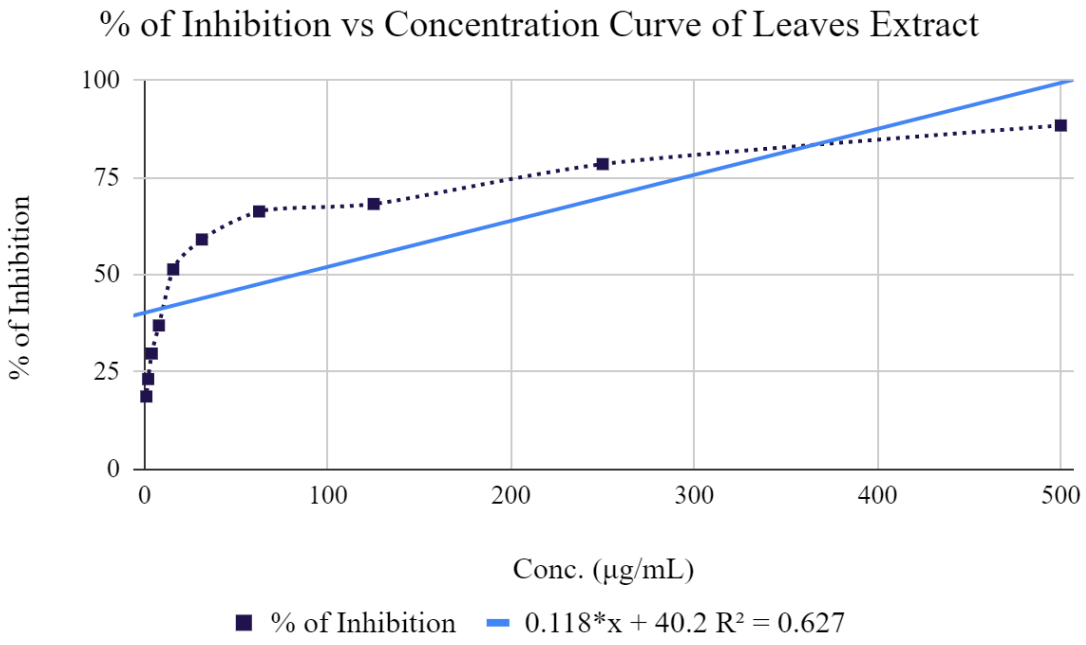

Figure 3.2: % Inhibition vs. Concentration Curve of *L. thorelli* Leaves Extract

Explanation: *L. thorelli* leaves extract was considerably efficient in scavenging DPPH free radicals from the test mixtures as it demonstrated an IC_{50} value of 83.05085 µg/mL. Whereas, IC_{50} value of ascorbic acid (standard) was 47.55245 µg/mL. It means that *L. thorelli* requires higher concentration to yield the equivalent antioxidant effect of the ascorbic acid.

3.1.2. Evaluation of Total Phenolic Content

Total phenolic content (TPC) test was used as a quantitative analysis tool. The higher the concentration of phenolic compounds, the greater the antioxidant capacity of any natural product. In the present study, TPC of the *L. thorelli* leaves extract was estimated spectrophotometrically from the standard curve of gallic acid which exhibited peak absorbance at a wavelength of 765 nm. Color change of test mixture from yellow to dark blue preliminarily conveyed phenolic presence in the extract in comparison to the dilutions of gallic acid solutions. These phenolic compounds are correlated with scavenging free radicals.

Table 3.3: Determination of Regression Equation from Gallic Acid Absorbance

Concentration (µg/mL)	Absorbance	Regression Equation	R^2 Value
100	0.836	0.00745x - 0.0106	0.918
75	0.419		
50	0.327		
25	0.228		
0	0.000		

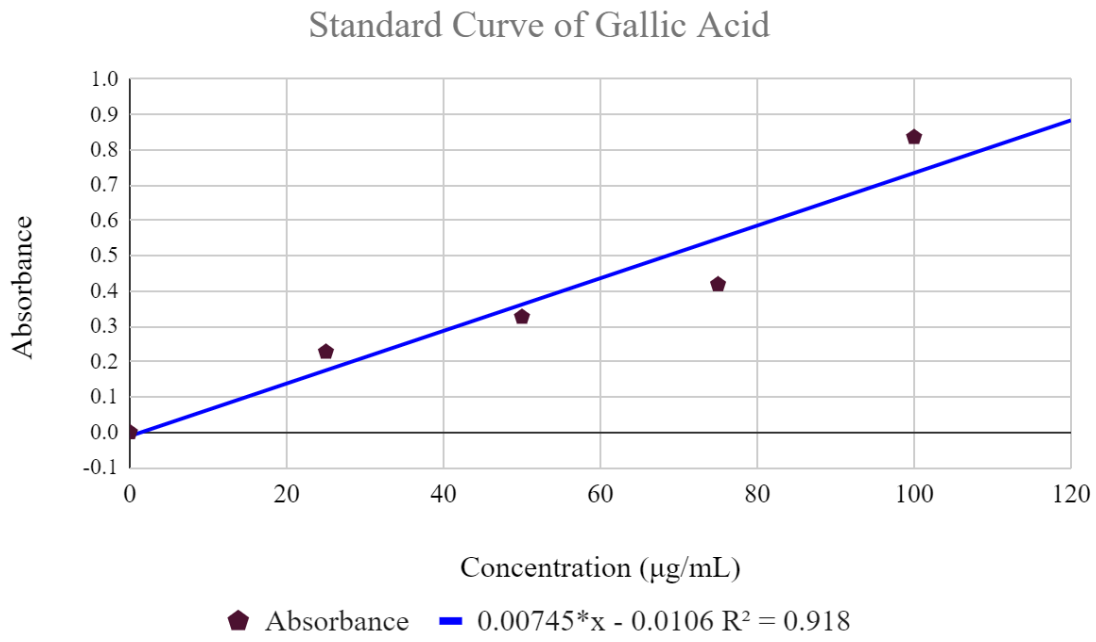

Figure 3.3: Standard Curve of Gallic Acid for TPC Test

Table 3.4: Determination of Total Phenolic Content of Test Sample

Name of Extract	Plant Part	Absorbance of Extract	Total Phenolic Content (mg/g GAE of dry extract)
Ethanol extract	Leaves of *L. thorelli*	0.903	122.6309

Explanation: Based on calculations from the regression equation of table 3.3 above, the concentration of phenolics in the ethanolic extract of leaves was determined to be 122.6309 mg/g GAE of dry extract. So, *L. thorelli* leaves extract contains a noteworthy amount of phenolic compounds which is associated with its antioxidant activity.

3.2. Cytotoxicity Property Analysis

3.2.1. Evaluation of Brine Shrimp Lethality Bioassay

The findings of this brine shrimp/*Artemia salina* lethality bioassay is expressed as the mortality percentage (%) of the nauplii observed at different conc. of the standard and the extract. Concentrations converted to their respective logarithmic values were plotted against corresponding mortality percentage (%) to construct a graph from where LC_{50} was obtained.

Table 3.5: Effects of Vincristine Sulfate on *Artemia salina* Nauplii

Conc. (µg/mL)	Logarithmic Conc.	Initial Count of Nauplii	No. of Dead Nauplii	No. of Live Nauplii	% Mortality	LC_{50} (µg/mL)
0.01953125	-1.70927	15	11	4	73.33	0.000145
0.0390625	-1.40824	15	11	4	73.33	
0.078125	-1.10721	15	12	3	80	
0.15625	-0.80618	15	13	2	86.67	
0.3125	-0.50515	15	13	2	86.67	
0.625	-0.20412	15	14	1	93.33	
1.25	0.09691	15	14	1	93.33	
2.5	0.39794	15	15	0	100	
5	0.69897	15	15	0	100	
10	1	15	15	0	100	

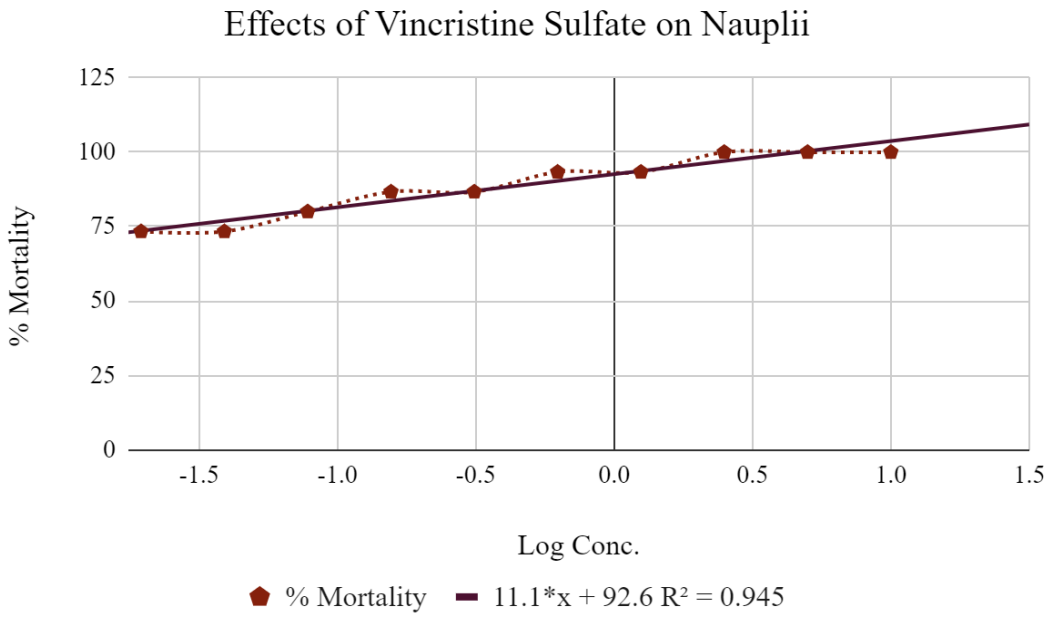

Figure 3.4: Percentage (%) of Mortality of Vincristine Sulfate

Table 3.6: Effects of *L. thorelli* on *Artemia salina* Nauplii

Conc. (µg/mL)	Logarithmic Conc.	Initial Count of Nauplii	No. of Dead Nauplii	No. of Live Nauplii	% Mortality	LC_{50} (µg/mL)
1.5625	0.19382	15	0	15	0	28861.40
3.125	0.49485	15	0	15	0	
6.25	0.79588	15	0	15	0	
12.5	1.09691	15	1	14	6.67	
25	1.39794	15	2	13	13.33	
50	1.69897	15	2	13	13.33	
100	2	15	3	12	20	
200	2.30103	15	3	12	20	
400	2.60206	15	4	11	26.67	
800	2.90309	15	5	10	33.33	

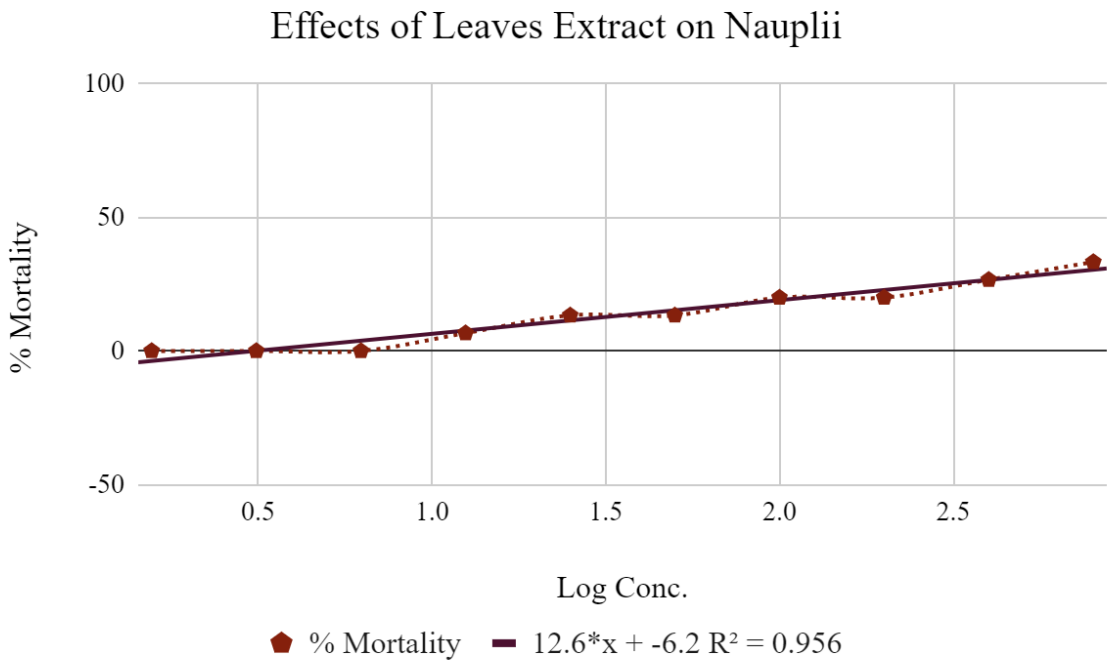

Figure 3.5: Percentage (%) of Mortality of Ethanolic Extract of *L. thorelli*

Explanation: Regression equations obtained from the above two graphs were employed to calculate the LC_{50} value. The LC_{50} value of *L. thorelli* leaf extract is 28861.40 µg/mL and 0.000145 µg/mL for vincristine sulfate (positive control). There seems to exist a high difference between these two values (0.000145 << 28861.40). So, it requires a huge amount of *L. thorelli* leaf extract to cause equivalent toxicity of vincristine sulfate, indicating that the extract is not as toxic as vincristine sulfate.

Chapter Four

Discussion

4. Discussion

Determination of bioactive compounds from medicinal plants highly depends on the type of solvent/menstruum used in the maceration technique. Therefore, characteristics of the solvent such as polarity, safety, reactivity, viscosity, recovery, boiling point etc. are skimmed through properly (Abubakar & Haque, 2020). Among the polar solvents, methanol and ethanol are widely used for extraction. This is because these solvents are relatively more efficient in separating polar compounds from the plant materials. Some nonpolar constituents are also removed and dissolved in these solvents (Das et al., 2010). Though methanol has higher extractive yield than ethanol and shows greater concentration of biologically active compounds, ethanol was picked for maceration of *L. thorelli* leaves. Because, methanol is comparatively more toxic than ethanol and any residual quantity of methanol can be hazardous to human and animal cells as well as be responsible for false positive results.

The current project was specifically organized and performed to ascertain antioxidant and cytotoxicity properties of *L. thorelli* leaf extract using several in vitro experimental approaches. Both qualitative (DPPH free radical scavenging assay, Brine shrimp lethality bioassay) and quantitative (Total phenolic content test) assays had been included which revealed considerable potentials of the *L. thorelli* leaves as a natural source of medicinal products.

Presence of antioxidant properties is associated with terminating free radicals by donating electrons. Free radical disruption is proven to have beneficial effects in certain human diseases, for example: atherosclerosis, diabetes, stroke, arthritis, cancer and neurodegenerative diseases. In addition, scavenging of free radicals improves stability of foods and increases nutritive value. Thus, antioxidant activity is of high importance both in medicinal and dietary sectors (Shovo et al., 2021). During the DPPH free radical scavenging experiment, *L. thorelli* leaves extract showed significant antioxidant activity by exhibiting IC_{50} value at 83.05085 µg/mL against ascorbic acid, for which the IC_{50} value was obtained at 47.55245 µg/mL. Moreover, quantitative analysis of total phenolic content gained high attention for the *L. thorelli* leaves extract. TPC test estimated the *L. thorelli* leaves extract to contain approximately 122.6309 mg/g GAE phenolic compounds of the dry extract. Result of the TPC test strongly supports the DPPH assay as phenolic compounds are directly correlated with free radical termination.

Cytotoxicity of the *L. thorelli* leaves extract was analyzed involving vincristine sulfate as the reference standard. Vincristine sulfate is an anticancer drug belonging to the group 'Vinca Alkaloids' and is highly effective at preventing the spread of malignant cells. Many plants were discovered to be reliable sources of new anticancer agents with lower side-effects. Cytotoxicity of a compound is ascertained impotent when value of median lethal concentration (LC_{50}) is found at the range of 500-1000 µg/mL, average at 100-500 µg/mL and potent at 0-100 µg/mL (Shovo et al., 2021). In the current study, LC_{50} of vincristine sulfate was obtained at 0.000145 µg/mL, indicating that it is a potent cytotoxic agent. Whereas, LC_{50} of *L. thorelli* leaves extract was obtained at 28861.40 µg/mL, demonstrating that the leaves extract had impotent cytotoxicity. In other words, the leaves extract was satisfactorily safe to administer even at high concentrations.

4.1. Conclusion

The globe continues to see tremendous growth in the appliance of medicinal plants as the promising source of self-treatment for minor health issues. As a result, safety of the medicinal plants have become a significant public health concern. Additionally, substituting natural antioxidants for synthetic ones will be advantageous from a variety of angles. Investigation of the *Lagerstroemia thorelli* ethanolic leaf extract conveyed compelling evidence of antioxidant activity. However, it is crucial to look into the root causes and mechanisms of the profound antioxidant effects. Since thousands of phytochemicals or bioactive compounds may occur simultaneously in the aforementioned leaves extracts that co-exist as multicomponent mixtures. Isolation, identification, structure elucidation and determination of their function are still needed to be outlined. Moreover, it is apparent from the brine shrimp lethality assay that the extract has impotent cytotoxic activity. Therefore, further in-depth studies of this medicinal plant are highly suggested to clarify the in-vivo potential of the leaves extract in the management of various human diseases resulting from oxidative stress complications and long term toxicity studies for ensuring a complete safety profile.

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